RNA INTERFERENCE AND HETEROCHROMATIN FORMATION IN FISSION YEAST

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Abstract

In *Schizosaccharomyces pombe* (fission yeast), centromeric DNA is packaged in heterochromatin domains that are important for normal centromere function. Heterochromatin acts as a platform to establish tight physical cohesion between sister chromatids which is important for their efficient segregation during anaphase. The assembly of this structure is known to require specific chromatin modifying factors as well as components of the RNA interference (RNAi) machinery.

Transcription from the centromeric outer repeats triggers Dcr1 to produce siRNAs which are loaded into Ago1 in the RITS complex. RITS then localizes to centromeric outer repeat DNA and recruits histone H3 lysine 9 methylation, thus promoting the formation of heterochromatin. RNAi is also involved in establishing heterochromatin domains at the silent mating type loci and sub-telomeric regions.

Fission yeast RNAi was proposed to act in regulating gene expression. It was suggested that RNAi targeted Long Terminal Repeats (LTRs) for heterochromatin silencing and in the process affected the transcription levels of nearby genes. This model was disproven since no evidence was found for RNAi activity against LTRs or any indication of the presence of heterochromatin overlying these sequences. Expression levels of genes supposedly targeted by this mechanism where not sensitive to loss of RNAi or to heterochromatin instability.

There is strong evidence suggesting that RNAi acts co-transcriptionally in order to promote heterochromatin formation. Thus, it is possible that RITS activity is interlinked with transcription-related processes such as cleavage/poly-adenylation, transcription termination and RNA turnover by the exosome complex. In order to investigate this hypothesis, the integrity of RNAi and heterochromatin was assayed in mutants for factors that are involved in all three pathways. Mutations on *dhp1* (termination), *pfs2* (cleavage and polyadenylation) *dis3* and *rrp6* (exosome) had negligible effects on RNAi activity and heterochromatin-mediated silencing with only the exosome showing some involvement in the downstream degradation of centromeric transcripts.

Conventional RNAi enforces post-transcriptional repression by targeting mRNA molecules for degradation. This is mediated by the endonuclease activity of Argonaute ("slicing"). Although the key residues for this activity are conserved between human Ago2 and *S. pombe* Ago1, the importance of this "slicing" activity to heterochromatin assembly was not clear. Mutations were made in putative catalytic residues on the endogenous *ago1* gene in order to address this question. These mutations severely affect the activity of RNAi in fission yeast and destabilize the heterochromatin structure at centromeres. Consequently, centromere function is affected and chromosome segregation is deficient. Ago1 localization to the centromeres is impaired in these mutants and cannot nucleate heterochromatin nucleation though siRNA production is not fully abolished. Thus, Ago1 slicing activity is crucial for sustainable RNAi and its role in heterochromatin integrity.

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List of abbreviations

ade – adenine

Ago - Argonaute

APC/C - anaphase promoting complex/cyclosome

ARS - autonomous replicating sequence

ATP - adenosine triphosphate

bp - base pairs

CBP - CREB-binding protein

CDE - centromere DNA element

cDNA - complementaty DNA

CENP-A - Centromere protein A

ChIP - chromatin immunoprecipitation

Chp1/2 - chromodomain protein 1 or 2

cid - caffeine-induced death

Clr4 - cryptic locus 4

cnt - central core sequence

CPF - cleavage and polyadenylation factor complex

cpm - count per minute

CPSF - cleavage and polyadenylation specifying factor

cs - cold sensitive

CUT - cryptic unstable transcripts

Dcr1 - Dicer

DDH motif - Aspartate-Apastate-Histidine motif

dis - defective in segregation

DMTs - DNA methyltransferases

DNA - Deoxyribonucleic acid

DNMT - DNA *de novo* methyltransferases

dNTP – deoxyribonucleotide triphosphate

dsRNA - double-stranded RNA

EDTA - ethylene diamine tetraacetic acid

EGTA - ethylene glycol tetraacetic acid

eIF - eukaryotic translation initiation factor

EN - endonuclease

ERV - endogenous retrovirus

FACT - "facilitates chromatin transcription"

FLD - flowering locus D

FOA - 5- fluoroorotic acid

FXR - Fragile X-related protein

GFP - green fluorescent protein

HAC - human artificial chromosome

HATs - histone acetyltransferases

HDACs - histone deacetylases

HOP-1 - homolog of presenilin

HP1 - heterochromatin protein 1

IAP - Intracisternal A-Particle

IES - internally eliminated sequences

imr - inner most repeats

IN - integrase

INCENP - inner centromere protein

IP - immunoprecipitation

IRC - inverted repeat at centromere

JmjC - Jumonji C

Kb - kilobases

KDa - kiloDaltons

LB - Luria Bertani medium

LHP1 - Like-Heterochromatin Protein 1

LINE - long interspersed nuclear element

LSD1 – lysine specific demethylase 1

LTRs - long terminal repeats

Mb - megabases

MBT - malignant brain tumor

ME - malt extract

MIP - methylation induced premeiotically

miRNA - microRNA

miRNP – microRNA-protein complex

mRNA - messenger RNA

MSK Kinase - mitogen and stress activated protein kinase

Mtases - methyltransferases

nt - nucleotide

otr - outer repeats

PAB - poly(A) binding protein

PADs - peptidylarginine deaminases

PAZ - PIWI/Argonaute/Zwindle domain

PBS - phosphate buffered saline

PEV - position-effect variegation

PEM – PIPES, EDTA and magnesium chloride solution

PEMS – PEM with sorbitol

PHD - plant homology domain

piRNAs - piwi-associated RNAs

PIPES - Piperrazine-N,N'-bis(2-ethanesulfonic acid)

PIWI - P-element induced wimpy testis

PMG - Pombe minimal medium

PR - protease

PTGS - post-transcriptional gene silencing

qPCR - real time (quantitative) PCR

RB - RNA binder

rDNA - ribosomal DNA

RDRC - RNA-dependent RNA polymerase complex

RdRP - RNA-dependent RNA polymerase

RIP - repeat induced point mutagenesis (previously known as rearrangement induced premeiotically)

RISC - RNA-induced silencing complex

RNA - ribonucleic acid

RNAi - RNA interference

RRM - RNA recognition motif

Rrp6 - ribosomal RNA processing 6

RT - reverse transcriptase

RT - room temperature

RT-PCR - reverse transcriptase polymerase chain reaction

SAGA - Spt-Ada-Gcn5-Acetyltransferase

SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SET - Supressor of variegation-Enhancer of zeste-Trithorax domain

SET1 - SET-domain protein 1

SHREC - Snf2/HDAC-containing repressor complex

Sim - silencing in the middle

SINE - short interspersed nuclear element

SIR - silent information regulator

siRNA - small interfering RNA

Su(var)3-9 - Suppressor of variegation 3-9

TAP - tandem affinity purification

TBE - Tris Borate EDTA buffer

TBZ - thiabendazole

TE - Tris-EDTA solution

TES – Tris, EDTA and sodium dodecyl sulphate solution

TEs - transposable elements

TFIID - Transcription initiation factor IID

TGS - transcriptional gene silencing

TIR - terminal inverted repeats

TLF2 - Terminal Flower 2

tlh1 - telomere-linked helicase gene 1

TRBP - human immunodeficiency virus transactivating response RNA-binding protein

tRNA - transfer RNA

ts - temperature sensitive

TSA - Tricostatin A

TSDs - target-site direct repeats

Tudor-SN - Tudor-streptococcal nuclease domain

UV - ultraviolet

VIG - vasa intronic gene

wtf - with-Tf

YES - Yeast extract supplemented medium

Histones modifications

The histone modification nomenclature uses the following rules: the histone name (e.g. H3, H4) followed by the position of the modified residue in the N-terminal tail (e.g. K4 for lysine 4; S10 for serine 10) and finally an abbreviation of the modification (e.g. me for methyl; ac for acetyl) and the number of such molecule groups on that residue (e.g. for methylation use 1 or nothing for mono-methyl; 2 for di-methyl; 3 for tri-methyl). For instance, tri-methylated histone H4 on lysine 4 becomes H3K4me3.

CHAPTER 1
INTRODUCTION

1.1. CHROMATIN

The genetic information encoded in the DNA sequences of eukaryotic cells is stored in a specialised structure called the chromosome. This entity is essentially a very long stretch of double stranded DNA that is packaged into a small, compact macromolecule that can be efficiently handled and segregated during cell division. Illustrating the degree of DNA packaging attained in chromosomes, the roughly 2 meters of DNA that a typical human cell contains are packaged into 46 chromosomes that fit a nucleus whose diameter does not exceed 6 μm. This feat is accomplished by packaging the DNA strand with a scaffold of histone proteins which together is called chromatin. The histone genes are some of the most well conserved genes within eukarya and so are the basic principles of chromatin organization. Chromatin is highly dynamic and its structure can react according to the different biological processes in activity in the cell, such as gene expression, DNA replication, cell division or DNA damage and repair.

1.2. CHROMATIN STRUCTURE

The basic structural unit of chromatin is the nucleosome, in which 146 bp of DNA is coiled around an octamer of core histone proteins (with 2 of each histone: H2A, H2B, H3 and H4). The periodic arrangement of the nucleosomes along a DNA strand with a small stretch of linker DNA in between nucleosomes constitutes the first level of packaging and it is usually referred to as the 10 nm fibre or the "beads on a string" structure. Chromatin can be found in this form over DNA regions which are heavily transcribed by RNA polymerases or bound to by multiple DNA-binding proteins. The next level of chromatin is the 30 nm fibre (Kornberg and Lorch 1999; Hayes and Hansen 2001). The 30 nm fibre is a more compact and rigid structure due to a closer arrangement of the successive nucleosomes in a solenoid or spiral with the nucleosomes facing outward and with the linker DNA in the centre. In many eukaryotes, the 30 nm fibre is associated with the presence of the histone H1 that binds the linker DNA between nucleosomes. The "linker" histone

H1 is not absolutely required for higher order chromatin organisation since structures similar to the 30 nm fibre can also be found on chromosomes of lower eukaryotes despite the absence of histone H1 in these organisms (Robinson and Rhodes 2006). The 30 nm fibre is found covering vast regions of the chromosome arms that are rich in protein-coding genes.

Chromatin can assume other higher order organizations depending on the region of the chromosome and the life cycle of the cell. Chromatin fibres can form wide loops that allow distant regions on the same chromosome or on different ones to come together in the same region of the nucleus (Dernburg, Broman et al. 1996; Heliot, Kaplan et al. 1997; Dekker, Rippe et al. 2002; Mahy, Perry et al. 2002; Chambeyron and Bickmore 2004). These regions may have common active processes such as transcription, splicing and ribosome biosynthesis that share common components which may drive these chromatin regions to come closer in space in order to optimise efficiency of each process. During cell division (mitosis and meiosis), the degree of chromatin compaction reaches its highest level. In prophase, chromosomes are further condensed around a protein scaffold that contains elements such as condensins (Harvey, Krien et al. 2002; Belmont 2006). The size of chromosomes is reduced to a minimum and become very rigid, thus occupying a smaller physical space and avoiding DNA strand entanglements. Consequently, the chromosomes can be efficiently aligned at the spindle midzone during metaphase and quickly segregated during anaphase. Upon cytokinesis in telophase, chromosomes decondense and assume other organisation orders.

The mechanisms that govern the transitions between the different organisation levels of chromatin have been the focus of intensive research. The interest lies in that chromatin structure is clearly connected to the activity of underlying DNA or the functional state of the chromosome. In fact, chromatin can govern or at least influence most if not all of these functions (Kornberg and Lorch 1999). Chromatin researchers have found that there are particular signatures associated with each chromatin organisation state. In addition to the core histones (H2A, H2B, H3 and H4) there are other histone variants that possess the highly conserved histone fold motif but have other features that differ from the core histones. In fact, these variants can replace core histones in the nucleosome octamer and this has implications into the function and structure of chromatin. Replacement of core histones by these histone variants, such as histone H3 by H3.3 or CENP-A, H2A by H2A.X or H2A.Z confers a mark that distinguishes the underlying nucleosomes from normal chromatin. This is reflected on the proteins which can associate with this distinct chromatin and

also the particular structural features it can acquire (Sullivan, Hechenberger et al. 1994; Jin, Cai et al. 2005).

Chromatin organization controls the accessibility to underlying DNA. Nucleosome density and position have an impact on many processes that require access to elements within the DNA sequence (Knezetic and Luse 1986; Lorch, LaPointe et al. 1987; Han and Grunstein 1988). A set of ATP-dependent enzymes called chromatin remodelers can alter the arrangement of nucleosomes from the chromatin fibres, destabilise nucleosomes or influence the higher order structural arrangement of chromatin by introducing superhelical torsion on the underlying DNA (Becker and Horz 2002). ATP-dependent chromatin remodelers can be divided into three families: Swi2/Snf2, ISWI and Mi-2/NuRD. They are found in both lower and higher eukaryotes (with the exception of Mi-2 members which are only known in humans and *Xenopus laevii*) where they play crucial roles in controlling gene expression, accessibility to DNA replication, and response to DNA damage (Sif 2004). Chromatin remodelers work concertedly with enzymes that modify key residues of core histones within the nucleosomes.

1.3. CHROMATIN MODIFICATIONS

A vast repertoire of post-translational modifications of core histone proteins has been described in recent years. Most of these modifications are found on the N-terminal tails of the core histone proteins which are physically accessible from outside the nucleosome. Specific residues in these tails can be the target of modifications such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination and proline isomerisation (Kouzarides 2007).

A large proportion of known chromatin modification was found to be associated with specific biological processes and/or particular chromatin states. The enzymes responsible for these modifications can target specific residues within the histone tail of a particular histone within the nucleosome. Hence, it was proposed that these post-translational modifications constitute the basis of a "histone code", an epigenetic layer of information that is responsible for controlling the chromatin structure and influence its activity (Strahl and Allis 2000; Jenuwein and Allis 2001). The functional context of nucleosomes becomes imprinted in the form of modifications "written" by specific modifying enzymes. In turn, this code is interpreted by specific proteins that either bind to

the modified nucleosomes or whose association to particular residues in the N-terminal tail becomes blocked by such modifications. Each set of histone modifications results in an apparatus of differentially associated proteins. Thereby, the histone modification code is "read" and used to determine the organization of that distinct chromatin domain and the activity of the underlying DNA. Histone acetylation, methylation and phosphorylation are the most common and best characterized chromatin modifications.

Histone acetylation

Acetylation is one of the most widespread modifications in the genome and has been detected at multiple residues on all four core histones, several histone variants such as H2A.Z, H3.3 and CENP-A (human). Acetylation is a reversible histone modification since the activity of histone acetyltransferases (HATs) can be counteracted by histone deacetylases (HDACs) (Grunstein 1997). Acetylated lysines on histone tails are known to be the recognized by proteins containing bromodomain motifs such as Bdf1p (yeast), Brd2 and Brd4 (mammals) (Ornaghi, Ballario et al. 1999; Dey, Chitsaz et al. 2003; Ladurner, Inouye et al. 2003; Matangkasombut and Buratowski 2003; Kanno, Kanno et al. 2004). Bromodomain-containing proteins are numerous in all eukaryotic genomes and are involved in diverse biological processes such as transcription, DNA replication and DNA damage repair (Chen, Tini et al. 2001). Histone H3K8 and K16 acetylation levels respond to nearby DNA double-stranded breaks (Downs, Allard et al. 2004; Jazayeri, McAinsh et al. 2004). Similarly, nucleotide excision repair of DNA pyrimidine dimers caused by UV radiation is known to involve H3K9 and/or K14 acetylation by Gcn5 in budding yeast (Teng 2002 ; Yu PNAS 2005).

Acetylation is a mark associated with active chromatin since many HATs participate in transcriptional co-activator complexes and support transcription events by RNA polymerases (Brownell, Zhou et al. 1996; Mizzen, Yang et al. 1996; Sterner and Berger 2000; Roth, Denu et al. 2001). Some of these factors include the human ACTR co-activator, TFIID transcriptional initiator (Mizzen, Yang et al. 1996; Chen, Lin et al. 1997), the budding yeast SAGA and elongator complexes that facilitate RNA polymerase II transcription (Grant, Duggan et al. 1997; Otero, Fellows et al. 1999). It has been proposed that extensive histone acetylation modifies the net charge of nucleosomes, which could loosen inter- or intranucleosomal DNA-histone interactions (Kornberg and Lorch 1999). This hypothesis is supported by the observation that acetylated histones are easier to displace from chromatin both *in vivo* and *in vitro* (Ito, Ikehara et al. 2000; Reinke and Horz

2003; Zhao, Herrera-Diaz et al. 2005; Chandy, Gutierrez et al. 2006; Hassan, Awad et al. 2006). Hence, histone acetylation can facilitate the passage of RNA polymerases along a chromatin template. In contrast, de-acetylated nucleosomes are linked to reduced transcriptional activity in fungi and metazoa alike (Braunstein, Rose et al. 1993; Grunstein 1997).

Histone phosphorylation

Phosphorylation is a highly dynamic histone modification that responds to processes such as mitotic chromosome condensation, transcription and DNA repair. Histone phosphorylation is controlled by specific kinases and phosphatases that can modify serine and threonine residues on the histone tails. This modification is recognized by proteins of the 14-3-3 family of phosphor-binding factors, most which are involved in signalling transduction and many other cellular pathways (Chen and Wagner 1994).

Histone H3T3 phosphorylation by the Haspin kinase occurs during mitosis and is involved in control of sister chromatid cohesion that is required for normal chromosome alignment during metaphase in metazoa (Dai, Sultan et al. 2005; Dai, Sullivan et al. 2006). In plants, histone H3T11 phosphorylation is involved in chromosome condensation during mitosis and meiosis (Houben, Demidov et al. 2005).

In animal cells, phosphorylation of histone H3S10 is performed by the MSK kinase to facilitate transcription activation of immediate-early genes such as *c-myc*, *c-fos* and *c-jun* when cells recover from quiescence (Mahadevan, Willis et al. 1991; Chadee, Hendzel et al. 1999; Thomson, Clayton et al. 1999; Soloaga, Thomson et al. 2003). This same modification is promoted by the Fyn kinase in response to DNA damage (He, Cho et al. 2005). Recently, it was discovered that H3S10 phosphorylation promotes de-repression of silent chromatin upon entry into mitosis (Fischle, Tseng et al. 2005; Hirota, Lipp et al. 2005). Phosphorylation of H2A in yeast and H2AX in other eukaryotes is linked to an initial response at sites of DNA damage. H2A/H2AX phosphorylation is required to recruit the INO80 chromatin remodeler (SWI/SNF family) and the NuA4 HAT complex to sites of DNA double-stranded breaks to participate in the repair of DNA double strand breaks (Downs, Allard et al. 2004; Morrison, Highland et al. 2004; van Attikum, Fritsch et al. 2004).

Histone methylation

Methylation is an important histone modification for determining changes in chromatin structure. Methylation has been documented for lysine and arginine residues in both histones H3 and H4. A single arginine residue on a histone tail can be methylated up to two times while a lysine can present up to three methyl groups. This suggests that different numbers of methyl groups on a histone residue can convey different signals or degrees of signal strength. The enzymes responsible for histone lysine methylation are the family of SET domain-containing methyltransferases such as SET1, G9a, *Su(var)3-9* and Clr4 (Rea, Eisenhaber et al. 2000; Lachner and Jenuwein 2002). This modification can be bound to by proteins containing any of several specialized domains such as the chromodomain, Tudor, PHD and MBT domains (Lachner, O'Carroll et al. 2001; Huyen, Zgheib et al. 2004; Kim, Daniel et al. 2006; Pena, Davrazou et al. 2006; Shi, Hong et al. 2006; Wysocka, Swigut et al. 2006).

The functional roles of histone methylation are diverse. Methylation of histone arginines and histone H3 lysines 4, 36 and 79 are marks that accompany active gene transcription. In budding yeast, Set1-mediated H3K4me3 is associated with the initiating form of RNA polymerase II at the 5' end of genes. Conversely, Set2 deposits H3K36 tri-methylation (H3K36me3) at the 3'end of protein-coding genes with elongating RNA polymerase II where it acts synergistically with the Rpd35 deacetylase to suppress cryptic transcriptional initiation sites (Carrozza, Li et al. 2005; Joshi and Struhl 2005; Keogh, Kurdistani et al. 2005). Methylation on histone H3K9, K27 and histone H4K20 are involved in repressing gene transcription and assembling heterochromatin (for more details see page 10). Methylated H3K9 is a binding site for the chromodomain protein HP1 (heterochromatin protein 1) (Bannister, Zegerman et al. 2001; Lachner, O'Carroll et al. 2001). HP1 binding to a chromatin region is coupled to strong transcriptional repression (Eissenberg, James et al. 1990; Eissenberg, Morris et al. 1992). H3K27 methylation accompanies the binding of Polycomb-family proteins to chromatin and their involvement in regulating developmental genes such as the homeotic genes in both plants and animals as well as X-chromosome inactivation in mammals (Cao, Wang et al. 2002; Czermin, Melfi et al. 2002; Kuzmichev, Nishioka et al. 2002; Muller, Hart et al. 2002; Hernandez-Munoz, Lund et al. 2005; Schwartz and Pirrotta 2007).

Unlike other transient modifications, such as phosphorylation and acetylation, methylation is regarded as a more stable modification, particularly tri-methylation (Martens, O'Sullivan R et al. 2005). Historically, it was considered that arginine and lysine methylation represented permanent modifications given their observed low rate of cellular turnover. In recent years, it has been shown that histone methylation can be reversed *in vivo* through histone demethylases which can act on

both methylated arginine and lysine residues. In 2004, it was discovered that histone H3 and H4 arginine methylation was antagonized by peptidylarginine deaminases (PADs) that catalize the conversion of methylated arginine to citrulline (Cuthbert, Daujat et al. 2004; Wang, Wysocka et al. 2004). LSD1 was the first histone lysine-specific demethylase to be characterized, which was found to be specific to methylated H3K4 and K9 (Shi, Lan et al. 2004). LSD1 was the first of a newly-discovered class of SWIRM domain-containing enzymes that can reverse histone methylation through a flavin-dependent oxidative mechanism. LSD proteins are involved in controlling gene expression, particularly in developmental programmes and cell fate. LSD1 is essential for viability of mouse embryos and is part of both transcriptional repressor and activator complexes which serve multiple roles in cell differentiation (Saleque, Kim et al. 2007; Wang, Scully et al. 2007). One of the three LSD1 homologues in *A. thaliana*, FLD (flowering locus D) has a role in timing of flowering development (He, Michaels et al. 2003). In *C. elegans*, the LSD1 homologue SPR-5 has been implicated in blocking the expression of HOP-1, a factor involved in the Notch signalling pathway specifically in the neural development (Eimer, Lakowski et al. 2002; Jarriault and Greenwald 2002). In *Schizosaccharomyces pombe* (fission yeast), Lsd1/Swm1 regulates the spreading of heterochromatin domains (H3K9 methylation) and is important to control expression levels of a subset of genes (Lan, Zaratiegui et al. 2007; Opel, Lando et al. 2007).

The mechanism of demethylation employed by LSD1-related enzymes is limited in that they can remove methyl groups from mono- and di-methylated lysines only and cannot process tri-methylated lysines. Jumonji C domain-containing proteins, such as JMJD1 to 3 (mammals), Lid (fruit fly), Jhd1 (budding yeast) and Jmj2 (fission yeast), are Fe(II) and α-ketoglutarate-dependent dioxygenases which remove the methyl groups from tri-methylated lysines on histone tails (Trewick, McLaughlin et al. 2005; Tsukada, Fang et al. 2006). Like LSD1-related proteins, Jumonji C (JmjC) domain proteins are found in large numbers widespread in different eukaryotic organisms, from yeast to animals and plants. Since the discovery of the histone demethylase activity of human JHDM1, similar findings have been made for other JmjC proteins in budding yeast (Rph1, Jhd2p), fission yeast (Jmj2), fruit fly (Lid) and mammals (RBP2, JHDM3A, JMJD6, PLU-1) (Klose, Yamane et al. 2006; Chang, Chen et al. 2007; Huarte, Lan et al. 2007; Klose, Gardner et al. 2007; Lee, Zhang et al. 2007; Liang, Klose et al. 2007; Yamane, Tateishi et al. 2007). JMJD6 has the peculiarity of being a JmjC-containing arginine demethylase (Chang, Chen et al. 2007). It is likely that the number of known histone demethylases will continue to increase since many more JmjC domain proteins remain to be characterized (Trewick, McLaughlin et al. 2005). Like in the case of LSD proteins, the

biological role of each of these JmjC enzymes depends on their target residues. While some have activity towards specific residues that are linked to gene repression, (H3K4: Lid, Jmj2, RBP2, Jhd2p, PLU-1; H3K36: Rph1, JHDM1), some can demethylate residues involved in both gene activation and repression and thus have a dual role in controlling gene expression (e.g. JHDM3A). In higher eukaryotes, JmjC proteins, like LSD proteins, are associated with developmental programmes (Lee, Zhang et al. 2007). In humans, both types of demethylase enzymes have been connected to endocrine regulation and different aspects of human disease, such as cancer and neurological disorders (Shi, Wang et al. 2007).

DNA methylation

Histones are not the only chromatin components that can be modified. DNA methylation is one of the most well described epigenetic modifications that can be found in all biological kingdoms. Up to this date, only budding yeast (*Saccharomyces cerevisiae*) and the nematode *Caenorhabditis elegans* are known to be devoid of DNA methylation. DNA methylation occurs at the C-5 or N-4 positions of cytosine and at the N-6 position of adenine and is catalyzed by enzymes known as DNA methyltransferases (MTases). In higher eukaryotes, only C-5 cytosine methylation is found. In bacteria, DNA adenine methylation is involved in control of gene expression in the context of cellular regulatory events, including those involved in bacterial virulence (Low, Weyand et al. 2001). DNA cytosine methylation together with methylation-sensitive restriction endonucleases (such as HpaII and NotI) constitutes part of the host restriction system that defends the bacteria against infection by bacteriophages (Low, Weyand et al. 2001). DNA methylation is imparted to bacterial DNA which protects it against the action of endogenous methyl-sensitive restriction endonucleases. Any invading DNA, either from plasmid or bacteriophage, is unmethylated and thus vulnerable to the restriction endonucleases.

Filamentous fungi also employ DNA cytosine methylation to suppress invading DNA elements. *Neurospora crassa* monitors DNA for repetitive content and methylates any repeated DNA sequences for subsequent directed mutagenesis by a process known as RIP (Repeat Induced Point Mutagenesis) (Selker, Cambareri et al. 1987; Cambareri, Jensen et al. 1989). A similar mechanism called MIP (Methylation Induced Premeiotically) is applied by *Ascobolus immersus* to mark repetitive DNA (Rhounim, Rossignol et al. 1992). In both fungi, 5-methyl-cytosine is a potent inhibitor of transcriptional elongation (Barry, Faugeron et al. 1993; Rountree and Selker 1997). Both

processes are believed to have evolved to deal with invasive multi-copy DNA sequences such as transposon and viral DNA. This functionality is present in plants, where DNA methylation plays a key role in controlling the proliferation of retrotransposable elements (Amedeo, Habu et al. 2000; Miura, Yonebayashi et al. 2001; Singer, Yordan et al. 2001). Large chromatin domains enriched in DNA methylation and histone H3K9 methylation can be found covering DNA regions that contain large numbers of transposable elements (Lippman, May et al. 2003; Lippman, Gendrel et al. 2004). DNA methylation across a promoter region in plants effectively blocks transcription initiation but unlike in filamentous fungi, it cannot shut down transcriptional elongation.

The genomic DNA in mammals is extensively methylated but it does not bear the sequence specificity that plant genomes display. Whereas in plants cytosine methylation can be found in multiple nucleotide contexts such as CpG, CpNpG and CpNpN, methylation in mammals is mainly occurs in the CpG (symmetrical) context. Despite this, mammalian cytosine methylation is found widespread across the genome, marking 70-80% of all CpG dinucleotides in the case of human somatic cells (Ehrlich, Gama-Sosa et al. 1982). The exceptions are particular regions of GC-rich DNA called "CpG islands" that are found residing in the vicinity of gene promoters (Bird 1987). These regions are differentially methylated and can serve as regulatory elements for gene transcription through the activity of Methyl-CpG binding domain proteins (MBDs) (Lewis, Meehan et al. 1992; Nan, Meehan et al. 1993). MBDs such as MeCP2 and MBD2 are associated with histone deacetylases and chromatin remodeler complexes that promote gene repression (Jones, Veenstra et al. 1998; Nan, Ng et al. 1998; Zhang, Ng et al. 1999). DNA methylation in mammals is tightly connected to developmental programmes of gene expression and is required for embryonic viability in vertebrates (Li, Bestor et al. 1992; Okano, Bell et al. 1999). DNA methylation is also required for silencing the inactive X chromosome in somatic cells (Sado, Fenner et al. 2000). The appearance of *de novo* DNA methylation on promoters of tumour suppressor genes is often found associated with carcinogenesis (Momparler 2003).

In higher eukaryotes, DNA is methylated by the action of DNA methyltransferases (DMTs) which can be classified into two groups. Maintenance DMTs such as DNMT1 (humans), MET1 and CMT3 (both from plants) copy the methylation patterns onto newly synthesized DNA during replication and thus perpetuates this epigenetic pattern (Yen, Vertino et al. 1992; Ronemus, Galbiati et al. 1996; Bartee, Malagnac et al. 2001; Lindroth, Cao et al. 2001). Members of the *de novo* class of methyltransferases such as DNMT3a, DNMT3b (humans) and DRM2 (plants) are responsible for methylating novel genomic sequences such as developmental genes or repeats

(Okano, Bell et al. 1999; Cao and Jacobsen 2002). Removal of DNA methylation patterns can occur passively throughout multiple cell divisions simply by inhibiting the maintenance class of methyltransferases, as in the case of post-zygotic loss of methylation on maternal chromosome in mammals (Howell, Bestor et al. 2001). In vertebrates, there is strong evidence pointing to active DNA demethylation events in early embryonic development although there is considerable controversy regarding the involved proteins and possible mechanisms. In plants, active demethylation is required for expression of imprinted genes in the endosperm. In *Arabidopsis thaliana* (thale cress), this process is catalyzed by the DNA glyoxylase/lyase DEMETER (DME) that promotes base excision of 5'-methylated nucleotides (Gehring, Huh et al. 2006). The DME-like proteins ROS1, DML2 and DML3 use the same mechanistic principle but have a broader role as general silencing antagonists that protect endogenous genes from deleterious DNA methylation (Penterman, Uzawa et al. 2007; Penterman, Zilberman et al. 2007).

Chromatin modifications and the code hypothesis

Each of the chromatin modifications described here is accompanied by both "writing", "reading" and "erasing" mechanisms. The modifications are interpreted by a set of specialized proteins that contribute to affect the chromatin structure or control the activity of specific pathways on the underlying DNA. Thus the histone code hypothesis is supported by this collection of evidence. The nature of such a code is the matter of intense debate due to the sheer number of different modifications and their association with distinct chromatin functional states. Marks such as H3K9 methylation and consequent binding of HP1 are generally associated with repressive chromatin but recently they were observed to occur within coding regions of heavily transcribed genes (Vakoc, Mandat et al. 2005). Similar observations were made regarding histone H3K36 acetylation, which generally favours transcription but if targeted to promoter sites can have the opposite effect (Strahl, Grant et al. 2002; Landry, Sutton et al. 2003). Examples like these forced researchers to reassess the "histone code" hypothesis as a broader premise that emphasizes the relevance of histone modifications to the functional state of the underlying chromatin but with an element of uncertainty regarding the nature of the actual "code". It is now understood that the role of a particular histone modification is determined by the type and position of the modified residue but is also highly dependent on the functional context of the underlying DNA, cell cycle stage and other chromatin modifications that accompany it (Kouzarides 2007; Li, Carey et al. 2007).

1.4. HETEROCHROMATIN

The chromatin arrangement in interphase chromosomes can be broadly classified into two main categories. Euchromatin is the most widespread form that encompasses all normally "active" or decondensed forms of chromatin. It contains most of the protein coding genes and other actively transcribed elements in the DNA and thus makes up the largest part of the chromosomes arms. These euchromatic regions are interspersed by domains of a distinct form of chromatin called heterochromatin that remains highly condensed in interphase. The largest concentration of heterochromatin is on the DNA regions surrounding the centromeres (peri-centromeres) and adjacent to telomeres. Whilst euchromatin provides a more open scaffold that allows access to DNA in order for processes such transcription to occur, heterochromatin is a dense, more rigid structure that provides less accessibility to underlying DNA (Allshire, Nimmo et al. 1995; Henikoff 2000; Richards and Elgin 2002; Maison and Almouzni 2004). These characteristics are important for its role in preserving the structural integrity of chromosome ends and its contribution to centromeric function. One of the characteristics of heterochromatin is that it exerts a strong repressive influence on gene expression, hence its general denomination of "silent chromatin" (Allshire, Nimmo et al. 1995; Henikoff 2000; Richards and Elgin 2002). Genes localized inside a domain of heterochromatin are silenced whilst genes positioned in the vicinity are subject to a variable repressive influence on their expression – an effect first described in *D. melanogaster* as position effect variegation (PEV) (Muller 1930; Schultz 1936; Spradling and Karpen 1990). This classic epigenetic phenomenon is believed to originate from the dynamics of heterochromatin assembly along chromatin fibres. Thus, heterochromatin can regulate gene expression and this is a property that is exploited by many eukaryotic organisms for controlling developmental genes and transposable elements (Cavalli 2002; Fisher and Merkenschlager 2002). One of the most significant examples of this function lies in the dosage compensation mechanism in female mammals though which one of the two X chromosomes is entirely inactivated in order to preserve X-linked gene dosage. Coating of the inactive X chromosome with the *Xist* RNA is accompanied by the recruitment of dense H3K9 methylation over the entire chromosome which then enforces transcriptional silencing of all its genes (Heard, Rougeulle et al. 2001). Heterochromatin contributes to chromosome integrity by protecting DNA regions against events such as recombination,

transposition of mobile DNA elements or other more drastic chromosome rearrangements (Grewal and Klar 1997; Henikoff 2000; Peng and Karpen 2007). In addition, heterochromatin can act as a scaffold for the assembly of specialized protein machineries, such as the centromeric cohesion which is responsible for physically tethering the centromeres of sister chromatids up until separation in the onset of anaphase (Bernard, Maure et al. 2001; Nonaka, Kitajima et al. 2002).

Heterochromatin-linked modifications

Euchromatin and heterochromatin are distinct not only in structural aspects but also in the repertoire of associated histone post-translational modifications. Nucleosomes in euchromatic domains present extensive acetylation of histone H3 and H4 (Figure 1-1). Histone acetylation accompanies newly-synthesized and assembled nucleosomes during replication but euchromatic nucleosomes are particularly enriched in this modification due to the activity of histone acetyltransferases (HATs) linked to the transcription machinery (Grunstein 1997). HATs such as Gcn5, CREB-binding protein (CBP) and p300 are co-activators that co-localise with numerous transcription factors to promoter regions and acetylate key nucleosomes (Ogryzko, Schiltz et al. 1996; Grant, Duggan et al. 1997; Roth, Denu et al. 2001). Complexes such as the Elongator complex acetylate histones in nearby nucleosomes during a transcription event to facilitate the passage of an actively transcribing RNA polymerase II complex along a chromatin template (Otero, Fellows et al. 1999). It has been suggested that histone acetylation contributes to relax chromatin structure by neutralizing the charge of nucleosomes and consequently weaken interactions between nucleosomes or between histones and the DNA backbone (Kornberg and Lorch 1999). Acetylation also allows for higher accessibility to DNA by transcription factors and efficiency in displacing nucleosomes during transcription. Transcription is also responsible for attracting histone H3 K4 methylation mediated by the methyltransferase SET1 to coding regions of highly transcribed genes. In contrast, nucleosomes in heterochromatin domains are generally hypoacetylated by virtue of specialized HDACs (Moazed 2001) (Figure 1-1). In budding yeast (*Saccharomyces cerevisiae*), the *SIR* genes (silent information regulator) encode proteins that promote formation of silent chromatin at telomeres, rDNA and mating type locus through histone deacetylation. Sir2 is a NAD-dependent histone deacetylase that removes acetyl groups from nucleosomes in order to allow for Sir3 and Sir4 to bind and enact transcriptional silencing (Imai, Armstrong et al. 2000; Gasser and Cockell 2001; Carmen, Milne et al. 2002; Hoppe, Tanny et al. 2002). Different types of HDACs are also

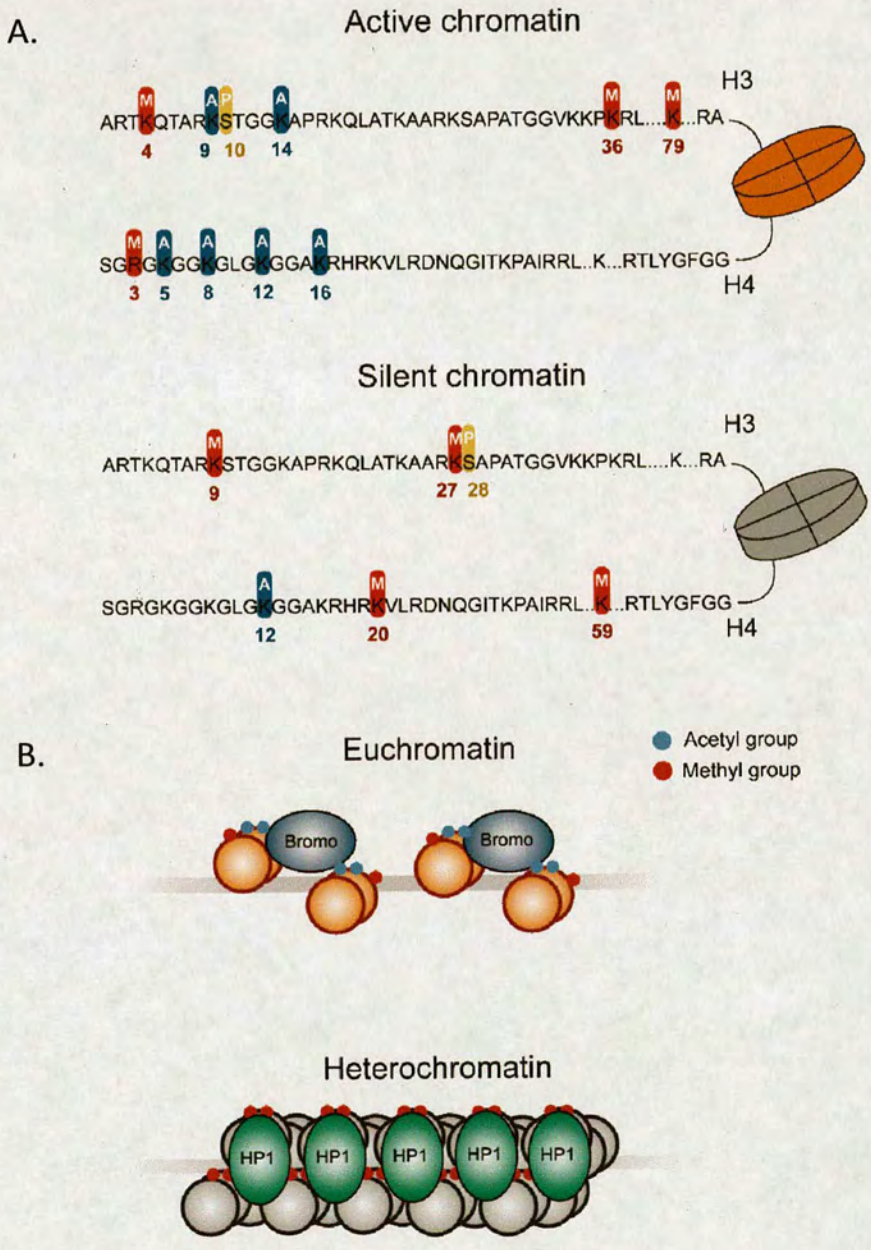


Figure 1-1: Histone tail modifications and chromatin states.

A. Histone H3 and H4 modification patterns found to be associated with transcriptionally active and silent chromatin in various organisms.

B. Basic representation of euchromatin and heterochromatin states. Euchromatic nucleosomes are acetylated and packaged in a more relaxed configuration that permits transcription. The acetylated (in blue) N-terminal tails provide a binding site for bromodomain-containing proteins. In comparison, the nucleosomes heterochromatin are more condensed and transcriptionaly. The histone tails are hypoacetylated and methylated (in red) to which heterochromatin protein 1 (HP1) is bound.

essential in promoting heterochromatin in fission yeast, plants and metazoa (Ekwall, Olsson et al. 1997; Grewal, Bonaduce et al. 1998; Olsson, Ekwall et al. 1998; Olsson, Silverstein et al. 1999; Sekinger and Gross 2001; Shankaranarayana, Motamedi et al. 2003). In addition, heterochromatin in these organisms presents high levels of histone H3K9 methylation (H3K9me2 and H3K9me3) (Figure 1-1). SET domain histone methyltransferases such as Su(var)3-9, its mammalian homolog Suv39h1, Clr4$^{Su(var)3-9}$ from fission yeast and KYP from *A. thaliana* are responsible for this modification in their respective organisms (Rea, Eisenhaber et al. 2000; Czermin, Schotta et al. 2001; Nakayama, Rice et al. 2001; Jackson, Lindroth et al. 2002). Heterochromatin in fission yeast and mammals is enriched in chromodomain proteins such as Su(var)2-5 in *Drosophila melanogaster* (fruit fly), HP1 in mammals and its fission yeast homolog Swi6^{HP1} that are key effectors of this chromatin structure (Eissenberg, James et al. 1990; Eissenberg, Morris et al. 1992; Ekwall, Javerzat et al. 1995). In plants, it is unclear which protein is the main H3K9methyl-binding heterochromatin protein. The only HP1 homolog known so far in *Arabidopsis thaliana* (thale cress) and *Solanum lycopersicum* (tomato), LHP1 (LIKE HP1) or TLF2 (Terminal Flower 2), is a Polycomb-related protein that also possesses sequence similarity to the chromodomain and chromo shadow domain from metazoan HP1 (Gaudin, Libault et al. 2001; Kotake, Takada et al. 2003; Zemach, Li et al. 2006). Even though some initial data suggested that this protein localized to regions in the periphery of centromeres and could bind H3K9me in vitro, a more detailed analysis showed that TLF2 preferentially localized to H3K27me3 domains mainly in euchromatic regions. Thus, it is unclear at this stage if plants have an equivalent of metazoan HP1 and fission yeast Swi6^{HP1} associated with heterochromatin. Heterochromatin domains also contain high levels of H4K20 methylation although this is only observed in plants, *D. melanogaster* and mammals. In addition to histone modifications, heterochromatin in plants displays extensive DNA CpNpG methylation while in metazoa heterochromatic DNA is also fully methylated (CpG). Thus, heterochromatin domains in each eukaryotic organism possess a distinct signature consisting of chromatin modifications and associated proteins that contrast with euchromatic features.

Even though heterochromatin can be established over varied DNA loci, there is a clear preference for the type of DNA sequences that attract the assembly of this particular chromatin state. Heterochromatin is frequently found overlying genomic regions that contain repetitive DNA. This trend is common to fungal, plants and metazoan genomes and represents a clear preference of all the diverse pathways that control heterochromatin formation for this type of genomic elements in a manner that is functionally conserved throughout eukaryotic organisms (Ye and Signer 1996;

Grewal and Klar 1997; Henikoff 2000; Gvozdev, Aravin et al. 2003; Martienssen 2003; Saveliev, Everett et al. 2003; Sun, Haynes et al. 2004; Martens, O'Sullivan et al. 2005).

1.5. SILENCING OF REPETITIVE DNA

Repetitive DNA is ubiquitous in eukaryotic genomes, where it constitutes a substantial part of all genomic DNA, from 8% in fungi to 40-50% of mammalian genomes and up to 80% in some plant species (e.g. whisk fern - *Psilotum nudum*). Repetitive DNA can be composed of short units with only a few nucleotides to several kilobases long elements that are repeated either organized in arrays or scattered throughout the genome. The rDNA clusters are a common example of several megabase-long arrays with more than 100 copies of rDNA genes with several kilobases each arranged in tandem. Major and minor satellite repeats are examples of classes of repetitive DNA with shorter repeat units (120-300 bp) that forms several megabase-long arrays at mouse centromeres (Karpen and Allshire 1997). Invariably, most of these sequences can be found associated with either heterochromatin or some other form of silent chromatin.

With the exceptions of the rDNA arrays, centromeres and telomeres, the function of a large part of the repetitive content found in eukaryotic genomes is not clear. It seems that for the most part these sequences have originated from the cumulative action of transposable elements (TEs) and retroviruses during evolution. Transposable elements are mobile genetic elements that colonise a host genome where they can survive and proliferate (Curcio and Derbyshire 2003). TEs are commonly divided into type I (RNA transposons or retrotransposons) and type II (DNA transposons) (Figure 1-2). The transposition process of retrotransposons requires the expression of a full-length RNA intermediate from which a cDNA copy is synthesised, using an endogenously encoded reverse transcriptase, for subsequent insertion. LTR-retrotransposons, such as the Ty elements in *S. cerevisiae*, IAP (Intracisternal A-Particle) and ERV (Endogenous Retrovirus) elements in vertebrates, are characterized by two flanking tandemly oriented long terminal repeats (LTRs) which accumulate transcription initiation and termination functions with primer binding regions for reverse transcription. Non-LTR retrotransposons, such as the LINE (Long Interspersed Nuclear Element) and SINE (Short Interspersed Nuclear Element) families of elements, rely on an internal promoter rather than flanking repeats to drive transcription events. DNA transposons, such as P, Ac, Tc, Mariner and other elements employing a DDE type transposase use terminal inverted

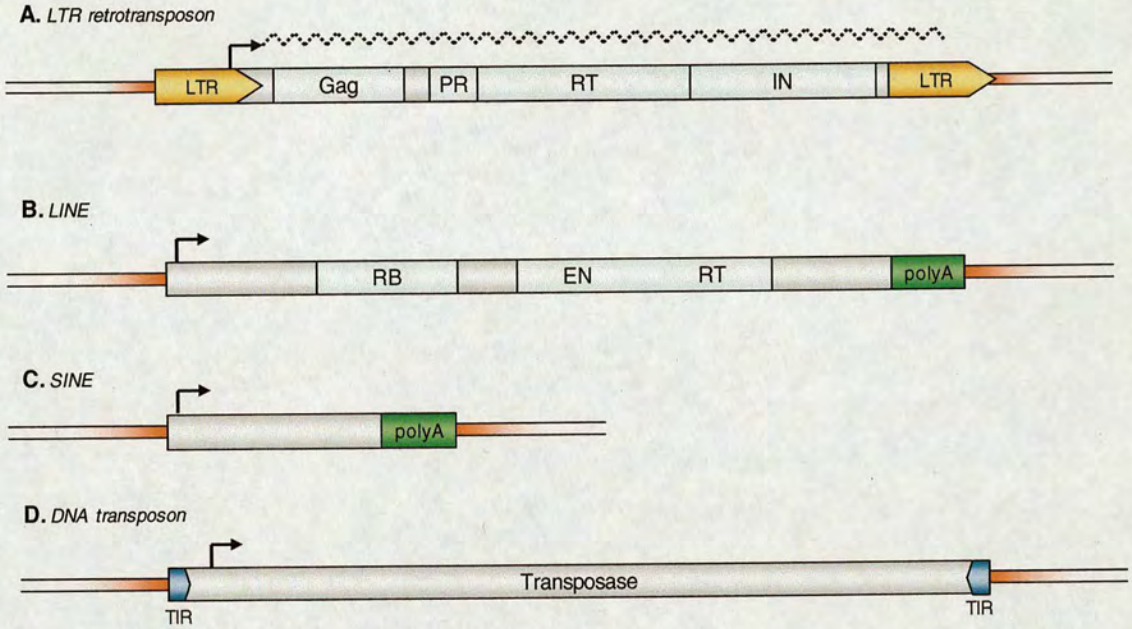


Figure 1-2: Typical organization of the different classes of TEs.

A TE insertion is commonly found flanked by target-site direct repeats (TSDs) of various sizes (red bars). With the exception of SINEs, the TE body (light blue) contain open reading frames for the several endogenously encoded proteins such as Gag, PR (protease), RT (reverse transcriptase), IN (integrase), RB (RNA binder), EN (endonuclease) and transposase.

A. LTR retrotransposons (Type I) derive their name from the two long terminal repeats (LTR– yellow arrow boxes). Transcription of the RNA intermediate (wavy line) is controlled by promoter and terminator elements present in the LTRs.

B. Both SINEs and LINEs commonly posses a 3' poly-A tail (green box). A LINE encodes an RNA chaperone (RB), reverse transcriptase (RT) and endonuclease activity (E) under the control of a RNA polymerase II promoter.

C. SINEs rely on the protein machinery expressed by LINEs in order to transpose. These elements highly abundant in mammalian genomes contain an internal RNA polymerase III promoter and often also transcriptional enhancers.

D. The terminal inverted repeats (TIR – blue arrow boxes) of a DNA transposon (Type II) serve as transposase binding sites. This family TEs highly abundant in plants, flies and nematodes contains a single open reading frame that encodes a transposase.

repeats (TIR) as recognition sites for this protein during the transposition process. Transposition of a type I element is generally described as a "copy-out, paste-in" or "copy-out, copy-in" process through which these TEs can progressively populate a genome in high numbers. Most type II TEs relocate from one locus to another using a "cut-out, paste-in" approach but some can also move by a variation of this mechanism called replicative transposition, through which a new copy of the TE is inserted at a new chromosomal location, leaving the original one intact.

Excessive TE activity may pose a fitness problem for the host. TEs can be powerful genome de-stabilisers for a variety of reasons. Transposition events frequently induce positional mutations at the insertion and vacation sites and extensive TE activity can lead to dramatic chromosomal rearrangements (Lim and Simmons 1994). In addition, TEs are known to distort gene expression patterns and to cause disruption or shuffling of coding sequences (Morgan, Sutherland et al. 1999; Maside, Bartolome et al. 2002; Iwashita, Osada et al. 2003; Han, Szak et al. 2004). Particularly in bacteria, their mobility between genomes allows for horizontal gene transfer which contributes for an increase in the effective gene pool (Frost, Leplae et al. 2005). For these reasons, TEs, despite their parasitic nature, are now recognized as important drivers of evolution of genes and genomes alike (Lim and Simmons 1994; Lev-Maor, Sorek et al. 2003). Secondly, proliferation of TEs is a major contributor to the large increase in genome size seen between species. TEs and retroviruses contain various forms of repeat sequences, which can serve as positional cues and binding sites for many of the components involved in the transposition process (Curcio and Derbyshire 2003). Very frequently, these repeats remain as evidence of a former transposition event or the remnants of degraded transposable elements and they can quickly accumulate in the span of a few generations (Yoder 1990; Curcio and Derbyshire 2003). For example, in *Zea mays*, more than 70% of total genomic DNA is made of TEs or related sequences (Meyers, Tingey et al. 2001). Whilst their purpose intrigued early molecular biologists, the notion that they constitute redundant genomic debris quickly gained support as no biological function could be associated with these sequences. It also became clear that TEs and viral sequences acted in their own interests (Doolittle and Sapienza 1980; Orgel and Crick 1980) which suggested that genomic repeat content arises from the activity of these elements regardless of any useful purpose to the host.

Mechanisms that silence TEs may then have evolved as a means to control the rate of genome evolution and improve the host's fitness by stabilizing both gene pool and genome size. The outcome of the activity of these homology-based pathways can manifest as post-transcriptional silencing, transcriptional silencing, site-directed mutagenesis or DNA elimination.

Heterochromatin is preferentially formed over repetitive sequences in plants and animals (Martienssen and Colot 2001; Smith, Shu et al. 2007). The packaging of TEs into silent chromatin represses their expression and blocks their ability to transpose. In *A. thaliana*, TEs are targeted precisely for DNA cytosine and histone H3K9 methylation mechanisms that lead to the formation of silent chromatin overlying these elements in such a way that does not spread to adjacent DNA regions (Lippman, Gendrel et al. 2004). Metazoan transposable elements are similarly engulfed in heterochromatin but, unlike plants, heterochromatin domains in animal chromosomes are more dynamic and can engulf adjacent regions in a stochastic manner, leading to position-effect variegation (PEV) of nearby genes (Muller 1930). Curiously, it is known from studies in *D. melanogaster* that PEV can be reproduced ectopically on an artificial tandem array of several copies of a *white* gene inside transposable P-elements (Dorer and Henikoff 1994). This PEV-like phenomenon is sensitive to suppressor of variegation mutants such as *Su(var)205* which is a mutation in HP1 in *D. melanogaster*. Subsequent studies further established that the silencing phenomenon observed on these artificial repeat arrays is due to heterochromatin assembly (Martin-Morris, Csink et al. 1997; Fanti, Dorer et al. 1998). Similar observations were made in *Nicotiana tabacum* (tobacco plant) and *A. thaliana* with the insertion of an array with multiple drug resistance genes (Matzke, Primig et al. 1989; Assaad, Tucker et al. 1993). The presence of multiple gene copies in the same locus coincided with a reduction in mRNA levels and increased DNA methylation. Both bodies of evidence underlie a general chromatin silencing model that focuses on repetitive DNA. However, the process of recognizing repetitive DNA is still a matter of intense debate since it seems to be the combinatorial result of multiple mechanisms acting separately (Matzke and Matzke 1995).

The cellular response against repetitive DNA is not restricted to arrays of repetitive sequences but also extends to repeated sequences that are scattered throughout the genome. This latter phenomenon is called "co-supression" and refers to studies made in petunia plants in which the introduction of multiple of copies of an overexpressing flavonoid transgenes (CHS and DFR) into the genome were shown not to result in intensified colour in most of the transformant plants as expected (Napoli, Lemieux et al. 1990; van der Krol, Mur et al. 1990). Instead, the increase the number of transgene copies led to the suppression of the endogenous DFR and CHS gene and the resulting petunia flowers were white or displayed white sectoring. This phenotype is reversible and the flowers can regress to wild-type colour provided that the gene copy number is decreased. Whilst PEV in *D. melanogaster* is the manifestation of a local repressive influence (*cis*), this

mechanism of transgene silencing acts in *trans* since it can downregulate multiple genes in several independent genetic loci simultaneously. At the same time, similar phenomena were being described in numerous plants species, the filamentous fungus *Neurospora crassa* and the nematode *Caenorhabditis elegans* (Cogoni, Irelan et al. 1996; Gaudet, VanderElst et al. 1996). It has since become clear that that the phenomenon of co-suppression is common to many eukaryotic organisms and is the result of several mechanisms acting against multi-copy DNA species in the genome. Namely, it was shown that co-suppression in plants could be enforced in both at the level of chromatin by DNA methylation (transcriptional gene silencing, TGS) (Van Blokland, Van der Geest et al. 1994) and at the level of mRNA stability (post-transcriptional gene silencing, PTGS) (Van Blokland, Van der Geest et al. 1994; Matzke and Matzke 1995). Whilst it was obviously dependent on sequence homology and copy number, the molecular identity of the trigger for transgene silencing was unclear and several mechanisms were suggested, including homology-based DNA-DNA interactions that could lead to a chromatin-based repression. Eventually, it was revealed that a similar mechanism to transgene silencing could be directed by RNA (Metzlaff, O'Dell et al. 1997). Research conducted in parallel in *N. crassa*, *C. elegans*, *Chlamydomonas reinhardtii* and *A. thaliana* pointed to the involvement of RNA-associated factors in the mechanism of transgene silencing (Wassenegger and Pelissier 1998; Cogoni and Macino 1999; Cogoni and Macino 1999; Dalmay, Hamilton et al. 2000; Wu-Scharf, Jeong et al. 2000). Introduction of dsRNA homologous to a reporter gene or expression of an antisense construct was shown to induce reporter silencing (Fire, Xu et al. 1998; Ngo, Tschudi et al. 1998; Waterhouse, Graham et al. 1998; Jensen, Gassama et al. 1999). The resulting method of silencing depends on the homology region: PTGS is enforced when the coding region is targeted and TGS is established when the homology lies in the promoter area, leading to DNA methylation at the promoter (Wassenegger, Heimes et al. 1994; Jones, Hamilton et al. 1999; Mette, Aufsatz et al. 2000).

Research on mechanisms behind transgene silencing continued to converge and eventually led to the discovery of an RNA-based mechanism that underlies several of these phenomena of repetitive DNA repression. Research conducted in *C. elegans* on the metazoan counterpart of this mechanism led to the description of the molecular details of what is now labelled as "RNA interference" (Fire, Xu et al. 1998). The insights gathered from that work and subsequent biochemical analyses of the functioning principle behind RNA interference unveiled an ancient pathway present in numerous eukaryotic organisms.

1.6. RNA SILENCING

"RNA interference" is the term ascribed to a post-transcriptional silencing mechanism first described in *Caenorhabditis elegans* (Fire, Xu et al. 1998; Ketting, Haverkamp et al. 1999). The basic machinery responsible for this phenomenon is identical in PTGS and TGS mechanisms discovered in plants (post-transcriptional and transcriptional gene silencing – PTGS and TGS - (Matzke, Primig et al. 1989; Napoli, Lemieux et al. 1990; van der Krol, Mur et al. 1990; Metzlaff, O'Dell et al. 1997), filamentous fungi (quelling) (Cogoni, Irelan et al. 1996) and in numerous other species. These closely related silencing phenomena are jointly referred to as "RNA silencing".

In all its manifestations, RNA silencing relies on 21-28 nt small RNA molecules to induce repression in a sequence specific manner (Hamilton and Baulcombe 1999; Hammond, Bernstein et al. 2000; Zamore, Tuschl et al. 2000; Elbashir, Harborth et al. 2001; Elbashir, Lendeckel et al. 2001; Elbashir, Martinez et al. 2001; Schwarz, Hutvagner et al. 2002). The pathway also employs the activity of three major components: Dicer, Argonaute and RNA-dependent RNA polymerase (RdRP) (Bohmert, Camus et al. 1998; Cogoni and Macino 1999; Tabara, Sarkissian et al. 1999; Bernstein, Caudy et al. 2001). The small RNA molecules, called small interfering RNAs (siRNAs), are generated from double-stranded precursor RNAs by the DEAD helicase/RNase-like III enzymes such as Dicer through cleavage into small duplexes (Bernstein, Caudy et al. 2001). These are then unwound and loaded into a multi-protein complex named RISC (for RNA-induced Silencing Complex) containing a PAZ/PIWI domain protein (or Argonaute) and several other additional factors (Hammond, Bernstein et al. 2000; Zamore, Tuschl et al. 2000). The interaction of the siRNA-loaded RISC targets a complementary sequence to the siRNA on which to exert repression (Figure 1-3).

The Argonaute family of proteins plays a central role in all the manifestations of RNA silencing found in both lower and higher eukaryotes. Argonautes can be found in all the different eukaryotic kingdoms as well as in Archaea and Eubacteria. Within RISC and related complexes, these proteins containing PAZ and PIWI domains are responsible for holding the small RNA molecule and overseeing its interaction with a target RNA (Hammond, Bernstein et al. 2000). Both the PAZ and PIWI domains can directly interact with RNA molecules regardless of their sequence. The PAZ domain binds to the 5' end of the siRNA molecule probably to facilitate the siRNA loading process (Song, Liu et al. 2003). The PIWI domain forms a specialized cleft where most of the siRNA

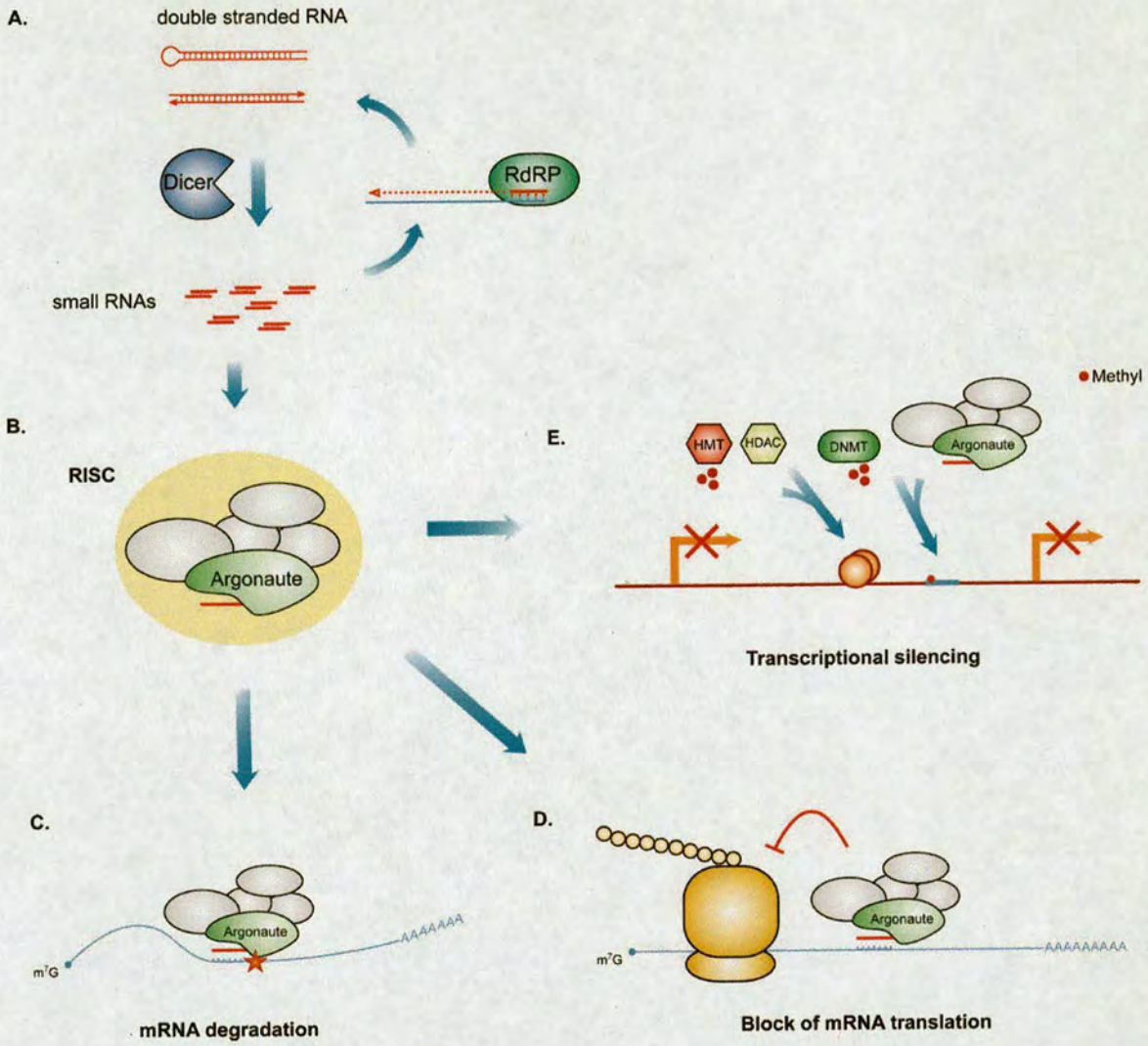


Figure 1-3: Basic mechanism of RNA silencing and its different forms of repression (from Almeida and Allshire 2005)

A. The pathway is triggered by double stranded RNA complementary RNA molecules or a single RNA hairpin or stem loop. DsRNA is processed by Dicer, into small duplex RNAs (21-28 nt). The RNA-dependent RNA polymerase (RdRP) generates more dsRNA from target or "aberrant" RNA.

B. The small RNAs are loaded into Argonaute in RISC (RNA-induced Silencing Complex). RISC uses the small RNA to a target RNA with high specificityto enforce silencing.

C. Argonaute can promote the degradation of the target RNA. This method is predominant in RNA interference and gene regulation by microRNAs.

D. Binding of Argonaute loaded with a microRNA to the 3' end of an mRNA can block its translation by the ribosome. **E.** Argonaute complexes can recruit chromatin modifiers such as histone deacetylases (HDACs), histone methyltransferases (HMTs) and *de novo* DNA methyltransferases (DMTs) to repress transcription on target DNA loci.

molecule resides and meets the opposite strand of the target molecule (Parker, Roe et al. 2004; Song, Smith et al. 2004). It also contains an RNase H-like fold that confers endonucleolytic activity to some members of the Argonaute family. This nuclease activity is called "slicing" and can manifest itself as a precise nick on the target strand within a siRNA-mRNA duplex (Hammond, Bernstein et al. 2000; Zamore, Tuschl et al. 2000; Lingel and Izaurralde 2004). This means that the minimal RISC activity resides in Argonaute's ability to perform the roles of mediator and effector in small RNA-mediated silencing (Liu, Carmell et al. 2004; Baumberger and Baulcombe 2005; Miyoshi, Tsukumo et al. 2005; Rivas, Tolia et al. 2005) (Figure 1-3C).

RdRP (for RNA-dependent RNA polymerase) amplifies the RNA silencing response by producing complementary strands of an single-stranded RNA molecule, giving rise to more dsRNA and subsequently more siRNAs (Cogoni and Macino 1999; Dalmay, Hamilton et al. 2000; Mourrain, Beclin et al. 2000; Smardon, Spoerke et al. 2000; Sijen, Fleenor et al. 2001). RdRP can perform this function in several different ways. SiRNAs can be used as primers for homing in on specific transcripts, thus enabling RdRP to provide positive feedback of existing siRNA signal (Lipardi, Wei et al. 2001; Nykanen, Haley et al. 2001). More recently, it has been proposed that recruitment of RdRP by RISC to an RNA molecule is enough to engage the RdRP in making short complementary transcripts in an unprimed manner (Sijen, Steiner et al. 2007). RdRP has been observed to also act in a primer-independent matter upon single-stranded transcripts that bear an hypothetical "aberrant" characteristic – this is particularly relevant in the context of antiviral defence in plants as it is believed that plant RdRPs contribute to the RNA silencing response against invading viral RNAs (Wassenegger and Pelissier 1998; Mourrain, Beclin et al. 2000; Xie, Fan et al. 2001; Makeyev and Bamford 2002). Although it is a key component of the RNA silencing pathway in plants, nematodes and fungi, no orthologs of RdRP have been identified in insects or vertebrates, despite evidence of its biochemical activity from *D. melanogaster* extracts (Palauqui, Elmayan et al. 1997; Lipardi, Wei et al. 2001; Vaistij, Jones et al. 2002).

RISC and related complexes also include multiple additional factors which can be species specific or relate to a single derivative of RNA silencing. The *D. melanogaster* proteins FXR (Fragile X-related protein), VIG (vasa intronic gene) and Tudor-SN (Tudor-streptococcal nuclease domain) are constituents of RISC and have known orthologs in several other organisms (Fagard, Boutet et al.

2000; Hammond, Bernstein et al. 2000; Caudy, Myers et al. 2002; Hannon 2002). TRBP (human immunodeficiency virus transactivating response RNA-binding protein) is a component of human RISC that is implicated in maturation and loading of siRNAs onto Argonaute (Chendrimada, Gregory et al. 2005; Haase, Jaskiewicz et al. 2005). The same function is performed in *C. elegans* by RDE-4 (Tabara, Yigit et al. 2002) and in *D. melanogaster* by R2D2 and Loquacious (Liu, Rand et al. 2003; Forstemann, Tomari et al. 2005; Saito, Ishizuka et al. 2005). RISC and derivatives often contain GW repeat proteins such as human GW182, *Arabidopsis* NRPD1b, *C. elegans* AIN-1 and *S. pombe* Tas3 (Verdel, Jia et al. 2004; Ding, Spencer et al. 2005; Pontier, Yahubyan et al. 2005; Rehwinkel, Behm-Ansmant et al. 2005; Pontes, Li et al. 2006; El-Shami, Pontier et al. 2007). Whilst these proteins may share common functionalities that assist Argonaute in its activity, it is likely that they reflect the specialization of the Argonaute-containing complexes on the various forms of RNA silencing.

There are three distinct methods by which small RNA-driven repression can occur. Conventional RNA interference results in cleavage of homologous mRNA molecules at the siRNA binding site by means of the endonuclease ("slicing") activity of Argonaute (Song, Smith et al. 2004) (Figure 1-3C). The activity of RISC is coupled to other mechanisms responsible for RNA turnover. More specifically, RISC localises to cytoplasmic GW/P bodies (Ding, Spencer et al. 2005; Jakymiw, Lian et al. 2005; Liu, Rivas et al. 2005; Liu, Valencia-Sanchez et al. 2005; Meister, Landthaler et al. 2005; Sen and Blau 2005). In these specialised sites for mRNA storage and turnover, the sliced mRNAs are degraded by a combination of 5'-3' exonuclease XRN2 and the 3'-5' exonuclease "exosome" complex (Orban and Izaurralde 2005; Eulalio, Behm-Ansmant et al. 2007). Slicing provides a form of circumventing the mRNA's natural protection against exonuclease degradation, namely the 5' end m^7G cap and 3' poly(A) tail. Argonautes can also trigger mRNA turnover by recruiting de-adenylating and decapping enzymes to the mRNA at GW/P bodies, leading to its degradation even in the absence of slicing (Behm-Ansmant, Rehwinkel et al. 2006; Giraldez, Mishima et al. 2006; Wu, Fan et al. 2006).

In addition to mRNA turnover, short RNAs can drive RISC-like complexes to inhibit translation of homologous mRNAs, effectively causing repression without affecting their stability (Figure 1-3D). This silencing method was first observed in the mechanism of microRNA (miRNA) regulation (Reinhart, Slack et al. 2000). MicroRNAs are a particular class of small RNA molecules found in multi-cellular eukaryotes that precisely regulate expression timing of a large number of genes, particularly those involved in cellular differentiation and developmental processes (Ambros,

Lee et al. 2003; Ambros 2004; Dugas and Bartel 2004; John, Enright et al. 2004). MicroRNAs are produced from specialized genetic elements that when transcribed originate stem loop RNAs which are recognized by a specialized machinery involving Dicer but is otherwise independent of RNAi/PTGS (Grishok, Pasquinelli et al. 2001; Lau, Lim et al. 2001; Lee and Ambros 2001). This processing mechanism leads to the precise release of a short RNA molecule identical to an siRNA that is loaded into a specialised form of RISC called the miRNP (microRNA protein complex) that contains specific Argonaute proteins (Mourelatos, Dostie et al. 2002). In plants, miRNAs target the coding region of genes and enforce post-transcriptional repression mostly by mRNA slicing in a manner similar to RNAi/PTGS. However the majority of known miRNAs in metazoa blocks the translation of mRNA targets by binding to conserved sites on their 3' untranslated regions. This is due to the fact that metazoan miRNAs are often imperfect matches with their targets (Reinhart, Slack et al. 2000). Consequently, miRNA-mRNA pairings contain mismatches or bubbles which can interfere with efficient mRNA slicing. Recently, it was suggested that *C. elegans* Ago2 enforces miRNA-mediated translational inhibition by binding to the m^7G cap of mRNAs through a newly identified motif and in the process precluding the recruitment of eIF4E, an essential translation initiation factor (Kiriakidou, Tan et al. 2007). In humans, miRNP is bound to eIF6, a conserved 60S ribosome-associated factor that is known to prevent the assembly of the 80S translationally competent ribosome (Chendrimada, Finn et al. 2007). This factor may be involved in effecting miRNP-mediated translational arrest, as it was shown that the depletion of eIF6 allowed the translation of several miRNA targets in both human and *C. elegans*.

Transcriptional Gene Silencing

The third form of repression performed by RISC-like complexes involves directing chromatin and DNA modifications upon complementary DNA loci (Figure 1-3E). This form of siRNA-mediated repression was initially noticed in plants where, together with PTGS, participates in a co-suppression response that is involved in defence against viral invasion (Wassenegger, Heimes et al. 1994; Metzlaff, O'Dell et al. 1997). Further analyses demonstrated that the same small RNA-based mechanism behind PTGS could induce chromatin modifications over a DNA locus when prompted by the introduction of complementary double-stranded RNA (Mette, Aufsatz et al. 2000). These chromatin modifications include dense DNA cytosine methylation that is coupled with

transcriptional silencing, particularly if the introduced RNA is targeting promoter regions (Jones, Hamilton et al. 1999). The same basic machinery, namely small RNAs, Dicer-like and Argonaute genes, is required for this phenomenon, which illustrates the direct link with RNA silencing (Lippman, May et al. 2003). However, upon removal of dsRNA stimulus, the chromatin modifications are found to be stable in subsequent generations (Jones, Ratcliff et al. 2001). Silencing by TGS is more stable since it relies on a set of epigenetic marks that possess additional conservation mechanisms, such as the mammalian Dnmt1 and plant MET1 maintenance DNA methyltransferases (Bestor, Laudano et al. 1988; Finnegan, Brettell et al. 1993). Therefore, RNA silencing through TGS has the potential to stably imprint gene repression, which suggests that it may participate in gene regulation events during development and/or cell differentiation. On the other hand, its capacity to direct the assembly of stable chromatin brings up the possibility that RNA silencing may be involved in the origin of constitutive heterochromatin domains, such as the centromere and telomeres.

In fission yeast, the RNA interference pathway does contribute to the formation and stability of constitutive heterochromatin domains at centromeres, mating type locus and subtelomeric chromatin. In this organism, the core RNA silencing components, Argonaute, Dicer and RdRP, are present in single copy and react to non-coding transcripts originating from repetitive DNA at these loci by producing complementary siRNAs (Reinhart and Bartel 2002). Disruption of the RNA silencing pathway not only stabilizes these non-coding transcripts but also leads to a reversion of the heterochromatin state that affects all of the constitutive heterochromatin domains to different extents (Volpe, Kidner et al. 2002). In the absence of RNAi, repression of genes placed at heterochromatin domains is alleviated and heterochromatin signatures such as histone H3K9 di-methylation and Swi6^{HP1} binding are reduced, particularly at centromeres. In addition, it has been shown that fission yeast RNAi also targets TE-related repetitive DNA for establishment of heterochromatin, leading to transcriptional repression of both TE-related sequences and of genes located in the vicinity (Schramke and Allshire 2003). These observations suggest that TGS in fission yeast has a role in regulation of gene expression.

Does siRNA-directed heterochromatin nucleation occur in other organisms apart from plants and fission yeast? There is considerable evidence suggesting a link between RNA silencing and chromatin modifications in metazoa. However, until now no mechanism for heterochromatin nucleation directed by small RNAs has been clearly described in these organisms. Most of the

collected evidence refers to specific a branch of the Argonaute protein family, the PIWI proteins. Recent developments have unveiled further connections between these proteins and chromatin modifications in ciliates and metazoa.

Members of the Argonaute family can be classified into two groups according to sequence similarity to either *A. thaliana* AGO1 or PIWI from *D. melanogaster* (Seto, Kingston et al. 2007). The former group includes all plant Argonautes, *S. pombe* Ago1 and most Argonautes that participate in siRNA- and miRNA-mediated silencing in other organisms. The PIWI proteins are predominantly found in ciliates, flies and vertebrates. PIWI proteins are associated with a particular class of small RNAs called piRNAs (for piwi-associated RNAs) which are longer than normal siRNAs (24-27 nucleotides as opposed to 21-22) and much less abundant than other classes of small RNAs, such as siRNAs and miRNAs (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam et al. 2006; Grivna, Beyret et al. 2006; Lau, Seto et al. 2006; Saito, Nishida et al. 2006; Vagin, Sigova et al. 2006). The activity of PIWI proteins and piRNAs appears to be closely connected to both invasive DNA and chromatin modifications. The mechanism of DNA elimination employed by the ciliate *Tetrahymena termophila* is one of the most well characterised examples of such activity (Mochizuki and Gorovsky 2004). *Tetrahymena* possesses two distinct nuclei, the diploid micronucleus and the polyploid, transcriptionally active macronucleus. During micronuclei conjugation, the development of the new macronucleus is accompanied by a maturation process characterized by extensive DNA elimination. Roughly 15% of the original DNA content is epigenetically programmed for deletion by histone H3K9 methylation (Taverna, Coyne et al. 2002). The recognition of these internally eliminated sequences (IES) is linked to Twi1p, a PIWI protein, and ~28nt small RNA molecules (Mochizuki, Fine et al. 2002). It was demonstrated that these small RNAs constitute the basis of a scanning system for micronuclear sequences unrepresented in the old macronucleus that programs these for elimination from the developmental macronucleus (Mochizuki and Gorovsky 2004; Mochizuki and Gorovsky 2004; Mochizuki and Gorovsky 2005).

In animals, PIWI proteins play crucial roles in pre-meiotic cells and are essential for germline maintenance (Cox, Chao et al. 1998; Carmell, Xuan et al. 2002; Cheng, He et al. 2002; Kuramochi-Miyagawa, Kimura et al. 2004; Carmell, Girard et al. 2007). In *D. melanogaster*, a large percentage of these piRNAs are specific to repetitive DNA, TEs and heterochromatin regions (Brennecke, Aravin et al. 2007). Null mutations on PIWI genes such as *aubergine* and *piwi* lead to increased expression of TEs, which supports the notion that this class of proteins is primarily

devoted to controlling TE proliferation (Brennecke, Aravin et al. 2007). TEs are particularly active during meiosis and their activity if uncontrolled may lead to meiotic collapse due to chromosome loss (Bourc'his and Bestor 2004). This provides an explanation for why PIWI proteins are required for the viability of germ cells. Whereas PIWI have a clear role in post-transcriptional repression of these elements, *piwi* mutants also have a mild effect on the localisation patterns of heterochromatin proteins 1a and 1b (HP1a and HP1b) (Pal-Bhadra, Leibovitch et al. 2004). It was recently reported that PIWI binds to HP1a and co-localise at some heterochromatic loci (Brower-Toland, Findley et al. 2007). Mutations within the domain within the PIWI protein that is responsible for this interaction abolishes silencing of a marker gene inserted in peri-centromeric heterochromatin and cannot rescue the loss of viability of a null *piwi* homozygote. There is also a described link between PIWI proteins and Polycomb family proteins in the co-suppression of multiple copies of *w-Adh* reporter genes (Pal-Bhadra, Bhadra et al. 1997; Pal-Bhadra, Bhadra et al. 2002). In mammals, mutations in Miwi2 and Mili proteins lead to loss of DNA methylation over TEs such as L1 (LINE-1) elements and subsequent increase TE expression (Aravin, Sachidanandam et al. 2007; Carmell, Girard et al. 2007). Given that the cell types in which PIWI proteins have a stronger presence undergo extensive epigenetic reprogramming, it has been suggested that PIWI and piRNAs contribute to this process. However, the mechanism of such involvement is still unclear and, for the moment, the association of this aspect of RNA silencing with chromatin modifications remains circumstantial.

A separate line of evidence suggests that Dicer is necessary for the correct function of centromeres in vertebrate cells (Fukagawa, Nogami et al. 2004). The requirement of Dicer for centromeric function may be linked to the stability of heterochromatin over pericentric repeat arrays, which in mouse embryonic stem cells was also shown to be Dicer-dependent (Kanellopoulou, Muljo et al. 2005). Furthermore, two recent studies demonstrated that siRNAs can direct site-specific DNA methylation in human cells (Morris, Chan et al. 2004), which serves as a proof of principle that RNA silencing can enforce transcriptional silencing in a similar fashion to plants but provides no insights into possible endogenous targets of siRNA-mediated transcriptional silencing in metazoa. Nevertheless, it is likely that RNA silencing maintains a semblance of its involvement in chromatin silencing found in plants and *S. pombe* and may still be linked to pericentric repeats and centromere function in a similar fashion.

1.7. THE CENTROMERE

Although repeats are widespread genomic features, they are found in higher numbers concentrated in specific regions of the chromosome. Arguably the largest of these regions is the centromere, where it was demonstrated that repetitive DNA plays an important role in its function. Centromeres are crucial for the assembly of the kinetochore, the machinery that attaches chromatids to the mitotic spindle (Cleveland, Mao et al. 2003). This multi-protein complex is responsible for organizing chromosomes on the metaphase plate and ensuring proper chromosome segregation during the cell cycle. The function of the centromere is crucial for the accurate passage of identical copies of genomic DNA to newly formed cells during division. If this process is disturbed, defective chromosome segregation ensues and may lead to unequal separation of chromosomes (aneuploidy) which is associated with some human diseases (Nicolaidis and Petersen 1998; Sen 2000).

The centromere itself is a cytologically visible feature of the chromosome that is distinct both at the level of DNA sequence and portfolio of associated proteins. Cytologically, it defines the primary constriction visible on condensed chromosomes (Flemming 1880). Centromeric chromatin is rich in specific proteins such as the histone H3 variant CENP-A, that defines the kinetochore assembly site (Van Hooser, Ouspenski et al. 2001), and factors like CENP-B proteins that associate with repeat DNA and contribute to the structure of pericentric chromatin (Masumoto, Masukata et al. 1989; Pluta, Mackay et al. 1995; Nakagawa, Lee et al. 2002). The kinetochore protein complex assembles at centromeres and provides physical attachment of the chromosome to the mitotic spindle. The underlying functional principle of centromeres is both essential and conserved throughout eukaryotes. Nevertheless, centromeres are found in a surprising variety in sizes, sequence and other structural features throughout eukaryotic organisms (Figure 1-4).

The simplest known centromeres are found in *Saccharomyces cerevisiae* (budding yeast) whose minimal centromeric sequence occupies only 125 bp (Cottarel, Shero et al. 1989; Cheeseman, Drubin et al. 2002) (Figure 1-4A). These well characterized centromeres contains 3 conserved regions (CDE I, II and III) that define the site of kinetochore attachment within a 220-250bp DNA segment that is protected from nuclease cleavage and is contained within a highly organized array of nucleosomes (Clarke 1998; Cleveland, Mao et al. 2003). The CDE elements are

common to all *S. cerevisiae* 16 centromeres. CDEI is a short sequence (8 bp) that serves as binding site for centromere binding factor 1, or Cbf1p (Mellor, Jiang et al. 1990). CDEII is an 78 bp AT-rich sequence that acts as a spacer between CDEI and CDEIII. As the only essential element of this centromere, CDEIII is an 80 bp AT-rich sequence that marks the deposition site for a single nucleosome containing Cse4p, the budding yeast CENP-A homolog. This "point centromere" precisely determines the site of kinetochore assembly and is required for faithful chromosome transmission. The core centromere, containing the three CDE elements, is flanked by DNA regions which are hyper-sensitive to DNAse I digestion whilst the core sequence itself is impervious to endonucleases provided that a functional kinetochore is in place (Saunders, Yeh et al. 1990). Even though the budding yeast centromere differs from other eukaryotes in terms of relying heavily on cis-acting sequences, the overlying kinetochore machinery is still very similar. The CBF3 complex, with its components Ndc10p, Cep3p, Ctf13p, Skp1p and Sgt1p, assembles over CDEIII and forms much of the inner kinetochore (Kitagawa, Masumoto et al. 1995; Kaplan, Hyman et al. 1997). A group of three other centromeric proteins, Ctf19p, Mcm21p and Okp1p, link the CBF3 complex with the remaining centromeric components, such as Cbf1p, Mif2 and Cse4p (Ortiz, Stemmann et al. 1999). Mif2 is distant homolog of the conserved kinetochore protein CENP-C (centromere protein C) that also binds to these centromeres (Meluh and Koshland 1997). Bir1p is part of the Cut17/Survivin family of chromosomal passenger proteins associated with Aurora kinase activity that binds to Ndc10 at the inner kinetochore (Yoon and Carbon 1999). In turn, the Aurora kinase homolog Ipl1p interacts with Sli15p, the homolog of the passenger protein INCENP. Ipl1p and Sli15p localize together to centromeres and were shown to also bind microtubules *in vitro* (Kang, Cheeseman et al. 2001). Proteins associated with the Spindle Pole Body, namely Ndc80p, Nuf2p, Spc24p and Spc25, were also shown to bind centromeres *in vivo* (Wigge and Kilmartin 2001). All these proteins are present in fission yeast while Ndc80 and Nuf2 are known to have homologs in humans. In addition, Slk19, Mtw1 and Dam1 provide additional links between the centromere and the mitotic spindle by binding to both the kinetochore and spindle microtubules (Jones, He et al. 2001). In contrast with *S. cerevisiae*, centromeres from other organisms such as fission yeast, plants and metazoa tend to consist of much larger regions of AT-rich and characteristically low complexity DNA. Furthermore, the identity and function of centromeres in those organisms is less restricted to *cis*-acting DNA elements when compared to budding yeast. The only characterized centromere in *Drosophila melanogaster* was found in the mini chromosome *Dp1187* that derives from the X

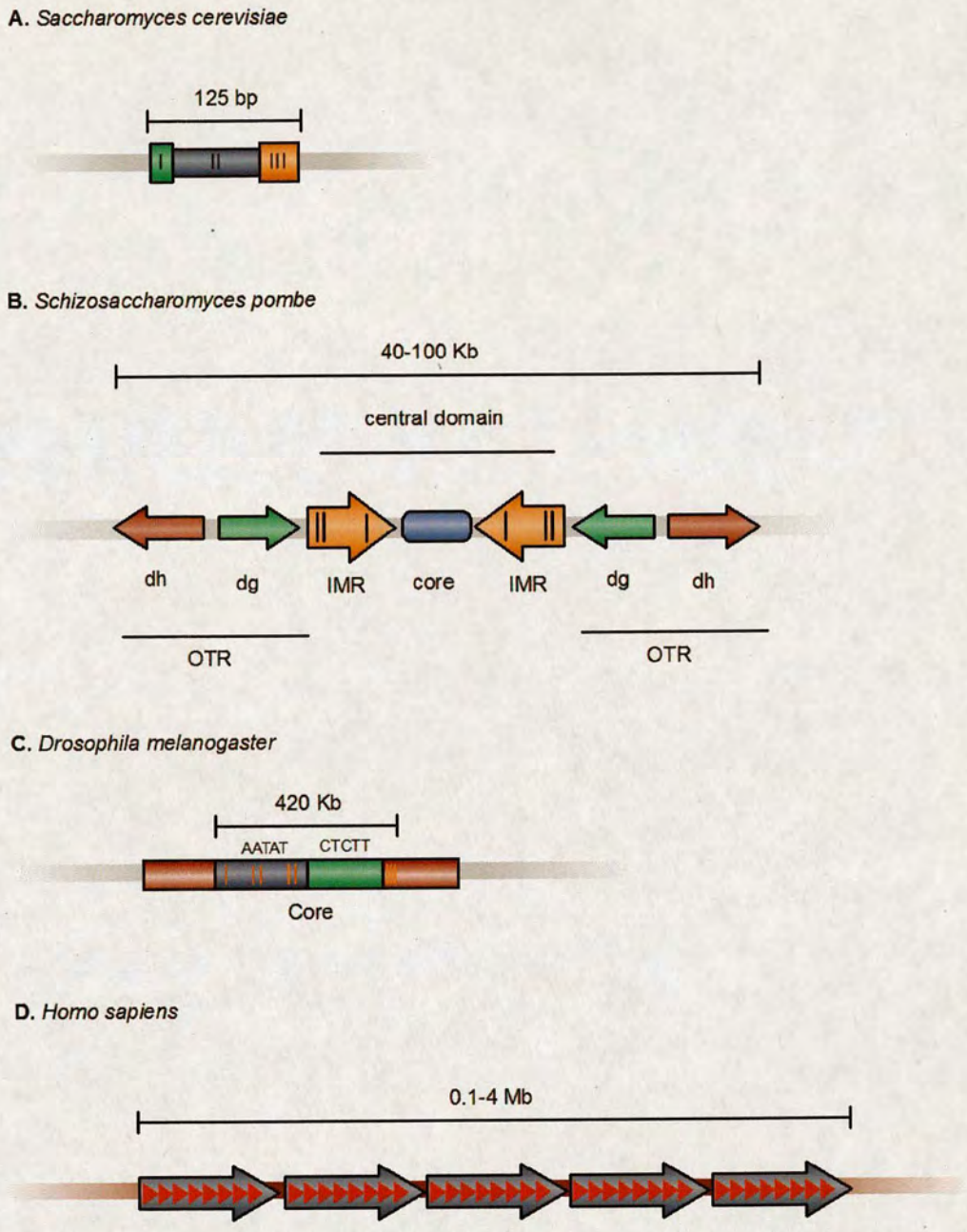


Figure 1-4: Examples of known centromeres.

A. The 125bp-long minimal centromere in budding yeast is composed by the CDE I, II and III boxes. The *S. cerevisiae* homolog of CENP-A Cse4 is found in the nucleosomes overlapping the CDE II box (in blue).

B. In *S. pombe*, the kinetochore assembles over the central domain, which consists of a non-repetitive central core (blue box) surrounded by the innermost repeats (*imr*; yellow box arrows). The outer repeats (*otr*; red and green box arrows) flank the central domain and are bound in heterochromatin.

C. The only defined centromere in fruit flies belongs to the minichromosome Dp1187. It consists of two arrays of 5bp repeats (blue and green boxes) interrupted by TEs (yellow lines). Cid^{CENP-A} is found over this core region which is surrounded by other repetitive DNA bound in heterochromatic (in red).

D. The human centromere consists of multiple tandem arrays (blue box arrows) of 120bp α-satellite repeats (red triangles). It is surrounded by extensive arrays of pericentric repeats bound in heterochromatin (red line). Assembly of the kinetochore occurs over a portion of the alphoid arrays.

chromosome (Figure 1-4C). Its core sequence is defined by a 220 Kb region made up of two arrays of 5 bp-long satellite repeats and several transposable elements that is surrounded by other repetitive DNA (Murphy and Karpen 1995; Sun, Wahlstrom et al. 1997). The AATAT and AAGAG satellites that comprise most of this centromeric DNA are also found elsewhere in the fruit fly genome, albeit in regions not associated with centromere function (Sun, Wahlstrom et al. 1997). Thus, it is unlikely that these repeats are sufficient to promote kinetochore assembly. The AAGAG block is thought to include the sites for kinetochore binding since the presence of these satellites is essential for centromere function. In contrast, deletions of AATAT repeats only have a mild effect on centromere activity, suggesting that these sequence act in more of a supportive role (Murphy and Karpen 1995). CENP-A^{Cid} occurs in these centromeres in a discontinuous fashion, forming small domains which are intercalated with nucleosomes containing canonical histone H3. The three-dimensional organization of this stretch of staggered chromatin joins CENP-A^{Cid} and H3 regions into two separate higher order domains that may have distinct functional properties (Blower, Sullivan et al. 2002).

The chromosomes of animals such as *Caenorhabditis elegans* and plants like *Luzula nivea* are distinct in that they are holocentric which means that they do not present a primary constriction. Instead of forming at one locus, the kinetochores are assembled diffusedly throughout the entire length of the chromosome (Maddox, Oegema et al. 2004). Consequently, spindle microtubules are attached to the chromosomes at multiple sites along the chromosome arms. Very little is known of the DNA sequences over which these diffuse centromeres function or of the mechanisms that control deposition of CENP-A. Unlike the majority of organisms, *C. elegans* does not require CENP-A^{HCP-3} to efficiently segregate chromosomes during meiosis and thus can assemble kinetochores in a more flexible fashion that does not require binding to specialized nucleosomes (Monen, Maddox et al. 2005). Thus, holocentric chromosomes may have evolved out of the requirement of a domain with special DNA sequences and nucleosomes for assembling a functional kinetochore.

Mammalian centromeres cover vast regions of DNA and are considerably longer than fruit fly centromeres. Mouse (*Mus musculus*) kinetochores assemble over uninterrupted tandem arrays of major (234 bp long) and minor (120 bp long) satellite repeats that can contain approximately 2500 copies. Similarly, the core region of human centromeres is composed of repeat arrays in which the basic unit is a 171 bp-long element called α-satellite. These are organized in high order

arrays of tandem α-satellite that can span a region from 100 Kb to several megabases (Figure 1-4D). Interspersed LINEs and Alu repeats are also found within the alphoid arrays and on surrounding pericentric regions, together with other types of satellite repeats (Willard 1998). Like in *D. melanogaster*, the human centromere is not fully occupied by CENP-A nucleosomes. These are organized into domains interspersed by histone H3 nucleosomes and only occupy between half to two thirds of the entire centromere (Warburton, Cooke et al. 1997; Blower, Sullivan et al. 2002; Lam, Boivin et al. 2006). The α-satellites are only found in primates and contain 17 bp long motifs that serve as recognition sites for the DNA binding centromeric protein B (CENP-B) (Masumoto, Masukata et al. 1989).This protein is thought to play a role in higher order structure of centromeric chromatin . Alphoid repeat arrays containing CENP-B binding motifs appear to be sufficient to determine centromere identity since it recruits the assembly of a functional kinetochore within a human artificial chromosome (HAC) when it is introduced into the cells as naked DNA (Ohzeki, Nakano et al. 2002).

Centromeres in plants share the same underlying organization principle with metazoan centromeres. Their activity is dependent on the CENP-A homolog CENH3 and they assemble over vast arrays of 180 bp repeats (Murata, Ogura et al. 1994; Round, Flowers et al. 1997; Copenhaver, Nickel et al. 1999; Heslop-Harrison, Murata et al. 1999). The size of the repeat arrays at plant centromeres is highly variable such as in *Oryza sativa* (rice) where centromeric arrays vary between 60 kb to 1.9 Mb in different chromosomes (Ananiev, Phillips et al. 1998; Cheng, Dong et al. 2002; Jin, Melo et al. 2004; Kato, Lamb et al. 2004). These arrays are also associated with rDNA repeat arrays and numerous TE, which are known to possess heterochromatin (Cheng, Dong et al. 2002). Similarly to other organisms, the plant centromeric repeat arrays recruit deposition of $CENH3^{CENP-A}$ (Nagaki, Cheng et al. 2004; Nagaki and Murata 2005).

In most of the cases portrayed above, the issue of centromere identity is not straightforward. Although the function of centromeres is conserved in all eukaryotic cells, the sequence composition and arrangement of centromeres are surprisingly variable, even between chromosomes of the same organism (Karpen and Allshire 1997). In the case of the human centromere, a large proportion of the alphoid arrays are redundant for kinetochore assembly and normal segregation (Wevrick, Earnshaw et al. 1990; Yang, Pendon et al. 2000). For instance, the fusion of human chromosomes 13 and 14 generates di-centric chromosomes in which one of the centromeres is active and the other is disabled. Within a cell population, both centromeres can be

found associated with a functional kinetochore, pointing towards a mechanism of centromere activation/inactivation that is stochastic and not strictly enforced by the presence of centromeric repeats. Whilst it is clear that centromeres are associated with repetitive DNA elements, centromere activity is not rigorously linked to these sequences. The complete removal of the entire native centromere from a chromosome may lead to the assembly of a functional kinetochore over a region that does not contain any centromeric sequences. These "neocentromeres" are known to occur in *D. melanogaster* and *H. sapiens* (Depinet, Zackowski et al. 1997; du Sart, Cancilla et al. 1997; Warburton and Cooke 1997; Williams, Murphy et al. 1998; Lo, Craig et al. 2001; Maggert and Karpen 2001). Neocentromeres have been reported to form over DNA loci displaying arrays of tandem arrays of AT-rich repetitive sequences, similar in characteristics to centromeric repeats, thus suggesting a link between the rare event of neocentromere formation and chromosomal regions that display similar features to centromeric DNA (du Sart, Cancilla et al. 1997). Despite the lack of underlying centromeric DNA, neocentromeres are faithfully propagated in subsequent divisions (Murphy and Karpen 1995; du Sart, Cancilla et al. 1997). Altogether, this evidence suggests that the identity of a centromere in higher eukaryotes is facilitated but not exclusively defined by primary DNA sequences like in *S. cerevisiae*. Instead, the centromere appears to rely on a particular epigenetic signature (Choo 1997; Karpen and Allshire 1997; Warburton and Kipling 1997).

Even though the sequence of centromeres is distinct between organisms and even between chromosomes within the same cell, the overlying organization of centromeric chromatin shares similar characteristics amongst most of the known centromeres. These underlying characteristics are epigenetic in the form of particular context of chromatin modifications that are propagated alongside functioning centromeres. With the exception of budding yeast and holocentric chromosomes, centromeric chromatin is comprised of a core region of nucleosomes containing the histone variant CENP-A. This observation is also valid in the case of kinetochores assembled over non-centromeric sequences such as the neocentromeres. Given that a form of CENP-A is present in all these organisms, it appears that specialized nucleosomes are essential for the efficient assembly of the kinetochore complex. Moreover, core centromeres are almost invariably found adjacent to domains of heterochromatin enriched in histone H3K9 and DNA methylation (Choo 2001). This is certainly true for centromeres in fission yeast, *D. melanogaster*, plant and mammalian centromeres. In fission yeast, a minimal centromere must contain at least a portion of repeat DNA that attracts heterochromatin together with a central region that elicits CENP-A deposition (Baum,

Ngan et al. 1994; Takahashi, Chen et al. 2000; Kniola, O'Toole et al. 2001). In this organism, centromeric heterochromatin is known to be required for establishment of cohesion at centromeres between sister chromatids, which in turn is important for faithful chromosome separation (Bernard, Maure et al. 2001; Nonaka, Kitajima et al. 2002). The centromere from *Dp1187* in *D. melanogaster* requires an additional 200 kb on either side of the 220 kb core region in order to be fully stable (Murphy and Karpen 1995). These adjacent regions covered in heterochromatin were suggested to act as support and to establish centromeric cohesion. Human artificial chromosomes contain only alphoid DNA but centromeric heterochromatin is known to form over these sequences adjacently to CENP-A domains (Nakashima, Nakano et al. 2005). In addition, CENP-B protein also localises to centromeric heterochromatin which is also a widespread feature of centromeres among eukaryotes (Pluta, Mackay et al. 1995). CENP-B is thought to promote heterochromatin formation and to enforce a higher order structure to underlying chromatin by dimerizing and pulling together bound DNA (Kitagawa, Masumoto et al. 1995). It has been suggested that CENP-B bears some similarity with transposase from TEs (Smit and Riggs 1996). While this claim is debatable, it suggests a TE-related origin for specialized centromeric repeats such as the human α-satellite and the fission yeast outer repeats. However, mice deprived of CENP-B are viable and do not display any obvious defects in chromosome segregation (Hudson, Fowler et al. 1998; Kapoor, Montes de Oca Luna et al. 1998). Furthermore, stable human neocentromeres such as *mardel (10)* form over regions that don't contain α-satellite repeats and lack CENP-B, suggesting that while CENP-B may contribute to kinetochore assembly, its role is not essential (du Sart, Cancilla et al. 1997; Saffery, Irvine et al. 2000). However, it does not rule out the possibility of pre-existing heterochromatin at the sites of neo-centromere formation, in which case that particular chromatin structure may be involved in its establishment. The presence of heterochromatin may also act as a stabilizer for the central CENP-A domain. In the case of the minimal centromere of *S. cerevisiae*, both the assembled kinetochore and the precise nucleosome disposition pattern can be disrupted by promoting transcription through the centromere (Hill and Bloom 1987). The process of transcription may temporarily abolish the protein-DNA interactions at the centromere and also modify the chromatin locally though the remodelling activity of the FACT complex or histone acetylation by the Elongator complex (Otero, Fellows et al. 1999; Mason and Struhl 2003). Hence, flanking heterochromatin domains may serve to protect the central core region of the centromere from incoming transcription events and thus preserve the distinct chromatin arrangement and plethora of chromatin-bound kinetochore proteins at centromeres.

1.8. FISSION YEAST CENTROMERES

The centromere of *Schizosaccharomyces pombe* (fission yeast) is one of the simplest and best characterized (40-100 kb) yet it bears similarity to the larger centromeres of multicellular eukaryotes (Kniola, O'Toole et al. 2001). It is composed mostly of repetitive DNA divided into the "outer repeats" (*otr*) and the "inner-most repeats" (*imr*) with an unconserved central core region. The central core is occupied by nucleosomes bearing the histone H3 variant Cnp1^{CENP-A} upon which the kinetochore is assembled. The flanking outer repeats are engulfed in silent chromatin whose nucleosomes bear extensive H3K9 methylation. This methylation is the product of the histone methyltransferase Clr4$^{Su(var)3-9}$ through a complex set of pathways that also involves RNA silencing. Thus, the fission yeast centromere bears the same basic chromatin layout observed in centromeres from higher eukaryotes and provides a valuable model with which to study the influence of epigenetic mechanisms in centromere establishment and function (Pidoux and Allshire 2004).

All three centromeres of fission yeast are based mostly on common DNA elements but still present individual diverging characteristics (Figure 1-5). The centromere from chromosome I (*cen1*) is the smallest one with only 45 kb while *cen2* is 65 kb-long and *cen3* reaches 110 kb in size. All centromeres possess a 4-7 Kb central core (*cnt*) region composed mostly of non-repetitive AT-rich DNA but the actual sequence is diverges between chromosomes. Central cores 1 and 3 share a 3.3 kb long region called "TM" that is 99% identical. Central core 2 has a smaller 1.5kb region that is only 48% identical to TM (Wood, Gwilliam et al. 2002). All three central cores are surrounded by two convergent *imr* repeats which are identical between the two copies of each centromere but completely distinct between centromeres. The "outer repeats" (*otr*) are composed of two basic repetitive units *dg* and *dh* (also called K and L repeats) with a short spacer sequence. The relative orientation of the *dg* + *dh* pair is different between *cen1* (divergent) *cen2* and *3* (tandem). Within each centromere, the sequence composition of *dg* and *dh* copies is virtually identical but show some divergence between centromeres. The *dg* sequences are 97% identical between all three centromeres but *dh* sequences only share 48% identity. Centromere 1 is the only one to present a symmetrical disposition of outer repeats. Centromere 2 possesses two pairs of *dg* + *dh* at the left arm and one at the right arm. Centromere 3 is the most asymmetrical, with 9 *dg* + *dh* pairs on the

left arm and 4 on the right arm. The different number of outer repeat pairs of each of the three centromeres accounts for most of the size differences. In addition, all three centromeres are flanked by IRC sequences that have been recently shown to perform as chromatin boundaries (Noma, Cam et al. 2006).

With the exception of the right arm of *cen1*, these boundary areas are associated with tRNA genes in various numbers. The *imr* repeats also contain functioning tRNA genes. Recently, these tRNA genes together with other RNA polymerase III-related motifs in the flanking IR sequences were shown to enforce chromatin boundaries that isolate the centromere from surrounding chromatin and the central domain from the outer repeats. These boundary elements appear to be important for maintaining both the organization of epigenetic characteristics within the centromere and its function in chromosome segregation (Noma, Cam et al. 2006; Scott, Merrett et al. 2006; Scott, White et al. 2007).

The number of outer repeats each centromere presents is highly variable, suggesting that the outer repeats are less important for centromere function. In fact, the central domain is essential for centromere function but it is not sufficient to recruit a functional kinetochore. Instead, studies in the synthesis of artificial mini-chromosomes have made clear that a minimally functional fission yeast centromere requires both the central domain and an adjacent fragment of outer repeats (Clarke, Amstutz et al. 1986; Niwa, Matsumoto et al. 1986; Chikashige, Kinoshita et al. 1989; Hahnenberger, Baum et al. 1989; Niwa, Matsumoto et al. 1989; Matsumoto, Murakami et al. 1990; Hahnenberger, Carbon et al. 1991). Even though the CENP-A containing central domain is the functional assembly site for the kinetochore, the identity of the centromere in fission yeast is also defined by the outer repeats.

Epigenetic features of fission yeast centromeres

There are several lines of evidence demonstrating that fission yeast centromeres are epigenetically regulated. The placement of genes in close vicinity to heterochromatin domains in animal cells causes their expression to become negatively affected to a variable extent. This phenomenon is termed Position Effect Variegation (PEV) and was first described in *D. melanogaster* (Muller 1930). In this study, a *white* reporter gene was placed either in the vicinity or within a

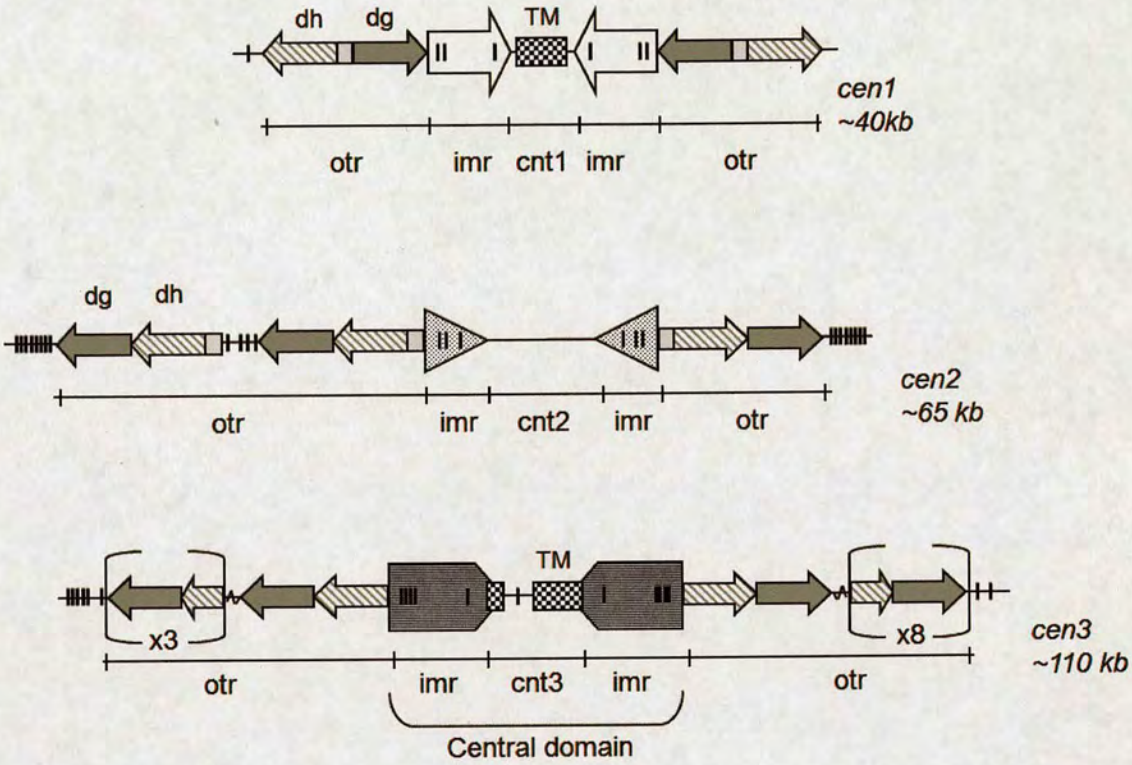


Figure 1-5: Organization of fission yeast centromeres.

In fission yeast, all three centromeres share the same basic structure. It comprises a non-repetitive central core (*cnt*) surrounded by the innermost repeats (*imr*). All three central cores are distinct but *cnt1* and *cnt3* are homologous in a region called TM (checkered box). The *imr* are completely distinct between centromeres but within each centromere the pair is identical. The central core together with most of the *imr* forms the central domain, where $Cnp1^{CENP-A}$ and the kinetochore assembles. The central domain is surrounded by arrays of *dg* (filled arrows) and *dh* (hatched arrows) which constitute the outer repeats (*otr*). The sequences of *dg* and *dh* are highly conserved in all three centromeres bu the number of *dg* + *dh* pairs found on the left and right arms vary between centromeres. The outer repeats are packaged in heterochromatin. The solid vertical lines represent multiple tRNA genes that serve as chromatin boundaries between the two distinct chromatin environments within the centromere.

domain of heterochromatin. This lead to the formation of flies whose composite eyes displayed a mosaic of red and white colour patches. The reason behind this phenotype lies in the stochastic variegation of *white* gene expression between different eye cells. Since then, it has been established that PEV is an inherent property of metazoan heterochromatin (Wreggett, Hill et al. 1994; Aagaard, Laible et al. 1999; Heard, Rougeulle et al. 2001). Similarly, expression of reporter genes introduced at the centromeric outer repeats in fission yeast is also negatively affected. For instance, the introduction of the *ade6+* marker gene into the central core causes cells to assume an *ade-* phenotype and in consequence to accumulate a red coloured metabolite (Allshire, Javerzat et al. 1994). This change is propagated through cell divisions even though no genetic change has occurred to the *ade6+* gene. However, a percentage of the colonies can also display the normal white colour, white sectors in red background or intermediate shades of pink. This indicates that repression of the *ade6+* gene at the outer repeats can be lifted in a stochastic manner. Similar studies demonstrated that the repressive effect is stronger at the outer repeats and that it varied depending on site of marker gene insertion within the outer repeats: weaker when the insertion was closer to the periphery of the centromere and stronger when it was buried deeper into the *otr* (Allshire, Javerzat et al. 1994; Allshire, Nimmo et al. 1995). This phenomenon is akin to PEV and strongly suggested that the outer repeats are coated with heterochromatin.

The second line of evidence supporting epigenetic regulation at centromeres derives from studies on the behaviour of centromeric marker gene insertions in the presence of the drug Tricostatin A (TSA), which is a known inhibitor of histone deacetylases (HDACs) (Ekwall, Olsson et al. 1997). In the presence of TSA, centromeric nucleosomes acquire a heritable increase in histone acetylation levels over centromeric chromatin. Coupled with this, silencing of marker genes at centromeres is alleviated and centromere function becomes crippled. Cells displaying alleviated silencing divide slowly and have a high rate of chromosome loss. This centromeric state is stable and is propagated through subsequent cell divisions even in the absence of TSA. However, after a number of generations, a fraction of the cells at each cell cycle is able to regain centromeric silencing and normal centromere function. Thus, the changes at centromeres caused by TSA treatment appear to be epigenetic. Given that hypoacetylation of histones H3 and H4 is a known hallmark of heterochromatin (Jeppesen and Turner 1993; Belyaev, Keohane et al. 1996), this evidence suggests that heterochromatin forms at fission yeast centromeres and that this fact is important for regulating the function of centromeres. In addition, several studies demonstrated

that mutants that affect silencing of marker genes at the centromere are often coupled to impaired centromere function (Allshire, Nimmo et al. 1995; Ekwall, Javerzat et al. 1995; Ekwall, Nimmo et al. 1996; Ekwall, Cranston et al. 1999; Pidoux, Richardson et al. 2003). This means that transcriptional silencing can be used as readout of centromeric integrity and that the function of the centromere is inherently linked to the epigenetic state of its chromatin.

The third line of evidence comes from observing the behaviour of fission yeast mini-chromosomes containing a minimal form of the centromere (Steiner and Clarke 1994). When transformed as naked DNA, the stability of the constructs relies on the establishment of a functional kinetochore, which appears to be a stochastic event. Transformation of such a construct (*Nbg*) into *S. pombe* gives rise to instances where cells retain it as a mini-chromosome and cells that lose it if plasmid selection is relaxed. Careful analyses of *Nbg* retrieved from both populations of cell showed that that no mutations, rearrangements or integration into the genome had occurred. Even when *Nbg* did not become stable, forcing the cells to retain it by selection can lead to a stabilization event in further cell divisions. Conversely, even when the kinetochore is already established mini-chromosomes may still be lost further on. The stability of these constructs within the cells appears to be a matter of probability and not of primary DNA sequence composition, suggesting that the formation of a functional kinetochore may rely on establishing a specific chromatin context that favours this process. Hence, this supports the hypothesis that centromere identity has epigenetic properties.

The central domain and associated factors

Within the fission yeast centromere, the Cnp1^{CENP-A} domain occupies the entire *cnt* region and overlaps with the most of the *imr* (Takahashi, Chen et al. 2000) (Figure 1-6). Micrococcal nuclease digestion analysis of the central domain shows that the disposition of these nucleosomes is not periodic as in most other parts of the chromosomes. Instead of producing a ladder pattern reflecting the staggered disposition of nucleosomes along the DNA fibre, the central domain pattern is smeared (Polizzi and Clarke 1991; Takahashi, Murakami et al. 1992). In contrast, the pattern of the chromatin overlying the outer repeats is regular, much the same as in other areas of the chromosome outside the centromere (Castillo, Mellone et al. 2007). The smeared pattern is linked to the presence of functional Cnp1^{CENP-A} in the cells since depletion of this protein using the

cnp1-1 allele causes a reversion of the nucleosomal pattern to a regular one (Takahashi, Chen et al. 2000). At the same time, cells experience severe chromosome segregation defects with consequent aneuploidy and cell death. Mutations in factors involved in Cnp1 deposition, such Mis6 and Sim4, also disrupt the particular nucleosomal pattern at the central core (Saitoh, Takahashi et al. 1997; Pidoux, Richardson et al. 2003). Thus, it was suggested that the CENP-A containing nucleosomes confer a specialized structure or spatial organization to the central domain chromatin that accounts for the unusual micrococcal nuclease digestion pattern. It is also possible that this pattern is a consequence of the presence of kinetochore proteins bound to the central domain in active centromeres.

Assembly of a functional kinetochore disrupts transcription of marker genes inserted in the central domain. Thus, transcriptional repression can be used as readout of the functional status of kinetochore. Indeed, this property was used as the basis of a genetic screen for factors involved in kinetochore stability. The Sim (Silencing In the Middle of the centromere) screen yielded Cnp1^{CENP-A} itself and Sim4, a coiled coil domain protein that is required for Cnp1^{CENP-A} deposition (Pidoux, Richardson et al. 2003). Sim4 is part of a 13-subunit complex comprised of Sim4, Mis6, Mis15, Mis17, Mal2, Dad1, and Ftp1 to 7 (Liu, McLeod et al. 2005). The Sim4 complex binds directly to the central domain and is believed to serve as a platform for the transient DASH complex at the kinetochore during mitosis (Liu, McLeod et al. 2005). Mal2, Mis6 and Mis12 were previously isolated as kinetochore factors in mini-chromosome loss screens (Takahashi, Yamada et al. 1994; Fleig, Sen-Gupta et al. 1996) Takahashi 1996). In addition to *sim4* and *cnp1*, mutations in *mal2* and *mis6* also alleviate marker gene silencing at the central core.

Mutants of these four genes together with the GATA-like factor *ams2* all cause a shift of the smeared micrococcal nuclease digestion pattern to a regular ladder typical of normal chromatin (Saitoh, Takahashi et al. 1997; Goshima, Saitoh et al. 1999; Jin, Pidoux et al. 2002; Chen, Saitoh et al. 2003; Pidoux, Richardson et al. 2003).

Deposition of Cnp1^{CENP-A} is linked to the functional state of the centromere but the mechanisms that control this process are still unclear (Marschall and Clarke 1995). Mis6 has been implicated as a loading factor since *mis6* mutant cells were shown to fail to incorporate newly synthesized GFP-Cnp1^{CENP-A} at the centromeres (Takahashi, Chen et al. 2000). This role appears not to be conserved by the budding yeast homolog Ctf3 since it is not required for loading of Cse4p^{CENP-A}

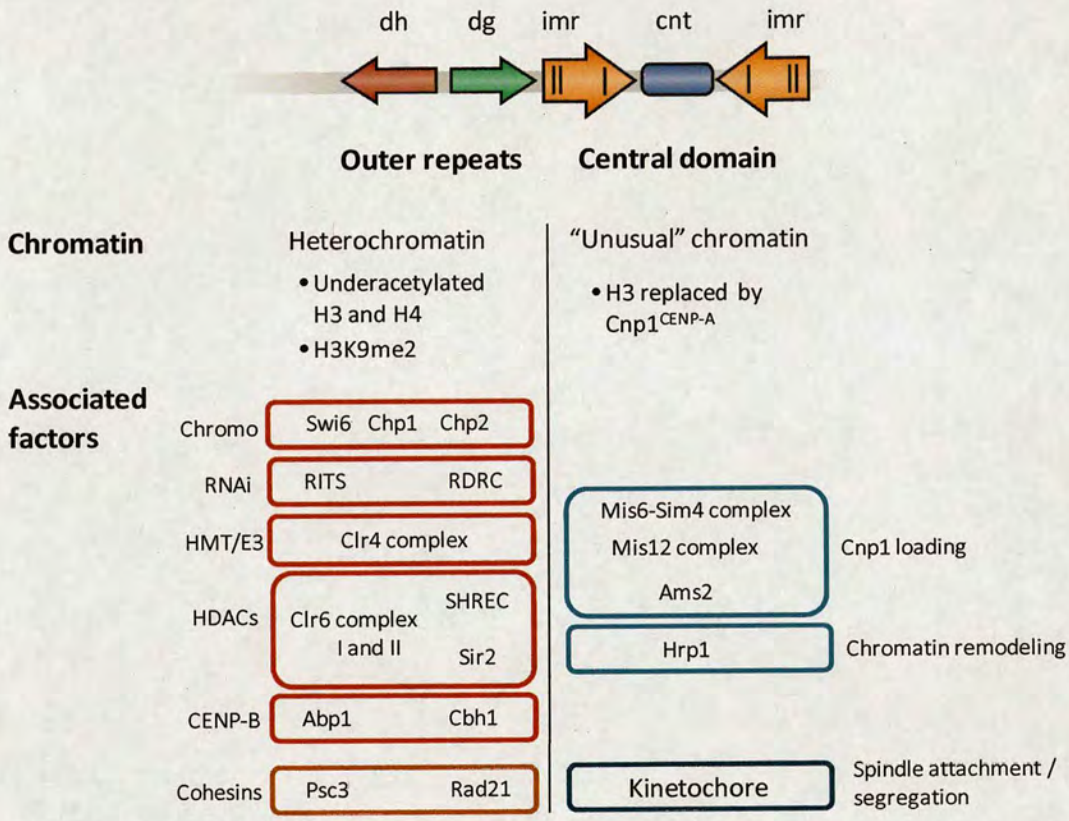


Figure 1-6: The two distinct chromatin domainsin of the fission yeast centromeres (adapted from Pidoux and Allshire 2005)

The nucleosomes overlying the outer repeat DNA (left) are hypoacetylated and enriched in H3K9 methylation (heterochromatin) while at the central domain Cnp1^{CENP-A} is replacing histone H3. Together with these distinct chromatin domainsare associated with a distinct set of factors. On the central domain, a series of protein complexes and the transcription factor (Ams2) function synergistically to load of Cnp1^{CENP-A} in the nucleosomes. Chromatin remodelling is also required for maintaining silencing at the central core. The kinetochore large protein complex is assembled over the central domain chromatin and is responsible for attachment to the spindle microtubules and for chromosome segregation.On the left in red boxes are the factors involved in establishment and maintenance of heterochromatin, namely chromodomain proteins (Chromo), components of the RNA interfence pathway (RNAi), histone methyltransferases (HMT), histone deacetylases (HDACs), ubiquitin E3 ligase (E3) and CENP-B proteins. Heterochromatin has functional implication for loading of cohesins at the centromere (orange box). SHREC stands for Snf2/Hdac-containing Repressor Complex, RITS is the RNA-induced Initiation of Transcriptional Silencing complex and RDRC is the RNA-Dependent RNA polymerase Complex.

(Measday, Hailey et al. 2002). Initial studies of the vertebrate Mis6 homologue CENP-I and a Sim4 related protein CENP-H suggested that neither were essential for association of CENP-A to centromeres (Nishihashi, Haraguchi et al. 2002). More recently, it was shown that in chicken DT40 cells, the CENP-H + CENP-I complex is required for the efficient deposition of CENP-A at centromeres. In vertebrates, CENP-H and CENP-I are present in a complex that contains 11 identified proteins (CENP-K to CENP-R, CENP-50, CENP-H and CENP-I) Mutant analysis of several of these factors shows that their function is required for faithful chromosome segregation. Some of the defects observed in CENP-H-I complex component mutants include arrest of cell cycle progression, failure to align chromosomes at the metaphase plate and mitotic spindle formation abnormalities. The same mutants showed impaired loading of newly-synthesized CENP-A-GFP at centromeres. CENP-K has weak similarity to Sim4 while CENP-L is a distantly related to Fta1, suggesting that the vertebrate CENPH-I complex is a functional homologue of the *S. pombe* Sim4 complex.

The Mis16-Mis18 complex is also required for $Cnp1^{CENP-A}$ deposition at fission yeast centromeres (Hayashi, Fujita et al. 2004). Mis16 has two homologues in humans, RbAp46 and RbAp48 (human retinoblastoma binding proteins) whose depletion causes a disruption in CENP-A localization to centromeres (Hayashi, Fujita et al. 2004). *In vitro* studies have shown that *D. melanogaster* RbAp48 can assemble $CENP\text{-}A^{CID}$ chromatin but so far no *in vivo* studies have validated these observations. Both the fission yeast and human complexes were implicated histone deacetylation activity. Mis16 and Mis18 are required for maintaining low levels of histone H3 and H4 acetylation at the central domain, which has been suggested as a pre-requisite for $Cnp1^{CENP-A}$ deposition (Hayashi, Fujita et al. 2004).

Ams2 is a GATA-like transcription factor that is also a factor involved in $Cnp1^{CENP-A}$ loading at centromeres (Chen, Saitoh et al. 2003). Unlike other factors involved in this process, *ams2+* is not an essential gene and it appears to regulate $Cnp1^{CENP-A}$ loading in a Mis12-independent manner. Ams2 controls the expression of histone genes in G1-S phase, which led to the suggestion that it has an impact in the timing of expression of histone genes required for $Cnp1^{CENP-A}$ deposition during replication (Takahashi, Takayama et al. 2005; Takayama and Takahashi 2007). It is known that $Cnp1^{CENP-A}$ can be loaded at centromeres in G2 phase, which provides a possible explanation for why *ams2-* cells are viable (Takahashi, Takayama et al. 2005). Ams2 shows peak association with chromatin during mitotic S phase and binds to centromere GATA-core sequences (Chen, Saitoh et

al. 2003), which may reflect a similar *modus operandi* to budding yeast Spt4p. Spt4p is also transcription factor which has been shown to be critical for restricting the localization of Cse4p^{CENP-A} at *S. cerevisiae* centromeres (Crotti and Basrai 2004).

It appears that the process of CENP-A loading is the result of the contribution of several converging mechanisms that have been preserved to various extents in other eukaryotic organisms. The conciliating feature of CENP-A loading is that it is associated with a functional kinetochore. While the machinery involved in the loading process may differ between species, it has been suggested that CENP-A loading is driven by the mechanical tension caused by the attachment of this structure to the mitotic spindle that "marks" the centromere for CENP-A deposition (Mellone and Allshire 2003).

Outer repeats and heterochromatin proteins

As previously mentioned, the outer repeats at the centromeres are coated in heterochromatin which represses transcription of inserted marker genes (Figure 1-6). This property was used to screen for factors that are involved in the establishment and maintenance of this particular structure and to study the impact of heterochromatin disruption in the process of chromosome segregation. The screen unveiled Clr4 (cryptic loci regulator 4), Rik1 and Swi6^{HP1} (trans-acting switch locus 6) as factors involved in maintaining silencing at the outer repeats (Allshire, Nimmo et al. 1995). Originally, these factors were first revealed in analyses of the *mat2* and *mat3* silent mating type loci, where heterochromatin is also formed (Ekwall and Ruusala 1994; Lorentz, Ostermann et al. 1994). In addition, cells bearing mutations on one of these three genes suffer from chromosome segregation defects (Allshire, Nimmo et al. 1995). While in late anaphase, mutant cells often display chromatids that were delayed in migrating to the poles of the mitotic spindle with the remaining chromatids. The phenotype is described as "lagging chromosomes" and is characteristic of cells defective in centromeric cohesion (Ekwall, Nimmo et al. 1996; Bernard, Maure et al. 2001). The same cells also present a significantly higher rate of mini-chromosome loss (Allshire, Nimmo et al. 1995). The silencing phenotypes of the three mutants also present important differences. The alleviated silencing phenotypes of *clr4* and *rik1* are stronger than that of *swi6* (Allshire, Nimmo et al. 1995). In addition, all three mutants also affect the remaining two constitutive heterochromatin loci in fission yeast: the telomeres and the silent mating type loci

mat2 and *mat3* (Ekwall and Ruusala 1994; Lorentz, Ostermann et al. 1994; Allshire, Nimmo et al. 1995). In addition to Clr4, Rik1 and Swi6, subsequent studies have identified a number of additional factors that contribute to maintain the heterochromatin over centromeric outer repeats.

Swi6

Swi6 is a protein that presents a chromodomain that is 48% identical to the same motif present in *D. melanogaster* HP1 (heterochromatin protein 1) (Lorentz, Ostermann et al. 1994). Of proteins bearing chromodomain such as HP1 and Polycomb, Swi6 resembles HP1 more closely by also sharing the chromo-shadow domain, a protein-protein interaction motif that is involved in homodimerisation (Cowieson, Partridge et al. 2000). $Swi6^{HP1}$ binds to chromatin at centromeres and at a number of other loci (Ekwall, Javerzat et al. 1995). Localization of this protein relies on the presence of $Clr4^{Su(var)3-9}$ and Rik1 (Ekwall, Nimmo et al. 1996). Like HP1, the chromodomain of $Swi6^{HP1}$ is known to bind nucleosomes which present di and tri-methylated H3K9, a mark of heterochromatin (Bannister, Zegerman et al. 2001; Nakayama, Rice et al. 2001). In the absence of Swi6, centromeric function is affected. Null mutants display lagging chromosomes in late anaphase and increased chromosome loss rate (Allshire, Nimmo et al. 1995; Ekwall, Javerzat et al. 1995). In addition, *swi6* mutant cells are sensitive to thiabendazole (TBZ), a microtubule-destabilizing drug (Ekwall, Nimmo et al. 1996). There is genetic evidence linking *swi6* with *nda3* (α-tubulin; synthetic sick), the mitotic spindle checkpoint factor *bub1,* the cohesin subunits *rad21* and *psc3* and the exosome component *dis3* (all synthetic lethal) (Bernard, Hardwick et al. 1998; Bernard, Maure et al. 2001; Nonaka, Kitajima et al. 2002; Murakami, Goto et al. 2007).

Clr4 complex

Chromatin overlying the centromeric outer repeats is enriched in histone H3 K9 di- and tri-methyl, both marks of heterochromatin. Clr4 is the fission yeast homolog of *D. melanogaster Su(var)3-9* that is responsible for this modification. Like Swi6, it contains a chromodomain that allows it to bind H3K9me. In addition, it contains the SET domain that has been implicated in the histone lysine methyltransferase activity (Rea, Eisenhaber et al. 2000). The SET domain deposits methyl groups on histone H3K9 and thus provides a binding site for $Swi6^{HP1}$ and other

chromodomain proteins. Clr4$^{Su(var)3-9}$ is responsible for H3K9 methylation in all constitutive heterochromatin loci in fission yeast (centromeres, sub-telomeric regions and silent mating type loci). In the absence of Clr4, Swi6^{HP1} localization to these loci is lost. Clr4$^{Su(var)3-9}$ is found in large multimeric complex that includes Rik1, Raf1, Raf2 and Pcu4/Cul4 (Hong, Villen et al. 2005; Horn, Bastie et al. 2005; Li, Goto et al. 2005; Thon, Hansen et al. 2005).

Rik1 is a known Clr4$^{Su(var)3-9}$ interactor that it is essential for fission yeast heterochromatin (Ekwall and Ruusala 1994; Nakayama, Rice et al. 2001). Rik1 is a WD propeller protein that also bears a CPSF-A domain, a motif that is found in the DNA damage response factor DDB1, the human spliceosome factor SAP130 and CPSF1 (Neuwald and Poleksic 2000; Brand, Moggs et al. 2001; Tuzon, Borgstrom et al. 2004). The CPSF-A motif derives its name from the first protein that was identified with it, namely the large subunit of the mRNA cleavage and poly-adenylation factor CPSF1 which plays a crucial role in the later stages of RNA polymerase II transcription and mRNA maturation (Murthy and Manley 1995; Brand, Moggs et al. 2001). Despite this information, the activity of Rik1 is still unknown. It is believed to act as protein interaction hub for the remaining Clr4 complex components but these implications in DNA damage responses and transcription haven't yet been explored.

Raf1 and Raf2 are two components that were identified in three separate mass spectrometry studies. The multiply named Raf1 (Rik1 associated factor 1)/Dos1 (delocalization of Swi6 1)/Cmc1/Clr8 is a previously known heterochromatin factor that contains four WD repeat motifs (Horn, Bastie et al. 2005; Li, Goto et al. 2005; Thon, Hansen et al. 2005). This protein has no strong sequence homologues outside fission yeast but it has been suggested as a possible functional homolog of the human DDB2 protein, again a DNA-binding factor involved in DNA damage response. Raf2, also known as Dos2, Cmc2 and Clr7, also has no close homologs in other species (Hong, Villen et al. 2005; Horn, Bastie et al. 2005; Li, Goto et al. 2005; Thon, Hansen et al. 2005). It has no discernable protein domains apart from a putative zinc-finger-like motif (Horn, Bastie et al. 2005; Thon, Hansen et al. 2005). Rik1 and Raf2 were found in a complex with Pcu4/Cul4, a cullin-dependent E3 ubiquitin ligase that is required for heterochromatin formation (Hong, Villen et al. 2005; Horn, Bastie et al. 2005). The contribution of this E3 ubiquitin ligase activity to heterochromatin assembly is unclear. DDB1 is an adapter protein that is required for ubiquitylation by Cullin 4A in response to the signalosome and DNA damage (Groisman, Polanowska et al. 2003). It is possible that Rik1 may also perform as an adapter in directing

Pcu4/Cul4 E3 ligase to a specific substrate to promote heterochromatin assembly. *In vitro* activity assays suggest that histone H2B is a substrate for Cul4-mediated ubiquitylation (Horn, Bastie et al. 2005). H2B ubiquitylation has been recently shown to promote efficient transcriptional elongation through chromatin and to have ties with DNA damage response (Sung, Prakash et al. 1988; Laribee, Fuchs et al. 2007; Tanny, Erdjument-Bromage et al. 2007). Thus, the connection between this histone modification and heterochromatin is not obvious but it is still possible that Cul4 may have preference for other substrates *in vivo*.

Histone deacetylases

The appearance of H3K9 methylation is preceded by histone deacetylation. To this end, three histone deacetylases (HDACs) are known to function in *S. pombe* in promoting silent chromatin at the outer repeats and other constitutive heterochromatin loci. Outer repeat nucleosomes are underacetylated in multiple residues in both histone H3 (K9, K14, K18, K23 and K27) and histone H4 (K5, K8, K12 and K16). This HDAC activity is absolutely crucial for transcriptional silencing in these heterochromatin domains(Ekwall, Olsson et al. 1997; Mellone, Ball et al. 2003). The Clr3, Clr6 and Sir2 HDAC proteins are all involved in maintaining transcriptional silencing over heterochromatin-bound repetetitive DNA at centromeres (Grewal, Bonaduce et al. 1998; Bjerling, Silverstein et al. 2002; Shankaranarayana, Motamedi et al. 2003). Clr3 deacetylates H3K14 while Sir2, a homolog of the NAD-dependent HDACs Sir2p from budding yeast, is specific for acetylated H3K9 (Shankaranarayana, Motamedi et al. 2003). Clr6 appears to be more promiscuous and is able to remove acetyl groups from several lysine residues in the tails of both histones H3 and H4 (Bjerling, Silverstein et al. 2002).

The activity of these three HDACs influences heterochromatin establishment but the impact of the loss of each of these HDACs varies depending on the heterochromatin locus. Together with Clr1, Clr2 and Mit1, Clr3 forms the SHREC (Snf2/Hdac-containing Repressor Complex) which participates in transcriptional silencing at the centromeric outer repeats, telomeres, silent mating type loci and rDNA loci (Sugiyama, Cam et al. 2007). Mit1 has a PHD motif and SNF2 helicase domains which are involved in the ATP-dependent chromatin remodelling activity described for SHREC. Unlike its budding yeast homolog Snf2p that is part of the SWI-SNF global transcriptional

activator, the chromatin remodelling activity of SHREC is required for its role in transcriptional silencing (Cote, Quinn et al. 1994; Henry, Campbell et al. 1994; Sugiyama, Cam et al. 2007).

Clr6 is a member of the class I HDACs that also include budding yeast Rdp3p and human HDAC1 to 3 (Grewal, Bonaduce et al. 1998). Clr6 is found in complex with two different sets of interactors which contain different proteins homologous to budding yeast Sin3p, a partner protein to Rpd3p (Kadosh and Struhl 1998; Nakayama, Xiao et al. 2003; Nicolas, Yamada et al. 2007). The Clr6 complex I, composed of Clr6, Prw1, Pst1 and Sds3, preferentially de-acetylates histones at promoter-containing intergenetic regions, thus repressing a specific set of genes and repetitive sequences (Nicolas, Yamada et al. 2007). Complex I plays an essential role in the cell while cells depleted of components of the complex II are viable. The presence of the Sin3p-like protein Pst1 is essential for cell viability but Pst2, a member of complex II, is not (Nicolas, Yamada et al. 2007). Complex I is also responsible for preventing de-repression of donor mating type genes, thus preventing haploid meiosis from occurring (Nicolas, Yamada et al. 2007). The ING-family protein Png2 can bind to complex I, forming complex I' which appears to be involved in DNA damage response (Nicolas, Yamada et al. 2007). Complex II, composed of Clr6, Prw1, Alp13, Cph1 and Cph2, is responsible for de-acetylating nucleosomes over coding regions, thereby preventing spurious transcription initiation within the genes that might lead to the production of truncated mRNAs or anti-sense transcripts (Nicolas, Yamada et al. 2007). Similarly to complex I', defects in complex II also affect the capacity of the cells to deal with DNA damage. Both Clr6 complexes I and II intervene at the centromeric outer repeats to enforce transcriptional silencing on both strands of DNA (Nicolas, Yamada et al. 2007).

The activity of the Sir2, the fission yeast member of the Sir2p-SirT1 ("sirtuins") family of NAD(+)-dependent histone deacetylases, has been shown *in vitro* to be specific for H3K9 and H4K16 residues (Shankaranarayana, Motamedi et al. 2003). The deacetylase activity of this protein family is induced by increased levels of NAD(+), the oxidated form of the co-enzyme NAD, which can occur as consequence of nutrient starvation (Tanner, Landry et al. 2000; Armstrong, Kaeberlein et al. 2002; Shankaranarayana, Motamedi et al. 2003). *In vivo*, Sir2 is essential for silencing at the telomeres and mating type locus (Shankaranarayana, Motamedi et al. 2003). Loss of Sir2 has an impact on transcriptional silencing at centromeric outer repats and rDNA arrays but to a much lower extent than other loci. (Shankaranarayana, Motamedi et al. 2003) More specifically, in *sir2Δ* centromeric silencing is lost at the *imr* but it is only weakly affected over the outer repeats,

suggesting that Clr3 and Clr6 might be the most predominant centromeric HDACs while Sir2 is mostly recruited to telomeres and mating type loci.

Chp1 and Chp2

In adittion to Swi6^{HP1} and Clr4, two other chromodomain proteins Chp1 and Chp2 bind to heterochromatic regions and are required for the stability of its chromatin structure (Halverson, Gutkin et al. 2000; Partridge, Borgstrom et al. 2000; Thon and Verhein-Hansen 2000; Sadaie, Iida et al. 2004). Like Swi6, Chp1 can bind to H3K9me2 via its chromodomain (Partridge, Scott et al. 2002). Unlike Chp2 and Swi6^{HP1} (~30 kiloDaltons), Chp1 is a much longer protein (100 KDa) that contains an RRM (RNA recognition motif) and a largely uncharacterized C terminus (Petrie, Wuitschick et al. 2005). Like Swi6, Chp1 forms multiple (up to 5) independent foci within the cell nucleus (Sadaie, Iida et al. 2004; Petrie, Wuitschick et al. 2005). This reflects the participation of Chp1 in the establishment of heterochromatin at centromeres, telomeres and silent mating type loci (Sadaie, Iida et al. 2004). Chp1 is particularly relevant in forming and maintaining heterochromatin domains over the centromeric outer repeats, where it is involved in RNA interference-mediated heterochromatin assembly (Verdel, Jia et al. 2004). Chp1, together with Ago1 (Argonaute) and Tas3, forms the RITS complex (RNA-induced transcriptional silencing) that is responsible for directing Clr4-mediated H3K9 methylation over the outer repeats in a mechanism that is dependent on Dcr1 (Dicer) and centromere specific siRNAs (Verdel, Jia et al. 2004). Chp1 also localizes to other constitutive heterochromatin domains, such as mating type locus and telomeres, even though its presence is not as required to maintain H3K9me2 and transcriptional silencing as at the centromere (Sadaie, Iida et al. 2004). Although Chp1 is redundant in maintenance of heterochromatin at the two latter loci, it is essential to efficiently establish *de novo* heterochromatin at these loci in a situation where H3K9me becomes totally depleted (Sadaie, Iida et al. 2004).

Chp2 is much closer to Swi6^{HP1} in terms of size and by the fact that it contains a chromoshadow domain (Halverson, Gutkin et al. 2000). Loss of Chp2 has a moderate impact in transcriptional silencing at centromeres, silent mating type loci and rDNA clusters (Thon and Verhein-Hansen 2000). Like Swi6, Chp2 is an integral part of heterochromatin structure and is required in order to maintain H3K9me2 at centromeres, particularly the residual levels observed in *chp1Δ* or other RNAi mutants (Sadaie, Iida et al. 2004). It is also involved in recruiting the HDAC Clr3

to the mating type loci, possibly reflecting its involvement in a heterochromatin maintenance pathway.

CENP-B homologues

Fission yeast has three homologs of the human CENP-B protein that binds centromeric α-satellite repeats (Masumoto, Masukata et al. 1989; Murakami, Huberman et al. 1996; Lee, Huberman et al. 1997; Baum and Clarke 2000; Nakagawa, Lee et al. 2002). Loss of Abp1, Cbh1 or Cbh2 has only a small impact on centromeric H3K9me2 levels and on transcriptional silencing but the phenotype is enhanced in double mutants, suggesting that the function of these proteins may be partially redundant (Irelan, Gutkin et al. 2001; Nakagawa, Lee et al. 2002). In fact, Abp1 and Cbh1 appear to be more relevant for centromere function than Cbh2. It has been confirmed that Abp1 physically associates with outer repeat DNA although its binding motif is unknown (Lee, Huberman et al. 1997; Nakagawa, Lee et al. 2002). It has been proposed that Abp1 and Cbh1 may function as *cis*-acting heterochromatin nucleating factors at the outer repeats in a similar fashion to how Atf1 and Pcr1 proteins act at the mating type locus (Nakagawa, Lee et al. 2002). Despite their role in heterochromatin, these proteins are likely to present other cellular roles since Abp1 binds to autonomous replicating sequence (ARS) elements and is also required for meiosis to be carried out (Murakami, Huberman et al. 1996).

Other heterochromatin factors

In addition to the proteins described in this section, there is a group of additional factors which are involved in heterochromatin formation and stability but whose mechanisms of action or purpose are unclear. Two of these proteins are Hip1 and Slm9 which, together with Hip3, form a complex that is related to the metazoan HIRA complex and the budding yeast HIR histone gene regulator (Sherwood, Tsang et al. 1993; Spector and Osley 1993; Blackwell, Martin et al. 2004; Greenall, Williams et al. 2006). In fact, all three fission yeast genes are required for establishing repressive chromatin at centromeres and mating type loci. HIRA is involved in nucleosome deposition independently of DNA replication but at this time it is unclear how this activity contributes to heterochromatin structure (Tagami, Ray-Gallet et al. 2004). Epe1 is a

heterochromatin stability factor and one of the 6 fission yeast members of the "jumonji" family of proteins (Ayoub, Noma et al. 2003; Trewick, McLaughlin et al. 2005). Epe1 localizes to the boundary areas of heterochromatin domains and so has been proposed to act as a chromatin boundary and anti-silencing factor (Zofall and Grewal 2006). This hypothesis has been disputed given that heterochromatin domains appear to expand and contract when Epe1 is overexpressed (Trewick, Minc et al. 2007). Another recent study shows a correlation in genome-wide expression levels between *epe1+* overexpressing cells and *clr6-1* null mutant cells (Hansen, Burns et al. 2005; Isaac, Walfridsson et al. 2007). This suggests that Epe1 functions at the level of histone deacetylation and thus affects both constitutive heterochromatin and gene repression by HDACs (Isaac, Walfridsson et al. 2007). The jumonji C (jmjC) domain is associated with Fe(II) and α-ketoglutarate dependent oxidative catalytic activity that is employed in DNA repair and histone demethylation (Trewick, Henshaw et al. 2002). It was recently demonstrated that jmjC containing proteins can remove all methyl groups of histone H3K36 and H3K9 in metazoan (Klose, Yamane et al. 2006; Chang, Chen et al. 2007; Huarte, Lan et al. 2007; Klose, Gardner et al. 2007; Lee, Zhang et al. 2007; Liang, Klose et al. 2007; Yamane, Tateishi et al. 2007). Epe1 was similarly proposed to act as a histone H3K9 demethylase that antagonizes heterochromatin but this activity has not yet been demonstrated (Trewick, McLaughlin et al. 2005).

Role of heterochromatin at centromeres

The heterochromatin domains formed over the centromeric outer repeats are not essential for kinetochrore-related centromere function. Reflecting this, many of the factors involved in forming and stabilizing centromeric heterochromatin are not essential for life. In fact, basic kinetochore function is maintained in the absence of $Clr4^{Su(var)3-9}$ or $Swi6^{HP1}$. However, heterochromatin domains play a part in centromere function during cell cycle.

Inter chomosome cohesion is established during S phase and is a crucial event for both mitosis and meiosis. It is required for maintain the sister chromatids or homologous chromosomes physically associated through the duration of prophase and metaphase, when kinetochores are attached to opposite poles of the spindle and metaphase alignment is achieved. In budding yeast mitosis, cohesion is supported by the cohesin subunits Smc1p and Smc3p that, together with Scc1p/kleisin, were proposed to form a ring that holds the two chromatin fibres together

(Uhlmann, Lottspeich et al. 1999; Haering, Lowe et al. 2002). Upon triggering of anaphase, the APC/C (anaphase promoting complex/cyclosome) activates the protease separase that opens the cohesin rings by cleaving Rad21/Scc1p, thus allowing the movement of chromosomes to the spindle poles (Uhlmann, Lottspeich et al. 1999; Uhlmann, Wernic et al. 2000). Cohesion is established throughout the entire chromosome arms and also at centromeres. The latter is particularly important for meiosis II because it holds the sister chromatids together between anaphase I and anaphase II which otherwise could not occur. Cohesion is established during S phase but, in addition, centromeric cohesion requires heterochromatin (Bernard, Maure et al. 2001; Nonaka, Kitajima et al. 2002). More specifically, deposition of Rad21^{Scc1} and Psc3^{Scc3} was shown to require the presence of Swi6^{HP1} and Clr4$^{Su(var)3-9}$ (Bernard, Maure et al. 2001; Nonaka, Kitajima et al. 2002). Cells lacking Swi6^{HP1} have defective centromeric cohesion but are rescued by arm cohesion which is established independently. Nevertheless, *swi6* null cells display premature sister centromere separation and have high incidence of lagging chromosomes in late anaphase.

As discussed previously, it is long known that the presence of outer repeat DNA is essential for the stability of mini-chromosomes in *S. pombe* (Clarke, Amstutz et al. 1986; Niwa, Matsumoto et al. 1986; Chikashige, Kinoshita et al. 1989; Hahnenberger, Baum et al. 1989; Niwa, Matsumoto et al. 1989; Matsumoto, Murakami et al. 1990; Hahnenberger, Carbon et al. 1991; Steiner and Clarke 1994). One of the proposed models for this requirement was that the heterochromatin-based cohesion established over mini-chromosomes was essential to maintain the two sister mini-chromatids together since arm cohesion was less intense due to reduced size of mini-chromosomes. Another possibility is that heterochromatin may be somehow involved in facilitating Cnp1^{CENP-A} deposition. In *S. pombe*, it is known that heterochromatin can be established over a 1.5 kb fragment of outer repeat DNA placed ectopically in the genome and that it can silence marker genes inserted in its vicinity (Partridge, Scott et al. 2002). A very recent study demonstrated the connection between heterochromatin, deposition of Cnp1^{CENP-A} and mini-chromosome stability (Folco, Pidoux et al. 2008). It was observed that on stable mini-chromosomes, histone H3K9me2 is deposited over the outer repeat DNA present while Cnp1^{CENP-A} is found over the adjacent central core region. However, the establishment of the Cnp1^{CENP-A} domain on the mini-chromosome when it is introduced into the cell requires the presence of Clr4$^{Su(var)3-9}$ and several other factors that participate in heterochromatin assembly (Folco, Pidoux et al. 2008). Once the Cnp1^{CENP-A} domain is established on the mini-chromosome, Clr4$^{Su(var)3-9}$ is no longer required to maintain it. This situation

mimics the fact that Cnp1^{CENP-A} domains at endogenous centromeres are unaffected by heterochromatin mutants. Even though centromeric heterochromatin and central domain chromatin represent distinct and independent domains, they collaborate in defining a functional centromere.

1.9. RNA INTERFERENCE AND HETEROCHROMATIN IN FISSION YEAST

The first evidence hinting to the involvement of RNAi in silencing at the outer repeats was the discovery of homologous siRNAs (Reinhart and Bartel 2002). In fission yeast, the main components of the RNAi machinery - Argonaute, Dicer and RdRP – are present in single copy. Following the first discovery of centromere-specific siRNAs in *S. pombe*, subsequent studies showed that disruption of any of the three main RNAi components does lead to defects in chromosome segregation, lagging chromosomes and higher rate of chromosome loss that arise from defective sister chromatid cohesion (Volpe, Kidner et al. 2002; Hall, Noma et al. 2003; Volpe, Schramke et al. 2003). Transcripts corresponding to centromeric outer repeats accumulate in these mutants, which suggest that RNAi is involved in silencing their expression (Volpe, Kidner et al. 2002). In fact, centromeric heterochromatin is abrogated in *ago1Δ*, *dcr1Δ* and *rdp1Δ*, preventing the establishment of pericentric cohesion (Hall, Noma et al. 2003; Volpe, Schramke et al. 2003). RNAi mutants have no impact in central core silencing, which shows that the activity of RNAi at centromeres is restricted to the outer repeat domains.

Heterochromatin marks are affected in RNAi mutants, particularly at centromeres. H3K9me2 levels over marker gene insertions at the *otr* are totally depleted and Swi6^{HP1} binding is also lost. The effect of RNAi mutants is not as dramatic over native *otr* sequences, where a modicum of H3K9me2 and Swi6^{HP1} binding is still detectable. Nevertheless, this crippled heterochromatin is unable to maintain transcriptional silencing or to support effective centromeric cohesion. On other loci, the loss of RNAi has less of an impact. A repetitive sequence residing in the *mat2/3* mating type locus (*cenH*) which bears homology to centromeric outer repeats is subject to a similar process of RNAi-dependent chromatin nucleation (Hall, Shankaranarayana et al. 2002). Nevertheless, the silent mating type loci are able to retain heterochromatin marks and transcriptional silencing in the absence of RNAi (Hall, Shankaranarayana et al. 2002). In fact,

nucleation of heterochromatin over this region is supported by an independent, *cis*-acting mechanism involving the ATF/CREB-like factors Atf1 and Pcr1 that recruit Clr3 directly (Jia, Noma et al. 2004; Kim, Choi et al. 2004; Yamada, Fischle et al. 2005). In circumstances where mating type loci heterochromatin is lost, the presence of RNAi contributes to efficient re-establishment of this heterochromatin domain. The telomere-linked helicase gene (*tlh1*) also bears two repetitive insertions which are homologous to outer repeats and is enriched in histone H3K9 methylation (Hansen, Ibarra et al. 2006). Methylation is lost and transcript levels of *tlh1* increase in RNAi mutants, indicating that this same pathway is recruited to silence this gene and establish sub-telomeric heterochromatin (Hansen, Ibarra et al. 2006). In contrast to centromeres, telomeric heterochromatin is mostly refractory to RNAi mutations since the telomere-linked proteins Taz1 and Ccq1 promote histone deacetylation and heterochromatin formation independently of RNAi (Cooper, Nimmo et al. 1997; Cooper, Watanabe et al. 1998; Nimmo, Pidoux et al. 1998; Kanoh and Ishikawa 2001; Hall, Noma et al. 2003; Sadaie, Naito et al. 2003; Sugiyama, Cam et al. 2007).

The current model for RNAi activity at centromeres suggests that assembly of centromeric heterochromatin is triggered by transcription of the outer repeats, which occurs naturally in wild-type cells (Figure 1-7). Centromeric transcripts are produced by RNA polymerase II from both strands of *dg* and *dh* repeats and form dsRNA molecules which are subsequently recognized and processed by Dcr1 (Dicer) (Djupedal, Portoso et al. 2005; Kato, Goto et al. 2005). The main effector complex of fission yeast RNA interference is termed RITS (RNA-induced Initiation of Transcriptional Silencing). It contains the Argonaute protein Ago1, the GW repeat protein Tas3 and the chromodomain protein Chp1 (Verdel, Jia et al. 2004; Partridge, DeBeauchamp et al. 2007; Till, Lejeune et al. 2007). Thus, the composition of RITS illustrates the connection of this RNA-based mechanism to chromatin in fission yeast. Ago1 is loaded with centromeric siRNAs that allow it to target the RITS complex to the centromeric *otr* (Noma, Sugiyama et al. 2004; Verdel, Jia et al. 2004). The localization of RITS elicits histone H3K9 methylation on overlying nucleosomes by the SET-domain methyltransferase $Clr4^{Su(var)3-9}$. These modified nucleosomes are bound by $Swi6^{HP1}$ which in turn enforces transcriptional silencing, thus forming heterochromatin and allowing centromeric cohesion to establish (Bernard, Maure et al. 2001; Nonaka, Kitajima et al. 2002).

RITS functions co-transcriptionally and in *cis*

Recent analysis of RITS activity strongly favours a model in which RITS binds to a nascent *otr* transcript by means of siRNA complementarity (Partridge, DeBeauchamp et al. 2007) (Figure 1-7). In turn, the localization of RITS to the target RNA molecule attracts the RDRC (RNA-dependent RNA polymerase complex) (Motamedi, Verdel et al. 2004; Sugiyama, Cam et al. 2005). RDRC contains the RNA-dependent RNA polymerase Rdp1, the helicase Hrr1 and the Trf4p-related RNA poly(A) polymerase Cid12 (Motamedi, Verdel et al. 2004). RdRP proteins participate in RNA silencing by generating a complementary strand to a target RNA molecule, thus generating more dsRNA for siRNA production by Dcr1 to amplify the RNA silencing response (Sijen, Fleenor et al. 2001). Similarly, RDRC is thought to serve as positive feedback to fission yeast RNAi by a similar mechanism to promote RITS-mediated heterochromatin formation. The localization of both RITS and RDRC to *otr* loci is sensitive to RNase treatment, suggesting that RNA molecules act as a platform for their recruitment (Motamedi, Verdel et al. 2004). In addition to this evidence, a genetic screen for factors involved in silencing unveiled *rpb2-m203*, a mutant in the second largest subunit of RNA polymerase II that affects transcriptional silencing at centromeric outer repeats but does not affect the expression of any heterochromatin-related factors (Kato, Goto et al. 2005). Furthermore, it has been reported that Ago1 co-immunoprecipitates with RNA polymerase II (Schramke, Sheedy et al. 2005; Schramke, Sheedy et al. 2005). This co-transcriptional action of RITS bears close resemblance to the functional connection in *A. thaliana* between AGO4, an Argonaute protein primarily involved in TGS, and RNA polymerase IV, a plant-specific DNA-dependent RNA polymerase (Herr, Jensen et al. 2005; Onodera, Haag et al. 2005). RNA polymerase IV (RNA Pol IVa) is required for producing RNAs that originate the AGO4-specific siRNAs (Herr, Jensen et al. 2005; Onodera, Haag et al. 2005; Pontier, Yahubyan et al. 2005; Pontes, Li et al. 2006). In addition, a distinct isoform of RNA pol IV holocomplex (RNA Pol IVb) interacts with AGO4 and is important for enforcing TGS (Pontier, Yahubyan et al. 2005; El-Shami, Pontier et al. 2007). Hence, it appears that the functional principle behind TGS is a crosstalk between Argonaute proteins and transcription machinery that enforces silencing at the chromatin level.

RNAi-mediated heterochromatin formation functions in a closed mechanistic loop in fission yeast. Deposition of histone H3K9me2 is largely assumed to be the product of RNAi activity but it is also the modification that allows Chp1 to bind to histone H3 tail with high affinity (Hall,

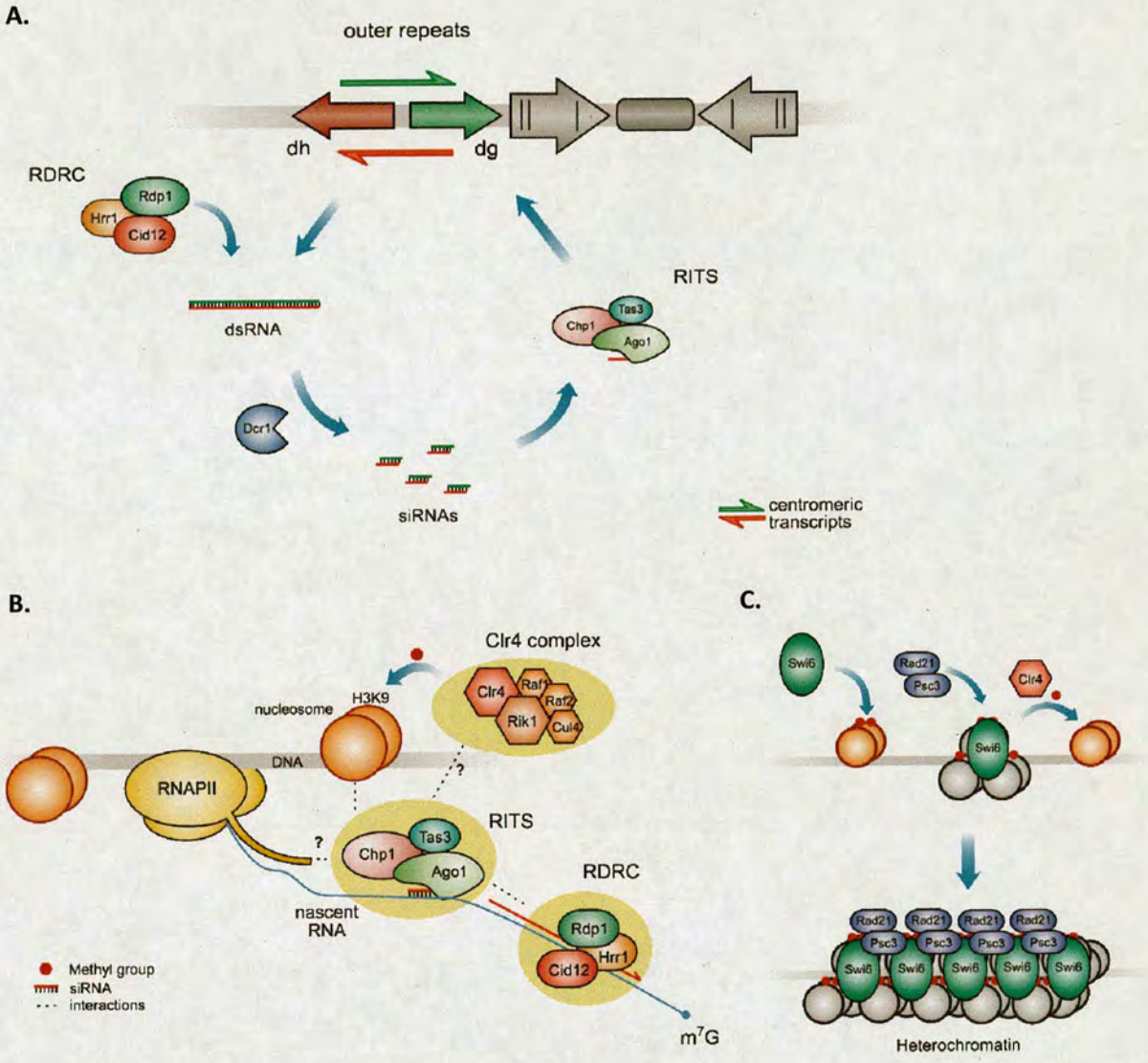


Figure 1-7: RNAi and heterochromatin assembly in S. pombe.

A. Centromeric transcripts originating from the outer repeats *dg* and *dh* form dsRNA which is then processed by Dcr1. The activity of RNA polymerase II is responsible for the presence of centromeric RNA but the RNA-dependent RNA polymerase complex (RDRC) also contributes to the formation of this double-stranded template. The resulting siRNAs are loaded into the RNAi effector complex in *S. pombe* called RITS (RNA-induced Initiation of Transcriptional Silencing) containing Ago1, Chp1 and Tas3, which then homes in on the chromatin locus.

B. Recent evidence suggests that target recognition at the outer repeats is mediated by RNA-RNA interaction between the loaded siRNA and a nascent RNA polymerase II (RNAPII) transcript. RITS interacts with RNA pol II and recruits the Clr4 histone methyltransferase complex to methylate lysine 9 of histone H3 (H3K9me2) on surrounding nucleosomes. The chromodomain of Chp1 is known to recognize this methylation mark and may contribute to tether the complex to this chromatin region.

C. Upon H3K9 methylation, binding of $Swi6^{HP1}$ ensues and promotes heterochromatin assembly by dimerization and/or recruitment of $Clr4^{Su(var)3-9}$ to methylate surrounding nucleosomes. The cohesin subunits Psc3 and Rad21 are recruited to the $Swi6^{HP1}$ scaffold thus establishing physical cohesion between sister chromatids.

Shankaranarayana et al. 2002; Partridge, Scott et al. 2002; Volpe, Kidner et al. 2002). The activity of the methyltransferase Clr4$^{Su(var)3-9}$ is not only essential for modifying histones but also to maintain RNAi activity (Noma, Sugiyama et al. 2004). The levels of centromeric siRNAs are severely depleted in the absence of *clr4+*, suggesting that H3K9me2 or the activity of Clr4$^{Su(var)3-9}$ is required for some functional feedback that allows cells to sustain RNAi activity. Thus, it was proposed that RNAi-mediated heterochromatin functions in *cis*, meaning that H3K9me2 allows RITS to bind to chromatin and control transcription locally (Noma, Sugiyama et al. 2004). RDRC is then recruited locally to promote siRNA amplification and re-inforce RITS activity (Motamedi, Verdel et al. 2004; Sugiyama, Cam et al. 2005). The consequent recruitment of Clr4-mediated H3K9methylation re-enforces the heterochromatin state. This model proposes that RNAi acts to support a heterochromatin nucleation mechanism that might be involved in expanding and stabilizing heterochromatin domains. However, it does not provide an explanation for the initial H3K9 methylation event that allows Chp1 binding and suggests that RNAi cannot function in *trans* (Grewal and Jia 2007). The latter point is controversial since, up to date, no *cis*-acting primary nucleation mechanisms have been clearly described for centromeric outer repeats. The matter is still unclear, much because fission yeast RNAi does not respond to experimental stimulation with artificial constructs as well as its counterpart in plants and metazoa. Despite the considerable genetic evidence linking fission yeast RNA interference with Clr4$^{Su(var)3-9}$ methyltransferase, there are currently no known protein-protein interactions or common components between the Clr4 complex and RITS or RDRC. The link between Clr4$^{Su(var)3-9}$ and HDACs such as Clr3 is more evident but there are also no known molecular links between any of the heterochromatic HDACs (Clr3, Clr6 and Sir2) with RNAi (Yamada, Fischle et al. 2005). Thus, the actual mechanism by which RITS induces Clr4$^{Su(var)3-9}$ to methylate histone H3K9 to form heterochromatin is still unknown.

Transcription is required to promote silencing – the paradox

There are two models that provide alternative explanations for the paradoxal requirement of transcription for establishment of silent heterochromatin. The first one is based on the description of a mutation in *rpb7*, one of the subunits of the RNA polymerase II complex. The *rpb7-1* mutant was isolated as part of a screen for suppressor of position effect at centromeres (or *"csp"* mutants) (Ekwall, Cranston et al. 1999; Djupedal, Portoso et al. 2005). The *rpb7* mutant affects RNAi

activity at the outer repeats upstream of Dcr1, at the level of production of centromeric transcripts (Djupedal, Portoso et al. 2005). This mutation, that modifies a residue (G150D) in the RNA-binding domain of Rpb7, was shown to affect the firing of an *otr*-specific promoter that is partially responsible for generating transcripts for centromeric siRNA production (Djupedal, Portoso et al. 2005). Interestingly, the *rpb7-1* mutant does not appear to affect general mRNA levels or RNA pol II competence in transcriptional elongation or termination (Djupedal, Portoso et al. 2005). Thus it was proposed that Rpb7 is a specialized RNA polymerase II component that participates in promoting transcription from promoters residing in a heterochromatic context in a form that is distinct from general mRNA expression. This model is supported by the characterization of plant RNA polymerase IV which is similarly responsible for producing transcripts involved in TGS of 5S rDNA gene clusters and *AtSN1* retroelements (Herr, Jensen et al. 2005; Onodera, Haag et al. 2005).

An alternative model proposes that transcription and establishment of fully silent centromeric heterochromatin are two temporally separated events in the fission yeast cell cycle. In wild-type cells *otr* transcripts can be faintly detected (Volpe, Kidner et al. 2002). However, based on literature it is not possible to attribute the source of this amount of *otr* RNA - whether it is due to low-level of transcription in all cells or stronger transcription in a few cells within a much larger population. In fact, most of the published molecular analyses on the various aspects of RNAi function, centromeric transcription and chromatin modifications were performed on cultures of un-synchronized cells, which contain a mixture of cells in G1, G2, S and M. Hence, it is possible that accumulation of *otr* transcripts occurs naturally on a narrow window of time during the cell cycle, after which heterochromatin is re-established and *otr* RNA is degraded. The condensed structure of the heterochromatic domains must be resolved in early S phase in order for replication of centromeric DNA to occur (Kim, Dubey et al. 2003). During DNA replication, segregation of old and newly-synthesized histone octamers occurs randomly, which effectively should result in an attenuation of H3K9me2 signal over the outer repeats (Jackson and Chalkley 1985). Consequently, transcriptional silencing may be relaxed in the stages following replication and re-activation of RNA polymerase II. Hypothetically, this would result in a peak of *otr* transcript production, to which Dcr1 would respond by generating siRNAs. Consequently, RITS would be recruited to the *otr* and reinforce H3K9me2 by recruiting $Clr4^{Su(var)3-9}$. RITS bearing siRNAs from previous iterations would also contribute to this outcome. Transcription from the *otr* would then cease as the consequence of heterochromatin being fully established in time for mitosis. Unless the behavior of RNAi is analyzed

on a cell cycle basis, such a mechanistic detail would not be evident. In fact, a recent publication addresses this issue at centromeres throughout the replication stage of the cell cycle of fission yeast (Chen, Zhang et al. 2008). Chen et al. describe that in S phase there is a brief period where centromeric repeats are preferentially transcribed by RNA polymerase II. This brief burst of transcription is linked with siRNA production and the recruitment of RNAi components and heterochromatin factors to the centromere (Chen, Zhang et al. 2008). Thus, these observations strongly support the model of a cyclic renewal of RNAi induction and recruitment of silencing factors to the centromere occurring at a particular stage of the cell cycle.

Interspersed repeats and gene silencing in fission yeast

Fission yeast is equipped with a form of RNA silencing that contributes to promote formation of heterochromatin, which in turn has a role in centromere identity and function in cell division (Volpe, Kidner et al. 2002; Hall, Noma et al. 2003; Volpe, Schramke et al. 2003). Heterochromatin assembly directed by RNAi is also important for controlling mating type switching, the main cellular differentiation process in fission yeast (Hall, Shankaranarayana et al. 2002). RNAi is also recruited to the rDNA arrays, which coincidently are also engulfed in heterochromatin, but to what purpose is still unclear (Cam, Sugiyama et al. 2005). Nevertheless, RNAi appears to act against repetitive DNA as other forms of RNA silencing do in many other systems. The novel functions assumed by this pathway in fission yeast may represent subversions of its original purpose of genome surveillance against repetitive DNA.

The genomic load of repetitive DNA in *S. pombe* is not limited to the aforementioned loci. The genome of fission yeast is populated by relatively small numbers of interspersed repeats that are derived from TEs, more specifically LTR retrotransposons (Bowen, Jordan et al. 2003). At the time when the research work described in this thesis began, previous work in our lab had proposed that interspersed LTR repeats are also targets for RNAi-mediated heterochromatin assembly (Schramke and Allshire 2003). It is critical to mention that the conclusions from this aforementioned work have since been retracted (Allshire 2005). They are mentioned in this thesis solely as a reference for the study described in Chapter 3.

S. pombe LTRs (Long Terminal Repeats) are 358 bp-long functional components of a class of TEs known as retrotransposons and they are also found in retroviruses. In the case of fission yeast, there are 275 LTRs scattered in the genome that derived from TEs of the Tf1 and Tf2 families (Bowen, Jordan et al. 2003). LTRs were shown to be transcribed in both strands and respond to RNAi mutants similarly to centromeric transcripts. LTRs also displayed enriched levels of H3K9 methylation and bound $Swi6^{HP1}$ in an RNAi and Clr4-dependent manner (Schramke and Allshire 2003).

LTR repression appears to influence expression of nearby genes. So far, 7 meiotic genes have been found to become upregulated in RNAi and heterochromatin mutants (Schramke and Allshire 2003). This influence on gene expression is also abolished once the nearby LTR repeat is deleted. The repressive effect is sensitive to the distance between the gene promoter and the LTR as no genes whose promoters are more than 10 kb away from an LTR were reported to be affected (Schramke and Allshire 2003). This phenomenon is highly reminiscent of PEV and it is likely to be caused that heterochromatin assembled over LTRs spreading and engulfing nearby genes. These findings are highly interesting since they suggest the existence of solo-repeat-based chromatin silencing mechanism that can widely employed to regulate gene expression on a lower eukaryote. The potential number of genes targeted for regulation by this mechanism is high since there are an estimated 800 genes located within 10 kb of a solo LTR.

The conclusions mentioned in the two paragraphs above have since been restracted As mentioned previously, the genomes of vertebrates, particularly mammals, are populated by hundreds of thousands of repeats scattered throughout the genome. Large numbers of these repeats are characterized by extensive histone de-acetylation, and display H3K9 di-methylation and DNA methylation, among other marks (Kondo and Issa 2003; Martens, O'Sullivan R et al. 2005). The chromatin modifications associated with TEs and related interspersed LTRs display a dynamic behaviour throughout differentiation stages of embryonic stem cells. There is also evidence supporting the activity of RNA interference against some of these sequences as part of a TE repression process that is active in mouse pre-implantation embryos(Svoboda, Stein et al. 2004). Coincidently, it is also during these stages of embryonic development and cell differentiation that significant epigenetic reprogramming occurs in the mammalian genome. In light of this, it is possible that a process of solo repeat-mediated gene repression, similar to LTR-mediated silencing in *S. pombe*, may occur in metazoa. Therefore, characterizing the mechanism of LTR silencing in *S.*

pombe could provide insights into solo repeat function and attain knowledge on the function of interspersed repetitive elements that may prove to be relevant for the genomes of higher eukaryotes.

1.10. SUMMARY AND AIMS

The epigenetic processes that control chromatin structure and the activity of underlying DNA respond to the presence of repetitive DNA. The connection between repetitive DNA and heterochromatin has functional consequences in chromosomal structure, centromere function, cell differentiation, viral defence and regulation of gene expression in a large range of eukaryotic organisms. Some of the mechanisms involved employ short RNA molecules and a conserved set of proteins (Argonautes, Dicer and RdRP) to recognize and direct specialized modification machinery to target DNA loci in order to alter the structural and functional parameters of the overlying chromatin. The same basic pathway is present in the unicellular eukaryote *S. pombe* in a minimal form that has been revealed to perform several key functions that have counterparts in higher eukaryotic organisms. Metazoa and plants are often equipped with several Dicers (2 in *D. melanogaster* and 4 in plants) and multiple Argonautes (4 in *D. melanogaster*, 8 in *H. sapiens* and 24 in *C. elegans*) that reflect the different specializations of RNA silencing but make molecular and genetic analysis difficult due to redundancy. In comparison, fission yeast is a genetically tractable system that contains only one copy of each of the three key RNA silencing genes, thus allowing for comprehensive and informative studies of the RNAi-mediated chromatin modification mechanism.

The work presented in this thesis focuses on exploring the mechanism and functional implications of RNA interference in fission yeast. The involvement of RNAi in LTR repression and gene silencing was investigated in order to confirm the existence of a gene regulatory mechanism based on this phenomenon and to further determine the extent of targeted genes in order to establish the functional impact of this mechanism on the biology of this organism. I monitored expression of genes nearby LTRs in wild-type and mutant backgrounds in order to determine if and which are under the control of RNAi and heterochromatin. RNAi activity towards LTRs was assayed as well as chromatin modifications over LTR loci. The observations collected throughout this study

challenge the report that gene expression is affected by a solo LTR-mediated regulation mechanism or that LTR themselves are targeted by RNAi or heterochromatin assembly.

A considerable part of the project was aimed at unravelling aspects of the mechanism by which RITS directs transcriptional silencing and heterochromatin assembly. One of the analysed aspects was the possible relationships between RNAi and other co-transcriptional processes, namely RNA polymerase II transcription termination, mRNA cleavage and polyadenylation. The integration of RNAi with RNA turnover by the exosome complex was also investigated. The connections between these different pathways was analysed in light of RNAi activity and its function at the centromere. For this purpose, I conducted transcriptional silencing and RNAi activity analyses on mutants for the cleavage and poly-adenylation factor *pfs2*, the rRNA maturation and transcription termination factor *dhp1* and exosome components *dis3* and *rrp6*. I concluded that neither cleavage & poly-adenylation nor transcription termination play a crucial role in RNAi-mediated heterochromatin formation. On the other hand, RNA degradation by the exosome appears to be involved in transcriptional silencing and to process centromeric transcripts, although the mechanistic details of this involvement are not clear.

Even though there is considerable evidence supporting the model that RITS acts co-transcriptionally, very little is known of the mechanism by which RITS enforces TGS and recruits chromatin modifications. I focused on Ago1 and investigated the functional relevance of its putative RNA slicing activity in TGS and heterochromatin assembly. For this purpose, I introduced point mutations predicted to abolish slicing activity in the *ago1* gene and analysed their effect in transcriptional silencing, RNAi activity and on the behaviour of RITS components. The results show that slicing is crucial for RNAi activity and consequently for siRNA-directed assembly of heterochromatin. Instead of representing a form of effecting siRNA-mediated repression, RNA slicing is an essential property of Ago1 without which it cannot perform any of its roles in the fission yeast RNAi pathway.

CHAPTER 2
MATERIALS AND METHODS

2.1. GENERAL SOLUTIONS AND MEDIA

PBS (1 litre):	10 g NaCl
	0.25 g KCl
	1.43 g Na_2HPO_4
	0.25 g KH_2PO_4
	Autoclaved
TE:	1 mM EDTA
	10 mM Tris-HCl, pH 8
	Autoclaved
20X TBE (1 litre):	Tris base 216 g
	Boric acid 110 g
	80 ml 0.5 M EDTA, pH8

Fission Yeast Media

All solutions were made up to the final volume with distilled H_2O. All the following solutions were sterilized by autoclaving, unless otherwise stated.

PMG agar (1 litre):	3 g Pthallic Acid
	2.2 g Di-sodium orthophosphate
	3.75 g Glutamic acid
	20 g D-Glucose anhydrous (Fisher Scientific)
	1.0 ml 1000x Vitamins
	0.1 ml 10.000x Minerals
	20 ml 50x Salts
	20 g Agar (OXOID)

PMG liquid (1 litre):	3 g Pthallic Acid
	2.2 g Di-sodium orthophosphate
	3.75 g Glutamic acid
	20 g D-Glucose anhydrous (Fisher Scientific)
	1 ml 1000x Vitamins
	0.1 ml 10.000x Minerals
	20 ml 50x Salts
YES agar (1 litre):	5 g Yeast Extract (DIFCO)
(no adenine)	30 g D-Glucose anhydrous (Fisher Scientific)
	0.2 g Arginine
	0.2 g Histidine
	0.2 g Leucine
	0.2 g Lysine
	0.2 g Uracil
	20 g Agar (OXOID)
YES liquid (1 litre):	5 g Yeast Extract (DIFCO)
	30 g D-Glucose anhydrous (Fisher Scientific)
	0.2 g Adenine
	0.2 g Arginine
	0.2 g Histidine
	0.2 g Leucine
	0.2 g Lysine
	0.2 g Uracil
50x Salts:	53.5 g Magnesium Chloride.$6H_2O$
	1 g Calcium Chloride.$6H_2O$
	50 g Potassium Chloride
	2 g Di-Sodium Sulphate

1000x vitamins:	0.5 g Pantothenic Acid
(100 ml)	1 g Nicotinic Acid
	1 g Inositol
	1 mg Biotin
	(Filter sterilized)
10.000x minerals:	5 g Boric Acid
	4 g $MnSO_4$
	4 g $ZnSO_4$
	2 g $FeCl_2.6H_2O$
	1.6 g Molybdic Acid
	1 g KI
	0.4 g $CuSO_4.5H_2O$
	(Filter sterilized)
Supplement stocks:	50x Adenine 5g/l
	100x Arginine 10g/l
	100x Histidine 10g/l
	100x Leucine 10g/l
	100x Uracil 10g/l (dissolved by adding NaOH)
Malt Extract plates:	20 g/l agar
	30 g/l malt extract (OXOID)
	250 mg/l Adenine
	250 mg/l Arginine
	250 mg/l Histidine
	250 mg/l Leucine
	250 mg/l Uracil
5-FOA plates:	PMG or YES-agar
	1g/l 5-FOA (Melford Laboratories)
	(added to melted agar below 60°C)

TBZ plates: YES-agar

TBZ (stock 10 mg/ml in DMSO) to 10 µg/ml or 20 µg/ml added to melted agar below 60°C

Phloxin B plates: PMG or YES-agar

2.5 µg/ml Phloxin B

2.2. FISSION YEAST PROTOCOLS

MEDIA AND GROWTH

Haploid strains of S. pombe grow at the following generation times.

Medium	Temperature ºC	Generation time
Yeast extract (rich)	25	3h
	28	2h 40min
	32	2h 10min
	36	2h
minimal	25	4h
	28	3h
	32	2h 20min

For mutant strains the generation times may be longer. The time required for the cell population to double in size can be calculated more precisely using the following equation:

$$T=\frac{\log(2^{t2-t1})}{\log(\frac{y}{x})}$$

T is the generation time from the table above while y is cells/ml at time t2 and x is cells/ml at time t1.

Temperature sensitive strains were grown at 25°C and 36°C was the restrictive temperature. Cold-sensitive strains were grown at 36°C and the restrictive temperature was 18°C. Some of the experiments were conducted at semi-restrictive temperature which was 32°C.

Liquid cultures

For physiological experiments, it is important that cultures are maintained in mid-exponential phase between 2 x 10^6 and 1 x 10^7cells/ml. To generate cultures in mid-exponential growth, a fresh colony or overnight patch of a strain of known genotype was inoculated in 10 ml of YES (or minimal medium if the strain carried a plasmid with an auxotrophic marker) and incubated during the night at the appropriate temperature for the culture to reach early stationary phase. The next day, this pre-cultured was used to inoculate a larger culture, taking into consideration the generation times.

The size of the culture flask was selected according to the required volume of culture:

Culture volume	Size of culture flask
up to 10 ml	25 ml
up to 50 ml	100 ml
up to 100 ml	200 ml
up to 125 ml	250 ml
up to 250 ml	500 ml
up to 500 ml	1000 ml
up to 1000 ml	2000 ml

The media used for growing S. pombe are described below. YES liquid or solid medium prepared with agar was used whenever possible for vegetative growth.

Temperature/cold sensitivity

Temperature sensitive (ts) and cold sensitive (cs) were checked by replica plating onto YES plates containing phloxin B and incubated at the restrictive temperature. Phloxin B is a dye that permeates the cell membrane of fission yeast cells but is actively pumped out. Sick or dead cells fail to do so and become stained by accumulating Phloxin B.

Cell counting

The density of fission yeast cell cultures was analysed using a Coulter Counter Z1 (Beckman Coulter). This device has the advantage that samples can be taken quickly during an experiment and the processed at leisure. The device plots a histogram of the cell volume within the population of counted cells, which can be used to assay the growth state of the cells as well as detect bacterial contamination

Alternatively, the haemocytometer was used. The haemocytometer is a specialized microscope slide in which 2 grids have been engraved in a central region that is 0.1 mm lower than the rest of the slide. Each grid comprises 25 large squares, each containing 16 smaller squares. A coverslip is applied to the slide and 10 µl of cell culture is pipette under the coverslip. Multiplying the total number of cells in the 25 large squares by 10^4 yields the number of cells/ml.

Auxotrophy

The auxotrophic markers most commonly used in *S. pombe* required adenine, arginine, histidine, leucine, lysine and uracil. Cells were grown in the presence of 100 mg/L (4 ml of 10 mg/ml stock solution per 400 ml medium). To test for auxotrophy in a certain strain, cells were spread on a medium plate to obtain single colonies after growth. Then the colonies were replica plated onto minimal medium containing or not the appropriate supplement. The plates were grown for 1-2 days and then examined for growth under the different conditions.

Serial dilution assay

To assess the growth of various mutant strains on different media or at different temperatures, cells from a fresh plate were resuspended in sterile dH_2O and diluted 10 fold in a 96-well microtiter plate. Cells were then spotted onto appropriate media using sterilized metal 'hedgehog' and plates were incubated at the desired temperature for a minimum of 3 days.

2.3. MOLECULAR GENETICS

Transformation

A minimum of 50ml of culture grown to a density of 1×10^7 cells/ml in YES (5×10^8 cells) was required for each transformation. Cells were collected from culture by spinning at 3200 rpm for 5 minutes at 20°C.

- **Lithium acetate**

  Cells were washed once in 10 ml 0.1 M LiOAc pH 4.95 and then resuspended in 10 ml 0.1 M LiOAc pH 4.95 and incubated at 32°C for 30 minutes. Cells were then resuspended at 10^9/ml in 0-1 M LiOAc pH 4.95. 150 µl of cell suspension was mixed with 1 µg DNA and 370 µl of PEG 3350 (50% solution dissolved in TE ph8) and then incubated for a further 30 minutes at 32°C. Subsequently, cells were heat-shocked at 42°C for 20 minutes and then resuspended in selective liquid media for at least 3 hours before plated on appropriate selective media.

- **Electroporation**

  Cells were washed three times with 10 ml of ice-cold 1.2 M sorbitol solution. After the final wash, the cells were resuspended at 10^9/ml. 200 µl of cell suspension was mixed with 100 ng of plasmid or 10 µg of linearized DNA in a chilled transformation cuvette. Cuvette was placed in slot of electroporator (Biorad) and was pulsed briefly with the settings 1.5 kV, 200 ohms and 25 µF. 500 µl of ice-cold 1.2 M sorbitol was added to the cuvette and mixed gently. The resulting suspension was spread onto 12 plates of appropriate media. Transformants appeared after 3-4 days of growth at 32°C. When transforming DNA fragments intended to integrate into the genome by homology recombination, 5-10 µg of fragment DNA was co-transformed with 10 ng

of LEU2$^+$ plasmid to minimize background. Cells were selected on –LEU plates before replica plating onto media selecting for the inserted DNA.

Genetic crosses

Crosses were carried out on nitrogen starved malt extract (ME) or pombe minimal medium (PMG). To cross two strains, a loopful of a freshly grown h+ strain and a loopful of freshly grown h- strain were mixed together on a plate. The cross was then incubated at 25°C (or 36°C if one of the strains was cold-sensitive) to allow conjugation. Fully formed ascii containing four spores are visible after 2-3 days of incubation at 25°C.

- **Random spore analysis**

All crosses performed for this thesis were processed by random spore analysis. A 2-3 day old cross was checked for the presence of ascii by light microscopy. A loopful of mating mixture was resuspended in 300 µl of 1 in 100 dilution of glusulase and incubated at 32°C overnight or at room temperature for 2 days. Glusulase is a crude snail gut enzyme that breaks down the vegetative cells and the wall of the ascus. Between 200 – 1000 spores were then plated on YES agar or selective media at appropriate temperature until colonies are formed.

2.4. DNA AND RNA PROTOCOLS

Preparation of genomic DNA

5 ml of stationary phase culture was pelleted at maximum speed in a benchtop centrifuge. The pellet was resuspended in 250 ml SP1 buffer containing 0.4 mg/ml Zymolyase 100-T and incubated at 37°C for 1 hour. Spheroplasted cells were pelleted at 8,000 rpm in a eppendorf centrifuge for 15 seconds. The pellet was resuspended in 500 µl TE and 50 µl 10% SDS was added, followed by vortexing and the addition of 165 µl 5 M KOAc. Samples were incubated on ice for 30 minutes and spun in a microfuge for 10 minutres. The supernatant was then added to 750 µl isopropanol and placed in dry ice for 10 minutes. Samples were spun for 10 minutes in a microfuge and the pellet was allowed to dry. Pellet was then resuspended in 300 µl TE containing 10 µg/ml RNase and incubated for 1 h and 30 minutes at 37°C. DNA was then extracted by

phenol/chloroform and precipitated with 3 volumes of ethanol and 1/10 volume of 3 M NaOAc. Genomic DNA was then resuspended in 20 µl TE.

Rapid preparation of genomic DNA (SPZ)

A small amount of cells was picked from a fresh plat using a sterile cocktail stick and were resuspended in a microfuge tube containing 20 µl of SPZ buffer + 0.5 µl Zymolyase 100-T (10 mg/ml). After mixing, tubes were incubated at 37°C for 20 minutes. 200 µl of sterile dH_2O was then added, the tubes were vortexed and 2 µl of the mixture was used in a 20 µl PCR reaction.

SPZ buffer: 1.2 M sorbitol, 100 mM sodium phosphate pH 7.4, 2.5 mg/ml Zymolyase 100-T. Stored at 20°C.

Preparation of total RNA

A 10 ml culture of cells was grown in YES or appropriate media to a density of up to 1×10^7 cells/ml. Cultures were pelleted by centrifugation, washed with TE and transferred to microfuge tubes prior to being resuspended in 300 µl RNA extraction buffer. 300 µl of acid-washed glass beads (Sigma) were added followed by 300 µl phenol/chloroform 4:1 pH 4.7 (Sigma). The microfuge tubes were shaken at high speed on a multi-head vortexer for 30 minutes at 4°C to lyse the cells, followed by centrifugation at 10,000 rpm at 4°C for 5 minutes and removal of supernatant. The supernatant was extracted with phenol/chloroform and then with chloroform. The RNA was precipitated with 3 volumes of ice-cold ethanol and centrifuged at 10,000 rpm for 20 minutes at 4°C. The pellet was air-dried and resuspended in 25 µl dH_2O or 50% formamide (freshly prepared, Sigma). 50% formamide preserves the integrity of the RNA but is not suitable for enzymatic procedures, such as cDNA synthesis by reverse transcriptase. The concentration of each sample of RNA was determined by spectrophotomery analysis on a Nanodrop ND-1000 (Thermo Fisher Scientific).

RNA extraction buffer: 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 mM NaCl, 1% SDS.

Reverse transcriptase PCR (RT-PCR)

For RT-PCR 1 µg of each sample of RNA was aliquoted into a microfuge tube. The samples were mixed with dH_2O to a final volume of 8 µl, boiled for 5 minutes at 95°C and allowed to cool down at room temperature for a few seconds. This step favours the breakage of RNA-DNA hybrids that are shielded from DNase digestion. 1 µl of DNase I (Invitrogen) and 1 µl of DNase buffer were added and the reaction was allowed to occur at 25°C for 1 hour. Once completed, the reaction was stopped with 1 µl 25mM EDTA followed by incubation at 65°C for 10 minutes. The DNase digestion was confirmed at this stage by using 1 µl on a PCR reaction. Once completed, 1 µg of oligo dT_{17} or other specific oligo was added to the samples along with dH_2O to a final volume of 24 µl. The samples were allowed to anneal for 10 minutes at 70°C and then placed on ice. Samples were collected by brief centrifugation at 4°C and returned to ice before adding 12 µl 5x Superscript II First Strand buffer (Invitrogen), 2 µl 2.5 mM dNTPs and 1 µl 0.1 M DTT. The samples were then split into two 19 µl aliquots and incubated for 5 minutes at 42°C. 1 µl Superscript II reverse transcriptase was added to one of each of the pair of tubes (marked +RT). The tubes were then incubated at 42°C for 50 minutes and then 70°C for 15 minutes until they were returned to ice. 1 µl of the final cDNA was assayed in a 20 µl PCR reaction to measure transcript levels.

Real time PCR (qPCR)

Real time PCR reactions were carried out using a customized PCR mixture containing AmpliTaq Gold polymerase (Applied Biosystems), SYBR Green I (Molecular probes), dNTPs (Roche Applied Sciences) and a variety of stabilizers and PCR enhancers. The mixture and PCR conditions were tested and optimized with a variety of cDNA and ChIP samples until it performed adequately when compared to commercial reagents.

- **Primers**

Primer oligos were designed for all the analysed genes and DNA loci using Beacon Designer 6 (Premier Biosoft). The software selected primer sequences in the basis of best annealing parameters (Tm 50°C or higher), product size (between 75 and 200 bp), no sequence homology in target area (BLAST analysis), low secondary structure and both self- and cross-annealing properties ($\Delta G > -3.0$ Kcal/mol).

- **Reaction set up**

The reactions were carried out in 25 µl volumes, with 12.5 µl of PCR mixture and 12.5 µl comprised of 4 pmol of each primer oligo, 2 µl of sample (diluted cDNA or ChIP DNA) and dH_2O. The reactions were carried out in 96-well optical PCR plates (Eurogentec) sealed with optical film (ABI Prism) in a Biorad iCycler PCR machine.

- **PCR programme**

The PCR programme consisted of an initial 10 minute long denaturation step at 95°C followed by 40 iterations of the following cycle: 15 seconds at 95°C, 30 seconds at 50°C, 30 seconds at 72°C. This programme was followed by a melt curve analysis in order to visualize possible amplification of primer dimers.

- **Standard curve**

Each pair of primer oligos was assayed by serial dilution analysis, in which a series of 10 fold dilution of a ChIP total DNA extract was amplified in triplicate. The average cycle threshold (Ct) was calculated for each dilution step and the standard amplification curve was derived by the iCycler software. These analyses provided the PCR efficiency rate for each primer pair that was later used in the quantifications:

$$E = 10^{\frac{-1}{m}}$$

E is the PCR efficiency value and m and the slope of the standard curve.

- **Quantification methods**

The quantifications were made by two methods. The first was quantification relative to standard DNA (in arbitrary units) and was applied to Chromatin IP samples. The undiluted standard used for serial dilution was set to "1" and all the samples were quantified in function of it. The internal ratios of target gene versus control gene in each sample were then compared between IP and Total samples to yield the the final enrichment values. The second method was the Pfaffl relative quantification model (Pfaffl 2001) :

$$Ratio = \frac{E_{\text{target}}\,^{(Ct\ \text{control} - Ct\ \text{mutant})}}{E_{\text{reference}}\,^{(Ct\ \text{control} - Ct\ \text{mutant})}}$$

Etarget is the PCR efficiency value for the oligos targeting the analysed gene while Ereference is the same value for the reference gene. Ct is the mean threshold cycle for each set of triplicate reactions performed on either control (e.g. wild-type) or mutant strain sample.

- **Reagents**

Custom qPCR reagent (for 5ml): 1385 µl dH_2O, 1000 µl AmpliTaq Gold buffer (10x), 1000 µl $MgCl_2$ (25 mM), 80 µl dNTPs (25 mM each), 25 µl SYBR Green I (1:100), 10 µl Fluorescein (10 µM), 800 µl DMSO, 600 µl 50% Glycerol, 50 µl 10% Tween 20, 50 µl AmpliTaq Gold (5U/ul).

The solution was mixed well before adding the enzyme. I made separate aliquots (600 µl) and keep at -20°C. Filter tips and freshly autoclaved water were used. Dedicated stocks were kept for all components of the mixture that were renewed frequently. Diluted SYBR Green I is unstable and its degradation product is a strong PCR inhibitor. Fresh dilutions of SYBR Green I in DMSO or TE ph 7.5 were prepared and stored at 4°C for a maximum of two weeks.

Preparation of small RNA

This protocol is basically a scaled up version of the previous one. A 50 ml culture of cells was grown in YES or appropriate media to a density of up to 1 x 10^7 cells/ml. Cultures were pelleted by centrifugation, washed with TE and transferred to microfuge tubes prior to being resuspended in 500 µl RNA extraction buffer. 500 µl of acid-washed glass beads (Sigma) were added followed by in 500 µl phenol/chloroform 4:1 pH 4.7 (Sigma). The microfuge tubes were shaken at high speed on a multi-head vortexer for 45 minutes at 4°C to lyse the cells, followed by centrifugation at 10,000 rpm at 4°C for 8 minutes and removal of supernatant. The supernatant was extracted with phenol/chloroform and then with chloroform. PEG8000 and NaCl were added to the supernatant up to 10% and 0.5M final concentration respectively and incubated on ice for 30 minutes. This caused the precipitation of the large rRNA, mRNA and genomic DNA molecules from the supernatant, leaving the smaller RNAs in solution. The supernatant containing only the small RNAs was precipitated by adding 3 volumes of ice-cold ethanol and incubating at -20°C for a minimum of 3 hours. The PEG-precipitated fractions were recovered by washing with 70% ethanol and resuspending in 25 µl dH_2O or 50% formamide. These are suitable for northern analysis and RT-PCR. The small RNA fractions are recovered by adding 1 µl of glycogen (20 mg/ml, Roche Applied

Science) and spinning at 10,000 rpm for 30 minutes at 4°C. The resulting pellets were washed once with 80% ethanol and spun again at 10,000 rpm for 15 minutes at 4°C. After careful removal of the supernatant, the pellet was air dried for 15 minutes and resuspended in 25 µl 50% formamide. The concentration of each sample of RNA was determined by spectrophotomery analysis on a Nanodrop ND-1000 (Thermo Fisher Scientific). Small aliquots of the large and small RNA fractions were loaded side by side and migrated on a standard, non-denaturing agarose mini-gel. Both large rRNA bands should appear in the large fraction lanes and not in the small RNA fraction. The only visible band in the small RNA lanes, consisted of 5S, 5.8S rRNAs and tRNAs, should migrate near the 100 bp DNA marker.

Samples were stored at -80°C.

RNA extraction buffer: 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 mM NaCl, 1% SDS.

Northern analysis

- **Large RNA analysis**
  - **Formaldehyde gel electrophoresis** (Sambrook and Russell 2006)

    This method was used for large RNA analyses described in chapters 3 and 4.

    Samples of 10 µg of total or large RNA were diluted in 3 volumes of sample loading buffer, vortexed and denatured for 10 minutes at 65°C before cooling on ice for 5-10 minutes. Samples were loaded on a 1% agarose-formaldehyde gel that had been cast and set in the fume hood. The gel was run at 80 V for a total of 4 hours. When the bromophenol blue dye had migrated to approximately 2 cm from the bottom of the gel, the run was stopped and the gel photographed under UV next to a fluorescent ruler.

    Running buffer: 1x HEPES ph 7.8

    Sample loading buffer (for 1 ml): 100 µl 10x HEPES pH 7.8, 500 µl formamide, 160 µl 37% formaldehyde, 170 µl 50% glycerol, 5 µl ethidium bromide (10 mg/ml), 65 µl dH_2O.

- **Glyoxal/DMSO gel electrophoresis** (Sambrook and Russell 2006)

This method was used for large RNA analyses described in chapter 5.

Samples of 10 µg of total or large RNA were diluted in 5 volumes (minimum) of glyoxal reaction mixture, incubated at 55°C for one hour and then chilled for 10 minutes on ice until ready to use. 1-2 µl of RNA loading buffer was added to the samples before loading onto the gel. The gel was run at 80 V for approximately 4 hours, until the dye front was 2 cm from the end of the gel. After running, the gel was photographed under UV next to a fluorescent ruler.

This method of RNA electrophoresis is more prone to RNA degradation by RNase contamination. Before casting the gel, all the required equipment was thoroughly washed with hot water and detergent, rinsed with 3% hydrogen peroxide, rinsed several times with ddH_2O and finally rinsed with 70% ethanol before air drying.

Running buffer: 1x BPTE electrophoresis buffer

Glyoxal reagent mixture (for 10 ml): 6 ml of DMSO, 2 ml of deionized glyoxal, 1.2 ml of 10x BPTE electrophoresis buffer, 0.6 ml of 80% glycerol, 0.2 ml of ethidium bromide (10 mg/ml). The mixture was separated into 500 µl aliquots and stored at -70°C. Aliquots were not used more than once.

10x BPTE electrophoresis buffer: 100 mM PIPES, 300 mM Bis-Tris, 10 mM EDTA. Final pH was approximately 6.5.

- **Transfer**

The gel was soaked in 5 volumes of 2x SSC for 20 minutes. In the meantime, the membrane (Hybond NX, GE Healthcare) was pre-conditioned by first soaking briefly in dH_2O and then in 10x SSC for 10 minutes. Inside a tray filled with 20x SSC solution, the following stack was assembled: a large gel tray upside-down, 3 large pieces of pre-soaked 3MM paper touching the 20x SSC solution to serve as wick, 3 gel-sized pieces of pre-soaked 3MM, the gel upside down, the membrane, 3 membrane-sized pieces of pre-soaked 3MM, a large stack of paper towels, a glass or hard plastic plate and finally

a weight of approximately 500 g. Care was taken while assembly the stack so that no air bubbles were trapped. The membrane was carefully laid on top of the gel and was not moved or adjusted afterwards due to contact transfer. The stack was insulated on all four sides of the gel with cling film to prevent the solution from bypassing the gel and short-cutting to the stack. The transfer was left for a minimum of 16 hours. After disassembly, the position of the gel was marked on the membrane with a pencil. The membrane was dried under a hood and cross linked twice using an Autocrosslink Stratalinker (1200 joules/cm^3). Both the transferred gel and membrane were photographed under UV to confirm the transfer and obtain a loading control from the membrane. After this, the membrane was stored at room temperature until required.

20x SSC: Dissolve 175.3 g of NaCl and 88.2 g of tri-sodium citrate in 800 ml of H_2O. Adjust the pH to 7.0 with a few drops of a 14 *N* solution of HCl. Adjust the volume to 1 litre with H_2O and autoclave.

- **Hybridization**

The membrane was re-soaked in 2x SSC and then placed in a hybridization bottle. In the case of glyoxal/DMSO treated RNA, the membrane was first incubated in TE pH 8 for 15 minutes and then washed with 2x SSC. Once re-soaked, the membrane was pre-hybridized in 25 ml of modified Church-Gilbert buffer at 65°C in a roller oven for one hour. While the pre-hybridization was ongoing, the radiolabelled probed was prepared.

Radiolabelled probes were prepared by the random priming method using the High Prime labelling kit (Roche Applied Science). 25ng of template DNA were added to H_2O to a total volume of 11 µl, denatured at 95°C for 10 minutes and then quickly chilled on ice for 5 to 10 minutes. 4 µl of High Prime reaction mixture were added to the denatured DNA along with 5 µl of 50 µCi [α^{32}P] dCTP (GE Healthcare). The reaction was quickly mixed, spun down and incubate at 37°C for 45 minutes. The reaction was stopped by adding 30 µl of 25 mM EDTA and incubating at 65°C for 2 minutes. The unincorporated nucleotides were removed using a Sephadex G25 microcentrifuge column (Microspin G25, GE healthcare). To estimate the incorporation rate, 1 µl samples are taken before and after running the probe through the column. These are

mixed with 49 µl H_2O and the whole volume is placed in a counter vial with 10 ml of scintillation fluid and allowed to settle. The vials are counted in a scintillation counter using the ^{32}P programme for a minimum of 2 minutes. The estimated incorporation rate is calculated by the ratio of the counts per minute (cpm) of the probe after clearing the column over the same before the column. Probes with less than 15% are discarded. The probe was denatured at 95°C for 10 minutes and then cooled down quickly on ice. The probe was quickly mixed with 1 ml of pre-warmed hybridization buffer and transferred to enough volume of hybridization buffer. Rapid mixing is required to prevent the labelled DNA from reanneling, which would cause the hybridization to fail. The final probe concentration used was 1×10^6 cpm/ml of hybridization buffer.

Once the pre-hybridization of the membrane was completed, the solution was exchanged by the probe and left incubating overnight at 65°C. Once the hybridization was completed, the membrane was washed as follows: rinsed briefly with 25 ml 2x SSC; washed twice with 25 ml of 2x SSC, 1% SDS for 5 minutes; washed twice with 25 ml of 0.5x SSC, 0.1% SDS for 10 minutes. The membrane was rinsed with 2x SSC to wash off the SDS, wrapped in plastic sleeve or Saran wrap and placed in a cassette with a Storage Phosphoscreen (GE Healthcare) for a minimum of 4 hours. After exposure, the phosphoscreen was scanned on a Storm phosphorimager (GE Healthcare).

Hybridization buffer (modified Church-Gilbert buffer): 0.5 M sodium phosphate pH 7.2, 7% SDS, 10mM EDTA.

- **Small RNA analysis**
  - **Mini denaturing PAGE and electrotransfer**

    This method was used for small RNA analyses described in chapters 3 and 4.

    Electrophoresis on 17.5% polyacrylamide 7M urea gel was performed on mini-protein gel apparatus (Hoefer) with 1.5mm comb and spacers. To prepare 30 ml of gel solution (enough for 2 gels), 12.6 g of urea (Gibco-BRL) was dissolved in 13.1 ml of 40% acrylamide:bisacrylamide 19:1 (Severn Biotech Ltd.) and 1.5 ml of 10x TBE while at 37°C. Once the solution was cooled, 15 ml of the gel solution was mixed with 240 µl of

10% ammonium persulfate (freshly prepared) and 11 µl TEMED. The solution was thoroughly mixed and poured into the gel apparatus. Once polymerized, the gel was set up with 0.5x TBE running buffer. The gel was pre-run for at least 30 minutes at 80 V. Prior to sample loading, the wells were thoroughly washed with a syringe and fresh 0.5x TBE.

Samples of at least 20 µg of small RNA were mixed with sample buffer, denatured at 95°C for 5 minutes and left on ice until loading. Once the samples were loaded using duckbill tips, the gel was run at 80 V until the dye front reached the bottom of the gel. The gel apparatus was disassembled and the gel was stained in 0.5x TBE containing 1 µg/ml ethidium bromide for 10 minutes. Subsequently, it was washed in fresh 0.5x TBE and then photographed in a UV transilluminator (Kodak).

The gel was transferred onto membrane using a Biorad wet electroblotting apparatus. The gel was equilibrated briefly in 0.5x TBE and then assembled in a blotting cassette. From the cathode (-) to the anode (+), the stack was thus assembled: soaked sponge, 3 pre-soaked gel-sized pieces of 3MM, pre-equilibrated gel, pre-soaked membrane (Hybond NX), 3 pre-soaked gel-sized pieces of 3MM, soaked sponge. The transfer was left at 100 V, 400 mA, 10 W for an hour at 4°C.

4x RNA sample buffer: 5 mM EDTA, 0.03% bromophenol blue, 50% glycerol, 50 mM Tris-Cl pH 7.7.

- **Large denaturing PAGE and transfer**

This method was used for small RNA analyses described in chapter 5.

The gel was prepared with the large Hoefer SE600 Ruby apparatus, using 1.5 mm comb and spacers. This gel system allows for better resolution that the previous system. The 8% polycacrilamide-urea gel was prepared with Sequagel sequencing gel solutions (National Diagnostics). For 50 ml (1 gel), 16 ml of Sequagel concentrate were mixed with 29 ml of diluent and 5 ml of 10x TBE 7.5 M urea. To polymerize, 400 µl of 10% ammonium persulfate (freshly prepared) were added together with 20 µl TEMED. The mixture was poured into the apparatus and the gel was allowed to polymerize for

30 minutes or longer. Once it was ready, the gel was set in the electrophoresis tank with 1x TBE. The wells were washed with fresh running buffer using a syringe and then the gel was pre-run at 150 V for 30 minutes.

Samples of at least 20 µg small RNA were mixed with equal volume of 2x FDE loading buffer, denatured at 65°C for 15 minutes and placed on ice until loading. Loading was performed with duck bill tips once the wells had been washed for a second time. The gel was run at 300 V for 2-3 hours until the dye front is 2 cm from the bottom. Once finished, the apparatus was disassembled and the gel cut with a scalpel above the xylene cyanol band. The top portion of the gel was stained in 1x TBE 1 µg/ml ethidium bromide for 10 minutes, destained in fresh 1x TBE for 20 minutes and photographed under the UV. The 5S, 5.8S rRNAs and tRNAs are visible in this section of the gel and provide a loading control. The bottom part of the gel contains all the RNA molecules with sizes below approximately 80 nt.

Prior to transfer, the bottom part of the gel is soaked in 10 mM sodium phosphate buffer ph 7 for 10 minutes and then washed in 20x SSC for another 10 minutes. In the meantime, a membrane fragment (Hybond NX, GE Healthcare) cut to the size of the gel was pre-conditioned by first soaking briefly in dH_2O and then in 10x SSC for 10 minutes. Inside a tray filled with 20x SSC solution, the following stack was assembled: a large gel tray upside-down, 3 large pieces of pre-soaked 3MM paper touching the 20x SSC solution to serve as wick, 3 gel-sized pieces of pre-soaked 3MM, the gel upside down, the membrane, 3 membrane-sized pieces of pre-soaked 3MM, a large stack of paper towels, a glass or hard plastic plate and finally a weight of approximately 500 g. Care was taken while assembly the stack so that no air bubbles were trapped. The membrane was carefully laid on top of the gel and was not moved or adjusted afterwards due to contact transfer. The stack was insulated on all four sides of the gel with cling film to prevent the solution from bypassing the gel and short-cutting to the stack. The transfer was left for a minimum of 16 hours. In the end, only a few paper towels are soaked due to reduced permeability of the polyacrylamide gel. After disassembly, the position of the gel was marked on the membrane with a pencil. The membrane was dried under a hood and cross linked twice using an Autocrosslink

Stratalinker (1200 joules/cm^3). After this, the membrane was stored at room temperature until required.

2x FDE sample buffer (for 10 ml): 10 ml deionised formamide, 200 µl 0.5M EDTA pH 8, 10 mg xylene cyanol, 10 mg bromophenol blue.

20x SSC (for 1 l): Dissolve 175.3 g of sodium chloride and 88.2 g of sodium citrate in 800 ml of H_2O. Adjust the pH to 7.0 with a few drops of a 14 *N* solution of HCl. Adjust the volume to 1 litre with H_2O and autoclave.

- **Hybridization**

The membrane was re-soaked in 2x SSC and then placed in a hybridization bottle where it was pre-hybridized in 25 ml of modified Church-Gilbert buffer at 42°C for one hour. While the pre-hybridization was ongoing, the radiolabelled probed was prepared.

Random-primed radiolabelled DNA probes were employed for siRNA detection. The procedure used in preparation of these probes was identical to the one for northern analysis of large RNAs (see above). In addition, a radiolabelled DNA oligo was used as loading control probe (snoR58). These were prepared using a T4 polynucleotidyl kinase (PNK) end-labelling kit (Roche Applied Sciences). Briefly, 0.4 µl of DNA oligonucleotide (10 µM) was mixed with 1 µl T4 PNK buffer (10x), 6.6 µl of H2O, 1 µl of PNK and 1 µl of 50 µCi [γ^{32}P] ATP. The reaction was incubated at 37°C for 45 minutes and then added to the probe mixture.

Once the pre-hybridization of the membrane was completed, the solution was exchanged by the probe and left incubating overnight at 42°C. Once the hybridization was completed, the membrane was washed at 50°C at least twice with 25 ml 2x SSC, 0.2% SDS. The membrane was rinsed with 2x SSC to wash off the SDS, wrapped in plastic sleeve or Saran wrap and placed in a cassette with a Storage Phosphoscreen (GE Healthcare) for a minimum of 4 hours. After exposure, the phosphoscreen was scanned on a Storm phosphorimager (GE Healthcare).

Hybridization buffer: PerfectHyb (Sigma) or modified Church-Gilbert buffer: 0.5 M sodium phosphate pH 7.2, 7% SDS, 10mM EDTA.

2.5. PROTEIN TECHNIQUES

Total protein extraction from fission yeast

A 10 ml cuture was grown to log phase in YES or minimal medium and cells were harvested by spinning in a benchtop centrifuge at 3000 rpm for 2 minutes. Pellet was resuspended in 1 ml PEMS and transferred to a microfuge tube. Pellet was then resuspended at 10^8 cells/ml in PEMS containing 0.4 mg/ml Zymolyase 100-T and incubated at 37°C for 20 minutes. Spheroplasted cells were then washed in PEMS and resuspended in 5 x 10^7 cells per 100 µl 2x sample buffer (containing freshly added PMSF). Samples were vortexed vigorously and boiled for 5 minutes at 95°C on a heat block. Samples were spun briefly to pellet cellular debris before loading on gel (10 µl / 5 x 10^6 cells per lane) or freezing at -20°C.

2x sample buffer: 2% SDS, 50 mM Tris-Cl pH 6.8, 2 mM EDTA, 10% glycerol, 0.03% bromophenol blue, 2% β-mercaptoethanol.

Alternative protein extraction method

Depending on the protein, the above protocol may result in excessive degradation. The following method is less aggressive and yields protein extracts of higher quality.

A 50 ml cuture was grown to log phase in YES or minimal medium and cells were harvested by spinning in a benchtop centrifuge at 3000 rpm for 2 minutes. The cells were washed with ice cold PBS and spun again at 3000 rpm for 2 minutes. Most of the PBS was removed while the remainder was used to ressuspend the cells and move them to a fresh tube. The PBS wash was repeated, the cells resuspended in PBS and then frozen in liquid nitrogen by dropping slowly the cell suspension using a pipette or syringe. The frozen pellets can be kept at -80°C.

A mortar and pestle were was put in an ice bucket full of dry ice and pre-chilled with liquid nitrogen before adding the cell pellets. The pellets were manually ground in the presence of liquid nitrogen for 20 minutes. The lysis rate was checked under the microscope until it was above an estimated 50% lysis. The powder was then resuspended in 250 µl 2x sample buffer and incubated for 10 minutes at room temperature. Samples were spun briefly to pellet cellular debris before loading on gel (5 to 10 µl) or freezing at -20°C.

2x sample buffer: 2% SDS, 50 mM Tris-Cl pH 6.8, 2 mM EDTA, 10% glycerol, 0.03% bromophenol blue, 2% β-mercaptoethanol.

SDS-PAGE (Laemmli 1970)

Proteins were separated on 1 mm thick discontinuous SDS-PAGE (sodium dodecyl suphate-polyacrylamide gel electrophoresis) with the Hoefer minigel apparatus. The percentage of polyacrylamide in the resolving gel was selected to allow optimum separation of proteins within the size range required.

Resolving gel (for 10 ml):

8%: 2.7 ml 30% acrylamide/bis-acrylamide mix (Sigma), 4.55 ml dH_2O, 2.5 ml 1.5 M Tris-Cl pH 8.8, 100 µl 10% SDS, 100 µl 10% ammonium persulphate (freshly prepared), 10 µl TEMED.

10%: 3.3 ml 30% acrylamide/bis-acrylamide mix (Sigma), 3.25 ml dH_2O, 2.5 ml 1.5 M Tris-Cl pH 8.8, 100 µl 10% SDS, 100 µl 10% ammonium persulphate (freshly prepared), 10 µl TEMED.

12%: 4 ml 30% acrylamide/bis-acrylamide mix (Sigma), 3.25 ml dH_2O, 2.5 ml 1.5 M Tris-Cl pH 8.8, 100 µl 10% SDS, 100 µl 10% ammonium persulphate (freshly prepared), 10 µl TEMED.

Stacking gel (for 10 ml, 2ml per gel):

5%: 1.7 ml 30% acrylamide/bis-acrylamide mix (Sigma), 6.95 ml dH_2O, 1.25 ml 1.5 M Tris-Cl pH 8.8, 0.1 ml 10% SDS, 1 µl 10% ammonium persulphate (freshly prepared), 10 µl TEMED.

The ammonium persulfate and TEMED were added only before pouring. The resolving gel was poured first, followed by 2 ml of the stacking gel into which the comb was inserted. Gels were run in 1x SDS running buffer at 180V for approximately 40 minutes. Gels were stained using SimplyBlue SafeStain (Invitrogen) to reveal protein according to manufacturer's instructions.

5x SDS running buffer (for 1 l): 30 g Tris base, 144 g glycine, 5 g SDS.

Western analysis

Proteins were transferred on Protran nitrocellulose (Schleicher & Schuell) using a Hoefer semi-dry electroblotter. The membrane floated on dH_2O, soaked in blotting buffer and then placed on top of 6 pieces of 3MM paper of the size of the gel. The SDS gel was placed on top of the membrane followed by 6 more pieces of 3MM paper soaked in blotting buffer. As each layer was added, bubbles were rolled out using a glass tube. Transfer was carried out at the constant amperage of 65 mA for 2 hours. The membrane was washed in dH_2O, followed by staining with Ponceau S solution (Sigma) to verify protein transfer. The membrane was washed with PBS followed by incubation in blocking buffer for 1 hour at room temperature with agitation. The membrane was then placed in a sealed bag and incubated with the primary antibody of interest in blocking buffer overnight at 4°C with agitation. The membrane was washed three times each for 10 minutes in PBS 0.1% Tween 20 and then incubated with the appropriate HRP-conjugated secondary antibody of interest in blocking buffer for 1 hour at room temperature with agitation. The blot was washed again three times in PBS 0.1% Tween 20, each for 10 minutes, followed by a final quick wash in PBS. Proteins were revealed using the Enhanced Chemi-Luminescence kit (GE Healthcare) following the manufacturer's instructions. The blot was exposed to Kodak BioMax Light film for 10 seconds up to 1 hour.

Blotting buffer: 20 ml 5x SDS running buffer, 60 ml dH_2O, 20 ml methanol
Blocking buffer: PBS with 5% Marvel dried non-fat milk, 0.1% Tween 20

Chromatin immunoprecipitation (ChIP)

- **Zymolyase method**

This protocol was employed for all ChIPs performed for this thesis with the exception of H3K9me2 ChIPs.

50 ml of exponentially growing cells (5×10^6 cells/ml; 2.5×10^8 cells per ChIP) were fixed for 5 to 30 minutes (depending on protein to be ChIP'd and temperature of culture) with 3% paraformaldehyde freshly prepared in YES (+ 10 *N* NaOH to neutralize). Fixation was stopped by the addition of 2.5 M glycine (20x) to cultures for 5 minutes at room temperature with agitation. Cells were then washed twice in 20 ml of ice-cold PBS, resuspendend in 1 ml PEMS

and transferred to a microfuge tube. Pellet was then resuspended at 10^8 cells/ml in PEMS containing 0.4 mg/ml Zymolyase 100-T and incubated at 37°C for 20 to 30 minutes. Cells were then washed twice in PEMS (pellets may be frozen at -20°C in "one ChIP" size aliquots at this point). Pellet was then resuspended in 300 µl of lysis buffer containing protease inhibitor cocktail (100x Sigma) and 2 mM PMSF. Lysates were then sonicated using a water bath sonicator for 4 x 5 minutes. This should result in shearing the chromatin to approximately 500 – 1000 bp. After sonication, lysate was adjusted to a total volume of 400 µl. Tubes were spun for 5 minutes at 13,000 rpm at 4°C, supernatant was removed to new tube and spun for 15 minutes at 13,000 at 4°C to remove debris. Cleared lysate was pre-cleared by adding 25 µl of Protein A or Protein G agarose beads (Roche Applied Sciences) and were incubated with gentle rocking for 1-2 hours at 4°C. Protein A/G agarose was washed 3 times in lysis buffer and made into a 50:50 (v/v) suspension of beads in lysis buffer. Protein A agarose was used for rabbit antibodies and Protein G agarose was used for mouse antibodies and monoclonals. After pre-clearing, beads were spun at 8,000 rpm for 2 minutes at 4°C and supernatant was transferred to a new tube using a duckbilled pipette tip (Sorenson BioScience Inc.). 40 µl of this pre-cleared lysate was frozen as 'crude input' sample. The appropriate amount of antibody was added to the remaining lysate for 4 hours to overnight at 4°C.

Beads were then spun at 8,000 rpm and washed for 10 minutes at 4°C with rocking with 1 ml of each of the following buffers: lysis buffer, lysis buffer with 500 mM NaCl, wash buffer, TE pH 8. After the washes, 250 µl TES was added to the beads while 210 µl TES was added to the 'crude input' sample and all tubes were incubated at 65°C between 6 hours and overnight to reverse the cross-linking. 30 µl of 10 mg/ml Proteinase K (Roche Applied Sciences) and 450 µl TE were then added and tubes were incubated at 37°C for 2 hours. Samples were then phenol/chloroform and chloroform extracted and DNA was precipitated with 1/10 volume 3 M NaOAc pH 5.5, 2.5 volumes of ice-cold ethanol and 1.5 µ of 10 mg/ml glycogen was added to facilitate precipitation. Samples were mixed thoroughly by vortexing and incubated on dry ice for 1 hour. DNA was recovered by centrifugation at 4°C for 30 minutes at maximum speed. The pellet was dried under the fume hood for 15 to 20 minutes. ChIP (IP) DNA was resuspended in 30 µl and crude input DNA (Total) in 300 µl TE. 2 µl of DNA were used in 20 µl PCR reactions with appropriate multiplex primer sets with added Mg^{2+}.

PEM: 100 mM PIPES, pH 6.9, 1mM EDTA, 1 mM $MgSO_4$

PEMS: 100 mM PIPES pH 6.9, 1 mM EDTA, 1 mM $MgSO_4$, 1.2 M sorbitol

Lysis buffer: 50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate (w/v)

Wash buffer: 10 mM Tris-Cl pH 8, 0.25 M LiCl, 0.5% NP-40, 0.5% (w/v) sodium deoxycholate, 1 mM EDTA

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

TES: 50 mM Tris-Cl pH 8, 10 mM EDTA, 1% SDS

- **Beadbeater method**

For anti-H3K9me2 ChIP (mAb 5.1.1, a gift from Takeshi Urano), the zymolyase ChIP protocol was modified as following. Cells were fixed 1% formaldehyde for 15 minutes (2.5 x 10^8 cells per ChIP). On washes with PBS, cell were transferred to a round-bottomed screw-capped tube. After the addition of lysis buffer, 500 µl of acid-treated glass beads (Sigma) were added and cells were bead-beaten on ice twice for 2 minutes in a Mini BeadBeater (Biospec). To isolate the supernatant from the cellular debris, the bottom of the screw-capped tube was pierced with a hot needle and placed inside a 15 ml falcon tube containing a microfuge tube for collection. The tubes were centrifuged at low speed (1000 rpm) for 1 minute and the lysate was collected in a fresh microfuge tube. Samples were then sonicated 3 times for 5 minutes (30 seconds max sonication, 30 seconds rest) in ice-cold water bath on a Bioruptor sonicator (Wolf Laboratories). 1µl of H3K9me2 antibody and 25 µl of pre-washed Protein G agarose beads were subsequently added to each sample and left at 4°C overnight with slow rotation. The remainder of the procedure (washes, de-crosslinking, DNA purification) followed the standard zymolyase protocol.

- **Analysis**

Most of ChIP DNA samples described in this thesis were analysed by multiplex PCR using the following programme: 94°C for 4 minutes; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute; 72°C for 5 minutes. In the experiment from Figure 3-6, using LTR specific oligos, the programme conditions were similar with the exception of a lower annealing temperature (50°C instead of 55°C). In the experiment from Figure 5-, the

programme comprised a shorter extension step of 72°C for 30 seconds and 35 cycles instead. Part of the ChIP analysis described in Chapter 3 was carried out by qPCR (see above).

FLAG Immunoprecipitation (IP)

This procedure was followed to perform FLAG IP mentioned in Chapter 5. It was adapted by Alexander Kagansky from the standard FLAG IP protocol from Mike Tyers' lab.

Special care was taken in that: all steps either on ice or at 4°C; lysates were not allowed to warm up under any circumstance, all tubes and rotors pre-cooled were pre-cooled so that no steps resulted in sample heating; the lysates were kept concentrated, on the order of 50 mg/ml.

A large culture of cells was prepared in 1 litre of 4x YES up to a cell density of 1.2×10^8 cells/ml. At this density the cells are still in exponential growth phase (Sharon White). Cells were collected by centrifugation at 4,000 rpm for 15 minutes at 4°C on a Beckman Avanti-J centrifuge using the JLA-10.500 rotor (Beckman Coulter). The cells were washed three times in ice-cold PBS with a total volume of 2 litres, all the times followed by centrifugation for 4,000 rpm at 15 minutes. At the last wash, the cells were moved to a 50 ml falcon tube, spun down at 3,000 rpm for 10 minutes at 4°C on a tabletop centrifuge and resuspended in approximately ¼ volume of lysis buffer containing protease inhibitors. This cell suspension was snap frozen by dripping into liquid nitrogen. The cell pellets were collected and stored at -80°C.

For the lysis procedure, the cell pellets were ground on a Retsch mortar grinder with liquid nitrogen for 30 minutes. The lysis efficiency was confirmed to be above 50% by analysing the cell powder in a light microscope. The powder was collected and stored at -80°C until used

For each IP, 5 g of cell powder were used. The powder was allowed to warm up to -20°C for 30 minutes and then mixed with 10 ml of pre-chilled lysis buffer containing protease inihibitors. The suspension was allowed to dissolve for 30 minutes at 4°C under vigorous rotation. The lysate was cleared of cellular debris by centrifucation at 3,000g for 5 minutes at 4°C. The supernatant was carefully decanted to a pre-chilled ultra-centrifuge tube and loaded onto a pre-cooled 70 Ti rotor. The supernatant was centrifuged at 17,000 rpm for 30 minutes at 4°C under vacuum in a Beckman

Optima preparative ultracentrifuge (Beckman Coulter). Once completed, the lysate was carefully removed by pipetting the clear liquid between the lipidic phase and the pellet.

In the meantime, monoclonal anti-FLAG M2 antibody (Sigma) was pre-coupled to Protein G Dynabeads (Invitrogen). For each sample, 4 µl of bead slurry was washed three times with ice cold PBS in a microfuge tube. Beads were washed by pipetting the suspension several times followed by magnetic trapping of the beads for 1 minute. Beads were resuspended in 0.5 ml of PBS and mixed with 8 µl of antibody and allowed to couple for 30 minutes at 4°C with rotation. The beads were washed twice with PBS and once with lysis buffer. The slurry was finally resuspended with 10 µl lysis buffer.

10 µl of pre-coupled anti-FLAG Dynabeads were added to the lysate and left incubating at 4°C for 1 hour. Beads were centrifuged briefly at low speed and collected with a 50 mL MagnaBot (Promega). Subsequently, the beads were resuspended in 500 µl fresh lysis buffer with inhibitors and moved to a microfuge tube. Tubes were placed in a microfuge MagnaBot (Promega) and the beads collected magnetically for 30 seconds. The solution was replaced 1 ml fresh lysis buffer with inhibitors and allowed to resuspend the beads by pipetting up and down 10 times. This wash was repeated 3 times in a similar fashion.

For western analysis, beads were cleared of unspecific elution with 50 µl 200 µg/ml HA peptide in PBS for 20 minutes, followed by elution with 50 µl of FLAG peptide 20 µg/ml in PBS for 20 minutes and a second elution with 50 µl 200 µg/ml FLAG peptide. Both FLAG elutions were pooled and 10 µl was mixed with 2X Sample buffer and loaded onto an SDS-PAGE (see above).

For mass spectrometry analysis, the beads were resuspended in 0.5 ml lysis buffer with 2mM MgCL2 but without EDTA. 2 µl (500 units) of benzonase (Novagen) were added to each sample, followed by incubation at 4°C under rotation for 15 minutes. Beads were magnetized, transferred to a fresh microfuge tube and then washed twice with 1 ml fresh lysis buffer. Beads were then resuspended in 0.5 ml BD buffer, transferred to a fresh tube and washed again with BD buffer. Beads were again magnetized and resuspended on 5 µl Tris-HCl pH 8.0, making sure all beads were collected at the bottom of the tube. 2.5 µl trypsin solution was added and the tube sealed with parafilm, followed by overnight incubation at 37°C with 14000 rpm agitation (Eppendorf Thermomixer). On the following day, beads were re-magnetized and the supernatant was carefully separated to a fresh tube. The supernatant was mixed with another 2.5 µl trypsin

solution and incubated overnight at 37°C without rotation. The sample was again magnetized and moved to a new tube, followed by the addition of 50 µl 0.1% Trifluoroacetic acid (in water) to acidify the peptides. The samples were then filtered with an RP tip and injected into a LTQ Orbitrap LC/MS hybrid mass spectrometer (ThermoElectron) coupled to a Nano-HPLC (Agilent, 1200) for peptide analysis (Juri Rappsilber). The peptide masses were cross-related to a predicted peptide library for *S. pombe* and identified using the software Mascot.

Lysis buffer:50 mM Hepes-NaOH pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40. Just before used the following was added: 5mM DTT, 1x EDTA-free protease inhibitors cocktail (Roche), 1x yeast protease inhibitors cocktail (Sigma), 0.2mM PMSF, 0.2mM benzamidine.

Trypsin solution: 100 ng/µl trypsin powder resuspended in 20mM Tris-HCl pH 8.0.

Buffer BD: 20mM Tris-HCl pH 8.0, 150 mM NaCl, 2mM $CaCl_2$.

2.6. MICROSCOPY

Immunostaining

- **General protocol**

20 ml of a cell culture was grown to a concentration of 5 x 10^6 cells/ml. Cells were fixed by the addition of 3.7% paraformaldehyde dissolved in culture medium (a 10x stock was dissolved at 65°C and cooled down to room temperature) and the culture was shaken at room temperature for the appropriate time. Cells were spun in a benchtop centrifuge at 18°C, washed once with 10 ml PEM, transferred to a microfuge tube and washed twice with PEMS. Cells were then incubated at 37°C for 90 minutes in PEMS containing 1 mg/ml Zymolyase 100-T (ICN) at a concentration of 10^8 cells/ml. After washing in 1 ml PEMS, cells were resuspended in 1 ml PEMS containing 1% Triton X-100 and incubated on the bench for 5 minutes. Cells were then washed once with PEM, resuspended in 500 µl PEMBAL and incubated on a rotating wheel for 1 hour at room temperature. Aliquots of cells were then taken to be incubated with the

appropriate dilution of primary antibody in 100 µl PEMBAL overnight at 4°C on a rotating wheel.

After incubation with primary antibody, cells were washed three times with 1 ml PEMBAL incubating for at least 30 minutes for each wash. The required secondary antibody (Molecular Probes Alexa anti-mouse, anti-sheep or anti-rabbit) conjugated to the desired fluorescent probe (Alexa Fluor 488 or 594) were added at the concentration of 1:1000 in 100 µl PEMBAL. Tubes were wrapped in foil and incubated for 4 hours at room temperature with rotation or overnight at 4°C. Cells were washed once for 30 minutes in PEMBAL and incubated for 5 minutes in PEM + 0.1% sodium azide containing 1 mg/ml DAPI (stock 500x stored at -20°C). Cells were finally spun and resuspended in 20 µl PEM + 0.1 % sodium azide. 2 µl of cells were spread in a thin layer on a poly-L-lysine coated glass slide and allowed to dry. 1 drop of mounting medium VectaShield (Vector Laboratories Inc.) was then applied to the slide and a coverslip was gently lowered at an angle over the slide to minimize the formation of air bubbles. The coverslips were sealed with transparent nail varnish and observed using an Axioplan 2 IE fluorescence microscope (Carl Zeiss MicroImaging Inc.) equipped with Chroma 83000 and 86000 filter sets, Prior ProScan filter wheel (Prior Scientific) and Photometrics CoolSnapHQ CCD camera (Roper Scientific). Image acquisition was controlled using Metamorph software (Universal Imaging Corp.).

PEM: 100 mM PIPES, pH 6.9, 1mM EDTA, 1 mM $MgSO_4$
PEMS: 100 mM PIPES pH 6.9, 1 mM EDTA, 1 mM $MgSO_4$, 1.2 M sorbitol
PEMBAL: 100 mM PIPES, pH 6.9, 1mM EDTA, 1 mM $MgSO_4$, 1% BSA (Sigma), 0.1% sodium azide, 100 mM lysine hydrochloride (BDH)

- **Formaldehyde-glutaraldehyde method for staining microtubules**

For immunolabelling of microtubules, the above protocol was modified as follows.

Cells fixed with 3.7% freshly prepared paraformaldehyde, followed by the addition of 0.0625% glutaraldehyde one minute later for a total of 10 minutes at room temperature. Cells were washed, spheroplasted and permeabilised with 1% Triton X-100 as described above. Free

aldehyde groups resulting from the glutaraldehyde fixation were then reduced by washing the cells three times for 10 minutes with 2 mg/ml sodium borohydride in PEM. Sodium borohydride solution was prepared immediately before use. Cells were then washed three times in PEM (care was taken as the pellet was light and difficult to precipitate at this point) and blocked with PEMBAL as described above. Aliquots of cells were then resuspended in 100 µl PEMBAL containing TAT anti-α-tubulin mouse monoclonal antibody at dilution 1:15 and incubated overnight at 4°C with rotation.

2.7. BACTERIAL METHODS

Escherichia coli DH5α and TOP10 bacterial cells were used for all the cloning performed in this thesis. Cells were grown at 37°C in LB medium, solid or liquid, supplemented with 30 µg/ml ampicilin for plasmid selection. Liquid cultures were grown at 37°C with 225 rpm agitation.

Bacterial media

LB (per litre): 10 g Bacto-peptone, 5 g Yeast extract, 10 g NaCl. Autoclaved

Antibiotics and concentration used in plates: ampicilin 30 µg/ml, carbenicilin 50 µg/ml, chloramphenicol 20 µg/ml.

Transformation of competent cells

Subcloning efficiency DH5α or One Shot TOP10 chemically competent cells were transformed according to manufacturer's instructions (Invitrogen). Briefly, a frozen aliquot of competent cells was thawed on ice for 10 minutes. 50-100 µl of cells were added to up to 5 µl of ligation mixture or 10 ng of plasmid DNA and incubated on ice for 30 minutes. Cells were heat shocked for 30 seconds at 37°C (DH5α) or at 42°C (TOP10) and then placed on ice for 2 minutes. 1000 µl of LB (DH5α) or 250 µl of S.O.C. medium was added to the cells and the tubes placed at

37°C for 1 hour at 225 rpm agitation. The cells were then spun down briefly on a microfuge at max speed for 30 seconds and resuspended in 200 µl of medium. 50 µl and 150 µl of cells were spread on pre-warmed LB-agar plates containing the appropriate antibiotic required for plasmid selection using sterile glass beads. Plates were incubated at 37°C until colonies appeared but not exceeding 16 hours.

Plasmid construction

Restriction enzymes were obtained from New England Biolabs and were used in the reaction buffer supplied by the manufacturer. Fragments were amplified using Platinum Pfx Polymerase (Invitrogen). Digestions were carried at 37°C for 2-4 hours, unless otherwise specified by the manufacturer. Digested fragments were purified from agarose gel using the QIAquick gel extraction kit (Qiagen), according to the instructions of the manufacturer. Ligations were carried out using T4 DNA ligase (Roche) in the supplied buffer overnight at 18°C.

Plasmid preps

2-3 ml bacterial cultures were grown overnight from a single colony in liquid LB containing antibiotic. Plasmids were isolated using the QIAquick Plasmid Mini Kit (Qiagen) following the manufacturer's instructions. For larger scales, 25 ml cultures were inoculated with freshly grown pre-culture and grown overnight. Plasmids were then isolated using the QIAquick Plasmid Midi kit (Qiagen) following the instructions of the manufacturer.

2.8. ANTIBODIES USED IN THIS THESIS

Western analysis:

Rabbit anti-Bip1 (Alison Pidoux) (1:10000)

Rabbit anti-myc A14 (Santa Cruz Biotechnology) (1:500)

M5 monoclonal anti-FLAG HRP-conjugated (Sigma) (1:500)

Anti-rabbit HRP-conjugated (Sigma) (1:10000)

Anti-mouse HRP-conjugated (Sigma) (1:10000)

Immunofluorescence

Sheep anti-Cnp1^{CENP-A} serum (1:500)

Mouse TAT anti-α-tubulin (Iain Hagan) (1:15)

Rabbit anti-myc A14 (Santa Cruz Biotechnology) (1:50)

Chromatin IP

Rabbit anti-myc A14 (Santa Cruz Biotechnology) (1:150)

Mouse mAb5.1.1 monoclonal anti-H3K9me2 (Takeshi Urano) (1:300)

Rabbit anti-Swi6 serum (1:30)

Immunoprecipitation

M5 monoclonal anti-FLAG (Sigma) (0.8 μg/ml)

2.9. PRIMERS USED IN THIS THESIS

Name	Sequence	Description
3-ade6	GGCCACCATAGACATAACTG	ChIP PCR primer for *otr1R(SphI):ade6+*
5-otr1-ade6	CTACTCTTCTCGATGATCCTGTA	ChIP PCR primer for *otr1R(SphI):ade6+*
fbp1_fwd	ACTTCAGCTAGGATTCACCTGG	ChIP PCR primer for *fbp1+*
fbp1_rev	TGTGACAATGTCAGTGTCG	ChIP PCR primer for *fbp1+*
OTR_A	CACATCATCGTCGTACTACAT	ChIP PCR primer for *otr (dg)*
OTR_B	GATATCATCTATATTTAATGACTACT	ChIP PCR primer for *otr (dg)*
ago1(mut)_fwd	ATGTCGTATAAACCAAGCTCAGAAAT AGCTTTACGTCCCGGTTAT	PCR primer for *ago1* mutagenesis
ago1(mut)_rev	TTAGCTCTATCAAGTAAATTGAAAAC AAAGATGTGGTATATGTAA	PCR primer for *ago1* mutagenesis
ago1_fus_long_Kan1_R	TTAATTAACCCGGGGATCCGTATTAT ACTGAGTAAATCAG	PCR primer for fusion of KanMX4 resistance cassette
ago1_fus_long_Kan2_F	GTTTAAACGAGCTCGAATTCATATTG ATTTAATTAAGTTT	PCR primer for fusion of KanMX4 resistance cassette
ago1_fus_long_Ura1_R	GCATACATATAGCCAGTGGGTCCAA CCTTGACATTAATCT	PCR primer for fusion of *ago1::ura4+* disruption cassette
ago1_fus_long_Ura2_F	GGTTATAAACATTGGTGTTGGTCTAT CACCAAAATACAATC	PCR primer for fusion of *ago1::ura4+* disruption cassette
ago1_fus_Nat_F	CTGATTTACTCAGTATAATACGGATC CCCGGGTTAATTAA	PCR primer for fusion of NatR resistance cassette
ago1_fus_Nat_R	AAACTTAATTAAATCAATATGAATTC GAGCTCGTTTAAAC	PCR primer for fusion of NatR resistance cassette
ago1_NatR_check_fwd	CGAAAGGTATTCGCAAATGTAATAA TCG	PCR primer for verifying NatR cassette insertion next to *ago1* locus
ago1_NatR_check_rev	GGGAGGTTCAAAATCAATGATTACT AC	PCR primer for verifying NatR cassette insertion next to *ago1* locus
ago1_seqfor1	AAGACTTATGTTGCGTTTGC	Sequencing primer for a region of *ago1* locus
ago1_seqfor2	GTAAGTTCCTAGAAATCGCA	Sequencing primer for a region of *ago1* locus
ago1_seqfor3	CGAAAGCAATCCCAGTTGAT	Sequencing primer for a region of *ago1* locus
ago1_seqfor4	ACGACCGAATCAGGGTTTCA	Sequencing primer for a region of *ago1* locus
ago1_seqfor5	CGATGCTTCCTATTGAATTCTGTTT	Sequencing primer for a region of *ago1* locus
ago1_seqfor6	ACACTCACTTCGTTGGGAAT	Sequencing primer for a region of *ago1* locus
ago1_seqfor7	AACAGGTGTTTCGATTGCTT	Sequencing primer for a region of *ago1* locus
ago1_seqfor8	CTCACCCCTATCAGTACGAT	Sequencing primer for a region of *ago1* locus
ago1_seqfor9	CCACAACTTTTACTTCCTTA	Sequencing primer for a region of *ago1* locus
ago1_seqrev1	TGGGTTAAGTGAATGTCATT	Sequencing primer for a region of *ago1* locus
ago1_seqrev10	TGCAAGACAGATACAAAATG	Sequencing primer for a region of *ago1* locus
ago1_seqrev11	CTCGTTAGGTAAAGAAATGA	Sequencing primer for a region of *ago1* locus
ago1_seqrev2	ATCCATTGATGCTTCTGATG	Sequencing primer for a region of *ago1* locus
ago1_seqrev3	CCAGGGAGGGGATTTCCATT	Sequencing primer for a region of *ago1* locus
ago1_seqrev4	GGGAACGTGAAACAGCCGTA	Sequencing primer for a region of *ago1* locus
ago1_seqrev5	ATATGGTTCAGGCGAATTTT	Sequencing primer for a region of *ago1* locus

ago1_seqrev6	AAATTCCAACGTCCACTAAC	Sequencing primer for a region of *ago1* locus
ago1_seqrev7	CCCGTTCTTTACTAATATGC	Sequencing primer for a region of *ago1* locus
ago1_seqrev8	AAGTAAAGAATCATTACGCC	Sequencing primer for a region of *ago1* locus
ago1_seqrev9	AGATGATTTTTGAATAGAGAAT	Sequencing primer for a region of *ago1* locus
ago1D580A_fwd	ACTCTTATTCTTGGTGGAGCTGTTTA TCACCCTGGGGTT	PCR primer for *ago1* mutagenesis
ago1D580A_rev	AACCCCAGGGTGATAAACAGCTCCA CCAAGAATAAGAGT	PCR primer for *ago1* mutagenesis
ago1D651A_fwd	CGTATTATCTATTTCCGTGCCGGTAC CTCGGAAGGACAA	PCR primer for *ago1* mutagenesis
ago1D651A_rev	TTGTCCTTCCGAGGTACCGGCACGG AAATAGATAATACG	PCR primer for *ago1* mutagenesis
ago1H617A_fwd	TCACGTTCCCAACCTCGTGCTCAAGA AGTGATTGAAGGA	PCR primer for *ago1* mutagenesis
ago1H617A_rev	TCCTTCAATCACTTCTTGAGCACGAG GTTGGGAACGTGA	PCR primer for *ago1* mutagenesis
ago1Kan1_fwd	TGAATACAATGTAAATTCACAA	PCR primer for fusion of KanMX4 cassette for *ago1* locus
ago1Kan1_rev	CCGCTCGAGTATTATACTGAGTAAAT CAG	PCR primer for fusion of KanMX4 cassette for *ago1* locus
ago1Kan2_fwd	TGCTCTAGAATATTGATTTAATTAAG TTT	PCR primer for fusion of KanMX4 cassette for *ago1* locus
ago1Kan2_rev	GAATATTAATCTGGCAACTT	PCR primer for fusion of KanMX4 cassette for *ago1* locus
ago1Ura_1_fwd	CTGAACAGGTTGGTAATGCT	PCR primer for fusion of *ago1::ura4+* disruption cassette
ago1Ura_1_rev	CCGCTCGAGTCCAACCTTGACATTAA TCT	PCR primer for fusion of *ago1::ura4+* disruption cassette
ago1Ura_2_fwd	TGCTCTAGAGTCTATCACCAAAATAC AATC	PCR primer for fusion of *ago1::ura4+* disruption cassette
ago1Ura_2_rev	ATTTCGTCGTGCAAAACCGT	PCR primer for fusion of *ago1::ura4+* disruption cassette
qAct1_fwd	GGTTTCGCTGGAGATGATG	qPCR primer for *act1*
qAct1_rev	ATACCACGCTTGCTTTGAG	qPCR primer for *act1*
qCnt1_fwd	CAGACAATCGCATGGTACTATC	qPCR primer for *cnt1* (central core 1)
qCnt1_rev	AGGTGAAGCGTAAGTGAGTG	qPCR primer for *cnt1* (central core 1)
qDgl_fwd	AATTGTGGTGGTGTGGTAATAC	qPCR primer for *otr1 (dg)*
qDgl_rev	GGGTTCATCGTTTCCATTCAG	qPCR primer for *otr1 (dg)*
qDhl_fwd	CTACGCTTGATTTGAGGAAGG	qPCR primer for *otr1 (dh)*
qDhl_rev	AAAGTATGAGTCGCAGAAGTG	qPCR primer for *otr1 (dh)*
qImr1_fwd	CTAATGCGGAGTAAGGCTAATC	qPCR primer for *imr1*
qImr1_rev	TGGACAGAATGGATGGATATTG	qPCR primer for *imr1*
qSPAC56F8.17c_fwd	TCATTGTCCAGGATCAGCTATG	qPCR primer for SPAC56F8.17c ORF
qSPAC56F8.17c_rev	TGGTCTTCTCTCGTAAAACAGG	qPCR primer for SPAC56F8.17c ORF
qSPAC56F8.14c_fwd	ATCTTGGCAGTACCGAGTG	qPCR primer for SPAC56F8.14c ORF
qSPAC56F8.14c_rev	TCTTGTTGACCATCGGCC	qPCR primer for SPAC56F8.14c ORF
qSPAC26H5.11_fwd	CGCCGCCCAAAGAGTTCC	qPCR primer for SPAC26H5.11 ORF
qSPAC26H5.11_rev	ATGCACAACATCGCCATTTAGC	qPCR primer for SPAC26H5.11 ORF

qSPAC26H5.07c_fwd	GGAAGACGATGATGGCGAGAAG	qPCR primer for SPAC26H5.07c ORF
qSPAC26H5.07c_rev	GTGGGGAGCGGTTTGGTTAATC	qPCR primer for SPAC26H5.07c ORF
qSPAC26H5.12_fwd	GCGTGGATCTTTCGAGTTGAC	qPCR primer for SPAC26H5.12 ORF
qSPAC26H5.12_rev	CGCTGAGTCGGATTAAGTGATG	qPCR primer for SPAC26H5.12 ORF
qSPAC26H5.13c_fwd	TGTTTCGGCGTCCAAATTGG	qPCR primer for SPAC26H5.13c ORF
qSPAC26H5.13c_rev	GGGATACAATGCCGTAAAGACC	qPCR primer for SPAC26H5.13c ORF
qSPAC26H5.10c_fwd	GCCGCAAGTACGAGGATATG	qPCR primer for SPAC26H5.10c (tif51) ORF
qSPAC26H5.10c_rev	GTCTCTTCACCCATAGCGG	qPCR primer for SPAC26H5.10c (tif51) ORF
qSPAC26H5.09c_fwd	CTGTGGTTTGGTGTATGACTTG	qPCR primer for SPAC26H5.09c ORF
qSPAC26H5.09c_rev	GTGGAGAACGATGCGGAAG	qPCR primer for SPAC26H5.09c ORF
qSPAC26H5.08c_fwd	GCTTCCGAAATCGCTTCTTC	qPCR primer for SPAC26H5.08c ORF
qSPAC26H5.08c_rev	TGTTGGTGTAAGGAGCAAGG	qPCR primer for SPAC26H5.08c ORF
qmeu6_fwd	AACATGATTCGGGATCTGCTG	qPCR primer for *meu6*
qmeu6_rev	GGCTCCTTGGGTTCCTCAG	qPCR primer for *meu6*
Ingela_oligo1	CTACTCTTCTCGATGATCCTG	PCR primer for *otr (dg)* probe for northern analysis
Ingela_oligo2	GTAGTACGACGATGATGTGTTTTC	PCR primer for *otr (dg)* probe for northern analysis
LTR_probe_fwd	ACTACGTTGCGTATCACTAT	PCR primer for LTR α and β subclade consensus
LTR_probe_rev	AGAACTGCGGTGAGTTTTCC	PCR primer for LTR α and β subclade consensus
LTR_30D11.02_fwd	CTATGCTCAGTTGCTACTTAT	PCR primer for LTR in SPAC30D11 locus
LTR_30D11.02_rev	GTAGAATTTAGTGTAAGCTACGC	PCR primer for LTR in SPAC30D11 locus
c26H5+0_fwd	CTCTTCAATCATACTTCACTGTTC	ChIP PCR primer for right of LTR in SPAC26H5
c26H5+0_rev	GCGTAGCGTTTGGATGTAAG	ChIP PCR primer for right of LTR in SPAC26H5
c26H5-0_fwd	CCATTATACTCTCCTGTTGAC	ChIP PCR primer for left of LTR in SPAC26H5
c26H5-0_rev	ATCGTACATTGAATCCGTTAG	ChIP PCR primer for left of LTR in SPAC26H5
CPC3	GGCTGGCCTGTTGAACAAGTCTGGA	Bioneer primer for KanMX4 insertion (3' end)
CPN1	CGTCTGTGAGGGGAGCGTTT	Bioneer primer for KanMX4 insertion (5' end)
CPN10	GATGTGAGAACTGTATCCTAGCAAG	Bioneer primer to check for KanMX4 insertion (5' end rev further into kanmx4)
rrp6_check_fwd	GAACTAATCATAAATAATTGCTTT	SPZ PCR primer for verifying *rrp6Δ* in Bioneer strain
rrp6_check_rev	GCATGCATTCTCAATTTCTTCTTATAGC	SPZ PCR primer for verifying *rrp6Δ* in Bioneer strain
cen_fwd	GAAAACACATCGTTGTCTTCAGAG	PCR primer for *otr (dh)*
cen_rev	CGTCTTGTAGCTGCATGTGAA	PCR primer for *otr (dh)*
L71	AGACTGTTGTTGAGTGCTGTG	ChIP PCR primer for *otr (dg)*
L72	ATTTGCCTGTTGTACATTTTTGC	ChIP PCR primer for *otr (dg)*
MM	TACGTTCAGTAGACGTAGTG	SPZ PCR primer for determining mating type
MP	ACGGTAGTCATCGGTCTTCC	SPZ PCR primer for determining mating type
MT1	AGAAGAGAGAGTAGTTGAAG	SPZ PCR primer for determining mating type
DF151	GACTGTTGTTGAGTGCTGTG	ChIP PCR primer for pH-cc2 (Diego Folco)
DF169	CGCAATTAATGTGAGTTAGC	ChIP PCR primer for pH-cc2 (Diego Folco)

2.10. *S. pombe* STRAINS USED IN THIS THESIS

FY#	Relevant genotype	
340	*h- ade6-210 leu1-32 ura4-DS/E TM-ura4+::R.int*	
872	*h? clr4-S5 ade6-210 leu1-32? ura4-DS/E TM1-ura4+::Rint*	
972	*h+ wild-type fission yeast*	
1082	*h? swi6::his1+ ade6-210 his1-102 leu1-32 ura4-DS/E TM-ura4+::Rint*	
1180	*h+ ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
1181	*h- ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
1645	*h+ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18*	
1646	*h- ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18*	
3132	*h- clr4::LEU2+ ade6-DN/N leu1-32 ura4-D18 otr1R(Sph)::ade6+*	
3190	*h+ ade6-DN/N leu1-32 ura4-D18 his1-101 arg3-D4 otr1R(SphI):ade6+*	
3191	*h- ade6-DN/N leu1-32 ura4-D18 his1-101 arg3-D4 otr1R(SphI):ade6+*	
3746	*h? ade6-DN/N leu1-32 ura4D18 his3-D1 arg3-D4*	
3747	*h? ade6-DN/N leu1-32 ura4D18 his3-D1 arg3-D4*	
4132	*h- ade6-210 arg3D his3D leu1-32 ura4DS/E*	
4133	*h+ ade6-210 arg3D his3D leu1-32 ura4DS/E*	
4835	*h- ade6-210 leu1-32 ura4-DS/E his3-D1 arg3-D4 TM-ura4+::Rint*	
5021	*h+ chp1-myc-LEU2+ ura4-DS/E his3-D1 arg3-D4 leu1-32*	
5023	*h- chp1-myc-LEU2+ ura4-DS/E his3-D1 arg3-D4 leu1-32*	
6215	*h- ago1::kanR ade6-210 ura4-DS/E*	
6218	*h- dcr1::kanR ade6-210 ura4-DS/E*	
6222	*h- rdp1::kanR ade6-210 ura4DS/E*	
6519	*h- dis3-54 leu1-32? =ALP344*	from M. Yanagida
7093	*h- ade6-M210 leu1-32 pfs2-3169 bub1+- GFP-kanR*	
7125	*h- pfs2-11 ade6-M210 Ch16 ura4-D18 his7*	
7127	*h- ade6-M216 ura4-D18 leu1 dhp1-1(ura4+)*	MP101 from K. Tatebayashi
8059	*h+ ago1::ura4+ ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
8061	*h+ ago1-H617A ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
8196	*h+ ago1-D580A ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
8197	*h+ ago1-D651A ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
10376	*h- chp1-3xFLAG-KanR ade6-216*	SPT5 from Shiv Grewal
11540	*h- dcr1::kanR his3-D3 arg3-D4 ura3-D18 ade6-210 his3-D1 arg3-D4 TM-ura4::Rint*	
11541	*h? pfs2-11 ade6-DN/N ura4-D18 his3-D1 arg3-D4*	
11542	*h? dhp1-1 ade6-DN/N his3-D1 arg3-D4*	
11543	*h? dhp1-1 ade6-DN/N ura4? his3-D1*	
11544	*h? dhp1-1 ade6-DN/N ura4? his3-D1 arg3-D4*	

11545	*h? pfs2-3169 ade6-DN/N ura4? his3-D1*	
11546	*h? pfs2-3169 ade6-DN/N ura4? arg3-D4*	
11547	*h? dhp1-1 otr1R(SphI)::ade6+ ade6-DN/N his1? his3? arg3-D4*	
11548	*h? dhp1-1 otr1R(SphI)::ade6+ ade6-DN/N his1? his3? arg3-D4*	
11549	*h? dhp1-1 otr1R(SphI)::ade6+ ade6-DN/N his1? his3? arg3-D4*	
11550	*h? dhp1-1 otr1R(SphI)::ade6+ ade6-DN/N his1? his3? arg3-D4*	
11551	*h? dhp1-1 otr1R(SphI)::ade6+ ade6-DN/N his1? his3? arg3-D4*	
11552	*h? dhp1-1 otr1R(SphI)::ade6+ ade6-DN/N his1? his3? arg3-D4*	
11553	*h? rrp6::kanR ade6-DN/N ura4-D18 leu1-32 his3-D1 arg3-D4*	*
11555	*h? rrp6::kanR otr1R(SphI)::ade6+ ade6-DN/N his1? his3? arg3-D4*	*
11556	*h? rrp6::kanR otr1R(SphI)::ade6+ ade6-DN/N his1? his3? arg3-D4*	*
11557	*h? rrp6::kanR otr1R(SphI)::ade6+ ade6-DN/N his1? his3? arg3-D4*	*
11558	*h+ natR-3xmyc-ago1+ ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
11559	*h+ natR-3xmyc-ago1::ura4+ ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
11560	*h+ natR-3xmyc-ago1-D580A ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
11561	*h+ natR-3xmyc-ago1-D580A ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
11562	*h+ natR-3xmyc-ago1-H617A ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
11563	*h+ natR-3xmyc-ago1-H617A ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
11564	*h+ natR-3xmyc-ago1-D651A ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
11565	*h+ natR-3xmyc-ago1-D651A ade6-210 leu1-32 ura4-D18 otr1R(SphI):ade6+*	
11566	*h? natR-3xmyc-ago1-D651A chp1-3xFLAG-KanR ade6-210*	
11568	*h? ago1-D580A ade6-210 leu1-32 ura4- otr1R(SphI):ade6+ chp1-myc-LEU2+*	
11569	*h? ago1-D580A ade6-210 leu1-32 ura4- otr1R(SphI):ade6+ chp1-myc-LEU2+*	
11570	*h? ago1-D651A ade6-210 leu1-32 ura4- otr1R(SphI):ade6+ chp1-myc-LEU2+*	
11571	*h? ago1-D651A ade6-210 leu1-32 ura4- otr1R(SphI):ade6+ chp1-myc-LEU2+*	

* *rrp6Δ* strains were derived from crosses of BG-0781 (Bioneer Corporation, Korea)

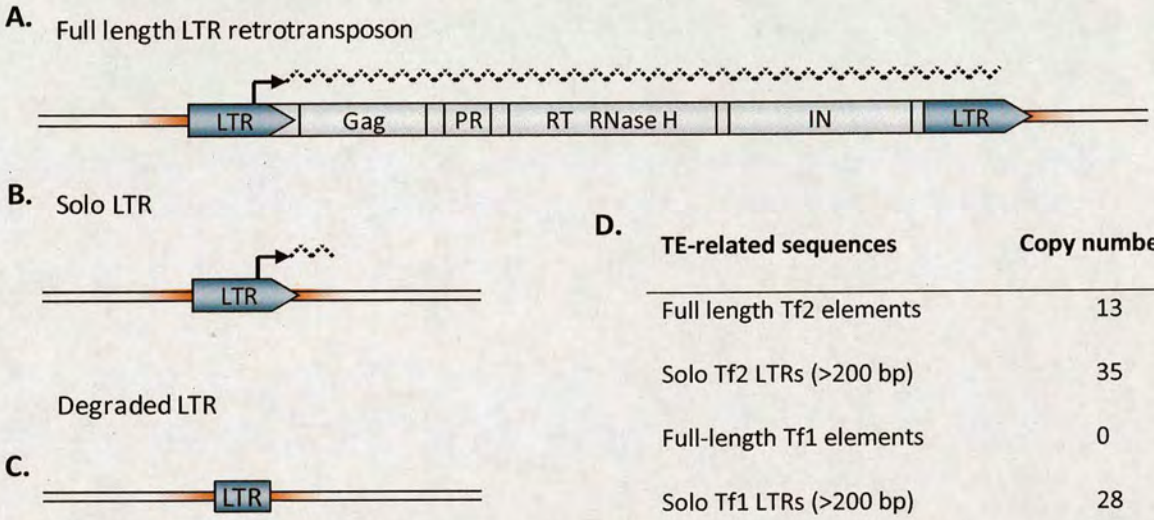
CHAPTER 3

GENE REGULATION VIA INTERPERSED REPEATS BY RNAi-MEDIATED CHROMATIN SILENCING

3.1. INTRODUCTION

In the 1950s, Barbara McClintock observed that a new kind of mobile genetic element had the property of altering gene function and affecting normal plant development (McClintock 1956). These elements turned out to be transposable elements (TEs). Nowadays it is known that TEs can affect genes by inserting within their sequence, causing disruption or truncation of protein coding sequences (Fedoroff 1989). In addition, TE insertions can have dramatic effects on expression levels of surrounding genes by disturbing the transcriptional activity of the affected regions (Morgan, Sutherland et al. 1999; Maside, Bartolome et al. 2002; Iwashita, Osada et al. 2003; Han, Szak et al. 2004). Since these elements are often a target for chromatin silencing in eukaryotes, it is possible that some of the observed changes of gene expression associated with TEs could result from transposon silencing events involving the formation of silent chromatin over such elements (Morgan, Sutherland et al. 1999; Lippman, Gendrel et al. 2004). TEs and related repetitive sequences are among the most highly represented classes of genomic elements in eukaryotic genomes. Repetitive sequences comprise 8% of fission yeast genome, 38-48% in mammalian genomes and up to 80% of total nuclear DNA in maize and many other plant species (SanMiguel, Gaut et al. 1998; Venter, Adams et al. 2001; Waterston, Lindblad-Toh et al. 2002; Wood, Gwilliam et al. 2002). Whilst a significant fraction of these sequences is found clustered at centromeres and telomeres, a large proportion is found interspersed along the chromosome arms in the vicinity of protein-coding genes. Considering that interspersed sequences may be targeted by RNA silencing and silent chromatin formation, can interspersed repeats regulate gene expression in a form that is dependent on RNA and chromatin silencing?

The fission yeast *Schizosaccharomyces pombe* serves as simple but tractable experimental model in which to study the function of repetitive DNA in gene control. Apart from possessing RNA interference and heterochromatin structure akin to higher eukaryotes, its genome contains



D.

TE-related sequences	Copy number
Full length Tf2 elements	13
Solo Tf2 LTRs (>200 bp)	35
Full-length Tf1 elements	0
Solo Tf1 LTRs (>200 bp)	28
Related LTRs (>200 bp)	111
Related LTR fragments (<200 bp)	75
Total LTRs	275

Figure 3-1: Retrotransposons and LTR sequences in the genome of *S. pombe*.

A. Diagram of the Tf1 and Tf2 LTR retrotransposons from fission yeast. These transposable elements (TEs) are ~5 kb long and are constituted by a single open reading frame that encondes Gag, protease (PR), reverse transcriptase, endonuclease (RT RNase H) and integrase (IN). They are flanked by two tandem long terminal repeats (LTR) that promote transcription of the TE and participate in the transposition process.

B. Solo LTRs are common throughout fission yeast chromosomes and result from deletion of TEs by homologous recombination events. Solo LTRs contain the TE promoter and have been reported to be transcriptionally active.

C. Many of the solo LTR sequences were accumulated from ancient TE invasion events and have since become considerably degraded by random mutations throughout evolution.

D. Summary of TE related sequences in the genome of fission yeast (FY972) (adapted from Bowen et al 2003). The genome of fission yeast carries 13 full length Tf2 LTR retrotransposons that are inactive but no full length Tf1 elements. It possesses a number of Tf1 and Tf2 solo LTRs and a large number of related LTRs that have originated from other unknown retrotransposons.

relatively small numbers of interspersed repeats. Previous work in our lab demonstrated that interspersed LTR repeats are also targets for RNAi-mediated heterochromatin assembly (Schramke and Allshire 2003). These LTRs (Long Terminal Repeats) are 358 bp-long functional components of a class of TEs known as retrotransposons which are also found in retroviruses. Scattered throughout the genome are 275 LTRs scattered from TEs of the Tf1 and Tf2 families, although only 26 are still associated with full length elements (Bowen, Jordan et al. 2003) (Figure 3-1). The vast majority of these repeats show obvious signs of degradation and are considered to be remnants of deleted TEs. LTRs contain promoters which allow transcription of the full-length TE in the first step of the retro-transposition process. These full-length transcripts are then primed for reverse transcription and the resulting cDNA is integrated at a novel locus through the action of a transposase. Solo LTRs are known to maintain promoter activity and indeed LTRs in *S. pombe* were found to be actively transcribing. However, similar to centromeric outer repeats, LTR transcripts were detected for both strands at increased levels in RNAi mutants. In fact, it was observed that LTRs display enriched levels of both H3K9me2 and Swi6^{HP1} but only while RNAi is active and Clr4$^{Su(var)3-9}$ is present (Schramke and Allshire 2003). Thus, it was proposed that RNAi acts on several or all LTRs to bring about silent chromatin assembly on LTRs (Schramke and Allshire 2003).

The most surprising finding about LTR repression was that it influenced the expression of nearby genes. While testing for effects of LTR heterochromatin on surrounding genes, 7 out of 11 meiotic genes were found to have increased transcription levels in the absence of RNAi (*ago1Δ*, *dcr1Δ*, *rdp1Δ*) or heterochromatin (*clr4-S5* and *swi6Δ*) (Schramke and Allshire 2003). As with other meiotic genes, these 11 genes are normally upregulated only during meiosis, which is triggered by nitrogen starvation, leading to sexual differentiation, conjugation and eventually sporulation of a zygote. This derepression of meiotic genes during the vegetative cycle was also shown to be dependent on the presence of the nearby LTR since disruption with a selectable marker reproduced the derepression effect on nearby meiotic genes. The promoters of all 7 affected genes were, at most, 6.6 kb away from an LTR while the remaining 4 unaffected genes were positioned further away from any neighbouring LTR (at least 10 Kb away). This is reminiscent of a position-based effect which, together with the requirement for Swi6^{HP1} and Clr4, strongly suggests that repression of these 7 genes during the vegetative cycle is caused by heterochromatin spreading laterally from the LTR to adjacent regions (Figure 3-2).

In this chapter I will describe the work I performed in exploring the process of LTR-mediated repression. Previously published data provided few clues to the molecular mechanisms ruling this

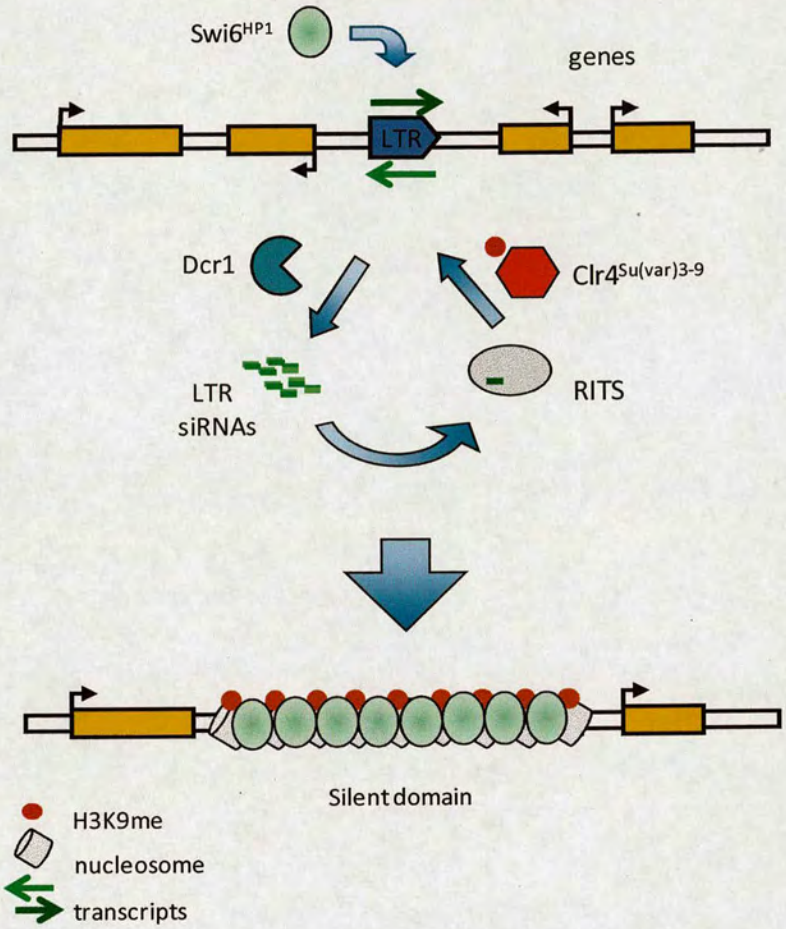


Figure 3-2: Original model for LTR-mediated gene silencing (Schramke and Allshire, 2003). Transcripts from both strands of LTRs (green arrows) are produced and lead to the formation of dsRNA. This is cleaved by Dcr1 into siRNA molecules that are loaded into Ago1 inside the RITS (RNA-induced Initiation of Transcriptional Silencing) complex. RITS then proceeds to recruit the $Clr4^{Su(var)3-9}$ methyltransferase to deposit H3K9me2 on overlying nucleosomes and recruit the chromodomain protein $Swi6^{HP1}$. This establishes a patch of heterochromatin over the LTR sequence that can spread to adjacent regions and silence nearby genes.

process. It was not known how components of RNA silencing, heterochromatin assembly and transcription acted together to bind LTRs in silent chromatin and affect the expression levels of nearby genes. Thus, this project was primarily aimed at characterizing the molecular basis of LTR-mediated repression by focusing the analyses on a few selected LTR-gene clusters. My approach consisted of studying the behaviour of both repeats and genes at the level of chromatin structure and transcription. However, the work that I present here fails to support the original observations and even challenges the existence of a phenomenon of LTR repression by RNAi and heterochromatin as well as any influence of such a mechanism on the activity of surrounding genes.

3.2. RESULTS

3.2.1. LTR-mediated gene repression is not reproducible

Previous work from our lab analysed the expression behaviour of 11 meiotic genes in function of RNAi and the presence of a nearby LTR (Schramke and Allshire 2003). Although 7 genes were reported to be repressed by means of RNAi and a neighbouring LTR sequence, the analyses did not encompass all the genes surrounding these same LTRs. It also did not provide any quantitative measure of the effect on gene expression. My first aim was to reproduce these original data and expand their coverage in order to investigate the repressive influence of a few given LTRs over all the genes found within a 10 kilobases of the LTR. By accurately measuring the expression levels of all genes present on several LTR loci, I hoped to describe the extent of the repressive influence of these repeats and derive common aspects of function. If indeed heterochromatin spreading was involved then several genes, particularly the ones located closer to the LTR, might be expected to display affected expression levels due to PEV.

For this purpose, a more accurate quantification method than the standard reverse transcriptase polymerase chain reaction (RT-PCR) was required to properly characterize known LTR-sensitive genes in more detail as well as to assay other potentially regulated genes. Therefore I used reverse transcriptase quantitative (real time) PCR (RT-qPCR) to measure the transcript levels of all the genes in my chosen LTR loci. Using this technique, it is possible to visualize the kinetics of the PCR reaction through successive cycles. Rather than deriving the amount of starting template from the final product of the reaction, as per standard gel-based RT-PCR, by RT-qPCR it is possible to estimate the amount of starting template by comparing amplification curves of test samples

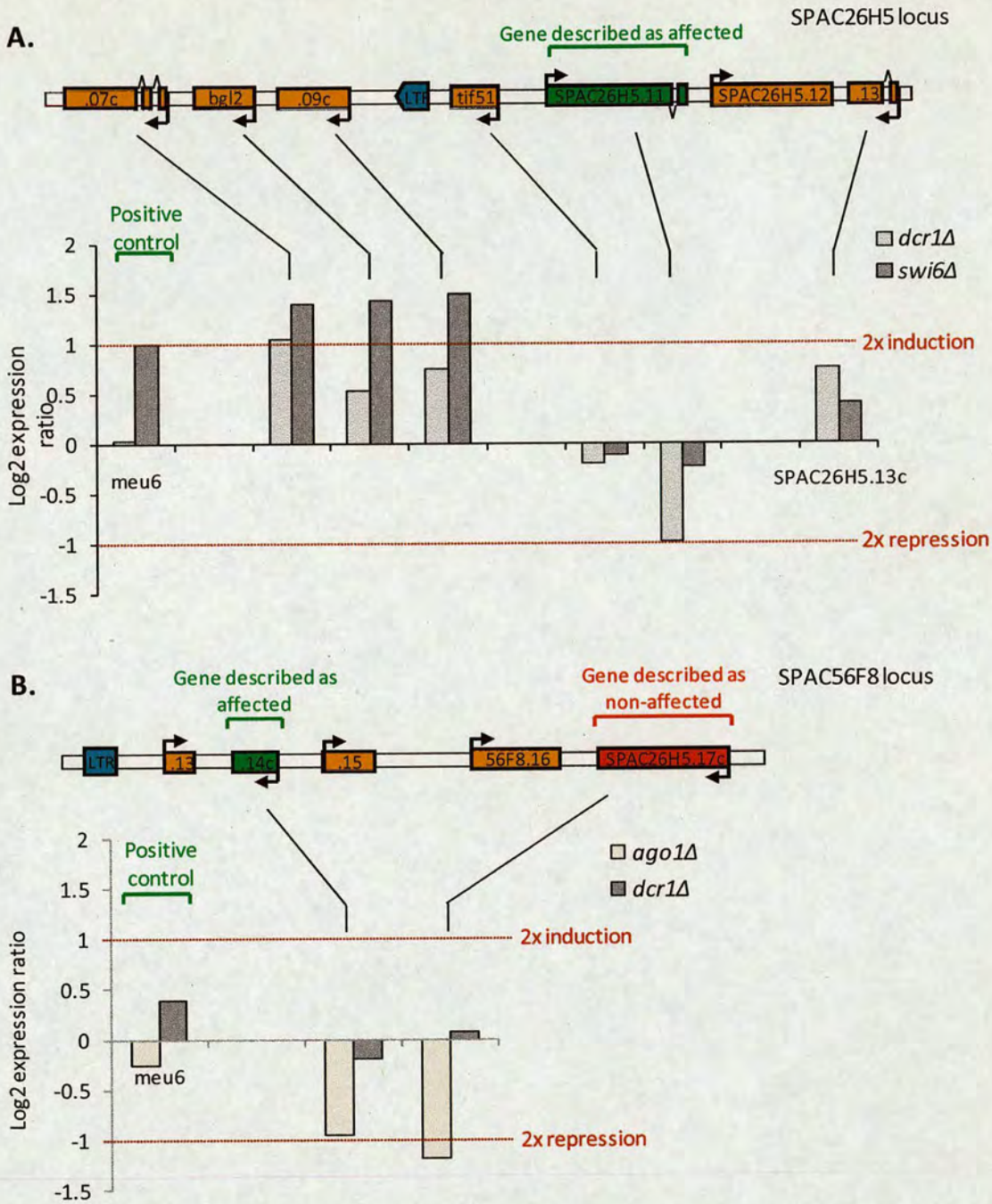


Figure 3-3: RT-qPCR analysis conducted in mutant strains for RNAi and heterochromatin assembly in two independent LTR loci. Relative expression ratios versus wild-type normalized to *act1* are depicted in the bar chart as $\log_2$-based values. Red lines mark the thresholds for two-fold variation in expression. The diagram above the graphs marks the relative position of the genes within each locus. ORFs marked in green were previously shown as LTR-sensitive, in red the LTR-insensitive genes and in orange the ones to which the behaviour towards LTRs is unknown. **A.** Expression analysis of ORFs of the SPAC26H5 locus in wild-type versus *dcr1Δ* and *swi6Δ*. Entries represent each of the genes analyzed in this locus, SPAC26H5.07 to 11. The last entry refers to SPAC26H5.13, which is located further than t10kb away from the LTR. The LTR-sensitive gene *meu6* was included as a positive control. **B.** Similar analysis performed on two genes of the SPAC56F8 locus, 14c and 17c, which had been reported as LTR-sensitive and –insensitive genes, respectively.

against a set of DNA standards. Thus real time PCR data is more accurate because it is less likely to be contaminated by differences in reaction efficiency or rate-limited reaction setups. It is also far more sensitive since it relies on detection of DNA product bound to the fluorescent dye SYBR Green II on the reaction plate rather than gel staining (Bustin 2000; Bustin 2002).

The initial plan was to monitor a total of four LTR loci encompassing 34 different ORFs in wild type and mutant backgrounds using real time PCR. The reagents used were a customized mixture with AmpliTaq Gold, several additives used to lower non-specific amplification and the fluorescent dye SYBR Green II to measure the amount of double stranded DNA produced throughout PCR cycling. All experiments were conducted in a Biorad iCycler system. Temperature gradients were used to determine the optimal annealing temperature of each primer pair. The PCR efficiency of each pair was determined using 10-fold serial dilutions of DNA standards (sheared genomic DNA) and by regression of the resulting standard curve. While performing these tests, I was able to verify that the setup performed adequately as it was capable of distinguishing 2-fold differences in starting amounts of template.

The analysis was conducted on two LTR loci: SPAC26H5 and SPAC56F8. Both loci contain what were previously described as LTR-sensitive genes (SPAC26H5.11 and SPAC56F8.14c) whilst SPAC56F8 also contains one gene known not to be affected by LTR repression (SPAC56F8.17c) (Schramke and Allshire 2003). Total RNA was extracted from cell cultures in log phase and used to synthesize cDNA with an oligo-dT primer. All samples were assayed in triplicate using specific primers and normalized against levels of *act1* (actin). The resulting expression ratios were calculated using the Pfaffl method for semi-quantitative PCR (Figure 3-3A) (Pfaffl 2001). The expression level of SPAC26H5.11 was not affected in *swi6Δ* and was even reduced rather than enhanced in *dcr1Δ*. Genes downstream of the LTR show increased transcription in the absence of $Swi6^{HP1}$ but this tendency is not reproduced in *dcr1Δ*. A gene described as LTR-regulated – *meu6* – showed no difference in expression levels in the absence of Dcr1. The same behaviour was observed for another supposedly LTR-regulated gene (SPAC56F8.14c) in a separate experiment (Figure 3-3B). By semi-quantitative RT-PCR assay, it was possible to detect accumulated centromeric transcripts in the strains used in this study, which confirms their defect in RNAi and heterochromatin silencing (Figure 3-4) (Volpe, Kidner et al. 2002). Thus, in this assay I was not able to detect any significant RNAi or heterochromatin-dependent effects on transcript levels of genes adjacent to LTRs.

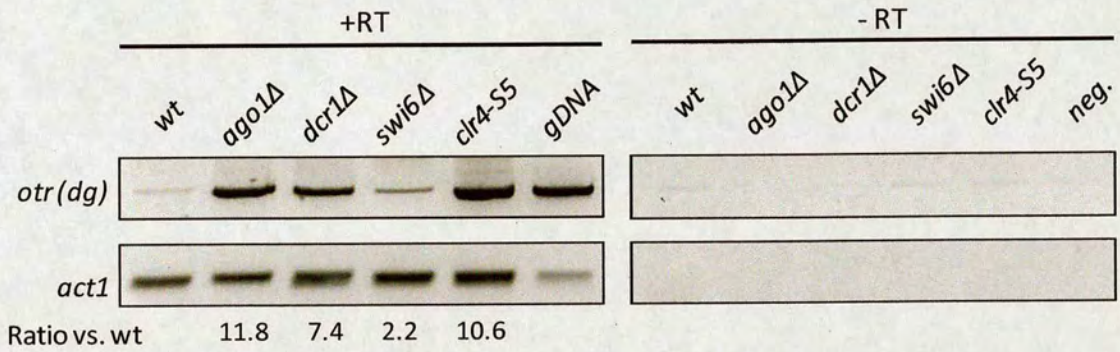


Figure 3-4: **RT-PCR analysis for centromeric transcripts in RNAi and heterochromatin mutants.** The accumulation of transcripts homologous to the outer repeats was measured for each sample by semi-quantitative PCR using poly(A) cDNA. The control reaction is shown as –RT (no reverse transcriptase). The positive control for the PCR reaction was performed with a sample of genomic DNA (gDNA). The amounts of *otr (dg)* transcripts detected for each mutant were first normalized to *act1* and are depicted here as overexpression ratios in comparison with wild type sample (wt). Both RNAi (*ago1Δ* and *dcr1Δ*) and heterochromatin mutants (*swi6Δ and clr4-S5*) visibly accumulate *otr* transcripts. As previously reported, the accumulation of otr RNA in *swi6Δ* is less proeminent than in *clr4-S5* and RNAi mutants.

3.2.2. Heterochromatin does not establish over LTRs and surrounding genes

The genes contained in the SPAC26H5 locus were also assayed for the presence of heterochromatin. The data obtained here refers to Swi6^{HP1} association only which is still sufficient to determine whether a particular region is enveloped in heterochromatin or not since Swi6^{HP1} is a component of this structure that is essential for silencing underlying genes (Lorentz, Ostermann et al. 1994; Nimmo, Cranston et al. 1994).

Chromatin immunoprecipitation was performed with anti-Swi6 serum and the enrichment was evaluated by semi-quantitative PCR using primers for the outer repeats (*otr*) and *fbp1* gene as an internal control. The degree of enrichment was determined by comparing the relative levels of these two DNA species in the immunoprecipitated fraction against the total extract (fig.5A). These same samples were assayed by real-time PCR using primers for the "*dg*" and "*dh*" outer repeats, the inner-most repeats (*imr*) and the central core (*cnt*) of the centromere 1, with *act1* as the euchromatic control. The results show that this assay is capable of detecting increased levels of Swi6^{HP1} in the heterochromatic regions of the centromere (Figure 3-5B). It also shows that this enrichment of Swi6^{HP1} over the centromeric outer repeats requires Dcr1, which illustrates the connection between RNAi and the stability of centromeric heterochromatin (Volpe, Schramke et al. 2003). However, no significant differences in enrichment were found for the genes surrounding the LTR in SPAC26H5 (Figure 3-5C). This suggests that, in these conditions, LTR heterochromatin is not engulfing surrounding genes. Since the LTR in SPAC26H5 belongs to a subclade of highly homologous LTRs, it is not possible to generate primers specific for this particular repeat. However, the resolution of chromatin immunoprecipitation is limited by the extent of chromatin shearing, which in general, does not go below 500 bp. It is then possible to measure the levels of Swi6^{HP1} protein over the LTR using primers to amplify the sequences regions immediately upstream and downstream of the LTR itself. By standard semi-quantitative multiplex PCR, no Swi6^{HP1} enrichment was detected over the LTR region (Figure 3-6A). Therefore, it is not likely that this LTR is coated in heterochromatin. In a separate experiment, the LTR adjacent to the SPAC30D11.02c heterochromatin-sensitive gene was tested for enrichment of Swi6^{HP1} in an attempt to reproduce the original observations on LTR heterochromatin (Schramke and Allshire 2003). By multiplex PCR, no noticeable enrichment of Swi6^{HP1} is observable over this sequence (Figure 3-6B). This result contradicts published results that showed Swi6^{HP1} localization to these repeats in wild type in a form sensitive to disruption of RNAi or heterochromatin assembly machinery (Schramke and Allshire 2003).

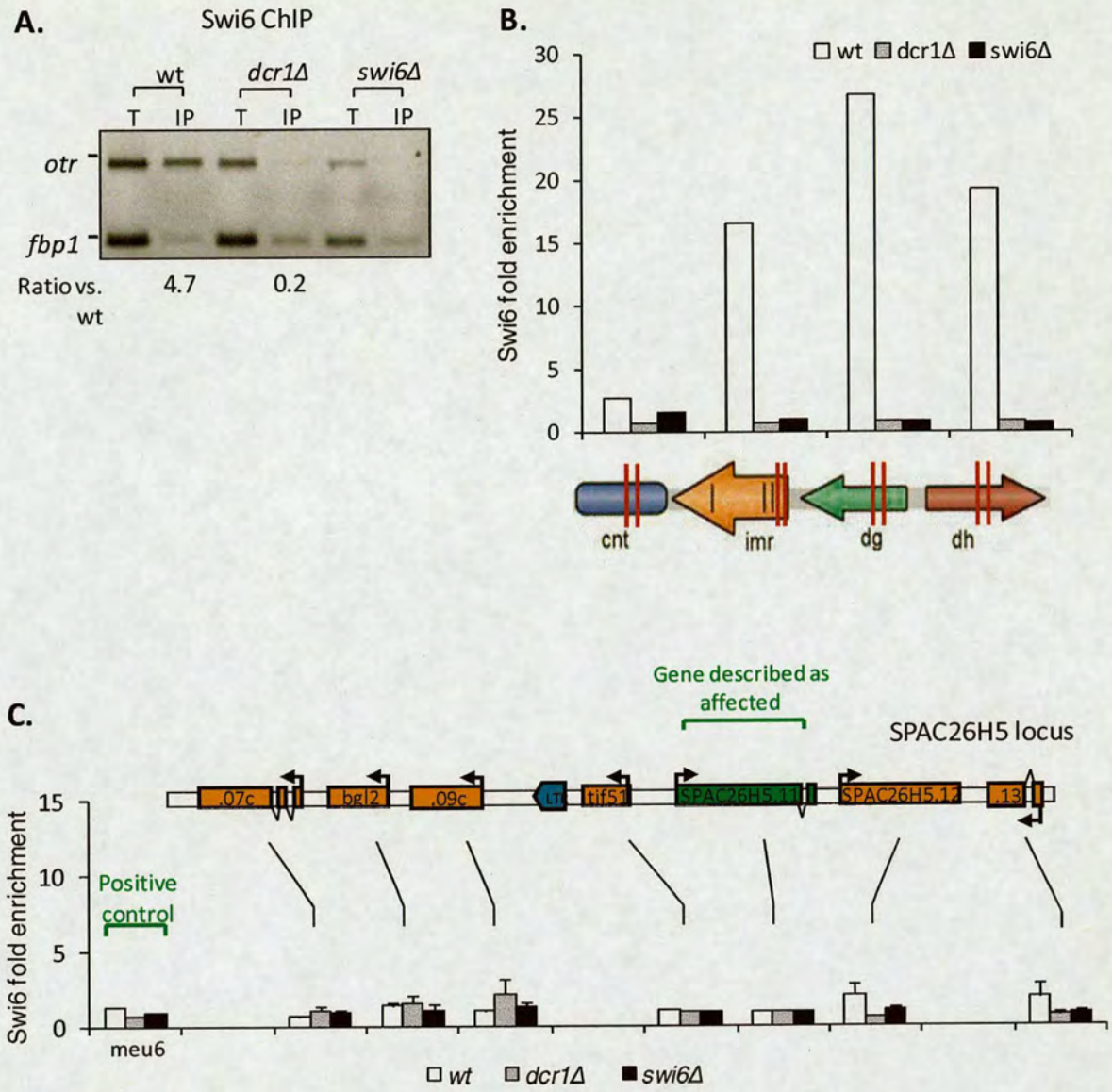


Figure 3-5: Swi6^{HP1} Chromatin IP (ChIP) analysis on SPAC26H5 locus.

A. Chromatin IP (ChIP) was perfomed on wild-type, *dcr1Δ* and *swi6Δ* cells using an antibody against Swi6^{HP1}. Enrichment levels of Swi6^{HP1} over centromeric outer repeats were assayed by multiplex PCR. The values were calculated by normalizing to the internal control (*fbp1*) and then compared the ratios from IP and total or input (T) samples.

B. The same samples were assayed by real-time PCR (qPCR) using primers for several regions at centromere 1: the central core (*cnt*), inner-most repeats (*imr*) and both types of outer repeats – *dg* and *dh*. Enrichment levels are normalized to *fbp1+*. The region of *imr* selected for this assay is still within the heterochromatic domain.

C. Levels of Swi6^{HP1} were assayed by a similar method on the open reading frames (ORFs) that surround the LTR in the SPAC26H5 locus. No significant enrichment was observed for any of analysed ORFs, including SPAC26H5.11 which was reported to be affected by LTR silencing. Similar results were obtained for the LTR-sensitive *meu6+* gene and the outlying ORF SPAC26H5.13. No measurements were made on the LTR sequence due its high redundancy and lack of locus-specific primers. Error bars represent standard deviation of replicate ratios (n=3).

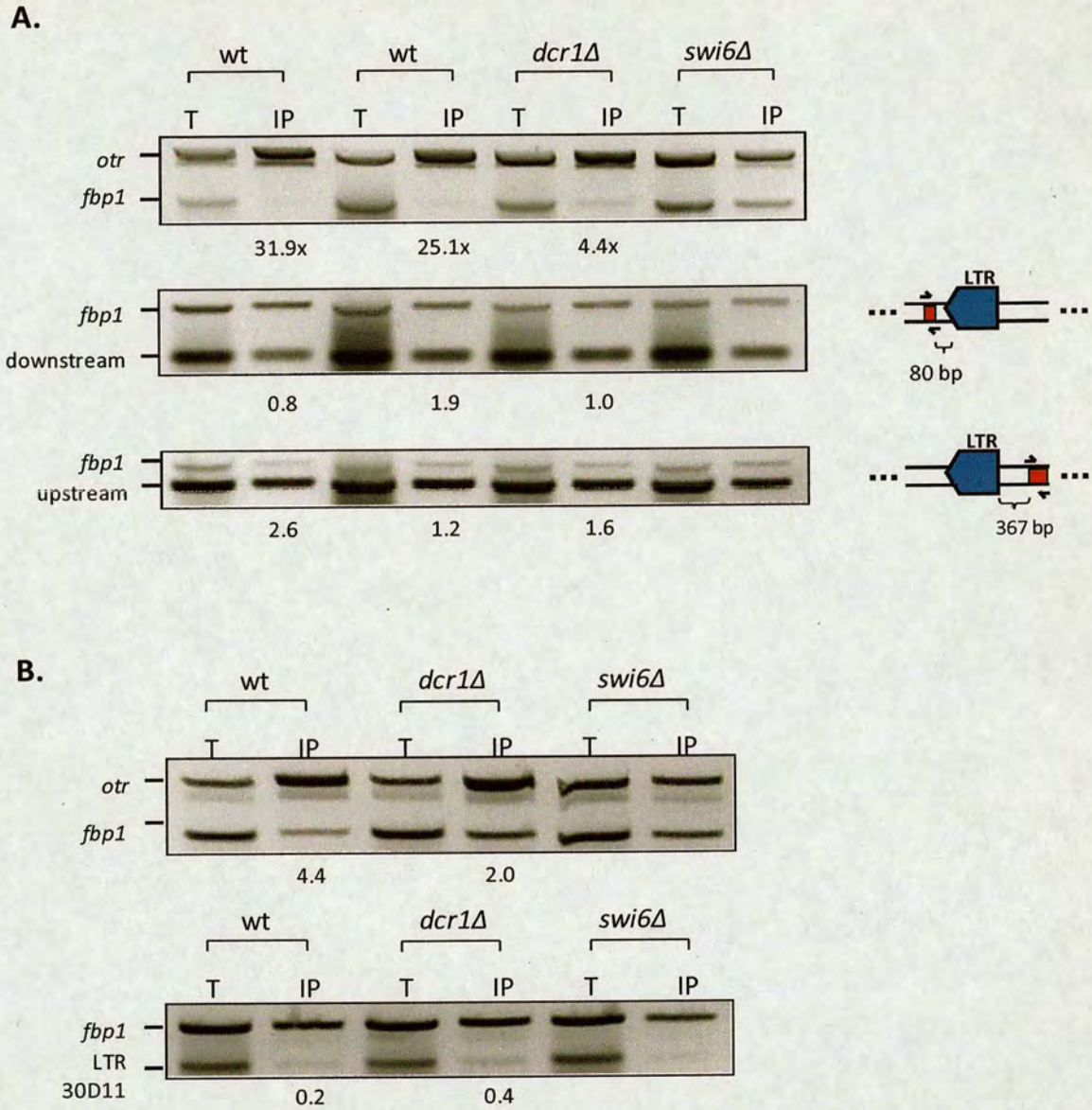


Figure 3-6: ChIP analysis of Swi6^{HP1} levels over LTR sequences.

A. The amount of Swi6^{HP1} over the LTR belonging to the SPAC26H5 locus was analyzed by multiplex PCR using primers that amplify regions directly downstream (3') and upstream (5') of its sequence. The enrichments were first normalised internally against *fbp1* and then calculated IP over input (T). Even though Swi6^{HP1} is readily detectable at centromeres (top panel), it is not detectable at sides the LTR (middle and bottom panels).

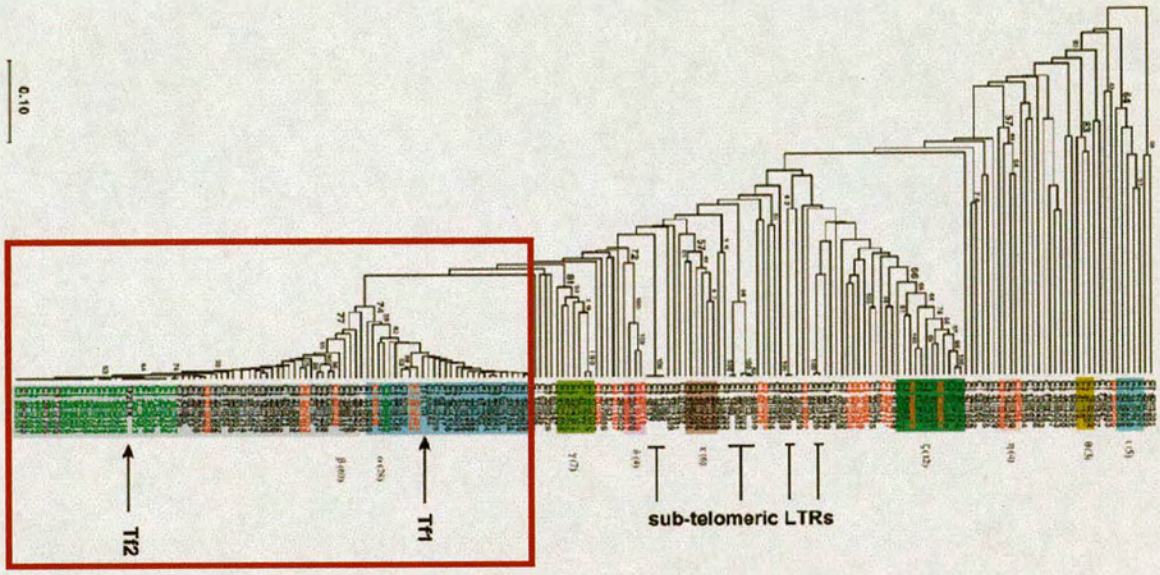
B. Similar experiment using thethered primers to measure enrichment levels over the LTR belonging to the SPAC30D11 locus which was previously described as target for RNAi and heterochromatin formation. The LTR primers used here are identifical to the ones used in Schramke and Allshire 2003. Swi6^{HP1} is detected at the centromeric *otr* (top panel) but not over the SPAC30D11 LTR, unlike previously reported.

3.2.3. LTR-specific transcripts are not detectable in RNAi mutants

The observation that LTR transcripts were visible in RNAi mutants but were significantly less abundant in wild type conditions provided one of the first clues hinting to an involvement of RNAi in LTR silencing and LTR-mediated gene repression. To reproduce this observation, I synthesized strand-specific cDNA from total RNA using primers that could recognize multiple LTRs (LTR consensus) for reverse transcription (fig 3.7). Subsequently, I performed PCR using the same primers to amplify the LTR fragments and resolved the products on an agarose gel stained with ethidium bromide. The results suggest that that sense transcripts for LTRs are found in higher amounts in RNAi mutants (*ago1Δ*, *dcr1Δ*, *rdp1Δ*) compared with wild type (Figure 3-8A). However no conclusion can be drawn from these results since this experiment suffered from difficulties in removing contaminating gDNA due to the high copy number of these repeats in spite of several adjustments being made.

The levels of LTR transcripts were also assessed by standard RT-PCR using oligo-d(T) primed cDNA. The samples were analyzed for LTR expression levels using primers for the LTR consensus or for a single LTR that was originally described as regulated by RNAi and heterochromatin. The results are depicted as expression ratios normalized to wild-type expression levels. Expression levels of LTRs were not found to be induced in mutants for RNAi and heterochromatin formation (Figure 3-8B). This suggests that the previous result represents a very small increase in LTR expression level that is only detected with cDNA primed with specific oligos. An alternative explanation is that LTR transcripts do not acquire a poly-(A) tail. Hence, analysis by Northern blot would allow me to detect any LTR transcripts regardless of their end modifications.

Northern blot showed no detectable increase in LTR expression in the absence of RNAi. Samples of total RNA from wild type, *ago1Δ* and *dcr1Δ* strains were analysed by Northern blot using probes specific for the centromeric outer repeats (dg) and the LTR consensus. Only the ribosomal RNA background was observed for the LTR hybridization in all trials of the procedure, with an exception of a faint smear running at approximately 4500 nt (Figure 3-9). The relative size of this smear suggests that it might represent transcripts from full length Tf2 TEs (4 kb long) but the low signal intensity and extensive smearing suggests that it might be background signal arising from unspecific binding of the probe to the membrane. Furthermore the signal is present in all samples in the same relative amounts when comparing the first wild-type and the mutant lanes. If indeed this smear



LTR consensus probe

(from Bowen et al. 2003)

Figure 3-7: **Phylogenic tree diagram showing the similarity hierarchy of the LTRs (>200bp) in *S. pombe* (Bowen et al. 2003)**. The depitcted sequences were divided into subclades according to sequence conservation. The two major subclades (alpha and beta, red box) contain the highest number of nearly intact LTRs in the genome. Primers were designed to amplify a 280bp homologous region common to all these sequences in order to obtain higher sensitivity in PCR and northern hybridizations.

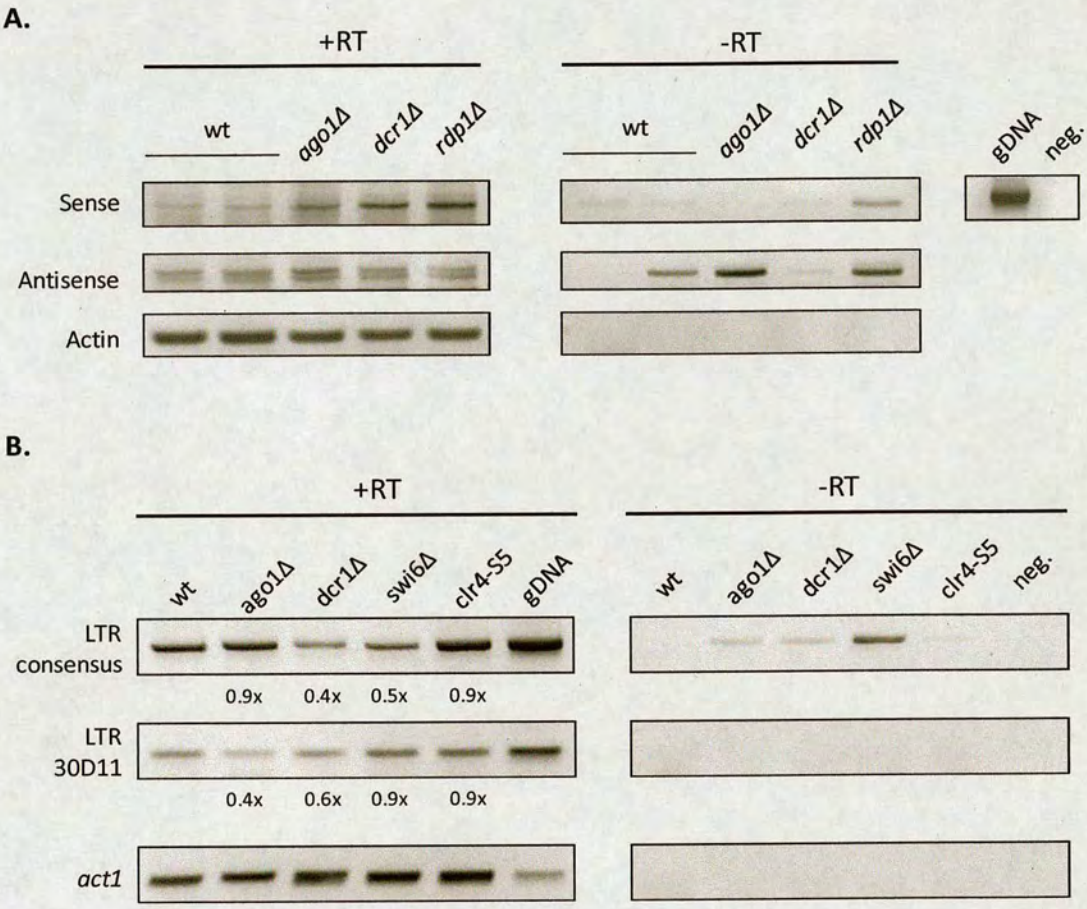


Figure 3-8: RT-PCR analysis for detecting LTR-specific transcripts.

A. Single-stranded RT-PCR using oligos that can recognize multiple LTRs (LTR consensus). Shown are the RT-PCRs specific to the sense and antisense strands of the LTR with *act1* as control. Due to the high copy number of LTR sequences in the genome (close to 300), I was unable to completely remove all homologous DNA from the samples which resulted in significant amounts of contamination visible in the –RT control PCR.

B. RT-PCR for poly-adenylated transcripts. Samples were assayed for levels of LTR transcripts using primers specific for multiple LTRs (LTR consensus) or a single LTR. The LTR from SPAC30D11 locus analyzed here was previously suggested to be silenced through RNAi and heterochromatin.

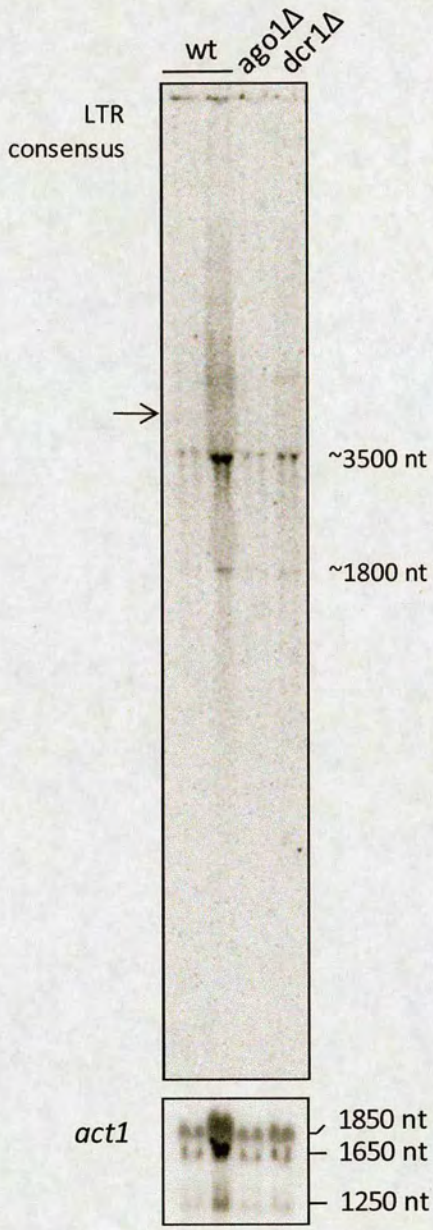


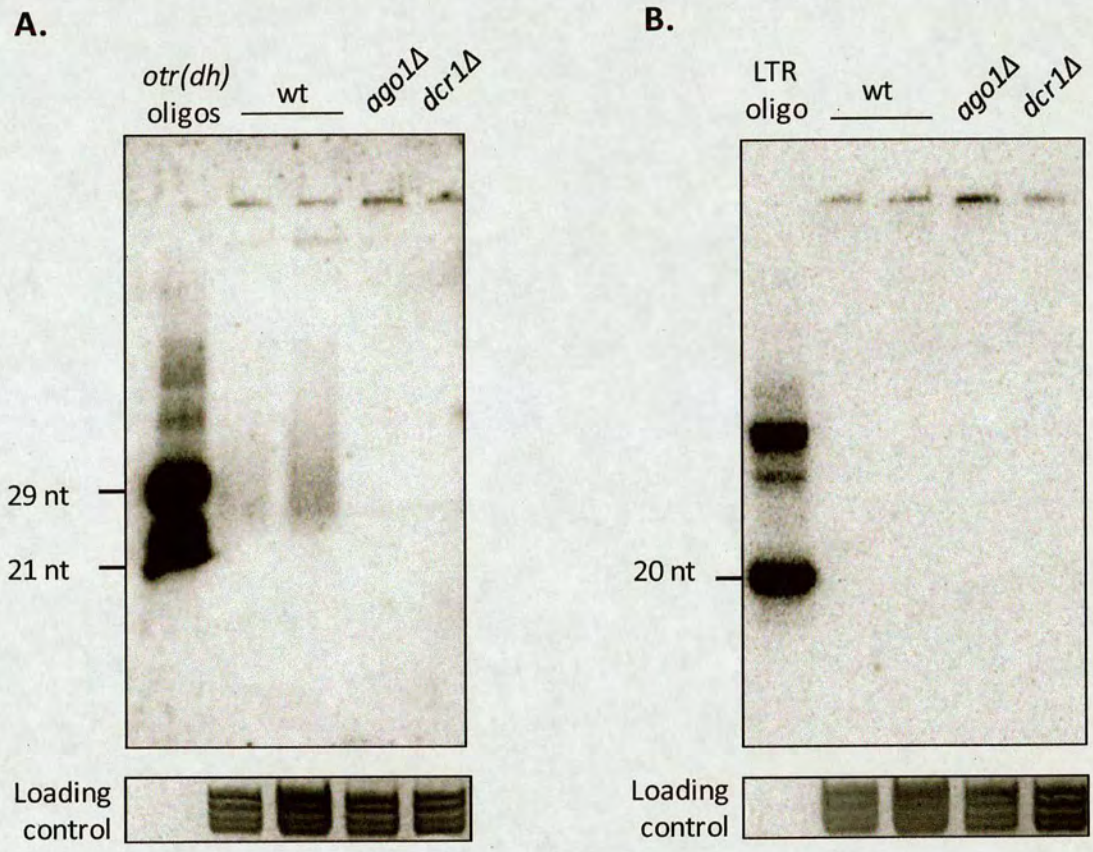
Figure 3-9: Northern analysis for LTR transcripts in wild-type and RNAi mutant cells. The two bands visible at approximately 3500nt and 1800nt correspond to the two larger ribosomal RNA molecules and were assumed to be background. The black arrow marks were a full length Tf-2 transposon transcript was expected to run (~4000nt). The panel below shows the result of a loading control hybridization using an *act1* specific probe. The several visible bands correspond to the three known isoforms of the *act1* mRNA in fission yeast (Mertins and Gallwitz, 1987).

represents Tf2 TE transcription, its levels are not affected by loss of RNAi. The presence of visible bands with the *act1* probe demonstrated that the hybridization conditions were adequate and that the RNA samples were not degraded (Figure 3-9, bottom panel). The multiple bands in the *act1* hybridization represent the 1850, 1650 and 1240 nucleotide long actin mRNAs found in *S. pombe* (Mertins and Gallwitz 1987). It is possible that these transcripts are found in very low amounts in the cells so the amount of RNA used in these blots may have been insufficient. Another possibility is that the LTR transcripts are short enough to have migrated out of the agarose gel. The most likely explanation is that LTR transcripts are present in very low or even negligible levels in wild-type and RNAi mutant cells, which explains the difficulties in detecting them. However this explanation clashes with the original postulate of LTR repression being mediated by RNAi (Schramke and Allshire 2003).

3.2.4. siRNAs from LTR repeats are not detectable

The conventional method of detecting siRNAs is by Northern blot using a probe whose sequence is identical to the siRNA target site. However, a major shortcoming of this technique is its reduced sensitivity, making it less adequate to detect small RNAs present in low amounts. In order to increase the chances of detecting LTR siRNAs by Northern blot, I opted for a probe that could recognize several of these repeats. I designed primers that could amplify a large number of closely related LTRs, with preference for the alpha and beta subclades (Figure 3-7) (Bowen, Jordan et al. 2003). These two subclades together contain a large number of full-length repeats in the total population of LTR that bear closer similarity to Tf1 and Tf2 LTRs, respectively. For this reason, it is likely that the transcriptional activity of the majority of these repeats is still intact, which could be crucial for producing dsRNA. The resulting probe is nearly equivalent in sequence to all 86 members of these two subclades. Additionally, in a separate hybridization I used a probe specific for a region within the centromeric outer repeats known to contain siRNAs, thus providing a positive control for the siRNA detection procedure.

By using a probe specific to a centromeric outer repeat region numbers of known centromeric siRNAs (siRNAs K to H) it was possible to detect by Northern blot small RNAs complementary to centromeric outer repeats with sizes in the range expected for siRNAs (Figure 3-9A and B) (Reinhart and Bartel 2002). As expected, these were only visible in wild type extracts and not on samples of *ago1Δ* or *dcr1Δ* samples (Volpe, Kidner et al. 2002). LTR-specific small RNAs were



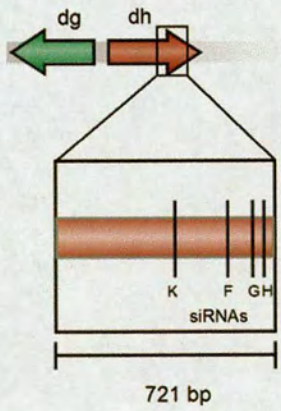


Figure 3-10: Northern analysis for centromeric and LTR small RNAs.

A. Samples of <100nt RNA from wild-type, *ago1Δ* and *dcr1Δ* were migrated in parallel with DNA oligos used in the PCR reactions for the corresponding probe. The signal corresponding to siRNAs can be seen as a smear migrating at ~23 nt and above. The diagram below depicts the location within the outer repeat *dh* of the probe used in this experiment.

B. Similar experiment using an LTR-specific probe. No signal was detectable in both wild-type samples, despite longer exposure time.

Loading control in both experiments was ethidium bromide staining of tRNAs and rRNAs from the gel.

not detected in any of the lanes, suggesting that LTRs are not targeted for siRNA production. As shown in the previous section, no signal for LTR was detectable by small RNA Northern blots. Furthermore, no LTR signal was detected at higher sizes that those expected for siRNAs which argues against the existence of short LTR transcripts at a rate that can be detected by this technique. Given that the sensitivity of the assay is questionable, I cannot exclude the possibility that LTR siRNA levels are present in wild-type cells in amounts below the threshold detection level of this Northern blot protocol. Only by performing RNAse protection assay would be possible to properly assess this possibility. However it is safe to say that LTRs are not targeted for siRNA production to the same extent as centromeric outer repeats are.

3.3. DISCUSSION

The data presented in this report is not sufficient to assess all reasonable hypotheses that could explain the contradictory evidence of my analyses with that of Schramke et al. It is sufficient to cast reasonable doubts on the existence or the details of the proposed phenomenon of LTR-mediated silencing. While this work was being conducted, two independent reports were published containing evidence that disputed the original description of LTR silencing by RNAi and heterochromatin (Cam, Sugiyama et al. 2005; Hansen, Burns et al. 2005). Hansen *et al.* performed genome-wide microarray expression analysis on mutants for RNAi factors *ago1*, *dcr1* and *rdp1*, the H3K9 methyltransferase *clr4* and histone deacetylases *clr3* and *clr6* as well as *clr1* (Hansen, Burns et al. 2005). The authors looked for genome elements that were overexpressed in cells depleted of RNAi and silencing pathways and found only 18 genes that responded to RNAi ablation with an expression induction over at least 1.4 fold. This group of 18 genes are located 10 kb or more away from any LTR sequence and do not include the 7 meiotic genes previously suggested to be LTR-regulated (Schramke and Allshire 2003; Hansen, Burns et al. 2005). In *the clr* mutants, the effect on genome expression levels is more dramatic, particularly on the *clr3Δ clr6Δ* double mutant cells that displayed a total 606 upregulated genes. These include several genes located in the vicinity of Tf2 transposons and their LTRs. The latter observation is interesting considering that transcript levels for the Tf2 transposons themselves are higher in *clr3Δ clr6Δ* double HDAC mutant cells. However the populations of genes that respond to histone deacetylase mutations do not overlap with the 18 RNAi-sensitive genes. Meanwhile the effect of RNAi mutants on Tf2 transcription levels is hardly detectable. This evidence prompted the authors to conclude that histone deacetylases are involved

in regulating expression of genes and transposable elements in a manner that is independent of RNAi-independent and H3K9 methylation (Hansen, Burns et al. 2005).

The second study is also a genome-wide survey of chromatin modifications (H3K9 methylation and H3K4 methylation) as well as localization of RITS components (Ago1 and Chp1) and $Swi6^{HP1}$ by ChIP-on-chip (Cam, Sugiyama et al. 2005). In addition, the authors assembled a library of sequences from purified *S. pombe* siRNAs and mapped their targets in the genome. Cam and colleagues failed to encounter any significant enrichment of LTR sequences for H3K9me2, $Swi6^{HP1}$ or any of the RITS components. The authors could not find any matches to LTRs or Tf1/2 transposable elements in the total 1292 individual siRNA sequences. Expression microarray experiments confirmed the observations from Hansen *et al.* in that the 7 genes supposedly regulated by LTRs are not overexpressed in the absence of heterochromatin (*clr4Δ*) (Cam, Sugiyama et al. 2005).

The evidence presented in this chapter, combined with these two independent reports, provide considerable arguments against the notion that LTRs are targeted by heterochromatin formation in an RNAi-dependent manner and that this has any effect on genes surrounding these LTR sequences. Given that the original observations could not be reproduced, the conlusions published in Schramke et al. were since retracted (Allshire 2005).

Even though RNAi and heterochromatin appear not to be involved, TEs and their associated LTRs in fission yeast appear to be under the control of a stress-related regulatory pathway that comprises inducible transcription factors of the Atf/CREB family and histone deacetylases. However this TE transcription regulation mechanism is not novel since it is long known that TEs can be induced in stress conditions in other eukaryotes (Pouteau, Huttner et al. 1991; Liu, Chu et al. 1995). This phenomenon might either reflect a complex cellular response to stress in which these elements play a role or a window of opportunity for TEs to further spread in the host genome (Liu, Chu et al. 1995; Grandbastien 1998). In the case of fission yeast this regulation process is clearly acting independently of RNAi and conventional heterochromatin. There is no conclusive evidence showing that this phenomenon can affect nearby genes by a spreading mechanism either. In the case of *wtf* (with-Tf) genes, it is not obvious if the promoters of *wtf* series of genes contain Atf1 binding sites or if these genes are under the control of LTR promoters themselves (Bowen, Jordan et al. 2003). The *wtf* genes themselves are expressed yet it is unclear what their function consists of since they are either pseudo-genes, non-coding genes or encode uncharacterized proteins (Mata,

Lyne et al. 2002; Bowen, Jordan et al. 2003). The regulatory interactions between TEs and associated *wtf* genes are a matter of interest and require further detailed studies.

CHAPTER 4

EXPLORING LINKS BETWEEN RNA PROCESSING AND RNA INTERFERENCE

4.1. INTRODUCTION

It is becoming increasingly clear that RNA silencing does not function in isolation from other molecular pathways within the cell. In conventional RNAi, the RISC complex localizes to cytoplasmic GW/P bodies in vertebrate cells whenever RNAi is actively degrading a target mRNA (Liu, Valencia-Sanchez et al. 2005; Rehwinkel, Behm-Ansmant et al. 2005). These bodies are involved in mRNA translational arrest and turnover and the association of RISC with these structures points to a connection between RNA silencing and these other RNA mechanisms in the cell (Liu, Valencia-Sanchez et al. 2005; Rehwinkel, Behm-Ansmant et al. 2005). It appears that these links are conserved in other eukaryotes since a genetic screen conducted in *C. elegans* for additional factors involved in RNAi (Kim, Gabel et al. 2005) identified several genes that are known to be involved in RNA processing. In the case of *S. pombe*, RITS (the effector complex: Ago1, Tas3 and Chp1) is thought to localize to the centromeric outer repeats and to interact with RDRC (RNA-dependent RNA polymerase complex) via a nascent RNA polymerase II transcript (Motamedi, Verdel et al. 2004). Thus, fission yeast RNAi functions within a co-transcriptional context with must be coordinated with other molecular events that are linked with RNA polymerase II transcription, such as pre-mRNA splicing, 5' end capping and 3' end polyadenylation. This may then explain for example why mutations in several splicing factors can affect the ability of the cells to maintain silencing of a reporter gene inserted into the centromeric outer repeats (Bayne, Portoso, Ekwall and Allshire – unpublished observations). However, it is not clear how pre-mRNA splicing or any other RNA processing pathway influences RNAi-mediated chromatin silencing.

It is possible that the mechanism of RNA silencing overlaps with many other RNA metabolism pathways by sharing common components, reaction intermediates or by competing for the same resources. For instance, the helicase Hrr1 and the poly(A) polymerase Cid12 (RDRC components) are essential for RNAi but are also similar to other factors involved in RNA processing and turnover (Motamedi, Verdel et al. 2004). While helicases are common in molecular pathways

that deal with RNA or DNA, Cid12 is more distinct in that it shares close homology with Trf4p. The latter is a member of a family of poly(A) polymerases that forms the TRAMP complex with Air2p and Mtr4p and is involved in targeting transcript degradation by the exosome complex in budding yeast (LaCava, Houseley et al. 2005; Wyers, Rougemaille et al. 2005). One of the functions of TRAMP and the exosome is to degrade "aberrant" transcripts formed by incorrect transcription, RNA end processing or folding. In a similar aspect, specific plant RNA-dependent RNA polymerases (RdRP) are believed to trigger PTGS (Post-Transcriptional Gene Silencing) of viral RNA by responding to an yet-unidentified characteristic of these molecules that distinguishes them from endogenous transcripts (Hamilton and Baulcombe 1999; Mourrain, Beclin et al. 2000; Yu, Fan et al. 2003; Schwach, Vaistij et al. 2005). The fact that RNAi and TRAMP-mediated degradation by the exosome both employ poly(A) polymerases suggests that the two mechanisms share the same functional principle for recognizing target RNA and consequently cooperate or compete for the same targets.

If RNAi is compromised in a mutant for an RNA metabolism factor, this will have an impact in centromeric heterochromatin and consequently the cells will display chromosome segregation defects could be expected. In fact, several factors involved in different aspects of RNA polymerase II transcription termination and 3' end processing are indeed known to be required for proper chromosome segregation. One such factor is Pfs2, an essential pre-mRNA cleavage and polyadenylation factor that is required to prevent transcriptional read-through in *S. pombe* (Wang, Asakawa et al. 2005). It is also essential for accurate chromosome segregation and entry into S phase (Wang, Asakawa et al. 2005). Mutation in *pfs2+* leads to chromosome segregation defects and activation of the spindle checkpoint, as shown by the accumulation of Bub1 and Mad2 foci in the nucleus (Wang, Asakawa et al. 2005). Pfs2 is a component of the yeast CPF (cleavage and polyadenylation factor) complex that is responsible for pre-mRNA cleavage at the co-transcriptional termination site and to promote the 3' end maturation of the pre-mRNA by polyadenylation. One of the components of the CPF is Cft1, a homologue of the human CPSF1 factor that binds to the AAUAAA conserved sequence in the pre-mRNA (Murthy and Manley 1995; Dichtl, Blank et al. 2002). Both CPSF1 and Cft1 have a conserved motif called CPSF-A that is also found in Rik1, a component of the $Clr4^{Su(var)3-9}$ complex that is required for heterochromatin assembly in fission yeast (Ekwall and Ruusala 1994; Allshire, Nimmo et al. 1995; Ekwall, Nimmo et al. 1996; Nakayama, Rice et al. 2001). Thus, it is possible that these two processes are functionally connected.

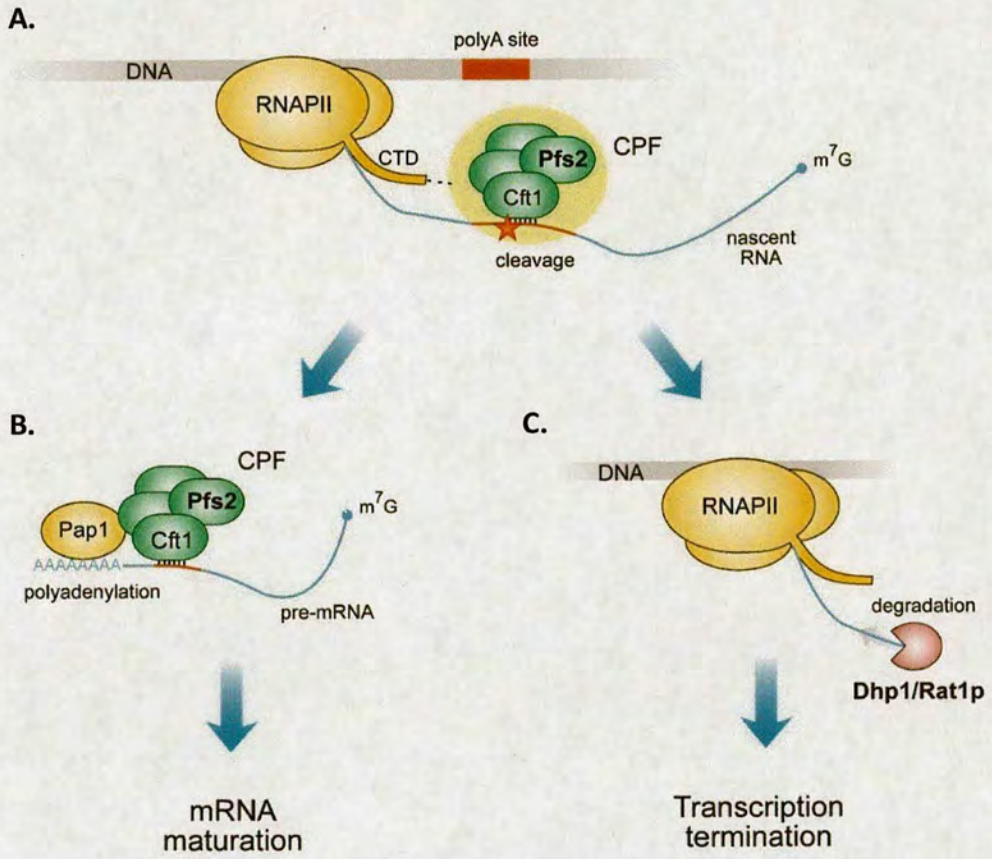


Figure 4-1: Overview of RNA polymerase II transcription termination and pre-mRNA 3' end processing.
A. An RNA polymerase II (RNAPII) transcribing through a gene will eventually reach the termination or poly(A) site (red box). This site encodes a conserved AAUAAA motif that is recognized by Cft1 which together with Pfs2 and other factors form the yeast CPF (cleavage and polyadenylation factor) (in red). The CPF cleaves the nascent transcript at the AAUAAA motif, which then initiates pre-mRNA 3' end maturation and signals RNA Polymerase II to terminate transcription.
B. CPF promotes the maturation of the pre-mRNA molecule by recruiting the poly(A) polymerase Pap1 to produce a poly(A) tail on the free 3' end of the pre-mRNA
C. After the cleavage event, RNA polymerase II continues to transcribe regardless and must be terminated. The Rat1p exonuclease (in purple) begins degrading the free 5' end of the RNA strand still attached to the polymerase. As it is more processive that elongating RNA polymerase II, Rat1p reaches the RNA polymerase II and signals it to disengage from the DNA template.
Highlighted in bold are two factors involved in the two mechanisms, Pfs2 and Dhp1/Rat1p. Mutants of these two factors were analysed for possible links of transcription termination and 3' end processing with RNAi and heterochromatin formation.

Dhp1 is also known to be involved in chromosome segregation. Dhp1 is the counterpart of the *S. cerevisiae* 5'-3' exonuclease Rat1p that is involved in diverse processes such as rRNA maturation and transcriptional termination (Henry, Wood et al. 1994; Kim, Krogan et al. 2004) (Figure 4-1C). Depletion of this essential protein in *S. pombe* leads to accumulation of polyadenylated transcripts in the nucleus but also causes defects in chromosome segregation (Shobuike, Tatebayashi et al. 2001). Previous work in our lab suggested that the *dhp1-1* temperature sensitive mutant has defects in centromeric heterochromatin formation and accumulates centromeric transcripts in similar fashion to RNAi mutants such as *dcr1Δ (*Douglas Robertson – unpublished observations).

Finally, the cold-sensitive mutant *dis3-54* (Defective In Segregation) was isolated in a mutagenesis screen for factors involved in mitotic chromosome segregation (Ohkura, Adachi et al. 1988). Dis3 is actually the homolog of *S. cerevisiae* Dis3/Rrp44p, an essential subunit of the 3'-5' exonuclease complex known as the exosome that is involved in transcript turnover and maturation of precursor RNA molecules (Mitchell, Petfalski et al. 1997; Suzuki, Noguchi et al. 2001). In *S. cerevisiae*, Dis3/Rrp44 is involved in directing the activity of the core exosome complex to process and degrade multiple RNA substrates in both nucleus and cytoplasm, which has far-reaching implications in general RNA metabolism in the eukaryotic cells (Dziembowski, Lorentzen et al. 2007) (Figure 4-2). The exosome is important for the quality control of transcription and 3' end processing of a large diversity of RNA species, including snRNAs, snoRNAs, rRNAs, tRNAs and mRNAs. In fission yeast cells, impaired Dis3 function results in severe defects in sister chromatid separation and failure to exit mitosis (Ohkura, Adachi et al. 1988). This phenotype is not directly reminiscent of a classic centromeric heterochromatin defect but it is conceivable that the severe phenotype includes defects in RNAi-directed heterochromatin formation.

In this chapter I describe the results obtained from investigating into the possible role of Pfs2, Dhp1 and Dis3 in processing of non-coding centromeric transcripts and centromeric heterochromatin integrity. The results demonstrate that RNA 3' end processing and transcription termination pathways do not have a significant influence on the RNAi pathway and on the stability of centromeric heterochromatin. Defective RNA termination only results in a subtle defect of centromeric silencing. Although the exosome does play a role in degrading centromeric transcripts downstream of the RNAi pathway, its direct involvement in silencing and heterochromatin stability is unclear.

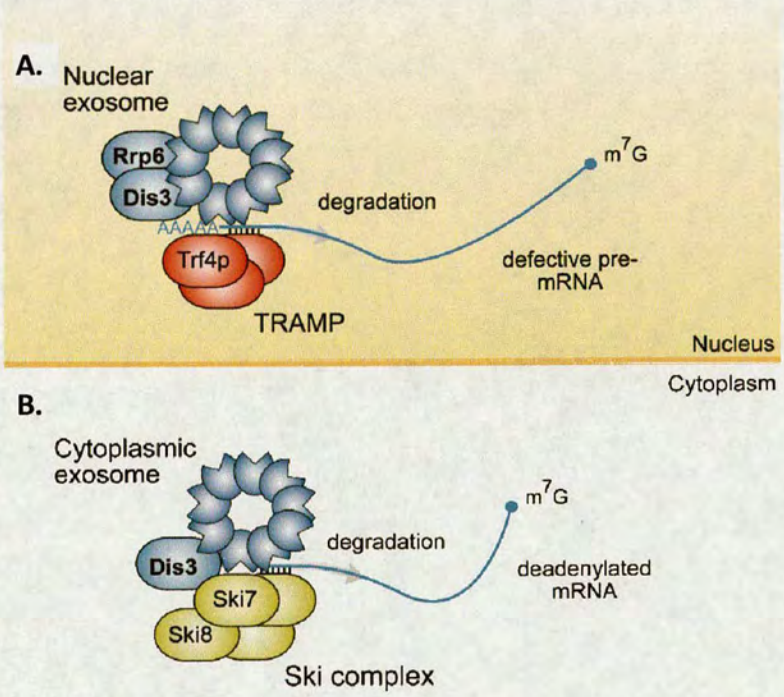


Figure 4-2: Diagram of the nuclear and cytoplasmic forms of the exosome complex (adapted from Houseley et al. 2006)

The exosome (in blue) is found in both nucleus and cytoplasm but the two forms of the exosome complex are slightly different in composition and required different targeting machinery.

A. In the nucleus, the core exosome is associated with Dis3 and Rrp6 and interacts with the TRAMP (Trf4p Air1p Mtr4p poly(A) polymerase) complex. TRAMP binds to a defective RNA species, such as a pre-mRNA (pictured), misfolded tRNA or rRNA, and begins polyadenylating its 3' end. This facilitates the loading of the exosome and initiation of 3' to 5' degradation. TRAMP is thought to interact with both Rrp6 and Dis3 on the surface of the nuclear exosome complex.

B. Unlike Rrp6, Dis3 is also found on the cytoplasmic form of the exosome complex. Targeting is provided by factors such as the Ski complex that recruit the exosome for degrading transcripts in the context of specific mRNA downregulation, mRNA turnover, nonsense-mediated and non-stop decay.

Highlighted in bold are the two exosome components for which mutant strains were analysed in this study, Dis3 and Rrp6.

4.2. RESULTS

4.2.1. RNA processing and turnover mutants have subtle defects in heterochromatin integrity

When the *ade6+* gene is inserted with the outer repeats of fission yeast centromeres, the gene is silenced by nearby heterochromatin (Ekwall, Cranston et al. 1999). Cells with the *ade6-* phenotype accumulate the upstream substrate which when oxidised turns colonies red. Silencing of ade6+ causes the cells to be phenotypically *ade6-* and thus accumulate the red-coloured metabolite P-ribosylaminoimidazole in vacuoles when they are grown in medium supplied with low adenine. When centromeric heterochromatin is disrupted, expression levels of the *ade6+* gene increase and the colony colour shifts from red to white to an extent that reflects the magnitude of the silencing defect. This provides a valuable assay to determine if a particular mutation affects RNAi and heterochromatin integrity at centromeres.

The RNA processing and turnover mutants assayed in this study are *pfs2-11*, *pfs2-3169*, *dis3-54* and *dhp1-1*. All the assayed mutants are temperature-sensitive with the exception of *dis3-54* which is cold-sensitive. This means that *dhp1-1*, *pfs2-11* and *pfs2-11* mutant cells can grow at 25°C (permissive temperature) but lose the function of these essential proteins when the temperature is shifted to 36°C (restrictive temperature) (Shobuike, Tatebayashi et al. 2001; Wang, Asakawa et al. 2005). Conversely, the permissive temperature for the *dis3-54* mutant is 34°C and the restrictive is 20°C (Ohkura, Adachi et al. 1988). The most informative temperature is 32°C which can be considered as semi-restrictive to all of these mutants because it allows the mutant cells to grow albeit deficiently.

The four mutants were crossed to create strains containing a copy of the *ade6+* gene inserted at outer repeats of centromere 1 (*otr1R(SphI):ade6+*) and an *ade6* allele with an internal deletion at the endogenous *ade6+* locus (*ade6-DN/N*). They were assayed for centromeric silencing defects at a range of temperatures between 18°C and 36°C in a serial dilution assay (Figure 4-3). The impact of the temperature on the growth rate of each mutant can be clearly seen here. This implies that these cells suffer from a loss of function of the mutated protein that is severe enough to affect cell growth when they are incubated at 32°C. The colour of the *pfs2-11 and pfs2-3169* mutant colonies at 32°C and 34°C is dark red similarly to wild-type colonies which indicates that centromeric silencing remains normal in these mutants. The colour of *dhp1-1* colonies is lighter at 25°C through 34°C but not at 18°C, suggesting that a slight alleviation of silencing occurs when

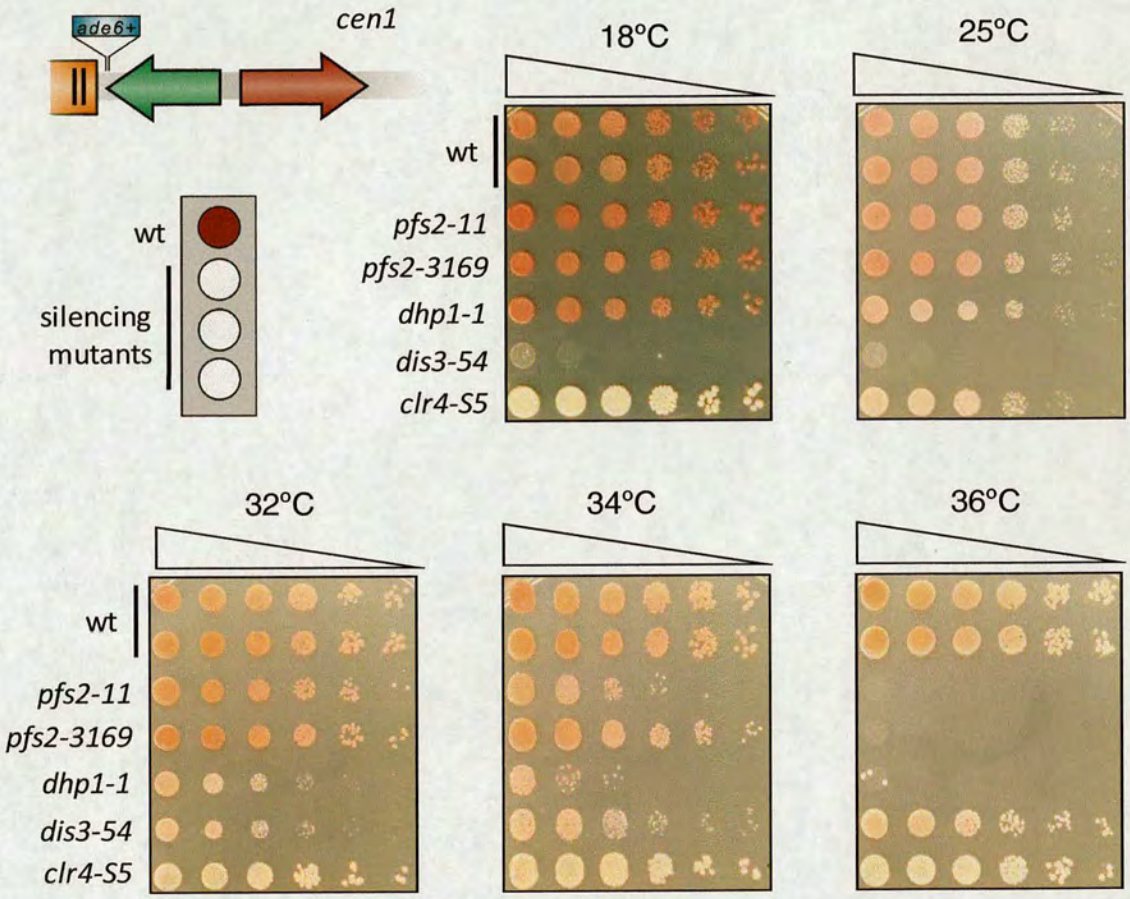


Figure 4-3: Silencing assay performed on *pfs2*, *dhp1* and *dis3* mutants.
All strains are carrying the *otr1R(SphI):ade6+* insertion at the centromere and the (top left diagram) while the endogenous *ade6* locus has an internal deletion (*ade6-DN/N*). Heterochromatin enforces silencing on the centromeric *ade6* whichs blocks the adenine biosynthesis pathway. This causes the cells to accumulate a red pigment when grown in media with limited adenine. Alleviation of silencing of *otr1R(SphI):ade6+* leads to loss of this red colour toward the normal white colour , providing a readout for silencing defects (top left panel).Fresh cells were plated in 10-fold serial dilution series on YES plates containing limiting adenine. Subsequently the plates were left to grow at 5 different temperatures ranging from 18°C to 36°C for 7 days (14 days for 18°C). The differences in growth are a consequence of the temperature-sensitive or cold-sensitive phenotypes of each mutant. Any defects in silencing are detected as a colour shift from red (wt – normal silencing) to white (*clr4-S5* – no silencing).

dhp1-1 cells are compromised. The same can be said for *dis3-54* cells, which display lighter colour in all temperatures where growth is visible. In sum, the results of the silencing assays suggest that *dis3-54* and *dhp1-1* mutations cause mild defects on transcriptional silencing at the centromeric outer repeats. However, this requires confirmation by molecular analysis.

To further determine if the *dhp1-1*, *pfs2-3169*, *pfs2-11* or *dis3-54* mutants have any effect on RNAi-directed silencing, I monitored levels of centromeric *otr* transcripts and the corresponding siRNAs in all mutants. RNA was harvested from cells grown at semi-restrictive temperature (32°C) at which both *dhp1-1* and *dis3-54* showed slight alleviation of silencing in the serial dilution assays. The levels of outer repeat transcripts were first addressed by semi-quantitative RT-PCR using oligo-d(T)-primed cDNA. Transcript levels were not found to be increased in relation wild-type levels with the exception of *dis3-54*, which displayed increased levels of transcripts (Figure 4-4A). Northern analysis was performed on the same samples using a probe specific for the *dg* outer repeats that overlapped the region assayed by RT-PCR (Figure 4-4B). The result confirms that the *dhp1-1*, *pfs2-11* and *pfs2-3169* alleles do not accumulate *otr* transcripts at a detectable level. In RNA extracted from *dis3-54* cells, centromeric transcripts were not readily detectable by northern analysis unlike for *dcr1Δ* and *clr4Δ* samples. This observation suggests that the increase in centromeric transcripts in *dis3-54* is mild and may occur by reasons other than defective RNAi or centromeric heterochromatin.

To further assess the integrity of the RNAi pathway in these mutants, the presence of centromeric siRNAs was also examined by northern analysis (Figure 4-5). Wild-type levels of centromeric siRNAs were detected in all mutants, arguing that there is no extensive defect in siRNA production in any of these mutants at semi-permissive temperature. Given that the levels of centromeric siRNA levels are maintained by a closed loop of RITS and RDRC acting together with Dcr1 and Clr4 at the outer repeats it is not likely that the pathway is being significantly affected by any of the RNA processing and turnover mutations at semi-permissive temperature.

4.2.2. RNAi pathway integrity in *dhp1-1*, *pfs2-11* and *pfs2-3169* mutants

The nature of the slight alleviation of silencing detected is different between *dhp1-1* and *dis3-54*. While *dis3-54* colonies display a lighter homogenous colour, *dhp1-1* ones are heterogeneous in colour (variegated) (Figure 4-6A). This could be indicative of switching between silent and active states of the centromeric *ade6+* gene. This is supported by the observation that

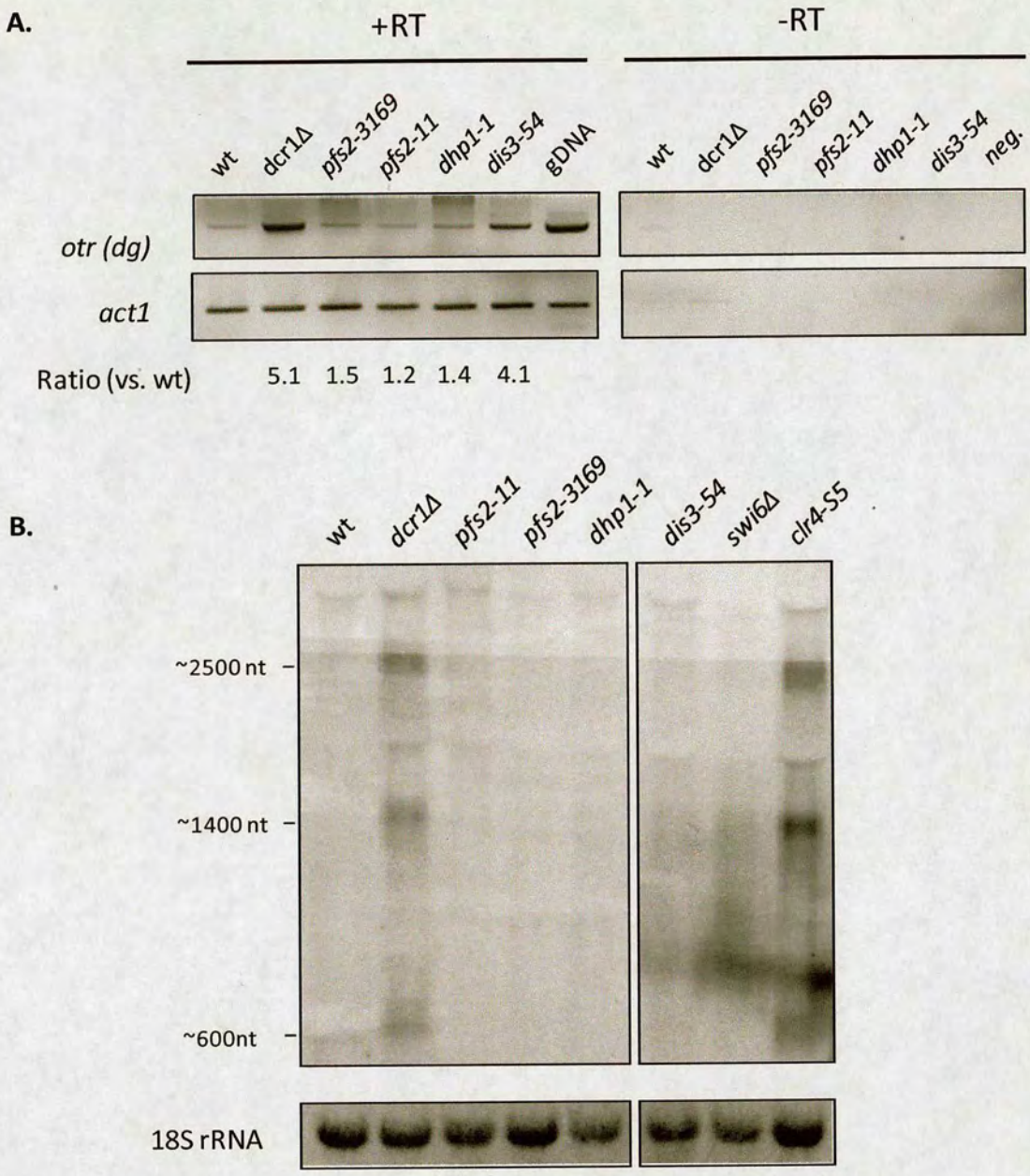


Figure 4-4: Analysis of *otr* transcripts on all mutants at semi-permissive temperature.

A. RNA was purified from cells grown at 32°C and used to synthesize cDNA using oligo-d(T). Semi-quantitative PCR was performed using primers for the *dg* region of the outer repeats and *act1* as a control. Ratios of expression were calculated vs. *wt* levels.

B. Samples of 10 ug of total RNA from control and mutant cells were assayed by northern analysis using a *dg*-specific radiolabelled probe. The three major sizes of transcripts from this region are visible at approximately 2500, 1400 and 600 nucleotides. The loading control was 18S rRNA visible on the membrane by ethidium bromide staining.

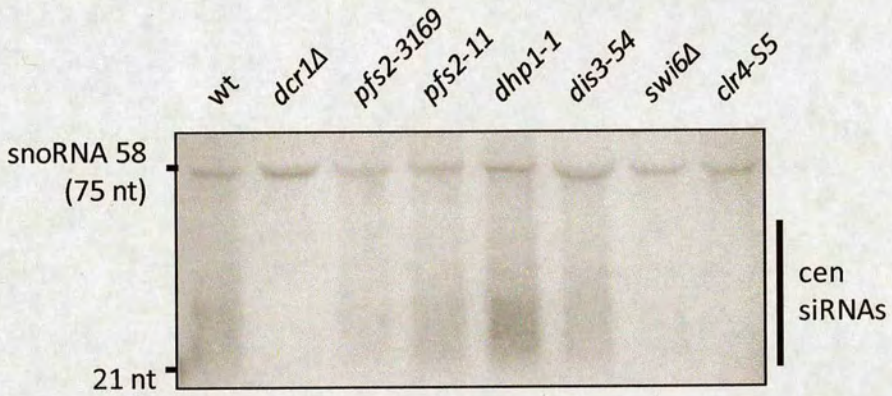


Figure 4-5: Levels of centromeric siRNAs on all mutants grown at semi-permissive temperature. RNA was extracted from *pfs2*, *dhp1* and *dis3* mutant cells and the lower molecular weight fraction was separated by differential precipitation. The resulting fractions were used run on a 15% polyacrylamide gel in the presence of Urea. After transfer the membrane was hybridized with a probe that recognizes siRNAs from the *dh* region of the centromeric outer repeats. Loading control was provided by the hybridization of an end-labelled oligo to snoRNA58.

the alleviated (white) sectors of the variegating colonies tend to overgrow the red sectors, suggesting that the white sectors result from *ade6+* phenotype rather than indirect effects upstream of *ade6*. The degree of this variegation was quantified by surveying the colour of a large number of single colonies grown at 25°C and 32°C for each mutant. Of all the tested mutants, variegation was only found in *dhp1-1* colonies particularly at 25°C where it is seen on 29% of all colonies analysed (Figure 4-6B). At 32°C the percentage of variegating colonies found is only 7% (Figure 4-6B).

The stability of the red and white status in *dhp1-1* cells was addressed more closely. Several colonies grown at 25°C were selected according to colour and plated at 25°C and 32°C and the resulting colonies were again categorised according to colour (Figure 4-7A). The purpose was to determine the relative stability of the silent and alleviated states by measuring how often white colonies would remain white or switch back to red and vice-versa. At 25°C all red colonies retained silencing while more than 50% of the white cells maintained their alleviated state. At higher temperature, white colonies were less stable and formed more red sectored colonies. At 32°C, the proportion of red and white colonies in both populations is similar regardless of the starting state. When the same growth assay was performed in liquid culture, subsequent northern analysis showed that no increase occurs in *otr* transcript levels regardless of cell colour (Figure 4-7B). Thus the *dhp1-1* mutation does not significantly affect transcript levels from the outer repeats, unlike mutants in RNAi and heterochromatin factors.

Although the function of mutant Pfs2, Dhp1 and Dis3 proteins may be affected at semi-restrictive temperature in the mutants analyzed here, it is not fully abrogated since the mutant cells are still partially viable. It is thus possible that under the conditions used, defects in defects in RNAi are too subtle to be detected using the standard assays. Shifting the mutant cell cultures to restrictive temperature should further inactivate the mutant proteins and thus may reveal more readily detectable RNAi defects. According to published data, cells containing the *pfs2-3169* allele activate the spindle checkpoint after 4 hours of growth at 36°C (Wang, Asakawa et al. 2005). This is indicative of cell cycle checkpoint activation caused by loss of Pfs2 function (Wang, Asakawa et al. 2005). The much tighter *pfs2-11* allele causes cell cycle arrest after only 1 hour of growth at 36°C (Wang, Asakawa et al. 2005). In *dhp1-1* cells, the mutant protein is degraded to undetectable levels after 2 hours of incubation at 36°C. This leads to loss of cell viability at 4 hours (Shobuike, Tatebayashi et al. 2001). To study the behaviour of RNAi under these conditions I performed time-course temperature experiments. Cultures of control cells and all mutants were grown at the

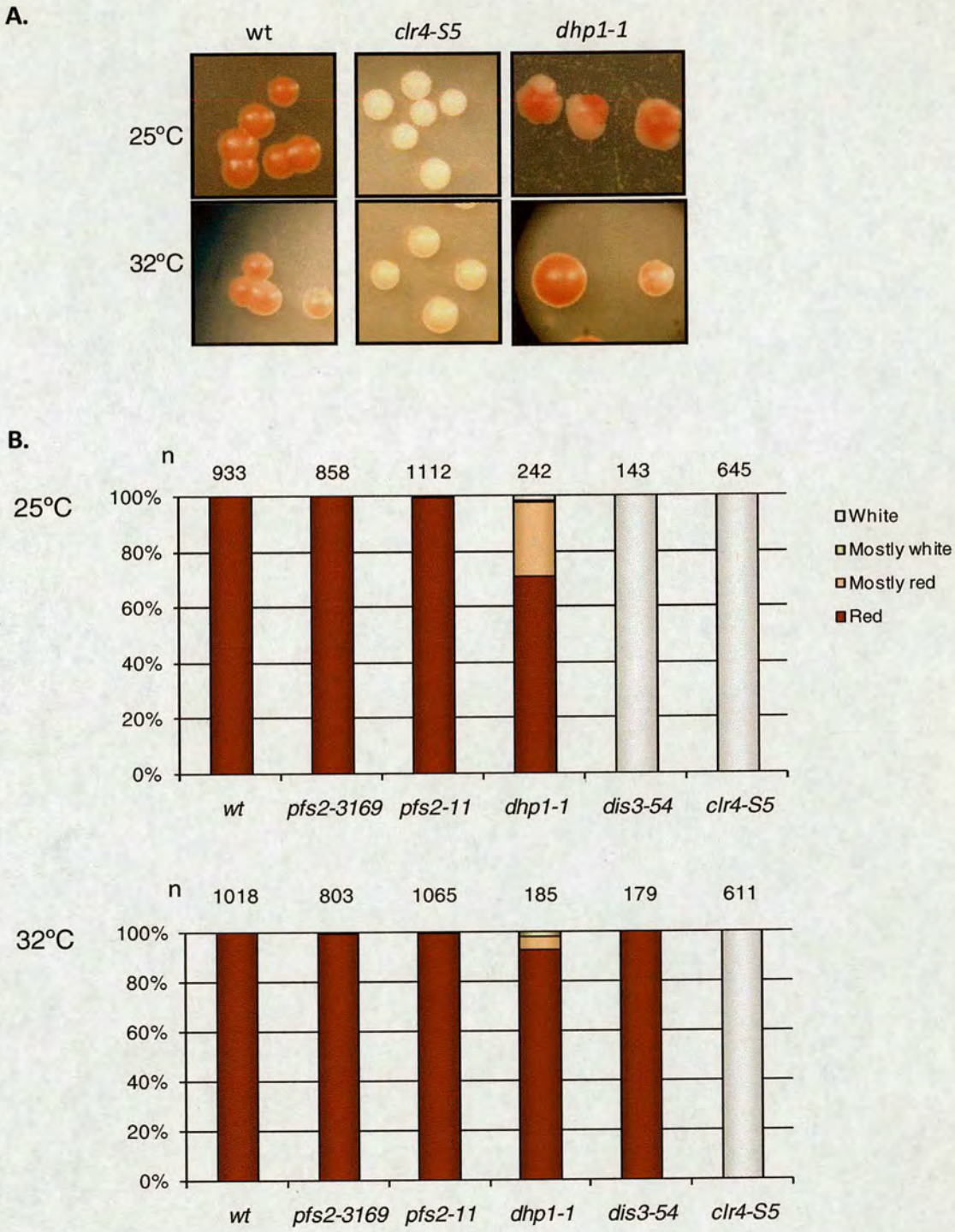
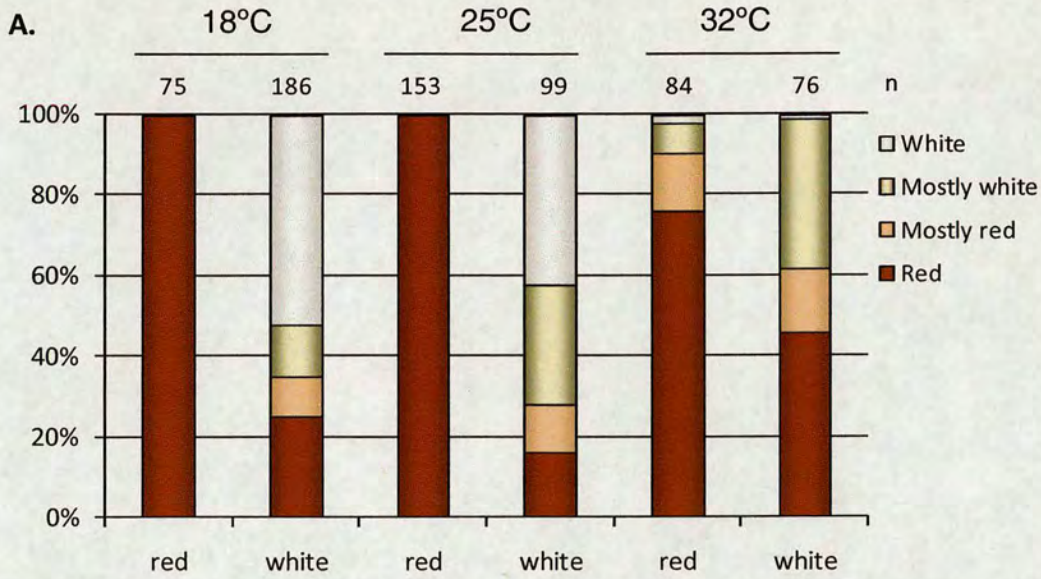


Figure 4-6: ***dhp1-1* colonies display variegated silencing. A.** Colony spread plating assay in YES with low adenine. While *wt* and *clr4-S5* colonies display an homogenously red and white colour respectively, dhp1-1 colonies have sectors with both colours. **B.** Analysis on frequency of variegation in colony spread assay performed at 25°C and 32°C. Colonies were classified into 4 categories according to relative percentage of white vs. red colour. The intermediate categories represent the variegated colonies in which one colour occupies more than 50% of the colour. In the case of *dis3-54*, colonies were allowed to grow for an additional 7 days in order to develop colour. The colour obtained at 25°C was peach and not white but showed no variegation. Total number of analyzed colonies for each mutant is indicated above the respective bar graph.



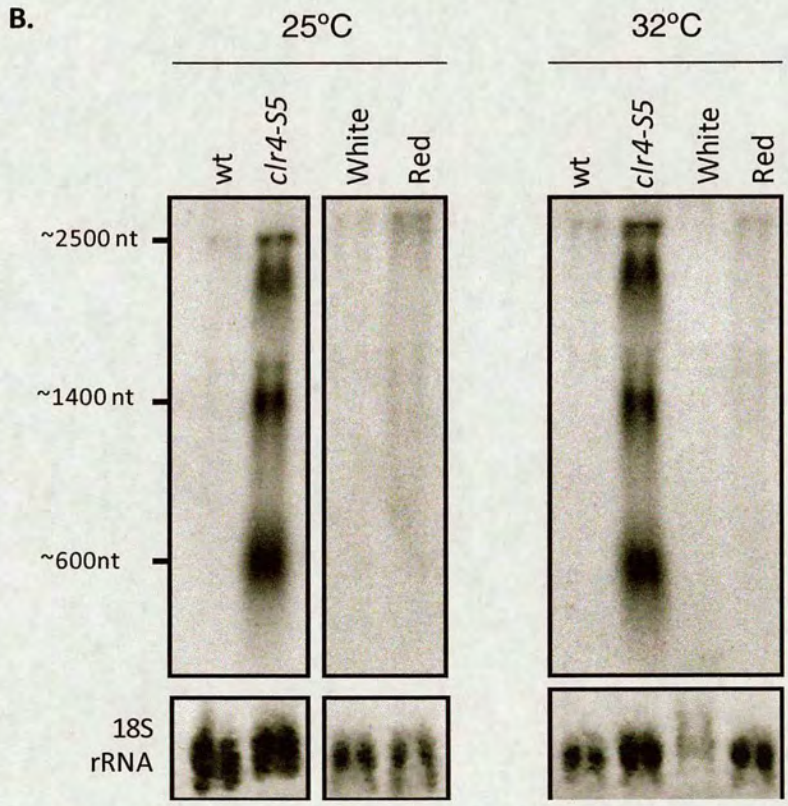


Figure 4-7: Analysis on variegated *dhp1-1* colonies. A. White and red *dhp1-1* were pooled separately, plated on YES medium with low adenine and incubated for 5 days at 18°C, 25°C and 32°C. The resulting colonies were classified into 4 categories according to colour. The intermediate categories represent variegated colonies that show a predominance (>50%) of either red or white colour. **B.** A similar procedure was used to pool white and red *dhp1-1* colonies and incubate them in liquid YES medium for 36 hours in order to extract RNA. Total RNA samples (10 µg) were obtained and used to perform Northern blot for *dg* transcripts as shown before. Wild-type and *clr4-S5* cells were used as controls. Loading control is provided by 18S rRNA stained with ethidium bromide on the membrane.

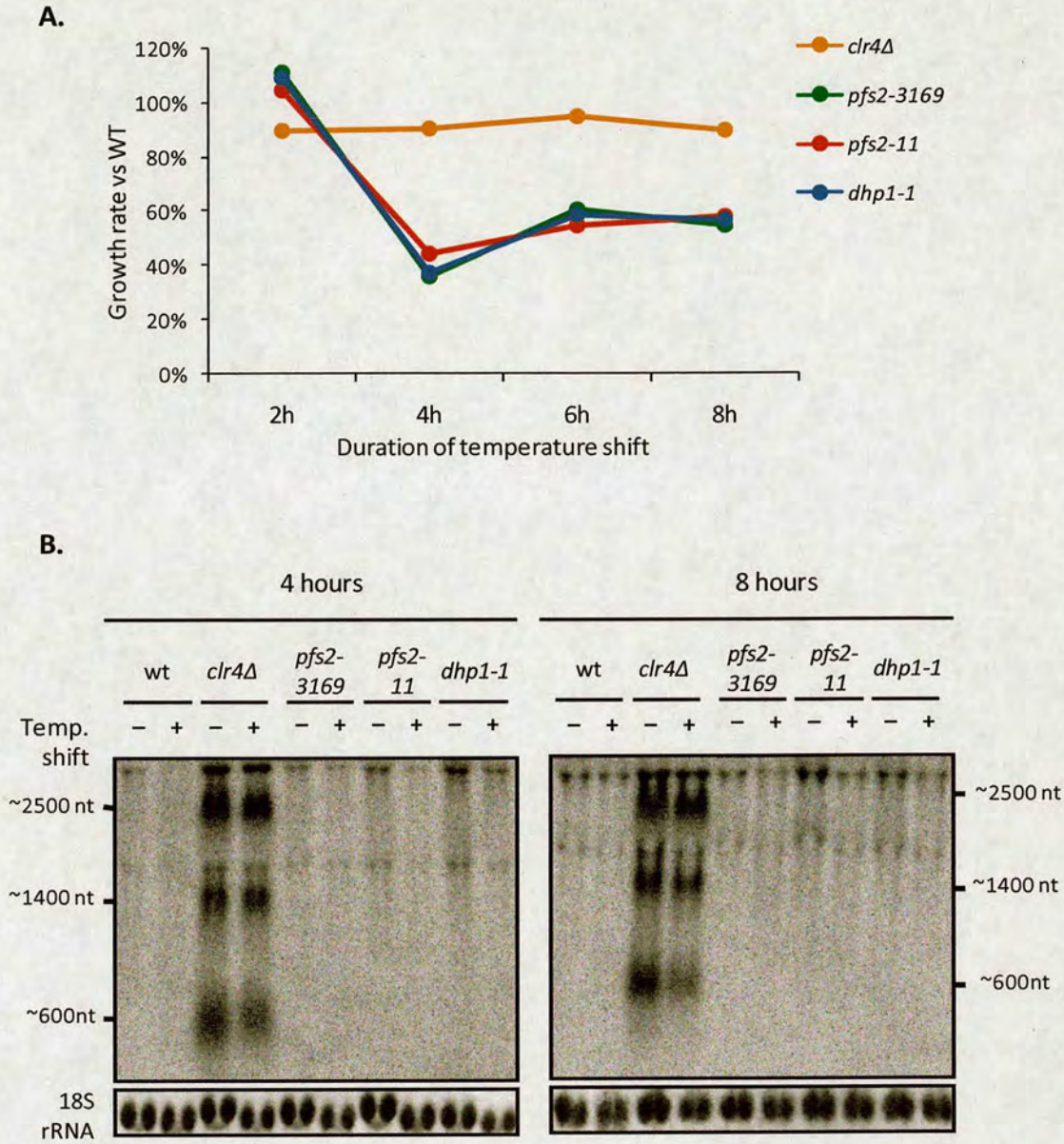


Figure 4-8: Time-course temperature shift experiment performed with *dhp1-1* and *pfs2-* mutant cells with cells with *wt* and *clr4-S5* as controls.

A. Cell cultures were grown continuously in liquid YES medium at 36°C hours for a total of 8 hours. All cultures were kept in exponential growth throughout the experiment. Growth rates for each mutant were calculated for each 2 hour period relatively to wild-type and depicted in the graph.

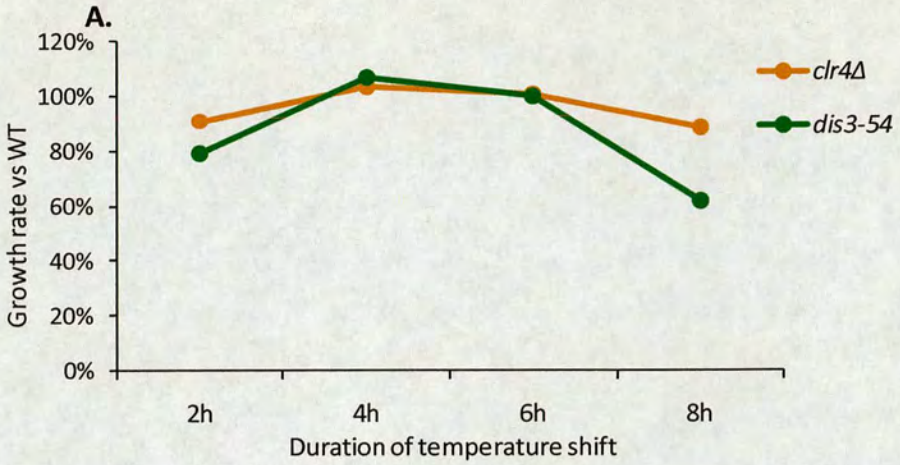
B. Samples of each culture were collected after 4 and 8 hours of 36°C incubation to perform RNA extraction and northern analysis. The resulting membranes were hybridized to a probe specific to the *otr* (*dg*). Loading control is provided by ethidium bromide staining of 18S rRNA on the membrane.

permissive temperature and then shifted to the restrictive temperature for 2, 4, 6 and hours. Cell growth was monitored for every two hour period and RNA was extracted at each time point. The levels of centromeric transcripts were then assayed by Northern analysis. I observed that the growth rate of *dhp1-1* and *pfs2* mutants decreases sharply to 50-60% of wild-type rate after 4 hours of incubation at 36°C (Figure 4-8A). This behaviour was expected due to the temperature-sensitive cell cycle defect described for these mutants. Northern analyses on samples taken after 4 and 8 hours are shown in Figure 4-8B. Even after 8 hours of temperature shift, no centromeric transcripts are detectable in samples of both mutants, indicating that RNAi and heterochromatin silencing are still intact. These data suggest that the RNA cleavage and polyadenylation factor Pfs2 and the transcription termination factor Dhp1 do not play any role in the processing of centromeric transcripts for RNAi.

4.2.3. RNAi and the exosome complex

The exosome mutant *dis3-54* was originally described as a leaky allele, which means that *dis3-54* cells retain significant viability at restrictive conditions (Ohkura, Adachi et al. 1988). This occurs despite the fact that *dis3+* is an essential gene in fission yeast. In *S. cerevisiae*, Dis3p/Rrp44p is essential for the activity of the exosome complex both *in vivo* and *in vitro* (Dziembowski, Lorentzen et al. 2007). It is then likely that the Dis3-54 mutant protein does not lose its function completely under restrictive conditions. In an assay similar to what was performed above, the behaviour of *dis3-54* cells in culture during an 8 hour long temperature shift to 18°C matches the original description of this mutation (Figure 4-9A). At this temperature, the growth rate of *dis3-54* cells measured for every two hour period remained similar to wild-type until after 6 hours of temperature shift. Between 6 and 8 hours, the growth rate of *dis3-54* decreased to 62% of the wild-type rate. The reduced growth is likely to be reflecting the known cell cycle exit defect caused by this mutation which indicates that Dis3 function became impaired after 8 hours at 18°C (Ohkura, Adachi et al. 1988). Even then no accumulation of centromeric RNA could be observed (Figure 4-9B). However, since the mutant phenotype is leaky one cannot rule out that accumulation of transcripts is occurring at these conditions albeit at a level not detectable by Northern analysis.

Considering that the *dis3-54* cannot be used to completely deplete the function of the exosome, I turned my attention to another mutation on a component of the exosome-mediated RNA degradation pathway. The budding yeast Rrp6p (Ribosomal RNA processing 6) is a non-



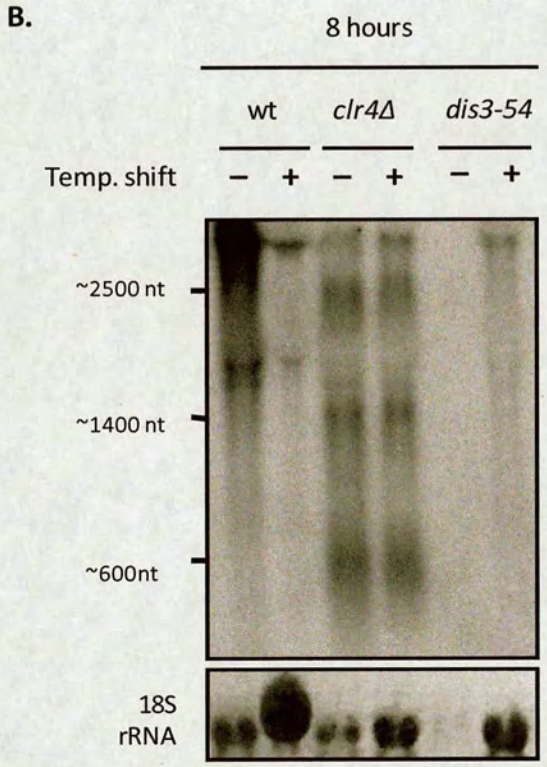


Figure 4-9: Time-course temperature shift experiment performed with *dis3-54* mutant cells with cells with *wt* and *clr4-S5* as controls.

A. Log phase cultures grown at 36°C in liquid YES medium were cooled down with ice/water bath and then incubated at 18°C hours for a total of 8 hours. All cultures were kept in log phase throughout the experiment. Growth rates for each mutant were calculated relatively to wild-type and depicted in the graph.

B. Northern blot analysis of RNA collected at the end of the experiment. The resulting membrane was hybridized to a probe specific to the *otr (dg)*. Loading control was provided by ethidium bromide staining of 18S rRNA on the membrane.

essential component of the nuclear form of the exosome that is involved in 5.8S ribosomal RNA 3' end processing as well as degradation of aberrant poly(A)-mRNAs and 3' extended read-through RNAs in the nucleus (Wyers, Rougemaille et al. 2005). Rrp6p only associates with the nuclear form of exosome, where it functions with the TRAMP complex to degrade a class of RNAs called cryptic unstable transcripts (CUTs) (Burkard and Butler 2000; van Hoof, Lennertz et al. 2000; Wyers, Rougemaille et al. 2005). The function of CUTs in budding yeast is yet unclear but they may share some characteristic with the non-coding centromeric *otr* transcripts from fission yeast. In budding yeast, Rrp6p-mediated processing or degradation of transcripts occurs in vicinity of the DNA locus where the transcripts were formed (Hilleren, McCarthy et al. 2001). This resembles the situation in fission yeast in which RITS localizes to centromeric *otr* loci in an RNA-dependent manner (Motamedi, Verdel et al. 2004). Furthermore TRAMP shares functional characteristics with the RDRC complex in the form of the poly(A) polymerases, suggesting that TRAMP-mediated degradation and RNAi might be connected processes (Motamedi, Verdel et al. 2004; LaCava, Houseley et al. 2005; Wyers, Rougemaille et al. 2005).

The *S. pombe* Rrp6 protein is closely similar to budding yeast Rrp6p. The fission yeast *rrp6Δ* null mutant is viable but displays slow growth at temperatures below 32°C and temperature sensitivity at 36°C (data not shown). This phenotype is very similar to the one observed in budding yeast *rrp6* null mutant cells, suggesting that the fission yeast Rrp6 is indeed the functional counterpart of budding yeast Rrp6p (Briggs, Burkard et al. 1998). To determine if defects of the nuclear exosome have an impact on integrity of RNAi and centromeric heterochromatin, RNA was extracted from *rrp6Δ* cells and assayed by northern analysis for levels of centromeric outer repeat RNA (Figure 4-9C). Transcripts originating from the *otr* were clearly detected in *rrp6Δ* but the pattern obtained is distinct from what is observed in RNAi and heterochromatin factor mutants. In *rrp6Δ*, the size of the detected *otr* RNA is less defined (smear) and migrates close to 600 nt. This smeared pattern is reminiscent of the poly-adenylated RNA intermediates that budding yeast *rrp6Δ* cells accumulate which would otherwise be degraded by the exosome (Houseley and Tollervey 2006). TRAMP marked these transcripts for degradation by polyadenylating their 3' ends, which cause them to appear as smears in northern analyses. It is likely then that the smeared band shown here corresponds to centromeric transcripts that were targeted for exosome-mediated degradation but were stabilized by the absence of Rrp6 protein.

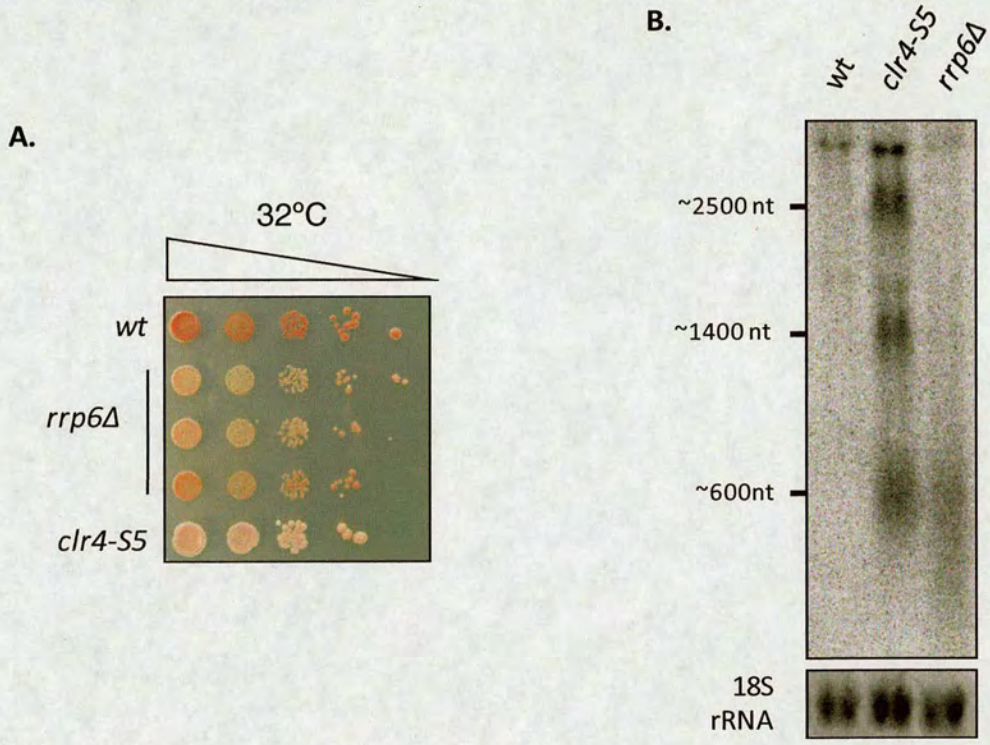


Figure 4-10: Centromeric silencing in *rrp6Δ* mutant.

A. Colour-based silencing assay performed on limiting adenine medium with three independent *rrp6Δ* clones containing *otr1R(SphI):ade6+*. Colour levels in wild-type and alleviated states are shown by the *wt* and *clr4-S5* controls.

B. Northern blot analysis of *rrp6Δ* mutant. Cells were grown at 32°C in preparation for RNA extraction. The membrane was hybridized to an *otr* (*dg)* specific probe. Loading control is provided by ethidium bromide staining of 18S rRNA on the membrane.

4.3. DISCUSSION

There are two significant caveats in investigating possible connections between mRNA 3' processing, turnover and transcription termination with other pathways using a loss of function or mutant approach such as the one described in this chapter. These three processes can have a significant impact on global gene expression and consequently affect multiple pathways in the cell. Hence any mutation that affects expression of RNAi or heterochromatin factors could have an indirect affect on RNAi or centromeric heterochromatin. The biggest challenge of this approach is to distinguish genuine functional links from indirect effects. In addition, the fact that most RNA processing & turnover processes are essential to the cell presents difficulties in studying them. Using temperature-sensitive alleles is not an ideal solution because it is difficult to evaluate their extent of the loss of function. Furthermore, because these are hypomorphic alleles the mutant proteins may not perform their function within normal parameters even under permissive conditions. In light of this, any conclusions drawn from this work must be done so conservatively, especially given the subtle silencing phenotypes here described.

Centromeric silencing assays based on the *otr1R(SphI):ade6+* insertion allow to determine the impact of a given mutation on silencing at centromeric outer repeats by variations of colony colour. Even though this assay is convenient, it has some an important shortcoming. It is possible that a mutation in a factor might affect the expression of genes that function in the adenine biosynthesis pathway upstream of *ade6+*. In this situation, the colony colour becomes attenuated in a way could be mistaken for affected silencing of *otr1R(SphI):ade6+*. Given that the mutants analysed here are involved in mRNA maturation and stability, it is likely that they may have an indirect effect on colony colour. A similar assay could be performed in selective conditions using medium completely lacking adenine. Thus, only cells which are phenotypically *ade6+* are able to grow efficiently in this depleted medium. Mutant cells which possess a defective pathway upstream of *ade6+* should not grow, thereby discriminating any false positives from the colour assay.

The results obtained indicate that transcription termination and the exosome give a subtle contribution to the normal functioning of RNAi and centromeric silencing. It is clear that functional Pfs2 is not required for the integrity of centromeric heterochromatin. The reason behind the chromosome segregation defects that arise from mutations in *pfs2+* must lie in other aspects of centromere function or chromosome structure. Kinetochore assembly and attachment to spindle microtubules might be compromised which would explain the appearance of Bub1 foci in the nuclei

of *pfs2* mutant cells, an indication that the spindle checkpoint activation is activated (Bernard, Hardwick et al. 1998; Wang, Asakawa et al. 2005). This connection is further strengthened by the observation that cell viability is further reduced in *pfs2*-11 cells when Bub1 is absent (Wang, Asakawa et al. 2005). However, it is unclear how Pfs2 could participate in the process of kinetochore assembly. An alternative explanation may reside in the connection of transcription and cohesion distribution along the chromosome arms, in which Pfs2 may be influential (Lengronne, Katou et al. 2004; Wang, Asakawa et al. 2005). Lacking decisive evidence, the simplest explanation for how *pfs2+* mutations may interfere with any of these two processes remains on the effect they may have on the level of expression of key factors.

Although Dhp1 and Pfs2 share a role in preventing read-through transcription and production of 3' over-extended transcripts, Dhp1 differs in that its loss seems to reflect on centromeric silencing. The effect is mild, causing only a percentage of cells in the population to lose silencing. The loss of silencing seems to be limited to the marker gene inserted in the centromeric outer repeats since outer repeat transcript levels do not increase. A proportion of white "active" cells can reacquire the silent state upon propagation. This suggests that the silencing defects observed are transient and that silencing of the marker gene is re-established even though Dhp1 function is compromised. Mutant Dhp1 protein was previously shown to be severely depleted after only a few hours at 36°C (Shobuike, Tatebayashi et al. 2001). Despite this, centromeric silencing is not affected to a greater extent at 36°C (Figure 4-4). Furthermore, centromeric silencing can be re-established itself even at semi-permissive conditions (32°C). Together, this shows that defective Dhp1 does not impair centromeric RNAi significantly. The slight defect in marker gene silencing may be caused by the incapacity to properly terminate RNA polymerase II transcription of the *ade6+* gene, which could in some way antagonize heterochromatin from spreading in from the adjacent *otr* sequences. If the reason for the *dhp1-1* silencing phenotype is indeed only due to altered chromatin dynamics at the centromeric *ade6* locus, this would also explain why the system is able to resume silencing in subsequent cell divisions. Alternatively, a change in the dosage levels of proteins such as Swi6 or Clr4 could explain the difference of heterochromatin dynamics and justify the variegation phenotype. It is conceivable that the *dhp1-1* mutation may affect expression levels of one or more of such key heterochromatin factors which would reflect on a drop in silencing efficiency throughout the entire centromere but only having a noticeable effect on the *ade6* gene. Chromatin immunoprecipitation experiments using antibodies against $Swi6^{HP1}$ and H3K9me2 could be employed to address this idea.

It is highly unlikely that the defective chromosome segregation phenotype of *dhp1-1* is caused by loss of centromeric heterochromatin. Firstly, the impact of this mutation on centromeric silencing is very mild and does not compromise either RNAi or the stability of heterochromatin to a significant extent. Secondly, *dhp1-1* cells appear to have a defect in sister chromatid separation during anaphase which is a distinct phenotype from the one observed when RNAi and/or centromeric heterochromatin are abrogated (Ekwall, Javerzat et al. 1995; Shobuike, Tatebayashi et al. 2001; Volpe, Schramke et al. 2003). Loss of centromeric heterochromatin leads to defective cohesion at the centromeres but this still allows sister chromatids to separate during anaphase. Although loss of centromeric heterochromatin leads to non-disjunction and chromosome loss, the cause is distinct from *dhp1-1*. This suggests that dhp1-1 affects a different aspect of the chromosome segregation apparatus.

The exosome complex participates in the process of centromeric silencing but it does not appear to be of critical importance. The silencing phenotype of *dis3-54* is the strongest of all mutants tested but it still only displays a mild alleviation of silencing. *Dis3-54* cells exhibit a homogenous colour which seems to reflect a stable alleviation of silencing that appears to become stronger in more restrictive growth conditions. However, this mutation is known to have a strong impact in cell cycle progression and as a consequence *dis3-54* cells grow very slowly compared to wild-type in semi-restrictive conditions (Ohkura, Adachi et al. 1988). It is therefore difficult to conclude from growth assays if the lighter colour of the *dis3-54* colonies corresponds to defective silencing or subtle changes in the expression of components of the adenine biosynthesis pathway upstream of *ade6+*. The purpose of studying rrp6Δ was to allow a distinction between effects on silencing caused by a defect of nuclear exosome function from the phenotype caused by both nuclear and cytoplasmic exosome defects (Dis3). Indeed *rrp6Δ* cells do display a defective silencing phenotype, suggesting that the exosome is involved in the process of chromatin silencing at the nuceleus. These cells also display a clear accumulation of centromeric *otr* transcripts albeit at a distinct pattern, suggesting that these *otr* transcripts are RNA intermediates that were marked for degradation. Rrp6 and the exosome then seem to be involved in turning over centromeric transcripts. Since centromeric silencing is partially affected in *rrp6Δ* and given the presence of *otr* siRNAs in *dis3-54*, it is likely that the exosome acts downstream of RNAi in processing centromeric transcripts. In other words, RNAi could act on nascent *otr* transcripts to promote heterochromatin assembly, followed by exosome-mediated turnover of these transcripts. The efficiency of transcript turnover could be relevant for heterochromatin integrity.

Since completing these analyses, the exosome has featured in several independent reports regarding its connection to RNAi and heterochromatin in fission yeast. It was shown by several groups that both Dis3 and Rrp6 are required to turn over transcripts that originate from the *otr* repeats (Irvine, Zaratiegui et al. 2006; Buhler, Haas et al. 2007; Murakami, Goto et al. 2007). This seems to correlate with the role of degrading general antisense transcripts that are produced throughout the genome suggested for the fission yeast exosome (Nicolas, Yamada et al. 2007). Both Dis3 and Rrp6 were also suggested to be involved in heterochromatin formation based on data indicating that H3K9 methylation levels become reduced in their absence (Murakami, Goto et al. 2007; Nicolas, Yamada et al. 2007). Although this fits with my data showing decreased centromeric silencing silencing in *dis3-54* and *rrp6Δ* the involvement of the exosome in this process remains unclear. One possible explanation for this genome-wide H3K9me2 decrease may relate to the budding yeast Rrp6p and its recently shown involvement in the control of core histone mRNAs (Reis and Campbell 2007). Thus, loss of Rrp6 might lead to overexpression of the core histones, which may lead to enhanced nucleosome deposition during S phase and a resulting dilution of H3K9me2 levels. Whatever impact the exosome may have on heterochromatin, it seems not to disturb the functioning of RNAi since both *dis3-54* and *rrp6Δ* cells have normal levels of *otr* siRNAs (Buhler, Haas et al. 2007; Murakami, Goto et al. 2007). In sum, the involvement of exosome in RNAi-mediated chromatin silencing seems to occur at the level of transcript turnover downstream of the RNAi pathway and heterochromatin formation. Exosome-mediated degradation of transcripts at heterochromatin domains may be important for the efficient RNAi-mediated nucleation of heterochromatin but not critical for the proposed co-transcriptional RNAi that supports continual production of centromeric siRNAs. Further studies are required to fully understand the role of the exosome on this complex phenomenon.

CHAPTER 5
SLICING RESIDUES ARE ESSENTIAL FOR AGO1 FUNCTION IN RNAi AND HETEROCHROMATIN ASSEMBLY

5.1. INTRODUCTION

In fission yeast, the process of RNAi-mediated heterochromatin formation is centred on noncoding RNA molecules that originate from the centromeric outer repeats (Volpe, Kidner et al. 2002; Motamedi, Verdel et al. 2004; Noma, Sugiyama et al. 2004; Buhler, Verdel et al. 2006). These molecules are used as seed for producing siRNAs and act as a platform for recruiting the RNAi machinery along with chromatin modifiers required for establishing a stable heterochromatin structure (Motamedi, Verdel et al. 2004; Noma, Sugiyama et al. 2004; Partridge, DeBeauchamp et al. 2007). Yet these transcripts are barely detectable in wild-type cells and only accumulate when RNAi or heterochromatin assembly pathways are disrupted. The accumulation of centromeric *otr* RNAs may be averted by wild-type cells in a number of ways: by preventing transcription initiation, blocking transcription elongation or degrading the final RNAs. RNAi prevents transcription initiation events at the centromeric outer repeats by promoting histone H3K9 methylation and the assembly of heterochromatin at centromeres. In fact, it was recently demonstrated that loss of RNAi and heterochromatin leads to increased transcription at the *otr* (Buhler, Verdel et al. 2006; Nicolas, Yamada et al. 2007). In addition, it is possible that RNAi contributes to deplete centromeric otr RNAs in another form. It is known that the loss of both Swi6 and Chp2, hence heterochromatin, is not sufficient to increase *otr* transcript accumulation to levels seen in mutants that affect the RNAi pathway (Volpe, Kidner et al. 2002; Sadaie, Iida et al. 2004). Therefore, RNAi may be acting to diminish the amount of *otr* RNA present in the cell by interfering with elongating RNA polymerase II at the *otr* loci or by actively degrading the resulting RNA. The latter scenario is more likely, given that RNAi in other organisms has evolved to silence gene expression by destabilising mRNA molecules (Tuschl, Zamore et al. 1999).

RNA interference is a method of post-transcriptional gene silencing (PTGS) that acts by cutting the strand of mRNA molecules selected on the basis of siRNA-mRNA complementarity

(Hamilton and Baulcombe 1999; Hammond, Bernstein et al. 2000; Zamore, Tuschl et al. 2000; Boutla, Delidakis et al. 2001; Elbashir, Lendeckel et al. 2001). Consequently the level of mRNA abundance for a particular gene is severely depleted with only a few molecules reaching the ribosomes where translation can occur (Hammond, Bernstein et al. 2000). This is the basis of the host defence pathway in which RNAi participates to repress viral or transposon proliferation at a post-transcriptional level (Ketting, Haverkamp et al. 1999; Tabara, Sarkissian et al. 1999). It is also the principle by which RNAi is employed as an experimental method of knock-down gene expression (Elbashir, Harborth et al. 2001). It was made clear since early on that the RNAi effector complex RISC was responsible for hastening the target mRNA's demise via a endonucleolytic cleavage event (Hammond, Bernstein et al. 2000). RISC cleaves a target RNA at the phosphodiester bond between nucleotides 9 and 10 in the siRNA-mRNA duplex (Elbashir, Harborth et al. 2001; Elbashir, Martinez et al. 2001; Schwarz, Hutvagner et al. 2002). However, the endonuclease itself responsible for this cleavage or "slicing" remained elusive until recently when it was demonstrated that it resides in the core of the RISC complex itself – the Argonaute protein (Lingel and Izaurralde 2004). Argonaute proteins contain a PIWI domain which is responsible for holding the siRNA molecule and mediating the interaction with the target RNAs (Cox, Chao et al. 1998; Parker, Roe et al. 2004; Song, Smith et al. 2004). Crystallographic studies on the Argonaute protein from *Pyrococcus furiosus* and the PIWI protein from *Archaeoglobus fulgidus* both revealed RNase H-like folds within the PIWI domain located close to the cleavage site of the target RNA molecule (Parker, Roe et al. 2004; Song, Smith et al. 2004). The residues involved in this fold are conserved throughout most of the eukaryotic Argonaute genes and several subsequent reports have proven that this domain indeed is responsible for the endonucleolytic activity of the target RNA during RNAi/PTGS (Liu, Carmell et al. 2004; Baumberger and Baulcombe 2005; Miyoshi, Tsukumo et al. 2005). Moreover, it is possible to reconstruct *in vitro* a minimal RNAi activity using a single Argonaute protein that once it is loaded with an siRNA can bind to a target mRNA and slice it. This illustrates the importance of slicing by Argonaute proteins for the mechanism of RNAi/PTGS (Gregory, Chendrimada et al. 2005; Rivas, Tolia et al. 2005).

There is an easily recognizable role for silencing by Argonaute in the context of transcript knockdown by RNAi or PTGS but the same cannot be said for transcriptional forms of RNA silencing. At the time the following work was initiated, it was not known if slicing activity of Argonaute was required for siRNA-directed chromatin silencing and if so, what role it would play (Baumberger and Baulcombe 2005). The RNase H fold in the PIWI domain of Argonaute proteins contains a motif of

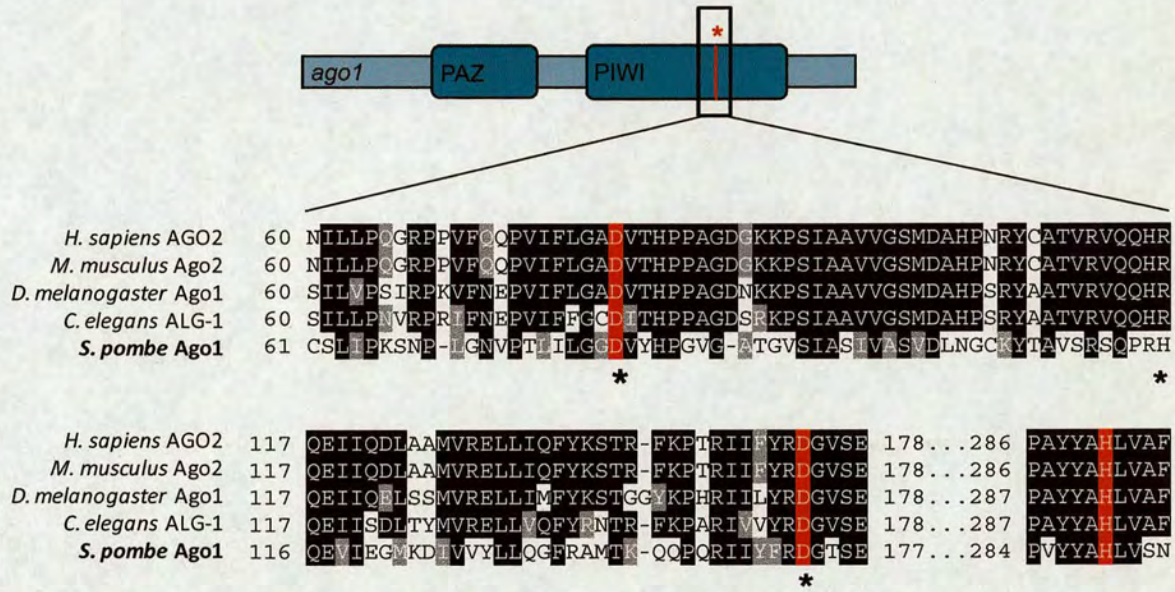


Figure 5-1: **ClustalW alignment of PIWI domain sequences found in Argonaute proteins from higher to lower eukaryotes.** The sequences belong to *H. sapiens* Argonaute 2, *Mus musculus* Ago2, Ago1 from *D. melanogaster*, Argonaute-like gene 1 from *C. elegans* and Ago1 from *S. pombe*. In red are marked the three highly conserved DDH residues that are important for the endonucleolytic activity of the PIWI domain. The three residues marked with * were individually mutated to alanine in *S. pombe* Ago1. Both D580A and D651A disrupt the DDH motif and are predicted to abolish slicing activity while H617A is a control mutation.

three residues (aspartate-aspartate-histidine, DDH) that is required for slicing activity. This DDH motif is present in the PIWI domain of the fission yeast Argonaute protein (Ago1), suggesting that Ago1 can slice target molecules (Figure 5-1). This suggests that Ago1 might contribute to lowering outer repeat transcript levels in wild-type cells by means of slicing. Since slicing is an endonucleolytic cleavage event, the remaining fragmented RNA strands must be degraded by additional nucleases in order for transcript turnover to occur (Orban and Izaurralde 2005). In fact, the results obtained in Chapter 4 demonstrate that the exosome is employed to efficiently turn over *otr* transcripts. The exosome is a 3'-5' exoribonuclease that requires a free 3' end in order to efficiently degrade a RNA molecule. Normally, the 3' ends of RNA polymerase II transcripts are protected by association of protecting proteins to the poly(A) tails (poly(A) binding proteins or PABs) and require either a 3' de-protection event or for the strand to be nicked internally to generate a new free 3' end (Mitchell and Tollervey 2000). Slicing of an *otr* transcript would provide a free 3' end to which the exosome could latch on to and begin degrading. This provides a model for one method in which RNAi might act to lower *otr* transcript levels: RNAi-mediated turnover of centromeric otr transcripts initiated by Ago1-mediated slicing leading to exosome-mediated degradation. Nevertheless, considering that the nascent *otr* transcripts may serve as a co-transcriptional platform from which RNAi can induce heterochromatin nucleation, the purpose of their cleavage associated with this same mechanism is not immediately clear.

In this chapter I describe the work I conducted to examine the role of the putative slicing activity of Ago1 in heterochromatin integrity. I constructed point mutants in key residues of the putative catalytic site in Ago1. Analysis of these mutants demonstrated that these residues are not only required to lower the levels of *otr* transcripts but are also crucial for the function of the entire RNAi pathway. In *ago1* slicing mutants (*ago1-sm*), centromeric heterochromatin is de-stabilized leading to loss of silencing and chromosome segregation defects. RNAi is severely compromised in these mutants, as levels of centromeric siRNAs are depleted and transcripts accumulate to levels similar to those seen in cells lacking RNAi. Localisation of the RITS complex to the centromeres is impaired and the cells expressing the mutant *ago1-sm* lose their capacity to establish *de novo* histone H3K9 methylation. The data presented suggests that Ago1 slicing activity has a fundamental role in the mechanism of RNAi in fission yeast.

5.2. RESULTS

5.2.1. Mutations in Ago1 slicing residues cause loss of silencing and increased TBZ sensitivity

To create point mutations in *ago1+*, the 1.7 kb long cassette containing the *ura4* marker gene was first inserted into the *ago1+* gene within the region encoding its PIWI domain via homologous recombination. Homologous recombination was then employed again to replace the 1.7 kb marker cassette with a PCR product containing the desired mutation, thus reconstructing the gene carrying a specific mutation but still under the control of its endogenous promoter. Three individual point mutations were generated: aspartate 580 to alanine (D580A), histidine 617 to alanine (H617A) and aspartate 651 to alanine (D651A). Both aspartate residues are part of the DDH motif which is required for slicing activity while the histidine 617 is an unrelated residue that was mutated for control purposes (Figure 5-1). The D580A and D651A mutations are predicted to disrupt the ability of Ago1 to slice, as it was demonstrated for Argonaute proteins in other organisms (Liu, Carmell et al. 2004; Parker, Roe et al. 2004; Baumberger and Baulcombe 2005; Miyoshi, Tsukumo et al. 2005). In the course of this chapter, the *ago1-D580A* and *ago1-D651A* mutants will be referred to jointly as "*ago1-sm*" (*ago1* slicing mutants). All three point mutations were generated at the endogenous *ago1* locus of a wild-type strain and also in a strain in which the N terminus of the *ago1+* gene was tagged with 3xmyc epitopes. This *3xmyc-ago1* construct is expressed under its native promoter and was previously reported as being fully functional (Noma, Sugiyama et al. 2004). All the results shown here were performed on strains carrying the 3xmyc epitope tag on the N terminus of *ago1*, unless otherwise noted. The majority of the assays described here were also performed on untagged strains to verify that the phenotype was not being affected by the presence of the N-terminal epitope tag.

All mutants were constructed in a strain background that include the *otr1R(Sph1):ade6+* marker gene insertion at the centromere, which is silenced by heterochromatin. Mutations in both aspartate residues (D580A and D651A) but not the histidine mutant (*ago1-H617A* – not shown) display alleviated silencing of *otr1R(Sph1):ade6+* (Figure 5-2A). The extent of the defect is similar to the one observed in a complete deletion of the *ago1* gene (*ago1Δ*). The results of silencing assays were identical in strains containing a myc-tagged or untagged *ago1* (not shown). Western analysis shows that $Ago1^{D580A}$ and $Ago1^{D651A}$ are stably expressed in the cells, showing only a slight reduction when compared to wild-type protein levels (Figure 5-2B). In addition, both D580A and D651A mutants have higher sensitivity to thiabendazole (TBZ), a microtubule destabilizing drug (Figure 5-

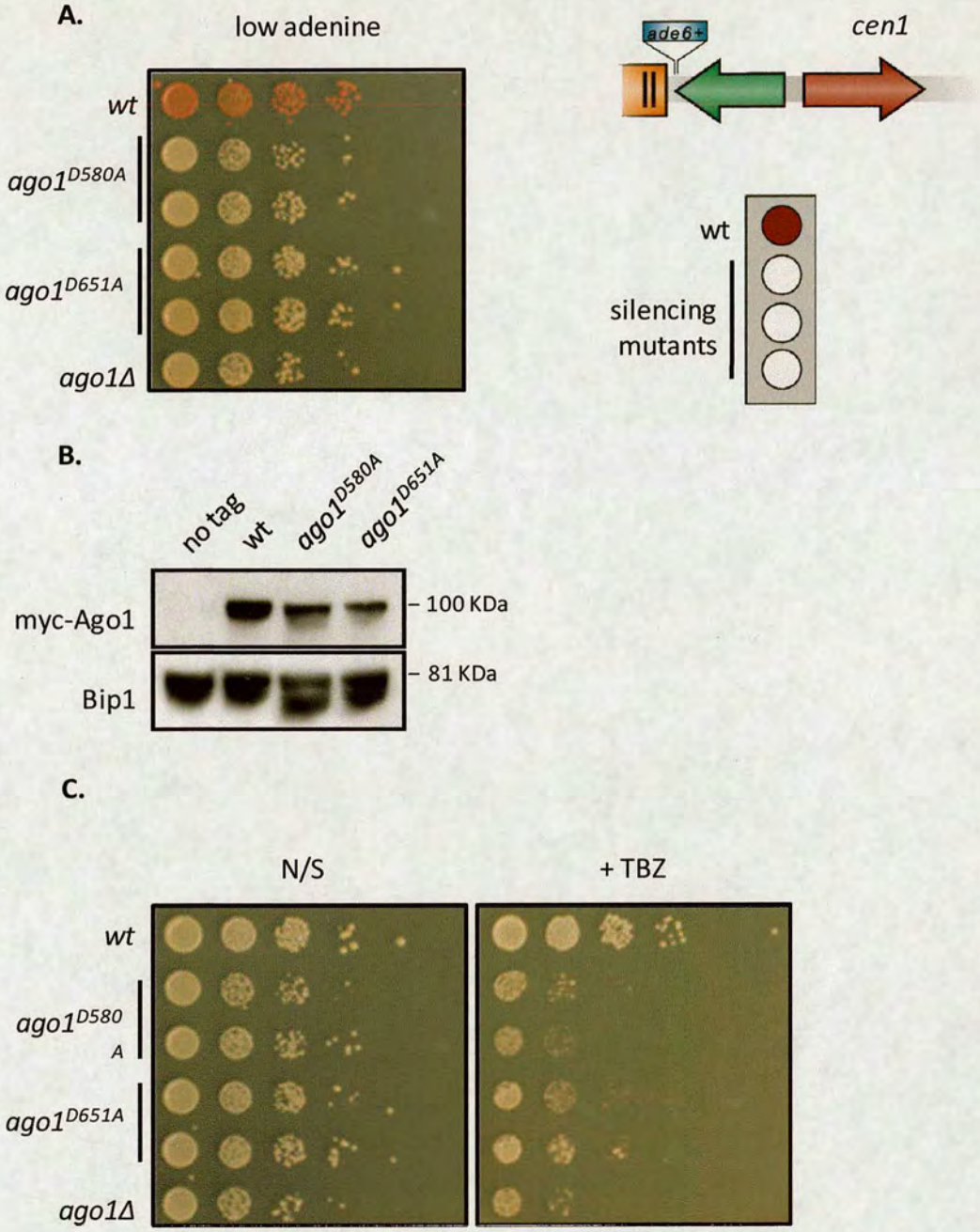


Figure 5-2: ago1^{D580A} and ago1^{D651A} (*ago1-sm)* mutants are defective in silencing at the centromeric outer repeats and have increased sensitivity to thiabendazole.

A. Colour-based silencing assay using *otr1R(SphI):ade6+* silent marker gene (top right panel). Defects in silencing of *otr1(SphI):ade6+* cause a shift in colony colour from red to white when the cells are grown in the presence of limiting adenine (top right panel). The assay was conducted with two independently isolated colonies of ago1^{D580A} and ago1^{D651A} mutants (*ago1-sm*) together with wild-type (silencing) and *ago1Δ* (no silencing) controls. **B.** Western analysis of Ago1 expression in wild-type and *ago1-sm* mutants. The membrane was probed with anti-myc antibody and with anti-Bip1 antibody, an abundant endoplasmic reticulum protein as a loading control. **C.** Growth-based sensitivity assay performed in the presence of Thiabendazole (TBZ), a microtubule destabilizing drug. Mutant strains with chromosome segregation defects, such as RNAi mutants, have increased sensitivity to TBZ.

2C). Increased TBZ sensitivity is observed in mutants whose centromeric heterochromatin is depleted and that suffer resulting chromosome segregation defects during mitosis, such as null mutants for RNAi or heterochromatin factors.

5.2.2. Centromeric heterochromatin is destabilized and cannot be established *de novo* in *ago1* slicing mutants (*ago1-sm*)

The alleviation of silencing and chromosome segregation phenotypes observed in the two *ago1* slicing mutants (*ago1-sm*) strongly suggests that centromeric heterochromatin has been compromised. To further investigate this, chromatin immunoprecipitation (ChIP) was performed with an antibody specific for histone H3 di-methylated on lysine 9 (H3K9me2). All mutants were assayed in duplicate and the level of enrichment was calculated in relation to the total or input extract by semi-quantitative PCR. Levels of this modification were assayed over *otr1R(SphI):ade6+* and on native centromeric *otr* sequences. H3K9me2 levels over the marker gene were found to be totally depleted in *ago1-sm* (D580A and D651A) but not in the control mutant (H617A), thus providing an explanation to the alleviation of *otr1R(SphI):ade6+* silencing (Figure 5-3A). Furthermore, the H3K9me2 levels over the native sequences at the centromeric outer repeats are also dramatically reduced in *ago1-sm* but to a lesser extent that the one observed over *otr1R(SphI):ade6+* (Figure 5-3B). A remnant of H3K9me2 at centromeric outer repeats is commonly observed in RNAi mutants and has been widely reported (Sadaie, Iida et al. 2004; Yamada, Fischle et al. 2005). It is unclear whether it is the result of a H3K9 methylation maintenance process or of a *cis*-acting heterochromatin nucleation pathway that is RNAi-independent.

RNAi is known to be required to establish H3K9me2 over centromeric repeats (Volpe, Kidner et al. 2002). Even though the residual H3K9me2 phenomenon is observed in all RNAi mutants, a heterochromatin establishment assay was performed using *ago1-sm* and control strains to determine if *ago1-sm* cells still retain any capacity to nucleate heterochromatin via RNAi. The assay is based on *de novo* assembly of chromatin structure and heterochromatin nucleation found to occur on plasmid DNA containing fragments of centromeric *otr* sequences. The cells were transformed with the plasmid pH-CC2 (Diego Folco) which derives from a previously published mini-chromosome construct (pSp-cc2-K'') (Baum, Ngan et al. 1994). The pH-CC2 plasmid contains a fragment of the outer repeats from centromere 1 (*dg*) placed adjacently to a copy of the central core from centromere 2 (*cc2*) (Figure 5-4A; see Figure 1-5, page 36). Upon transformation into

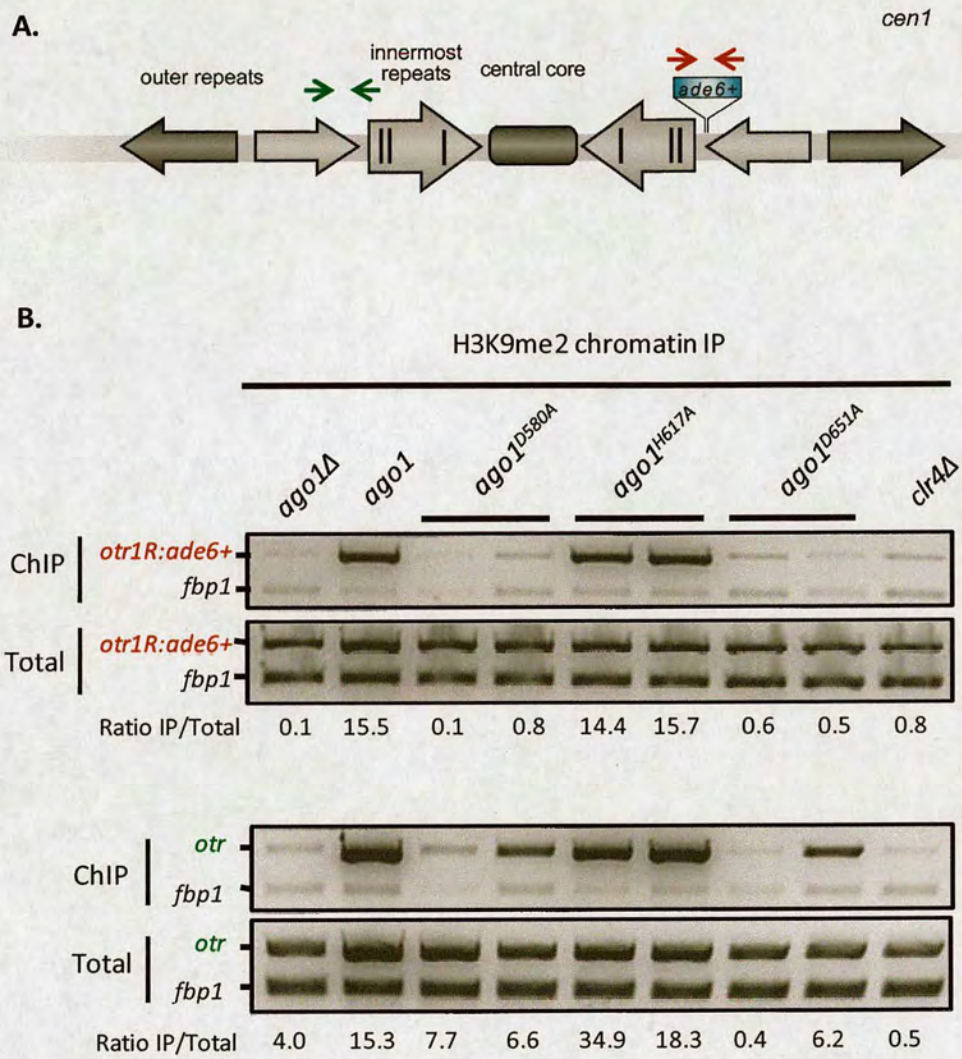


Figure 5-3: **Heterochromatin analysis of *ago1-sm mutant* cells.** Chromatin immunoprecipitation (ChIP) was performed on *ago1-sm* and control cells using an antibody specific to H3K9me2, a histone mark characteristic of heterochromatin.

A. ChIP enrichment levels were measured by PCR using primers for *otr1R(SphI):ade6+* (red arrows) and the native centromeric outer repeat sequences (green arrows).

B. The samples were analysed by multiplex PCR for H3K9me2 levels at the two genomic regions of interest (top panel in red, *otr1R(SphI):ade6+*; bottom panel in green , native *otr*) and the euchromatic control gene *fbp1*. The internal ratios versus fp1 were compared between ChIP and Total (input) samples to yield an enrichment ratio, which reflects the amount of H3K9me2 present over the region of interest.

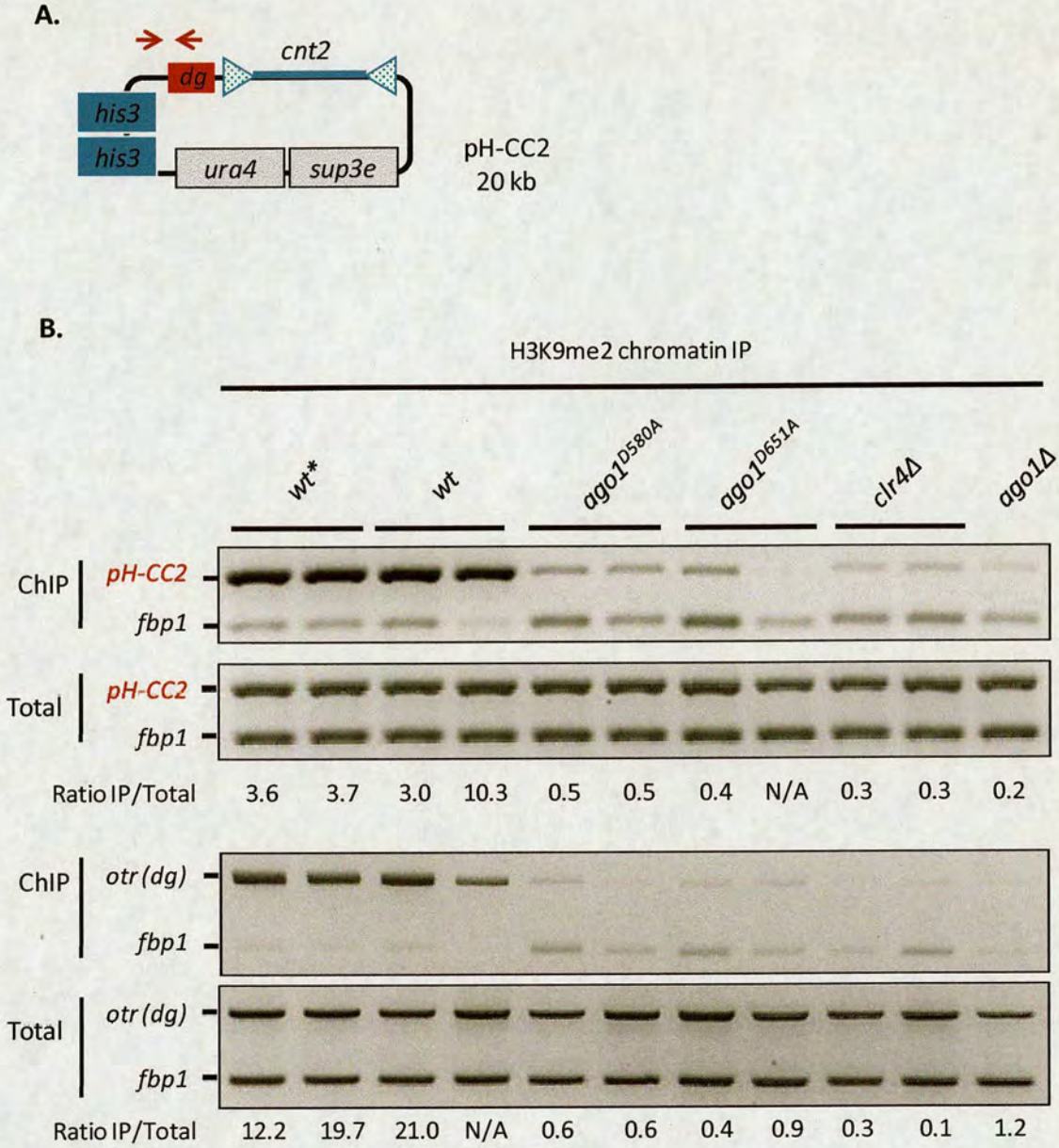


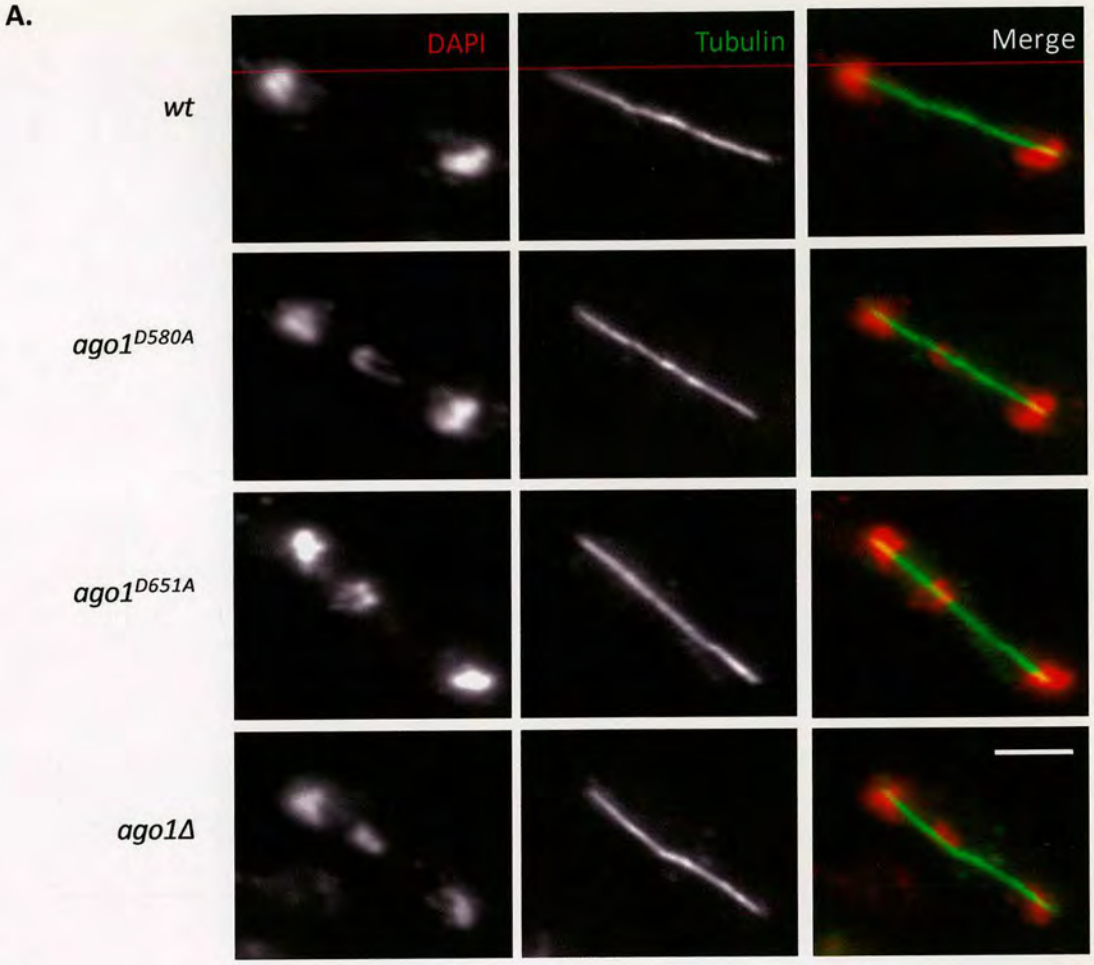
Figure 5-4: Heterochromatin *de novo* establishment assay.

A. Wild-type and mutant cells were transformed with a plasmid (pH-CC2) containing a fragment of *otr* from centromere 1 (*dg*, red box) and the central core from centromere 2 (*cnt2*, in blue). Chromatin immunoprecipitation (ChIP) was performed using anti-H3K9me2 antibody and the samples were analysed by multiplex PCR with oligos specific to the junction between the *otr* fragment and the plasmid backbone (red arrows) as well as the euchromatic control gene *fbp1*. **B.** The samples were analysed by multiplex PCR for H3K9me2 levels in comparison to the endogenous *fbp1+* gene. Top panel shows the results obtained for the plasmid *otr* DNA fragment while the bottom panel depicts the results obtained for native *otr* sequences at centromeres. The internal ratios vs. *Fbp1+* were compared between IP and Total samples to yield an enrichment ratio, which reflects the amount of H3K9me2 deposited on the plasmid *otr* fragment. The *wt** samples were obtained from wild-type cells with untagged *ago1*, while *wt* cells have *myc-ago1*. The enrichments of H3K9me2 over plasmid DNA are similar in both sets of samples, showing that the 3xmyc tag on Ago1 does not hinder the capacity of the cells to form heterochromatin over pH-CC2.

fission yeast cells as naked DNA, this plasmid becomes a stable mini-chromosome (Baum, Ngan et al. 1994; Folco, Pidoux et al. 2008). The chromatin assembled over the plasmid DNA is enriched in histone H3K9me2 over the plasmid *dg* sequence and in Cnp1^{CENP-A} over the *cc2* sequence (Folco, Pidoux et al. 2008). Wild-type, *ago1-sm* and control cells were transformed with pH-CC2 plasmid and grown under selective conditions. ChIP was then performed using an antibody specific H3K9me2 to measure rates of heterochromatin nucleation over the *dg* sequence fragment on the plasmid DNA (Figure 5-4). The enrichment displayed in all wild-type samples shows that cells are able to assemble heterochromatin on the plasmid over the outer repeat fragment. In comparison, cells containing *ago1-sm* or *ago1Δ* mutations display no H3K9me2 enrichment on the plasmid. This shows that *de novo* nucleation of heterochromatin on a centromeric sequence requires Ago1 function. Since *ago1-sm* cells are equally depleted of the capacity to form heterochromatin on the plasmid as *ago1Δ*, the slicing residues must be crucial for this process.

5.2.3. Ago1 slicing mutants have high incidence of lagging chromosomes

The loss of centromeric heterochromatin explains the increased TBZ sensitivity phenotype of *ago1-sm* mutants. Defective heterochromatin leads to loss of sister chromatid cohesion specifically at centromeres (Nonaka, Kitajima et al. 2002; Bernard, Drogat et al. 2006). Premature separation of sister centromeres in mitosis results in defective chromosome segregation and elevated chromosome loss rates (Bernard, Maure et al. 2001; Nonaka, Kitajima et al. 2002). Cells without centromeric heterochromatin are still able to progress into anaphase but display chromosomes that lag in their movement to the spindle poles (lagging chromosomes) (Ekwall, Nimmo et al. 1996). To examine the cause of the observed TBZ sensitivity, *ago1-sm* cells were fixed and stained by immunofluorescence using an antibody specific to α-tubulin to visualise the mitotic spindle while DAPI was used to stain DNA. Lagging chromosomes were indeed found to occur frequently in *ago1-sm* cells undergoing anaphase (Figure 5-5A). Quantification of the incidence of lagging chromosomes in late anaphase spindles shows that the values are significantly higher than wild-type cells in *ago1-sm* and very similar to the frequency detected in *ago1Δ* (Figure 5-5B). Together these data show that *ago1-sm* mutations cause a loss of centromeric heterochromatin and have an equivalent impact on chromosome segregation as a complete *ago1* deletion (*ago1Δ*).



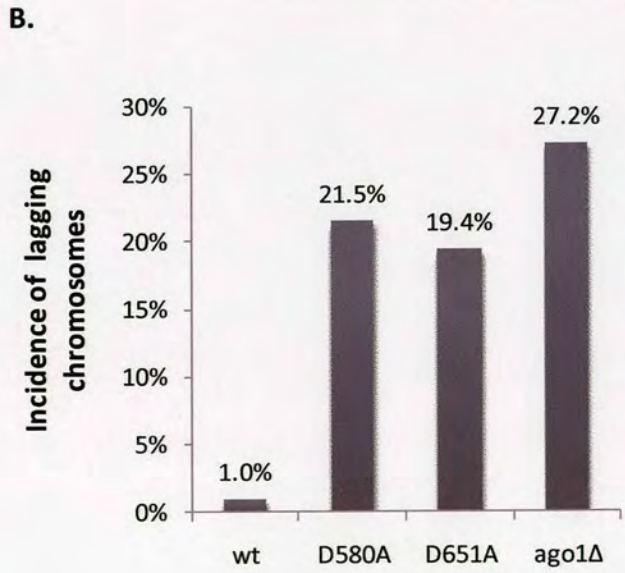


Figure 5-5: ***ago1-sm*** **cells have lagging chromosomes.**

A. Wild type and *ago1* mutant cells were grown at 32°C, fixed and immunostained with anti-TAT1 α-tubulin antibody and DAPI for staining DNA. The cells portrayed here are in late anaphase, showing two DAPI masses at opposite ends of the microtubule spindle. Both *ago1-sm* and *ago1Δ* mutants display high incidence of cells showing lagging chromosomes –DAPI masses whose segregation movement was delayed. White bar represents 4 µm.

B. Quantification of the cells in late anaphase displaying lagging chromosomes. A total of 200 cells in anaphase were counted for each sample and the results are displayed as percentage of phenotype incidence over total number of cells in anaphase.

5.2.4. RNAi activity is blocked in *ago1-sm* mutants downstream of Dcr1 activity

All the evidence obtained up to this point concerning the *ago1-sm* mutants point to a complete loss of Ago1 function in these mutants. This only implies that mutant Ago1-sm protein cannot perform its function to a point where it can promote heterochromatin formation. To obtain a more detailed image of the state of RNAi activity in *ago1-sm* mutants, northern analyses were performed to measure the levels of centromeric *otr* transcript (Figure 5-6A). Again, *ago1-sm* mutant cells accumulate centromeric outer repeat transcripts at the same level as in *ago1Δ*. Conversely, northern analysis of siRNAs that are raised against the centromeric outer repeats shows that *ago1-sm* cells are depleted in centromeric siRNA levels to below one tenth of the wild-type levels (Figure 5-6B). These results show that RNAi is ineffective in *ago1-sm* mutants but do not indicate a clear reason to why the pathway is being affected. This is mostly due to the fact that fission yeast RNAi functions in a closed positive feedback loop, in which the outcome of its activity (heterochromatin) promotes reinforcement of the molecular intermediates (siRNAs) (Noma, Sugiyama et al. 2004). The closed loop nature of fission yeast RNAi is clearly illustrated by the loss of centromeric siRNAs in *clr4Δ* (Noma, Sugiyama et al. 2004). The following series of experiments attempt to rule out individual deficiencies within RNAi mechanism in order to ascertain at which point the Ago1-sm protein introduces a defect.

Although the process is not as efficient as in higher eukaryotes, fission yeast RNAi can be programmed to act on a novel target using an artificial trigger (Sigova, Rhind et al. 2004). This trigger is an inverted repeat construct homologous to a target gene that once transcribed folds back in a "hairpin" structure producing a stretch of dsRNA that Dcr1 can cleave into siRNAs. More specifically, a GFP hairpin construct was shown to induce GFP repression in an RNAi-mediated fashion (Sigova, Rhind et al. 2004). It was demonstrated that this hairpin construct could lead to the production of GFP siRNAs that could reduce the GFP expression levels within a population of cells. I used this hairpin construct to investigate whether *ago1-sm* are competent in siRNA production. The advantage of this system is that, unlike endogenous siRNAs, GFP siRNA production does not seem to be significantly affected by the absence of components of the RDRC (RNA-dependent RNA polymerase complex: Rdp1, Hrr1 and Cid12) and does not require intact heterochromatin (Motamedi, Verdel et al. 2004; Sharon White and Femke Simmer – unpublished observations). Most likely this is due to the high level of expression of the GFP hairpin construct since it is produced from the strong nmt1 promoter (Maundrell 1990; Maundrell 1993). This system provides an entry point to the closed loop of RNAi to perfom analysis on RNAi function in *ago1-sm* cells.

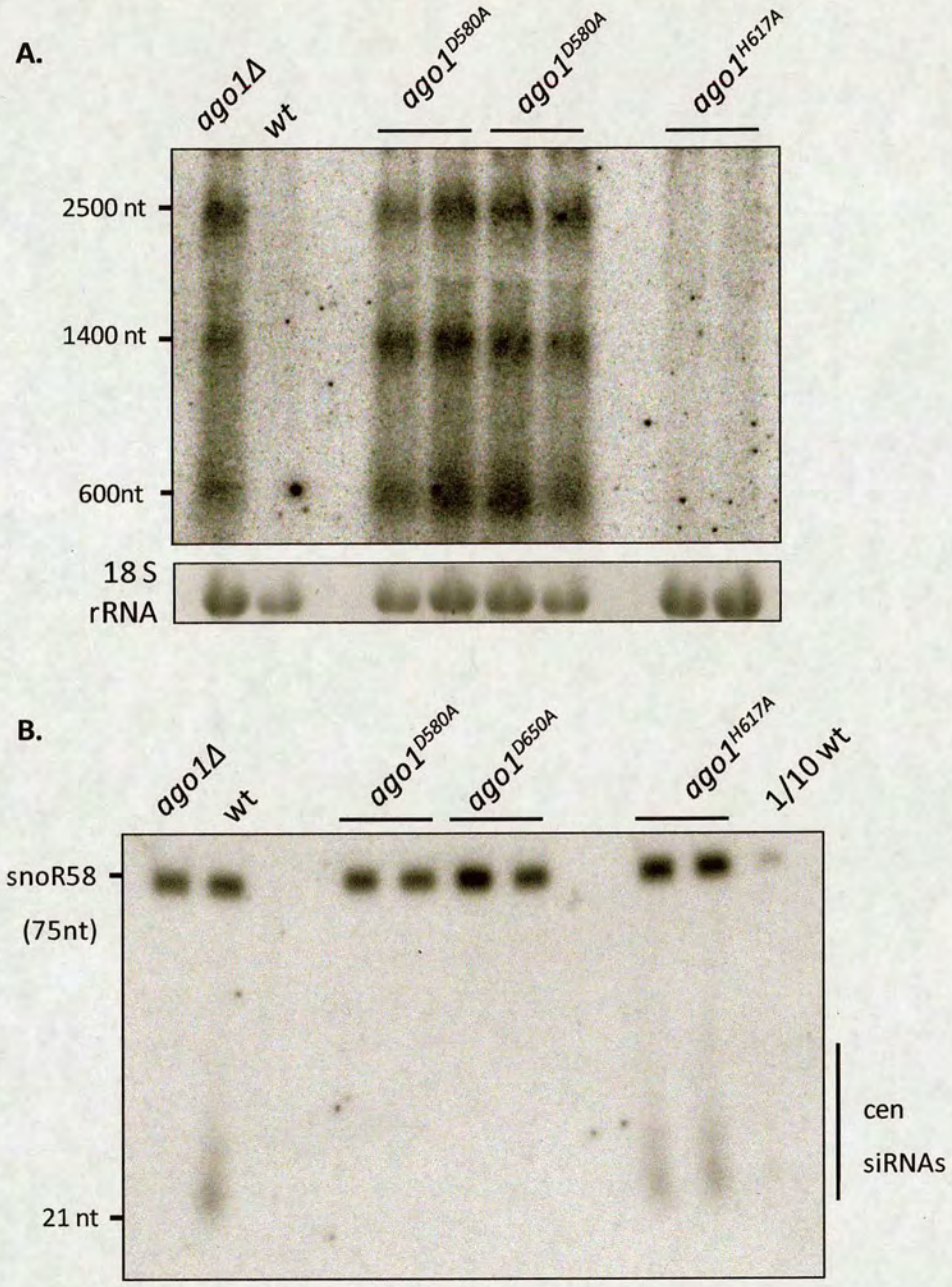


Figure 5-6: ***ago1-sm* mutant cells accumulate centromeric *otr* transcripts and are depleted in centromeric siRNAs.**

A. Northern analysis of centromeric transcripts in *ago1-sm* compared to wild-type, *ago1Δ* and *ago1*^{H617A}. Total RNA from all samples was transferred to a membrane and hybridised to a probe specific to the centromeric *otr (dg)*. Loading control is provided by Ethidium bromide staining of 18S rRNA on the membrane. **B.** Northern analysis of centromeric siRNA levels in the same strains. Low molecular weight RNA samples for each strain were transferred and hybridised to a similar probe used to measure transcript levels. In addition, loading control is provided by a snoR58-specific hybridization probe. To illustrate the sensitivity of the assay, a lane was loaded with a ten-fold smaller RNA sample from wild-type cells (2 µg for this lane, 20 µg for the others).

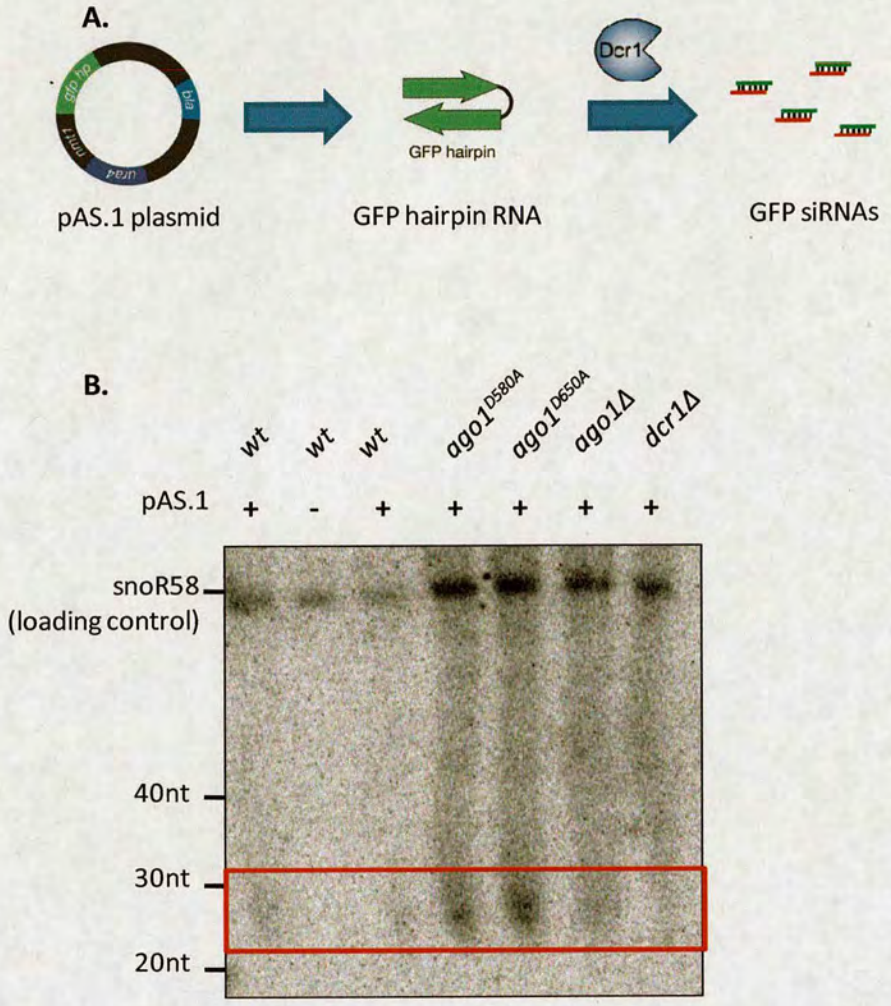


Figure 5-7: *ago1-sm* mutant cells can generate siRNAs from artificial hairpin dsRNA.

A. Diagram depicting Dcr1-dependent GFP siRNA production triggered by a plasmid expressing an artificial hairpin RNA containing GFP sequences (pAS.1) (adapted from Sigova et al. 2004). This provides the basis of the siRNA production assay to be performed on *ago1-sm* cells.

B. Northern analysis of GFP-specific siRNA levels on *ago1-sm* cells with wild-type, *ago1Δ* and *dcr1Δ* controls in the presence or absence of the pAS.1 hairpin plasmid. Loading control is provided by the hybridisation signal of a snoR58-specific probe. Red box marks the regions were the Dcr1-specific GFP signal migrates. The smeared GFP signal of larger size is always detected in samples from cells where the hairpin is present and is believed to originate from RNAi-independent degradation of hairpin RNA.

Cells were transformed with the plasmid containing the GFP hairpin construct (pAS.1) and were assayed by northern analyses for the presence of GFP siRNAs (Figure 5-7). A high level of smeared hybridization signal at 30-75 nt can be seen in cells expressing the GFP hairpin even in the absence of Dcr1 (Sharon White, Femke Simmer, my results). This probably results from its high level of expression and degradation. However, the hybridization signal is more intense between 20 and 30 nt which correspond to GFP siRNAs. This GFP signal can be seen in wild-type samples only in the presence of the pAS.1 plasmid but it is not detected in *dcr1Δ*, showing that it is a product of Dcr1 activity. GFP siRNA signal can also be detected in *ago1-sm* samples showing that siRNA production by Dcr1 is not affected in these mutants. GFP siRNA levels are somewhat lower in *ago1Δ* but still detectable, demonstrating that Dcr1 cleavage of GFP dsRNA can occur in vivo in the absence of Ago1 protein. Hence, it is likely that centromeric siRNA production is not blocked in *ago1-sm* mutants and only the downstream amplification dependent on RDRC (RNA-dependent RNA polymerase complex) is affected. RDRC-dependent siRNAs make up the bulk of the signal detected by Northern blot in wild-type samples as evidenced by the total loss of siRNA signal documented for deletions of RDRC components or catalytically dead mutants of the Rdp1 RNA dependent RNA polymerase) (Motamedi, Verdel et al. 2004; Sugiyama, Cam et al. 2005). Hence *ago1-sm* mutants might be affecting RDRC activity in amplifying siRNA signal thus leading to defective RNAi.

5.2.5. RITS localisation to centromeres is impaired in *ago1-sm* mutants

All the different aspects of the *ago1-sm* phenotype may be explained by defective recruitment of key machinery to outer repeat chromatin. Defective downstream interaction with the H3K9 methyltransferase $Clr4^{Su(var)3-9}$ and the remaining components of the $Clr4^{Su(var)3-9}$ complex (Raf1, Raf2, Rik1, Cul4, may lead to loss of heterochromatin. Consequently this leads to higher transcription rate at the centromeric outer repeats and the subsequent accumulation of *otr* RNA. The drop in siRNA levels can be explained by an inability to recruit RDRC to sustain dsRNA production. It is possible that RDRC requires a slicing event in order to process a single-stranded RNA into a dsRNA substrate that Dcr1 can use. However, all these hypotheses are based on the assumption that RITS (RNAi effector complex; Ago1, Tas3 and Chp1) is still able to localise to the centromeric outer repeats. Regardless of the capacity of Ago1 to slice, the mutant Ago1 protein should still be able to use an siRNA to bind to a nascent transcript. Hence, Ago1-sm should still localise to centromeric chromatin. To verify this, I fixed *ago1-sm* cells for immunolocalisation analysis using antibodies against $Cnp1^{CENP-A}$ protein and myc epitope as well as staining DNA with

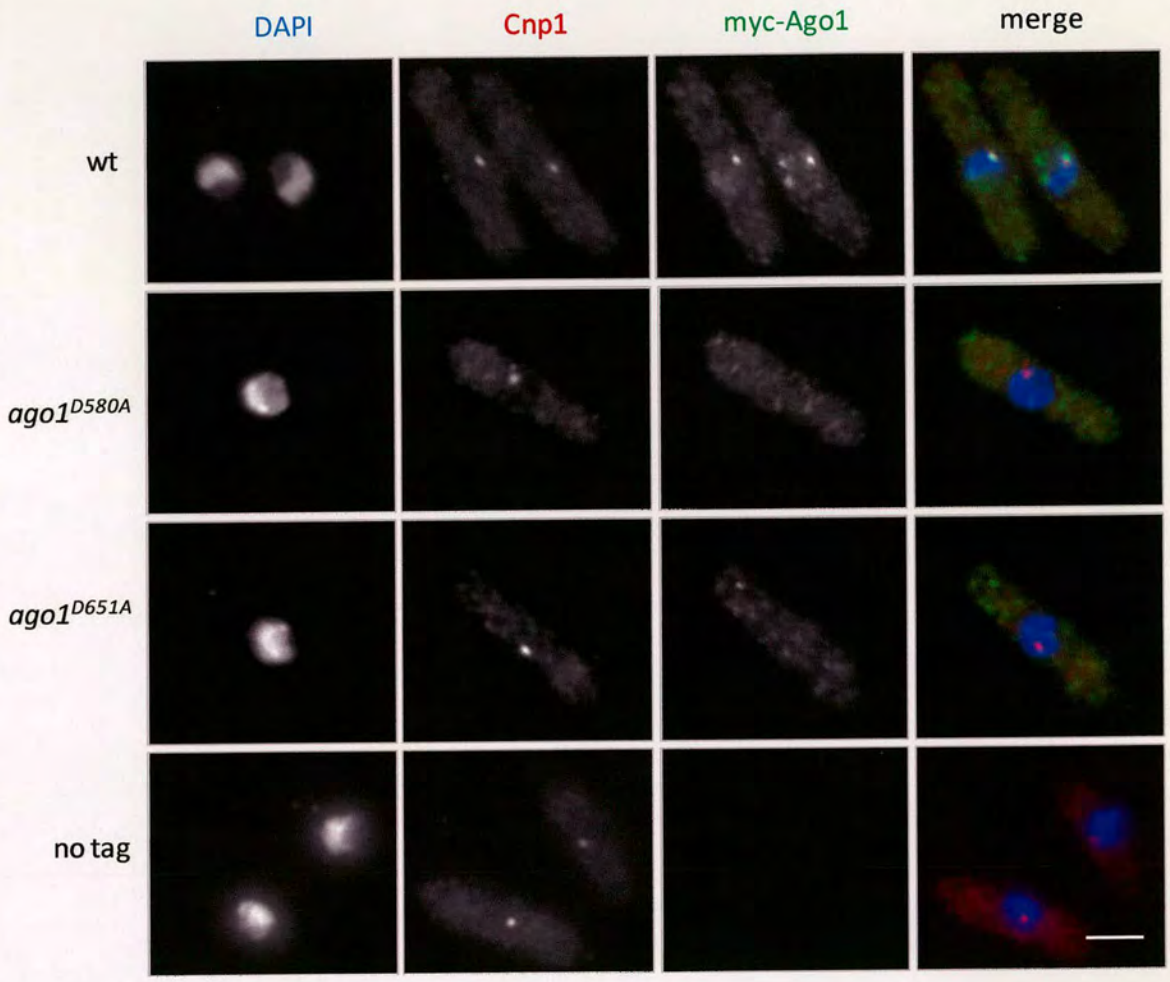


Figure 5-8: Ago1 localization to centromeres is disrupted in *ago1-sm* mutants.
Myc-tagged wild-type and mutant *ago1* strains were fixed and stained by indirect immunofluorescence using anti-Myc. Centromeres were marked using anti-Cnp1 antibody while DNA was stained using DAPI. Fluorescence signal intensity was normalized for each channel between all images. White scale bar represents 4 µm.

DAPI. Cnp1^{CENP-A} is loaded onto chromatin specifically over the central domains of centromeres, which makes it a suitable marker to visualise centromeric chromatin by microscopy. In interphase, all centromeres are clustered together in the vicinity of the spindle pole body, which causes the Cnp1^{CENP-A} signal to appear as a single dot in the cell nucleus (Funabiki, Hagan et al. 1993; Takahashi, Chen et al. 2000). Control and *ago1-sm* cells were imaged by fluorescence microscopy to observe the localisation pattern of Ago1 in wild-type and mutant cells (Figure 5-8). The resulting images show that Ago1 localises mainly to three foci in the nucleus during interphase: the largest focus represents the clustered centromere as shown by the Cnp1^{CENP-A} staining and the two remaining ones are the telomere clusters in the nuclear periphery (Ekwall, Javerzat et al. 1995; Noma, Sugiyama et al. 2004). However this pattern is completely lost in *ago1-sm* mutant cells. Western analysis shows that expression of Ago1 protein in *ago1-sm* mutants occurs in similar levels to wild-type *ago1+* expression (Figure 5-2B). However, the mutant Ago1 proteins fail to form nuclear foci. Hence, the *ago1-sm* mutations are introducing a defect that prevents Ago1 from being recruited to the centromeric chromatin.

The RITS complex is composed of Ago1, Tas3 and Chp1 proteins that co-localise to different heterochromatin loci within the cell nucleus (Noma, Sugiyama et al. 2004; Verdel, Jia et al. 2004). The localisation pattern of RITS components to centromeres is dependent on RNAi (Verdel, Jia et al. 2004). However, the localisation of Tas3 and Chp1 to other heterochromatic loci, such as the silent mating type loci or telomeres, is not dependent on Dcr1 or Ago1 (Petrie, Wuitschick et al. 2005). Unlike in the case of the centromeric outer repeats, RNAi does not play a predominant role in the deposition of H3K9me2 over these other heterochromatic loci. Consequently, Chp1 can bind to H3K9me2 at the mating type loci and telomeres even in the absence of functional RNAi (Petrie, Wuitschick et al. 2005). In order to investigate the impact of *ago1-sm* mutations on the localisation of the Chp1 to centromeres, ChIP (chromatin IP) was performed with anti-Chp1 antibody in control and *ago1-sm* cells. Preliminary results show that levels of Chp1 at the centromeric outer repeats appear to decrease in *ago1-sm* mutants (Figure 5-9). Chp1 levels in *ago1-sm* mutants are reduced but not completely abrogated when the signal is compared to *clr4Δ*. This result is consistent with the H3K9me2 ChIP data that shows that *ago1-sm* and *ago1Δ* cells retain a remnant of H3K9me2 over centromeric outer repeats (Figure 5-3). To further investigate the behaviour of Chp1 in *ago1-sm* mutants, immunolocalization analysis was performed using strains carrying untagged *ago1+* along with *chp1-13xmyc*. The cells were fixed and immunostained with anti-myc antibody to reveal

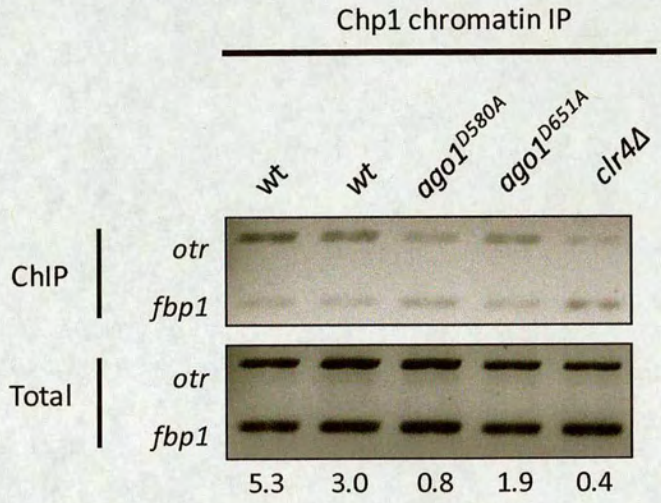


Figure 5-9: Chp1 levels are reduced at centromeric outer repeats in *ago1-sm*. Chromatin immunoprecipitaion (ChIP) was performed on *ago1-sm* as well as wild-type and *clr4Δ* cells using anti-Chp1 antibody. Levels of Chp1 were determined by multiplex PCR using primers for the centromeric outer repeats (*otr*) and the control gene *fbp1*. Enrichment ratios were calculated in ChIP versus control reactions and are shown below each panel.

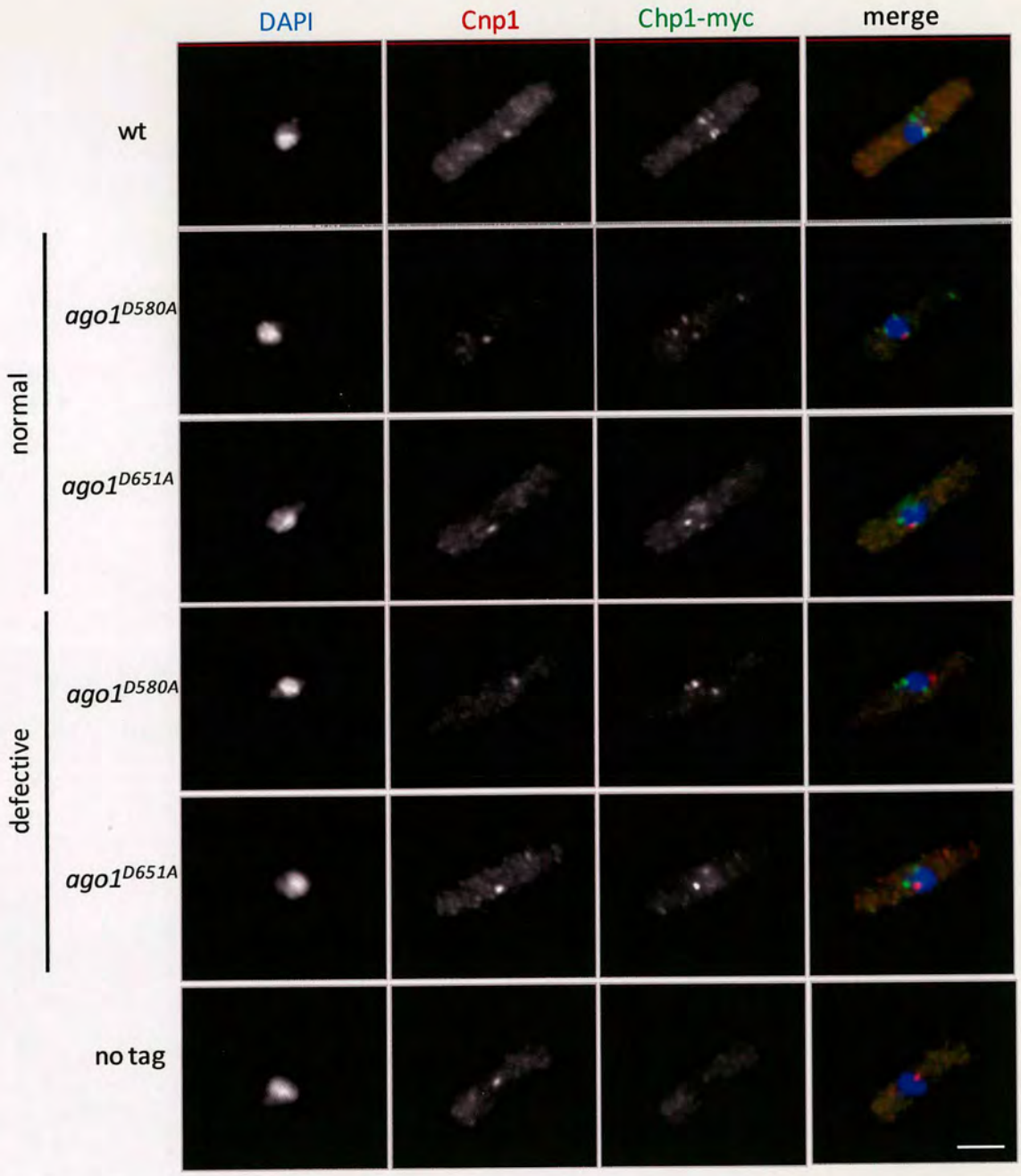


Figure 5-10: Chp1 localization in *ago1-sm* cells. Chp1-13xmyc localisation in wild-type and *ago1-sm* (*ago1*^{D580A} and *ago1*^{D651A}) cells is shown here by indirect immunofluorescence imaging using anti-myc antibody. The centromere cluster was marked with anti-Cnp1^{CENP-A} antibody and the DNA stained with DAPI. The cells were analysed for the presence or absence of a Chp1 focus adjacent to the Cnp1^{CENP-A} focus that marks the position of the centromeres. Two cells are portrayed for each of the two *ago1-sm* mutant strains to illustrate the heterogenous Chp1 localization phenotype observed in these mutant cells (normal and defective). Fluorescence signal intensity was normalized for each channel between all images. White scale bar represents 4 µm.

Chp1, anti-Cnp1^{CENP-A} antibody to mark the clustered centromeres and DAPI to stain the DNA (Figure 5-10). Wild-type cells display multiple Chp1-13xmyc foci, which correspond to the diverse genomic loci which are coated in heterochromatin (centromeres, silent mating type loci and telomeres). In comparison, *ago1-sm* cells display a heterogeneous phenotype, with a subpopulation of cells displaying normal Chp1 localisation at centromeres while others display reduced or undetectable amounts of centromeric Chp1. Localisation in the other foci does not appear to be affected, which is consistent with the fact that RNAi is mostly redundant in nucleating heterochromatic at telomeres and silent mating type loci (Hall, Shankaranarayana et al. 2002; Hall, Noma et al. 2003; Jia, Noma et al. 2004). It is difficult to estimate the degree of heterogeneity of Chp1 localisation in *ago1-sm* cells since it was not extensively analysed. However, my observations suggest that a significant portion of the cells within a population are able to maintain Chp1 protein at the centromeric outer repeats. Given that RNAi mutants display significant amounts of H3K9me2 remaining at centromeres, it is possible that this signal detected by ChIP originates from a small subpopulation of cells that retain H3K9me2 and consequently Chp1 at centromeres rather than a uniform reduction of H3K9me2 throughout the entire population.

A possible explanation of the phenotypes observed is that mutation in Ago1 on D580 and D651 (*ago1-sm*) not only inhibit slicer activity but also affect the ability of Ago1 to form a functional RITS complex together with Chp1 and Tas3. To verify this, considerable effort was invested in purifying the RITS complex from cells using immunoprecipitation techniques. A tag-based approach was attempted using Tandem Affinity Purification (TAP) and FLAG immunopurification by placing the appropriate tags on Chp1 and Tas3 (Puig, Caspary et al. 2001; Verdel and Moazed 2005). Despite the existence of several published methods to purify this complex, my efforts have been unsuccessful due to high instability of the Chp1 protein, which was found to be promptly degraded during any of these procedures (my observations). However, one of the procedures used allowed the purification of Chp1-FLAG. The resulting sample was subjected to gel-free mass spectrometry analysis (LC-MS/MS) in collaboration with Alexander Kagansky and the laboratory of Juri Rappsilber. The results obtained reveal that Ago1-sm and Tas3 are detected in immunoprecipitates of Chp1-FLAG (not shown). This suggests that the RITS complex is still intact in *ago1-sm* cells. However, no inference could be made on the relative stability of the complex in wild-type and mutant situations since this procedure is not quantitative. A similar experiment using relative or absolute quantitative MS approaches such as the SILAC method (stable isotope labelling with amino acids in cell culture) may allow us to determine if the association dynamics of RITS are affected in *ago1-sm* cells

although this approach could still be affected by the inherent instability of Chp1 (Ong, Blagoev et al. 2002).

5.3. DISCUSSION

The experiments described in this chapter support the hypothesis that the putative slicing domain in *S. pombe* Ago1 protein plays a fundamental role in the activity of RNA interference in this organism. Mutations predicted to abolish slicing activity lead to a complete loss of RNAi function phenotype that is mostly undistinguishable from a complete *ago1* gene deletion or indeed mutations in other components of the RNAi-directed chromatin modification pathway. The stability of Ago1 protein is not compromised and preliminary data suggest that its interactions with RITS components are not severed. Nevertheless, heterochromatin at centromeres is destabilized in *ago1-sm* mutants leading to chromosome segregation defects. The loss of centromeric heterochromatin is a consequence of the collapse of RNAi function at centromeres as observed by the accumulation of centromeric *otr* transcripts, loss of centromeric siRNAs and de-localisation of components of the RITS complex, with Ago1 as the most notable one. Thus both *ago1-sm* mutations not only affect the integrity of centromeric heterochromatin but also cause a defect in the processing of noncoding centromeric RNAs to siRNAs. It appears that the defect is introduced at a stage in which RITS is loaded with siRNA and activated prior to localising to centromeric chromatin.

One of the key questions faced while researching these mutants was if fission yeast Ago1 is indeed capable of siRNA-directed endonucleolytic cleavage or "slicing". Previous analyses had shown that Argonaute proteins from *H. sapiens*, *A. thaliana* and *D. melanogaster* can slice an RNA molecule *in vitro* using a complementary siRNA (Liu, Carmell et al. 2004; Baumberger and Baulcombe 2005; Miyoshi, Tsukumo et al. 2005; Rivas, Tolia et al. 2005). In fact, I attempted to develop a similar *in vitro* assay using Ago1 purified from fission yeast. However, at an early stage of this work, two other labs published results that confirm that Ago1 has slicing activity (Irvine, Zaratiegui et al. 2006; Buker, Iida et al. 2007). In both cases, recombinant fission yeast Ago1 was shown to cleave a target RNA in an ATP-independent manner using a siRNA molecule as a guide. Any mutation to Ago1's DDH motif, including D580A and D651A (*ago1-sm*), abrogated this enzymatic activity. In agreement with the data shown here, both publications describe similar

findings regarding the impact that these mutations have on RNAi and centromeric heterochromatin.

There are notable differences between the published studies and the data described here. Irvine et al. claim that Ago1 levels at the outer repeats are not significantly affected in *ago1-sm* (Irvine, Zaratiegui et al. 2006). While the silencing assays performed by Irvine et al. use untagged *ago1-sm* mutants similar to the ones described in this chapter, Ago1 chromatin IP was performed on cells expressing ago1 with an hemaglutinin peptide (HA) tag on its C terminus (Ahmet Dehli and Greg Hannon) in order to use anti-HA antibody to immunoprecipitate the protein (Irvine, Zaratiegui et al. 2006). Observations made in our lab suggest that the construct in question is not fully functional since silencing at centromeric outer repeats appeared to be defective in cells expressing Ago1-HA. Unfortunately, Irvine *et al.* did not perform centromeric silencing assays on the strains carrying the *ago1-HA* construct. Consequently, the authors assumed that Ago1 was still able to localise to centromeres despite the *ago1-sm* mutations. Based on this evidence, the authors concluded that slicing activity of Ago1 is involved in mediating co-transcriptional silencing at the centromeric repeats and recruiting chromatin modifications (Irvine, Zaratiegui et al. 2006).

From my analyses, it appears that Ago1 slicing activity is required for the RNAi pathway at a stage upstream of its co-transcriptional activity at the centromeric outer repeats. The observations by Bukher et al. agree with my findings that Ago1 localization to centromeric chromatin is affected in *ago1-sm* mutants (Buker, Iida et al. 2007). The latter authors went further and suggested a model for the basis of the RNAi defect in *ago1-sm* cells. When an siRNA duplex is loaded onto an Argonaute protein, the molecule must be unwound and one of the strands discarded so that Argonaute can use the remaining strand to bind to a target RNA molecule (Elbashir, Lendeckel et al. 2001; Nykanen, Haley et al. 2001). It was previously shown *in vitro* that slicing activity could facilitate this process of siRNA maturation (Matranga, Tomari et al. 2005). Argonaute slicing of the discarded strand (passenger) favoured the kinetics of release and allowed for Argonaute loaded with single-stranded siRNA to be more readily available for recognizing a target RNA (Matranga, Tomari et al. 2005). Buker *et al.* suggest that fission yeast Ago1 similarly slices an siRNA passenger strand during loading and that in the absence of slicing activity, Ago1 becomes blocked with a duplex siRNA that cannot recognize a target transcript (Buker, Iida et al. 2007). The authors present supporting evidence for this model in the form of hybridization signal corresponding to duplex centromeric siRNAs detected in Ago1-sm immunoprecipitated samples. This model offers an explanation for why the localisation of Ago1 to centromeres and other loci in the nucleus is

disrupted in the absence of slicing activity, which leads to RNAi collapse and subsequent de-stabilization of centromeric heterochromatin.

In spite of this and similar studies, the actual purpose of Ago1 slicing in the process of RNAi-mediated heterochromatin formation remains unclear. Even though it has been shown that Ago1 can slice *in vitro*, there is no strong evidence demonstrating that Ago1 indeed slices centromeric transcripts. An indication of transcript slicing comes from the analysis made by Irvine et al (Irvine, Zaratiegui et al. 2006). The authors demonstrate that transcripts that originate from outer repeat sequences extend into a marker gene insertion at the centromeric outer repeats. Furthermore, the authors observed that such transcripts are only detected if Ago1 slicing activity is abolished or if the exosome component Rrp6 is absent (Irvine, Zaratiegui et al. 2006). The authors conclude that Ago1 slicing must be involved in initiating the degradation of these transcripts and that the exosome is involved in mediating their turnover (Irvine, Zaratiegui et al. 2006). However, it is difficult to distinguish the effects of Ago1 slicing from the increased transcription rate caused by loss of silencing. Furthermore, outer repeat transcripts have variable sizes, multiple promoters and undefined termination sites, making any detailed centromeric transcript analysis exceedingly complex. Using the endogenous centromeric sequence might then be inadequate for determining if Ago1 indeed slices target transcripts *in vivo*. Alternatively, an approach based on a synthetic RNAi system might provide clearer results. The artificial GFP hairpin mentioned in this chapter is a convenient form of inducing RNAi activity (Sigova, Rhind et al. 2004). A reporter gene, such as a fusion of GFP with the *ura4* reporter gene, provides a target whose silencing can be monitored by fluorescence-based detection methods, western analysis or growth assays in medium depleted of uracil (Halim Boukaba and Femke Simmer). More importantly, such a reporter construct has a single promoter, terminator and a defined transcript size. In addition to silencing assays, northern analysis should allow the detection of any reporter transcript size shifts caused by RNAi activity. If Ago1 slicing of this target transcript indeed occurs, it should be readily detectable either in wild-type conditions or in TRAMP/exosome mutants such as *cid14*, *rrp6Δ* or *dis3-54* that stabilize RNA intermediates that have been marked for degradation (Ohkura, Adachi et al. 1988; Briggs, Burkard et al. 1998; LaCava, Houseley et al. 2005; Wyers, Rougemaille et al. 2005; Buhler, Haas et al. 2007).

The detection of products of slicing activity *in vivo* would confirm that Ago1 performs this enzymatic activity in the course of its mechanism. However, it is not sufficient to determine the relevance of slicing for promoting heterochromatin formation. In fact, there is no published evidence that specifically indicates whether recruitment-based form of action for RITS or a more

active role for Ago1 involving slicing is the more crucial aspect for nucleating heterochromatin. An artificial tether linking the RITS complex directly to DNA might allow the question of whether RITS recruitment is sufficient to attract Clr4-mediated H3K9 methylation to be addressed. Such an approach has been successfully employed to force the targeting of the RITS complex to an RNA species (Buhler, Verdel et al. 2006). This was accomplished by expressing a fusion of Tas3 with the λN binding domain together with a modified *ura4* gene containing 5 Box-B sites (Buhler, Verdel et al. 2006). Consequently, Tas3-λN is recruited to ura4-BoxB RNA molecules and the cells produce ura4-specific siRNAs, resulting in silencing of the *ura4-5xBoxB* gene and heterochromatin being established over its locus (Buhler, Verdel et al. 2006). The authors claimed that this evidence provided proof for co-transcriptional silencing by RITS. However, based on the design of this experiment, they cannot rule out that binding of Tas3-λN with *ura4-5xBoxB* RNA may occur elsewhere in the nucleoplasm and not solely at the *ura4-5xBoxB* locus. Consequently, this may lead to siRNA production, which would trigger RITS to find the *ura4-5xBoxB* locus and induce silencing. The fact that this system requires RDRC components (RNA-dependent RNA polymerase complex) in order to silence *ura4* further suggests that the production of siRNAs must occur before *ura4* becomes silent, thus supporting the alternative scenario (Buhler, Verdel et al. 2006).

Whereas an RNA tethering method may not provide a definitive way to target the RITS complex to a defined locus, a DNA tethering method may be more effective. In fact, a DNA tethering system has been developed in the lab using the DNA-binding domain from Gal4 (GBD) and an array of *GAL4* upstream activating sequences (UAS) from *S. cerevisiae* to serve as a biding site (Chien, Buck et al. 1993) (Alexander Kagansky and Kirstin Scott). Using this set-up, it was possible to induce formation of heterochromatin at an ectopic locus by forcefully recruiting $Clr4^{Su(var)3-9}$ to methylate the surrounding histones, leading to a drop in expression levels of adjacent marker genes (Kagansky et al., unpublished observations). This system is refractory to the loss of RNAi components and seems to rely mostly on factors associated with $Clr4^{Su(var)3-9}$ activity, such as members of the Clr4 complex and histone deacetylases. Potentially, the same tethering system can be employed to tether RITS to a DNA region. The question remains whether such a forced localization suffices to recruit Clr4-mediated H3K9 methylation and if Ago1 slicing can influence the outcome in any way.

CHAPTER 6
FINAL DISCUSSION

6.1. THE LINK BETWEEN RNAi AND TRANSCRIPTION

The involvement of the RNA interference pathway in promoting heterochromatin assembly in fission yeast has been made clear but it is still difficult to discern the details of the molecular mechanism by which this is achieved. In fission yeast, RNA interference functions in a closed self-reinforced loop, combining chromatin binding proteins, histone modifiers, RNA polymerases and multiple nucleases (Volpe, Kidner et al. 2002; Noma, Sugiyama et al. 2004; Verdel, Jia et al. 2004). The outcome of this mechanism is a sustained chromatin environment at the centromeric outer repeat DNA that is structurally distinct and transcriptional silent (heterochromatin) (Hall, Noma et al. 2003; Volpe, Schramke et al. 2003). However, due to the complexity of the pathway, the order of molecular events leading to the formation of heterochromatin via RNAi is unclear. Often, the molecular marks of RNAi function rely on the presence of a catalytic activity that is conceptually placed downstream in the pathway. For instance, the amount of centromeric siRNAs in the cells is sensitive to the presence or absence of the $Clr4^{Su(var)3-9}$ methyltransferase, showing that the chromatin outcome of RNAi is important for sustaining its level of activity (Noma, Sugiyama et al. 2004). RNAi-mediated heterochromatin assembly mechanism in fission yeast is complex but it is still the simplest know form of this pathway that can be studied. *S. pombe* possesses all three core RNAi genes in single copy and a form of heterochromatin that resembles the one in higher eukaryotes, which provides an opportunity to study and dissect the molecular details of TGS in a simpler biological model that allows for robust genetic and biochemical approaches.

The case for a link between RITS and nascent transcription at centromeres is very compelling. It was proposed that RNAi establishes its influence on chromatin through the presence of RNA polymerase II and newly made transcripts. The localization of RITS and RDRC to outer repeat chromatin supports this hypothesis, especially given that the co-localization of these two complexes to the *otr* is sensitive to RNAse treatment. RNA polymerase II is involved in mediating chromatin silencing by RITS because the *rpb2-m203* mutant shows defects in transcriptional silencing but not in producing *otr* transcripts or maintaining centromeric siRNAs. The implication is

that RNAi-mediated chromatin silencing is required to interact with RNA polymerase II at some stage in order to nucleate heterochromatin.

Evidence from other organisms supports the existence of a connection between Argonaute proteins and DNA-dependent RNA polymerases in mediating transcriptional gene silencing (TGS). In *A. thaliana*, RNA polymerase IV plays a key role in TGS along with Argonaute 4 (AGO4), DICER-like protein 3 (DCL3) and RNA-dependent RNA polymerase 2 (RDR2). RNA polymerase IV exists in two isoforms, depending on the associated GW repeat protein, NRPD1a and NRPD1b. The RNA polymerase IVa isoform cooperates with DCL3 and RDR2 in the biosynthesis of the longer siRNAs (23-24 nt instead of 21-22 nt) that mediate TGS through AGO4. In turn, RNA polymerase IVb isoform associates with AGO4 through the direct interaction between the Argonaute protein and NRPD1b. This association is required for siRNA-directed *de novo* DNA methylation. The only known example of siRNA-directed TGS in mammals functions by transfection of siRNAs specific to the promoter of a reporter gene (Morris, Chan et al. 2004). This causes the accumulation of methylation on CpG dinucleotides, histone H3K9 and K27 and, consequently, silencing of the reporter gene. AGO1, the Argonaute protein found to be responsible for this transcriptional silencing phenomenon, was found to interact with unphosphorylated RNA polymerase II (Kim, Villeneuve et al. 2006). Transcription of the promoter region is required for mediating TGS, suggesting that the interaction between AGO1 and RNA polymerase II is functionally relevant for TGS (Kim, Villeneuve et al. 2006). In summary, research from three distinct organisms suggest a direct connection between RNA silencing and RNA polymerase machinery in mediating siRNA-directed transcriptional silencing.

The method by which Argonaute proteins recruit chromatin modifications to establish silent chromatin domains is not as clear. In human cells, the artificially introduced siRNAs that mediated TGS were shown to be associated with a protein complex containing the *de novo* methyltransferase DNMT3A (Kim, Villeneuve et al. 2006). In turn, DNMT3A is known to interact with HDAC1 and Suv39h1 (H3K9 methyltransferase), suggesting that AGO1 may recruit these chromatin modifiers to promote assembly of heterochromatin (Kim, Villeneuve et al. 2006). Although many chromatin modifiers involved in TGS are known in both *A. thaliana* and *S. pombe*, currently there is no evidence demonstrating a direct interaction between Argonaute proteins and chromatin modifying enzymes or suggesting an alternative method of recruitment of heterochromatin assembly machinery (Lippman, May et al. 2003).

In this chapter I will discuss the existing evidence on possible links between RNAi and transcription in fission yeast. I will analyse some of the proposed methods by which RITS may signal

the assembly of heterochromatin and how Clr4 might be recruited to the DNA loci where RNAi is active in light of the results described in this thesis.

6.1.1. Transcription termination and RNAi in fission yeast

In fission yeast mutants for RNAi and heterochromatin factors, the levels of RNA polymerase II at the centromeric outer repeats increases noticeably. Naturally, this occurs due to alleviation of silencing of these DNA domains that leads to higher rate of transcription firing from the *otr* promoters. In addition, it has been suggested that RITS (the effector complex: Ago1, Tas3 and Chp1) could signal transcriptional termination from a centromeric *otr* RNA template. For that effect, RITS should be able to somehow signal RNA polymerase II to disengage. In normal mRNA expression, this is performed by the CPF (yeast cleavage and polyadenylation factor complex) that recognizes a termination signal in the nascent pre-mRNA, binds to it and cleaves the transcript (Shatkin and Manley 2000; Dichtl and Keller 2001). Then, the same complex proceeds to promote maturation the 3' end of the newly separated mRNA by recruiting PAP (poly(A) polymerase) and PABs (poly(A) binding proteins). Alternatively, cleavage is performed by a ribozyme (CoTC in budding yeast) that is encoded in many genes at the termination site (Teixeira, Tahiri-Alaoui et al. 2004). Once polymerase transcribes through the ribozyme, it folds and cleaves itself, generating new 3' and 5' ends. The remaining RNA strand still attached to the RNA polymerase II plays a role in terminating transcription. In the "torpedo" model described in budding yeast, the Rat1p 5'-3' exonuclease engages on the free 5' end and begins its degradation (Kim, Krogan et al. 2004). The processing rate of Rat1p nuclease is much higher than the elongation rate of RNA polymerase II and eventually catches up with the polymerase complex. Rat1p then somehow signals RNA polymerase II to disengage and transcription is effectively terminated (Kim, Krogan et al. 2004).

The hypothetical slicing of a nascent transcript by RITS would be a similar event to the nascent RNA cleavage that precedes transcription termination. Hence, RNA polymerase II may be forcefully disengaged from *otr* DNA as a consequence of Ago1 slicing activity. While the "torpedo" mechanism hasn't been demonstrated in fission yeast, the *dhp1-1* mutant phenotype bears strong resemblances to budding yeast *rat1-* phenotype (Shobuike, Tatebayashi et al. 2001). Overexpression of Dhp1 also rescues the temperature-sensitive phenotype of a *rat1-ts* mutant, suggesting that both proteins share the same function in the cell (Sugano, Shobuike et al. 1994). However, as it was shown in Chapter 4, a temperature-sensitive mutation on Dhp1, the fission

yeast homologue of Rat1p, has only a very mild effect on centromeric silencing. Therefore, transcriptional silencing at the centromeric outer repeats does not seem to rely on transcription termination by Dhp1.

Based on the analyses described in Chapter 4 using mutants of factors that are involved in transcription termination, it appears that defective termination of RNA polymerase II transcription does not present an obstacle for the function of RNAi at centromeres. The only evidence that suggest that a transcription termination factor may play a role in heterochromatin integrity comes from the analysis of *hrp1Δ* (Walfridsson, Bjerling et al. 2005). Hrp1 is a fission yeast protein that belongs to the CHD-Mi2 family of ATP-dependent chromatin remodelers (Jin, Yoo et al. 1998; Yoo, Jin et al. 2000). Hrp1 was also identified as a fission yeast factor involved in transcription termination (Alen, Kent et al. 2002). Its budding yeast homologue Chd1p modifies the positioning of nucleosomes at the 3' end of genes in order for RNA polymerase II termination to occur efficiently at the poly(A) site (Alen, Kent et al. 2002). In *hrp1Δ* cells, silencing at centromeres, including the outer repeats, is affected (Walfridsson, Bjerling et al. 2005). Recently, Hrp1 was found to be associated with the histone deacetylases Clr6 and the histone demethylases Swm1 and Swm2 (Lan, Zaratiegui et al. 2007; Opel, Lando et al. 2007). Hrp1 appears to be required to silence a subset of genes in euchromatic regions (Opel, Lando et al. 2007). It is possible that Hrp1 is required to efficiently disengage RNA polymerase II from DNA to allow the establishment of a repressive chromatin environment, such as in the case of centromeric outer repeats. Hrp1 also has an impact in both $Cnp1^{CENP-A}$ deposition and silencing at the central core, suggesting it seems that Hrp1 has a general influence in centromeric chromatin and not specifically on heterochromatin assembly. Further analyses are required to determine the nature of the influence of Hrp1 in transcriptional silencing at the centromere. In addition, investigations of the activity of RNA polymerase II found at the centromeric outer repeats might provide more definitive answers regarding the hypothetical involvement of transcription termination in RNAi in transcriptional silencing at centromeric heterochromatin domains.

6.1.2. A possible connection between Rik1 and the CPF complex

There are very few clues suggesting how the Clr4 complex may be called to intervene at the centromeric outer repeats. None of its constituents is known to have strong interactions with RNAi components or participate in alternate complexes that may have a functional connection with RNA interference. However, there might links between Clr4 and RNAi through the transcription process, namely at the stage of mRNA cleavage and polyadenylation. Such link was proposed between the Clr4 complex member Rik1 and the cleavage and polyadenylation factor complex (CPF) (Ekwall and Ruusala 1994; Shatkin and Manley 2000; Dichtl and Keller 2001). Rik1 contains a WD propeller domain that is predicted to serve as dedicated protein interaction domain (Neuwald and Poleksic 2000). In addition, Rik1 possess the CPSF-A motif that shows homology to the C-terminus region of the human cleavage and polyadenylation specifying factor (CPSF1) (Murthy and Manley 1995). In yeast, the CPSF1 homologue (Cft1) resides in the CPF complex and is responsible for recognizing the AAUAAA polyadenylation signal in the nascent mRNA (Dichtl, Blank et al. 2002). The yeast CPF (cleavage and polyadenylation factor) complex combines two roles in mRNA 3' end processing: co-transcriptional cleavage and polyadenylation (Dichtl and Keller 2001). It was suggested that Rik1 may interact with the CPF complex and hence be recruited to nascent RNAs undergoing cleavage and polyadenylation. In Chapter 4 of this thesis, mutants for the CPF component Pfs2 were assayed for defects in transcriptional silencing at the outer repeats (Wang, Asakawa et al. 2005). The results of the analysis of *pfs2-3169* and *pfs2-11* demonstrate that the complete function of the CPF complex is not a primary requirement for transcriptional silencing at the outer repeats. The analyses were performed in restrictive conditions in which the mutant cells were shown to present signs of transcriptional read-through, which is accounted by impaired function of the CPF complex in transcription termination (Wang, Asakawa et al. 2005). In the conditions used, *pfs2* mutant cells do not show evidence of defective silencing in the heterochromatin domains at the centromere, further suggesting that CPF-mediated transcription termination is not required for silencing or maintenance of heterochromatin at the centromeric outer repeats. Given that CPF acts epistatically with Dhp1/Rat1p to promote transcription termination, this constitutes further evidence that transcription termination machinery is not required for the integrity of heterochromatin at centromeres.

Based on data on *pfs2-3169* and *pfs2-11* mutants, it is not possible to rule out the possibility that the remaining components of the CPF complex can still associate with centromeric

outer repeats. If true, this may be sufficient for Rik1 association with *otr* transcripts. Further analyses are required on mutants for other components of the CPF, such as Cft1, Pap1 or Dis2, in order to definitely establish whether the CPF is anyway relevant to the process of heterochromatin assembly (Dichtl and Keller 2001). RNA-immunoprecipitation experiments using tagged CPF components could allow to test whether centromeric *otr* transcripts are recognized and bound by the CPF complex.

6.2. RNA SLICING BY *S. pombe* Ago1

The precise role of Argonaute proteins in mediating TGS (transcriptional silencing) has not yet been fully determined in fission yeast. The endonuclease (slicing) activity of Argonaute proteins has been shown to be the key method of effecting silencing in the case of RNA interference in animals and PTGS in plants (Liu, Carmell et al. 2004; Baumberger and Baulcombe 2005; Miyoshi, Tsukumo et al. 2005). Slicing is not the only method of enforcing repression by Argonaute proteins. In miRNA-driven repression, Argonaute proteins are present in the miRNP (microRNA protein complex) that can block the translation of the target mRNA (Lee and Ambros 2001; Morris, Chan et al. 2004; Chendrimada, Finn et al. 2007; Kiriakidou, Tan et al. 2007). Alternatively, miRNPs localize to cytoplasmic P bodies where the target mRNA is decapped and degraded (Behm-Ansmant, Rehwinkel et al. 2006; Wu, Fan et al. 2006). For these two possible outcomes orchestrated by miRNA-loaded Argonaute complexes, slicing activity is not crucial. In the case of transcriptional silencing, chromatin modifying enzymes such as $Clr4^{Su(var)3-9}$ in fission yeast are responsible for promoting the assembly of silent chromatin that blocks transcription (Volpe, Schramke et al. 2003; Verdel, Jia et al. 2004). However the method of recruitment of chromatin modifiers to loci where TGS occurs is not clear. Until recently, the precise role of Argonaute was equally unclear since slicing activity had not been characterized in the context of TGS.

The results presented in Chapter 5 suggest that Ago1 slicing is required for RNAi-mediated heterochromatin assembly in fission yeast. However, it also shows that slicing is required for sustained levels of centromeric siRNAs and for normal localization of Ago1 to the *otr* loci. Mutations in residues predicted to affect the endonucleolytic activity of Ago1 (D580A and D651A, referred to as *ago1-sm*) were shown to affect localization of both Ago1 and Chp1 to the centromere and to cripple the cells in the ability to assemble heterochromatin *de novo* on a naked template. Based on

these data, it is not possible to conclude if Ago1 slicing of a nascent *otr* transcript occurs naturally and if this has any consequences to the process of heterochromatin assembly. Two separate fission yeast studies have confirmed these observations (Irvine, Zaratiegui et al. 2006; Buker, Iida et al. 2007). These contain *in vitro* evidence that show that Ago1 is indeed a competent slicer (Irvine, Zaratiegui et al. 2006; Buker, Iida et al. 2007). In addition, one of the reports proposed that slicing is required to activate the siRNAs loaded into Ago1 before RITS can use them to recognize centromeric *otr* transcripts (Buker, Iida et al. 2007). A form of bypassing RITS activation by Ago1 slicing is required in order to determine if slicing contributes to chromatin silencing at a downstream stage.

The role of Argonaute slicing in TGS was also investigated in plants. Similar mutations introduced to the ones made in S. pombe Ago1 were introduced in *A. thaliana* AGO4 (Qi, He et al. 2006). AGO4 is an Argonaute protein involved in TGS in *Arabidopsis* by directing DNA methylation (mostly non-CpG) to targets based on siRNA complementarity (Zilberman, Cao et al. 2003; Chan, Zilberman et al. 2004; Xie, Johansen et al. 2004; Zilberman, Cao et al. 2004). Similarly to slicer Argonautes in other organisms, AGO4 can slice a target *in vitro* but loses this nuclease activity if mutations are introduced in the residues that form the DDH motif (Asp-Asp-His) in the PIWI domain (Qi, He et al. 2006). In order to determine the role of slicing in TGS, both wild-type and DDH mutant AGO4 were introduced in plants in an attempt to rescue a null *ago4-1* background mutation. Surprisingly, the results showed that AGO4 DDH mutants could restore non-CpG methylation to a subset of AGO4 target loci provided that *ago4-1* plants managed to retain complementary siRNAs (Qi, He et al. 2006). In other loci for which *ago4-1* plants lost both siRNAs and methylation, only wild-type AGO4 managed to efficiently restore silencing (Qi, He et al. 2006). The most important conclusion from this work is that DNA methylation can be directed by an Argonaute protein that is incapable of slicing. These observations suggest that Argonaute-mediated slicing of a target RNA is not a pre-requisite for recruiting chromatin modifying machinery to DNA loci. This mechanistic insight from *A. thaliana* may constitute a general principle of TGS in eukaryotes, including fission yeast.

What caused the difference in results between the analysis of *A. thaliana* AGO4 and the study on *S. pombe* Ago1 described in this thesis? *A. thaliana* differs from *S. pombe* in that it encodes for multiple Argonaute proteins which may cooperate or act redundantly in siRNA biosynthesis and TGS (Lippman, May et al. 2003; Qi, He et al. 2006). In the case of loci such as *AtMu1*, other *A. thaliana* Argonaute proteins such as AGO1 may participate in maintaining *AtMu1* siRNA levels in the absence of AGO4 (Qi, He et al. 2006). In *S. pombe* cells, where only one

Argonaute gene is present, an *ago1-sm* mutation effectively abolishes slicing from the entire Argonaute protein molecules in the cell. This implies that transcriptional silencing may be affected in *ago1-sm* cells mainly due to the failure of these cells to sustain centromeric siRNA levels. In this case, the introduction of a wild-type *ago1* gene in addition to *ago1-sm* could hypothetically restore centromeric siRNA levels. In these circumstances, outer repeat silencing would be restored but it would be interesting to determine if the Ago1-sm mutant protein now reacquires function and localizes to the outer repeat loci. If so, it would indicate that Ago1-sm can perform its role without nuclease activity. If the protein still fails to localize, then slicing may still play a role in bringing about TGS to centromeric outer repeats. The answer to the question of whether slicing of a nascent transcript is required for RNAi-dependent heterochromatin nucleation in fission yeast seems close at hand and it is likely that it may be revealed in the near future.

6.3. FISSION YEAST RNAi AND THE EXOSOME

Whenever an Argonaute protein slices a target mRNA in order to enforce post-transcriptional silencing, the remaining RNA strands are degraded by the combined activity of the 5'-3' exonucleases such as Xrn1 and the 3'-5' exonuclease complex called the exosome (Hsu and Stevens 1993; Muhlrad, Decker et al. 1994; Beelman and Parker 1995; Mitchell, Petfalski et al. 1997; Zhang, Williams et al. 1999). The exosome is a multi-functional complex composed of 9 exoribonucleases (Rrp40p, Rrp41p, Rrp45p, Rrp46p, Rrp43p, Mtr3p, Rrp42p, Rrp4p, Csl4p) that are conserved from Bacteria to yeast and higher eukaryotes. In addition to these 9 core subunits, the exosome possesses the RNAse II-like Dis3/Rrp44p and the nuclear specific Rrp6 subunits that are unique to eukaryotes (Mitchell, Petfalski et al. 1997). The exosome complex participates in multiple RNA-related processes involving maturation, surveillance and turnover both in the nucleus and cytoplasm. Its substrates include rRNA, tRNA or snoRNA precursors, prematurely terminated or otherwise aberrant mRNAs and other RNA species which are marked for degradation (Houseley, LaCava et al. 2006). In performing these roles, the exosome is activated and recruited to target RNA molecules by supporting proteins, such as Ned8p, Ski factors and the TRAMP complex (Houseley, LaCava et al. 2006). In budding yeast, the TRAMP complex is composed of a poly(A) polymerase Trf4p or Trf5p, the DExH box helicase Mtr4p and a zinc-knuckle protein Air1p or Air2p (LaCava, Houseley et al. 2005; Wyers, Rougemaille et al. 2005). TRAMP facilitates turnover of RNA molecule in the nucleus by polyadenylating its 3' end, which in turn makes the RNA a better substrate for the

exosome. Trf4p is a homologue of the fission yeast family of Cid proteins (caffeine-induced death) that include Cid12, an RNAi component (Wang, Toda et al. 2000; Motamedi, Verdel et al. 2004). The putative poly(A) polymerase Cid12 is required for RNAi function and to sustain heterochromatin at centromeres (Motamedi, Verdel et al. 2004). The function of Cid12 within the RDRC complex is unclear but it has been suggested that its role may be similar to the one of Trf4p in TRAMP – to recruit the exosome to centromeric transcripts.

The results of my investigation on the possible participation of the exosome complex in RNAi and heterochromatin assembly are reported in Chapter 4 of this thesis. The stability of heterochromatin silencing at the outer repeats was evaluated in fission yeast cells carrying *dis3-54* and *rrp6Δ* mutations. Mutation in *dis3*, the fission yeast homologue of *DIS3/RRP44*, is predicted to affect all forms of the exosome while *rrp6Δ* only affects the nuclear form of the exosome complex (Ohkura, Adachi et al. 1988; Mitchell, Petfalski et al. 1997; Hilleren, McCarthy et al. 2001). In budding yeast, both exosome proteins are required for TRAMP-mediated targeting of RNA for degradation (LaCava, Houseley et al. 2005; Wyers, Rougemaille et al. 2005). My results show that the exosome is called in to process transcripts that derive from the outer repeat loci. Silencing of *otr1R(SphI):ade6+* is moderately affected in both *dis3-54* and *rrp6Δ*, indicating that either chromatin silencing is alleviated in these mutants or that the exosome is failing to degrade *otr1R(SphI):ade6+* transcripts. Other reports have shown that in the two exosome mutants, similar increases in transcript levels occur for other heterochromatic loci such as *mat2/3* locus and sub-telomeric *tlh2* gene (Buhler, Haas et al. 2007; Nicolas, Yamada et al. 2007). This phenotype is also observed in mutants for TRAMP complex, *cid14Δ* (the fission yeast counterpart of Trf4p) and *mtr4Δ*, strongly suggesting that TRAMP is directing the turnover of these RNAs by the exosome (Buhler, Haas et al. 2007).

Does RNAi require the function of TRAMP and the exosome to nucleate heterochromatin? Here, the results from TRAMP and exosome mutants diverge greatly. The levels of centromeric siRNAs appear not to be affected in *rrp6Δ* or *dis3-54* but are severely depleted in *cid14Δ*. Silencing of *imr1R:ura4+* (*ura4+*insertion at the centromeric innermost repeats) is attenuated in *cid14Δ* cells. Surprisingly, histone H3K9 methylation and Swi6^{HP1} levels at centromeres and telomeres in *cid14Δ* are similar to wild-type levels, arguing that Cid14 has no influence in heterochromatin integrity (Buhler, Haas et al. 2007). In comparison, the same heterochromatin marks are stable at centromeres in *dis3-54* cells but *rrp6Δ* cells show a strong decrease in genome-wide levels of histone H3K9 methylation (Murakami, Goto et al. 2007; Nicolas, Yamada et al. 2007). Based on this evidence, is difficult to determine the nature of the involvement of TRAMP and the exosome in the

process of RNAi-mediated heterochromatin formation. Common ground is only found in the involvement of all these factors in degrading transcripts that derive from heterochromatic loci, such as centromeres, telomeres and mating type locus (Buhler, Haas et al. 2007; Murakami, Goto et al. 2007; Nicolas, Yamada et al. 2007). At the level of RNAi function, the involvement of exosome and TRAMP is less clear. Cid14 appears to favour siRNA production, possibly by collaborating with Cid12 in the RDRC (RNA-dependent RNA polymerase complex) in amplifying the amount of centromeric siRNAs (Buhler, Haas et al. 2007). In *dis3-54* and *rrp6Δ*, the hallmarks for RNAi activity are unaffected yet silencing is affected in both mutants (Murakami, Goto et al. 2007; my observations, Chapter 4) and heterochromatin is destabilized in *rrp6Δ* (Nicolas, Yamada et al. 2007). Since RNAi is unaffected in exosome mutants, it is unclear how the exosome might be supporting heterochromatin. Judging from the genome-wide reduction of H3K9 methylation, it seems that *rrp6Δ* is exerting a general effect at the chromatin level that is felt even at telomeres and mating type locus, where RNAi is less relevant for heterochromatin nucleation (Buhler, Haas et al. 2007; Nicolas, Yamada et al. 2007). The possibility of an indirect effect of exosome mutants in key chromatin factors has not been ruled out yet and still provides the simplest explanation for these phenotypes. Further studies on the role of poly(A) polymerases and exosome subunits chromatin structure are required so that their involvement in the processes of RNAi and chromatin silencing may become clearer.

6.4. FISSION YEAST RNAi AND TRANSPOSABLE ELEMENTS

For the remainder of this chapter, I will discuss the function of RNAi at loci other than the constitutive heterochromatin domains (centromere, telemere and silent mating type locus). More specifically, it was proposed that RNAi targeted solo LTR (long terminal repeats) sequences for silencing and heterochromatin assembly along the chromosome arms. One of the proposed biological functions for this phenomenon was to control TE proliferation in the fission yeast genome.

The evidence described in Chapter 3 indicates that fission yeast does not target LTR sequences with RNAi or heterochromatin assembly. Nevertheless, Tf1 and Tf2 elements are controlled by other forms of transcriptional silencing in fission yeast. A recent report demonstrated that all three fission yeast class IV (sirtuin) histone deacetylases (Hst2, Hst4 and Sir2) localize to DNA loci containing Tf2 retrotransposons (Durand-Dubief, Sinha et al. 2007). Hst4 in particular is

required to repress the transcription of full-length Tf2 elements and was also shown to bind solo LTRs (Durand-Dubief, Sinha et al. 2007). Hence, histone deacetylation is employed by fission yeast to silence transcription from transposable element promoters and prevent their proliferation. Similar observations have been made regarding the role of the Clr6 HDAC complex II in controlling transcription of Tf2 transposons and solo LTRs (Nicolas, Yamada et al. 2007). The HIRA-like proteins Hip1 and Slm9 have also been implicated in controlling Tf2 transcription (Greenall, Williams et al. 2006). The HIRA complex functions as a transcriptional co-suppressor and is involved in establishing silent chromatin in metazoans and budding yeast (Sherwood, Tsang et al. 1993; Spector and Osley 1993; Lorain, Quivy et al. 1998; Magnaghi, Roberts et al. 1998). In fission yeast, Hip1 and Slm9 are important for transcriptional silencing at centromeric outer repeats and mating type region (Blackwell, Martin et al. 2004). In *S. cerevisiae*, the homologous HIR proteins are also required to silence expression of the Ty retrotransposons (Qian, Huang et al. 1998). Hence, it is likely that fission yeast assembles a form of silent chromatin that represses expression of transposable elements. Since neither RNAi nor histone H3K9 methylation appear to be involved, it appears that this form of transcriptional silencing is distinct from heterochromatin and resembles the silent chromatin found in budding yeast more closely (Grunstein 1997).

Thus, in fission yeast cells RNA interference is present but appears not to be acting against TEs in order to prevent their proliferation. However, this does not imply that fission yeast RNAi is unable to act on Tf1/2 TE elements. In wild-type cells, these elements are repressed by chromatin silencing mechanisms based on HDACs and chromatin remodelers and so very little Tf1/2 RNA is actually made. How would fission yeast RNAi react if transcriptional silencing of Tf2 elements was lost? Preliminary results from Elizabeth Bayne in our group working in collaboration with the lab of David Baulcombe have provided some insights into the behaviour of *S. pombe* in such circumstances. Large-scale siRNA purification and sequencing analysis was performed on RNA samples from wild-type cells and from cells in which the lysine 9 residue on histone H3 has been mutated for arginine (K9R) or alanine (K9A) (Mellone, Ball et al. 2003). These strains possess only one copy of each of the 4 core histones, meaning that for H3K9A and H3K9R strains all histone H3 proteins in the cell bear the mutation. In these mutants, H3K9 methylation is effectively abolished and transcriptional silencing at the centromeric outer repeats is lost (Mellone, Ball et al. 2003). However, the mutations do not cause the total collapse of the RNAi since centromeric siRNAs are still detected, albeit in reduced amounts (Sharon White, Elizabeth Bayne – unpublished results). From the results of the sequencing analysis, it is clear that K9 mutant cells now produce siRNAs targeting Tf2 transposons whereas wild-type cells do not. This indicates that RNAi is triggered by

TEs in circumstances where silent chromatin formation is impaired. At the moment, it is not known if the Tf2-specific siRNAs target these TEs at a post-transcriptional level, transcriptional level or both. Nevertheless, this evidence suggests that fission yeast RNAi can respond to TE over-expression and most likely prevent their proliferation. It also demonstrates the potential of large scale siRNA sequencing for unravelling new *in vivo* targets of RNAi in fission yeast.

6.5. RNAi AND GENE REGULATION IN *S. pombe*

In fission yeast, the involvement of RNAi in the process of heterochromatin assembly has clear implications in centromere function and contributes to the regulation of mating type switching. Unlike in many other organisms when RNA silencing is clearly an important mechanism of controlling gene expression, there is very little evidence pointing towards an involvement of fission yeast RNAi in the regulation of gene expression. In their genome-wide analysis, Cam et al. documented the existence of a number of heterochromatin "islands" scattered along the chromosomes arms that overlap with genes (Cam, Sugiyama et al. 2005). The chromatin over these "islands" is enriched in H3K9 methylation and Swi6 binding but is also bound by the RITS components Chp1 and Ago1 (Cam, Sugiyama et al. 2005). Thus, it is likely that RNAi contributes to nucleating these heterochromatin "islands". The set of genes associated with these heterochromatin "islands" appear to be upregulated during meiosis but, according to the authors, they are not overlapping with the 7 meiotic genes proposed to be regulated via LTR and RNAi-mediated heterochromatin assembly (Schramke and Allshire 2003; Cam, Sugiyama et al. 2005). Further studies should reveal if these genes are purposefully regulated by this mechanism in meiosis or another functional context. In published studies on gene expression using RNAi mutants, the number of affected genes appears to be relatively small (up to 18 genes) (Cam, Sugiyama et al. 2005; Hansen, Burns et al. 2005). Comparatively, the set of fission yeast genes upregulated in *clr3Δ*, *clr6Δ* (HDACS) and *clr4Δ* (H3K9 HMT) is considerably larger and divergent from the group of genes upregulated on RNAi mutants (Cam, Sugiyama et al. 2005; Hansen, Burns et al. 2005; Wiren, Silverstein et al. 2005). Thus, it is likely that transcriptional gene silencing in fission yeast occurs primarily through recruitment of SHREC (Clr3 and Mit1 histone deacetylases and chromatin remodeler complex), Clr4 and Clr6 complexes and does not involve RNAi (Hong, Villen et al. 2005; Horn, Bastie et al. 2005; Li, Goto et al. 2005; Thon, Hansen et al. 2005; Nicolas, Yamada et al. 2007; Sugiyama, Cam et al. 2007).

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RNA silencing and genome regulation

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Closely related RNA silencing phenomena such as posttranscriptional and transcriptional gene silencing (PTGS and TGS), *quelling* and RNA interference (RNAi) represent different forms of a conserved ancestral process. The biological relevance of these RNA-directed mechanisms of silencing in gene regulation, genome defence and chromosomal structure is rapidly being unravelled. Here, we review the recent developments in the field of RNA silencing in relation to other epigenetic phenomena and discuss the significance of this process and its targets in the regulation of modern eukaryotic genomes.

Introduction

RNA silencing is a general term for a particular collection of phenomena in which short RNA molecules trigger repression of homologous sequences. It is a highly conserved pathway, found in a large variety of eukaryotic organisms, and its main characteristic is the use of small RNA molecules of 21–28 nucleotides that confer high specificity to the target sequence. Originally, it was described as part of a 'co-suppression' phenomenon in plants [1–3] or 'quelling' in *Neurospora crassa* [4] and was later attributed to a posttranscriptional gene silencing process (PTGS; see Glossary) occurring in the presence of complementary RNA molecules that would bind and form double-stranded RNA [5]. A closely related effect described in *Caenorhabditis elegans* as 'RNA interference' (RNAi) [6,7] also requires long double-stranded precursor RNAs to induce and sustain efficient posttranscriptional repression of homologous sequences.

In RNA silencing, double-stranded RNA (produced by various mechanisms) enters the 'canonical pathway' after cleavage into small (21–28 nt) RNA duplexes by the helicase/RNase-like III *Dicer* [8]. Following unwinding, a single-stranded small RNA (small interfering RNA: siRNA) becomes part of protein complexes in which PAZ/PIWI domain proteins (PPD or *Argonaute*) are central players [9,10] (Figure 1a,b). These RNA-induced silencing complexes (RISC) then target homologous mRNAs and exert silencing either by inducing cleavage ('slicing') or, as in the case of micro-RNA-loaded RISC (see below), by also eliciting a block to translation (Figure 1c,e). RNA-dependent RNA polymerase (RdRP) also plays a role in nematodes [11], plants [12,13] and fungi [14,15] but is apparently not required or detectable in the genomes of flies and vertebrates. RdRP amplifies the RNAi/PTGS response by generating more double-stranded RNA from single-stranded targets that can then enter and continue to stimulate the RNA silencing pathway (Figure 1a). This positive-feedback system is crucial in plants and worms to amplify the siRNA signal transmitted from cell to cell and to mount a systemic form of silencing [16,17].

It is now evident that the core machinery required for RNA silencing plays crucial roles in cellular processes as diverse as regulation of gene expression, protection against the proliferation of transposable elements and viruses and modifying chromatin structure. While it appears that the basic pathway has been conserved, specialization has adapted the common RNA silencing machinery for these different purposes. This is implied both by the diversity of *Argonaute* proteins found in different species, such as *C. elegans* (more than 20), *Arabidopsis thaliana* (10) [18] and humans (8) [19] and also by the distinct phenotypic effects that arise from disrupting different *Argonaute* genes [20,21]. This specialization is most obvious in plants, which also encode multiple RdRP and Dicer-like proteins that are relevant for distinct small RNA pathways [22]. Here, we discuss these different pathways and the various levels through which small RNAs can influence the activity of the genome.

Regulation of gene expression – microRNAs

MicroRNA regulation is a clearly specialized branch of the RNA silencing pathway that evolved towards gene regulation, diverging from conventional RNAi/PTGS. MicroRNAs are a specific class of small RNAs that are encoded in gene-like elements organized in a characteristic inverted repeat. When transcribed, microRNA genes give rise to stem–looped precursor RNAs from which the

Glossary

5-Me-C 5-Methylcytosine
DNMT DNA *de novo* methyltransferase
dsRNA Double-stranded RNA
HDAC Histone deacetylase
HMT Histone methyltransferase
H3K9ac Histone H3 acetylated on lysine 9
H3K9me2/3 Histone H3 di/tri-methylated on lysine 9
LTR Long terminal repeat
PEV Position effect variegation
PPD PAZ/PIWI domain
PTGS Posttranscriptional gene silencing
RdRP RNA-dependent RNA polymerase
RISC RNA-induced silencing complex
RITS RNA-induced transcriptional silencing complex
RNAi RNA interference
siRNA Small interfering RNA
TE Transposable element
TGS Transcriptional gene silencing
TIR Terminal inverted repeat

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microRNAs are subsequently processed [23–25]. The released miRNAs are incorporated into RISC-like complexes containing a particular subset of Argonaute proteins that exert sequence-specific gene repression. The presence of these small RNAs was originally found to govern the expression timing of specific sets of developmental genes in *C. elegans* [26]. In the past few years, the number of genes encoding miRNAs identified in various systems has grown enormously, and it is now clear that hundreds of miRNAs regulate the expression timing of a large, but still underestimated, pool of genes [27,28]. A major challenge that remains is the accurate and comprehensive identification of all genes regulated by microRNAs. To date, miRNAs have not been described in simpler unicellular eukaryotes, suggesting that their evolution might be intimately linked to gene regulation in multicellular organisms. However, RNA-mediated silencing is present in both multi- and unicellular eukaryotes and performs a variety of other key functions.

Defence – transposable elements and viruses

RNA silencing was first recognized by its effect on the expression of multicopy transgenes. This curious phenomenon was then interpreted as a process of genome defence against foreign 'invading' sequences. In fact, it was observed in the early 1990s that, in plants, co-suppression or PTGS could play a role in defending against viral invasion [29]. Known core components of the RNAi pathway were found to be required for repressing transposable elements (TEs) in several eukaryotes:

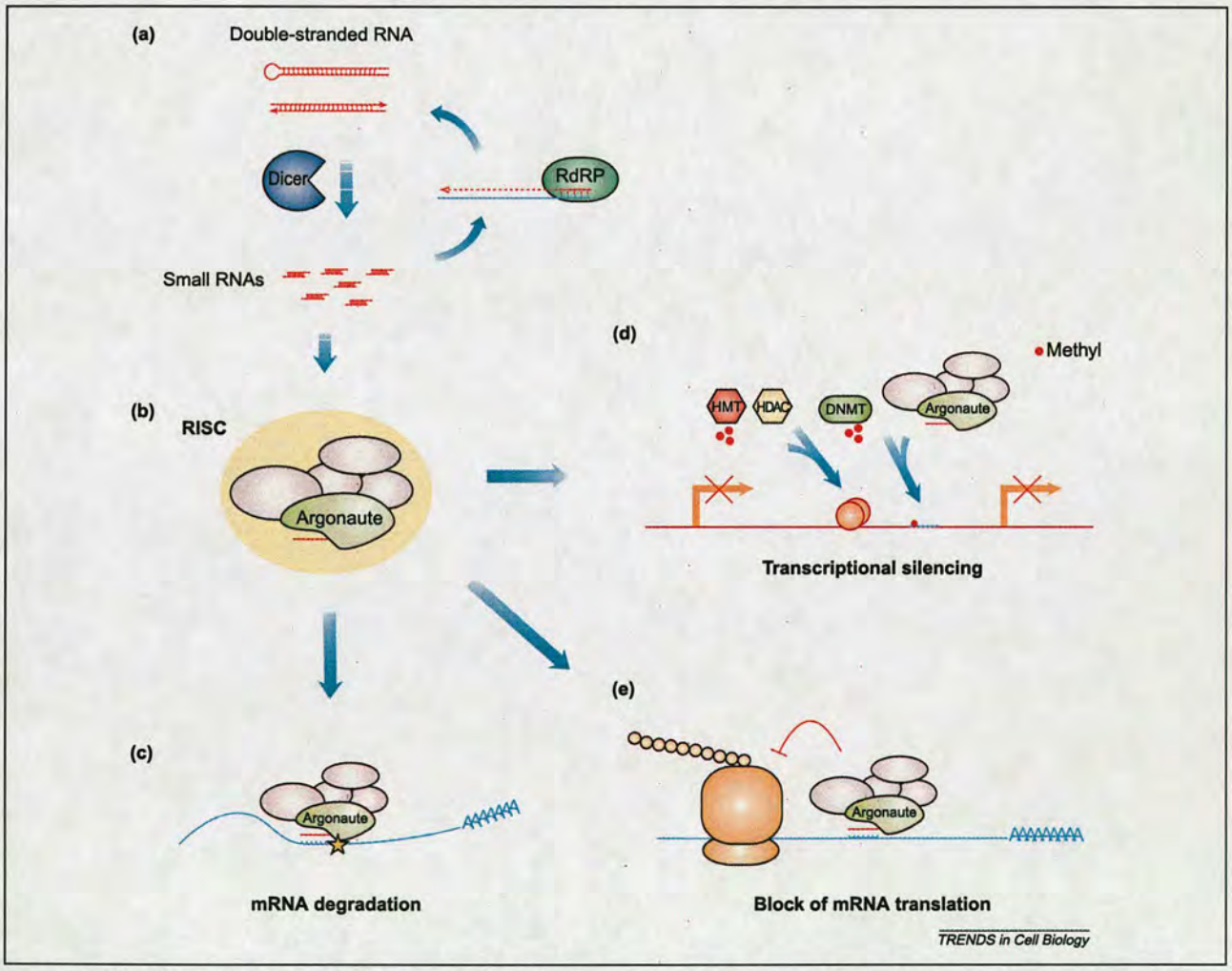

Figure 1. The different forms of RNA silencing. **(a)** Double-stranded RNA molecules derived from complementary transcripts or from a stem–loop structure are recognized by Dicer (in blue) and cleaved into small RNAs. The RdRP protein (in green) acts in a positive-feedback loop for the siRNA signal by producing complementary strands of the target RNA molecule, either by recognition of its 'aberrant' nature or by using small RNAs as primers [61], thus generating more homologous double-stranded RNA for Dicer processing. **(b)** The RISC complex, primed with a small RNA, can exert silencing in a variety of forms. In all cases, the small RNA confers target specificity, whereas the protein components within the RISC complex effect, or recruit mediators of, repression. **(c)** The conventional RNAi, PTGS or quelling pathway is depicted on the left, where the RISC complex associates with the target mRNA and employs the RISC 'slicing' activity of *Argonaute* protein to cleave the transcript [82,83] **(d)**. RISC can also induce transcriptional gene silencing (TGS) by using the siRNA specificity to direct silent chromatin modifications over homologous DNA loci. Target DNA (magenta line) and overlapping histones become methylated through the recruitment of DNA *de novo* methyltransferase (DNMT), histone deacetylase (HDAC) and histone methyltransferase (HMT) activities by a variant of the RISC complex, which can result in the shutdown of transcription. **(e)** A typical miRNA-loaded RISC does not affect mRNA turnover but binds to the 3′-UTR of the target transcript (blue line) and effectively blocks its translation by an unknown manner. It has been found recently that specific miRNAs can direct target mRNA cleavage and that an siRNA-loaded RISC can also block mRNA translation (see above), which suggests that it is the nature of the small RNA sequence, rather than the composition of RISC, that defines which process occurs [84,85].

C. elegans [30,31], *Chlamydomonas reinhardtii*. [32], *Drosophila melanogaster* [33] and now *Mus musculus* [34]. Since then, reports of small RNAs homologous to TE sequences have expanded to a larger variety of organisms [35–37], clearly implicating RNA silencing as both a conserved and widespread form of regulating transposon activity.

The term 'transposon' or 'transposable element' (TE) defines a selfish DNA entity capable of using a genome as an ecosystem where it can survive and proliferate. This definition can also be applied to a viral DNA sequence integrated in the host genome. TEs are powerful genome-destabilizing factors for a variety of reasons. Transposition events frequently induce positional mutations at the insertion and excision sites, and extensive TE activity favors recombination events that can lead to dramatic chromosomal rearrangements [38]. Although TEs are believed to contribute significantly to genome evolution, uncontrolled TE activity can be potentially detrimental to the fitness of the host [39,40]. Therefore, mechanisms that silence TEs have evolved to stabilize the genome.

Transposable elements and heterochromatin

In general, TEs and related DNA sequences are often found in chromatin domains that are transcriptionally silent and structurally distinct from the open euchromatic regions [41]. These heterochromatic regions have conspicuous features, which can include dense methylation of DNA (5-methylcytosine; 5-Me-C), hypo-acetylation of lysine residues in the N-terminal tails of histones H3 and H4 and methylation of specific lysine residues such as lysine 9 on histone H3 (H3K9me2/3). Some of these modifications create binding sites for particular proteins that, in general, promote transcriptional repression and the formation of silent chromatin or heterochromatin [42,43]. The packaging of TEs into heterochromatin represses their expression and blocks their ability to transpose. Hence, the assembly of TEs into this 'silent' chromatin is an effective way of inhibiting TE proliferation that has been employed by many eukaryotes. Because this form of regulation based on chromatin structure is independent of the primary DNA sequence, specialized mechanisms for recognizing these parasitic elements must be required to selectively trap them in heterochromatin. It is now evident that the formation of this heterochromatin is linked to the process of RNA silencing.

RNA silencing reaches chromatin

The same pathway that acts to repress genes posttranscriptionally can enforce modification of homologous chromatin in a way that alters its structure and consequently its function. Transcriptional gene silencing (TGS) (Figure 1d) was initially observed in plants and was associated with repression of exogenously introduced transgenes and viral suppression [44]. Remarkably, the presence of dsRNAs homologous to the promoter or the coding region in the DNA result in robust silencing that persists even after the trigger has been removed [45,46]. The TGS response triggered by double-stranded RNAs results in the complete transcriptional shutdown of a gene and is associated with *de novo* DNA methylation on the homologous DNA sequences.

TGS indeed appears to be employed to silence/inhibit the activity of several classes of TEs in plant genomes. Apart from the characteristic Dicer-like, *Argonaute* and small RNAs, the persistence of TE DNA methylation in *Arabidopsis thaliana* requires chromatin-modifying factors such as histone deacetylases, methyltransferases, DNA methyltransferases and *SWI2/SNF2*-related chromatin remodeling components – some of which are also required for the persistence of TE siRNAs [37] and for PTGS [47]. This underscores the intimate relationship between RNA silencing and chromatin regulation in plants and their role in repression of TEs [37,48,49].

Furthermore, it is becoming increasingly clear that TGS is a common form of general RNA silencing rather than a particular feature of RNA-mediated silencing in plants. Small RNAs are also known to direct chromatin modifications in other organisms. For instance, in the ciliate *Tetrahymena thermophila*, small RNAs are used to mark particular DNA sequences for elimination from the transcriptionally active macronucleus, most of which are of a repetitive nature [86,87]. In the fission yeast *Schizosaccharomyces pombe*, it has been clearly demonstrated that RNA silencing acts to facilitate chromatin modifications over repetitive sequences for the purpose of TE silencing, as in plants, but also impacts upon basic chromosomal functions [15,50,51].

Chromosomal function – the fission yeast centromere

In fission yeast, silent chromatin assembled over the outer repeat arrays at the centromeres is required for proper chromosome segregation during mitosis. The high density of cohesin complexes associated with this silent chromatin ensures that sister chromatids are held tightly together at centromeres after DNA replication and up until the onset of anaphase [52,53]. RNA silencing must play a direct role in this process in fission yeast as deletion of any gene encoding key RNAi components leads to defects in chromosome segregation. In fact, RNAi effector proteins are required to establish and maintain this pericentromeric heterochromatin and thus prevent premature sister-chromatid separation [15]. In addition, RNAi also acts to initiate a similar form of silencing at the mating-type locus in *S. pombe* [54]. It is thought that transcription from both strands of the outer repeats at the centromeres (*dg-dh/K-L*) and the related *cenH* element from the mating-type locus results in homologous dsRNA that then enters the RNA silencing pathway, resulting in the production of complementary small RNAs. Incorporation of these small RNAs into a variant of the RISC complex called RITS (RNA-induced transcriptional silencing complex), containing Ago1 (*Argonaute*), Chp1 (chromodomain protein) and Tas3, directs H3K9me2 methylation over homologous chromatin [55] (Figure 2). This requires an RdRP, the action of histone deacetylases and the histone methyltransferase Clr4 (SET domain protein, related to the mammalian *Suv39*) that forms a binding site for the HP1 (heterochromatin protein 1) ortholog Swi6 and Chp1 [42]. In turn, binding to H3K9me2 of Swi6 and Chp1 promotes spreading of the silenced chromatin state as well

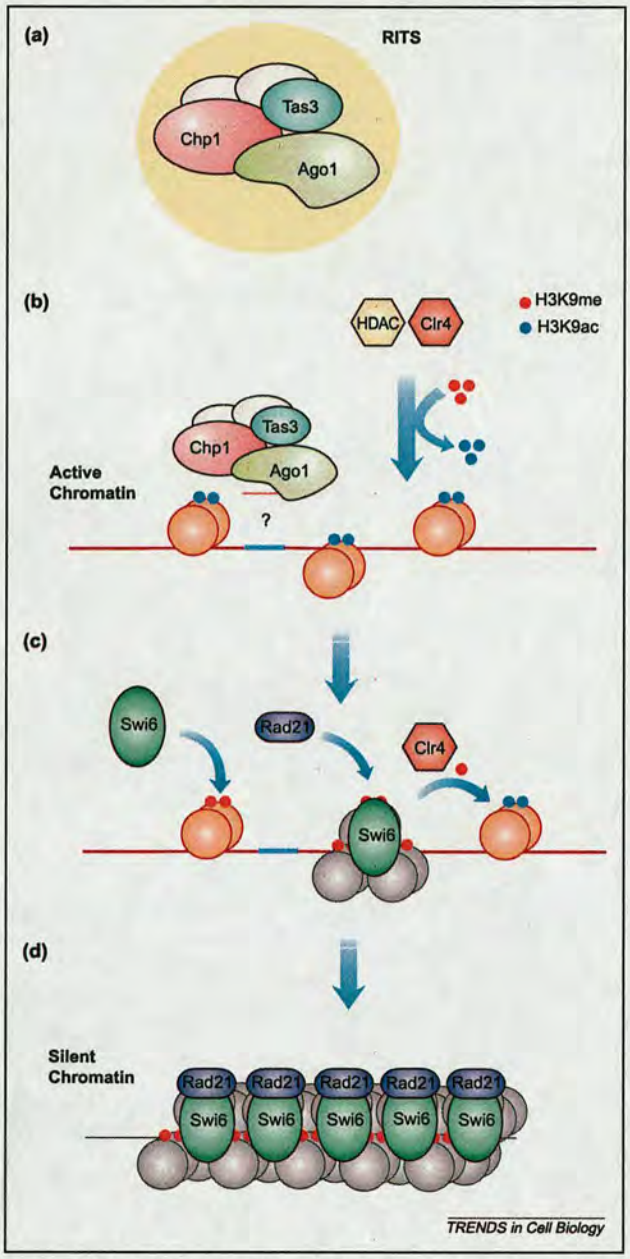

Figure 2. RNA-mediated heterochromatin formation in fission yeast. **(a)** The RISC-variant RITS complex with its known components: the *Argonaute*/PPD protein Ago1, the chromodomain protein Chp1 and Tas3. **(b)** RITS attracts Clr4 and an unknown HDAC to deacetylate and methylate histone H3-K9 over target DNA (blue line). It is still unclear whether the target recognition process involves RNA–DNA interactions between the small RNA and the target DNA or RNA–RNA interactions with a nascent transcript. **(c)** Nucleosomes bearing H3K9me are preferentially bound by Swi6/HP1, which promotes the recruitment of heterochromatin proteins, such as the cohesin subunit Rad21, and allows spreading of the heterochromatin domain to upstream and downstream regions. **(d)** This results in the assembly of a patch of heterochromatin that is rich in Swi6/HP1 and Rad21, as well as being transcriptionally silent.

as allowing the recruitment of the Rad21 cohesin and physical cohesion [52].

Expression of a synthetic hairpin RNA producing dsRNA (a conventional RNAi inducer in many systems) taps into this mechanism to promote silencing by directing histone H3 K9 methylation and recruitment of Swi6 and cohesin over a normally expressed euchromatic locus [50]. This demonstrates that the generation of siRNAs from a dsRNA precursor is sufficient to target chromatin modification to a homologous locus and also indicates that the primary DNA sequence does not play a role in specificity. Thus, the process of RNA-directed transcriptional gene silencing provides DNA targeting properties that facilitate the placement of histone modifications at specific loci for the purpose of TE repression in plants and fungi.

Repeats attract RNA silencing

To grasp the biological relevance of RNA-directed chromatin modifications, it is important to investigate the nature of the DNA sequences that generate the endogenous siRNAs that influence chromatin structure. To date, all natural targets for RNAi-mediated heterochromatin formation appear to involve TEs or repetitive DNA. This suggests that RNA silencing recognizes an intrinsic property common to these sequences in the context of centromeric function or transposon/viral control. But what could this defining characteristic be?

It has been suggested that *S. pombe* outer centromeric repeats, as well as the satellite sequences found around metazoan centromeres, resemble or are derived from TE sequences. Some centromeric repeats are bound by CENP-B proteins, which bear close resemblance to transposases encoded by the pogo superfamily of TEs [56]. Moreover, regions in the terminal inverted repeat (TIR) of the *Tigger* TE match almost perfectly the DNA binding motif recognized by CENP-B in human centromeric α-satellite repeats [56]. The implication is that perhaps all the currently known targets for RNAi-mediated heterochromatin formation are derived from TEs. Thus, in *S. pombe*, RNA silencing might be directed towards TE-derived repetitive DNA sequences by default. In this case, it appears that the cell has exploited a natural form of repeat silencing based on genome defence mechanisms (inhibition of transposition) to promote gene silencing. This now acts to ensure that specific chromatin structures are assembled over the outer repeat regions (flanking the kinetochore) at centromeres and the related sequences at the mating-type locus, which are now important for centromere-specific cohesion and the regulation of cell mating type.

But what triggers an RNA silencing response against such sequences? As dsRNA is the general substrate for the canonical RNAi pathway, it seems likely that a dsRNA is responsible for triggering RNA-mediated heterochromatin formation. Invariably, transcriptional activity is coupled to the transposition cycle of most TEs. Even isolated TE-derived repeats, such as solo LTRs, can remain transcriptionally active [57]. Since transcription alone is not sufficient to render such elements as targets, some process must generate a dsRNA substrate. Intuitively, two transcription events on opposite strands converging on any given sequence could generate complementary transcripts that would combine and form dsRNA (as used in various organisms to direct knockdown of gene expression: Figure 3a). Alternatively, complementary strands could be transcribed from different copies residing at distinct locations in the genome and subsequent

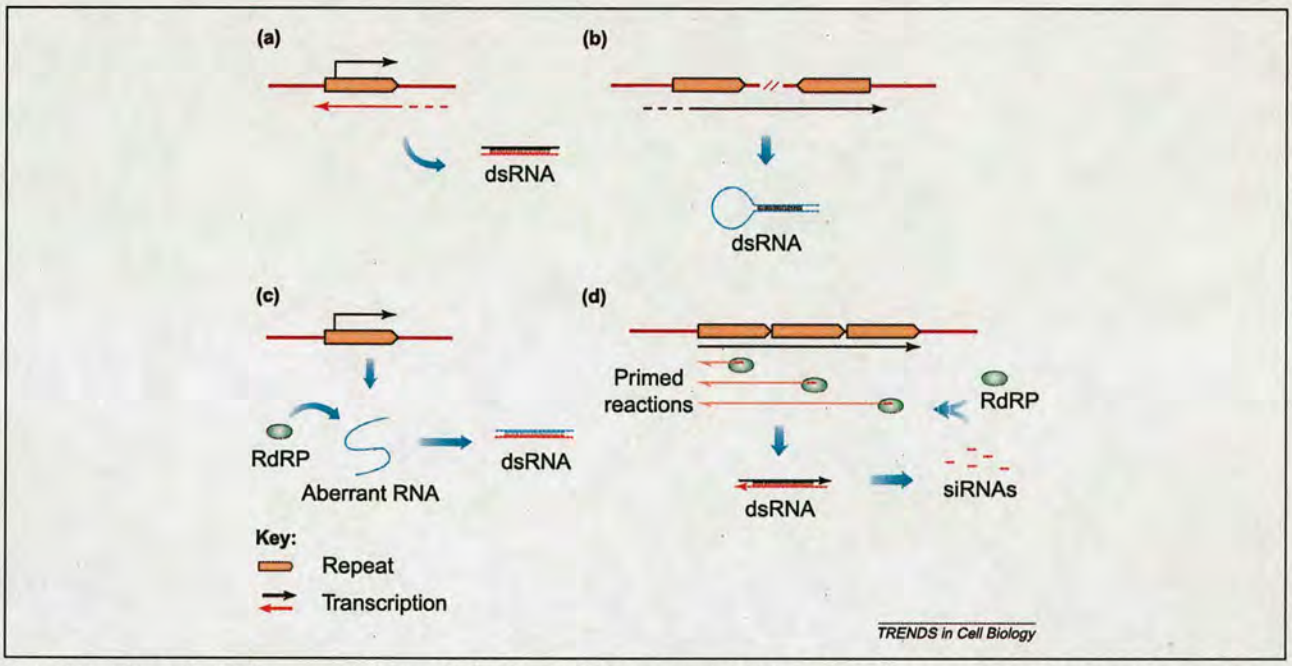

Figure 3. Possible ways by which transposable elements (TEs) and repeat dsRNA are generated. **(a)** Transcription of a repeat or TE (orange 'arrow box'), either from an internal promoter (black arrow) or leaking/originating from flanking sequences (red arrow), that occur in both sense and antisense strands can generate dsRNA. **(b)** Transcription through an inverted repeat disposition of repeats, such as the TIR repeats within DNA transposons, could give rise to an RNA molecule (in blue) that snaps back and adopts a stem–loop or hairpin structure, exposing segments of dsRNA. **(c)** RdRP recognizes the 'aberrant' nature of a TE/repeat transcript and uses it as a template to generate the complementary strand. **(d)** Transcription through a tandem array of repeats produces a transcript that bears multiple sites for production of complementary strands (red arrows) by RdRP (green) using repeat-specific siRNAs (in red) as primers. Extension of the complementary strand over multiple repeats generates a long dsRNA molecule that can be cleaved into higher numbers of repeat-specific siRNAs than in the case of an isolated repeat.

hybridization would allow the formation of a dsRNA substrate. Another simple way of obtaining dsRNA is by transcribing an inverted repeat, which produces a transcript that can snap back and form a stem–loop or hairpin structure (as with endogenous microRNA precursors: Figure 3b). This has been suggested as one source of dsRNA specific for *C. elegans* Tc1/Mariner TEs, which bear terminal inverted repeats in their structure [58]. Although siRNAs against Tc1/*Mariner* TIR repeats appear to be more abundant, transcripts from both strands of these TEs are produced, in the same way as has been observed for centromeric repeats and also interspersed LTRs in *S. pombe* [15,50,58]. The presence of siRNAs specific to most regions within these TEs in *C. elegans* suggests that full-length TE dsRNAs contribute to the induction of RNAi against these TEs. The origin of the convergent, read-through and/or complementary transcription events involved in TE dsRNA formation is obscure. They might arise from the activity of promoter sequences within the repeats, but transcriptional 'leakage' from flanking genes and from flanking cryptic promoters could contribute to the production of a TE homologous dsRNA pool.

An alternative explanation is that RdRP can in some way recognize transcripts coming from TEs or viral sources as 'aberrant' or 'foreign' transcripts and use them as templates to generate dsRNA [12,59,60] (Figure 3c). This idea is supported by the observation that the RdRP can produce dsRNA *in vitro* from a ssRNA template in a primer-independent manner [61]. More recent work suggests that transcripts lacking a 5′ cap are targeted by RdRP, although it is still possible that RdRP is attracted to other characteristics, such as premature termination or absence of polyadenylation, or a combination of features [62]. In the case of tandem repeat arrays, such as those commonly associated with pericentromeric regions, it has been suggested that this arrangement results in the production of transcripts that serve as more efficient RdRP substrates, thus ensuring the stability of the assembled heterochromatin over these regions [63] (Figure 3d). However, RdRP-independent strategies have presumably arisen in flies and vertebrates to maintain TEs and repeats under the influence of RNA silencing.

RNA-induced chromatin silencing in metazoans

SiRNAs act to target histone and/or DNA modifications to homologous sequences in plants, ciliates and fission yeast. But do noncoding RNAs play a pivotal role in gene silencing and chromatin modifications in metazoans? Clearly X-inactivation in female mammals requires expression of *Xist* RNA in *cis* to effect chromatin modifications that result in gene silencing [64]. In addition, imprinting of paternally derived *Igf2r* requires expression of the associated *Air* noncoding RNA [65]. Likewise, chromosomal rearrangements that result in antisense transcription of the gene encoding α-globin lead to DNA methylation of its promoter region and transcriptional silencing [66]. However, there is no evidence linking these phenomena to the process of RNAi. Nevertheless, several recent reports imply that the RNA pathway can mediate both chromatin modifications and gene silencing in metazoans.

As mentioned above, the placement of a gene close to domains of constitutive heterochromatin such as those residing at pericentromeric regions leads to variable expression (PEV). Unstable repression is thought to be due to the stochastic dynamics associated with heterochromatin assembly along chromatin fibres. This classic epigenetic effect can be imitated in euchromatic regions in fruit flies by arrays of a reporter gene such as *mini-white*, which also display variable expression. The RNAi/PTGS pathway affects the formation of silent chromatin over these arrays since the *piwi*, *aubergine* (both *Argonaute* homologs) and *spindle-E* (*homeless*: an RNA helicase) mutations alleviate their silencing [67]. The most likely explanation is that siRNA derived from *mini-white* array-generated dsRNA directs the assembly of heterochromatin over the *mini-white* sequences. It is not known whether these same mutations can alleviate silencing of a marker embedded in centromeric heterochromatin, but they do result in loss of H3K9me2/3 and in the redistribution of HP1 from centromeric regions.

A link between RNAi and TE silencing is also evident in *Drosophila* as siRNAs homologous to TE, satellite and microsatellite DNA have been detected [35]. While it is not known if these small RNAs exert repression at a transcriptional level, it is clear that their cognate sequences are normally associated with heterochromatin and are subject to RNA silencing in *D. melanogaster*.

A recent study suggests that RNA silencing is also involved in sister-chromatid cohesion in vertebrates, similar to what is observed in *S. pombe*. A chicken DT40 cell line containing human chromosome 21 was engineered creating a conditional allele allowing Dicer (and thus the RNAi pathway) to be turned off [68]. Cells depleted of Dicer displayed a mitotic phenotype, with disrupted HP1 and Rad21 localization, premature sister-chromatid separation and chromosome mis-segregation [51]. This implies that RNA silencing is also involved in the formation of pericentric heterochromatin in vertebrate cells and that this acts as a platform to promote efficient cohesion at centromeres.

A more direct test of the link between RNAi and chromatin modification in metazoans has come from the application of siRNAs to human cell lines. One study demonstrated that siRNAs homologous to the promoter of an integrated GFP reporter construct can induce transcriptional silencing of the gene encoding GFP [69]. Cytosine methylation at one site within the EF1A promoter was shown to increase after transfection of the homologous siRNAs. The effect was reversed by treatment with inhibitors of DNA methylation and histone deacetylation. A more comprehensive study conducted by Kawasaki *et al.* [70] underscored the ability of siRNAs to induce DNA and chromatin modifications in human MCF7 and mammary epithelial cells. Both transfection of siRNAs or expression of hairpin precursor RNAs homologous to the promoters of either the *E-cadherin* or *erbB2* genes resulted in effective gene silencing accompanied by DNA methylation and histone H3 K9 methylation.

To recap, chromatin modifications can be directed by small RNAs in fungi, plants and metazoans. The process involves components of the RNAi machinery that appear to be utilized to provide sequence specificity by homing in on targets bearing homology to siRNAs carried by the RNAi effector complex. This is related to the process that acts on transcripts derived from outer centromeric repeats in fission yeast and appears to be a conserved mechanism that acts at centromeric regions in vertebrates to ensure tight physical cohesion and normal chromosome segregation.

Transposable elements and repeats can influence gene regulation

The action of RNA silencing on centromeric repeat transcripts is important in defining structures and functions associated with these chromosomal regions. However, a large proportion of repetitive sequences are not concentrated in pericentromeric regions but are scattered throughout the genome. TE insertions are known to have dramatic effects on expression levels of surrounding genes by disturbing the transcriptional activity of the affected regions. Moreover, it now seems likely that observed changes of gene expression associated with TEs could result from transposon silencing events involving the formation of silent chromatin on such elements [71]. In light of this, it is interesting to re-evaluate the action of RNA silencing and TEs in terms of their consequences for gene activity.

A clear demonstration of transposon silencing affecting gene expression comes from the analyses of retrotransposons containing long terminal repeats (LTRs) in *S. pombe*. Most of the ~300 Tf1/2 LTRs are dispersed along chromosome arms as solo elements in various states of decay. Only a few (26) remain associated with full-length retrotransposons [72]. In one case, repression of a few nearby meiotically regulated genes during vegetative growth was shown to be connected to RNA silencing by LTR sequences [50]. The mechanism of this repression has not been completely unravelled, but LTRs are subjected to RNA-mediated chromatin silencing, resulting in H3K9me2 methylation and Swi6 association. One possibility is that binding of Swi6/HP1 to H3K9me2 promotes recruitment of additional Swi6/HP1 and chromatin modification factors to surrounding histones, which stabilizes the silent chromatin domain and also allows it to expand laterally and engulf neighboring genes [73–75]. Indeed, Swi6 was found to be required for repression of these nearby meiotic genes, suggesting that the LTR acts as a nucleation site from which silent chromatin spreads out and represses nearby genes, in the same way that it can spread from a region of a gene targeted by artificially induced siRNAs [50].

In plants, H3K9me and DNA methylation seem to be largely confined to transposon sequences or promoters of silenced genes and do not in general engulf neighbouring genes [49,63]. However, repression of a few genes in *Arabidopsis* that harbor insertions of repetitive sequences was found to be dependent on *DDM1* (a *SWI2/SNF2* chromatin remodeling factor required for maintenance of DNA methylation and H3K9me over TE sequences) [76].

Repeats and silent chromatin modifications are intimately linked in mammalian somatic cells – tandemly repeated satellite DNA as well as mobile genetic elements

and their DNA remnants are characterized by extensive histone deacetylation, H3K9, H3K27 and H4K20 methylation as well as 5-Me-C DNA methylation [77,78]. RNA-mediated silencing is fully active during early stages of embryonic development and cellular differentiation but inactive during the later stages of development and in the soma. Thus, it remains active during the stages where epigenetic reprogramming processes occur, before the establishment of cell fate [79]. The presence of Dicer is crucial for mouse embryonic viability, but it is also involved in repressing LTR-retrotransposons in mouse pre-implantation embryos [34,80]. Recent investigations of chromatin status over repetitive elements in the mouse genome have revealed that the modifications associated with TEs and related interspersed LTRs display a dynamic behaviour throughout differentiation stages of embryonic stem cells [78]. This is in contrast to the relatively stable H3K9me3 and H4K20me3 modifications associated with pericentromeric repeats. Given that RNA-dependent heterochromatin assembly appears to occur in vertebrates, it is seems quite likely that RNA silencing plays a key role in establishing transcriptional repression of these sequences upon determination of cell fate [81]. However, it is not clear whether transcriptional repression of these sequences plays a role in the process of cellular differentiation.

Concluding remarks

Noncoding RNA is the central player of an ancient and conserved form of silencing. Although the different forms of RNA silencing were initially unearthed as seemingly distinct phenomena, basic machinery is held in common between PTGS, TGS, *quelling* and RNAi. In addition, these same components are conserved in a large variety of organisms and thus must have arisen early in eukaryotic evolution. Since its discovery several years ago, the biological relevance of RNA-directed silencing mechanisms is rapidly becoming clear, and it is already evident in distinct processes such as chromosomal structure, genome defence and gene regulation. Despite the large body of information available on RNA silencing pathways, important questions still remain unanswered. Issues such as the total number of endogenous targets of siRNAs and microRNAs in the genome or the amount of crosstalk between the different manifestations of RNA silencing are currently being addressed and might yet reveal further surprises.

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so in the wild. The cognitive demands for inventing such traditions thus appear to be easily met, not only by chimpanzees but also by other great apes. If inventions occur easily, a high rate of invention could in principle contribute to making the distribution of traditions disjunct.

In practice, however, there is a problem with this explanation. Chimpanzees are an old species: they closely resemble bonobos, a morphologically derived sister species that split off at least 1 million years ago [12]. If chimpanzees have been inventing and passing on traditions even for as short a period as 1 million years, the distribution of traditions would be limited by the rate of invention only if the rate of invention were vanishingly low — much less than 1 in every 10,000 years for example. The fact that chimpanzees have invented traditions while being observed by humans suggests that every population should have had ample opportunity to acquire it. So the rate of repeat invention appears too high to account for the distribution of a series of idiosyncratic sets of chimpanzee traditions.

If invention alone cannot explain why the unpredictable location of traditions, we are forced to think about a little-studied topic: extinction. The obvious explanation for why Kibale chimpanzees do not dip for ants, Gombe chimpanzees do not hand-clasp-groom, or Bossou chimpanzees do not use leaf-napkins is that, although their ancestors did, the tradition died out. Why extinctions should happen regularly is unclear. Long-term studies will be needed to test how population bottlenecks, alternative fashions, individual personalities or other factors might promote rates of tradition extinction. Understanding the extinction of chimpanzee traditions holds promise for explaining why ape culture has never blossomed as it did, critically, for humans.

Unfortunately the opportunities for studying apes are disappearing rapidly due to extinction not just of traditions, but of whole populations. But on the positive side, Ebo nut-smashing is only one of many recent tool-using discoveries that in the 21st century include chimpanzee tool-kits in the Congo and the first gorilla tools in the wild, as well as capuchin monkey stone-tool-use in Brazil [13–15]. There is still an opportunity to learn much about the distribution of cultural variants, let alone why they are vulnerable to extinction.

Happily, as Morgan and Abwe [7] hint, the process of studying populations like Ebo often leads to the establishment of a long-term research program, one of the most effective ways to promote conservation. Their discovery thus promises to benefit both science and conservation. If the new tradition proves idiosyncratic Ebo will become a site of particular interest but whatever is found there, the big picture is clear: the cultural primatology of central Africa is still in its infancy.

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Molecular Biology: Silencing Unlimited

Heterochromatin domains are essential for normal chromosome functions. The Eri1 ribonuclease is a negative regulator of the RNA interference machinery; recent studies have shown that, in fission yeast lacking Eri1, heterochromatin formation is more promiscuous.

Ricardo Almeida, Alessia Buscaino and Robin C. Allshire

Heterochromatin is the portion of nuclear chromatin that maintains a condensed state during the cell cycle and that provides specific functions at various chromosomal locations, such as centromeres and telomeres. In the fission yeast, *Schizosaccharomyces pombe*, heterochromatin is formed at distinct chromosomal regions: centromeres, the mating type locus, telomeres and ribosomal

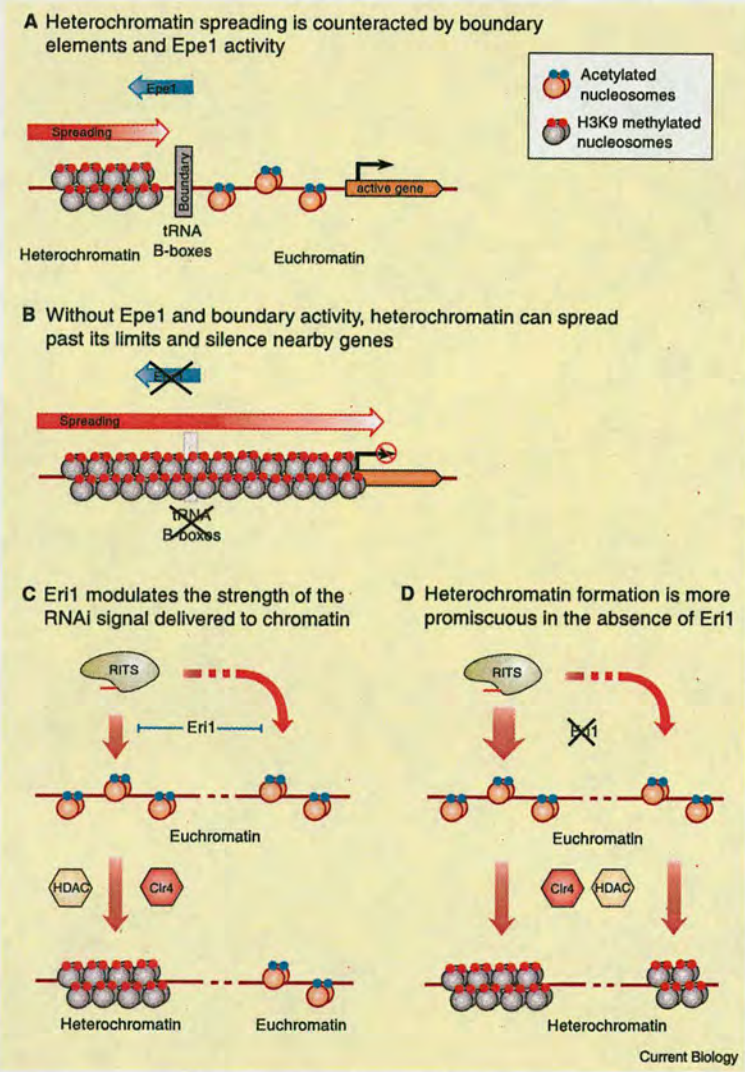

Figure 1. Heterochromatin formation and spreading are tightly controlled in fission yeast.

(A) Heterochromatin spreading (red arrow) is antagonized by two different known processes: boundaries (grey box) containing either tRNA or B-box motifs; the 'anti-silencer' factor Epe1 (blue arrow). (B) In the absence of these two processes, heterochromatin is allowed to expand past its normal limits and repress genes in euchromatin (in orange). (C) RNA interference is involved in determining the sites of heterochromatin formation (nucleation). The RITS complex (in green) containing siRNAs (in red) recognizes a target locus and induces deacetylation and H3K9 methylation by HDAC and Clr4 (hexagons). Eri1 antagonizes RNAi activity and limits its ability to nucleate. (D) In the absence of Eri1, the RNAi pathway is more active and can induce heterochromatin formation in chromosomal loci which are not typically engulfed in this structure (on the right).

(r)DNA arrays. A common feature is that these regions are all composed of repetitive sequences which may facilitate their assembly into transcriptionally silent chromatin. Several histone modifications and histone binding proteins are required to maintain the silent state: the nucleosomes are typically underacetylated and methylated on lysine 9 of histone H3 (H3K9me), which creates a binding site for chromo-domain proteins such as Swi6 (HP1).

An elegant series of studies has shown that the heterochromatic repeats are transcribed by RNA polymerase II and that these transcripts themselves are processed by Dicer, a component of the RNA interference (RNAi) machinery, into short-interfering (si)RNAs. The production of siRNAs is essential for targeting the 'RNA-induced initiation of transcriptional gene silencing' (RITS) complex — composed of Ago1, Chp1 and Tas3 — to heterochromatin repeats. This in turn leads to the recruitment of the histone methyltransferase Clr4. The consequent methylation of H3 on lysine 9 by Clr4 allows binding of the chromo-domain proteins Swi6 and Chp1, forming a nucleation site from which heterochromatin can spread outwards along the chromatin fibre [1].

Cells need to restrict heterochromatin to specific domains in order to avoid repression of essential genes. But how is the silencing machinery targeted solely to specific loci? And how is heterochromatin contained and prevented from spreading into other regions of the genome? One possibility is that components of heterochromatin recognize and bind specific sequences within the heterochromatic domains that are absent in euchromatin. However, this does not explain how a euchromatic marker gene is silenced when it is placed inside a block of heterochromatin. Another possibility is that specific boundary elements are located at the borders between heterochromatin and euchromatin. These elements might act as buffers to impede the spreading of heterochromatin to neighbouring chromatin. A third possibility is that specific factors act as 'anti-silencers'. Such proteins might antagonize RNAi-mediated heterochromatin assembly at particular steps in the pathway. The balance between 'silencer' and 'anti-silencer' activities might ensure the normal distribution of heterochromatin and euchromatin domains. Perturbations could enhance or reduce the formation of silent chromatin.

Several recent papers [2–5] report evidence that these last two mechanisms operate in

S. pombe. Two of the new studies [2,3] demonstrate the existence of chromatin boundaries surrounding heterochromatic loci in fission yeast. Transfer (t)RNA genes and B-box motifs were found to be functional components of these boundary elements and their activity prevents heterochromatin marks from oozing out into surrounding domains (Figure 1A,B). Other analyses suggest that, in addition to boundary elements, 'anti-silencer' factors play an important role in the negative regulation of heterochromatin. In particular, it has been shown that Epe1, a JmjC domain protein, counteracts repressive chromatin by facilitating the recruitment of RNA polymerase II to heterochromatic loci via Swi6 (Figure 1A,B) [4].

As reported recently in *Current Biology*, Iida *et al.* [5] have shown that the fission yeast orthologue of the *Caenorhabditis elegans* gene *Enhancer of RNA Interference 1* (*eri1*) has 'anti-silencer' activity. The worm protein ERI-1 was initially shown to be a nuclease that can degrade siRNAs *in vitro*, and it was suggested that it might diminish the pool of active siRNAs in the cells [6]. Consistent with this, worms lacking ERI-1 display enhanced RNAi silencing [6]. Iida *et al.* [5] showed that *S. pombe* Eri1 binds to and degrades double-stranded RNA *in vitro*. Mutation of Eri1's catalytic domain leads to increased levels of centromeric siRNAs which are associated with RITS complexes. Although cells lacking Eri1 display no change in the levels of silent chromatin modification over centromeric repeats, H3K9 methylation and the silencing of marker genes inserted in these repeats are noticeably increased, suggesting that the formation of heterochromatin via RNAi is enhanced on marker genes [5].

This role for Eri1 in opposing silencing is reinforced by another recent study [7] in which the Tas3 component of RITS was artificially tethered to *ura4* mRNA. The *ura4*$^+$ gene of fission yeast is located in euchromatin and is normally constitutively expressed. However, coercing the recruitment of RITS to the *ura4* transcript resulted in silencing of *ura4* expression in a manner that is dependent on RNAi, H3K9 methylation and Swi6. Thus, diverting the RNAi machinery to a transcript can trigger siRNA synthesis, silencing and heterochromatin formation on homologous chromatin.

Surprisingly, despite the fact that siRNA homologous to the *ura4* transcript are found within the RITS effector complex, a second copy or *ura4*$^+$ at a distinct location in the genome is not silenced unless the Eri1 nuclease is also absent (Figure 1C,D). In most systems the RNAi machinery homes in on target RNAs by complementarity with the siRNAs borne by the RISC effector complex. Yet it seems that in fission yeast RNAi is constrained, so that unlike in other organisms it is unable to silence identical sequences in the genome. The reasons for, and mechanism of, this restricted form of RNAi are unknown but it is clear that Eri1 contributes to it [7].

But how does Eri1 exert its negative influence on RNAi? It has been suggested that Eri1 might degrade siRNAs or the endogenous non-coding transcripts involved in triggering RNAi. A different scenario is supported by two recent publications [8,9] that show that Eri1 is required for RNAi activity against several endogenous somatic genes in *C. elegans*. These observations suggest that Eri1's negative effect on RNAi may be a consequence of competition for resources of this pathway, as Eri1 stimulates siRNA production against those genes, which in turn diminishes the intensity of RNAi response to other stimuli [8,9]. Applying the same reasoning to fission yeast, it is possible that RNAi might have another unknown regulatory role in which Eri1 is a central player. Indeed Iida *et al.* [5] observed that overexpressing Eri1 is toxic to the cells, a fact that cannot be simply explained by the loss of heterochromatin or RNAi, as none of these functions is essential in this organism. Although the authors suggest that toxicity may be due to this unspecific nuclease activity affecting the stability of other cellular RNAs, high Eri1 levels might instead cause excessive degradation of specific target RNAs, which in turn would compromise cell growth.

Taken together these new reports suggest that different, parallel mechanisms restrict heterochromatin to specific domains. Heterochromatin is not essential in fission yeast but we expect that an excess of it might be deleterious to the cell. Surprisingly, loss of Eri1 or Epe1 has no apparent affect on cell viability [3,4]. In a way, this could mean that the 'anti-silencers' are stemming a trickle rather than a flood — the cell's capacity to assemble more heterochromatin may well be limited due to low levels of key proteins such as Swi6. On the other hand, the cell may possess other undiscovered 'anti-silencers' that act redundantly with Eri1. All should fall in place once it is clarified how the different anti-silencers, such as Eri1 and Epe1, together with boundary elements negatively regulate heterochromatin formation and whether they cooperate with each other.

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Ocean Ecology: Don't Fence Me in

New research that combines ocean circulation and genetic models to predict population structure of corals will help conservation efforts in tropical reef ecosystems.

Simon R. Thorrold

The precarious future of coral reefs throughout the world's tropical oceans has generated unprecedented interest in the use of marine protected areas (MPAs) to conserve these unique habitats [1,2]. Occasionally it is possible to conserve an entire ecosystem, as has recently been proposed for the Northwestern Hawaiian Islands, but more commonly a number of smaller areas are designated for varying levels of protection. But which areas should be designated for MPAs, and where should fishing or other extractive activities be allowed? Satisfying answers to this question has flummoxed marine ecologists because it depends critically upon some knowledge of dispersal distances (connectivity) in populations of reef organisms (Figure 1). As they report in this issue of *Current Biology*, Galindo, Olson and Palumbi [3] used an oceanographic model to generate a larval connectivity matrix among almost 100 reef sites in the Caribbean region. The matrix was then used to estimate gene flow among the locations in a simple genetic model that incorporated life history characteristics of reef-building coral. Model predictions matched well with empirical data on genetic variation in Caribbean corals, suggesting that the ocean circulation model provides a reasonable facsimile of realized larval dispersal.

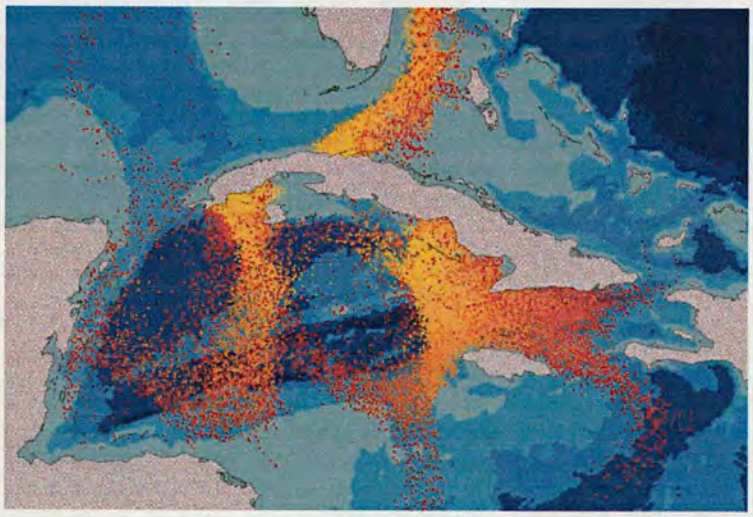

Figure 1. Ocean circulation models are being used increasingly to determine population connectivity during the pelagic larval phase of marine fish and invertebrates.
The models allow for visualization of complex dispersal patterns — in this instance the distribution of early stage virtual larvae (yellow dots) and 30-day old late stage virtual larvae (red dots) released from historical spawning sites of lane snapper (*Lutjanus synagris*) around Cuba. (Image courtesy of Claire Paris, University of Miami.)

Biodiversity in the ocean realm, as in terrestrial environs, is generated and maintained by barriers to dispersal. But while it is intuitively obvious that mountain ranges act to constrict animal movements on land, physical barriers in the ocean are much more difficult for humans to discern. A further complication arises because dispersal of most coral reef fish and invertebrates occurs primarily during a relatively short pelagic larval phase. Once pelagic, larvae are subject to diffusion, turbulence and advection in oceanic water masses that can potentially lead to dispersal of hundreds of kilometers [4]. But it has proved extremely difficult to either measure the frequency with which long distance movements during the larval phase occur, or alternatively to identify dispersal barriers that may act to isolate populations over ecological or evolutionary time. Data on ecological connectivity is critical, however, for spatial management of fisheries and the control of invasive species, while gene flow over evolutionary time scales will determine genetic structure and patterns of biodiversity in marine ecosystems.

Marine invertebrate and fish larvae are notoriously difficult to track in the field because they are invariably tiny and are quickly diluted in vast volumes of water [5]. Instead, Galindo *et al.* [3] tackled the problem by following particles — 'virtual larvae' — in Caribbean Sea currents derived from the Miami Isopycnal Coordinate Ocean Model (MICOM). Particles were deemed

so in the wild. The cognitive demands for inventing such traditions thus appear to be easily met, not only by chimpanzees but also by other great apes. If inventions occur easily, a high rate of invention could in principle contribute to making the distribution of traditions disjunct.

In practice, however, there is a problem with this explanation. Chimpanzees are an old species: they closely resemble bonobos, a morphologically derived sister species that split off at least 1 million years ago [12]. If chimpanzees have been inventing and passing on traditions even for as short a period as 1 million years, the distribution of traditions would be limited by the rate of invention only if the rate of invention were vanishingly low — much less than 1 in every 10,000 years for example. The fact that chimpanzees have invented traditions while being observed by humans suggests that every population should have had ample opportunity to acquire it. So the rate of repeat invention appears too high to account for the distribution of a series of idiosyncratic sets of chimpanzee traditions.

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