Silent chromatin at the middle and ends: lessons from yeasts

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Eukaryotic centromeres and telomeres are specialized chromosomal regions that share one common characteristic: their underlying DNA sequences are assembled into heritably repressed chromatin. Silent chromatin in budding and fission yeast is composed of fundamentally divergent proteins that assemble very different chromatin structures. However, the ultimate behaviour of silent chromatin and the pathways that assemble it seem strikingly similar among Saccharomyces cerevisiae (S. cerevisiae), Schizosaccharomyces pombe (S. pombe) and other eukaryotes. Thus, studies in both yeasts have been instrumental in dissecting the mechanisms that establish and maintain silent chromatin in eukaryotes, contributing substantially to our understanding of epigenetic processes. In this review, we discuss current models for the generation of heterochromatic domains at centromeres and telomeres in the two yeast species.

Introduction

Centromeres

The centromere is essential for proper segregation of chromosomes in mitosis and meiosis and is therefore of vital importance for genetic stability. It is the DNA region in which the kinetochore is formed, a structure that allows chromosomes to associate with spindle microtubules. Centromere function and its many associated proteins are conserved, yet centromere specification is not always hard-wired to the DNA sequence and displays dramatic plasticity (reviewed in Sullivan et al., 2001; Allshire and Karpen, 2008). Centromeres can have different structures depending on their size, the number of kinetochore microtubules they interact with and whether or not they are surrounded by pericentric heterochromatin.

Both Schizosaccharomyces pombe (S. pombe) and Saccharomyces cerevisiae (S. cerevisiae) are monocentric eukaryotes with localized centromeres, in contrast to holocentric organisms such as Caenorhabditis elegans, in which kinetochores form along the entire chromosome. A conserved feature of all centromeres is the special histone H3 variant, called Cnp1 in S. pombe and Cse4 in S. cerevisiae, which is found exclusively within the core centromeric region (Smith, 2002). In most other aspects, budding and fission yeast centromeres are quite different. In S. cerevisiae, complete centromere function is specified by only 125 bp of DNA comprising three distinct centromeric DNA elements (CDE I, II and III). The 15 bp of CDE III is most important as it attracts a complex containing sequence-specific DNA-binding proteins (Ndc10, Cep3, Ctf13 and Skp1). This complex dictates the assembly of the single Cse4-containing nucleosome, which spans the middle AT-rich CDEII element (Meluh et al., 1998; Furuyama and Biggins, 2007). Directly analogous elements are absent in S. pombe. Rather, centromere structure comprises a central core domain (cnt) bearing Cnp1 nucleosomes surrounded by a long inverted repeat. Each centromeric flank can be divided into two regions: the inner repeats (imr), which are specific to each of the three centromeres, and the outer repeats (otr), which are composed of elements known as dg and dh (Bjerling and Ekdall, 2002). The arrangement of dg and dh repeats with respect to each other and to the central core differs at each of the three fission yeast centromeres. Notably, the otr regions in S. pombe are assembled into silent heterochromatin, which is important for proper centromere function (see also accompanying Focus Review by Torras-Llort et al.).

Telomeres

The telomere assumes a ‘cap’ structure that maintains and protects the ends of eukaryotic linear chromosomes (Zakian, 1996). Telomeres impede chromosomal fusion (end-to-end joining) by blocking activation of the DNA damage checkpoint response and locally impairing double-strand break repair. Most importantly, telomeres and the RNA-directed enzyme telomerase ensure the addition of TG repeats that are otherwise eroded with each successive round of cell division. Collectively, these functions stabilize chromosome ends and contribute to genomic stability. Importantly, the telomeres of both budding and fission yeasts are assembled into silent chromatin structures (Huang, 2002; see also accompanying Focus Review by Luke and Lingner).

Telomeric DNA consists of three main parts: a short single-stranded (ss) 3’ overhang, double-stranded (ds) telomeric repeats and the subtelomeric region. The ss overhang and double-stranded stretch in S. cerevisiae comprise ~300 bp of an irregular TG_{1-3} repeat that lies terminal to subtelomeric sequences. The subtelomeric regions include up to four tandem copies of Y’ elements, short internal TG_{1-3} repeats

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and an X element composed of imperfect repeats and a conserved 437 bp core (Zakian, 1996).

The telomeric repeats of \textit{S. pombe} are also 300 bp long, but are somewhat more degenerate. They consist mainly of TTACA(G)n (where \(n = 1-8\)), and contain interspersed repeats of TTAGGG and TTACCGG, each with two Gs. The repeats at both ends of chromosome III are immediately flanked by repeats of ribosomal RNA genes, whereas chromosomes I and II share similar subtelomeric sequences that contain open reading frames (ORFs). Telomere-linked helicases (tih) are encoded by the most distal ORFs in the subtelomeric regions of chromosomes I (tih1+) and II (tih2+). These putative helicases are members of the recQ family and display extensive sequence homology with the \(dh\) and \(dg\) repeats found at centromeres (cenH like)(Wood et al., 2002; Mandell et al., 2005). Interestingly, the \textit{S. cerevisiae} \(Y\) elements also encode a DNA helicase, which is expressed primarily in meiosis (Louis and Haber, 1992; Yamada et al., 1998). In \textit{S. pombe}, there is conservation among neighbouring ORFs in addition to the homology shared by \(tih1+\) and \(tih2+\), indicating that the two subtelomeric regions resulted from a duplication.

Although the terminal telomere sequence is associated with non-histone proteins forming a ‘telosome’, subtelomeric regions in both fission and budding yeast are nucleosomal (Vega-Palas et al., 1998; Wieren et al., 2005). Important is the presence, or absence, of post-translational modifications on the histone tails of subtelomeric nucleosomes. In \textit{S. cerevisiae}, lysines at positions 9, 14, 18, 23 and 27 on H3, at positions 5, 8, 12 and 16 on H4, at position 7 on H2A, and at positions 11 and 16 on H2B are hypoacetylated in subtelomeric chromatin (Thompson et al., 1994; Braunstein et al., 1996; Suka et al., 2001). Moreover, two specific and universally conserved marks of active or open chromatin, H3K4me and H4K16ac, are absent from telomeres in both yeasts. Although \textit{S. cerevisiae} has no H3K9me at all, this modification is characteristically present throughout fission yeast heterochromatin, including pericentric DNA, subtelomeric domains and at silent mating-type loci (Nakayama et al., 2001). Nucleosomes bearing H3K9me are also typically hypoacetylated on H4K16 and H3K14.

In budding yeast, the hypoacetylated status of histone tails seems to be sufficient to favour the binding of the silent information regulatory (SIR) complex, Sir2-3-4, which in turn ensures a heritable downregulation of transcription of subtelomeric genes. This is called telomeric position effect, or TPE (see below). Sir2, a conserved NAD-dependent histone deacetylase, can act on all lysines of the H3 and H4 tails, but particularly targets H4K16ac (Blander and Guarente, 2004), as well as H3K9me in \textit{S. pombe} (Shankaranarayana et al., 2003). Other markers of active chromatin, notably di- and trimethylated forms of histone H3K9, antagonize the binding of the SIR complex and impair repression of subtelomeric genes (van Leeuwen et al., 2002). Thus, the predominant pattern of histone modification at budding yeast telomeres is an absence of active marks, whereas \textit{S. pombe} requires the positive signal provided by H3K9me. Intriguingly, in fission yeast Sir2 cooperates with Ctr3 to eliminate acetylation marks on both H4K16, H3K9 and K14, which allows for subsequent methylation of H3K9 (Wieren et al., 2005).

In addition to subtelomeric histones, a sequence-specific factor binds the TG-rich telomeric repeats. In almost every species these repeat-binding factors share a myb-like DNA-binding domain (Konig and Rhodes, 1997). In budding yeast, the terminal repeats are bound by the repressor activator protein 1 (Rap1), whereas in \textit{S. pombe} the analogous protein is called Taz1. In addition, \textit{S. pombe}, similar to man, has a Rap1 homologue that lacks the DNA-binding domain. Fission yeast Rap1 associates with telomeric repeats through Taz1, again analogous to the association of human Rap1 with Trf1. The additional telomere-associated proteins can be divided into two classes: those that mediate end maintenance by controlling telomerase accessibility, and those that promote silent chromatin. Ku, a heterodimer that binds all DNA ends regardless of sequence, has a special role at telomeres: it contributes both to controlling telomerase and to promoting silent chromatin. In addition, budding yeast Ku has a crucial role in anchoring telomeres to the nuclear envelope (NE), which further facilitates the nucleation and spread of chromatin-mediated gene silencing (see below) (Hediger et al., 2002; Taddei et al., 2004, 2009). In the absence of yKu, TG repeats in yeast shorten, subtelomeric repression is lost and strains become temperature sensitive (Fisher and Zakian, 2005).

The epigenetic nature of centromeres and telomeres

Epigenetics is the study of heritable changes in gene function that occur without a change in the sequence of the DNA. Centromere assembly and propagation provide a unique example of an epigenetic process as protein structures are assembled onto DNA and then stably propagated through numerous cell divisions in a DNA sequence independent manner. Despite their variation in size and sequence composition, the epigenetic aspect of centromeres is highly conserved.

The epigenetic nature of centromeres is manifest in the fact that—although there are different requirements for centromere establishment—a functional centromere is transmitted epigenetically to daughter cells. Even the \textit{S. cerevisiae} centromere shows epigenetic behaviour. Specifically, mutations in certain kinetochore proteins were shown to abolish de novo establishment of the \textit{S. cerevisiae} centromere, although functional centromeres could be stably propagated for over 25 generations in this background (Mythreye and Bloom, 2003). Moreover, mutations in the core CDE element reduced the association of cohesin with naïve centromeres, but had little effect on established centromeres (Tanaka et al., 1999). In \textit{S. pombe}, plasmids with minimal centromeric DNA establish functional centromeres stochastically, but once the functional state is attained it is propagated faithfully (Steiner and Clarke, 1994). Finally, a recent study also showed that heterochromatin and RNA interference (RNAi) are required to establish, but not to maintain, CENP-A\textsuperscript{case} chromatin at fission yeast centromeres (Folco et al., 2008).

Whether the telomeric functions of capping and end-replication behave epigenetically is unclear, yet telomere-associated gene silencing is one of the classic examples of semi-stable, yet heritable, transcriptional repression (Figure 1) (Gottschling et al., 1990). Both native subtelomeric genes and reporters integrated into telomere proximal zones succumb to transcriptional silencing through chromatin-mediated mechanisms. Despite the fact that the subtelomeric repression of transcription in budding and fission yeast share many heterochromatin-like features, the molecular mechanisms of repression differ significantly, as explained below.
ADE2 gene from its endogenous, euchromatic locus produce colo-

1990) in S. cerevisiae example of a telomeric position effect (TPE) (Gottschling

and imr

& 125-bp centromere of
domains of repetitive DNA at their centromeres. Thus, the
cated genes are influenced by nearby heterochromatin.

Position effect variegation

Position effect variegation (PEV) is a universally conserved
epigenetic phenomenon through which inserted or translo-
cated genes are influenced by nearby heterochromatin.

Thereafter, the ensuing expression status of the gene is

epigenetic phenomenon through which inserted or translo-
iautonomous. In budding yeast, the SIR

Figure 1 Variegated expression of a gene on packaging into a
euchromatic structure. (A) Cells expressing the wild-type

ADE2 gene from its endogenous, euchromatic locus produce colo-

1990) in S. cerevisiae example of a telomeric position effect (TPE) (Gottschling et al., 1990) in S. cerevisiae is shown here.

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form a zygote, undergo meiosis and sporulate. Thus, the

robustness of the species requires heritable repression of at

least one set of mating-type determining genes.

The mechanisms that ensure mating-type repression in

budding yeast, also serve to mediate position-dependent

repression at telomeres (Aparicio et al., 1991; reviewed in

Huang, 2002; Rusche et al., 2003). In an analogous manner,

mechanisms that repress recombination and transcription at

fission yeast centromeres contribute to silencing at the mat-
ing-type locus and TPE. The repression of mating-type

information in both species is robust and extremely stable,

whereas TPE is strongly variegating. This variegation is

manifest as an ability to switch at a low frequency between

‘on’ and ‘off’ states and then propagate either state for many

generations (Figure 1).

The other criteria that define epigenetic repression and

which are fulfilled by flies, S. cerevisiae and S. pombe are as

follows:

(1) correlation with an altered chromatin structure that

spreads outwards from a site of nucleation, silencing

independently of the promoter concerned;

(2) reduced accessibility for large molecules or complexes;

(3) presence of hypoacetylated histones and/or specific

marks that bind structural chromatin components;

(4) an involvement of nucleosome-binding non-histone com-

plexes that are limiting in abundance and show sensitiv-

ity to gene dosage; and

(5) heritability through either mitotic or meiotic division.

Screens in flies, S. pombe, and S. cerevisiae have identified

mutations that enhance or suppress heterochromatin-induced

silencing, classically called E(var)s and Su(var)s (Muller,

1930; Wakimoto, 1998). Hundreds of suppressors of PEV

have been identified to date, and these have proven to be

useful tools to study heritable repression, as well as centro-
mere and telomere biology (Allshire et al., 1995; Pidoux and

Allshire, 2004). Some of the mutated genes encode for

histone modifying enzymes, heterochromatin proteins

(HPs) or histone variants (reviewed in Huang, 2002; Rusche

et al., 2003). Notably, genetic approaches such as these have

allowed the field to create a general definition of heterochro-
matin, although the molecular mechanisms may be clearly

distinct in different organisms.

Silent chromatin assembly in budding yeast

The assembly of silent chromatin is a multistep process,

starting with the nucleation of a nucleosome-binding repres-

sion complex at specific regulatory sequences and its subse-
quent spread into neighbouring sequences. Pioneering

studies on the ordered assembly of silent chromatin have

been carried out in S. cerevisiae and have provided a founda-
tion for understanding epigenetic repression (reviewed in

Rusche et al., 2003). In brief, the formation of silent chromatin

in budding yeast requires the association of a heterotrimeric

nucleosome-binding SIR complex that contains Sir2, Sir3 and

Sir4 proteins in 1:1:1 stoichiometry (Cubizolles et al., 2006).

The complex is recruited to DNA by interactions with pro-

teins that bind to chromosome ends or to specific regulatory

sites called silencers. At budding yeast telomeres, the SIR

complex is recruited by Rap1 and the yKu heterodimer. The

Rap1 protein binds once every 18 bp within the TG repeat,

and each Rap1 molecule provides a binding site for Sir4 (Luo
Sir4 recruitment is further catalysed by the yKu70/80 heterodimer, which is associated with the telomere through its DNA-end binding function independently of Sir4 (Gravel et al., 1998; Martin et al., 1999). Importantly, Sir4 binding to Rap1 is antagonized by Rif1/Rif2 (Mishra and Shore, 1999).

Sir4 is necessary for the recruitment of the entire SIR complex, although once nucleated, excess Sir3 can propagate along nucleosomes without Sir4 (Hecht et al., 1996). Sir2's NAD-dependent histone deacetylase activity keeps telomeric nucleosomes in a hypoacetylated state (Imai et al., 2000). Sir2 binds neither DNA nor histones with high affinity, but once recruited by Sir4, Sir2-mediated deacetylation can create a high-affinity binding site for Sir3. Sir3 has dimerization capacity and in complex with Sir2-4, results in the spread of the SIR complex outward from the nucleation site (Hecht et al., 1996; Liaw and Lustig, 2006). Sir3 contributes to the specificity for deacetylated histone tails, whereas Sir4 enhances the affinity of the complex through its ability to bind DNA (Martino et al., 2009) (Figure 2).

During the deacetylation reaction catalysed by Sir2, NAD is hydrolysed and generates a by-product called O-acetyl-ADP-ribose (O-AADPR; Tanny et al., 1999; Tanner et al., 2000). This by-product can enhance the stability of the SIR–nucleosomal complex and may provoke a conformational change of the SIR-bound nucleosomal fibre (Tanny et al., 1999; Tanner et al., 2000; Tanny and Moazed, 2001; Liou et al., 2005; Martino et al., 2009). Although these in vitro results are compelling, questions remain as to how this works in vivo, because Sir2 deacetylation activity could be replaced in modified yeast by a class I catalytic domain that does not generate O-AADPR, with only minor loss of transcriptional repression (Chou et al., 2008).

Transcriptional silencing itself is thought to arise from steric hindrance of positive regulators of transcription, by the interaction of the SIR complex with nucleosomes (Hecht et al., 1995). SIR complex association also leads to the sequestration of the silent chromatin at the NE through association with Esc1 (Gartenberg et al., 2004; Taddei et al., 2004). Both the binding of the SIR complex to nucleosomes and the recruitment of silent chromatin to the NE, have been shown to render silent chromatin less accessible to the transcription machinery and to the action of enzymatic probes, such as a bacterial DNA methyltransferase or restriction endonucleases (Gottschling, 1992; Singh and Klar, 1992; Loo and Rine, 1994).

Despite this sequestration, certain classes of DNA-binding proteins seem able to access silent chromatin. For example, recognition sites for the FLP and Cre recombinases located within budding yeast silent chromatin domains are accessible to these enzymes when expressed at high levels (Holmes and Broach, 1996; Cheng et al., 1998). Moreover, promoters within a silenced domain can remain accessible to proteins of the transcription machinery, although the factors that stimulate elongation seem to be excluded (Sekinger and Gross, 2001; Gao and Gross, 2008). Fission yeast heterochromatin may also be accessible to the transcription machinery, because heterochromatin defects have been attributed to specific RNA pol II mutants (Dupedal et al., 2005; Kato et al., 2005). In addition, small interfering RNAs (siRNAs) have been identified, which match pericentromeric heterochromatin (Reinhart and Bartel, 2002; Cam et al., 2005; Buhler et al., 2008). Consistently, it was shown that though transcription of the ‘forward’ strand of pericentric DNA repeats was inhibited by heterochromatin formation, the ‘reverse’ strand seemed to be transcribed equally in both wild-type and heterochromatin-deficient strains (Volpe et al., 2002). This might suggest that transcription can cooperate with RNA decay mechanisms to keep heterochromatic regions repressed. The implications of this are discussed in more detail below.

**Heterochromatin assembly in fission yeast**

The assembly of heterochromatin in fission yeast, similar to that in budding yeast, involves orchestrated changes in chromatin modifications. After deacetylation of the histone H3 N-terminus by the class I and II histone deacetylases Clr3 and Clr6 (homologs of the HDACs Hd1a and Rpd3, respectively), and the class III NAD-dependent deacetylase Sir2, the methyltransferase, Clr4, methylates histone H3 at lysine 9, creating a binding site for the Swi6, Chp1 and Chp2 chromodomain proteins (Grewal et al., 1998; Partridge et al., 2000; Nakayama et al., 2001; Bjerling et al., 2002; Shankaranarayana et al., 2003; Motamedi et al., 2008). Swi6 and Chp2 are homologous to HP1 proteins, a conserved family of chromatin factors that recognizes methylated H3K9 in all species (Jacobs et al., 2001; Jacobs and Khorasanizadeh, 2002). Similar to the SIR complex, sequential cycles of Swi6 binding and Clr4 recruitment have been proposed to mediate the spreading of H3K9 methylation along the chromatin fibre (Nakayama et al., 2001; Grewal and Moazed, 2003).

Recent studies have begun to elucidate mechanistic details of assembly and maintenance of these heterochromatic structures. Specifically, it has been shown that the fission yeast chromodomain proteins Swi6, Chp1 and Chp2, although found at both centromeric and telomeric heterochromatin, contribute in distinct ways to heterochromatin assembly at...
these loci (Thon and Verhein-Hansen, 2000; Partridge et al., 2000, 2002). First, Chp1 contributes to de novo assembly at all sites of heterochromatin, yet contributes to the maintenance of repressed chromatin exclusively at centromeres (Sadaie et al., 2004). This may stem from the fact that different heterochromatic regions are more or less dynamic; centromeric domains seem to be less stable and more in need of establishment events.

Much similar to the situation in *S. cerevisiae*, the nucleation of heterochromatin in fission yeast requires cis-acting recruitment events (Figure 2), such as the recruitment of *S. pombe* Rap1 by Taz1 (Kanoh and Ishikawa, 2001; Zhang et al., 2008). Again similar to *S. cerevisiae*, recruitment pathways are partially redundant: the Taz1–Rap1 interaction is compensated by a second Taz1-dependent pathway that nucleates methylation of H3K9 by Clr4 (Kanoh et al., 2005). At the

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mating-type locus, an element called REII recruits ATF/CREB family proteins and helps to nucleate heterochromatin (Jia et al., 2004), whereas two further elements, REI and cenH elements (similar to dg and dh repeats found at the centromere) function cooperatively to enhance heterochromatin formation at the mating-type locus (Ayoub et al., 2000). The cis-acting nucleation sites at centromeres seem to be less well defined. Indeed, recent evidence suggests that transcription of pericentromeric dg and dh repeats has a critical function in heterochromatin assembly (Figure 2). It seems that, in addition to specific DNA sequences, transcription and/or non-coding RNAs (ncRNA) can provide an initial scaffold for the formation of heterochromatin (Cam et al., 2009). This observation, coupled with the fact that strains defective in RNA processing mechanisms compromise PEV (Buhler et al., 2007; Houseley et al., 2007; Murakami et al., 2007; Vasiljeva et al., 2008; Wang et al., 2008), have challenged the paradigm that heterochromatin excludes transcription.

Transcriptional scaffolds for the assembly of silent chromatin

Although it seems paradoxical, transcription may well be a prerequisite for the assembly and maintenance of some forms of silent chromatin. Although we know little about the underlying mechanisms that link RNA to chromatin, there is growing evidence that ncRNAs can contribute to epigenetic inheritance (Bernstein and Allis, 2005). One of the most prominent examples is the ncRNA Xist that is involved in X chromosome inactivation in mammalian females (Leeb et al., 2009; Senner and Brockdorff, 2009). Xist nucleates a repressive chromatin state in cis for almost an entire chromosome. ncRNAs have also been linked to certain forms of gene repression in budding yeast. For instance, a non-coding antisense RNA has been implicated in transcriptional silencing of Ty1 retrotransposons (Berretta et al., 2008), and antisense transcription has been shown to regulate chromatin-dependent silencing of the PHO84 gene in an aging yeast culture (Camblong et al., 2007). The PHO84 antisense RNA is normally kept at a low level by the nuclear exosome, an RNAse complex with 3′–5′ exonucleolytic activity. When this antisense RNA is degraded, PHO84 sense mRNA is present in maximal amounts, yet under stress conditions the antisense ncRNA accumulates and recruits the exosome to the PHO84 gene, reducing the sense message. The PHO84 ncRNA then seems to recruit a histone deacetylase to the locus to further inhibit sense transcription (Camblong et al., 2007). Although this is neither an SIR-dependent nor a heritable state of repression, it does underscore the role of RNA in the suppression of mRNA accumulation.

Finally, ncRNA has recently been detected to bind chromosome ends in which it contributes to the regulation of telomerase. The non-coding telomeric repeat-containing RNA (TERRA) is transcribed towards the chromosomal end in humans, mouse, hamster, zebrafish and in both budding and fission yeasts (Azzalin et al., 2007; Luke et al., 2008; Schoeftner and Blasco, 2008). Its role in gene repression is unclear, and the disruption of TPE through loss of SIR factors actually increased the level of TERRA, meaning that its presence is inversely correlated with repression (see also accompanying focus review by Luke and Lingner, in press). In contrast to this, it was found that fission yeast centromeric dg and dh transcripts are positively correlated with the assembly of heterochromatin in an RNA-dependent manner (Buhler and Moazed, 2007; Grewal and Elgin, 2007; Zarattiegui et al., 2007).

One major difficulty in assigning function to ncRNAs is to distinguish between the effects of transcription per se and a function of the transcript itself. It is possible that RNA Pol II transcribes non-coding DNA to remodel chromatin and that the resulting ncRNA is a non-functional by-product. Indeed, genes can be activated by transcription through promoter regions making DNA sequences more accessible to the transcription machinery (Hirota et al., 2008). It is also possible that genes become silenced as a consequence of transcription interference (Martens et al., 2004; Hongay et al., 2006). Finally, RNA could also actively recruit modifying enzymes that help assemble a higher-order chromatin structure. Interestingly, some HP1 proteins themselves have affinity for RNA (Muchardt et al., 2002), and recent work on the fission yeast Sir6 showed that it specifically interacts with heterochromatic transcripts. This led to the proposal that its RNA-binding activity serves the general function of retaining heterochromatic RNAs on chromatin (Motamedi et al., 2008).

RNAi-mediated heterochromatin assembly

In fission yeast several lines of evidence argue that RNA serves as a scaffold for the assembly of heterochromatin. In particular, the RNAi pathway contributes to repression at fission yeast centromeres, in which siRNAs, together with long ncRNAs, are essential for the formation of heterochromatin at pericentric dg/dh repeats. RNAi is a conserved silencing mechanism that is triggered by double-stranded RNA (dsRNA) (Bartel, 2004; Hannon, 2002). The mechanism of silencing involves the generation of small RNA molecules of ~22 nucleotides from the longer dsRNAs by an RNAse III-like enzyme called Dicer (Bernstein et al., 2001). These siRNAs then load onto an effector complex called RNA-induced silencing complex (RISC). RISC complexes contain Argonaute, which is a member of the conserved Argonaute/PIWI family of proteins that are required for RNAi in a variety of systems (Caudy et al., 2002; Hammond et al., 2001; Hutvagner and Zamore, 2002; Mourelatos et al., 2002; Zamore, 2001). siRNA-programmed RISC targets cognate mRNAs for degradation (Caudy et al., 2003). In a related process, small RNAs, called miRNAs, are produced from hairpin RNA transcripts by Dicer enzymes and programme RISC for translational repression of target mRNAs (Pillai, 2005). In some organisms, the RNAi response also requires an RNA-directed RNA polymerase (RdRp) that may be involved in amplifying dsRNA using siRNAs as primers (Dalmay et al., 2000; Sijen et al., 2001).

Although absent in S. cerevisiae, the key components of RNAi, Dicer and Argonaute, and an RNA-dependent RNA polymerase, are found in S. pombe. Deletion of the genes encoding any of these proteins (Der1, Ago1 and Rdpi, respectively) results in loss of H3K9 methylation and Sir6 localization at centromeres (Volpe et al., 2002). Moreover, siRNAs corresponding to centromeric repeats have been identified (Reinhart and Bartel, 2002; Cam et al., 2005; Buhler et al., 2008). Importantly, RNAi turned out to contribute to the initiation of heterochromatin assembly at all heterochromatic loci, but it is only required for maintenance at centromeres (Figure 2) (Jia et al., 2004; Sadaie et al., 2004; Kanoh et al., 2005). Thus, Chp1 and the RNAi machin-
ery seem to be functionally linked. Strikingly, Chp1 resides in a complex together with Ago1, a newly identified factor Tas3, and centromeric siRNAs. This complex has been termed RNA-induced transcriptional silencing (RITS) complex and is required for silencing and high H3K9 methylation levels at centromeric dg and dh repeats (Verdel et al., 2004).

Further biochemical analysis of the *S. pombe* RNAi proteins resulted in the identification of two additional RNAi effector complexes that are important for centromeric heterochromatin formation: the Argonauta siRNA chaperone (ARC) complex and the RNA-directed RNA polymerase complex (RDRC) (Motamedi et al., 2004; Buher et al., 2007). RDRC has RNA-dependent RNA polymerase activity and is thought to amplify siRNAs (Motamedi et al., 2004). As in the RITS complex, ARC contains siRNAs bound to Argonauta. However, the siRNAs found in ARC are mostly double stranded, suggesting that ARC is a precursor complex involved in siRNA maturation. The RITS complex contains single-stranded siRNAs (Buker et al., 2007), which have been proposed to act as specificity factors for association with chromatin. In principle, siRNAs could target specific chromatin regions by base pairing with either DNA or nascent RNAs (Grewal and Moazed, 2003).

Studies over the past years have provided support for a model in which siRNAs act as guide molecules to target histone modifying enzymes to chromatin through base pairing between siRNA and pre-mRNA, during RNA elongation by RNA pol II. This would allow nascent RNA to serve as a scaffold for the recruitment of histone modifying enzymes (Figure 2). Support for this model comes from artificial tethering of RITS to the transcript of a normally euchromatic gene. Tethering of the RITS complex to *ura4*+ RNA through a site-specific RNA-binding protein (N protein of phage *λ*) results in heterochromatin assembly and silencing of the cognate *ura4*− gene. This tethering also results in the generation of *ura4*−-specific siRNAs, and silencing requires proteins associated with both RNAi and heterochromatin (Buhler et al., 2006).

**Downregulation of RNA Pol II in fission yeast**

Recent work in fission yeast has shed new light on the mechanism of H3K14 deacetylation by the Ctr3 HDAC and its relative contribution to chromatin-mediated gene repression. Affinity purification of Ctr3 showed a complex termed SHREC (Snf2/Hdac-containing repressor complex) (Sugiyama et al., 2007). Core components of SHREC include Ctr1, Ctr2, the Ctr3 histone deacetylase and the Mit1 chromatin-remodelling protein. SHREC seems to act downstream of heterochromatin assembly (H3K9 methylation) to catalyse the deacetylation of H3K14. This in turn seems to limit transcription by impairing access of DNA Pol II to heterochromatin (Figure 2) (Bjelner et al., 2002; Sugiyama et al., 2007). SHREC itself is targeted to heterochromatin loci by different ligands, two of which are Chp2 or Ccq1. Chp2 seems to be particularly important at centromeres, whereas Ccq1 functions only at the telomere, together with Tas1 (Kanoh et al., 2005; Sugiyama et al., 2007; Motamedi et al., 2008). Briefly, at centromeres the recognition of H3K9me3 by Chp2 recruits SHREC to facilitate histone H3 deacetylation, which in turn coincides with reduced presence of RNA pol II. On the other hand, Ccq1 circumvents the requirement for H3K9 methylation. Similarly, Atf1/Pcr1 are required for Ctr3 targeting to a nucleation site at the mating-type locus, although it is not clear that this acts through physical recruitment of SHREC (Yamada et al., 2005).

The observation that RNA Pol II occupancy at heterochromatic loci increases on deletion of SHREC components implies SHREC in the restriction of promoter access. However, deletion of all the three chromodomain proteins (Swi6, Chp1 and Chp2) is needed to reach the level of derepression achieved in a *clr4A* strain. This argues that RNA Pol II restriction is only part of the silencing mechanism (Motamedi et al., 2008). The rest may involve RNA decay mechanisms that operate in cis. Importantly, the RNA degradation seems to be different from classical post-transcriptional gene silencing, because it depends on the status of the chromatin from which the RNA is transcribed (Buhler et al., 2006, 2007). Therefore, this is referred to as co-transcriptional gene silencing (CTGS, Figure 3).

**Chromatin-dependent RNA degradation**

At heterochromatic loci in which RNAi is essential for silencing, RNA degradation could theoretically be mediated by the RNAi machinery (Noma et al., 2004; Buhler et al., 2007). Consistent with this idea, recombinant fission yeast Ago1 has siRNA-guided endonucleolytic activity (‘slicer’ activity), and siRNAs originating from centromeric RNAs as well as centromeric reporter gene insertions have been detected (Irvine et al., 2006; Buhler et al., 2007; Buker et al., 2007). Intriguingly, heterochromatic siRNA levels increase upon deletion of SHREC components, suggesting that RNAi is compensating for the loss of TGS in these mutant strains (Sugiyama et al., 2007). Furthermore, silencing of heterochromatin has also been shown to require the TRAMP polyadenylation complex and exosome-mediated RNA degradation (Buhler et al., 2007; Murakami et al., 2007; Wang et al., 2008). Importantly, exosome and TRAMP mutant yeast strains show loss of heterochromatin gene silencing without any obvious defects in heterochromatin formation. Furthermore, highly unstable ncRNAs from silent chromatin regions can be detected in *S. cerevisiae*, which has entirely lost the RNAi pathway (Wyers et al., 2005; Houseley et al., 2007; Vasiljeva et al., 2008). This suggests that CTGS is likely to be a conserved RNA-turnover mechanism that can also function independently of the RNAi pathway to keep heterochromatin silent and further corroborates the concept of CTGS as a heterochromatic gene silencing pathway acting downstream of heterochromatin assembly (Figure 3; Buhler, 2009).

**Heterochromatic microenvironments in the interphase nucleus**

A striking feature of repetitive DNA and the silent chromatin it engenders is the propensity to stick together to form foci within the nucleus. These are called telomere clusters, or in the case of centromeres, chromocenters. Both can be found around the nucleolus or along the inner face of the NE in yeast and other organisms (reviewed in Akhtar and Gasser, 2007; de Laat and Grosveld, 2007). The result of this spatial arrangement is that the heterochromatin associated with simple repeat DNA creates a subnuclear compartment that sequesters silencing factors and silenced chromatin from the rest of the genome. The relevance of this phenomenon for both TPE and regulation of the rest of the genome were
elegantly shown in budding yeast, in which the components that anchor heterochromatin could be identified and ablated by genetic techniques (Taddei et al., 2004, 2009).

Recent work has elaborated a function for these subcompartments. First, with respect to silencing, subcompartments seem to favour repression by overcoming natural restrictions on TPE that are imposed by the limiting abundance of silencing factors (Taddei et al., 2009). Indeed, overexpression of Sir2, Sir3 and Sir4 group wise, or Sir3 or Sir2 alone, enhances repression of reporter genes at telomeres or the HM loci (Maillet et al., 1996). Consistently, native HMR and HML silencers were shown to out-compete telomeres or the HM loci (Maillet et al., 1996).

In an important study, the tethering of a silencer-flanked reporter construct near telomeric foci was found to improve repression in an SIR-dependent manner (Andrulis et al., 1998). Finally, repression at silencer-proximal genes far from telomeres was facilitated by SIR factor overexpression, as well as by compromising telomere anchorage (Maillet et al., 2001; Gartenberg et al., 2004; Taddei et al., 2009).

Thus, although a perinuclear anchoring is not absolutely necessary for SIR-mediated repression, it clearly contributes to its efficiency and propagation (Figure 4).

How is attachment at the NE achieved? There are two pathways of anchoring in budding yeast, one of which is enhanced by formation of silent chromatin (Sir4-Esc1, Figure 4A), whereas the other is efficient in its absence (yKu–Mps3). For the generation of telomeric subcompartments this latter pathway is very important, as it allows telomere juxtaposition before heterochromatin formation. The pathway that requires yKu tethers telomeres to the NE through telomerase RNA, and the telomerase subunits, Est2 and Est1 in S-phase cells (Schober et al., 2009), thanks to the ability of the telomerase cofactor Est1 to bind the integral NE protein Mps3 (Uetz et al., 2000; Antoniacci et al., 2007). Mps3 is a member of the conserved SUN domain family, which contains inner NE proteins that interact with both chromatin and the cytoskeleton in many species. In budding yeast it docks yKu70/80-Tlc1-Est1 and Sir4 at the NE (Bupp et al., 2007), whereas it binds other proteins of the nuclear lumen in other species (reviewed in Fridkin et al., 2009).

Figure 3 Chromatin-dependent gene silencing mechanisms operate at a transcriptional and/or post-transcriptional level. (A) Silencing of heterochromatin can be achieved by either shutting off transcription (TGS) or by degradation of heterochromatic RNAs (CTGS). In contrast to classic post-transcriptional gene silencing (PTGS), CTGS depends on the status of chromatin from which the gene is transcribed and is therefore referred to as ‘co-transcriptional’. (B) RNAi-mediated degradation of heterochromatic RNAs. Argonaute-containing complexes can be physically linked to heterochromatin through chromodomain proteins. One histone-octamer is shown in grey. The chromodomain protein binds to methylated K9 (orange) of the unstructured N-terminal tail of histone H3. The siRNA (blue) guides Argonaute to the heterochromatic RNA through base-pairing interaction and induces ‘slicing’. (C) Heterochromatic gene silencing mediated by a non-canonical polyA-polymerase and the exosome. RNAs transcribed from heterochromatic regions are identified by Cid14/Trf4 and marked as aberrant with a short polyA tail. This serves as a signal for the exosome to degrade the RNA.
Interfering with this pathway perturbs telomere position and yKu-mediated anchoring in S-phase cells, but not in G1, because Est1 is stable only in S phase (Larose et al., 2007). Intriguingly, the perturbation of telomere anchoring has more effects than simply the loss of TPE. Cells lacking both functional yeast Ku and Esc1 are viable, yet the dispersed SIR complexes have promiscuous effects on the transcriptome, and most notably at promoters implicated in ribosome biogenesis (Taddei et al., 2009; Zhu et al., 2009). At the same time the endogenous subtelomeric genes are derepressed. Thus, the sequestration of silencing factors in perinuclear foci has functional consequences for genome-wide gene regulation.

Much less is known about the mechanisms that tether heterochromatic regions of mitotically dividing fission yeast to the NE, although all three heterochromatic domains show perinuclear localization (Funabiki et al., 1993). The three centromeres and the MAT' locus localize at the nuclear periphery by attaching to the spindle pole body (SPB). The telomeres are also found at the nuclear periphery but on the opposite side of the SPB in the proximity of the nucleolus in two to four clusters (Funabiki et al., 1993; Alfredsson-Timmins et al., 2007). This organization depends on heterochromatin (Ekwall et al., 1996; Alfredsson-Timmins et al., 2007) and can also be affected by mutations in the key factors of RNAi (ago1, dcr1 and rdp1) (Hall et al., 2003). However, despite the loss of centromeric repression, centromere clus-
tering was unaffected in these RNAi mutants, and telomere clustering was lost without affecting telomeric silencing. Moreover, despite a loss of clustering, telomeres remained associated with the NE in RNAi mutants (Hall et al., 2003). Thus, in S. pombe, telomere–telomere interactions, but not centromere–centromere interactions depend on RNAi. Although RNAi is essential for telomere clustering, other pathways—possibly a redundant anchorage pathway such as the Ku pathway in budding yeast—position telomeres at the NE.

Apart from heterochromatin, other genomic elements are able to organize chromatin spatially. Notably, genomic domains designated as chromosome-organizing clamps (COC) are able to organize chromatin spatially. Notably, genomic elements designated as chromosome-organizing clamps (COC) are tethered to the nuclear periphery in a heterochromatin–unoccupied manner in S. pombe. This is mediated by the TFIIIC transcription factor complex that normally recruits RNA polymerase III (Noma et al., 2006), yet these sites are not occupied by RNA polymerase III. The functional consequences of tethering COCs to the nuclear periphery are less clear, but they may have a boundary function that could impact complex chromosomal processes such as gene regulation, DNA replication and recombination (Noma et al., 2006). Interestingly, TFIIIC is also known to bind to several sites across the S. cerevisiae genome, called ETC loci, which are similarly independent of RNA polymerase III localization (Moqtaderi and Struhl, 2004).

Meiosis entails major re-arrangements of the nuclear organization of fission yeast chromatin. Chromocenters are detached from the SPB and change places with telomeres, in preparation for the horsetail movements when the meiotic recombination takes place (Chikashige et al., 1997). In meiotic prophase, Taz1 is required for stable association between telomeres and SPB, and loss of the association leads to strong negative phenotypes (Cooper et al., 1998). Indeed, meiotic recombination is reduced, and both spore viability and the ability of zygotes to re-enter mitosis are impaired. Finally, mutations in the RNAi machinery provoke a mild but consistent disruption of meiotic telomere clustering and SPB integrity (Hall et al., 2003). To date it is unclear exactly to which extent meiotic and mitotic elements of nuclear organization are conserved in S. pombe. This awaits a careful genetic dissection of the localization machinery.

Flagging up damage and telomeres

Additional elements and pathways contribute to the perinuclear localization of budding yeast telomeres and centromeres, many being incompletely explained. Some pathways of positioning seem to be linked to the cellular response to a double-strand break, which raises the issue of whether the nuclear periphery influences recombinational repair or telomerase elongation, or both. Data are still scarce on this issue, but it seems that Mps3 may help suppress or regulate certain forms of recombination, such as break-induced repair (Gartenberg, 2009). This could be relevant for telomeres and damage, for instance, when there is no donor sequence for repair by homologous recombination. It remains to be seen exactly how and why telomerase wins over recombination pathways when chromosomal breaks bear TG repeats. This is an important question to solve if we are to understand the molecular structure of a chromosomal end.

Conclusions

Much of our understanding of chromatin-mediated repression comes from the study of model organisms. The two yeasts discussed here have widely different mechanisms of silencing, yet both contribute important principles of action, which have and will continue to guide studies in more biomedically relevant organisms. Budding yeast provides important paradigms for nucleation, propagation and questions of dosage dependence for heterochromatin, whereas S. pombe has contributed many of the models currently explored on how RNA contributes to transcriptional repression in the nucleus. Even principles of nuclear organization are likely to have parallels in higher eukaryotic cells, although other internal nuclear structures may replace the NE as an organizing principle. The power of genetics and population-wide statistics, which are so easy in yeast, will ensure that these organisms remain at the forefront of epigenetic research.

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