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Long-range silencing and position effects at telomeres and centromeres: parallels and differences

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Abstract. Most of the human genome is compacted into heterochromatin, a form that encompasses multiple forms of inactive chromatin structure. Transcriptional silencing mechanisms in budding and fission yeasts have provided genetically tractable models for understanding heritably repressed chromatin. These silent domains are typically found in regions of repetitive DNA, that is, either adjacent to centromeres or telomeres or within the tandemly repeated ribosomal DNA array. Here we address the mechanisms of centromeric, telomeric and locus-specific gene silencing, comparing simple and complex animals with yeast. Some aspects are universally shared, such as histone-tail modifications, while others are unique to either centromeres or telomeres. These may reflect roles for heterochromatin in other chromosomal functions, like kinetochore attachment and DNA ends protection.

Key words. Silencing; PEV; yeast; SIR protein; epigenetics; *Drosophila*; telomeres; rDNA.

Comparison of long-range silencing with heterochromatin

Long-range silencing generates a heritable, transcriptionally inactive chromatin structure that is associated with stable posttranslational modifications of histones, such as methylation or hypoacetylation (for reviews [1–3]). Such repression is not promoter specific, but rather region specific, and can act over large stretches of DNA. A repressed state can persist through meiotic and mitotic cell divisions and usually leads to late replication in S phase. Whereas gene silencing shares the key properties of inaccessibility and epigenetic inheritance with heterochromatin, transcriptionally silent domains are not always cytologically visible, even though this trait was traditionally used to distinguish heterochromatin from euchromatin [4]. The absence of a cytologically distinct hetero-chromatin is particularly pronounced in budding yeasts, which nonetheless has provided a powerful model for mechanisms of chromatin-mediated repression.

It is helpful to distinguish different types of heterochromatin; the most common are called facultative (as in tissue-specific domain inactivation) and constitutive, which generally refers to noncoding satellite repeats. Facultative heterochromatin can be specific to one of two homologous chromosomes, or to a certain cell type or developmental stage, and leads to the repression of unique sequences rich in genes. Constitutive heterochromatin remains condensed in almost all somatic cells of a given organism and usually involves repetitive, noncoding DNA sequences. Such gene-poor regions are typically, although not exclusively, located near centromeres or telomeres and can induce repression of nearby genes in an epigenetic manner. This phenomenon is called position effect variegation. The precise function of simple repeat DNA is poorly understood, but it is likely to serve a structural role in chromosome pairing and segregation (reviewed in [5, 6]), which only indirectly results in transcriptional silencing. Here we compare the silencing that occurs in *Saccharomyces cerevisiae* (budding yeast) with the constitutive heterochromatin found at the centromeres of *Schizosaccharomyces pombe* (fission yeast) and higher eukaryotes, like *Drosophila* and human beings (see fig. 1). By using 'heterochromatin' as a collective term to describe a range of compact interphase chromatin structures that are distinct from transcriptionally active and structurally accessible 'euchromatin,' we can indeed include silent loci in yeast in this category.

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Budding yeast silencing

Silent domains within *S. cerevisiae* chromosomes generally cover only a few kilobases, a distance that is not surprising given the compact organization of the budding yeast genome. Except for the irregular TG repeat at telomeres, budding yeast lacks simple repeat DNA, and genes have few introns and lie within 1–2 kb of each other. Perhaps because of the complexity and sheer size of higher eukaryotic genomes, many more proteins are implicated directly or indirectly in the formation of heterochromatin in complex organisms than in yeast. Indeed, constitutive heterochromatin constitutes >30% of the genome in *Drosophila* and >55% in humans and mice, whereas heritably silent chromatin represents <1% of the total budding yeast genome.

Nonetheless, three distinct chromosomal regions of *S. cerevisiae* confer a heritable state of transcriptional repression (i.e., epigenetic silencing) on otherwise functional promoters: (i) the mating-type loci *HML* and *HMR* (*h*omothallic *m*ating-type locus *l*eft or *r*ight), (ii) telomeres and (iii) the rDNA array (reviewed in [7, 8]). Genes located near or within these domains are either completely inert for transcription or exhibit a variegated state of repression that is relatively stable within a single cell. Importantly, repression is position, and not promoter, specific. Maintenance of silencing is essential for preserving mating competence in yeast, because derepression of the *HM* loci leads to expression of both mating-type programs, allowing haploid cells to acquire the properties of a nonmating diploid cell. On the other hand, the relaxation of silencing at the rDNA repeats leads to recombination and excision of rDNA repeats [9] which correlates with a shortened life span [10]. In *S. pombe* (fission yeast), centromeres are composed of large repetitive sequence elements that also confer position-dependent repression of Pol II-transcribed genes. This centromere-induced repression, although absent in budding yeast, is the most widely conserved type of heterochromatin and thus will be examined first.

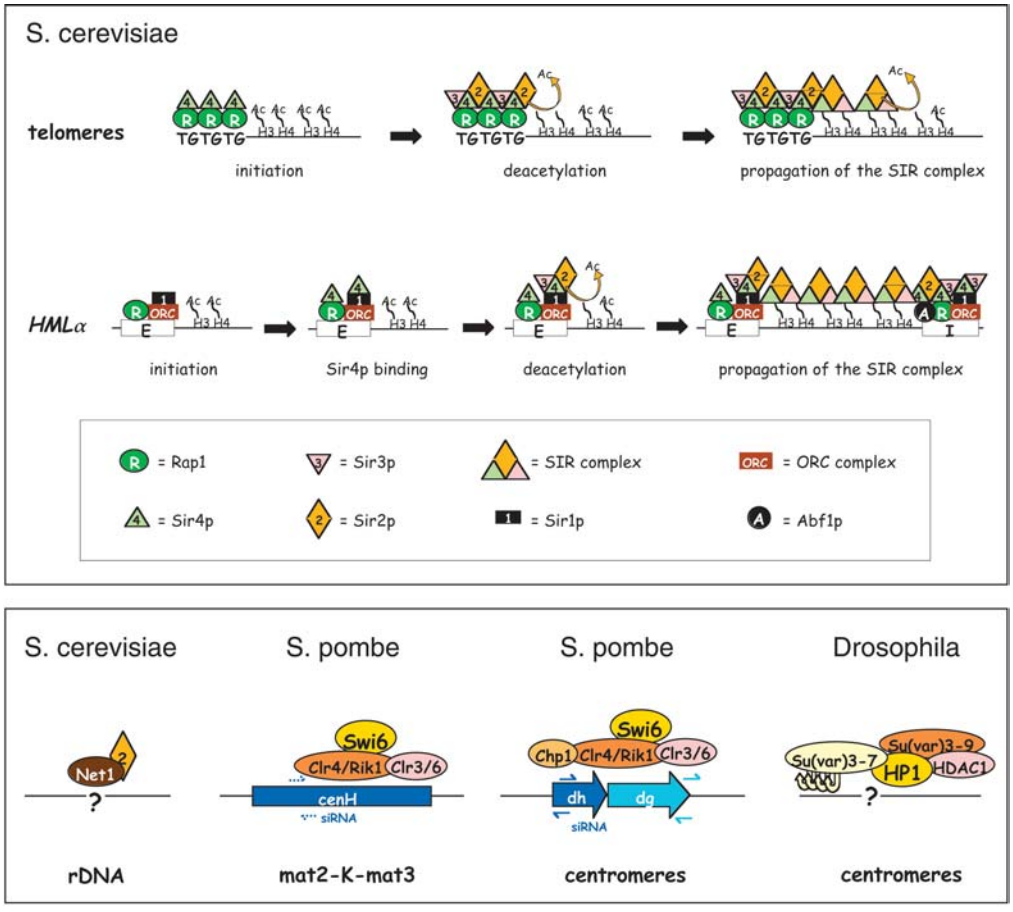

Figure 1. *Trans*-acting factors required for repression. *Top*: Propagation of silencing at budding yeast telomeres and *HM* loci involves multiple steps. *Bottom*: Key elements for targeting repression in diverse organisms. The *cis*-acting sequences necessary for the binding of *trans*-acting factors have not yet been determined for the budding yeast rDNA or for ribosomal repression or PEV in *Drosophila*. In fission yeast, siRNA are required at least for the initiation of repression and in addition for the maintenance of repression at centromeres. Only the sequence of siRNA necessary for centromeric silencing is known. Localization of Swi6 at the silent mating-type locus and at centromeres requires Clr4/Rik1, Clr3/Clr6 and Chp1, whereas HP1 independently initiates methylation in a small domain of the chromocenter.

Centromeres

Repetitive centromeric sequences and centromeric identity

The centromere is an essential chromosomal landmark that provides a site for the attachment of mitotic and meiotic spindle microtubules, which in turn mediate mitotic chromosome segregation. Centromeric activity in budding yeast is conferred by a specific sequence of roughly 125 bp, whereas centromeres of fission yeast and higher eukaryotes are composed of large repetitive DNA domains packaged into a heterochromatic structure (for details see table 1; for centromere reviews [11, 12]). Because these 'regional' centromeres cannot be easily delineated on the basis of their sequence, it has been difficult to determine the minimal size of a functional centromere in most organisms.

In fission yeast, *Drosophila* and mammals, heterochromatin is an integral part of the centromere, and the establishment and maintenance of heterochromatin correlates with centromere function [5]. Centric heterochromatin may not only provide a site for microtubule attachment, but may also contribute to the nucleation of sister chromatid cohesion [13]. The occasional creation of new centromeres (i.e., neocentromeres) at novel chromosomal sites that lack sequence homology with natural centromeres [14, 15] suggests that the structural organization of centromeric domains, rather than the DNA sequence per se, is the primary relevant parameter for their function. Centromere choice may also reflect complex parameters such as timing of replication, subnuclear positioning and/or other heritable features of chromatin structure [11, 12, 16].

One conserved heritable feature of centromeres is the presence of a special histone H3 variant, which is found exclusively within the core centromeric region. This special histone is called CENP-A in mammals (*cen*tromeric *p*rotein A), Cid (*c*entromere *id*entifier) in *Drosophila*, Cse4 in *S. cerevisiae* and Cnp1 in *S. pombe,* and it replaces histone H3 in specialized centromeric nucleosomes (reviewed in [11, 16]). The presence of this H3 variant appears to be crucial for kinetochore assembly and distinguishes the inner plate of the centromere from pericentric heterochromatin, which contains normal histone H3. In an attempt to explain this distribution, a recent study found that core regions of *Drosophila* centromeres replicate as isolated domains early in S phase, prior to the replication of flanking heterochromatin. Moreover, histone H3-containing nucleosome assembly was shown to be inhibited during the replication of these core centromeric sequences [17]. One suggestion for the perpetuation of this special centromeric chromatin is therefore that pericentromeric heterochromatin sequesters centromeres from an H3-specific assembly machinery, promoting incorporation of the centromere-specific variant [17]. Alternatively, a histone exchange factor may replace H3 with a H3 variant in a localized and replication-independent manner.

Pericentromeric heterochromatin mediates PEV

Pericentromeric heterochromatin and its associated phenomenon of position-effect variegation (PEV) are only observed in organisms having large regional centromeres. A mere 125 bp is necessary and sufficient for the assembly of a functional *S. cerevisiae* centromere, and this sequence is not flanked by heterochromatin, nor does it silence genes. In contrast, now-classic studies by H. J. Müller (reviewed in [18, 19]) showed that *Drosophila* genes that were transposed by natural or induced genetic rearrangements to sites near pericentric heterochromatin frequently assume a variegated pattern of expression. This chromosomal position effect can spread over distances of 1 Mbp or more, generally reflecting a gradient of gene inactivation that is inversely correlated with distance [18]. Recent data show that the local context of a gene also influences the degree of PEV, and suggest that the repression process can also be discontinuous and modulated by promoter strength [20]. Indeed, some genes are specifically adapted to be expressed exclusively in a heterochromatic context [19]. Genes that encode proteins structurally important for heterochromatin have been identified in screens for dominant mutations that enhance or suppress PEV, called E(var)s and Su (var)s, respectively [18]. Approximately 120 modifiers of PEV have been identified to date, and more can be expected.

Initiation of PEV at the molecular level is poorly understood, although the products of three Su(var) genes, HP1 [*h*eterochromatin *p*rotein 1 encoded by *Su(var)2-5*], Su(var)3-7, and Su(var)3-9 are strong candidates for structural components of pericentric heterochromatin in flies. All three colocalize to pericentric regions on polytene chromosomes, coimmunoprecipitate as members of a protein complex and interact in two-hybrid assays [21–22]. Only the Su(var)3-7 protein has affinity for DNA, it binds through a cluster of N-terminal zinc fingers. Deletion studies however show that the Su(var)3-7 C-terminal domain mediates targeting to pericentric heterochromatin and that HP1 localization occurs independent of the Su(var)3-7 N-terminus [23]. The cluster of zinc fingers may nonetheless mediate RNA binding (see below). Although transgene inactivation by PEV is correlated with HP1 deposition, this deposition alone is not sufficient to confer gene repression. Indeed, HP1 also localizes to over 200 euchromatic sites on polytene chromosomes, indicating that additional conditions must be met for promotion of a transcriptionally inactive chromatin state [24, 25].

In the case of fly PEV, the spread of centric heterochromatin can be assessed cytologically on the basis of a vis-

Table 1. The different heterochromatic loci found in human, fruit flies, fission yeast and budding yeast.

	Human	Fruit fly	Fission yeast	Budding yeast
Centromeres	**silent**	**silent**	**silent**	**not silent**
DNA sequence	tandem array of 171 bp of monomer α-satellite repeats	simple satellites and single complete transposable elements	a 15-kb central core with unique sequences (cnt and imr) flanked by 20–100 kb of repeats (otr with dh and dg repeats)	three conserved regions CDE-I TCACATGAT CDE-II 80–90 bp >90% AT CDE-III TGATTTCCGAA
size	>2 Mb to several Mb	>2 Mb to several Mb	from 35 kb to 120 kb	~125 bp
silencing proteins	HP1α and HP1β, SUV39H1	HP1, Su(var)3-7, Su(var) 3-9, Su(var) and E(var) proteins; to lesser extent Sir2	Clr1-4, Clr6, Rik1, Swi6, Chp1, Mis6, Csp, siRNA of dg and dh repeats	
deacetylases?	TSA sensitive	Rpd3 antagonizes,TSA sensitive	TSA sensitive	
telomeres	**silent**	**silent**	**silent**	**silent**
DNA sequence	tandem repeats of TTAGGG	terminal tandem arrays of HeT-A (~6 kb) and TART (~12 kb) retroposons + several kb of complex satellite termed TAS	~300 pb of TTACAGG repeats + at least 19 kb of TAS (composed of large unit of 0.9–1.2 kb made of 86–89 bp tandem repeats)	~300 pb of TG_{1-3} repeats + and subtelomeric regions, made up 0–4 Y element (middle repetitive element of 5.2 or 6.7 kb) interspersed with TG_{1-3} and ending with an X element (containing STR, and a core X)
size	from 2 to 50 kb	>20 kb	>19 kb	from 800 bp up to 8.5 kb
silencing proteins	?	Su(z)2	rat1, lot3, taz1/lot2, rap1 and to lesser extent Clr1-4, Clr6, Swi6, Rik1	Sir2-4 (SIR complex), Rap1, Ku heterodimer, H3 and H4
deacetylases?	TSA sensitive	?	TSA sensitive	Sir2 essential, Rpd3 antagonizes, Hos2 has minor effects
Mating-type loci	**silent**		**silent**	**silent**
DNA sequence	inactive X chromosome	male-specific upregulation of X-linked genes	the interval mat2-K-region-mat3 (the K region contains a 4.3-kb cenH domain homologous to the centromeric dg and dh repeats)	HMRa and HMLα flanked by E and I silencers (containing specific binding sites for Rap1, ORC and Abf1)
size	149 Mb		~20 kb	1.6 kb and 2 kb, respectively
silencing proteins	XIST RNA	Activators include MSL1, MSL2 and RNA	Clr1-4, Clr6, Rik1, Swi6, for initiation dependent on siRNAs	Sir1, Sir2-4 (SIR complex), Rap1, ORC, Abf1, to lesser extent H3 and H4 tails
rDNA locus	**?**	**?**		**silent**
DNA sequence			up to 50% of two rDNA arrays composed of of ~70–100 tandem repeats of a 10.4-kb unit	up to 50% of one rDNA array composed of ~200 tandem repeats of a 9.1-kb unit
size			two times ~1 Mb	at least 1.8 Mb
silencing proteins			Swi6, Clr4, Chp1, Chp2	Net1 and Sir2 (RENT complex), H2A and H2B dosage

ible change in the banding pattern of polytene chromosomes. Nonetheless, this *cis*-spreading model of centric heterochromatin does not adequately explain the ability of PEV to repress genes located several megabases away [18]. Such phenomena led to the suggestion that *trans*-interactions between different heterochromatic regions and the three-dimensional organization of chromosomes in the interphase nucleus may be important for PEV [18, 26]. Indeed, in many cells *Drosophila* centromeric heterochromatin is clustered at the 'chromocenter,' a zone of ~25% of the nuclear volume that is readily visualized by the detection of the HP1 protein [27]. In some cell types, *Drosophila* interphase chromosomes also exhibit a Rabl orientation, in which the centromeres and the bulk of heterochromatin are positioned at one end of the nucleus and telomeres at the opposite end [18]. In sum-

mary, two epigenetic mechanisms appear to contribute to the cell-to-cell phenotypic variation that is typical for PEV: the *cis*-spreading effect of adjacent heterochromatin and a *trans*-effect due to chromosomal interactions that themselves may involve by heterochromatin binding factors.

HP1 and its target, methylated lysine 9 of histone H3, are conserved

What targets the complex of HP1/Su(var)3-7/Su(var)3-9 to pericentric heterochromatin? Answers have been provided in part by recent studies showing that the hypoacetylation and subsequent methylation of a particular lysine (K_9) in histone H3 is a conserved marker for heterochromatin that provides a specific target for the binding of HP1 and related proteins [28]. In this aspect, fission yeast, flies and mammalian cells have all contributed to a coherent model of heterochromatin assembly.

The *S. pombe* homologue of HP1, Swi6, is found at pericentric heterochromatic regions, the imr and otr repeats (see table 1). Similarly, of the three human HP1 homologues, two, HP1$^{\alpha}$ and HP1$^{\beta}$, localize to heterochromatin, whereas a third one, HP1$^{\gamma}$, is primarily found at euchromatic sites (reviewed in [28]). All HP1-like proteins have a characteristic N-terminal chromodomain (CD), a short variable hinge region and a C-terminal chromoshadow domain (CSD), which mediates self-dimerization and protein-protein interaction. Although HP1 appears to bind specifically to >20 proteins, its most crucial binding partner may well be the methylated lysine 9 (K_9^{me}) of histone H3, which it binds through its chromodomain [29, 30]. Importantly, recent studies have shown that Su(var)3-9 is a conserved histone methyltransferase (HMTase) specific for H3 K_9 [31–33]. Consistently, both a mutation in the CD domain of HP1 and a deletion of Su(var)3-9 lead to the delocalization of HP1 from heterochromatin.

Although, H3 K_9^{me} is recognised by HP1, the two markers do not always colocalize [34–36]. For example, the inactive X chromosome contains extensive H3 K_9 methylation, yet no HP1 is bound to this chromosome [35, 36]. Moreover, as mentioned above, HP1 localizes to certain euchromatic bands in polytene chromosomes that show no staining for H3 K_9^{me} [36]. Although conclusions from these immunoanalyses must take into account whether the antibodies recognize di- and trimethyl forms of H3 K_9 equally, they clearly show that H3 K_9^{me} is not a sufficient condition for HP1 recruitment. Previous studies had shown that HP1 becomes delocalized from the pericentric foci in RNAse-treated cells [34, 35, 37]. This observation was extended by demonstration that the HP1 hinge domain binds single- and possibly double-stranded RNAs and that this hinge, together with the CD domain, is sufficient to ensure the proper localization of HP1 [38]. In conclusion, a combination of a specific histone-tail modification and an as yet unidentified RNA apparently targets HP1 and presumably triggers the establishment of a repressed heterochromatin structure. This minimal model certainly does not exclude the participation of other components, for example, the recently characterized HP2 protein [39].

Methylation at histone H3 K_9 and the formation of heterochromatin

The three centromeres of *S. pombe* chromosomes are composed of large inverted-repeat structures that surround the central core domain (cnt), called the imr (*i*n*r*most *r*epeat) and otr (*o*u*t*er *r*epeat). Reporter genes inserted at any of these domains are subject to position-effect variegation (reviewed in [40]), although those inserted in cnt are only weakly repressed compared with those inserted in imr and otr repeats. The initial *trans*-acting factors identified as necessary for the maintenance of heterochromatin were Clr1-4, Clr6, Swi6 and Rik1 [40].

Genetic studies in fission yeast have been crucial for identifying the key molecular events that lead to the formation of pericentric heterochromatin. Consistent with data discussed above, the *S. pombe* mechanism starts with covalent modifications of histone tails, that is, deacetylation and methylation, which appear to act in concert to establish a "histone code" that signals heterochromatin assembly [1]. Notably, the fission yeast homologue of Su(var)3-9, Clr4, has been shown to possess an intrinsic H3 K_9 methylation activity that can propagate this modification throughout the imr and otr repeats [32]. The deacetylation of H3 K_9 and K_{14} must occur prior to methylation, however, in a reaction mediated by Clr6 and Clr3, homologues of the histone deacetylases Rpd3 and Hda1. The final player recruited is Swi6, the closest homologue to HP1 in fission yeast. Because deletion of the Clr4 methyltransferase abolishes Swi6 localization at centromeres, one can infer that as in flies, the binding of Swi6 to centromeres depends on H3 K_9 methylation. The converse is not true, consistent with the idea that Swi6 (HP1) acts downstream of Clr4 (the H3 K_9 methyltransferase). Finally, deletion of either Rik1 or Chp1, a second chromodomain protein mainly confined to the otr regions, has been shown to abolish both H3 K_9 methylation and centromeric Swi6 localization [32, 41].

These observations suggest a progressive model for heterochromatin assembly based on an ordered series of histone modifications. Deacetylation of histone H3 by Clr3 and Clr6 creates conditions favoring the deposition of H3 K_9 methylation by the recruitment of Chp1, Rik1 and Clr4. H3 K_9^{me} is a specific binding site for Swi6, which in turn assembles nucleosomes into heterochromatin. HP1-like molecules like Swi6 may act in concert with the pas-

sage of the replication fork, because mutations in DNA Polα [32] also affect the centromeric localization of Swi6, and vertebrate HP1 associates with the replication-associated nucleosome assembly factor CAF1 [42]. In addition to replication factors, other *trans*-acting factors were identified in genetic screens for mutations that result in the disruption of centromeric silencing in a subdomain-specific manner. For example, silencing in the otr repeats is affected by mutations in 11 *csp* loci (*c*entromere *s*uppressor of *p*osition effect; [43]), whereas Mis6 affects silencing only at the imr and cnt domains to which it binds [41].

The observations that HP1 localizes to a restricted internal domain of the chromocenter in Su(var)3-9-deficient flies and that a chimeric HP1 containing the CD of Polycomb (Pc) relocalizes with Su(var)3-9 to Pc binding sites [21] suggest that the competence to nucleate pericentric heterochromatin is gained through HP1 binding. The spreading of pericentric heterochromatin, on the other hand, requires both HP1 and Su(var)3-9. In addition, the distribution of H3 K_9^{me} is diffuse throughout the nucleus when HP1 is absent [21], indicating that methylation and HP1 promote their localization mutually and spread in a self-perpetuating manner.

A role for RNAi in the establishment of heterochromatin

Three recent studies have solved the enigma concerning the role of RNA in the targeting HP1 for heterochromatin nucleation, by showing that small interfering RNAs (siRNA) are necessary for heterochromatin formation at fission yeast centromeres [44–46]. The siRNAs are end products of RNA interference (RNAi), a mechanism that processes double-stranded RNA into short sense and antisense RNA oligonucleotides of 21–25 nts. These siRNAs in turn inhibit the accumulation of homologous messenger RNA (mRNA) transcripts of cognate genes (reviewed in [47]). Intriguingly, deletion of the RNAi machinery genes *ago1* (Argonaute), *dcr1* (Dicer) and *rdp1* (RdRP) has been found to be correlated with the loss of pericentric heterochromatin, induction of transcription of pericentric reporter genes, loss of H3 K_9^{me} and delocalization of Swi6 [44]. The work proposes that in wild-type cells, the reverse strand of the dh repeat is transcribed, leading to a rapid cleavage of the ensuing dsRNA by the RNAi machinery. As a result, in RNAi mutants dh reverse transcripts accumulate. Naturally, the dh forward transcript also accumulates in these mutants, reflecting a loss of silencing. Consistently, in *swi6* mutants the forward dh transcript accumulates but the reverse one does not [44]. Finally dh and also dg siRNA are essential for the appropriate targeting of Clr4, Rik1 and Chp1 to centromeres and for the maintenance of pericentromeric heterochromatin [44, 45].

Establishment and maintenance of pericentric heterochromatin in human and *Drosophila* cells will probably similarly require the RNAi machinery, because the genes required for RNAi are conserved and the localization of HP1 and H3 K_9^{me} to pericentric heterochromatin is sensitive to RNAse [35]. Some aspects of this mechanism may also be conserved in the phenomenon of female-specific X-chromosome inactivation, a complex developmentally regulated event that requires transcription of the X-chromosome-specific Xist RNA and its antisense strand. X inactivation and PEV differ in many ways, however, because Xist RNA is not required for maintenance once repression has been established, and methylation of the inactive X is both Su(var)3-9 and RNAse insensitive. Finally, HP1 does not bind on the inactive X chromosome [35]. Nonetheless, X inactivation may present a paradigm for other instances of facultative or cell-type-specific heterochromatin.

Telomeres

Telomeres and telomeric position effect

The ends of linear chromosomes are stabilized by a structure called the telomere. If telomeres were to rely exclusively on conventional DNA polymerases to complete their replication, they would undergo terminal attrition and loss of genetic information. This problem has been solved in most eukaryotes by the ribonucleoprotein enzyme called telomerase, which extends the TG-rich strand of telomeric DNA by means of a self-templating mechanism (for review [48]). Thus, telomeres typically end in tandem arrays of G-rich telomeric repeats. In budding and fission yeast the terminal TG-rich repeats are irregular and extend only 300 bp, although they abut other middle-repetitive subtelomeric motifs (see table 1). *Drosophila* telomeres are exceptions to this general structure, as chromosomes end with retrotransposon-like elements and length is apparently maintained by recombination and retrotransposition [49].

Telomeres do not simply function as buffer zones to prevent the loss of essential sequences but have specialized functions that protect DNA ends. These capping functions impede chromosomal fusion (end-to-end joining) and degradation, which would otherwise lead to genomic instability. Paradoxically, telomeres exploit some of the same cellular machinery that mediates nonhomologous end-joining events at internal double-strand breaks, such as the Ku heterodimer, the Mre11/Xrs2/Rad50 complex and the flap-processing complex of Rad27 and Dna2 (reviewed in [50]). At telomeres these are thought to help recruit or facilitate telomerase action rather than promote recombination. When they are not being replicated, telomeres may have increased local folding, which could also facilitate the silencing of nearby promoters.

The SIR complex mediates silencing at budding yeast telomeres

As mentioned above, the extreme ends of *S. cerevisiae* chromosomes contain ~300 bp of an irregular TG_{1-3} repeat lying terminal to subtelomeric sequences. These include up to four tandem copies of Y′ elements (i.e., a middle repetitive sequence of either 5.2 or 6.7 kb), short internal TG_{1-3} repeats, and X element composed of subtelomeric repeats (STRs) and a conserved 437-bp core (fig. 2; [51]). Whereas the TG-rich portion of the telomeres (called the telosome) is free of nucleosomes, the subtelomeric repeats are nucleosomal [52]. Of the sequence-specific proteins that recognize the TG repeats (i.e., Cdc13, Tel2, Rap1), only Rap1 is absolutely required for the budding yeast telomeric-position effect (TPE), but the Ku heterodimer (Ku70 and Ku80), which binds telomeres because of its affinity both for free DNA ends and Sir4, contributes to silencing in at least two ways (see details below).

Rap1 has been suggested to recruit the two silent information proteins, Sir3 and Sir4, to telomeres, catalyzing the first step of a pathway that would lead to propagation of the silencing-proficient SIR complex (i.e., Sir2, Sir3 and Sir4, [53]). Recent data show, however, that Rap1 initially recruits Sir4 alone, because Sir4 can be detected at telomere ends in the absence of Sir2 or Sir3 [54, 55]. Given that the converse is not true, Sir4 appears to have an essential initiator function for the recruitment or stabilization of Sir2 and Sir3 at telomeres. The spreading of the SIR complex along subtelomeric nucleosomes is fully dependent on an intact SIR complex, on the nicotinamide adenine dinucleotide (NAD)-dependent deacetylase activity of Sir2 [54, 55], and on the deacetylated N-terminal tails of histones H3 and H4 [53]. This multiple-step pathway could be envisioned thus: First, Rap1 binds specifically to the TG repeats. Second, Rap1 recruits Sir4, which in turn recruits Sir2 and stabilizes the Rap1-Sir3 interaction. Finally, the full SIR complex would be able to spread along the TG-rich sequences because of affinity of Sir3 and Sir4 for the hypoacetylated tails of H3 and H4. This spread may require the active deacetylation of histone tails adjacent to the SIR complex by Sir2, because such spreading appears to be counteracted by a histone acetylase called Sas2 [56, 57]. The action of Sir2 deacetylase activity on adjacent nucleosomes may in fact be necessary for the cooperative, self-propagating nature of the linear and nucleosome-mediated spreading of SIR complexes along the chromosome.

To date only H3 K_9^{ac}, H3 K_{14}^{ac} and H4 K_{16} ac have been demonstrated to be substrates of Sir2 in vitro [58]. Be-

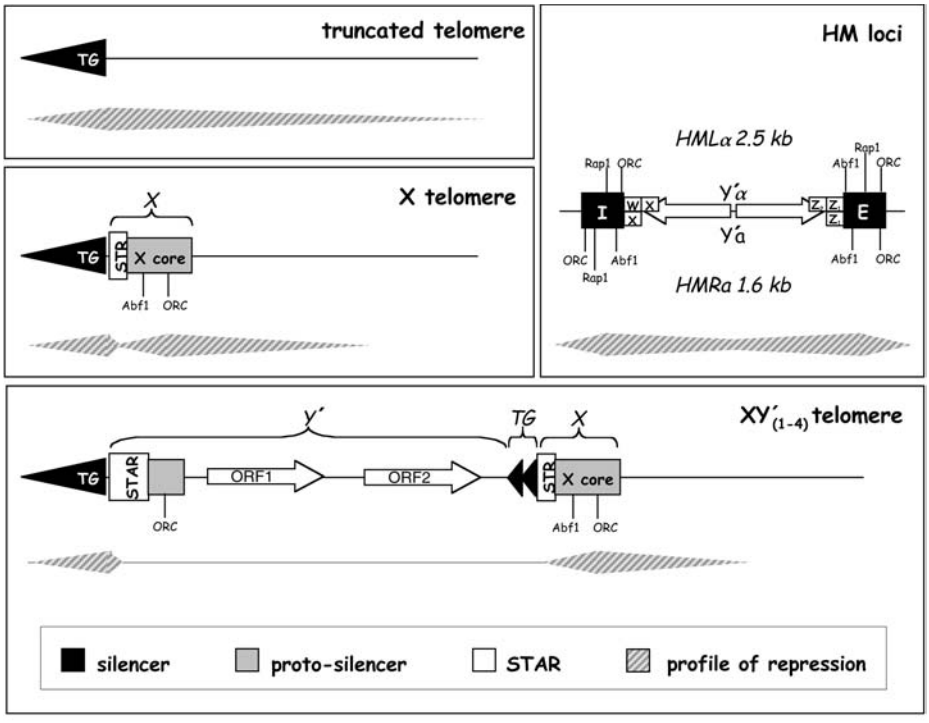

Figure 2. Protosilencers, STARs, silencers and profile of repression at budding yeast telomeres and *HM* loci. In budding yeast, native telomeres are composed of X and Y′ elements (i.e., $X_1Y'_{0-4}$). The X and Y′ elements each contain a protosilencer that can cooperate with telomeres to induce repression, as well as a STAR (*sub*telomeric *a*nti-silencing *r*egion, see [107]). Therefore, if Y′ elements are present at telomeres, they are flanked by STARs and insulated from repression. Repression is only observed at the X core element and ~2 kb centromere proximal. The *HM* loci are flanked by two silencers and are thus efficiently repressed, as even partial silencers cooperate to promote repression in this context [108].

cause Sir2 deacetylates a large variety of acetyl-lysine-containing proteins with equal efficiency, both the subnuclear environment and proteins that bind Sir2 are likely to help determine substrate specificity in vivo. Lysines at positions 9, 14, 18, 23 and 27 on H3 and positions 5, 8, 12 and 16 on H4 have been shown to be underacetylated in silent chromatin, as have lysine 7 on H2A, and lysines 11 and 16 on H2B [59]. This result suggests either that other histone deacetylases are required to maintain telomeres in their hypoacetylated state or that the histones have been assembled into silent chromatin in an underacetylated state and have been shielded from the action of acetylases. Since few deacetylase mutants other than SIR2 derepress TPE, the latter is more likely true.

Finally, as mentioned above, a further catalyst for Sir4 recruitment to telomeres is the yKu heterodimer, which is associated with termini both through its end-binding function and its affinity for Sir4 [60, 61]. Strains deficient in *ku70* or *ku80* lose telomeric repression, which can be restored through extreme telomere lengthening (i.e., an increase in Rap1 binding sites), by an increase in the dosage of Sir4 or by relief of the competition imposed by Rif proteins (Rap1-interacting factors) for the access of SIR proteins to Rap1 [62]. In brief, the Ku heterodimer aids but does not replace Rap1 in the recruitment of SIR proteins to telomeres. In addition, Ku plays an important role is anchoring telomeres to the nuclear periphery [63, 64], a localization that helps create subnuclear compartments enriched for Rap1 and SIR factors [65]. The importance of this nuclear organization for TPE is discussed below.

In summary, the establishment of silent chromatin at yeast telomeres requires the initial recruitment of Sir4 by Rap1 and yKu. Sir4 may be sufficient to recruit the intact Sir2-3-4 complex to TG_{1-3} repeats. The spreading of this SIR complex along nucleosomes, however, depends on a careful balance of the three SIR proteins and the deacetylase activity of Sir2, reminiscent of the means used by HP1 and Su(var)3-9 to initiate the formation of heterochromatin in *S. pombe*, *Drosophila* and mammalian cells. To date, however, no methylated forms of H3 K_9 have been detected in budding yeast, nor have homologues to the RNAi enzymes been reported in budding yeast. Thus, although conceptually similar, the mechanisms for repressed chromatin assembly at centromeres and telomeres are genetically distinct.

Barriers, insulators and the propagation of the repressed state

The most widely accepted model of telomeric silencing suggests that repressive chromatin propagates continuously inward from the telomere and diminishes with increasing distance from the terminus [53, 66]. Although this is true at artificial telomeres (i.e., terminal truncations lacking subtelomeric repeats), the extent of repression at native telomeres seems more limited [67]. For example, at native ends the subtelomeric Y elements are resistant to silencing along their entire length, although repression can be reestablished at the proximal X element by elements known as protosilencers (fig. 2; [67, 68]). Indeed, the Y elements appear to be flanked by insulators or barrier elements that protect sequences within the domain from repression. Thus, silencing at native telomeres seem in fact discontinuous, with maximal repression being found adjacent to the protosilencers element (an ARS consensus) in the subtelomeric core X element and extending inward with decreasing strength for ~3 kb. Although SIR proteins can be coimmunoprecipitated with the Y repeats, it is not clear whether they propagate continuously along its length or bind to flanking clusters of Rap1or Ku molecules [69]. Recent studies suggest that the extent of Sir3 spreading is determined by the balance of two antagonistic histone-modifying enzymes: the Sir2 deacetylase and the Sas2 histone acetyltransferase [56, 57]. Surprisingly, the deletion of *SAS2* leads to a loss, rather than improvement, of TPE at truncated telomeres possibly due to the uncontrolled spreading of Sir proteins inwards [56].

Yeast telomeres are anchored at the nuclear periphery and have a fold-back structure

In budding yeast, telomeric components such as the yKu complex mediate the tethering of telomeres with structural components of the nuclear envelope, leading to the clustering of telomeres into a limited number of discrete foci at the nuclear periphery (see references in [63–65, 70, 71]). Telomere clustering is correlated with an accumulation of SIR proteins and Rap1 in perinuclear foci, and the ensuing high concentration of SIR proteins has been shown to be a critical factor for efficient subtelomeric repression (reviewed in [70]). Despite the increased local concentration of SIR proteins at telomeres, their overexpression singly or in pairs (e.g., Sir3 and Sir4) still improves repression efficiency at telomeres, indicating that the SIR complex is limiting under normal growth conditions [65, 66].

Live imaging of GFP-*lac* repressor-tagged telomeres designed to determine whether the peripheral clustering of telomeres facilitates silencing, or whether silent chromatin itself is responsible for the creation of telomeric foci, has been performed under a range of mutant and wild-type conditions [64]. The deletion of *SIR4* leads to a slight but reproducible drop in telomere anchoring, although the extent of delocalization varies from end to end [64, 71]. Telomere displacement is even more striking in strains lacking the yKu70 or yKu80 protein [63, 64], but because SIR proteins are themselves displaced from

telomeres in *ku*-deficient strains [63], and because a subpopulation of the Ku heterodimer is displaced from telomeres in *sir*-deficient strains [60], determining whether yKu and SIR complexes define two independent anchoring pathways required restoration of telomeric silencing in a strain lacking yKu. This restoration was achieved by deletion of the *RIF1* gene, which encodes a Rap1-binding factor that competes with Sir3 and Sir4 for access to Rap1 [62]. In a *rif1 ku70* double mutant, the perinuclear anchoring of the lac^{op}-tagged Tel VI-R was restored, coincident with the restoration of TPE, in a SIR-dependent manner. This SIR-mediated telomere anchoring is cell-cycle dependent, being more pronounced in S-phase than in G1-phase nuclei [64], a dependence that is strikingly correlated with an ill-defined S-phase requirement for the establishment of silent chromatin (see below). In summary, telomere anchoring studies indicate that SIR-mediated tethering functions in parallel to the Ku-mediated pathway, which itself anchors telomeres in a repression-independent manner. Thus the clustering of telomeres into foci can both precede and result from silencing.

In contrast to initial suggestions [72, 73], the myosin-like proteins 1 and 2 play no significant role in the Ku-mediated anchorage pathway, as monitored by either fluorescence in situ hybridization (FISH) or live telomere-imaging techniques [64, 74]. Indeed, telomeres lengthen and TPE improves in *mlp1 mlp2* cells [74]. Although the membrane-associated partner for Ku remains obscure, a candidate perinuclear anchor for Sir4 has been identified as Esc1 ([75]; A. Taddei and S. M. Gasser, unpublished). Recently, de Bruin and colleagues have provided data suggesting that budding yeast telomeres not only cluster but fold back upon themselves [76]. Initial support for this idea was based on the observation that Rap1, as well as the Ku heterodimer, are not only recovered associated to the terminal TG-rich sequences but also bind repressed subtelomeric nucleosomes extending several kilobases from the TG repeats [60, 77, 78]. Although *trans*-interactions between telomeres could account for this result, a recent study based on a Gal4-mediated gene-activation assay argues that chromosomal termini fold inward to allow long-range protein-protein interactions in *cis*, in a Sir3-dependent manner [76].

Parallels between mammalian and yeast telomeres

Homologues of Sir3 and Sir4 have been found to date only in Trypanosomes and other Ascomycetes. Nonetheless, certain parallels can be drawn between budding yeast, mammalian and fission yeast telomeres. Indeed, among these organisms both the TG-rich repeats and some of the ligands for these repeats (i.e., homologues of Rap1, Cdc13 and the Ku heterodimer) are conserved, although *Drosophila* telomeres again are a special case, for which no sequence-specific proteins have been identified to date.

Mammalian TG-repeat tracts are conserved in sequence (TTAGGG) but are variable in length, showing organism- and cell-type-specific diversity. Lengths range from 5–30 kb in humans and up to >150 kb in mice. As in budding yeast, the fission yeast TG repeat covers <500 bp, although subtelomeric repeats can extend up to 19 kb. A Rap1-like factor is conserved in both human and fission yeast, yet in these organisms it lacks the conserved Myb-related DNA binding domain [79], and thus it does not bind TG repeats. Other related Myb-box-containing factors, such as TRF1 and TRF2 in humans [80, 81] and Taz1 and Teb1 in *S. pombe* [82, 83], bind the repeated sequence and mediate the association of human or fission yeast Rap1 to telomeres. Throughout evolution the structural DNA binding motif of telomeric proteins is conserved: yeast Rap1 binds to an iterated consensus by means of two internal Myb-like DNA binding domains [79], whereas the human TRF1 and TRF2 and the *S. pombe* Taz1 homodimerize, allowing the Myb domain of each subunit to contact one repeat unit [81, 82]. TRF2 is thought to play an additional role in protecting the telomere end from double-stranded-break repair factors, by aiding the formation of t loops [84, 85]. In t loops the single-stranded G-rich tail of the repeat folds back to anneal with internal repeats, forming a stable terminal loop [84]. Although t loops have been observed in *Trypanosoma* and mouse cells, as well as in humans, the frequency of their occurrence remains unclear, and it is likely that telomeres are stabilized by more than one mechanism [85].

Given their high degree of structural conservation, it was reasonable to speculate that telomeric silencing should also be conserved. Indeed, TPE exists in fission yeast [86], *Drosophila* [87], and in human somatic cells [88, 89]. To date little is known about the proteins that function in human and *Drosophila* TPE, but a luciferase reporter has been shown to be expressed 10-fold less efficiently when integrated near a truncated telomere in HeLa cells than when at internal sites. This telomere-proximal repression was sensitive to a general inhibitor of histone deacetylases, called trichostatin A, which does not inhibit the activity of the Sir2 family of NAD-dependent deacetylases [88]. Therefore, although Sir2 is conserved and may affect *Drosophila* PEV [90], there is no evidence that it affects TPE in organisms other than budding yeast.

In *Drosophila,* TPE and PEV appear to be qualitatively different, because pericentric insertions are generally much more repressed than telomeric insertions. Consistently, none of the 25 genes that influence the severity of centromeric variegation *in trans* have been found to affect TPE (reviewed in [91]). Moreover, all telomere-associated repressed transgenes analyzed to date have been shown to be embedded in or adjacent to telomere-associ-

ated sequences (TAS) and not within the retrotransposons HeT-A and TART [87, 92, 93]. This result may indicate that only the TAS repeats are packaged into a repressive chromatin structure [87].

Interestingly, HP1 was shown to associate with fly telomeres, even those lacking TAS, HeT-A or TART retrotransposons [24]. Because HP1 mutants show increased levels of telomere-telomere fusion [24], it was suggested that HP1 might be involved in telomere stabilization, rather than in telomeric silencing (fly telomeric silencing is reviewed in [93]). Like those of budding yeast, *Drosophila* telomeres are found clustered at the nuclear periphery [94], although their mechanism of anchoring and the importance of this clustering for telomeric repression are unknown.

The telomeres of fission yeast are also subject to transcriptional silencing, which results in the variegated expression of integrated reporter genes [86, 95]. Whereas TPE is only slightly affected by mutations affecting centromeric *trans*-acting factors (i.e., Clr1-4, Clr6, Swi6 and Rik1; [40]), another group of factors, *rat1*, *lot2*, *lot3/taz1* and *rap1,* specifically affect repression in telomeric sites [95, 96]. In *lot2* and *taz1* mutants, telomeres no longer associated with the spindle pole body at the premeiotic horsetail stage, although it is not known whether these mutations significantly impair telomere clustering in interphase of vegetatively growing cells [95, 97].

Silent mating-type loci

Both budding and fission yeasts are able to switch mating type because they maintain transcriptionally silent copies of both mating-type determinants in their haploid genomes. The repression at these silent loci is very similar to that found at either the telomeres or the centromeres of the species concerned. That is, repression of the *S. cerevisiae* mating-type loci requires Rap1 and the SIR proteins, whereas Clr1-4, Clr6, Rik1 and Swi6 serve this role in *S. pombe.* It is unclear whether the silencing mechanisms initially evolved for mating-type maintenance or for centromeric and/or telomeric functions.

Repression of silent mating-type loci in budding yeast

In budding yeast, haploid cells are 'sexed' with either **a**-type or α-type mating preferences, and this is determined by the *MAT* locus (*ma*ting-*t*ype locus) at which either **a1** and **a2** or α1 and α2 mating-type genes are expressed. Two related loci found near the left and right telomeres of chromosome III, called *HML*α and *HMR***a**, contain identical copies of the mating-type genes that are transcriptionally repressed. These silent copies allow mating-type switching to occur, a genetic event that changes the information present at the *MAT* locus through gene-conversion with either the *HML*α or *HMR***a** silent loci serving as donors (*HML*α donating its sequences to *MAT***a**, and *HMR***a** to *MAT*α). In haploid cells the *HM* loci must remain transcriptionally silent, because simultaneous coexpression of **a** and α mating-type gene products results in a nonmating **a**/α diploid cell.

The *HM* loci are flanked by two specific *cis*-acting sequences, the E and I silencers (<250 bp) on which the establishment and maintenance of their repression depends [98, 99]. Each silencer is composed of a specific combination of at least two of the three possible *cis*-acting sites that bind the *trans*-acting factors Rap1, ORC and Abf1. These *trans*-acting factors act as 'docking surfaces' for the Sir2-4 proteins, either directly or through Sir1, as for Orc1. Like telomeric silencing, *HM* repression requires an intact complex of Sir2, 3 and 4, whereas Sir1 facilitates the establishment of repression by helping recruit Sir4 to the silencer (reviewed in [7, 8]. Because the deletion of *SIR1* leads to semistable silencing and mimics certain alleles of Rap1 or mutations in the ARS sequence, it is thought primarily to aid the establishment of repression and not long-term maintenance like other SIR proteins [100].

Repression at telomeres in budding yeast can be considered a rudimentary or less robust form of its *HM* silencing. For example, whereas telomeric silencing is totally dependent on both histone H3 and H4 tails, *HML*α silencing is affected only by mutations in H4, and *HMR***a** repression does not require either the histone H3 or H4 N-termini [101–104]. If *HM* repression is weakened, e.g. by silencer mutation it also becomes fully dependent on histone tail integrity [105]. In addition, whereas telomeric silencing is exquisitely sensitive to SIR protein dosage [66] or to expression of subdomains of these proteins, *HM* repression is only mildly affected. Again, it is not true in silencer mutated strains [106].

The key to the robustness of *HM* silencing is redundancy. Redundancy is apparent in the organization of the *HM* loci, in that each domain is flanked by two silencers, even though a single silencer is sufficient to repress an adjacent promoter [98, 99]. Moreover, telomeric TG tracts which have a similar function, are found only on one side of the silenced gene. Intriguingly, natural protosilencers are found in subtelomeric repeats of native telomeres and cooperate with TG-bound Rap1 to promote repression [67, 68, 107, 108], but these are often counteracted by other subtelomeric sequences with antisilencing activities, called STAR elements (fig. 2; [67, 68, 107, 108]). Because the level of repression initiated at a silencer decreases proportionally with the distance to the silencer [66, 107, 108], the closer a promoter is to the nucleation sites, the more efficient its repression.

Further redundancy occurs within the structure of the E and I silencers themselves, as three of the four silencers

bind all three proteins, Rap1, Abf1 and the ORC complex, although any pair of these is sufficient to promote SIR recruitment in an appropriate context [99]. Finally, redundancy is also reflected in the chromosomal localization of the *HM* loci, as each is situated close to a telomere, a position that contributes to silencing efficiency [65]. Indeed, transposing *HM* loci to more internal sites affects their silencing properties. An 'internal' *HMR***a** locus is still silenced in wild-type backgrounds, it becomes sensitive to mutations in the histone H4 N-terminus [105, 109], whereas an 'internal' *HML*α can be repressed only if *SIR1*, *SIR3* and *SIR4* are co-overexpressed [65]. FISH data confirm that the proximity of the *HM* loci to telomeres favors their association with telomeric foci, where the concentration of SIR factors is high [71, 110]. Intriguingly, an *HML*α cassette carried on a centromere (CEN) plasmid is efficiently silenced in wild-type backgrounds, perhaps because centromeres cluster near the nuclear periphery (and hence near telomeric SIR pools) due to their interaction with microtubules extending from the spindle pole body [110].

Recent experiments show that *HM* silencing can be established in the absence of DNA replication, although it does require passage through S phase (for a review [111]). This finding provides an intriguing correlation with the S-phase-specific ability of SIR proteins to bind the nuclear envelope, suggesting a functional relationship between the establishment of repression and perinuclear position. Elegant recombination experiments have shown that both silencers and SIR proteins are essential for the stable maintenance of *HM* repression, even in cells arrested in a specific phase of the cell cycle [112–114]. The heterochromatin at silent mating type loci, despite its robust character, is therefore a dynamic structure with a continuous requirement for SIR proteins and the binding of an initiation complex at silencers, in both dividing and nondividing cells.

Repression of silent mating-type loci in fission yeast

Mating-type control in fission yeast resembles that in budding yeast, except that the active mating type loci are called mat1 P (plus) and M (minus) rather than **a** and α, and the mechanisms of repression is closely related to the Swi6-dependent centromeric heterochromatin. The *mat2* and *mat3* loci contain the same information as the *mat1-P* and *mat1-M*, respectively, but in a transcriptionally silent state (reviewed in [40]).

Repression at the silent mating-type region covers ~20 kb of chromatin, encompassing the *mat2*-K-*mat3* interval. It is bounded by two elements, the inverted repeats IR-L and IR-R, that delimit repression. As in *S. cerevisiae*, prototrophic markers introduced within this silent domain are subject to stable transcriptional repression. As at centromeres, mutations in *clr1-clr4*, *clr6*, *swi6* and *rik1* affect mating-type silencing [40]. A mechanistic link between the functional organization of centromeric and mating-type heterochromatin has been argued on the basis of sequence organization, because the K region contains a 4.3-kb cenH sequence homologous to the otr repeats found at centromeres [115]. Indeed, as in centromeric heterochromatin, mating-type silencing in fission yeast requires H3 K_9^{me} and Swi6 localization to the repressed *mat2-K-mat3* domain [32, 116]. Like the dg repeat at centromeres, cenH serves to nucleate repression at both ectopic and endogenous mating-type loci, and it does so by preferentially recruiting Clr4 [46]. The subsequent spreading of H3 K_9^{me} depends on Swi6, although here the resemblance to centromeric repression breaks down slightly: only the establishment of mating-type repression and not its maintenance appears to require the RNAi machinery [46].

Silencing rDNA tandem arrays

Although most repetitive arrays of DNA tend to be packaged into a transcriptionally silent state, the tandemly repeated rRNA gene array (rDNA) is an exception. Ironically, rRNA genes are among the most highly transcribed sequences in the nucleus, yet in many organisms these genes maintain close connections to heterochromatin. In *Drosophila,* rDNA is adjacent to centric heterochromatin, and in many other species, nucleoli and non centromeric heterochromatin are juxtaposed in interphase cells. In both budding and fission yeast, rDNA stability and expression are regulated by chromatin structure, which imposes positioned nucleosomes throughout the rDNA domain, rendering promoter regions inaccessible for transcription [117–120]. Indeed, although ~40% of the rRNA genes in *S. cerevisiae* are transcribed at a given time, the rest are actively repressed. The suppression of transcription and of homologous recombination between rDNA repeats requires the NAD-hydrolyzing deacetylase Sir2, as well as interacting partner called Net1 [9]. Unfortunately, compared with the extensive knowledge we have about mating type and telomeric and centromeric repression, the precise mechanism of rDNA repression is relatively obscure.

Repression of rDNA in budding yeast requires Sir2

As in other eukaryotes, *S. cerevisiae* ribosomal RNA genes encode a 35S RNA precursor. Unlike that in vertebrates, the 5S RNA gene is present with the 35S gene on a 9.1-kb unit, which is present in ~200 copies at one chromosomal location, on chromosome XII. This is found within a single large nucleolus.

The repression of *trans*-genes (i.e., Pol II transcribed genes) integrated into the rDNA (RPE for *r*DNA *p*osition

*e*ffect) has a high degree of variegation and a fairly unstable pattern of inheritance. RPE requires a multiprotein complex in which Sir2 is bound to Net1 and Cdc14 but not Sir3 or Sir4 [119, 121]. Net1 mediates the targeting of Sir2 to the rDNA, whereupon Sir2 triggers repression [121]. Net1 has an addition role, regulating exit from mitosis by sequestering the Cdc14 protein phosphatase in the nucleolus until telophase [122, 123]. Because of this dual function, the rDNA-silencing complex is referred to as the RENT complex (*re*gulator of *n*ucleolar silencing and *t*elophase exit). Because both Net1 and Sir2 are associated with DNA fragments throughout the length of rDNA repeats (Sir2 being particularly enriched at the NTS1/5S region), the Net1/Sir2 complex is thought to be able to spread along rDNA chromatin [69]. Indeed, a recent study has shown that Sir2-mediated silencing can spread a short distance, between 300 and 500 bp in a directional manner, beyond the rDNA repeats [124]. The direction of spreading is determined by the orientation of the PolI promoter, and silencing ironically appears to require some degree of PolI-mediated transcription. It is not known which component of the RENT complex binds DNA or nucleosomes or whether the rRNA or a particular PolI-associated factor is implicated in the repression mechanism.

Perhaps the most clear-cut function of Sir2 in the rDNA is to suppress recombination of the multiple rDNA repeats SIR2 deletion leads to rapid loss of rDNA repeats and eventually to cellular senescence [9, 10]. This phenomenon, like the silencing of PolII-transcribed genes, is tightly correlated with the establishment of a stable chromatin structure [118], and both the strength of rDNA repression and the suppression of recombination are directly proportional to *SIR2* dosage [9, 106, 125]. Although Sir4 is not directly required for RPE, it does regulate the amount of Sir2 available to the rDNA locus through its ability to bind and sequester Sir2. Upon deletion of *SIR4*, all detectable Sir2 relocalizes to the nucleolus and improves rDNA silencing, whereas *SIR4* overexpression titrates Sir2 away, decreasing rDNA repression efficiency [69, 119, 125]. Indeed, any event that influences the amount of Sir2 available for rDNA function is expected to modulate rDNA repression.

Sir2 is an NAD-dependent deacetylase

As mentioned above, the budding yeast Sir2 protein is the founding member of an evolutionarily conserved family of NAD-dependent deacetylases, which is characterized by an insensitivity to the standard deacetylase inhibitor trichostatin A [54]. Sir2-like proteins, Sirtuins, have been identified in species ranging from *Archea* and eubacteria to humans, showing variable numbers of homologues in each species [126]. Although some members, such as budding yeast *SIR2*, *HST1* and *HST3/HST4;* fission yeast *hst4*$^+$; and *Drosophila* Sir2, have roles in transcriptional silencing [90, 127–130], the biological functions of most other eukaryotic Sirtuins remain unknown. Intriguingly, like yeast Sir2, the *Caenorhabditis elegans* Sir2 appears to be involved in life-span regulation [131]. Other highly abundant homologues, such as yeast Hst2 and the human Sir2T2, are cytoplasmic [132–134] and are presumably engaged in other functions. Recent data suggest that mammalian Sir2T2 preferentially deacetylates α-tubulin in vitro, and unlike HDAC6, is able to deacetylate polymerized microtubules. Lowering enzyme levels in vivo lowered acetylated α-tubulin levels, suggesting that this may be one of its physiological substrates [135].

Additional studies indicate that nuclear Sir2 homologues also modify substrates other than histones. Human SIRT1 and the related mouse Sir2a enzymes are able to deacetylate p53 in vitro, while the mouse Sir2a acts on TAF_I68, a component of the transcriptional initiation complex for RNA polymerase I (reviewed in [136]). Current results present supportive, but not compelling, evidence that these latter two constitute physiological targets. Budding yeast strains lacking all five Sir homologues have a slight decrease, rather than an increase, in acetylated histone levels [137], again suggesting that most Sir2 family members modify substrates other than histone tails. Finally it has been proposed that the by-product of Sir2-mediated deacetylation, *O*-acetyl ADP ribose, may also have a biological function, acting as a second messenger within the cell [138].

Recent studies have shown that enzymatically inactive *sir2* mutants are defective for all types of silencing, including RPE [58, 139, 140], although the loss of silencing may have very different causes. Budding yeast cells appear to contain two distinct Sir2-containing complexes: the so-called „TEL complex“ contains Sir4 and two additional proteins, whereas the „RENT complex“ includes Net1 and six other proteins [141]. Whereas the TEL complex has a robust NAD-dependent histone deacetylase activity, the RENT complex has but weak NAD-dependent deacetylase activity and a vigorous NAD-independent one [141]. This pattern may reflect the presence of either of two histone deacetylases, Hos1 and Hos2, because the disruption of either of these enzymes leads to strong hyperacetylation of histone H4 K_{12} in the rDNA in vivo [142]. In this context it is striking that the deletion of *SIR2* does not trigger drastic changes in the acetylation status of H4 K_{16} in the rDNA domain [142], suggesting that other histone deacetylases play more important roles in rDNA repression. Accumulating evidence thus indicates that the importance of Sir2 activity and the substrates it alters are different for the telomeric and rDNA repression mechanisms.

A similar form of transgene repression occurs within the tandems arrays of 10.4-kb rDNA repeat units in fission yeast [120]. Four chromodomain proteins have been

found to participate in repression of reporter genes integrated into the rDNA, Chp1, Chp2, Swi6 and Clr4, although whether they act directly or indirectly is not yet known. In both budding and fission yeasts, further studies are needed to determine the relationship between stable rDNA chromatin structure and other mechanisms that establish long-range silencing at other sites.

Conclusions

A number of common features allow us to define parallels within the mechanisms that promote chromatin-mediated silencing in yeast and those that establish visually identifiable heterochromatin and its associated PEV in other organisms. Histone-tail modifications are a fundamental feature of both assembly mechanisms, but other modifications are also likely to play a role. Recent discoveries have allowed us to link histone-tail modifications to gene products that were previously identified as modifiers of position-effect variegation. The discoveries have been particularly powerful in the analysis of fission yeast centromeric and mating-type silencing. Although it is still not clear what role heterochromatin plays in the functions that are unique to centromeres and telomeres, a further analysis of mutant proteins involved in these structures will yield these answers. By analyzing both the similarities and the differences between yeast and humans, centromeres and telomeres, and constitutive and facultative heterochromatins, we are rapidly approaching a molecular definition of chromosomal states that were recognized by microscopists over 100 years ago.

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