Using genomics and proteomics to investigate mechanisms of transcriptional silencing in *Saccharomyces cerevisiae*

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**Abstract**

Silent chromatin in budding yeast is characterized by the presence of a specialized chromatin modification complex consisting of silent information regulator (Sir) proteins, closely packed pairs of nucleosomes, and hypoacetylated and hypomethylated histones. How this specialized chromatin is established, maintained and inherited has been extensively studied. Less investigated are the determinants that constrain its linear spread along the chromatin fibre and the manner by which it represses gene transcription. Here we review the essential features of SIR-mediated heterochromatin, and discuss genomic and proteomic approaches for discerning the composition of its boundaries and for elucidating the mechanisms by which it silences transcription.

**Keywords:** Sir proteins; silent chromatin; histone modifications; chromosomal boundary elements; RNA polymerase II; downstream inhibition model of silencing

**INTRODUCTION**

Transcriptional silencing is mediated by specialized chromatin structures that function in a regional, rather than promoter-specific, manner to repress gene expression. Such structures confer stable repression upon target genes, and are epigenetically inherited. This epigenetic mode of inheritance arises from specific features of chromatin (such as covalent histone modifications and the presence of non-histone proteins; discussed subsequently) that have the capacity to re-establish silencing where it existed during the previous cell cycle. How silent chromatin is assembled, maintained and inherited have been extensively investigated in the budding yeast, *Saccharomyces cerevisiae* [1]. Here we review its salient properties, and discuss recent genomic and proteomic approaches that have led to a deeper understanding of the boundaries that demarcate silent from active chromatin, and how similar strategies could be applied to unravel the molecular mechanisms by which silent chromatin represses transcription.

**SILENCING IS DISTINCT FROM GENE-SPECIFIC REPRESSION**

Silencing, as defined above, is distinct from a second type of negative transcriptional regulation, gene-specific repression. The latter is exemplified in yeast by the Snf6-Tup1 corepressor [2]. The two phenomena are similar in that both of them exert inhibitory effects on the gene being repressed in a way that prevents successful transcription by RNA polymerase II, and both employ nucleosome-modifying enzymes [in particular, histone deacetylase complexes (HDACs)]. They differ in that gene-specific repression targets only one gene...
promoter, and is mediated through operators that are located at or near the transcription start. Silencing, in contrast, involves assembly of a higher-order chromatin structure that can linearly spread over the chromosomal fibre and affect the expression of multiple genes. Interestingly, both gene-specific repression and silent chromatin-mediated repression can be relieved in response to environmental cues, although relief of repression in the case of Ssn6-Tup1 occurs through inactivation of sequence-specific DNA binding proteins [2], whereas derepression of silent chromatin stems from covalent modification of the chromatin modifying complex itself [3, 4].

**SIR PROTEINS TRIGGER FORMATION OF SILENT CHROMATIN**

In yeast, silent chromatin packages ~10% of the total genomic DNA and is concentrated in three general locations: the silent mating-type loci, the telomeres and the nucleolus. The products of four genes, termed Silent Information Regulators (SIRs), are critical for repressing transcription at the HMR and HML silent mating-type loci [5], sites of the strongest repression. Three of these proteins, termed Sir2, Sir3 and Sir4, form a chromatin modification complex that is recruited by sequence-specific proteins (ORC, Rap1 and Abf1) that bind to cis-acting silencers that flank each HM locus. The fourth Sir protein, Sir1, physically interacts with Orc1 (the largest of six subunits of ORC) and Sir4, facilitating the local recruitment of a Sir2/Sir3/Sir4 complex [6–8]. The Sir complex horizontally propagates via interactions with other Sir complexes as well as with nucleosomes bearing deacetylated lysines within the N-terminal tails of histones H3 and H4. Deacetylation of H4 Lys16 appears to be critical for spreading [9], which occurs in a stepwise fashion and involves sequential cycles of deacetylation catalysed by Sir2, a NAD⁺-dependent histone deacetylase [10, 11]. Indeed, deacetylation of H4 Lys16 and the resultant production of O-acetyl-ADP-ribose are necessary for the formation of the trimeric complex between Sir3 and Sir2/Sir4 [12]. Sir3 and Sir4 may play additional roles in formation of higher-order structures [13] and in anchoring silenced chromatin domains to the nuclear periphery, where Sir proteins are locally concentrated [14].

Mechanistically similar, although less robust, silencing occurs at telomeres; a related form of silencing, weaker than the other two and involving recruitment of a distinct Sir2-containing complex, is present within the ribosomal DNA (rDNA) repeats [1, 7, 15]. At all three loci, silenced chromatin has roles beyond transcriptional repression, including inhibition of genetic recombination and stabilization of chromosomal structure. The weaker silencing at telomeres reflects the fact that telomeric silencing, unlike HM silencing, is unstable and can spontaneously revert to a derepressed state [16]. It is thus not the level of repression that is less efficient at telomeric loci, but rather the stability of the silenced state, leading to the presence of two epigenetically distinct populations: those in which the telomeric gene is transcriptionally repressed, and those in which it is expressed.

It is noteworthy that although Sir1 is associated with centromeres [17], these structures do not exhibit characteristics of centromeric heterochromatin found in higher eukaryotes. Similar to metazoans, however, silent chromatin of S. cerevisiae is associated with the nuclear periphery, a consequence of the association of Sir4 with both the perinuclear protein Esc1 and the telomeric structural protein yKu80 [18], whose perinuclear ligand is unknown. Notably, neither the yKu complex (yKu70–yKu80) nor Esc1 is essential for HM silencing [18] although their elimination does lead to a loss of telomeric silencing [14].

**THE ROLE OF HISTONE MODIFICATIONS AND VARIANTS IN TRANSCRIPTIONAL SILENCING**

The role of histone modifications (and their corresponding enzymes) in SIR-dependent silencing has been extensively studied. A mutation in the histone methyltransferase, Set1, that prevents tri-methylation of H3 Lys4, perturbs telomeric silencing [19], as do mutations that block Dot1-mediated methylation of H3 Lys79 [20] and Rad6-mediated ubiquitylation of H2B [21, 22]. Deletion of the H2A variant, Htz1, similarly diminishes silencing at either HMR or telomeres [23]. In contrast, a null mutation in an HDAC, Rpd3, was found to enhance silencing [24]. If the effects of these chromatin modifiers were direct, then methylation of H3, ubiquitylation of H2B, acetylation of H3 and H4, and incorporation of Htz1...
into nucleosomes would all be predicted to enhance recruitment of Sir proteins to silent chromatin. However, genomic and proteomic analyses suggest that the effects of these mutations are indirect, and that the aforementioned modifications have the opposite effect. For example:

(i) There is a gradient of H4 Lys16 acetylation along a yeast chromosome, ranging from a hypoacetylated state near the telomeres to hyperacetylated in more distant regions. The dynamic balance of hyperacetylation/hypoacetylation is regulated by the opposing activities of the Sas2 HAT and the Sir2 HDAC [9, 25]. These observations support early findings that nucleosomes packaging the silent HM loci are hypoacetylated relative to active genes, as well as data suggesting that silencing per se and not merely the absence of transcription correlates with histone hypoacetylation [26].

(ii) Htz1 is located in euchromatic regions of the genome, and concentrated within positioned nucleosomes flanking the transcription start sites of most Pol II genes [27]. Its presence helps prevent the ectopic spread of silent chromatin [28]. Htz1 promotes full gene activation but does not generally impact repression [29]. Htz1 is acetylated at several Lys residues within its N-terminus, particularly Lys14, by the NuA4 and SAGA complexes [30, 31]; such acetylation contributes to blocking the spread of silent chromatin at telomeric heterochromatin boundaries [32].

(iii) Ninety percent of H3 molecules are endogenously methylated by Dot1 [33]. This observation, along with the facts that Sir3 exhibits reduced binding to telomeric loci in dot1Δ cells [33] at the same time that the number of Sir3 foci increases ~10-fold [34] indicate that H3 Lys79 methylation is not directly required for silent chromatin but rather is associated with active chromatin [33]. Indeed, Dot1 activity prevents the stable association of Sir proteins with many potential ‘proto-silencers’ (DNA elements harbouring binding sites for ORC or Rap1 proteins) dispersed throughout the genome [35, 36].

(iv) H2B ubiquitylation locally triggers Set1- and Dot1-mediated H3 methylation [21, 22]. As a result, Ubp10, a ubiquitin protease, is continuously required at telomeres to maintain a low level of ubiquitylation of H2B, which in turn ensures a low level of H3 Lys4 and Lys79 methylation and a high level of Sir2 occupancy [37].

Therefore, in yeast as in multicellular organisms, methylation of H3 (at Lys4 and Lys79) and acetylation of H4 (at Lys16) are associated with transcriptional activity [reviewed in 38]. The enzymes responsible for these modifications—Set1, Dot1 and Sas2, respectively—as well as Swr1, the enzyme responsible for replacement of H2A with Htz1 [39, 40], are continuously required to prevent the inappropriate binding of Sir proteins to euchromatin. Since Sir proteins are present in limiting quantities, mutations that interfere with genomic methylation or acetylation (or Htz1 deposition) tend to destabilize silencing at telomeres, where, as discussed previously, the intrinsic silencing is less stable than that present at the HM loci.

Consistent with this paradigm, conditional expression of Sir3 results in progressive inactivation of HMRα1; concomitantly and in parallel, levels of H3 acetylation diminish. H3 Lys4 and Lys79 de-methylation and full silencing takes several generations after Sir3 induction [41]. This indicates that the pathway of heterochromatin formation involves two phases, an ‘initiation’ phase resulting in a chromatin state that partially inhibits transcription but still retains histone methylation marks, and a ‘maturation’ phase resulting in a chromatin state containing approximately one Sir2/3/4 complex per nucleosome.

**COMPOSITION OF THE BOUNDARIES DEMARCATING ACTIVE FROM SILENT CHROMATIN**

**Molecular and genetic approaches**

Chromatin immunoprecipitation (ChIP) in combination with various genetic approaches has shown that SIR-mediated silent chromatin is prevented from spreading into active regions of the genome by cis-acting boundary elements [42–45]. Particularly well characterized is a barrier located ~1 kbp to the right of the HMR locus. This barrier consists of a tRNA gene, whose nucleosome-free promoter and associated Pol III initiation complex play a pivotal role in restricting the spread of Sir proteins [42, 43].
Also critical at this boundary are the SAGA and NuA4 histone acetyltransferase (HAT) complexes [45]. The presence of other factors, including the histone variant Htz1 [28], chromatin remodelling enzymes [44] and acetyl lysine-binding (bromodomain-containing) proteins [46] may contribute as well. At the HML locus, rightward spreading of silent chromatin is prevented by the presence of the I silencer, which establishes a boundary through its ability to promote unidirectional silencing [47]. The same may be true for the leftward spread of heterochromatin at HMR, where the E silencer appears also to be unidirectional, at least in its native context [48]. Interestingly, at an ectopic chromosomal locus, the HMRE silencer functions in an orientation-independent fashion [49].

Genomics and proteomics approaches
Chait and colleagues used a concerted genomics and proteomics strategy to dissect the composition of protein complexes that by genome-wide localization were found to be targeted to sites proximal or adjacent to silent chromatin [50]. These workers demonstrated that there are two boundary complexes, one containing DNA pol epsilon (among other proteins) and the other containing the ATP-dependent remodelling enzyme Isw2, the HAT Sas3 and the bromodomain-containing AAA ATPase, Yta7. Interestingly, both complexes are associated with nucleosomes exhibiting a distinctive modification state (H4 hyperacetylated at Lys16 while hypoacetylated at Lys5, Lys8 and Lys12) as determined by electrospray ionization mass spectrometry. The Isw2 complex associates with silent chromatin boundaries throughout the cell cycle, while the pol epsilon complex associates with chromatin only during the portion of the cell cycle when silent chromatin is duplicated and segregated. The identified boundary complexes play an important role in the epigenetic regulation of genes flanking the HM loci, as well as genes located in subtelomeric regions, including members of the FLO gene family [50].

THREE MODELS TO EXPLAIN HOW SILENT CHROMATIN REPRESSIONS TRANSCRIPTION
Despite the rather detailed understanding by which Sir heterochromatin is assembled, propagated and inherited, surprisingly little is known regarding the mechanisms by which it represses gene transcription. Three models have been proposed to account for Sir2/Sir3/Sir4-mediated silencing. Importantly, these may not apply to the silencing observed in rDNA for integrated Ty1 transposons or Pol II reporter genes [51, 52]. Silencing in rDNA chromatin requires neither Sir3 nor Sir4, and thus may not involve spreading. Rather, it appears to involve competition between RNA polymerases I and II, and perhaps also localization in a nucleolar compartment inaccessible to the Pol II machinery [53, 54]. The three models of HM and telomeric silencing are:

(i) Steric hindrance: The original model, it is based on several observations derived from study of the HM loci. First, silent chromatin is resistant to the activity of a variety of endogenous enzymes, including the HO endonuclease [55, 56], DNA repair enzymes [57] and ectopically expressed DNA methyl transferases [58, 59]. Second, a domain of ~3-kbp encompassing HMR evinces impaired accessibility to restriction endonucleases in isolated nuclei [60]. These and other observations led to the idea that silent chromatin represses transcription by physically blocking access of the DNA to sequence-specific activators, general transcription factors (GTFs) and RNA polymerase II (schematically summarized in Figure 1A).

(ii) Downstream inhibition: This model was suggested by structural studies conducted on hsp82 heat shock transgenes whose transcription was silenced by flanking ectopic HMRE silencers [49, 61]. Despite dramatically reduced basal transcription (100-fold for the most robustly silenced hsp82 allele), regulatory sites for the gene-specific activator [heat shock factor (HSF)] and TBP were maintained in an accessible chromatin state [61]. Moreover, as assessed by genomic footprinting, both regulatory elements—HSE1 and TATA—were strongly occupied under hyperrepressed conditions, resembling their state at the euchromatic, wild-type gene [49]. Indeed, ChIP analysis indicated that HSF, TBP and Pol II occupancy levels were close to wild-type [62], suggesting that silent chromatin represses hsp82 transcription at a step downstream of Pol II recruitment.
Such a transcriptional block must occur early, since even very short transcripts were undetectable [62].

It is possible that hsp82 transgenes are silenced in a novel manner, since heat shock promoters are unusual in that their chromatin configuration is held in an open state under non-inducing conditions, with Pol II and GTFs already recruited [63]. Arguing against this, the hyperrepressed HMR\textsubscript{a1} locus was likewise found to be permissive to TBP and Pol II binding [62]. In agreement, high-resolution mapping of the HMR locus revealed that the a1/a2 bidirectional promoter is accessible to micrococcal nuclease digestion, while its linked coding regions are assembled into closely packed pairs of positioned nucleosomes [64]. (HML evinces a similar chromatin organization [56].) Taken together, these studies support a model in which SIR-mediated silent chromatin permits binding of the gene-specific activator and formation of a pre-initiation complex (PIC) containing, at minimum, TBP and Pol II (Figure 1B). Cohabitation of the transcriptional machinery with components of silent chromatin may be mechanistically related to the way in which enzymes that mediate homologous recombination, site-specific recombination and retrotransposon integration all gain access to silent DNA within either HM locus [65–68].

(iii) PIC Interference: In agreement with the downstream inhibition model, Chen and Widom recently reported that the accessibility of both an exogenous exonuclease and Dam methyltransferase to the silenced HM loci was only 2-fold reduced compared their accessibility with euchromatic regions [69]. In addition, these authors found that a sequence-specific DNA binding protein (LexA) readily bound its cognate sites in silenced chromatin [69]. However, their ChIP assays failed to detect the presence of several components of the PIC—including TFIIB, TFIIE and Pol II—at the promoters of either HMR\textsubscript{a1} or HML\textsubscript{a1} [69]. These observations prompted an alternative model in which silent chromatin represses transcription by preventing the recruitment of GTFs and RNA polymerase II following

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**Figure 1:** Three models of SIR-dependent transcriptional silencing. (A) Steric hindrance. The gene-specific activator is prevented from gaining access to its cognate DNA site by silenced chromatin, preventing recruitment of the PIC. (B) Downstream inhibition. Both the gene-specific activator and components of the PIC, including TBP and RNA polymerase, gain access to the DNA, yet transcription is blocked at a downstream step. (C) PIC interference. The gene-specific activator gains access to its DNA binding site, yet recruitment of the PIC to the core promoter is blocked. Key: A, activator protein; shaded rectangles, factor-inaccessible (hypomethylated, hypoacetylated) nucleosomes; cross-hatched rectangles, factor accessible nucleosomes; circle, Sir2; speckled bar, Sir3; filled bar, Sir4; zig-zag line, DNA. The colour version of this figure can be found in www.bfgp.oxfordjournals.org
binding of the sequence-specific activator (Figure 1C).

The PIC interference model is intuitively appealing, as it offers a satisfying explanation for how silent chromatin represses transcription, namely, by blocking recruitment of the Pol II transcription machinery. Nonetheless, a recent high resolution genome-wide analysis of RNA polymerase II density by D.A. Brow and colleagues supports a basic tenet of the downstream inhibition model. These workers observed genome-average levels of Pol II within each silenced HM locus, as well as within the promoters of heterochromatic, telomere-linked genes [70]. In contrast, Pol II density was significantly reduced in the coding regions of the telomeric genes [70]. Together with the detailed analyses described above [49, 62], these ChIP-on-chip data support the notion that Pol II is recruited to silent gene promoters. But if Pol II is recruited to HM and telomeric promoters, how is transcription prevented? Transcription is a multistep process that can be regulated at different levels, including PIC assembly, open complex formation, promoter clearance, elongation and termination. Any step in this process could, in theory, be impaired by SIR-mediated silencing. Given the multiplicity of factors involved in transcription initiation, promoter clearance and elongation [reviewed in 71], it is conceivable that SIR targets one or more of these factors in addition to chromatin in triggering silencing. Indeed, mammalian Sir2 has been shown to negatively regulate the activity of TAF68 [72] and p53 [73, 74] through deacetylation. Sir proteins could also physically interact with the transcription machinery, in analogy with two heterochromatin-associated protein complexes in Drosophila, HP1 and PRC1 [75, 76].

A PROTEOMICS APPROACH TO CLARIFYING THE MOLECULAR BASIS FOR TRANSCRIPTIONAL REPRESSION

A powerful approach for furthering our understanding of the mechanism by which SIR-mediated chromatin silences transcription is proteomics. A recent global proteomics analysis of soluble complexes in S. cerevisiae [77] is revealing. As expected, Sir2 associated with a large number of partner proteins, consistent with previous work showing the existence of two independent Sir2 complexes, one nucleoplasmic and concentrated at the nuclear periphery (Sir2/3/4), and the other localized in the nucleolus (Sir2/Net1/Cdc14) [reviewed in 78]. Included amongst its partners is Rpbi0, a subunit of Pol II, and Pob3, a subunit of FACT, a Pol II-associated complex that facilitates elongation through chromatin [79]. Equally intriguing, Def1, a Pol II degradation factor required for ubiquitylation and proteolysis of arrested Pol II elongation complexes [80] and Pta1, an mRNA cleavage and polyadenylation factor that binds the phosphorylated CTD of Pol II [81], were found to interact with Sir3 [77].

It might be useful to extend this type of analysis to encompass DNA-associated complexes, in analogy to the approach taken by Chait and co-workers in their analysis of boundary elements discussed previously. Specifically, one could site-specifically excise the silenced HMR (or HML) locus from chromosome III, using a conditionally expressed recombinase (strategy pioneered by Gartenberg and colleagues [67] and schematically summarized in Figure 2). Silent HMR or HML chromatin ‘circles’ could then be affinity-purified from nuclear extracts of induced cultures using IgG-sepharose beads. This could be achieved by virtue of the binding of an ectopically expressed lacI protein whose lacO operator sites have been engineered as a tandem array adjacent to either the HMR or HML locus (modelled after the strategy of Belmont and colleagues [82]). Proteins isolated from these purified mini-circles could then be subjected to partial proteolysis, followed by mass spectrometric analysis. Of considerable interest would be a comparison of isogenic SIR+ and sir2Δ strains with respect to both GTF abundance and modification state. This assay could be also readily extended to an examination of a telomeric heterochromatin-silenced gene. It might be anticipated that, given the epigenetic nature of silencing (mosaic pattern of inheritance and the fact that transcriptional silencing is an all-or-none phenomenon), the GTF composition at telomeric promoters will be a hybrid between the fully off and fully on states. At the more robustly silenced HM loci, the GTF composition will represent almost exclusively the off state.

It is with much anticipation that we await the day when proteomic analysis of purified silent chromatin, such as envisioned here, is a reality.
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References


**Figure 2:** Proteomics strategy to elucidate the composition of silent chromatin. Silent domain bearing a tandem array of LacO operator sites (dots) is flanked by two loxP sites. Ectopically expressed, TAP-tagged lacI binds to the LacO array as indicated. Cre recombinase is then conditionally expressed, excising the domain as an extrachromosomal ring (ECR), which can be affinity-purified from nuclear extracts using standard TAP procedures [83]. Proteins isolated from the purified ECRs can then be resolved on one or two-dimensional gels and their identities determined by mass spectrometry.

**Key Points**

- A characteristic feature of silencing complexes is their capacity to linearly spread over the chromatin fibre.
- Histone H3 methylation at either Lys4 or Lys79 marks nucleosomes for active transcription.
- Other marks of active chromatin include acetylated H3 and H4 and ubiquitylated H2B.
- Boundary elements are required to prevent the spread of the Sir2/3/4 complex.
- A recent genome-wide map of Pol II density lends support to the downstream inhibition model.


