H3K9me-Independent Gene Silencing in Fission Yeast Heterochromatin by Clr5 and Histone Deacetylases

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Abstract

Nucleosomes in heterochromatic regions bear histone modifications that distinguish them from euchromatic nucleosomes. Among those, histone H3 lysine 9 methylation (H3K9me) and hypoacetylation have been evolutionarily conserved and are found in both multicellular eukaryotes and single-cell model organisms such as fission yeast. In spite of numerous studies, the relative contributions of the various heterochromatic histone marks to the properties of heterochromatin remain largely undefined. Here, we report that silencing of the fission yeast mating-type cassettes, which are located in a well-characterized heterochromatic region, is hardly affected in cells lacking the H3K9 methyltransferase Clr4. We document the existence of a pathway parallel to H3K9me ensuring gene repression in the absence of Clr4 and identify a silencing factor central to this pathway, Clr5. We find that Clr5 controls gene expression at multiple chromosomal locations in addition to affecting the mating-type region. The histone deacetylase Clr6 acts in the same pathway as Clr5, at least for its effects in the mating-type region, and on a subset of other targets, notably a region recently found to be prone to neo-centromere formation. The genomic targets of Clr5 also include Ste11, a master regulator of sexual differentiation. Hence Clr5, like the multi-functional Atf1 transcription factor which also modulates chromatin structure in the mating-type region, controls sexual differentiation and genome integrity at several levels. Globally, our results point to histone deacetylases as prominent repressors of gene expression in fission yeast heterochromatin. These deacetylases can act in concert with, or independently of, the widely studied H3K9me mark to influence gene silencing at heterochromatic loci.

Introduction

The mating-type region of the fission yeast *Schizosaccharomyces pombe* affords a well-defined system to investigate how heterochromatin histone modifications affect gene expression [1] (Figure 1A). The region comprises three cassettes, *mat1*, *mat2-P* and *mat3-M*. *mat1* contains and expresses either the P- or M- mating-type genes and thereby determines the mating-type of a cell. *mat2-P* and *mat3-M* contain the same genes and internal promoters of transcription as *mat1*, however these two cassettes are not expressed. They act as repressors of gene expression in fission yeast heterochromatin. These deacetylases can act in concert with, or independently of, the widely studied H3K9me mark to influence gene silencing at heterochromatic loci.


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Approximately 20 kb of DNA spanning *mat2-P*, *mat3-M* and the intervening K region are heterochromatic. Heterochromatin in this region is defined by H3K9me, the presence of chromodomain proteins, and hypoacetylation. Several histone deacetylases (HDACs) act in the region, in particular Chl3 and Chl6 [3,4]. H3K9me is catalyzed by Clr4, the sole H3K9 methyltransferase in *S. pombe* [5]. It is bound by Clr4 itself [6] and by three other chromodomain proteins, Swi6, Chp1, and Chp2 [7]. Clr4 is a Su(var)3-9 homolog and Swi6 and Chp2 are HP1 homologs.

Numerous studies have examined the mechanisms of recruitment of Clr4 to the mating-type region. A large region between *mat2-P* and *mat3-M*, cenH, is homologous to centromeric repeats [8]. Like centromeric repeats [9], cenH produces non-coding RNAs and small interfering RNAs [10]. It has been suggested that the non-coding RNAs are capable of attracting RNA interference (RNAi) factors to the region to somehow facilitate the establishment of H3K9me [11]. RNAi however is not absolutely required for H3K9me in the mating-type region since RNAi mutants lacking an essential RNAi component like Dcr1, Ago1, or Rdq1, are not distinguishable from wild-type cells unless heterochromatin
is artificially disrupted [7,11]. Even when heterochromatin is artificially disrupted, RNAi mutants are capable of re-establishing wild-type levels of H3K9me in their mating-type region [11]. The phenotype of the RNAi mutants can be explained by a redundant recruitment of Clr4 through the CREB-like transcription factor Atf1 bound at two sites near the mat3-M cassette [12,13]. The recruitment of Clr4 by Atf1/Pcr1 might be via a direct interaction between Clr4 and Atf1/Pcr1 [12] or it might be facilitated indirectly by histone deacetylation following the association of Clr3 and Clr6 with Atf1/Pcr1 [13,14]. Positive feedback loops strengthen H3K9me in the mating-type region, in particular Swi6 facilitates H3K9me in the centromere-proximal half of the mating-type region that includes mat2-P [11].

Other redundancies in the silencing mechanisms operating in the mating-type region are made obvious by two classes of epistasis analyses. One class of experiments combined mutations in the HDACs Clr3 and Clr6 [3]. The second class of experiments combined cis- and trans-acting mutations. These latter experiments involve two small elements, REII and REIII, adjacent to mat2-P and mat3-M respectively (Figure 1A). When combined with a mutation in Clr4 or other mutations in the Clr4 epistasis group, deletion of either REII or REIII causes a strong expression of the adjacent cassette [15,16,17]. This indicates the existence of a class of factors acting redundantly with Clr4 to silence mat2-P and mat3-M through REII or REIII. We present here the first characterization of a factor in this class, Clr5.

Results

Relative contributions of H3K9me and histone deacetylation to gene silencing in the mating-type region

The mat2-P cassette contains two genes, Pi and Pc, transcribed from an internal promoter [2] (Figure 1A). Whether these genes are expressed or not can be conveniently assayed in cells containing a stable, unswitchable, mat1-M cassette (mat1-Msmt-0). Because mat1-Msmt-0 cells cannot switch to mat1-P, they form colonies containing only cells of the M mating-type that fail to mate and sporulate due to the absence of compatible mating partners of the P mating-type in the same colony. The

Figure 1. Prominant role of histone deacetylation in the repression of mat2-P. (A) Schematic representation of the mating-type region. The region between IR-L (inverted repeat left) and IR-R (inverted repeat right) is heterochromatic. Binding sites for the Ste11 transcription factor located between cenH and REIII is not represented. A second Atf1 binding site located between cenH and REIII is not shown. The smt-0 mutation prevents switching of the mat1-M cassette allowing the expression of mat2-P to be assayed by iodine staining of colonies or by RT-PCR. Primers used for RT-PCR analysis are indicated by arrowheads below mat2-P and mat3-M. REII: repressor element II; REIII: repressor element III; cenH: centromere homology. (B) Iodine staining of wild-type (PG1789), clr4D (SPK450), clr3D (PG3564), swi6-115 (SPK29), clr6-1 (SPK467) and clr3A clr6-1 (PG3577) strains propagated on MSA sporulation plates. Dark iodine staining is due to haploid meiosis and reflects mat2-P expression. (C) Assay of mat2-P transcript levels by RT-PCR. RNA was prepared from strains induced to enter the meiotic program by 5 hours of nitrogen starvation in PM-nitrogen liquid medium. The strains are as in B.
uns switchable M colonies are not stained by iodine vapors, a stain specific for S. pombe spores. In this strain background expression of mat2-P from the normally-silenced region leads to haploid meiosis and spore formation. Hence the derepression of mat2-P can be monitored as an increase in iodine staining of mat1-MMS1-0 colonies, or by RT-PCR estimating the level of mat2-P transcripts in mat1-MMS1-0 cell cultures. As shown in Figure 1, the lack of Clr4 or Swi6 does not increase mat2-P expression significantly. This observation implies that the Clr4/Swi6 pathway of heterochromatin assembly is largely dispensable for the transcriptional repression of the mat2-P mating-type cassette.

Previous studies have indicated that a ure4+ reporter gene placed near mat2-P is tightly repressed in wild-type cells and derepressed by mutations in Clr4 or Swi6 [15]. However, even though it permits growth in the absence of uracil, remarkably little ure4+ transcript is present in the mutants [17]. The pronounced residual repression of ure4+ in Clr4 or Swi6 mutants is consistent with the effects observed here on mat2-P expression.

Unlike H3K9 methylation, several enzymes catalyze histone deacetylation redundantly. Impairing the Clr3 and Clr6 deacetylases simultaneously leads to full derepression of mat2-P evidenced by dark iodine staining of mat1-MMS1-0 colonies, high levels of haploid meiosis, and accumulation of mat2-P transcripts (Figure 1B and 1C). This derepression shows that histone deacetylases contribute strongly to the transcriptional repression of mat2-P. In contrast, deletion of the H3K9 methyltransferase Clr4, which prevents all H3K9me accumulation, causes no detectable elevation in mat2-P expression in these assays. These data suggest that silencing factors operating redundantly with the Clr4/Swi6 pathway remain to be identified.

Genetic screen for silencing factors independent of Swi6 and Clr4

We set up a genetic screen for factors acting redundantly with Swi6 and Clr4 (Figure 2). The screen was conducted in the S. pombe strain SPK29 following insertional mutagenesis with an S. cerevisiae LEU2 gene. SPK29 cells contain the unswitchable mat1-MMS1-0 allele, an S. pombe ure4+ reporter gene near mat2-P (Δ[Xhol]-ura4+), and a non-functional swi6+ gene (swi6-115). SPK29 mutants in which mat2-P is expressed were sought by screening for colonies stained darkly by iodine vapors under conditions of nitrogen starvation. Five mutants displaying a stable dark-staining phenotype and high levels of haploid meiosis were isolated among approximately 400,000 Leu+ colonies screened.

In two of the isolated mutants LEU2 was inserted in the mating-type region, in mat2-P and in its REI silencing element, respectively (SPK141 and SPK127 mutants; data not shown). Insertions disrupting REI are expected to display a cumulative effect with swi6-115 [15]. The remaining three LEU2 insertions defined a genetic locus linked to the mating-type region that we named clr5. (cryptic loci regulator 5; SPK129, SPK137 and SPK142 mutants; Figure 2A and 2B). mat2-P is strongly derepressed in clr5::LEU2 swi6-115, clr5::LEU2 clr3Δ or clr5::LEU2 clr6A double mutants but not in any of the single mutants (Figure 2). These phenotypes imply that Clr5 acts upon mat2-P in a pathway different from Clr3, Swi6 and Clr4 otherwise no cumulative effects would be seen when the mutations are combined. In contrast, no cumulative effects were observed in the mating-type region when clr5::LEU2 was combined with clb6-1, suggesting Clr3 and Clr6 act in the same pathway (Figure 2C and 2D). These epistatic relationships were clearly observed when examining mat2-P transcription, and they also seemed to apply to the cenH element (Figure 2D and see below). Although centromeric transcripts were detected at the same time as cenH transcripts in Figure 2D, potential effects of Clr5 at centromeres were not investigated further.

Clr5 contains a conserved domain defining a new protein family

The clr5::LEU2 insertion sites in SPK129, SPK137 and SPK142 were mapped by inverse PCR identifying the clr5 locus as the predicted open reading frame (ORF) SPAC29B12.08 (see Figures S1 and S2 for details). We refined the definition of SPAC29B12.08 by experimentally mapping an intron close to the 5’ end of the gene that was missing in the original database annotations. We also identified three mutations in SPAC29B12.08 obtained in independent mutant screens for a clr5+ phenotype (Figure S1). Deleting the complete clr5 ORF produced phenotypes indistinguishable from the original clr5::LEU2 insertions (see below). Clr5 tagged at its C-terminus with GFP localized predominantly in nuclear dots. It appeared to be at least partially excluded from the nucleolus (Figure 3).

The N-terminal part of the predicted Clr5 protein contains a domain conserved in fungal species (Figure 4A). To our knowledge, this domain had not been noticed before even though >100 family members containing this domain could be identified by BLAST searches at NCBI, a few of which are displayed in Figure 4. In most cases, the domain was found close to the N-terminus of the protein. The second distinguishable feature of Clr5 is that the central and C-terminal portion of the protein display unstructured properties (Figure 4B). Comparing Clr5 with its predicted homologs in Schizosaccharomyces japonicus and Schizosaccharomyces octosporus, the closest sequenced relatives of S. pombe, we observed a much higher sequence conservation in the N-terminal part of the three proteins than in their C-terminal part as expected for structured vs. unstructured regions (Figure 4B). Many proteins with Clr5-related N-terminal domains contain unstructured regions in their C termini, like Clr5. Others contain Ankyrin repeats (Figure 4C).

Transcriptional signature of clr5Δ mutant

clr5+ mutants display a growth defect (Figure S1) that is not simply explained by the derepression of the mating-type region but rather suggests additional targets of Clr5. In an attempt to identify these targets, we examined the transcription profile of cells lacking Clr5.

The expression profile was established in h clr5Δ cells. The h background is routinely used for microarray analyses i.e. [18]. In this specific case, it ensures that the variations observed between h clr5Δ cells and the h clr5+ control strain are not due to indirect effects through mat2-P derepression since mat2-P is lacking in h cells.

A striking overlap was observed between genes upregulated in clr5Δ cells and in cells overexpressing the master regulator of cell differentiation Ste11, or in cells in which the meiotic program had been induced (Figure 5A, 5B, and Figure S3). Ste11 is a transcription factor regulated by phosphorylation and by positive transcriptional feedback as cells respond to pheromones, prepare for mating, and undergo meiosis. In wild-type cells Ste11 activates the transcription of a series of genes involved in mating and sporulation including the two M-specific genes contained in mat1-M and the two P-specific genes contained in mat1-P. Our microarrays suggest that Ste11 itself, and possibly some of its downstream targets, are repressed by Clr5.

The fact that the same promoters of transcription are present in mat2-P and mat3-M as in respectively mat1-P and mat1-M including Ste11-binding sites (Figure 1A) raised the possibility that the increased expression of mat2-P in clr5Δ swi6-115 cells results from
increased Ste11 activity in these cells. However, induction of Ste11 by nitrogen starvation in mat1-Msmt-0 swi6-115 cells (Figure 2A), or expressing Ste11 from the thiamine-regulatable nmt1 promoter in these cells (Figure 5C), did not lead to the high frequency of haploid meioses caused by clr5D in the same genetic background, indicating the effects of clr5D in the mating-type region are not simply due to derepression of Ste11.

In addition to its effects on ste11+ and downstream effectors, we found that Clr5 acts together with the Cln6 deacetylase on a number of other targets (Figure 5A). The overlapping function of Cln5 and Cln6 is fully consistent with the epistasis analysis presented above suggesting that Cln5 and Cln6 repress the mating-type region together (Figure 2A and 2D). Cln5 and Cln6 also have non-overlapping roles in gene regulation consistent with Cln6 participating in various protein complexes.

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**Figure 2.** Cln5 acts in the same pathway as the HDAC Cln6 and represses mat2-P independently of Swi6, Cln3, and Cln4. (A) SKP29 and mutants obtained by insertional mutagenesis in SKP29. Colonies formed on MSA sporulation plates were stained with iodine (top panels). All strains contain the mat1-Msmt-0 cassette hence like in Figure 1 staining correlates with mat2-P expression. Cells from the same strains were imaged by DIC (middle panels) or fluorescence microscopy following DAPI staining (bottom panels). Spores are visible in DIC and as multiple DAPI-stained nuclei in clr5-129 swi6-115 (SKP129), clr5-137 swi6-115 (SKP137), and clr5-142 swi6-115 (SKP142) double mutants but not in the swi6-115 (SKP29) unmutagenized strain. (B) Real-time RT-PCR quantification of mat2-P transcript presented as mat2-P/actin ratios normalized to wild-type levels. RNA was prepared from cells propagated for 5 hours in ME. Strains from left to right: PG1789, SKP29, SKP129, SKP137, SKP142 and SKP368. (C) Epistasis analysis. mat1-Msmt-0 colonies formed on MSA sporulation plates were stained with iodine. Full derepression of mat2-P is observed when defective clr5 and clr3 or clr4 alleles are combined indicating Cln5 acts in a pathway distinct from Cln3 and Cln4. In contrast, no cumulative effect is seen when combining defective clr5 and clr6 alleles indicating Cln5 and Cln6 act in the same pathway, at least for their effects in the mating-type region. Top panel: PG1789, SKP450, PG3564, SP1240, PG3577. Bottom panel: SKP368, SKP447, SKP415, SKP493. (D) mat2-Pc and transcripts with centromere homology originating from centromeres (dh) or the mating-type region (cenH) were detected by RT-PCR using the same strains as in C.

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**Figure 3.** Localization of Cln5-GFP. Cells were propagated in EMM2+supplements to early log phase. Cln5-GFP was expressed from the endogenous clr5 locus, under control of the clr5 promoter. The strain was FY15231.

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Figure 4. Features of the Clr5 protein. (A) The N-terminus of Clr5 (first 120 amino acids) was compared to NCBI and Broad Institute databases by BLAST. Protein sequences retrieved in the searches were aligned using Multalin and manually annotated. Twenty four sequences are displayed below S. pombe, from top to bottom gi|213401369| S. japonicus; SOCG_04578 S. octosporus; gi|116202587| C. globosum; gi|145610619| M. grisea; gi|156060797| S. sclerotiorum; gi|171682396| P. anserina; gi|189207214| P. triticirepentis; gi|169619663| P. nodorum; gi|85085946| N. crassa;
Since the Clr3 and Clr6 deacetylases act redundantly on many genes [10] we compared the expression profiles of clr3A and clr6A clr6-1 mutants (Figure 5D and 5E). This comparison identified several genes with correlated expression values. In total 28 genes were commonly upregulated in the two mutants. By analyzing the genomic distribution of these genes we found a region spanning 11 subtelomeric genes that were upregulated in clr3A mutants (9 of 11 genes), clr3A clr6-1 double mutants (7 of 11 genes) and in clr6-1 mutants (5 of 11 genes; Figure 5E). Genes in this region are also induced during the meiotic program as a response to nitrogen starvation [19], and recently this region was found to favor neo-centromere formation [20] indicative of a unusual chromatin structure.

Range of action of Clr5 in the mating-type region

Heterochromatin spans ~20 kb in the mating-type region. mat2-P is close to the centromere-proximal edge of the heterochromatic domain, mat3-M close to its centromere-distal edge, and ~15 kb of heterochromatin separate the two cassettes (Figure 1A). Clr5 was identified because it represses mat2-P. We investigated whether Clr5 also represses mat3-M and/or reporter genes placed between mat2-P and mat3-M.

Whether Clr5 represses mat3-M was assayed using cells containing a stable mat1-P allele (mat1-PA17; Figure 6A). Expression of mat3-M was monitored in these cells by measuring haploid meiosis – driven by the co-expression of mat1-P and mat3-M, and by RT-PCR. The RT-PCR conditions we used failed to detect mat3-M transcripts in the clr3A and clr4A single mutants, however we observed occasional haploid meioses in clr3A or clr4A colonies indicating a low level of mat3-M transcription occurs in these mutants. In the double clr3A clr5A and clr4A clr5A mutants, both haploid meioses frequency and mat3-M transcript levels were increased. These effects of Clr5 at mat3-M appeared much less pronounced than the effects of Clr5 at mat2-P as judged by the iodine staining of mat1-PA17 clr3A clr4A colonies compared with mat1-Matr-0 clr3A clr4A colonies, however the abundance of mat3-M transcripts was clearly increased in the double mutants (Figure 6A). These observations show that Clr5 contributes to the repression of mat3-M – albeit to a comparatively low level – and that, at mat3-M like at mat2-P, repression by Clr5 is redundant with repression by Clr3 or Clr4 (Figure 6A).

As mentioned above the transcriptional repression of transgenes placed in the mating-type region is alleviated in mutants belonging to the Clr4/Ste6 pathway, but the transcript levels are not as high as when the genes are transcribed from a euchromatic location [17,21,22]. It is therefore possible to ask whether factors of interest contribute to the repression redundantly with Clr4 or Ste6 by examining the mat4P transcript levels in double mutants. We observed that mat4P inserted near mat2-P (Figure 1; mat2-P(XbaI)::ura4P) was more strongly expressed in the clr5-142 swi6-115 double mutant than in either single mutant (Figure 6B and Figure S4). We also observed increased accumulation of cenH transcripts in the clr5-142 swi6-115 and clr5-142 clr4A double mutants (Figure 2D and Figure 6B). These widespread effects strengthen the conclusion that Clr5 does not act solely through Ste11 to activate the mating-type genes specifically.

Clr5-responsive cis-acting elements

The RNAi pathway has been proposed to recruit Clr4 to the mating-type region by acting upon non-coding transcripts generated from the cenH element. Consistent with this proposal, deletion of cenH affects H3K9me in the mating-type region. Cells lacking cenH adopt one of two semi-stable epigenotypes: one similar to wild type displaying normal levels of H3K9me and one similar to the clr4A mutant characterized by reduced H3K9me [11,23,24]. The fluctuations between two phenotypes can be understood in the frame of models postulating that the establishment and maintenance of heterochromatin proceed through distinct mechanisms. One such model would be that cenH facilitates the establishment of H3K9me in wild-type cells without being necessary to the subsequent maintenance of the H3K9me state. The fluctuations between two epigenetic states can be followed experimentally using reporter genes, for example replacement of cenH with ade6P leads to variegated ade6P expression [25]. Noticeably, mat2-P remains silent in cenH::ade6P cells regardless of the expression state of ade6P (Figure 6C) in agreement with H3K9me being dispensable for the repression of mat2-P. Our observations with clr3A clr4A mutants suggested that combining clr3A with cenH should lead to a cumulative derepression of mat2-P. Indeed, deleting clr5 in cenH::ade6P cells increased the expression of mat2-P (Figure 6C). Furthermore, as with cenH::ade6P single mutants, fluctuations between two phenotypes still occurred. Similarly, deleting clr5 in a clr4A background released the repression of mat2-P in a variegated manner (Figure S5). We conclude from these observations that Clr5 insures a cenH/RNAi-independent silencing in the mating-type region.

We tested in a similar manner whether Clr5 exerts its effects through the REII or REIII silencing elements found near mat2-P and mat3-M respectively by combining clr5A with deletions of these elements. Deleting clr5 in cells lacking the mat3-M-adjacent element REIII lead to a small cumulative, variegated, derepression of mat3-M (Figure 6D) placing clr5 in a pathway different from the REIII pathway. In contrast to the situation with cenH or REIII, deleting clr5 in cells that lack REII did not increase the expression of mat2-P (Figure 6D). This supports the notion that Clr5 acts through REII, a proposition substantiated by the effects of clr5A on ectopic silencing reporters (see below) and by the fact that an REII insential mutant had been obtained in the same genetic screen as the clr5A::LEU2 mutants.

REII-mediated silencing at an ectopic site requires Clr5

To further test whether REII and Clr5 participate in the same silencing mechanism, we asked whether REIImediated silencing at an ectopic site depends on Clr5. Insertion of a cenH sequence adjacent to an ade6P reporter gene at an ectopic site confers partial heterochromatin silencing on ade6P [26]. Changes in the expression state of ade6P can be monitored at the colony level by a color test. Cells expressing ade6P produce white colonies while cells that fail to express ade6P produce red colonies or sectors due to the accumulation of a red byproduct in the adenine biosynthetic pathway. Hence, establishment of silencing can be monitored as a change from white to red and loss of silencing as a change from red to white. Silencing of ade6P::cenH is established at a very low
Figure 5. Transcription signature of clr5 Δ mutant. (A) and (B) The list of genes upregulated >2 fold in clr5 Δ cells was compared with the list of genes upregulated >2 fold in respectively clr6-1 cells [18], cells over-expressing Ste11 [65], and cells induced to undergo meiosis by 4 hours of nitrogen starvation [19]. P-values reflect the significance of gene list overlaps. (C) Over-expressing Ste11 from the pREP1-ste11 plasmid does not confer the same sporulation phenotype as deleting clr5 to a swi6-115 mutant. Sporulation was assayed on MSA medium lacking leucine and thiamine. mat1-Msmt-0 cells were PG1789 (wt); SPK29 (swi6-115); SPK464 (clr5 Δ) and SPK142 (clr5-142 swi6-115). A switching-competent h90 strain was used as an additional control for sporulation, WT139. (D) As A and B but comparing with clr3 Δ clr6-1 double mutant. (E) Transcriptional signature (mutant/wt ratios) of genes from a subtelomeric region of chromosome 1 (this study), [18]. Asterisks represent missing data points. Stippled lines indicate 2 fold
up- or down-regulation. The inset examines the distribution of genes upregulated >2 fold in the clr5Δ mutant (average of two arrays) for part of chromosome 1, plotting the probability of the observed distribution in a 20-gene sliding window. The orange line represents a P value of 0.05, while the red line represents a P value of 0.001. The peak is a 20-gene window centered on SPAPJ695.01c (P = 1.1e⁻⁸).

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Histone modifications in clr5 mutants

The genetic interactions between clr5, clr3, clr6, and clr4 suggested the chromatin structure of the mating-type region might change in some of the double mutants, accounting for changes in gene expression. Hence, H3K9 methylation (H3K9me2) and
Figure 7. Clr5 mediates gene silencing at an ectopic site in an REII-dependent manner. (A) Schematic representation of constructs inserted at the ade6 locus to monitor the effects of cenH, REII, REIII, and selected trans-acting factors on ectopic silencing. (B)–(D) Silencing of the REII-ade6
cenH ectopic construct depends on Clr5. (B) clr5+ and clr5Δ strains with REII-ade6
cenH were propagated on plates poor in adenine (AA with 15 mg/l adenine). On these plates, cells in which REII-ade6
cenH is repressed form red or pink colonies and cells in which REII-ade6
cenH is expressed form white colonies. Cells from white (C) or red (D) colonies were replated on medium with a low adenine concentration and white, pink, and red colonies were counted, hereby determining the proportion of cells that had changed their epigenetic state. At least 200 colonies were counted on each plate. The ade6
cenH WT strain was AP2374; the REII-ade6
cenH strains marked REII were WT: AP270; clr5Δ: AP2354; dcr1Δ: AP2403; the REII-ade6
cenH strains marked REIII were: WT: AP1665; clr5Δ: AP2346, dcr1Δ: AP2406. doi:10.1371/journal.pgen.1001268.g007

Discussion

The mechanisms by which H3K9me is brought about in defined chromosomal regions of fission yeast have been extensively studied in the last decade. Perhaps because of this widespread interest, H3K9me tends to be equated with heterochromatin while histone deacetylation in the same regions has often been presented as a simple pre-requisite for H3K9me. Recent studies have
proposed an additional, more direct, role of histone deacetylation in heterochromatic gene silencing [14,27–30]. However, this role has been discussed exclusively in the context of H3K9me, that is, histone deacetylation has been presented only as a facilitating factor for, or consequence of, H3K9me. Arguing against these widespread views we found that some essential properties of heterochromatin are largely independent of H3K9me and rely instead on deacetylation and on a hitherto uncharacterized factor, Clr5. These H9K9me-independent mechanisms of repression act in parallel and/or cooperate with H3K9me-dependent mecha-
acetylation, examined at meiosis was much more strongly affected in the double

\[ \text{clr4} \] lacking Clr4 (Figure 1, Figure 2, Figure 6, Figure 8; data not shown) in wild-type cells. Their repression was largely retained in cells and 

\[ \text{mat2-P} \]

are tightly repressed by heterochromatin in \( S. \) pombe. Histone modifications to ensure a very tight repression of the mating-type genes in 

\( S. \) pombe

have a broad impact on global gene expression in fission yeast \([10,31–33]\). The experiments presented here document critical effects of HDACs at the silent mating-type cassettes as well. 

\[ \text{mat2-P} \] and \[ \text{mat3-M} \] are tightly repressed by heterochromatin in wild-type cells. Their repression was largely retained in cells lacking Clr4 (Figure 1, Figure 2, Figure 6, Figure 8; data not shown) showing H3K9me is not necessary for silencing. Repression was much more strongly affected in the double \( \text{clr3} \) \( \text{clr6} \) HDAC mutant. Histone acetylation, examined at \[ \text{mat2-P} \], was as expected increased in the \( \text{clr3} \) \( \text{clr6} \) mutant correlating with \[ \text{mat2-P} \] expression (Figure 8). Whether increased acetylation was the sole cause for derepression of the mating-type region in the \( \text{clr3} \) \( \text{clr6} \) mutant is unclear since this mutant also lacked H3K9me at \[ \text{mat2-P} \] (Figure 8D) leaving open the possibility that loss of silencing results from a combination of increased acetylation and reduced H3K9me. More generally full expression of \[ \text{mat2-P} \] was observed only in mutants lacking both a H3K9me pathway component (Swi6, Clr3 or Clr4) and a component belonging to the REII/Clr6/Clr5 epistasis group (Figure 1, Figure 2, Figure 6, Figure 8) highlighting the redundancies and cross-talks which take place between deacetylation and H3K9me pathways to elicit full silencing in the mating-type region. Relatively little is known regarding the mechanisms by which histone modifications facilitate or inhibit gene expression in any eukaryote. In fission yeast, Clr3 preferentially deacetylates H3K14ac and Clr6 deacetylates several lysines of histone H3 and H4 [4]. Both enzymes repress transcription by limiting the access of Pol II to heterochromatin \([14,27–30]\). The H3K9 methyltransferase Clr4 has also been reported to restrict Pol II access to heterochromatin [34]. This might be through a direct effect of H3K9me, or it might be through the ability of Clr4 to indirectly recruit HDACs. Clr4 fails to associate with \[ \text{mat2-P} \] in \[ \text{swi6} \] mutants [14] suggesting it also fails to associate with \[ \text{mat2-P} \] in \[ \text{clr4} \] mutants. Other studies indicate the chromodomain protein Chp2 bound to H3K9me recruits the Clr3-containing complex SHREC while Swi6 recruits the Clr6-containing complex Clr6 CII \([28–30]\). HDACs and HMTs are found in complexes in higher eukaryotes and HP1, like Swi6 or Chp2 in \( S. \) pombe, can bridge H3K9me with HDACs \([35,36]\) indicating transcriptional repression by H3K9me might generally occur through the action of HDACs. In the present case, the ability of Clr4 to indirectly recruit HDACs might account for its redundant effects with Clr5.

Genes placed in heterochromatic regions can remain sensitive to transcriptional activation. For example in \( S. \) cerevisiae, a \( URA3 \) gene inserted near a telomere is silenced by the Sir proteins and histone deacetylation but its expression can be stimulated by increased levels of Ppr1, a transcriptional activator of \( URA3 \) [37]. Similarly, lack of Ppr1 increases \( URA3 \) silencing at the silent mating-type loci in \( S. \) cerevisiae mutants partially deficient for silencing [38] By analogy, increased expression of the \( stel1^{+} \) gene in \( clr3A \) mutants suggests a mechanism for the high haploid meiosis observed in for example \( clr3A \) \( clr4D \) mutants. Namely, the loss of H3K9 methylation combined with the presence of an activated transcription factor increases transcriptional activity at the normally-silent mating-type cassettes. Arguing against this simple model, we found that overexpressing \( stel1^{+} \) in \( swi6-115 \) cells starved for nitrogen does not lead to high levels of haploid meiosis (Figure 5C), indicating the effects of \( clr3A \) in the mating-type region are not solely due to increased \( stel1^{+} \) expression in this mutant. Our data do not exclude more complex models where down-regulation of the \( stel1^{+} \) gene or of the Ste11 protein activity by Clr5 would contribute to silencing in the mating-type region.

Our observations expand current models for silencing in the mating-type region (Figure 9). We propose that Clr5 and deacetylation – of histones and possibly other as-yet-unidentified substrates of Clr3 or Clr6 - repress \[ \text{mat2-P} \] via the REII element. Independently, deacetylation would proceed from Atf1-binding sites near \[ \text{mat3-M} \] as proposed by others [12,13] and perhaps through some other DNA element in REII distinct from the Atf1-binding sites [16]. The effects of Clr5 and Atf1 would not be strictly local, however each factor would predominantly affect the region close to its cognate cis-acting element. H3K9me spreading from the cenH nucleation site would further facilitate deacetylation and gene repression throughout the region \([14,28–30]\). Even in the absence of Clr4 and H3K9me, a substantial repression would be achieved, sufficient to prevent haploid cells from undergoing meiosis.

It has previously been proposed that REII and REIII might be transposon remnants capable of mediating silencing in cis like LTRs do in the case of retrotransposons, through histone deacetylation [39]. Our data suggest that the function of Clr5 at REII might be evolutionarily comparable to the function of Atf1 at REIII. Clr5 and Atf1 are functionally related in several other ways. In addition to being both required for transcriptional repression in the mating-type region \([12,13]\) (this study) both Atf1 and Clr5 regulate \( stel1^{+} \) [40] (Figure 5). Through these points of action, both factors prevent untimely meiosis. Atf1 is responsible
for other chromatin-mediated effects unrelated to transcription for example effects on recombination and transposition [41,42]. Similarly, Clr5 has other functions than those described here such as a role in DNA repair suggested by the hypersensitivity of **clr5Δ** cells to DNA-damaging agents [43]. This role in the resistance to DNA damage might be performed together with Clr6, like gene repression, since **clr6-1** mutants are also sensitive to DNA-damaging agents [44]. Clr5 might furthermore affect genome integrity through its control of a large region prone to neocentromere formation [20] (Figure 5). Unlike Atf1, Clr5 does not belong to a well-described family of transcription factors, however all known characteristics of Clr5 are compatible with a role in chromatin organization and transcription. For instance Clr5 localizes to the nucleus, the transcription profile of the **clr5Δ** mutant is consistent with Clr5 regulating transcription through deacetylation, and the predicted physical characteristics of Clr5 are also compatible with a role in transcription.

The Clr5 protein is predicted to contain a large disordered region. Intrinsically unstructured proteins (IUPs) are a large group of proteins that lack well-defined secondary and tertiary structures, reviewed in [45,46]. Many IUPs interact with other proteins via their disordered region, which has been proposed to undergo induced folding upon interaction with a binding partner [46]. Transcription factors are abundant among IUPs for example Jum, p53, Myb, and CREB contain unstructured domains. Similar to histone tails, their disordered nature allows access for various covalent modifications such as phosphorylation, ubiquitination, and acetylation, facilitating the concomitant folding and interaction with binding partners.

In addition to its large predicted disordered region the Clr5 protein contains a hitherto undescribed domain in its N-terminal region. This domain and its N-terminal location are conserved among a family of fungal proteins of currently unknown function.

The strains used in this study and their genotypes are listed in Table S1. Some were published previously as indicated [15,16,18,26,52,53,54]. The **clr5Δ** ORF was replaced with the **hpl1Δ** gene, which confers resistance to hygromycin B, by transforming SPK29 with a PCR product amplified from pCR2.1-hpl1 [55] with GTO-312 (TTACATTTTCCGGGGGTGTAACCTGGATC-GCTCTATTTTACATTTAACAGTGTTGTCATTGGTCG-ACTAAATTGTACAGACTTTTCACCCCTACCATTTAGGA-CACCACATGACCAATCCTG66CACTTCAAATC-TGCA-ACCTTGAACACACTTAAAGCAAGGAAAGGTGGAT-CAAGGATCCCGGGTTATATTAA) and GTO-313 (TAGG-CAGGTAGAGGCAGGTGTAAGAAATAATTAATAAAA-ATCCTAATAATTAATTGAGAACACAAACAAACCAGGAAATG-GCAGGAAAACCAACCATAAAGATAGGATTATGATAA-ATATTGAGAAGACACTTAAAGGTGGATCCCGGGTTATATTAA) producing strain SPK148.

**Table S1.** Some were published previously as indicated [15,16,18,26,52,53,54].

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and GTO-266 (CTTCGACAACAGGATTACGACC) and 25 PCR cycles to amplify ura4 and ura4-DS/E transcripts; GTO-223 (GAAACACATGTTGTCTCAGAG) and GTO-226 (TCGTCGTGATGCGATGTA) and 27 PCR cycles to amplify RNA originating from centromeric repeats or centH on OKR70 (GGCATCGACCTTCTACACAG) and OKR71 (GAGTCCAAAGACATCGATG) and 23 PCR cycles for actin. No-RT controls were conducted with GTO-223 and GTO-226 and 27 PCR cycles for all RNA preparations used in RT-PCR. No products were observed in these reactions.

Real-time RT-PCR displayed in Figure 1 was performed as described [61] to detect mat2-Pr using JPO-976 (TTGATAATAGTTATGGCCCTTTACATG) and JPO-977 (TGTTAGAATTGGCTGACTAATTT). Real-time PCR displayed in Figure 8 was performed using a Qiagen Quantitect SYBR Green RT-PCR kit for the reactions and a BioRad CFX96 PCR machine and BioRad software for the analysis. Dilution series of RNA prepared from a k0 strain were used to determine the range of exponential amplification which was found to extend to at least 30 cycles. All reactions were set up in triplicate except for the no-RT controls for which only one reaction was set up per sample. The mat2-Pr transcript was amplified with JPO-976 and JPO-977 using 75 ng of total RNA as template for each sample. The actin transcript was amplified with q-act-FOR (GGTTTCCGCTGGAATGGTATG) and q-act-REV (ATACCACGCTTGCTTTGAG) using 75 ng of total RNA as template for each sample. No mat2-Pr transcript was detected under these conditions for the wild-type (PG1789); clr4 (PG3633); clr3 (PG3564); and clr5 (PG3577) RNA preparations. No actin transcript was detected under these conditions for the wild-type (PG1789); clr4 (PG3633); clr3 (PG3564); and clr5 (PG3577) RNA preparations. No actin transcript was detected under these conditions for the wild-type (PG1789); clr4 (PG3633); clr3 (PG3564); and clr5 (PG3577) RNA preparations.

Values reported in Figure 8 for the relative increase in fold (Table S2). Use of either list produced essentially the same results. The significance of gene list overlaps was calculated using a Bonferroni multiple testing correction. Two lists of genes were compared with previously published microarray experiments. Cloning and sequencing of clr5 cDNA and clr5 mutant alleles

cDNA from exponentially growing wild-type cells (PG1789) was amplified using OKR86 and JPO998 as described above. A PCR product of approximately 600 bp was gel purified (Qiagen) and cloned into pCRII-TOPO (Invitrogen). The cloned cDNA was sequenced to identify the exon boundaries of clr5. To identify possible mutations within clr5 in the esp mutants [16], full-length genomic clr5 was amplified using primers OKR-95 (ATTCGGGATGAGCGAAGATGGAGATGTCG) and OKR-96 (CTGGTAGGCTGACCTAAACGAAAAGGACTACATC-TGGG), 18 PCR cycles and the Phusion polymerase (Finnzymes). PCR products from duplicate DNA samples from wild-type, esp1, esp2, esp3 and esp4 cells were TOPO-cloned and sequenced.

DAPI staining and microscopy

Cells propagated on ME plates for 3–4 days at 32°C were scraped, washed in 500 µl PBS, and incubated at room temperature for 10 min in 8 µg/ml DAPI/PBS solution. The suspension was diluted approximately 20 fold in PBS and 150 µl were spun (Cyto-Tek, Samura) onto poly-lysine coated slides (Sigma). The slides were air-dried and one drop of Vectashield (Vector Labs) was added before applying the cover slip. Images were obtained using a Zeiss AxioskopII microscope fitted with a CoolSNAP HQ camera. All images were taken at maximum resolution, using 100x objective and IPLab software (Scanalytics).

Localization of Clr5

Clr5 tagged at its C terminus with GFP [52] was expressed from the endogenous clr5 locus and used for localization studies. Cells were propagated to early log phase in supplemented EMM2 medium. Images were obtained using the 100x objective of a Zeiss Axioimager fluorescence microscope equipped with a Hamamatsu Orca-ER digital camera and Volocity 5.0

DNA and protein sequence analyses

Sequence analyses were performed using online available BLAST [63], ClustalW (www.ebi.ac.uk/clustalw/), IUPred (http://iupred.enzim.hu/), and services from the Sanger Institute (www.sanger.ac.uk) and Broad Institute (www.broad.mit.edu).

ChIP analyses

Cells were grown overnight in YES in a 30°C shaking incubator, diluted to 3.5×106 cells/ml in malt extract medium (ME) and incubated for a further 5 hr to induce nitrogen starvation. Chromatin immunoprecipitation was performed as previously described [61], but using 1% fixation and antibodies that recognize H3K9me2 (Abcam) or H3K9Ac (Millipore). Briefly, 3×107 cells were fixed with 1% paraformaldehyde for 18 min at room temperature prior to washing with PBS, permeabilization of the cell wall with zymolase 100T (0.4 mg/ml in PEMS), and incubation at 36°C for 20 min. Following extensive washing with PEMS, cell pellets were resuspended in 400 µl ChIP lysis buffer and sonicated (3x, 10s each). After pre-clearing with Protein A- agarose beads, the lysates were used for immunoprecipitation overnight with each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody. Antibody-protein complexes were purified using Protein A- agarose beads, washed, and reverse-crosslinking of each antibody.
were JPO-976 and JPO-977. Data were normalized to the cbh4A strain for the K9Me ChIPs, and to wild type for the K9Ac ChIPs.

Supporting Information

Figure S1 LEU2 insertions at the cb5 locus mapped by inverse PCR, and phenotypes of cb5a strains. (A) Position of LEU2 insertions relative to cb5 ORF in SPK129, SPK137, and SPK142. JPO998 and OKR 86 are primers used for the cb5 transcript analysis in C. The white lollipop indicates SPAC29B12.08 ORF start site proposed in databases (NCBI; Sanger Center); the black lollipop the start site suggested by our experiments. Black flowers show the location of the three LEU2 insertions. Genetic analysis of the previously isolated esp1 and esp2 mutants [24] demonstrated that these contained mutations tightly linked to cb5. Sequencing of cb5 in these mutants revealed single base pair mutations leading to an amino acid change in esp1 (R45A; allele renamed cb5-1059) or W50stop in esp2 (allele renamed cb5-1058). Similarly, sequencing the hitherto unpublished esp4 allele obtained in a similar screen found a short array of mutations leading to a frameshift in the beginning of the Ctrl5 unstructured domain. (B) Sequence of the three cb5:LEU2 insertion sites. Bases are numbered as in cosmid SPAC29B12. In each case, as seen from the alignments, a few nucleotides of cb5 were deleted by the integration event. (C) A size difference of about 600 bp between the PCR products obtained from cDNA or genomic DNA (gDNA) demonstrates mRNA splicing of cb5. (D) The cb5 intron displays conserved 5’ and 3’ splice motifs. Consensus splice motifs [66] are indicated in the shaded boxes. The nucleotide position refers to the position in SPAC29B12. W = T or A, Y = T or C (pyrimidines), and N = any base. (E) RT-PCR was performed using primers JPO-998 and OKR86 to examine cb5 transcript in wild-type and cb5-142 cells. (F) Analysis of mat2-Px transcript in wild-type (PG1789), cb5a (SPK464), sw6-115 (SPK29), and cb5a sw6-115 (SPK458) cells was performed as in Figure 1. (G) Tetrad dissection of a heterozygous diploid cb5::cb5A on YES medium. The cb5A progeny form smaller colonies than the cb55 progeny. Found at: doi:10.1371/journal.pgen.1001268.s001 (0.98 MB TIF)

Figure S2 cb5 coding and predicted protein sequence. An intron in the cb5 gene is indicated in red. Found at: doi:10.1371/journal.pgen.1001268.s002 (0.87 MB TIF)

Figure S3 Effect of cb5a on the expression of genes in the pheromone-response pathway. Expression ratios obtained in two micro-array experiments comparing cb5a to wild type are presented. Ratios greater than 2-fold are indicated in red. Pheromone induced genes controlled by the master regulator Ste11 and their relationships are depicted as described [67–71]. Ctrl5 regulates many genes in that pathway either directly or indirectly via Ste11 regulation. Found at: doi:10.1371/journal.pgen.1001268.s003 (1.26 MB TIF)

Figure S4 Expression of war4+ in the mating-type region. Northern blot of war4 transcripts originating from the mating-type region (mat2-P::XbaI::war4+) or euchromatic war4 locus (war4-DS/E) in cb55 (cb55 1Pu-1058; PG1210) or cb5 mutant (cb5-1058; PG1214; cb5-1059; PG1179) cells. All cells are sw6-115. Each cb5 mutation has a cumulative effect with the mutation in sw6, increasing the expression of mat2-P::XbaI::war4+ relative to war4-DS/E. Found at: doi:10.1371/journal.pgen.1001268.s004 (4.70 MB TIF)

Figure S5 Cumulative gene silencing by the RNAi pathway and Ctrl5. Ten-fold serial dilutions of switchable mat1 Msmt-0 mat2- P::XbaI::war4+ cells mutated in the RNAi pathway (dcr1A) or cb5 (cb5-142) were spotted on MSA sporulation medium. (A) No sporulation was observed on ura3-containing medium, a medium supporting growth of all cells plated independent of the expression state of their mating-type region. This indicates that mat2-P can be repressed in all mutants examined. (B) and (C) Variegated sporulation was observed in some of the mutants on ura3-free medium. Uracil-free medium selects for cells with a partially or totally derepressed mating-type region. Haploid meioses were not detected in wild-type or dcr1A cells on ura3-free medium indicating mat2-P remains silent in these cells. Very low levels of haploid meioses were detected in cb5-142 mutant and higher levels in the dcr1A cb5-142 double mutant. These observations are consistent with Ctrl5 repressing the mating-type region in a pathway different from the RNAi pathway. wt: PG1789; dcr1A: SPK425; cb5-142: SPK368; dcr1A cb5-142: SPK423; dcr1A cb5-142: SPK424. Found at: doi:10.1371/journal.pgen.1001268.s005 (3.91 MB TIF)

Figure S6 Inverse PCR design. Primers and restriction sites used for the amplification of LEU2-containing DNA for mutagenesis, or for the subsequent inverse PCR reactions. Found at: doi:10.1371/journal.pgen.1001268.s006 (3.39 MB TIF)

Figure S7 Statistical analysis for data presented in Figure 5E. The proportions of genes upregulated >2-fold in the cb5a mutant were determined along each chromosome in a sliding window of 20 consecutive genes and the probability of the observed proportions being due to chance was estimated and plotted for each window as detailed in Materials and Methods. The orange line represents a P value of 0.05 while the red line represents a P value of 0.001. The region on chromosome 1 (shown in Figure 5E) is significant for both lists. (A) A list of genes whose averaged expression between the duplicate microarrays was increased >2 fold in cb5a compared to wild-type. The peak in chromosome 1 is a 20-gene window centered around SPAPJ695.01c (P = 1.05 e-0). (B) A list of genes whose expression was increased >2 fold in both microarrays. The peak in chromosome 1 is centered on SPAPJ695.01c (P = 7.44 e-3). The peak in chromosome 2 is a 20-gene window centered on SPBC23G7.12c at the mating-type region (P = 2.46 e-3). Both gene lists are in Table S2. Found at: doi:10.1371/journal.pgen.1001268.s007 (0.08 MB PDF)

Table S1 List of strains and their genotypes. Found at: doi:10.1371/journal.pgen.1001268.s008 (0.09 MB DOC)

Table S2 Lists of genes used in Figure 5E and Figure S2. Found at: doi:10.1371/journal.pgen.1001268.s009 (0.01 MB PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: KRH IH SS AC GT. Performed the experiments: KRH IH SS JW VH AC GT. Analyzed the data: KRH IH SS JW BM JF JF AG CT. Contributed reagents/materials/analysis tools: KRH IH SS JW BM JF JF AG CT. Wrote the paper: KRH JF JF AG CT.

References


