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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 heterochromatic 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 donors for gene conversions of mat1 in a process leading to mating-type switching. The tight gene silencing of mat2-P and mat3-M is essential for the viability of vegetative cells because co-expression of the P and M mating-type information triggers meiosis in starved cells [2]. P and M co-expression normally occurs only in heterozygous (mat1-P/mat1-M) diploids where it causes meiosis and sporulation, a natural process facilitating survival in harsh conditions. Co-expression of the P and M mating-type information in haploid cells on the other hand, as might happen following expression of mat2-P and mat3-M, leads to haploid meiosis and cell death [2].

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 Ctr3 and Ctr6 [3,4]. H3K9me is catalyzed by Clr4, the sole H3K9 methyltransferase in S. pombe [5]. It is bound by Ctr4 itself [6] and by three other chromodomain proteins, Swi6, Chp1, and Chp2 [7]. Chp1 is a Su(var)3-9 homolog and Swi6 and Chp2 are HP1 homologs.

Numerous studies have examined the mechanisms of recruitment of Ctr4 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 Rdcp1, 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

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**Author Summary**

In eukaryotes some histone modifications are preponderantly associated with silent chromosomal domains, however the extent to which distinct modifications contribute to the silencing of gene expression is often not known. A well-studied chromosomal domain in which histone modifications have been extensively characterized is the fission yeast mating-type region. There, histone hypoacetylation and histone H3 lysine 9 methylation (H3K9me) are associated with a domain refractory to gene expression. Contrary to a general assumption, we found that genes naturally present in the mating-type region of wild-type strains remain repressed in the absence of the H3K9 methyltransferase Clr4. Their repression depends on histone deacetylases and on a hitherto uncharacterized factor, Clr5. Our results reveal an unsuspected robustness in the silencing mechanism, where H3K9me and deacetylation cooperate to ensure that the genes naturally present in the mating-type region remain silent in conditions where their expression would otherwise kill the cells.

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**Figure 1. Prominent 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 within the mating-type cassettes are indicated by brown arrows; a binding site for Atf1 in REII is indicated by a green arrow. A second Atf1 binding site located between cenH and REIII is not represented. 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), clr4 Δ (SPK450), clr3 Δ (PG3564), swi6-115 (SPK29), clr6-1 (SPK467) and clr3 Δ 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.

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unswitchable 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-Msmt-0* colonies, or by RT-PCR estimating the level of *mat2-P* transcripts in *mat1-Msmt-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 *ura4* 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 *ura4* transcript is present in the mutants [17]. The pronounced residual repression of *ura4* in Clr4 or Swi6 mutants is consistent with the effects observed here on *mat2-Pc* 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-Msmt-0* colonies, high levels of haploid meiosis, and accumulation of *mat2-P* transcript (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 H9K9me 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-Msmt-0* allele, an *S. pombe* *ura4* reporter gene near *mat2-P* (Δ*XhoI*::*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 were identified [15]. The remaining three *LEU2* insertions defined a genetic locus unlinked to the mating-type region that we named *clr5* (cryptic loci regulator 5; SPK129, SPK137 and SPK142 mutants; Figure 2A and 2B).

*mat2-Pc* is strongly derepressed in *clr5::LEU2 swi6-115, clr5::LEU2 clb3A* or *clr5::LEU2 clb4A* 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 Clr5 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 the *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 Clr6 deacetylase on a number of other targets (Figure 5A). The overlapping function of Clr5 and Clr6 is fully consistent with the epistasis analysis presented above suggesting that Clr5 and Clr6 repress the mating-type region together (Figure 2A and 2D). Clr5 and Clr6 also have non-overlapping roles in gene regulation consistent with Clr6 participating in various protein complexes.

Figure 2. Clr5 acts in the same pathway as the HDAC Clr6 and represses mat2-P independently of Swi6, Clr3, and Clr4. (A) SKP29 and mutants obtained by insertional mutagenesis in SPK29. 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 (SPK129), clr5-137 swi6-115 (SPK137), and clr5-142 swi6-115 (SPK142) double mutants but not in the swi6-115 (SPK29) 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, SPK29, SPK129, SPK137, SPK142 and SPK368. (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 Clr5 acts in a pathway distinct from Clr3 and Clr4. In contrast, no cumulative effect is seen when combining defective clr5 and clr6 alleles indicating Clr5 and Clr6 act in the same pathway, at least for their effects in the mating-type region. Top panel: PG1789, SPK450, PG3564, SP1240, PG3577. Bottom panel: SPK368, SPK447, SPK415, SPK493. (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.

Figure 3. Localization of Clr5-GFP. Cells were propagated in EMM2+ supplements to early log phase. Clr5-GFP was expressed from the endogenous clr5 locus, under control of the clr5 promoter. The strain was FY15231.

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Figure 3. Localization of Clr5-GFP. Cells were propagated in EMM2+ supplements to early log phase. Clr5-GFP was expressed from the endogenous clr5 locus, under control of the clr5 promoter. The strain was FY15231.
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**
- **S. japonicus**
- **S. octosporus**
- **C. globosum**
- **M. grisea**
- **S. sclerotiorum**
- **P. anserina**
- **P. triticirepentis**
- **P. nodorum**
- **N. crassa**
- **N. fischeri**
- **A. clavatus**
- **A. nidulans**
- **P. marneffei**
- **P. marneffei**
- **C. globosum**
- **T. stipitatus**
- **M. canis**
- **C. posadasii**

(B) Disorder tendency of the Clr5 protein as a function of amino acid position. The identity is indicated at the bottom of the graph.

(C) Domain distribution of the Clr5 protein in different species.
Since the Clr3 and Clr6 deacetylases act redundantly on many genes [10] we compared the expression profiles of clr3Δ and clr6Δ 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 clr3Δ mutants (9 of 11 genes), clr6Δ 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-P17; 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 clr3Δ and clr4Δ single mutants, however we observed occasional haploid meioses in clr3Δ or clr4Δ colonies indicating a low level of mat3-M transcription occurs in these mutants. In the double clr3Δ clr4Δ and clr3Δ clr5Δ 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-P17 and clr5Δ mat3-M (or clr4Δ) colonies compared with mat1-Mat1-0 clr5Δ mat3-M (or clr4Δ) colonies, however the abundance of mat3-M transcript 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/Swi6 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 Clr3 or Swi6 by examining the nap6Δ transcript levels in double mutants. We observed that nap6Δ inserted near mat2-P (Figure 1; mat2-P(XbaI::nap6Δ)) was more strongly expressed in the clb5-142 swi6-115 double mutant than in either single mutant (Figure 6B and Figure S4). We also observed increased accumulation of cenH transcripts in the clb5-142 swi6-115 and clb5-142 clr4Δ 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 epitypes: one similar to wild type displaying normal levels of H3K9me and one similar to the clr4Δ 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 ade6Δ leads to variegated ade6Δ expression [25]. Noticeably, mat2-P remains silent in cenH::ade6Δ cells regardless of the expression state of ade6Δ (Figure 6C) in agreement with H3K9me being dispensable for the repression of mat2-P. Our observations with clr3Δ and clr4Δ mutants suggested that combining clr3Δ with cenHΔ should lead to a cumulative derepression of mat2-P. Indeed, deleting clr5Δ in cenHΔ cells increased the expression of mat2-P (Figure 6C). Furthermore, as with cenHΔ single mutants, fluctuations between two phenotypes still occurred. Similarly, deleting clr3Δ in a cenHΔ 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 Clr3 exerts its effects through the REII or REIII silencing elements found near mat2-P and mat3-M respectively by combining clr3Δ with deletions of these elements. Deleting clr5Δ in cells lacking the mat3-M-adjacent element REIII lead to a small cumulative, variegated, derepression of mat3-M (Figure 6D) placing clr5Δ in a pathway different from the REIII pathway. In contrast to the situation with cenH or REII, deleting clr5Δ in cells that lack REII did not increase the expression of mat2-P (Figure 6D). This supports the notion that Clr5 acts through REI, a proposition substantiated by the effects of clb5Δ on ectopic silencing reporters (see below) and by the fact that an REII insertional mutant had been obtained in the same genetic screen as the clr3Δ-LEU2 mutants.

**REI-mediated silencing at an ectopic site requires Clr5**

To further test whether REI and Clr5 participate in the same silencing mechanism, we asked whether REI-mediated silencing at an ectopic site depends on Clr5. Insertion of a cenH sequence adjacent to an ade6Δ reporter gene at an ectopic site confers partial heterochromatic silencing on ade6Δ [26]. Changes in the expression state of ade6Δ can be monitored at the colony level by a color test. Cells expressing ade6Δ produce white colonies while cells that fail to express ade6Δ produce red colonies or septa 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 ade6Δ-cenH is established at a very low
Figure 5. Transcription signature of clr5Δ mutant. (A) and (B) The list of genes upregulated >2 fold in clr5Δ 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 clr5Δ to a swi6-115 mutant. Sporulation was assayed on MSA medium lacking leucine and thiamine. (D) As A and B but comparing with clr3Δ 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
acetylation (H3K9Ac) were examined at the REII element and mat2-P in single and double mutants (Figure 8). The expression of mat2-P was measured in the same strains (Figure 8A and 8B). This experiment gave the following insights in the molecular mechanisms responsible for the effects observed in the various mutants.

First, as predicted from the phenotypic analysis described above, lack of H3K9me2 is not sufficient to derepress mat2-P. This could be seen in the cht3A and cht4A mutants, both of which lacked H3K9me2 at REII and mat2-P, yet failed to express mat2-P to a detectable level (Figure 8A and 8C). Deletion of cht3 in either of these strains backgrounds lead to a >50 fold increase in mat2-P expression indicating Cht5 is necessary for the H3K9me-independent repression of mat2-P in cht4A and cht34 cells (Figure 8B and 8D), a result also corroborating our genetic analysis. Cht5 itself showed no sign of directly affecting H3K9me2, the level of H3K9me in cht5A or cht5-142 was not significantly different from wild-type (Figure 8C; please note that cht3-142 is in all likelihood a loss of function allele due to LEU2 insertion at the beginning of the gene. No cht5 transcripts were detected in cht5-142 cells (Figure S1).

Changes in H3K9Ac were also observed at REII and mat2-P in the various mutants examined. The greatest increase in H3K9Ac occurred in the cht5A cht6-1 double mutant consistent with the two HDACs acting redundantly on this substrate. Furthermore H3K9Ac increased in the cht4A cht5A and cht3A cht5A double mutants relative to each single mutant (which were not significantly different from wild-type) supporting the idea that Cht5 acts together with an HDAC. One should bear in mind when interpreting these data that histone deacetylases tend to be promiscuous affecting more than one of the numerous nucleosomal lysines that are subject to acetylation and they might furthermore deacetylate proteins other than histones hence changes other than H3K9Ac might take place in the mutants we examined and also affect gene expression.

Finally, only strains with both abnormally low H3K9me and abnormally high H3K9Ac expressed mat2-P. These were the cht4A cht5A, cht3A cht5A and cht3A cht6-1 double mutants. Strains lacking H3K9me but showing no increase in H3K9Ac (cht3A and cht4A) failed to express mat2-P. Conversely, a small increase in H3K9Ac that was not accompanied by loss of H3K9me in the cht6-1 cht5A double mutant did not lead to mat2-P expression. These results epitomize the redundancy of silencing mechanisms at the mat2-P cassette.

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-

Figure 8. Chromatin modifications and mat2-Pc expression in clr5 mutants. (A) and (B) mat2-Pc RNA levels in various mutants. RNA was prepared from cells starved for nitrogen for 5 hr to induce expression of the mating-type genes. Changes in mat2-Pc expression relative to wild-type (PG1789) were estimated by real-time PCR and plotted, using actin for normalization. The means of two biological experiments are displayed. Strains analyzed in (A) were WT: PG1789; clr4D: SPK450; clr3D: PG3564; clr6-1: SP1240; clr5D: PG3631; clr5-142: SPK368; and clr3D clr6-1: PG3577. Strains analyzed in (B) were WT: PG1789; clr4D: SPK450; clr3D: PG3564; clr6-1: SP1240; clr5D: PG3631; clr5-142: SPK368; and clr3D clr6-1: PG3577. (C) and (D) Chromatin Immunoprecipitation (ChIP) analysis of H3K9me2 at the REII and mat2-Pc locus of the mating-type region compared with the adh1+ locus measured by real-time PCR. Enrichment of H3K9me2 was normalized to the values derived for a strain that lacks H3K9me2 (clr4D, SPK450). Values represent the means of two independent ChIP experiments except for clr6-1 mutant were only one ChIP experiment is shown. Strains analyzed in (C) were as in (A). Strains analyzed in (D) were as in (B). (E) and (F) Chromatin Immunoprecipitation (ChIP) analysis of H3K9Ac at the REII and mat2-Pc locus of the mating-type region compared with the adh1+ locus measured by real-time PCR. Values were normalized to the wild-type strain (PG1789) and represent the means of two independent ChIP experiments. Strains analyzed in (E) were as in (A). Strains analyzed in (F) were as in (B). doi:10.1371/journal.pgen.1001268.g008
Figure 9. Model for gene silencing in the mating-type region. Both Clr5 and Atf1 repress gene expression by promoting deacetylation in their respective target regions, Clr5 directly or indirectly via the REI element (this study), and Atf1 via Atf1-binding sites near mat3-M [12,13]. The effects of Clr5 and Atf1 gradually decrease as the distance from their respective cis-acting element increases. An additional layer of silencing is orchestrated by Clr4. Clr4 can be recruited by direct binding to Atf1 [12] or through the RNAi-dependent cenH nucleation site [11]. H3K9me catalyzed by Clr4 permits binding of the chromodomain proteins Swi6 and Chp2 and spreading of histone deacetylation [14,28–30]. Inactivation of both the Clr4 and Clr5 pathways is required for mat2-P expression. While the clr4A clr5A combination derepresses mat2-P (this study), this is not the case for the clr4A clr6-1 combination [3]. To account for these different phenotypes, we suggest that Clr3 can partially substitute for Clr6 in a Clr5-dependent manner. doi:10.1371/journal.pgen.1001268.g009
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 clr5 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 unfolding upon interaction with a binding partner [46]. Transcription factors are abundant among IUPs for example Jun, 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. Many proteins in this family are in the same size-range as Clr5, of proteins that lack well-defined secondary and tertiary structures, among a family of fungal proteins of currently unknown function. Many proteins in this family are in the same size-range as Clr5.

The predicted high flexibility of the unstructured domain of Clr5 also mediates protein interactions. The predicted high flexibility of the unstructured domain is consistent with its relatively low sequence conservation between the Clr5 homologues in S. pombe, S. japonicus, and S. octosporus. Clr5 is the first member of its family with an assigned function. Its involvement in a well characterized biological process amenable to genetic analysis provides valuable tools to unravel questions relevant not only to the regulation of gene expression but also to the fields of structural biology and molecular evolution.

**Methods**

*S. pombe* strains and media

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 clr5 ORF was replaced with the *hph1* gene, which confers resistance to hygromycin B, by transforming SPK29 with a PCR product amplified from pCR2.1-hph1 [55] with GTO-312 (TTACATGTTCGGGGGTGATACGGTGGACGGATCCGGGTATCCGGGTTAAATTA) and GTO-313 (TACGGATGAGGGCAATGTGAGAAATATTAAAAATCTCATAAGTATAGCCAGAAGCAGGAGGATCGTGTGATCCGGGTATCCGGGTTAAATTA) producing strain SPK458.

Targeted integration of *ade6* and *mat* loci into the *ura4* locus were essentially as described before [26]. The REJIII containing sequence in AP1665, AP2346 and AP2406 is the 482 bp fragment described in [16]. Media were prepared as described previously [24,56,57].

**Mutagenesis**

*S. cerevisiae* LEU2-containing plasmid pJJ283 [58] was digested to completion with BamHI and HindIII (New England Biolabs). 18 bp random ends were added to the LEU2 fragment by PCR using primers N-OKR 76 (NGGATCCCTTGCATCTGATG) and N-OKR77 (NGGATCCCTTGCATCTGATG) (Figure S6). Insertional mutagenesis was essentially performed as a standard fission yeast transformation [56,59], by transforming SPK29 with the LEU2 PCR products with random ends. Following the transformation, cells were plated onto AA-lev plates and incubated 3–5 days at 33°C before being replicated onto MSA supplemented with adenine and uracil to induce meiosis in the Leu+ transformants.

**Inverse PCR**

Inverse PCR was performed essentially as described previously [59] using the Expand Long Template PCR System (Roche) and primers OKR78 (CTGGGGATACGGCGCCCGGTTGTTGTCC) and OKR79 (ACTACAGATTCTCTATATGGTATGGTCAAGTGGTGAAGGTAA) in the cases where the genomic DNA had been digested with HindIII; OKR83 (GGGAGAAAACTTGGAGGAGAACCCATCAGG) and OKR78, or OKR84 (GGGATACGGGGGGGTCCTGGGAG) and OKR79 for the EcoRI digests; and OKR82 (GGCATCACAATTTGCTGGAGGTCCCTTG) and OKR79 for the HindII digest (Figure S6).

**RNA extraction and transcript analysis**

Total RNA was extracted as described previously [60,61]. For the clr5 transcript analysis, the clr5 mRNA was reverse-transcribed using Superscript II (Qiagen) in a reaction containing OKR86 (GAGTGGCATTGGTTGCATGCAGC) to prime cDNA synthesis and 25 μg of total RNA produced from PG1789. Diluted cDNA was amplified with Expand High Fidelity Polymerase (Roche) using OKR86 and JPO998 (CATTGGCTTCCAAAGATGAA) for the analysis of other transcripts, cultures of wild-type or mutant strains growing exponentially in YES medium were harvested and starved for nitrogen in PM medium for 5 hours at 32°C in a shaking incubator to induce sexual differentiation. RT-PCR was performed as described in [17], with OKR93 (CGCTGCTTATATGTTATTTATTTATTTATTTATTATTTGTCGTCG) and OKR94 (CTATCAGAGATTGGAGGAGGCAGTGTTCCTAGGCG) and 24 PCR cycles to amplify the mat2-Pr transcript; GTO-353 (CTCTTCAATTCATTAGAATACTTGAGGTGAGT) and GTO-355 (GCTTTGCCACATCTCTCTCAACGAGCTTG) and 24 PCR cycles to amplify the mat3-Pr transcript; GTO-265 (GCTATTTACGTAGAGGTAGG)
and GTO-266 (CTTCGACAACAGGATTACGACC) and 25 PCR cycles to amplify ure25 and ure25-DS/E transcripts; GTO-223 (GAAAACACATCTGGTCTCTCAAG) and GTO-226 (TCGTCTTGATGCTCGATGGA) and 27 PCR cycles to amplify RNA originating from centromeric repeats or cenH4 or OKR70 (GGCGTAGCAGTTTCTACACG) and OKR71 (GAGTGC- CAAGACGATACAGTG) 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 mat2-2Pt using JPO-976 (TTGGATATA-TAGTATGCCTCAGAATCC) and JPO-977 (TGTTAGA-CCTTGCCCTGTTCAAAATT). 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 k∞ 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 mat2-2Pt transcript was amplified with JPO-976 and JPO-977 using 75 ng of total RNA as template for each sample. The mat2-2Pt transcript was amplified and detected under these conditions for the wild-type (PG1789); cb4A (SPK450); cb3A (PG3564); cb6-1 (SP1240); cb5dA (PG3631); cb5-142 (SPK368); and cb6-1 cb5-142 (SPK493) RNA preparations. Values reported in Figure 8 for the relative increase in mat2-2Pt transcript for the cb3A cb6-1 (PG3577); cb4A cb5dA (PG3630) and cb3A cb5dA (PG3633) RNA preparations are therefore likely to underestimate the real values.

Micro-array analysis

A cb5 D (SPK10) and a cb5dA (SPK573) strain were propagated in liquid EMM2 medium, and harvested at a cell density of ~5.0×10^6 cell/ml. RNA extraction and micro-array analysis were performed as described previously [62] in duplicate. The GeneSpring software package was used for data analysis and comparisons with previously published microarray experiments. The significance of gene list overlaps was calculated using a objective and IPLab software (Scanlytics).

ChIP analyses

ChIP experiments were performed as previously described [61], but using 1% fixation and antibodies that recognize H3K9me2 (Abcam) or H3K9Ac (Millipore). Briefly, 3 uL of genomic DNA was amplified using primers OKR-95 (ATTCCC-GGGGATGCAGAAGGATGGGATCTG) and OKR-96 (CTTCGAGCTGACCTAAGGGAAGGATCTACCTG), 18 PCR cycles and the Phusion polymerase (Finnzymes).

PCR products from duplicate DNA samples from wild-type, esp1, esp2, esp5 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 resuspended, 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 polyl-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 Coolspool HQ camera. All images were taken at maximum resolution, using 100x objective and IPLab software (Scanlytics).

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).

H3K9me-Independent Gene Silencing by 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.

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.

Cloning and sequencing of clr5 cDNA and clr5 mutant alleles

cDNA from exponentially growing wild-type cells (PG1789) was amplified using ORK86 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 (TTTCCGGGATGCAGAAGGATGGGATCTG) and OKR-96 (CTTCGAGCTGACCTAAGGGAAGGATCTACCTG), 18 PCR cycles and the Phusion polymerase (Finnzymes). PCR products from duplicate DNA samples from wild-type, esp1, esp2, esp5 and esp4 cells were TOPO-cloned and sequenced.
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 cbh5 locus mapped by inverse PCR, and phenotypes of cbh5A strains. (A) Position of LEU2 insertions relative to cbh5 ORF in SPK129, SPK137, and SPK142. JPO998 and OKR 86 are primers used for the cbh5 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 cbh5. Sequencing of cbh5 in these mutants revealed single base pair mutations leading to an amino acid change in esp1 (R45A; allele renamed cbh5-1059) or W50stop in esp2 (allele renamed cbh5-1058). 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 Clr5 unstructured domain. (B) Sequence of the three cbh5:LEU2 insertion sites. Bases are numbered as in cosmid SPAC29B12. In each case, as seen from the alignments, a few nucleotides of cbh5 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 cbh5 transcript. (D) The cbh5 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 cbh5 transcript in wild-type and cbh5-142 cells. (F) Analysis of mat2-Px transcript in wild-type (PG1789), cbh5A (SPK464), swt6-115 (SPK29), and cbh5A swt6-115 (SPK458) cells was performed as in Figure 1. (G) Tetrad dissection of a heterozygous diploid cbh5+/cbh5A on YES medium. The cbh5A progeny form smaller colonies than the cbh5A progeny.

Found at: doi:10.1371/journal.pgen.1001268.s001 (0.98 MB TIF)

Figure S2  cbh5 coding and predicted protein sequence. An intron in the cbh5 gene is indicated in red.

Found at: doi:10.1371/journal.pgen.1001268.s002 (0.87 MB TIF)

Figure S3  Effect of cbh5A on the expression of genes in the pheromone-response pathway. Expression ratios obtained in two micro-array experiments comparing cbh5A 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]. Clr5 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 wua4* in the mating-type region. Northern blot of wua4 transcripts originating from the mating-type region (mat2-P/XbaI:wua4*) or euchromatic wua4 locus (wua4-DS/E) in cbh55 (cbh5*: PG1210) or cbh5 mutant (cbh5-1058; PG1214; cbh5-1059; PG1179) cells. All cells are swt6-115. Each cbh5 mutation has a cumulative effect with the mutation in swt6, increasing the expression of mat2-P/XbaI:wua4* relative to wua4-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 Clr5. Ten-fold serial dilutions of unswitchable mat1-Msmt-0 mat2- P/XbaI:wua4* cells mutated in the RNAi pathway (dcr1Δ) or cbh5 (cbh5-142) were spotted on MSA sporulation medium. (A) No sporulation was observed on uracil-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 uracil-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 dcr1Δ cells on uracil-free medium indicating mat2-P remains silent in these cells. Very low levels of haploid meioses were detected in cbh5-142 mutant and higher levels in the dcr1Δ cbh5-142 double mutant. These observations are consistent with Clr5 repressing the mating-type region in a pathway different from the RNAi pathway. wt: PG1789; dcr1Δ: SPK425; cbh5-142: SPK368; dcr1Δ cbh5-142: SPK423; dcr1Δ cbh5-142: 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 cbh5A 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 cbh5A compared to wild-type. The peak in chromosome 1 is a 20-gene window centered around SPAPJ695.01c (P = 1.05e-6). (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 = 7.44e-5). The peak in chromosome 2 is a 20-gene window centered on SPBC23G7.12c at the mating-type region (P = 2.46e-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)

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Author Contributions

Conceived and designed the experiments: KRH IH SS AC GT. Performed the experiments: KRH IH SS JWVH AC GT. Analyzed the data: KRH IH SS JWVH RAM JFP AC GT. Contributed reagents/materials/analysis tools: KRH IH SS JWV H RAM JFP AC GT. Wrote the paper: KRH JB RAM JFP AC GT.

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