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The Establishment of Gene Silencing at Single-Cell Resolution

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Abstract

The establishment of silencing in *Saccharomyces cerevisiae* is similar to heterochromatin formation in multi-cellular eukaryotes. Previous batch culture studies determined that the *de novo* establishment of silencing initiates during S phase and continues for up to 5 cell divisions for completion. To track silencing phenotypically, we developed an assay that introduces Sir3 protein into individual *sir3* mutant cells synchronously and then detects the onset of silencing with single-cell resolution. Silencing was completed within the first one to two cell divisions in most cells queried. Moreover, we uncovered unexpected complexity in the contributions of a histone acetyltransferase (Sas2), two histone methyltransferases (Dot1 and Set1), and one histone demethylase (Jhd2) to the dynamics of silencing. Our findings revealed that removal of methyl modifications at H3 K4 and H3 K79 were important steps in silent chromatin formation, and that Jhd2 and Set1 played competing roles in the process.

Keywords

Chromatin Dynamics; *DOT1*; *KMT4*; *HML*; *HMR*; *SAS2*; *KAT8*; *SET1*; *KMT2*; *JHD2*; *KDM5*; Single-cell assay; Silencing; *SIR1*; *SIR2*; *SIR3*; *SIR4*; Mating Type

Introduction

Silencing is distinct from classic gene repression in its ability to block transcription throughout a chromosomal region. Chromatin domains that restrict gene expression are widespread in multi-cellular organisms, playing crucial roles in development, cell-identity,

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and position-effect variegation of trans-genes. In *S. cerevisiae*, silencing blocks expression of cryptic mating-type genes at *HML* and *HMR* loci 1-3. Loss of silencing in haploid cells leads to concomitant expression of transcription factors encoded by both **a** and **α** mating-types, resulting in sterility characteristic of **a/α** diploids⁴. Hence, silencing of *HML* and *HMR* is needed for a robust mating ability.

Silencing of *HML* and *HMR* loci requires flanking regulatory sites termed silencers, proteins that bind sequence motifs within silencers, and Sir proteins which localize both to silencers and the intervening silenced chromatin⁵⁻⁸. Although silencing is constitutive in yeast, conditional or inducible alleles of the Sir proteins have revealed orchestrated events that establish silencing *de novo*. During establishment, Orc1 bound to silencers recruits Sir19. A complex of Sir2/3/4 follows through its interactions with Sir1, Rap1, Abf1 and histones¹⁰⁻¹². Once recruited to silencers, Sir2 deacetylates a critical K16 acetyl mark on histone H4^{13,14} (and potentially also H3 K9, H3 K14, and H4 K56¹⁵⁻¹⁷), a process required for Sir2/3/4 complexes to bind throughout the locus^{11,15,6,7,18}. Following the deacetylation of H4 K16, methyl marks on H3 K4 and H3 K79 disappear in later steps of silent chromatin formation^{18,19}.

The establishment of silencing requires events restricted to certain phases of the cell-cycle²⁰⁻²³. Upon Sir protein induction, transcripts from *HML* and *HMR* decline as cells progress past S phase but not in cells arrested in G1 by **α**-factor or in S phase by hydroxyurea^{21,23}. The majority of Sir proteins bind to their target regions within the first one to two cell divisions following Sir protein induction causing the bulk of *HMR*-derived transcripts to decline on a similar timeline. Still, one study concluded that up to 5-cell divisions (15 hrs) are required for complete repression of transcription and for Sir proteins to saturate *HML* and *HMR*¹⁸. These findings inspired two opposing hypotheses for how events at the individual-cell level could account for observations made on batch cultures. The maturation hypothesis involves a multi-step process characterized by intermediate chromatin states perhaps with progressive decreases in transcription at different stages. In contrast, the stochastic hypothesis envisions individual cells adopting the silenced state in an all-or-nothing switch, initially producing a mixed population of silenced and un-silenced cells, but eventually resolving in a fully silenced population. These models need not be mutually exclusive.

As measured biologically, the consequence of *HML* and *HMR* silencing is a unique and robust mating phenotype. However, molecular experiments define silencing as the point at which mRNA transcripts from the silenced locus become undetectable, or the point at which Sir protein association with chromatin becomes saturated^{18,20-22}. Molecular measures may be a misleading mark of the phenotypic state of the cell because 1) it is unknown to what extent mRNA from *HML* and *HMR* must be reduced to achieve robust mating ability; 2) heterochromatin itself, once formed, might recruit more Sir proteins than are needed for phenotypic changes; and 3) upon Sir-induction in G1, Sir protein binding and spreading can occur, yet transcription persists²¹. Therefore ChIP measurements are a useful but imperfect measure of the silenced state. Therefore, we have defined *HML* and *HMR* silencing by its functional role – the point at which a cell regains a unique mating type. For these reasons,

we monitored the number of cell divisions required for cells to adopt the phenotypic hallmarks of silencing: mating pheromone sensitivity.

We hypothesized that structural differences between euchromatin and silenced chromatin could reflect either steps in the establishment process or consequences of silencing. For example, Sir2 deacetylation of lysine residues on H3 and H4 is critical for silencing establishment. However, in *S. cerevisiae*, silenced chromatin lacks other post-translational histone modifications. For example, upon establishment of silencing, lysine methylation at H3 K4 and H3 K79 decreases following drops in H4 K16 acetylation¹⁸. However, it is unclear whether removal of H3 K4 and H3 K79 methylation promotes silencing or whether the loss of these marks is a consequence of silencing. To resolve this issue, we assayed the kinetics and pattern of silencing establishment in single cells lacking *dot1*, *set1*, *sas2*, or the JmjC-domain-containing family of demethylases.

Results

A pedigree assay revealed the kinetics of silencing establishment

To assay the establishment of silencing in single cells, we introduced Sir3 protein into un-silenced *sir3* cells through mating, a process that involves both cytoplasmic and nuclear fusion. By this technique, Sir3 protein was delivered into nuclei containing an actively transcribed *HML* locus. The genotypes of our strains allowed the transcribed or silenced state of *HML* to be reported as sensitivity or resistance to α -factor (Fig. 1). When *HML* silencing was complete, cells arrested division and altered their morphology to form shmoo in response to α -factor. Therefore, the number of cell divisions of the resulting diploid zygote prior to arrest represented the number of cell divisions required to establish silencing at *HML*. This technique improved upon past approaches in three ways: 1) A single-cell approach can differentiate between the maturation and stochastic hypotheses of silencing establishment; 2) leaky, variable sources of Sir protein were avoided as the un-silenced cell contained no conditional, inducible, epitope-tagged or temperature-sensitive alleles of Sir3; 3) since mating is restricted to START in G1, the initial exposure of cells to Sir protein was synchronized in all zygotes.

To perform the assay, cells of the genotype *hml mat hmr SIR3* (Strain 1) provided a source of Sir3 protein (Fig. 1). Lacking all genes for mating-type transcription factors, these cells mate as **a** cells, which is the default mating type. Conversely, the query strain (Strain 2) of genotype *HMLa mat hmr sir3* expressed *a1* and *a2* transcripts from the un-silenced *HML* locus. Upon mating, the two strains formed a diploid zygote containing only **a** mating-type information (encoded at *HML* of Strain 2). Cell division in these cells was resistant to α -factor until the Sir3 protein functionally silenced *HML* at which point the diploids became sensitive to α -factor.

In the first experiment, we assayed 643 zygotes and 2,353 progeny for up to three cell divisions (Fig. 2). In no case did diploid cells shmoo immediately after mating. In contrast, 13.7 % of zygotes formed a pair of shmoo (the defining hallmark of silenced *HML*) after dividing only once. In these cases, the zygote (*Z'*) and its first daughter (*D1*) were sensitive to α -factor, and remained sensitive for the duration of the experiment (Fig. 2, Pattern 1).

Therefore, in this subset of cells, all events needed for silencing occurred within one cell cycle.

Interestingly, in 12.6 % of lineages, silencing was established asymmetrically with the daughter cell (D1) silencing first and the zygote (Z') continuing to divide once more (Fig. 2, Pattern 2). The reciprocal pattern in which Z' silenced after the first division, but D1 did not, was rare (8 out of 643 lineages; Fig. 2, Pattern 3). In these asymmetrical patterns, the establishment of silencing in the mother and daughter cells at the two-cell stage was independent of one another. Thus, there was no obligate coupling of the fates of their two *HML* loci. Notably, the two asymmetrical patterns were unequally represented.

The majority (65.3%) of lineages produced shmoo in all cells after two cell divisions. This pattern produced four silenced granddaughter cells (Z'' , D2, D1', D1-1) (Fig. 2, Pattern 4). In 46 out of 643 lineages, one grand-daughter cell continued division one more time before arresting. Barring those exceptions, two cell divisions represent the maximal time required for cells to silence *HML*.

If the establishment of silencing were a purely stochastic as a function of cell division, then the probability of silencing would be equal at any point in the pedigree. However, the probability of silencing changed with each division and depended on whether a cell was a mother or daughter cell (Fig. 2c). For example, the Z' zygote had a 14.9 % chance of establishing silencing after the first cell division (proportion of pedigrees with Pattern 1 and 3), whereas the D1 daughter cell had a 26.3% chance of establishing silencing at the same point (proportion with Pattern 1 and 2). These probabilities rose to over 90% in the Z'' , D1', D1-1, and D2 cells that had not silenced in the previous division. Therefore, these results were inconsistent with silencing being established with a fixed probability per cell cycle.

The inferred silencing of *HML* required Sir3 introduction, an α -factor source, and an *HML* locus competent for silencing. The absence of any of these components resulted in cells that divided indefinitely (data not shown). In summary, silencing progressed as a function of a cell's history and its identity (as either a mother or a daughter cell), did not occur with a fixed probability per cell division, and was complete within two cell divisions in most cells.

Sir3 was not limiting for the establishment of silencing

The data above were from diploid cells carrying one copy of the *SIR3* gene. Although *sir3* is recessive, we considered the possibility that *SIR3* hemizygosity might affect the kinetics of silencing, and that the rate of establishment might be hastened by a super-stoichiometric quantity of Sir3. To test this idea, we performed the pedigree assay using a derivative of Strain1 bearing *SIR3* on either a single-copy (CEN-ARS) or multi-copy (2 μ) plasmid. These strains expressed *SIR3* mRNA at roughly five and ten times the wild-type level, respectively, of *SIR3* transcript (Fig. 3a). Sir3 over-expressors did not establish silencing with significantly different kinetics than their isogenic wild types (Fig. 3b). Thus Sir3 levels were not limiting for establishment.

Chromatin-modifying enzymes impacted the kinetics of silencing establishment

Given that histone methylation and acetylation are reduced or missing from silent chromatin in *S. cerevisiae*, we assayed the kinetics of silencing establishment when both strains lacked the chromatin-modifying enzymes Dot1, Set1, Sas2, or the JmjC- and JmjN-domain-containing histone demethylases. We followed silencing patterns of over a hundred pedigrees for each mutant as well as wild-type controls on the same plate. Loss of *DOT1* and *SET1* significantly hastened silencing establishment whereas the loss of *SAS2* or *JHD2* significantly delayed it (Figure 4; contingency tables are in Supplemental Fig. 1; and mosaic plots as Supplemental Fig. 4a).

Dot1 (also known as Kmt4) was identified by the loss of telomeric silencing upon either its over-expression or loss-of-function²⁴ and was later shown to catalyze all methylation states of H3 K79²⁵⁻²⁷, a core nucleosome residue that marks euchromatin when methylated^{25,28}. Sir proteins are thought to have a lower-affinity for nucleosomes methylated at H3 K79²⁹. In addition, the Dot1 protein itself antagonizes silencing by competing with Sir3 for a binding site on histone H430³¹. In the pedigree assay, 32.5 % of *dot1* pedigrees established silencing in both cells after just one cell division, a roughly 2-fold increase over the wild type (Fig. 4). Still, over 95% of *dot1* mutants were silenced within the first two cell divisions. Therefore, Dot1, and by inference methylation on H3 K79, slowed the establishment of silencing.

H3 K4 mono-, di-, and tri-methylation is catalyzed by Set1 (also known as Kmt2), a member of the COMPASS complex that tracks along with RNA Pol II, creating a pattern of H3 K4 mono-, di- and tri-methylation along the length of transcribed genes³²⁻³⁴. The *set1* mutation, and a consequent loss of H3 K4 methylation, leads to growth defects³⁵, aberrant activation at some genes, repression defects at others³⁶⁻³⁹, and silencing defects^{40,41}. In our studies, *set1* cells exhibited accelerated establishment of silencing, though not as much as in the *dot1* mutant (Fig. 4). By inference, Set1 inhibited or antagonized the establishment of silencing.

Jhd2, a member of the Jmj-C family of histone demethylases, catalyzes the removal of all three H3 K4 methylation states, thereby opposing Set1 enzymatic activity in budding yeast⁴²⁻⁴⁶. Indeed, *jhd2* cells were slow to establish silencing, a phenotype opposite that of *set1* (Fig. 4). In contrast, removal of three other JmjC- and JmjN-containing proteins showed minimal to no effects on the establishment of silencing (Supplemental Fig. 3). Thus, the acceleration of silencing establishment in *dot1* and *set1* cells, and the retardation in *jhd2* mutants reflected specific effects of these enzymes on silencing kinetics.

Sas2 (also known as Kat8) catalyzes the acetylation of N-terminal tail residues in histones H3 and H4 and plays a role in gene activation. This enzyme also catalyzes the H4 K16 acetylation that is removed by Sir2 to produce silent chromatin. Therefore, one might expect *sas2* cells to establish silencing more expeditiously than wild-type cells because *sas2* cells lack a mark refractory to Sir protein binding. However, *sas2* cells were actually slow to establish silencing: only 1.9 % of *sas2* cells established silencing after the first cell division, in contrast to 12.7 % of wild-type cells (Fig. 4) and roughly 10 % of pedigrees failed to establish silencing even after 3 rounds of cell division. Our results closely mirrored

the delay in the association of Sir3p with the *HML* and *HMR-loci* reported in batch cultures of *sas2* cells¹⁸ and recapitulated findings that populations of *sas2* cells exhibit a slightly variable expression of *HML* at the single-cell level^{47,48}.

Cells with the *dot1*, *set1*, and *sas2* deletions produced phenotypes in steady-state that were often less severe than their phenotypes in silencing establishment. Compared with their strong defect in telomeric silencing, cells with *dot1* mutations had minimal effects on *HML* and *HMR* expression. Cells lacking *DOT1* retained wild-type mating ability (Fig. 5a) and successfully silenced an *HMRa1::URA3* reporter (Fig. 5c). However, as recently published elsewhere, *dot1* deletion enhances the silencing defects of some silencing-compromised mutations like *sir1*⁴⁹ (Fig. 5a,c). In addition, the slowed kinetics of silencing establishment in *jhd2* mutant cells had no discernable effect on the strength of silencing, once established, at either locus. We observed no impact on mating efficiency in *jhd2* mutants or over-expressers (Fig. 5b), and direct qRT-PCR analysis of *HML-a2* and *HMR-a1* expression revealed minimal detectable transcription from these loci (Fig. 5d). Therefore, though *jhd2* cells were initially slow to establish silencing, their silenced chromatin was as effective at silencing as the chromatin of wild-type cells once it had formed.

Discussion

The establishment of silencing as measured by a phenotype

By investigating silencing in dividing populations of single cells, we characterized the dynamics of silent chromatin formation, thereby testing aspects of the maturation and stochastic models. We excluded purely stochastic models by demonstrating that a cell's probability of silencing *HML* depended upon that cell's identity (zygote or daughter) and history (first or second cell cycle) during establishment. In addition, our findings supported aspects of the maturation hypothesis, but along a much shorter timeline (one to two cell divisions) than initially expected.

The relationship between the phenotypic measures reported here and the underlying molecular events reported elsewhere can be compared albeit with some limitations. After one complete cell cycle, previous studies reported that transcripts from *HMR* decrease to 9.8 % to 12.5 % of wild-type levels^{18,21,50}. Those decreases in mRNA quantity, measured at the population level, correlated with the 86.7 % of cells that retain the un-silenced phenotype after one cell division in our study. We inferred that mRNA measurements from batch culture studies reflected an admixture of two processes: a fraction of cells that had achieved phenotypic silencing, and a fraction of cells that had reduced transcript levels but not enough to pass the more stringent test of silencing used in this study. After two cell divisions, mRNA levels were reported to decrease to 2.5 %¹⁸ to 5 %⁵⁰ of full expression, correlating rather well with the 7 % of cells remaining in the un-silenced state in our studies.

The residual decrease in transcripts from *HML* and *HMR* measured between 3 and 5 cell divisions by Katan-Khayakovich et al.¹⁸ could result from a mixture of influences. These might include differences between the assays as a small fraction of cells slow to induce Sir3 from the inducible (*GALI*) promoter could account for persistent transcripts from the *HML* and *HMR* loci. Alternatively, it is possible that after the phenotypic changes measured in our

studies have occurred, the levels of mRNA transcript may continue to decline. Likewise, the super-stoichiometric Sir protein binding detected in later cell cycles¹⁸ may occur after phenotypic silencing is complete by the cell-based assay. This process may be similar to the Polycomb Group proteins (PcG) of *Drosophila melanogaster* that form heterochromatin to maintain the silencing of HOX genes after initial repression occurs by promoter-specific regulators^{51,52}.

Previously, Xu et al. measured the fluorescence recovery (2hrs) after photo-bleaching (FRAP) of fluorescent reporters from *HML* and *HMR* and determined that transcriptional ability is lost in an all-or-nothing capacity upon Sir-protein induction⁴⁷. We added to their findings by quantifying the phenotypic changes produced by the transcriptional changes they observed. Further, we quantified the probability that a cell will adopt a silenced phenotype depending on their place within a growing pedigree tree. Taken together, the work of Xu et al., Katan-Khayakovich et al., and this study illustrate that silencing in batch culture initiates in individual cells turning off transcription at slightly variable rates thereby creating mRNA levels within cells that transition between the fully transcribed and fully silenced states. Once internal mRNA levels decline to a threshold level, silencing establishes phenotypically. Though Sir protein binding may continue to increase in later stages of silent chromatin development, the phenotypic changes are complete within as few as two cell divisions.

There is a formal possibility that the phenotypic changes measured in our assay over-estimated the number of cell divisions required for transcripts from *HML* to decline. For cells to respond to α -factor, they must degrade proteins whose synthesis is controlled by α genes and undergo morphological changes in addition to undergoing transcriptional silencing. However, previous studies of homothallic mating-type inter-conversion showed that cells can switch from an α mating type to an **a** mating type within one cell cycle and that turn-over of mating-type associated mRNA and protein is quite rapid (less than 5 minutes for α 2) as compared to the 90 – 120 minute cell cycle^{53,54}. Therefore, it is likely that phenotypic changes occurred quickly following mRNA decline. Also, our assay may not have been capable of detecting silencing were it to occur prior to the first cell division because cells are only able to respond to α -factor during the G1 phase of the cell cycle. However, previous molecular data indicates that, upon Sir protein induction, cell-cycle progression past early S-phase is required for any detectable reduction in transcripts from the *HML* locus. Hence, the cells that showed sensitivity at the 2-cell stage represent the earliest observable transition to the silenced state. Therefore, the cellular assay used in this study to detect the onset of silencing was a close reflection of the transcriptional decline at *HMLa* but was logically expected to occur slightly after those molecular events.

The pattern of silencing establishment within a pedigree

We found a strong bias for synchronous establishment in mother-daughter pairs, implying a close concordance between the mother and daughter cells' fates. However, in cases where the fates of the two cells (*Z'* and *D1*) did not occur synchronously, the daughter cell was more likely to establish silencing while the mother cell continued to divide. This subtle

difference may reflect a difference between the timing of mother and daughter cell cycles or a biased segregation of silent chromosomes towards transmission into the daughter cell.

Chromatin modification and silencing establishment

Trimethylation of H3 K4 is associated with gene activation and is pre-dominantly found in the 5' region of euchromatic genes. Methylation of H3 K79 also demarks euchromatin, but more ubiquitously. Cells lacking histone methyltransferase enzymes (for these methyl marks) adopted the silenced state more readily than wild-type cells, whereas cells lacking a demethylase were slower. This suggests that de-methylation could be a rate-limiting step in the formation of silent chromatin. Formally, it is possible that the impact of Dot1, Set1 and Jhd2 on silencing could result from an indirect effect. However, the hypo-methylation of H3 K4 and H3 K79 within silenced chromatin suggested a direct connection. It is interesting to note that asymmetrical patterns of silencing were more common in the *dot1* and *set1* mutants (Pattern 2 and Pattern 3 in Fig. 2 and 4). Although the foundation of this difference remains unclear, the predominance of symmetric events in wild-type cells could potentially reflect the replication-coupled dilution of the chromatin marks that inhibit silencing.

Like histone methylation, histone acetylation is associated with euchromatin in budding yeast. In our assay, *Sas2* promoted efficient silencing establishment even though the acetylation catalyzed by this enzyme must be removed in the establishment process. There are two competing hypotheses for how *Sas2* and specifically H4 K16 acetylation affect silencing. One possibility is that the loss of H4 K16 acetyl marks in the *sas2* mutant creates additional chromatin sites permissive for Sir complex binding thereby diluting Sir proteins concentration at *HML* and reducing both the speed and the effectiveness of silencing. Alternatively, the active de-acetylation of H4 K16 by Sir2 may guide the Sir complex into an optimal conformation to promote silencing^{55,29,56}. Although our data do not distinguish which hypothesis for *Sas2*'s role is correct, they do demonstrate a role for *Sas2* in enhancing the kinetics of the establishment of silencing.

In steady-state cultures of *dot1*, *set1* and *sas2* cells, the strength of silencing at *HML* and *HMR* are mildly reduced (detectable in some mutants only in sensitized strains). This finding originally inspired the hypothesis that a re-localization of Sir proteins in these mutants weakens silencing. However, during the establishment process, acetyl marks promoted silencing and methyl marks delayed it. How can the chromatin modifying proteins have similar effects on silencing during steady-state growth yet opposite effects on the establishment of silencing? It is possible the impacts of these modifications on silencing establishment are direct, whereas the effects of these marks on steady-state silencing are indirectly linked to a re-distribution of Sir proteins within the genome²⁵. Alternatively, the transition from the active state to the silenced state may reflect a balance between the strength of transcription of the genes at *HML* and *HMR* versus the strength of silencing at those locations. By this hypothesis, the rapid rate of silencing establishment in *dot1* and *set1* mutants may indicate that their ability to maintain active transcription is compromised. Whatever the mechanism, the rate of silencing establishment in chromatin mutants provided a welcome new phenotype revealing their effects on dynamic aspects of gene regulation.

Though *jhd2* cells showed pronounced delays in silencing establishment, they had no defects in steady-state silencing. As such, *jhd2* mutants have never been isolated from screens for loss-of-silencing phenotypes. Because genomes of all organisms have evolved to respond dynamically to changing environments, genetic screens with the capacity to reveal dynamic phenotypes are likely to contribute new insight to well-studied phenomena.

In our assay, two-cell-cycles required 4 – 6 hours of time. Our data did not distinguish whether the two cycle requirement represents the need for a fixed amount of time, a fixed number of cell divisions, or a mix of both. For now, this issue is unresolved.

In summary, by studying the establishment of silencing in individual cells, we have disproven purely stochastic models and have limited the maturation hypothesis to a timeline in which events required for establishing silencing are complete in the vast majority of cells within two cell cycles. We found that euchromatic methyl marks slow the establishment of silencing. Finally, we note that the need to remove euchromatic marks provides an elegant explanation for the long-enigmatic discovery of the grand-parental effect on silencing establishment⁵⁷, a phenomenon in which bi-stable populations of *sir1* cells switch from the transcribed to the silenced state as four-synchronously switching grand-daughter cells.

Materials and Methods

Plasmids and Strains

All yeast strains were constructed in the W303 background (Table 1). Mutations were generated using the one-step integration of knockout cassettes^{58,59}.

JRY8828 contained a marker replacement of the *MAT* locus that was amplified from pKAN-MX using the primers oEO27 and oEO28. The *hmr* : *HYG-MX* replacement was constructed using a fragment amplified out of pAG32 using the primers oEO30 and oEO36. The *hml* : *NAT-MX* cassette replaced the *HML* locus with a fragment amplified out of pAG25 using oEO32 and oEO33. Genotypes of all strains in this study were confirmed using marker selection, diagnostic PCR of both the 5' and 3' ends, RT-PCR, and DNA blot hybridizations.

Strain 1 (JRY8828) and Strain 2 (JRY8829) were the parent strains for all isogenic chromatin-modification mutants.

Strains over-expressing *JHD2* or *DOT1* under the *TDH3* promoter were constructed by amplifying the *TDH3* promoter from genomic yeast DNA using oEO122 and the fusion primer oEO124. The marker KanMX was amplified from the pKAN-MX plasmid using oEO121 and oEO123. Both fragments were amplified for 24 cycles and cleaned using the Qiagen PCR purification kit. To create a *KanMX*: *TDH3promoter* fusion product appropriate for replacing the *JHD2* regulatory region, the two fragments were used as template for overlap-extension PCR using primers oEO119 and oEO120 for 20 cycles. Strains over-expressing *DOT1* under the *TDH3* promoter used the same template fragments, but the primers oEO125 and oEO126 in place of oEO119 and oEO120. Both fragments were transformed into JRY2334 and JRY4013, and the resulting transformants were checked by

diagnostic PCR, DNA sequencing, and Immuno-blot for increased H3 K4 methylation and H3 K79 methylation.

Pedigree Assay

Strain 1 (JRY8828), Strain 2 (JRY8829) and JRY2728 were streaked onto fresh YPD plates and grown overnight at 30°C. For the pedigree assay, the agar in a YPD plate was cut in half. One half was used for mating haploids to produce zygotes, and the other half was used for the α -factor sensitivity assay. On the mating half of the plate, 25 pairs of individual Strain 1 and Strain 2 cells were arranged in contact with one another using a micromanipulator to allow mating. On the other half, *MATa* cells (JRY2728) were spread in a thick line to produce a source of α -factor. Upon mating, the resulting zygotes (typically 10 – 20) were moved into close proximity of the α -factor -source and were allowed to divide at 30°C. Cells were monitored every 1 – 1 ½ hours by microscopy. With every cell division, mother and daughter cells were separated and arranged so that their identities and histories could be tracked. After a maximum of three cell divisions, or upon completion of the assay (all cells resulting in a shmoo) the pedigree pattern resulting from each zygote was recorded. In assay of strains lacking gene for particular chromatin modifying enzymes, both parents were deficient for the same gene. Their pedigree patterns were compared, on the same plate, to zygotes from a control mating of Strain 1 and Strain 2. Because there did not appear to be any plate-specific or day-specific systematic effects on the observed patterns, the results of several plates were pooled such that each mutant and corresponding wild-type dataset included the patterns of roughly 100 pedigree lineages.

Quantitative RT-PCR

Total RNA was harvested from 50 OD units (A600) of cells using the hot-phenol method. Total RNA was cleaned of DNA using Amplification-grade DNase I (Invitrogen) and purified using the RNeasy Minelute kit (Qiagen). cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR and oligo(dT) primer (Invitrogen). Quantitative PCR on the resulting cDNA was performed using an MX3000P machine (Stratagene) and the DyNAmo HS SYBR Green qPCR kit (NEB in Figure 3 and Invitrogen in Figure 5). *a1* transcripts were amplified using primers oBO29 and oBO30. *a2* transcripts were amplified using primers oEO258 and oEO259. Actin was amplified using act1f and act1r. Amplification values for all primer sets were normalized to actin (*ACT1*) cDNA amplification values and depicted relative to wild-type levels.

Quantitative Mating Assay

Efficiency of mating was assayed as previously described².

Testing for Phenotype-Genotype Associations

See Supplemental Materials and Methods.

Software

The pedigree assay data were analyzed using the R language and environment for statistical computing and graphics⁶⁰. Details on each of the functions used in this manuscript can be obtained from the R documentation and help files.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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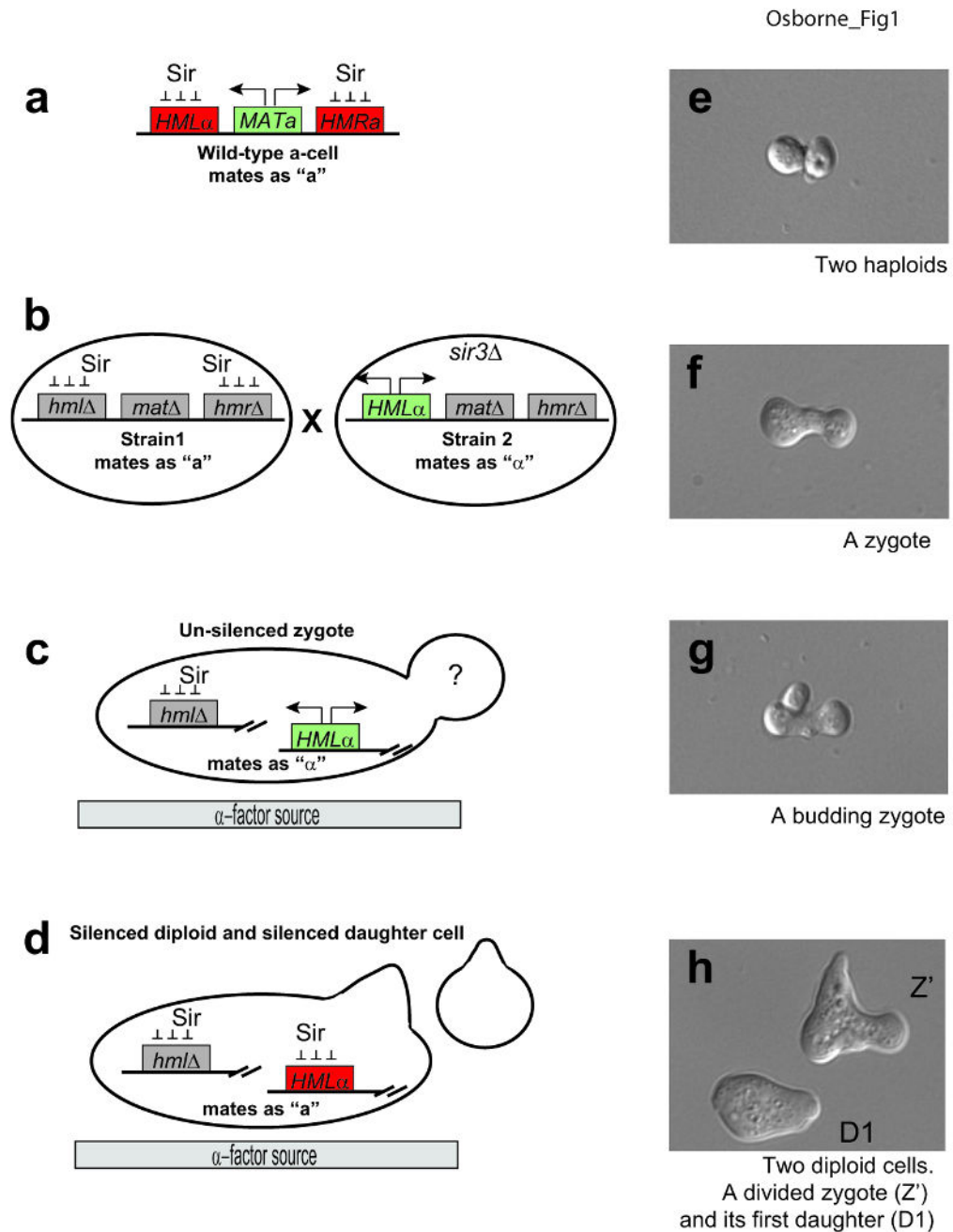


Figure 1. A pedigree assay to measure the establishment of silencing as a function of cell divisions

(a) Wild-type cells contain cryptic copies of *a1* and *a2* transcription factor genes at *HML* whereas copies of these same genes at *MAT* are transcribed. (b) In the pedigree assay, Strain 1 (JRY8828) containing a wild-type copy of *SIR3* was mated to a *sir3*-deficient Strain 2 (JRY8829). (Shown prior to mating in e, and after mating in f). (c) Using a micromanipulator, zygotes were moved to an α -factor source where they divided (pictured

in **g**) until *HML a1* and *a2* transcription factor genes were functionally silenced. (**d**) Upon silencing of *HML*, cells became sensitive to α -factor and arrested as shmoo (pictured in **h**).

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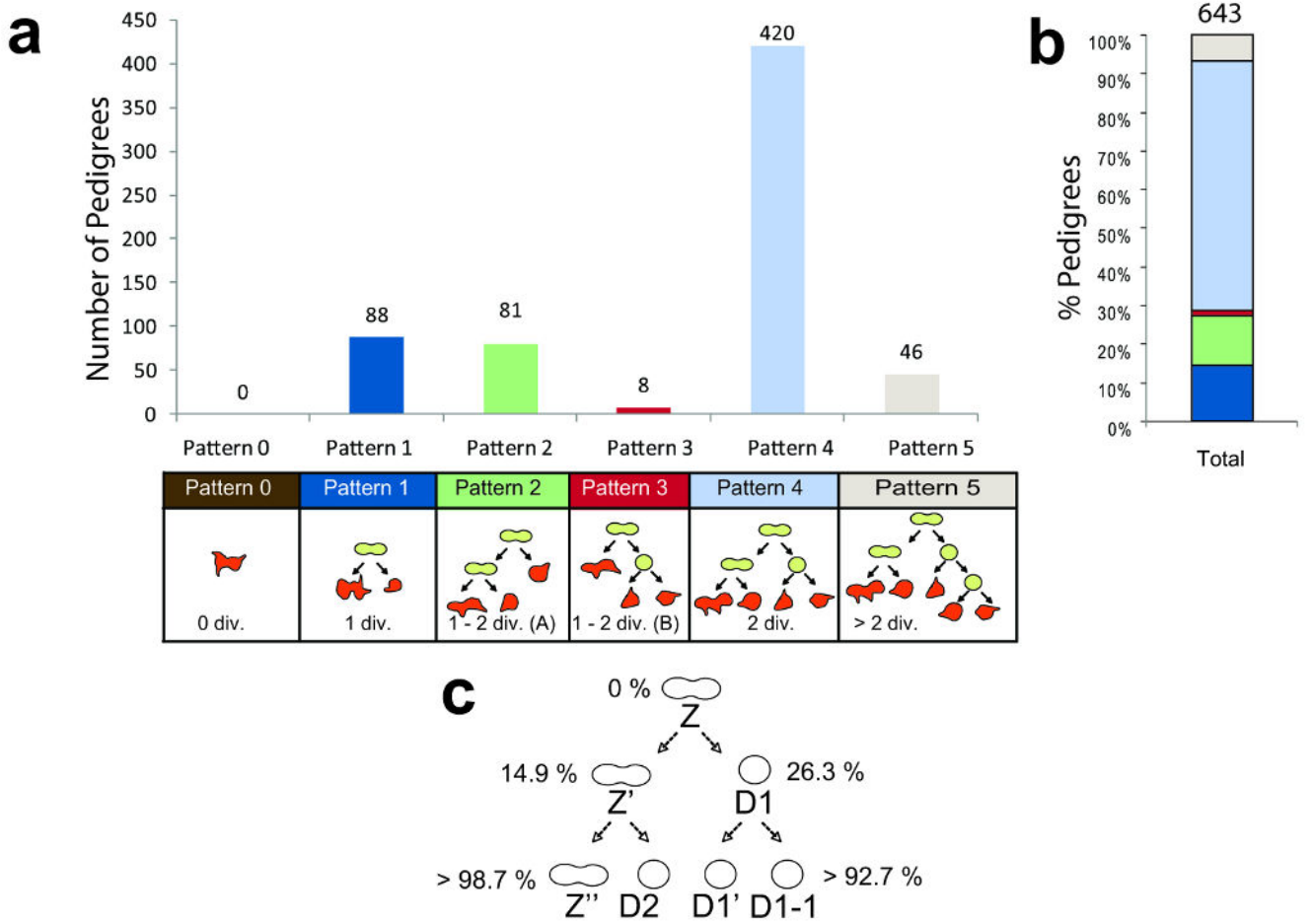


Figure 2. Strain 1 (JRY88828) and Strain 2 (JRY8829) fusion products established silencing after 1 – 3 cell divisions

(a) Barplot of pedigree pattern counts. Zygotes and their daughters were tracked by microscopy to determine their pattern of arrest, and by inference, *HML* silencing. Upon silencing, cells arrested division as shmoo at different points in their lineage, producing five possible patterns of silencing. No zygote ever silenced *HML* prior to cell division (Pattern 0). Pattern 1 – Pattern 5 represent silencing events produced between 1 – 3 cell divisions. In Pattern 5, the extra division could have occurred in any of the four granddaughter cells, not necessarily the D1-1 cell as depicted. The data represented 643 zygotes and all their descendants. **(b) Proportional stacked plot.** This figure depicts the same pedigree pattern counts as in Fig. 2a as a stacked plot. **(c) Pedigree notation and silencing probability.** The names of cells are given here as they are produced in a dividing pedigree lineage. The probability that a given cell of this type was silenced is shown adjacent (computed from the data in Fig. 2a).

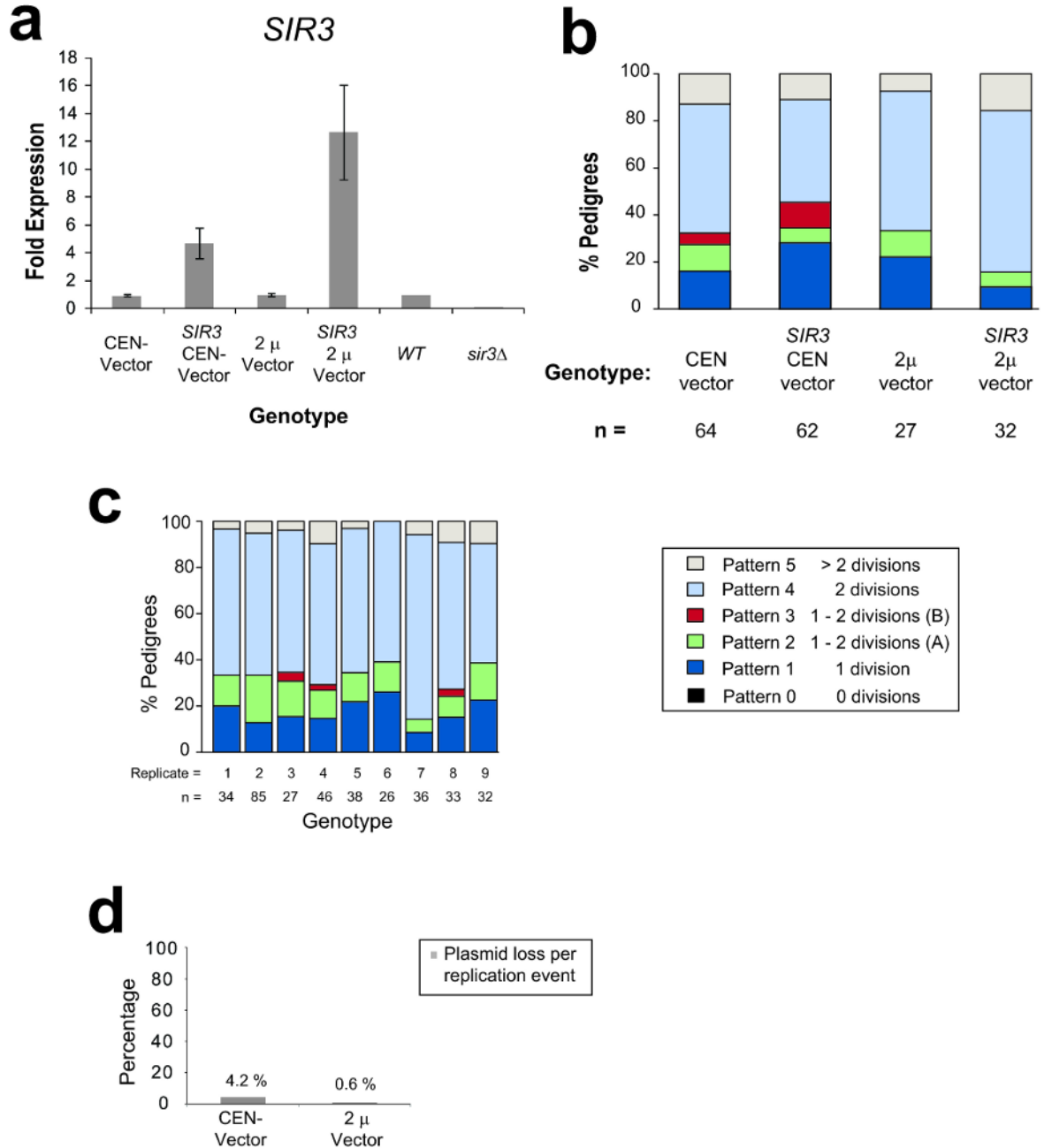


Figure 3. Sir3 over-expression studies

(a) Expression of *SIR3* by qRT-PCR. An additional copy of Sir3 on either a *CEN-ARS* (single-copy) or 2μ (multi-copy) plasmid in Strain 1 caused an over-expression of *SIR3* transcript (JRY8847 – JRY8850, using JRY8828 and JRY8829 as controls). **(b) Silencing *HML* using an over-abundance of Sir3.** Pedigree profiles of silencing establishment using Sir3 over-expression strains are shown compared to strains silenced with the native *SIR3* and empty vectors. From left to right, the strains were JRY8847- JRY8850 \times JRY8829. There was no significant association between pedigree pattern and Sir3 expression levels. That is,

the likelihood ratio test nominal p-values of 0.247 (CEN-ARS) and 0.545 (2μ) were not statistically significant. **(c) Variation between replicates.** Technical experimental replicates of Strain1 (JRY8828) and Strain 2 (JRY8829) in the pedigree assay were performed on zygotes from temporally coincident assays. Several replicates are depicted to illustrate the variation between Strain1 and Strain2 in the pedigree assay. The likelihood ratio test was performed for the pairwise comparison of the 9 groups of wild-type control pedigree assays: the 36 nominal p-values ranged from 0.181 to 0.999 with a mean of 0.626 suggesting that the differences between the profiles of *SIR3* over-expression lines to their corresponding control pedigrees are similar to the variation within wild-type assays (Supplemental Fig. 2a). **(d) CEN-ARS and 2μ plasmid loss rates.** The plasmid loss per cell division of the two *SIR3* over-expression plasmids is shown.

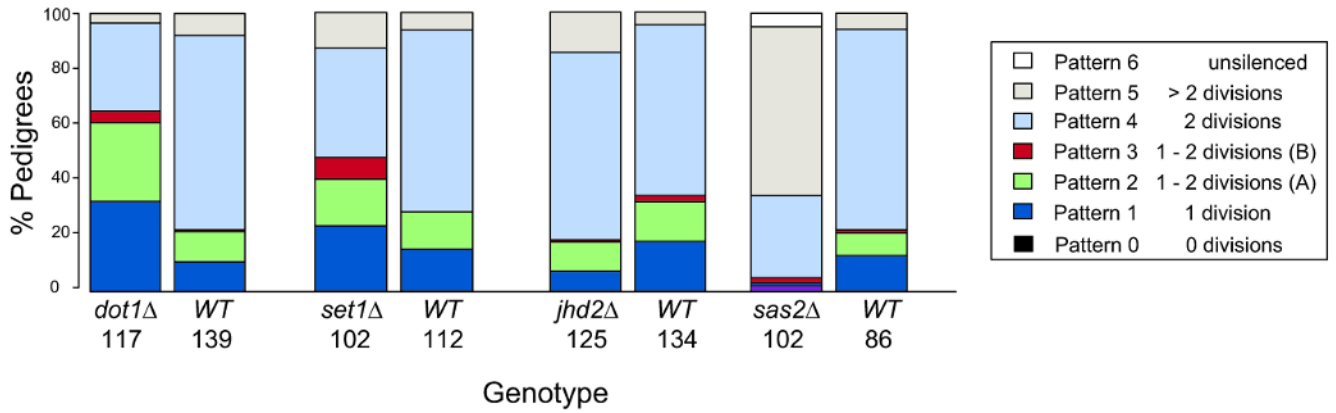


Figure 4. Silencing of cells with mutations in chromatin modifying enzymes

Yeast strains isogenic to Strain 1 (JRY8828) and Strain 2 (JRY8829) and lacking either *dot1*, *sas2*, *set1* or *jhd2* were assayed for their kinetics of silencing using the pedigree assay. They were compared to silencing in zygotes from wild-type Strain 1 and Strain 2 silencing on the same plates. Pedigree patterns generated from these strains are displayed using barplots. The likelihood ratio test was applied to detect associations between pedigree pattern and genotype. All four mutants were significantly different from wild type: $p\text{-value}_{dot1} = 4.59 \text{ E-}10$; $p\text{-value}_{sas2} < \text{E-}16$; $p\text{-value}_{set1} = 9.80 \text{ E-}5$; $p\text{-value}_{jhd2} = 4.22 \text{ E-}3$. As a benchmark, pairwise comparisons between the four groups of wild-type assays yielded six nominal p -values ranging from 0.179 to 0.900, with a mean of 0.610 representing the low variability amongst wild-type samples (Supplemental Fig. 2). The number of pedigrees tabulated for each comparison is indicated beneath the genotypes.

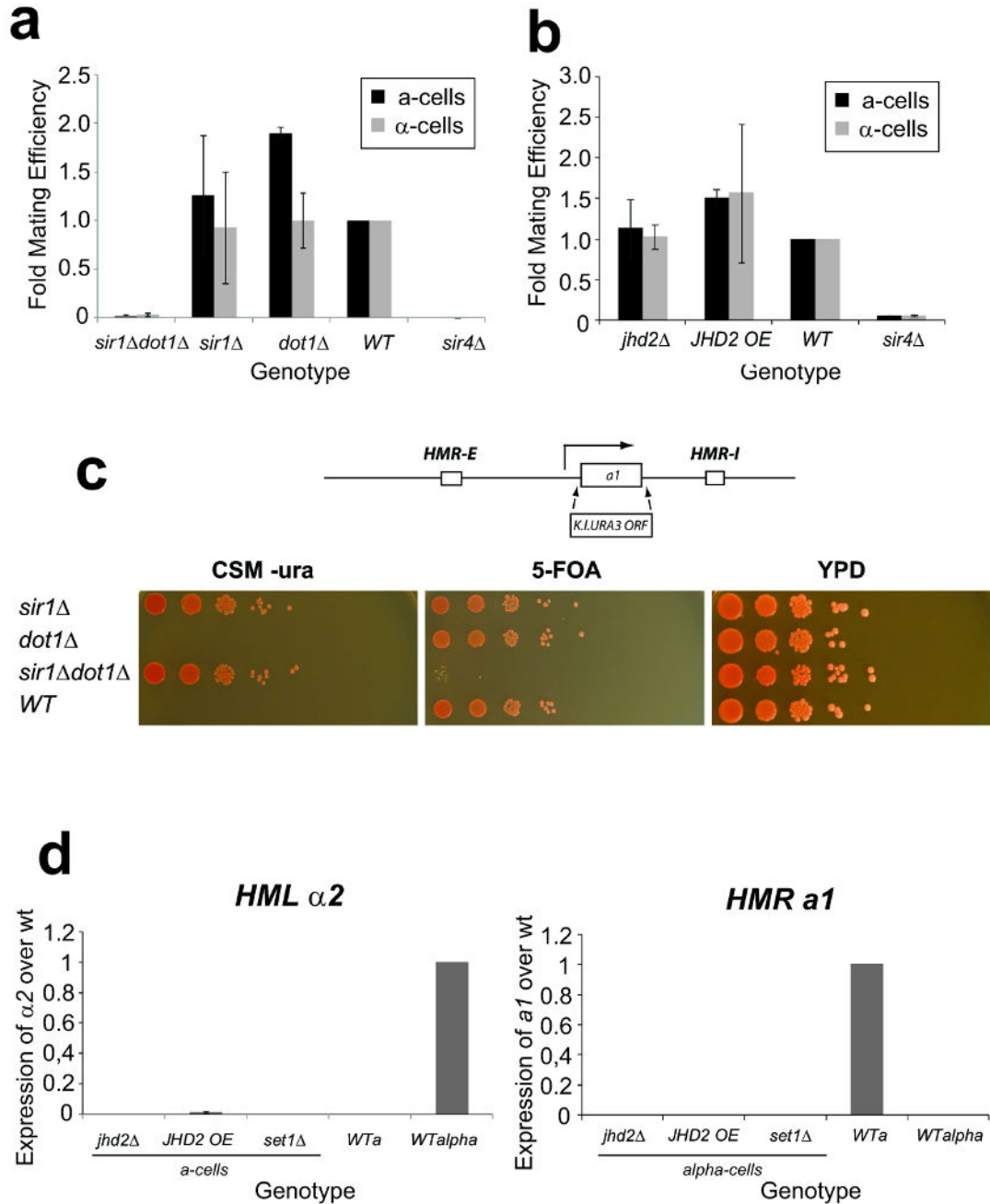


Figure 5. Loss of *DOT1* enhanced the *sir1* loss-of-silencing phenotype

(a) Mating ability of *sir1 dot1* double mutants. *sir1* and *dot1* single and double mutant cells (JRY8873, JRY4621, JRY8874, JRY8875, JRY8957, JRY8958) were tested for their ability to mate with tester strains (JRY2726, JRY2728) by quantitative mating efficiency assay and compared to wild-type (W303-1a, W303-1b) and *sir4* (JRY3411, JRY3841) strains. **(b) Mating ability in *jhd2* and *JHD2* over-expressing cells.** *jhd2* strains (JRY8843, JRY8844) and *JHD2* over-expressing yeast (JRY8884, JRY8885) were tested as in (a) for their ability to mate. **(c) Silencing of a *URA3* reporter in cells lacking**

SIR1 and DOT1. A strain replacing the *Kluyveromyces lactis URA3* open reading frame for *a1* at *HMR* was tested for *HMR* expression on CSM – ura plates and for growth on 5-fluoroorotic acid (5-FOA) plates to which strains expressing *URA3* are sensitive. Isogenic *sir1* , *dot1* , and *sir1 dot1* double mutants were diluted to 1 OD and 1:10 serial dilutions, spotted onto appropriate plates, and grown at 30° C for 2 days (JRY8876 – JRY8833). **(d)**

Expression of *a2* and *a1* in cells lacking or over-expressing *JHD2* by qRT-PCR.

Transcript levels of *a1* from *HMR* were measured in cells that lacked or over-expressed Jhd2 (*MATa* background, JRY8844 and JRY8885) using qRT-PCR. *set1* strains (JRY8889) and wild-type strains (W303-1a, W303-1b) served as controls. *HML a2*-gene expression in cells in a *MATa* cells (JRY8843, JRY8884, JRY8888, W303-1a, W303-1b) was also assessed.

Results were expressed as the average fold-expression over actin relative to *MAT* expression in biological triplicates.