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# Yeast heterochromatin stably silences only weak regulatory elements by altering burst duration

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## SUMMARY

Transcriptional silencing in *Saccharomyces cerevisiae* involves the generation of a chromatin state that stably represses transcription. Using multiple reporter assays, a diverse set of upstream activating sequence enhancers and core promoters were investigated for their susceptibility to silencing. We show that heterochromatin stably silences only weak and stress-induced regulatory elements but is unable to stably repress housekeeping gene regulatory elements, and the partial repression of these elements did not result in bistable expression states. Permutation analysis of enhancers and promoters indicates that both elements are targets of repression. Chromatin remodelers help specific regulatory elements to resist repression, most probably by altering nucleosome mobility and changing transcription burst duration. The strong enhancers/promoters can be repressed if silencer-bound Sir1 is increased. Together, our data suggest that the heterochromatic locus has been optimized to stably silence the weak mating-type gene regulatory elements but not strong housekeeping gene regulatory sequences.

## Graphical abstract

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AUTHOR CONTRIBUTIONS

K.W.: construction of reagents; experimental investigation; development, testing, and validation of methodology; data analysis; data curation; and editing of the manuscript. N.D.: construction of reagents, development and testing of methodology, and reviewing and editing of the manuscript. A.B.: construction and maintenance of equipment and writing scripts for image analysis. S.A.: construction and maintenance of equipment, supervision, and reviewing and editing of the manuscript. R.T.K.: conceptualization of experiments; construction of reagents; experimental investigation; supervision; and writing, reviewing, and editing of the manuscript.

SUPPLEMENTAL INFORMATION

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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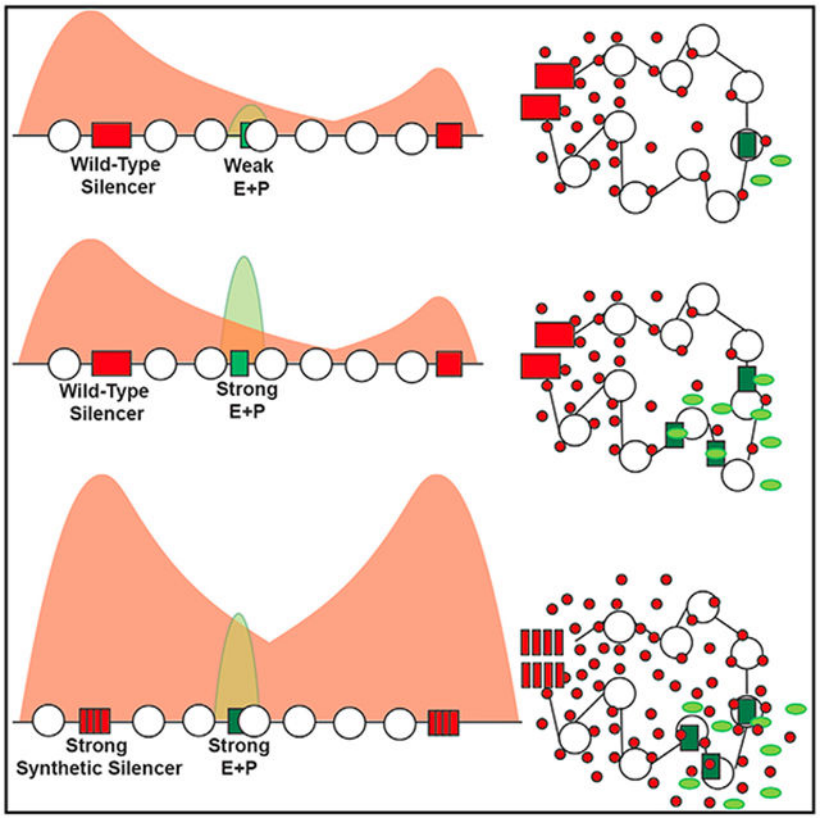


## In brief

Wu et al. show that Sir proteins stably silence only weak regulatory elements but not strong elements. This is due to limiting amounts of the Sir proteins being recruited by silencers and the presence of chromatin remodelers. Permutation analysis shows that silencing regulates enhancers and promoters by altering transcription burst duration.

## INTRODUCTION

The DNA in a eukaryotic nucleus is packaged with histones and non-histone proteins to form chromatin. The interplay between transcription factors (TFs) and nucleosomal packaged DNA sequences ultimately leads to stable programs of gene expression.[1]

### Transcription factors and transcription activation

Regulated transcription of genes in *Saccharomyces cerevisiae* requires sequence-specific transcription activators as well as the general TFs (GTFs). The latter bind sequences in the core promoter and mediate the formation of the preinitiation complex, while sequence-specific TFs bind to upstream activating sequence (UAS) enhancers to regulate transcription.[2,3] Most yeast TFs are not essential for viability but are required for growth in stress conditions,[4] although there are a handful of essential yeast TFs referred to as general regulatory factors (GRFs).[5-9]

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Genes in yeast have been classified into distinct groups based on their enhancer architecture and TF requirements. The largest class is constitutively transcribed at very low levels, typically generating between one and three mRNAs/h.[10-12] The second class comprises the stress-responsive genes that are transcribed at very low levels in rich media but are induced under stress conditions and are usually regulated by a single key TF.[11,13] The growth/housekeeping genes are genes whose expression is highest during rapid growth and that encode ribosomal proteins and glycolytic enzymes. This class of genes is regulated by combinations of the GRFs.[11,14-21]

Nucleosome architecture is integral to gene regulation by TFs, and the preinitiation complex cannot assemble at a core promoter if that element is packaged into a nucleosome.[22,23] Nucleosome profiling in *S. cerevisiae* has revealed a high degree of organization at the regulatory regions of most genes, with well-positioned +1 and −1 nucleosomes that flank a nucleosome-depleted region (NDR) of variable width and depth encompassing the UAS enhancer and core promoters.[8,24-28] The removal and/or the maintenance of an NDR requires some TFs[21,29,30] as well as specific chromatin-remodeling factors.[31,32]

In eukaryotes, transcription occurs in bursts due to the thermodynamics of transcription-activator and GTF binding as well as nucleosome mobility. Gene transcription can be divided into three steps. First, the frequency of transcription initiation (burst frequency) represents activator binding, nucleosome remodeling, and preinitiation complex formation. Once transcription is initiated, multiple polymerases can be loaded during a transcription burst. Finally, the transcription burst is of a limited duration, and so the gene switches from an active to a quiescent state.[33-37] TF binding affects burst frequency, while TF residence time determines burst duration.[38-49] Nucleosomes play a role in bursting, since they influence accessibility of sequences and residence time of TFs and GTFs bound to DNA.[50]

## Sir proteins and silencing

The opposite of gene activation is gene repression. Transcriptional gene silencing is a form of repression involving a chromatin state that leads to the stable and heritable inactivation of genes. Silencing in *S. cerevisiae* is mediated by DNA sequence elements called silencers, and at the *HMR* locus, the *HMR-E* and *HMR-I* silencers flank the *MATa1* and *MATa2* genes. *HMR-E* is bound by ORC, Rap1, and Abf1 proteins, while *HMR-I* is bound by Abf1 and ORC.[51,52] The *HMR-E*-bound ORC proteins aid in the binding of Sir1 to the *HMR-E* silencer.[53-55] Sir1, along with the other silencer-bound proteins, helps recruit Sir2, Sir3, and Sir4. The Sir2/Sir4 complex is responsible for the deacetylation of lysine 16 in histone H4 in nucleosomes, which promotes the binding of Sir3 to unacetylated nucleosomes.[52] Sir-protein-bound chromatin inhibits the binding of either TFs or GTFs,[56,57] although some studies suggest that silenced chromatin blocks events that occur after transcription initiation.[58,59]

The transcriptionally silent state is highly stable, and in wild-type cells, silencing of the *MAT* genes is rarely lost.[60] In cells with mutations in either Sir1 or the silencers,[61,62] the fidelity of silencing is reduced, and two populations of cells are observed, some cells with the gene fully silent and some with the gene fully active. Furthermore, these expression

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states, once established, are propagated across several cell generations, leading to two metastable populations of cells resulting in bistable expression states.[61-63]

Classical studies on silencing have focused on a set of UAS enhancer and core promoters of stress-inducible or mating-type genes that are controlled by single transcription activators (reviewed in Rusche et al.[51] and Gartenberg and Smith[52]). Housekeeping genes constitute around a third of the genes in yeast, but the ability of these genes to be silenced has not been investigated. In this article we analyze silencing of a set of UAS enhancers and core promoters of varying strengths and test different classes of elements: housekeeping, stress-induced, and weak constitutively expressed genes. We show that the wild-type silencers weakly repress highly transcribed housekeeping genes by altering the burst duration. While weak repression of the housekeeping gene elements was observed with the native silencers, this repression was not stably propagated, and the partial repression did not result in bistable expression states. While the native silencers were unable to effectively silence housekeeping genes, results with synthetic silencers show a correlation between the amount of Sir1 bound to a silencer and the repression of these strong enhancer/promoters.

Analysis of permutations of UAS enhancers and core promoters showed that sequences in both the enhancer and the core promoter determine the extent to which a regulatory sequence is susceptible to gene repression. Mutant analysis highlighted a role for chromatin remodelers in preventing a regulatory element from being repressed, suggesting that inhibiting nucleosome mobility may be a mechanism by which the Sir proteins silence genes. Our data suggest that the silenced locus has been optimized for just enough silencing to stably repress the mating-type-gene UAS enhancers and core promoters but not for the silencing of strong activating regulatory sequences.

## RESULTS

### Construction of yeast reporter strains to measure silencing

To measure the susceptibility of various enhancers and promoters to silencing, we expanded the modular system developed recently to study gene activation.[64,65] This allowed us to build various permutations of regulatory elements and investigate the functional relationship between silencers, UAS enhancers, and core promoters (Figure 1A). The system we generated involves silencers flanking the coding regions (CDSs) of various reporters: *MATa1*, *URA3*, Venus + PEST (proline [P], glutamic acid [E], serine [S], threonine [T]) + NLS (nuclear localization signal), and 14xPP7bs-*PHO5*. Immediately upstream of the CDS, we inserted UAS enhancer + core promoter segments (*MATa1*, *PDC1*, *PGK1*, *RPL28*, *TDH3*, *ACO1*, *CDC19*, *ADE2*, *GAL1*, and *MATalpha2*) that we had previously characterized for gene activation.[65] In this article, the term “UAS enhancer” is defined as a genetic element that interacts with specific TFs to stimulate transcription from a separable core promoter. We define a core promoter by its occupancy by GTFs based on chromatin-immunoprecipitation-sequencing (ChIP-seq) data.[66] While the transcription rate mainly determines the mRNA abundance in yeast, modulation of mRNA stability can also be a factor,[67] and the 3′ untranslated regions (UTR) play a role in mRNA stability and abundance.[65] To minimize this variable, we placed the *PGK1* 3′ UTR and transcription terminator downstream of all the reporter CDSs. These constructs were integrated at one of

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two sites in the yeast genome using CRISPR-Cas9: the heterochromatic *HMR* locus near the right telomere of chromosome III or the euchromatic *LEU2* gene in the middle of the left arm of chromosome III.

### *HMR* only partially represses housekeeping gene enhancers/promoters

A haploid yeast strain of the alpha mating type will mate with cells of the opposite mating type to form diploid cells only if the *MATa1* gene at the *HMR* locus is transcriptionally repressed. Derepression of the *MATa1* gene renders haploid alpha-mating-type cells unable to mate and form diploid colonies. We built constructs where the *HMR* locus with its native *HMR-E* and *HMR-I* silencers flanked the *MATa1* CDS fused to the 10 different UAS enhancers and core promoter sequences, and these were integrated at the *HMR* locus. The strains were analyzed using a semi-quantitative mating assay with 10-fold serial dilutions of cells (Figure 1B, at *HMR*). The data show that the *MATa1*, *MATalpha2*, *ADE2*, and *GAL1* enhancer/promoters were efficiently repressed. Among the other regulatory elements, there was partial but varying degrees of repression of the *ACO1*, *PGK1*, *PDC1*, *CDC19*, and *TDH3* elements, while the *RPL28* regulatory elements resisted silencing almost completely.

The enhancer and promoter elements that were partially derepressed at *HMR* became further derepressed when the silencing cassettes were instead integrated at the *LEU2* locus. *ACO1*, *PGK1*, and *PDC1* regulatory elements were derepressed to a greater extent, and the *TDH3* and *CDC19* regulatory elements now resisted silencing completely (Figure 1B, at *LEU2*). The four cassettes that were fully repressed at *HMR* remained repressed at *LEU2*. Altogether, these results indicate that robust silencing is position dependent and the *HMR* site is more efficient in silencing compared with the *LEU2* site.

Quantitative mating analysis revealed a gradient of repression dependent on the enhancer and promoter sequences. The *MATa1*, *ADE2*, and *GAL1* regulatory elements were silenced in most cells, whereas the *PGK1* enhancer/promoter was repressed in ~10% of the cells, *PDC1* and *ACO1* were repressed in ~1% of the cells, and *CDC19* showed repression in only 0.1% of the cells, while the numberswere even lower for *TDH3* and *RPL28* enhancers/promoters (Figure 1C).

The complete or partial repression observed with these constructs was Sir dependent. In a strain lacking Sir3, repression was completely lost for all the cassettes tested except for *ADE2* and *GAL1* (Figure 1B, *sir3*Δ). This is unsurprising, because silencing was monitored in synthetic dextrose medium with supplements, in which both genes are known to be transcriptionally inactive even in the absence of Sir proteins.

### Fluorescence cytometry shows that partial repression of housekeeping gene enhancers and promoters is not bimodal

Based on classic studies on the *MAT*, *URA3*, and *ADE2* enhancers/promoters, silencing is believed to be an all-or-nothing phenomenon exhibiting bistable expression states.[61,68] The partial repression observed for *TDH3*, *PGK1*, *PDC1*, *ACO1*, and *CDC19* in the mating assay raised the question of whether cells with these elements also exhibited bistable expression states. Fluorescence cytometry is well suited to analyze variation in gene expression in a cell population. To investigate the partial silencing, we built strains where the Venus

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CDS containing an NLS and a PEST sequence was linked to the different enhancers/promoters and integrated at the *HMR* locus. Logarithmically growing cells were analyzed for expression of Venus after appropriate gating using a flow cytometer (Figure 2). We demarcated cells as being silent relative to the expression values of the *MATa1* enhancer/promoter, where greater than 99% of these cells were considered repressed (Figure 2A). For the other enhancer/promoter cassettes, cells with fluorescence values within the *MATa1* regulatory element repressed gate were considered repressed, while the remainder of the cells were considered active, and we quantified the population frequency of these cells. The frequency of non-silenced cells was plotted (Figure 2B), and the data show that the *MATa1*, *ADE2*, and *GAL1* enhancers/promoters were fully repressed, while partial repression was seen for the other enhancers/promoters, with values ranging from ~15% active for *ACO1* to ~90% active for *RPL28* and *TDH3*.

When the same analysis was done with *HMR* cassettes containing Venus integrated at the *LEU2* locus, we observed similar results but with increased frequency of activation of some of the cassettes (Figure 2B). This trend was most obvious for the intermediately expressed regulatory elements—*CDC19* expression frequency increased from 0.67 when the locus was at *HMR* to 0.79 when the locus was at *LEU2*, and *PDC1* expression increased from 0.67 to 0.98 and *PGK1* from 0.41 to 0.60. These results are like the data obtained with the mating assay showing position-dependent effects on repression.

The protein expression profiles of individual cells in a population can also be informative with regard to mechanisms of regulation. Monitoring the expression histograms of these cells undergoing silencing did not show a bimodal peak profile that would be expected for bistable expression states (see Figure 2A). The histograms showed a single peak of expression, although for some of the housekeeping genes we observed a skewed normal distribution with a lagging edge extending toward the repressed state. Analyzing these peaks showed a continuum of protein expression values for the different enhancers/promoters, indicating that the partial repression observed was not an all-or-nothing phenomenon, but the amount of fluorescent protein varied within individual cells.

## Partial repression of housekeeping gene enhancers/promoters is not stably inherited

The *URA3* gene is commonly used as a reporter to measure Sir-mediated silencing.[68] Repression of *HMR::URA3* measured by colony formation on medium containing 5-fluororotic acid (5-FOA) measures repression throughout the cell cycle and over multiple generations. Cell growth on medium containing 5-FOA therefore identifies cells where the gene is sufficiently repressed to allow growth on 5-FOA, and the ability to form colonies on 5-FOA indicates heritability of the repressed state.

We built the *HMR* cassette with the different enhancers/promoters driving expression of the *URA3* CDS. These cassettes, integrated at *LEU2*, were monitored by growth on medium lacking uracil (–uracil) or medium containing 5-FOA. All strains grew normally in synthetic complete media (Figure 3). In medium lacking uracil, we did not observe any growth for strains with the *GAL1* enhancer/promoter, very weak growth for the *ADE2* enhancer/promoter, and robust growth for the *MATa1*, *MATalpha2*, *ACO1*, *PGK1*, *PDC1*, *CDC19*, *TDH3*, and *RPL28* enhancers/promoters. On the other hand, in medium containing 5-FOA,

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we observed robust growth for strains with the *GAL1*, *ADE2*, *MATa1*, and *MATalpha2* enhancers/promoters but no growth for the remaining six enhancer/promoters. These results indicated that the partial repression observed for the housekeeping gene (*ACO1*, *PGK1*, *PDC1*, *CDC19*, *TDH3*, and *RPL28*) enhancer/promoter was not at all stably inherited. On the other hand, *MATa1*, *MATalpha2*, and *ADE2* growth on both –uracil and 5-FOA media is typical of bistable expression states as reported.[68] The fact that we failed to see a bimodal distribution for these four regulatory elements with Venus (Figure 2A) is most likely a function of the sensitivity of detection by cytometry. These data, in conjunction with the cytometry data, suggest that the stability of the silenced state is not simply a function of silencer and silencing proteins but is also affected by the strength and properties of the enhancers/promoters undergoing repression and of the gene product being used as the reporter in silencing assays.

## Sir-mediated repression operates on both enhancers and promoters

Some studies have shown that the Sir proteins block access to TFs that bind UAS enhancers, while other studies find that the Sir proteins block GTFs from binding the core promoter sequences.[56,57,69,70] To measure the individual contributions of the UAS enhancer and the core promoter in resisting repression, we adopted the approach we used recently to delineate and characterize the strengths of the enhancers and promoters in gene activation.[65] We built a matrix of seven enhancers linked to seven core promoters and asked if the extent of repression of these 49 constructs was solely a function of enhancers, was solely core promoter dependent, or depended on both elements. Measurements of the Venus reporter showed that enhancers and promoters influenced expression to different degrees (Figure 4A). Enhancer strength clearly was a major determinant in the ability of that regulatory element to resist Sir-mediated repression. The *TDH3* enhancer was unable to be silenced when linked to different promoters, as were the *RPL28*, *PDC1*, and *CDC19* enhancers, while the *PGK1* and *ACO1* enhancers had intermediate ability to resist silencing. Remarkably, certain core promoters significantly influenced the ability of the strong enhancers to resist repression. For example, repression of the *TDH3* enhancer was significantly increased when this element was linked to the *MATa1* or *ACO1* promoter, suggesting that the promoter is also a target of Sir-mediated repression. Collectively, these data suggest that Sir proteins affect the activity of both enhancers and core promoters. The effectiveness of Sir-mediated repression of the *MATa1* enhancer and core promoter suggests that silencing is optimized to regulate this element.

## Measurement of transcription foci in cells undergoing silencing

Sir-mediated gene silencing operates at the level of transcription, but the assays with *MATa1*, *URA3*, and *Venus* reporters analyzed protein level/enzyme function and measured different aspects of silencing. Mating measures silencing in the G1 phase of the cell cycle, colony formation on 5-FOA measures persistent repression over many generations, while Venus fluorescence measures levels of expressed protein. In addition, the different protein reporters have distinct functions with different maturation times and thresholds of activity, detection, and sensitivity that can color interpretations.

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We therefore decided to directly assay mRNA synthesis in live cells and visualize transcription. We utilized an array of 14 binding sites for the bacteriophage coat glycoprotein PP7 and inserted these repeats in the 5′ UTR of the *PHO5* CDS.[39,45] This construct was linked to the different UAS enhancers and promoters in the silencing cassette. The transcribed mRNA forms stem loops in the 5′ UTR, allowing the bacteriophage protein PP7 to bind. Nascent mRNAs can thus be tracked in real time in individual cells that are constitutively expressing the PP7-GFP fusion protein using a sensitive high-resolution wide-field fluorescence microscope (Figure 4B). We can therefore directly visualize and quantify loci that are not silent and are being transcribed.[45] We analyzed approximately 400 cells per cassette and quantified the number of PP7-GFP transcription foci in these cells. Between 60% and 80% of the cells with *RPL28*, *TDH3*, and *CDC19* enhancers/promoters exhibited transcription foci. The frequency was lower for *PDC1* and *PGK1* cells, while only ~3% of *ACO1* cells had foci. We did not observe any foci for the *MATa1*, *ADE2*, and *GAL1* elements.

In a *sir3*Δ strain, the number of expression foci increased 2- to 3-fold for the moderately repressed *PDC1*, *PGK1*, and *ACO1* cassettes and increased to a lesser degree or not at all for the very strong regulatory elements (*TDH3*, *RPL28*, and *CDC19*), suggesting that these regulatory elements were undergoing robust transcription even in the presence of Sir proteins. Collectively our data highlight the fact that, while the different reporters (*MATa1*, *URA3*, Venus, and PP7-PHO5) measure different aspects of silencing, the regulatory elements linked to these reporters behave in a more or less consistent manner and maintain their relative rank in their susceptibility to repression.

## Measuring duration of transcription bursts

Measuring transcription foci quantifies the number of cells where the *HMR* locus is derepressed, but is not informative with regard to the duration of time the locus is active. To measure duration, we resorted to the use of a multi-focus microscope (MFM) and coupled this instrument with the PP7bs-PHO5-GFP system.[71] The MFM simultaneously acquires images of multiple focal planes at a single moment in time, thus reducing photobleaching, and allows the repeated imaging of a single cell over a period of time. However, previous work using this system has shown that the lower exposure time and reduced intensity of the laser allows the detection of only transcription foci with three or more engaged RNA polymerases.[45,72] Therefore, the presence of fluorescent foci using the MFM identifies loci with multiple transcriptionally engaged RNA polymerases, although the numbers obtained are an underestimate of the actual number of transcribing RNA polymerases. While not optimal, this system allows us to differentiate periods when the locus is highly active from periods when the locus is less active or inactive. Cells were visualized every 15 s for 10 min, and the presence of a transcription focus was highlighted (Figures 5A and 5B). For the three regulatory elements analyzed (*TDH3*, *PGK1*, and *RPL28*), the MFM data identified some cells in the population where we did not detect any transcription over a 10-min period, while there were other cells where we did observe transcription foci. In cells with a focus, transcription was intermittent, suggesting bursting (Figure S1). For each cell, the duration of time that the locus was undergoing transcription was quantified as the frequency of activity, and the data were plotted as a boxplot. The same analysis was also performed on *sir3*Δ

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strains (Figure 5B). The loss of Sir3 led to an increase in the total time when fluorescent foci were observed in all three strains, although the effect was less pronounced for the *RPL28* regulatory sequences (Figure S1). Since the presence of a detectable fluorescent focus indicates a cell with multiple engaged RNA polymerases, these data collectively suggest that Sir3 likely functions by reducing the time when specific sequences are open and accessible and that silencing likely functions, in part, by blocking the repeated initiation of transcription, thus reducing burst duration.

Our data suggest that Sir proteins influence burst duration. At active genes, enhancers regulate burst frequency, while core promoters regulate burst duration.[33,35,73,74] We therefore investigated repression of the *TDH3* enhancer when this enhancer was coupled to four different core promoters of differing strengths. We linked the *TDH3* enhancer to the *MATa1*, *PGK1*, *RPL28*, or *TDH3* promoter. Measuring the frequency of transcription foci in approximately 300 cells per cassette showed that the number of foci was reduced significantly when the *TDH3* enhancer was linked to the *MATa1* core promoter, increased when linked to *PGK1* promoter, but remained unchanged with the *RPL28* promoter (Figure 4C).

We next investigated transcription burst duration of these four cassettes using the MFM. For these strains, the duration of time that the locus was undergoing transcription was quantified as the frequency of activity. There were insignificant changes when the *PGK1* and *RPL28* promoters were linked to the *TDH3* enhancer, but there was a reduction in the duration of the active state when the *MATa1* core promoter was linked to the *TDH3* enhancer (Figures 5C and S2). Collectively, these data suggest that the core promoters influence burst duration and the Sir proteins function to regulate this step during silencing.

## Effects of chromatin remodelers on gene repression

Transcription bursting is regulated by nucleosomes, since most TFs and the GTFs cannot bind their recognition sites if those sites are packaged into nucleosomes.[22,23] During gene activation, histone-modifying and chromatin-remodeling complexes create a chromatin architecture that is nucleosome depleted, thus favoring transcription. We built strains where subunits of either chromatin remodelers or histone modifiers *RSC* (chromatin structure remodeling), *ISW2* (imitation switch), *SAS-I* (something about silencing), and *DOT1* (disruptor of telomeric silencing) were deleted. We monitored the expression of four different regulatory elements (*MATa1*, *PGK1*, *RPL28*, and *TDH3*) driving expression of three different reporters: *MATa1*, *URA3*, or Venus (Figure 6).

We first analyzed the *MATa1* reporter under the control of the four different enhancers/promoters in the four mutants (*dot1*Δ, *sas2*Δ, *isw2*Δ, and *rsc2*Δ) (Figure 6A). The reporter remained repressed when it was under the control of the *MATa1* enhancer/promoter in the different mutant backgrounds. With the *RPL28* enhancer/promoter, the reporter remained fully derepressed, and its expression did not change in the mutants. There was an increase in repression of the reporter under the control of the *TDH3* enhancer/promoter when Isw2 and Rsc complexes were absent, while the *PGK1*-linked reporter showed a small reduction in silencing in the *dot1*Δ and *sas2*Δ strains.

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The same analysis was also performed with *URA3* as the reporter (Figure 6B). Compared with the wild-type strain, we saw little change in growth on medium containing 5-FOA in the various mutant backgrounds. These results suggest that the increase in repression of the *TDH3* regulatory sequences seen in Rsc and Isw2 mutants (based on the mating assay) was not stably propagated.

We then measured repression using the Venus reporter and quantified the changes using cytometry (Figure 6C). Our data once again showed an enhancer/core promoter-dependent effect. The *MATa1* regulatory sequences remained silent in the mutant backgrounds. The *RPL28* regulatory sequences remained resistant to repression even upon loss of various chromatin cofactors. This is consistent with data showing that the NDR at ribosomal-protein-coding genes is mediated primarily by the GRFs.[75] In contrast, *TDH3* and *PGK1* regulatory sequences were dependent to some extent on RSC and ISW complexes in resisting silencing. Loss of these chromatin remodelers resulted in a small reproducible increase in repression. There was also an increase in repression of the *TDH3* regulatory elements in a *sas2*◈ strain. These results suggest that the formation/maintenance of a specific chromatin architecture at the *TDH3* and *PGK1* enhancers/promoters may play a role in their ability to resist Sir-mediated repression. While the effects reported here are reproducible, they could also be due to indirect effects, because the mutants analyzed affect the expression of many different genes in the yeast genome. Furthermore, the *URA3* reporter and the 5-FOA assay are known to be prone to indirect effects when combined with chromatin and replication mutants.[76]

## Increasing silencer-bound Sir1 enabled repression of strong enhancers and core promoters

Our data suggest that the strong housekeeping gene regulatory sequences resist being repressed. One question is whether this is an intrinsic property of the gene regulatory sequences or a function of relative balance between activators and repressors. Sir1 binds *HMR-E*,[55] while the loss of Sir1 leads to decreased recruitment of the other Sir proteins to the silenced locus.[77] We therefore decided to investigate the effects of increasing the number of Sir1 binding sites at *HMR* silencers. We constructed synthetic silencers where the wild-type ORC binding site of the *HMR-E* silencer was replaced with four binding sites for Gal4, and the ORC binding site at *HMR-I* was replaced with five binding sites for Gal4. We placed the Venus reporter linked to the various regulatory elements between these two synthetic silencers and integrated this cassette at the *HMR* locus (Figure 7A). Cells containing this cassette also expressed Gal4-Sir1 fusion protein under an inducible *MET17* enhancer/promoter such that Gal4-Sir1 was expressed only in medium lacking methionine. Expression and binding of Gal4-Sir1 to the synthetic silencer cassettes led to robust repression of all regulatory elements, although the *RPL28* regulatory element was repressed to a lesser degree compared with the other elements (Figure 7B). These results indicate that even the strongest enhancers and core promoters can be repressed, provided the amount of Sir1 bound to the silencer is increased significantly.

We were curious about the quantitative relationship between silencer strength and gene repression. While these elements could be repressed by a locus containing nine binding

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sites for Gal4-Sir1 (four at *HMR-E* and five at *HMR-I*), we decided to vary the number of Gal4-Sir1 binding sites. We built synthetic silencer constructs with either four or one binding site for Gal4 at *HMR-E* coupled with *HMR-I* silencers with five, one, or zero binding sites, cumulatively leading to nine, six, five, four, two, or one binding sites. We analyzed the *RPL28*, *TDH3*, *PGK1*, and *MATa1* regulatory elements driving the expression of Venus. The data showed that, as the number of binding sites for Gal4-Sir1 increased, there was greater repression in the presence of Gal4-Sir1 compared with in its absence (Figure 7C). These results indicated that a key limiting factor in the ability of the native silencers to repress the strong housekeeping gene regulatory elements was the inability of the native silencers to recruit sufficient amounts of Sir1 and possibly the other Sir proteins.

## DISCUSSION

Heterochromatic gene silencing is considered a stable, heritable, and effective form of gene inactivation mediated by a chromatin structure that inhibits expression of most genes regardless of the transcription activator or RNA polymerase involved.[51,52,78,79] The wild-type silencers very effectively silence the *MAT* genes by Sir-protein binding to nucleosomes, which likely prevents nucleosome sliding and/or removal, thus blocking the formation of stable transcription complexes. Reducing levels of the Sir proteins via deletion of Sir1, mutations in Rap1 binding sites at the silencers, reducing levels of Sir proteins, or moving the silenced locus to a euchromatic site leads to metastable silenced states.[61,62,80] Under these conditions, in a fraction of cells, stable transcription complexes can form, allowing these genes to remain active for extended periods of time. The bistable expression state can revert to monostable silencing upon increasing the levels of Sir proteins.[77] Bistability is also observed in instances where wild-type silencers regulate stress-responsive and -inducible genes such as *URA3* or *ADE2*.[63] These genes are activated by single TFs, and the bistable state is dependent on the activators binding their cognate sites, thus helping form stable transcription complexes at the promoter, thus resisting repression.[62,63,68] In contrast, housekeeping genes have large complex UAS enhancers. Optimal transcription of these genes requires binding of multiple TFs, while suboptimal expression is observed when some of the binding sites are mutated.[81] When these regulatory elements are placed at a silent locus, rather than observing bistable expression states, we observe robust but variable levels of transcription that are subtly affected by Sir proteins: an analog rheostat response directed by the UAS enhancers and promoters. The elements escape silencing to varying degrees, possibly due to varying levels and time of occupancy by the TFs or GTFs or variable nucleosome mobility. These data collectively suggest that Sir-mediated repression in wild-type cells is optimized to stably silence only weak regulatory elements such as the *MATa1* regulatory elements.

### How might the Sir proteins repress different enhancers and core promoters?

The mechanism by which TFs activate genes is a function of the underlying regulatory sequences as well as the TFs themselves. Different genes have different requirements for histone modifying and remodeling complexes during activation.[32,82] This genetic property of the regulatory system is unlikely to change when a regulatory element is transposed to a silenced domain. It is therefore likely that the Sir proteins negatively influence

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transcription of all genes while being agnostic with respect to the TFs and mechanisms involved. One common step in transcription of eukaryotic genes is the modification and mobilization of nucleosomes from regulatory sequences. It is therefore possible that silenced chromatin blocks this step. Histone acetylation by modifying enzymes is required for remodeler activity.[83] Similarly, remodeler binding to nucleosomes via their bromodomains is influenced by histone acetylation.[84] Interestingly, Sir3 interacts with nucleosomes via its bromo-adjacent homology domain, making contacts with the unacetylated H4 tail and H2B,[85] and these are also the sites required for chromatin remodelers binding to nucleosomes. These observations raise the possibility that Sir proteins repress genes by directly affecting the ability of chromatin remodelers to bind to and move nucleosomes, in turn changing the probability distribution of active and repressed chromatin configurations and thereby altering the temporal kinetics of TF binding, preinitiation complex formation, and transcription. This would be consistent with studies of RSC and silencing[86] as well as with observations showing that Sir-bound nucleosome have lower turnover rates compared with euchromatic nucleosomes.[87,88] This suggests that heterochromatin-mediated inhibition of transcription might not operate at the level of the accessibility of chromatin domains in the nucleus but at the level of the mobility of nucleosomes at the silenced chromatin domains.

Our analysis of housekeeping genes undergoing silencing demonstrated bursts of transcription followed by periods of low activity or quiescence, and this was altered in a Sir-dependent manner. At the molecular level, we can speculate on how transcription bursting affects silencing. We posit that the Sir proteins repress transcription by binding nucleosomes and affecting their mobility and turnover. For the very weak and stress-induced genes, the presence of nucleosomes over key regulatory sequences and the stabilization of these nucleosomes by the Sir proteins could result in an all-or-nothing expression phenotype,[70,89] because the Sir proteins would impede nucleosome mobilization by chromatin remodelers. On the other hand, the housekeeping gene regulatory sequences utilize multiple TFs. Some of these factors recruit chromatin remodelers, while others can bind their sites even when these sites are packaged in nucleosomal DNA.[28,75] The directed induction of active nucleosomal configurations by these GRF TFs allows these elements to resist and overcome Sir-mediated repression for variable lengths of time. Since burst durations are controlled by the core promoter and burst durations change in the presence of Sir proteins, the parsimonious model would be that Sir proteins bound to nucleosomes alter the position of the +1 nucleosome or the residence time of the preinitiation complex or both. It is therefore possible that the Sir proteins influence the frequency of chromatin configurations by directly hindering chromatin-modifying and -remodeling enzymes and indirectly hindering TF or preinitiation complex formation, leading to repression of weak and stress-induced genes that are dependent upon these remodeling/modifying complexes.

### How do increased levels of Sir1 at a synthetic silencer induce repression of even the strong housekeeping gene regulatory elements?

The silencers are critical for silencing, and loss of these elements immediately abrogates silencing.[90,91] Abf1, Rap1, and ORC bind the silencers, and any two of these are sufficient for robust silencing.[92] The function of Abf1 is to create directionality in silencing

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via its ability to bind/recruit chromatin remodelers, thereby generating evenly spaced nucleosomes.[28,93] Rap1 and ORC/Sir1 recruit Sir4/Sir2 and Sir3 to the silenced domain, thus enabling these proteins to deacetylate and bind evenly spaced nucleosomes. The strength of the silencer is likely a function of the binding affinities of these proteins for silencer DNA in the context of nucleosomes and for the interactions of ORC with Sir1.

There are several possible explanations why strong housekeeping genes can be repressed by increasing levels of Sir1 at the silencer. First, the binding affinity and residence time of Gal4-Sir1 for Gal4 binding sites at the synthetic silencer is unlikely to be the same as the binding affinity and residence time of Sir1 for ORC at the native silencer, and therefore, the dwell times of these two proteins at the silencer could be different, thereby affecting the efficiency of silencing. Second, increasing the number of Sir1 molecules at the silencer may lead to increased local concentrations of the Sir2, Sir3, and Sir4 proteins at the silencers. Loss of Sir1 can be compensated for by overproduction of Sir3 and Sir4,[94] and recent analysis in cells mutated for Sir1 shows that a key function of Sir1 is robust recruitment of the Sir proteins to the silencers.[77] The increase in Sir protein concentration at silencers is unlikely to alter the residence time of the Sir complexes bound to individual nucleosomes (since this is a concentration-independent property), but the increased concentration could affect the search times required by the Sir proteins to find, deacetylate, and bind unacetylated nucleosomes. This, in turn, would affect the ability of histone acetylases and chromatin remodelers to find, acetylate, and mobilize nucleosomes, thus altering the parameters for activation that, in turn, would alter the probability distribution of Sir-bound nucleosomes and silencing.

Collectively, our data suggest that yeast silenced chromatin developed to repress and stably silence transcription from weak constitutively active gene regulatory elements in a manner that modulates transcription burst duration and possibly frequency. Whether the same property holds true for silencing in larger eukaryotes requires further analysis. HP1-mediated constitutive heterochromatin and Polycomb-mediated facultative heterochromatin use different repressor proteins, and therefore it is not known if housekeeping regulatory elements are able to resist these forms of repression. But consistent with our results are the observations that locus control regions in mammalian cells can overcome gene silencing (reviewed in Kamakaka[95]). Furthermore, the demonstration that expressing a small number of mammalian pioneer TFs is sufficient to overcome facultative heterochromatin-mediated silencing and cell differentiation[96,97] is also consistent with our model. It should also be noted that the boundaries between competing chromatin domains in all organisms are often populated by housekeeping genes.[7,98-104]

## Limitations of the study

Stable transcriptional silencing has traditionally been monitored using reporter genes, but different reporters measure different aspects of gene silencing.

The use of the *URA3* reporter is prone to indirect effects, especially when combined with chromatin and replication mutants.[76]

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Most reporters measure protein activity, and each of these proteins has distinct functions with different maturation times and thresholds of activity, detection, and sensitivity.

The MFM does not detect single mRNA transcripts in the cell, and thus the transcription data using this approach are an underestimate of the actual number of transcription events.

These studies characterized Sir-mediated silencing, which is distinct from HP1- and Polycomb-mediated repression.

## STAR★METHODS

### RESOURCE AVAILABILITY

**Lead contact—**Further information and requests for resources and reagents should be made to and will be fulfilled by the lead contact, Rohinton Kamakaka (Rohinton@ucsc.edu).

**Materials availability—**All materials described in this manuscript are available upon request without restrictions from Rohinton Kamakaka (Rohinton@ucsc.edu).

**Data and code availability—**All images generated and reported in this paper have been deposited (see key resources table for DOIs) and are available for further analysis. These data will also be provided by the lead contact upon request.

This manuscript does not report original code.

Any additional information required to reanalyze the data reported in this manuscript are available from and will be provided by the lead contact upon request.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

All *Saccharomyces cerevisiae* strains used in this study were derived from the W303 strain background, generated either by integration or by crosses amongst isogenic strains, and are listed in Table S1.

Strains were grown in Yeast extract Peptone +2% Dextrose (YPD) or Yeast Minimal +2% Dextrose (YMD) with supplements (Leu, Ura, Trp, Lys, Ade, and His) at either 30°C or 23°C.

### METHOD DETAILS

**Semi-quantitative mating and growth assays—**Strains were grown overnight on YPD plates to generate single colonies. Cells from the colonies were used to inoculate 5 mL of YMD media with supplements (Leu, Ura, Trp, Lys, Ade, and His) (YMD + all) and grown overnight at 30°C on a roller drum. Cell culture density was measured ($A_{600}$) and cultures were diluted to 1 OD $A_{600}$ in YMD media without any supplements (YMD - all) then serially diluted 1:10 and 1:100. The resulting 1, 0.1, and 0.01 OD $A_{600}$ cultures were spotted onto appropriate selection plates using a stamper/cell frogger and allowed to grow in a 30°C incubator for 2–3 days before photography.

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For the mating assays, cultures of *MATa his4* and *MATaα. his4* strains were grown overnight in YPD media. 1.1 OD $A_{600}$ of cells in 250 μL YPD were spread evenly over a YMD - all plate and plates were allowed to dry. Tester strains were spotted onto this cell lawn.

For the analysis of growth on 5-fluororotic acid (5-FOA) media, cells were grown after dilution on YMD + all plates containing 1 g/L FOA., while uracil selection was performed on YMD plates with media lacking uracil (Leu, Trp, Lys, Ade, and His).

**Quantitative mating assay—**Tester strains were grown in 5 mL YMD + all media for 12–14 h at 23°C on a roller drum. At the same time, mating lawn strain (*MATa his4*) was grown in YPD, also for 12–14 h at 23°C. Cell densities ($A_{600}$) were measured for tester and mating lawn strains. Tester strains were diluted to 1 OD $A_{600}$/mL in YMD-all and from there serially diluted 1:10, resulting in suspensions of 1,0.1,0.01,0.001, and 0.0001 $A_{600}$/mL. The mating lawn culture was diluted in YPD to 2 OD $A_{600}$/mL.

100 μL of each diluted tester strain was mixed with 300 μL of the prepared mating lawn suspension, and all 400 μL of the mixture were subsequently plated onto YMD - all plates. In parallel, 100 μL of each diluted tester strain was mixed with 300 μL YMD and all 400 μL were plated onto YMD + all plates as growth controls. Cells were allowed to grow at 30°C for 3 days, after which colonies were counted. Three independent colonies were analyzed per strain.

**Fluorescence cytometry—**Strains were grown overnight on YPD plates. Cells were then transferred into 2-3 separate wells of microtiter plates (creating 2–3 replicates) containing 100 μL YMD + all supplement media and grown overnight at 30°C without shaking. 10 μL of each of these cultures were then used to inoculate deep well (2 mL) microtiter plates with each well containing 590 μL YMD + all. For the Gal4-Sir1 induction experiments, the YMD + all media used henceforth contained 3% dextrose rather than 2%, and with or without methionine supplementation as appropriate. All cultures were then grown overnight at 30°C on a shaker set to 600 rpm. 10 μL of these overnight cultures were then transferred into wells containing 590 μL of fresh YMD + all media, and again grown overnight at 30°C on the shaker.

Cell cultures were then measured for density and were each diluted to 0.2 $A_{600}$ OD/mL in 500 μL of YMD + all media and grown in deep well microtiter plates at 30°C for 3–4 h on the shaker. 400 μL of these cultures were filtered through a Nitex screen into fresh deep well microtiter plates. Finally, 200 μL of the filtered cultures were then transferred into wells of a standard microtiter plate and taken to an Attune NxT flow cytometer for analysis.

The Attune NxT flow cytometer with BRV6Y laser was controlled by the Attune cytometric software version 5.3.0. Data for approximately 10,000 cells were recorded per sample. Analysis of these data was performed using FlowJo software. Initial gating was performed for forward and side scatter, based on cell size and intracellular composition, followed by Boolean AND gating prior to the quantification of fluorescence.

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**Multi-focus microscopy—**Cell cultures were grown in YMD + all media at 30°C on a roller drum overnight, back-diluted to an OD $A_{600}$ of 0.2/mL, and returned to the roller drum for 4 h. 1 mL of each cell culture was then pelleted and resuspended in 20 μL of fresh YMD + all media, from which 3 μL was applied to a prepared 1.5% agarose YMD + all pad on top of a microscope depression slide, cover-slipped, and sealed with a 1:1:1 mixture of Vaseline, lanolin, and paraffin (VALAP).

Images were acquired on a high-resolution multi-focus microscope as previously described,[72] which allowed for the simultaneous acquisition of two-dimensional images across 9 different focal planes spaced 144 nm apart. The diffraction-limited axial resolution of the ~525 nm fluorophore was ~600 nm with a 1.4 NA. The objective was a 60× 1.3 NA silicone-immersion lens, and the total magnification was around 180×, diffraction-limited to ~200 nm. Using a two-Z-step acquisition, we were able to capture multiple focal planes of cells across a depth of 3 μm per image with minimal photobleaching. For each field of view, an image was captured every 15 s for 10 min, totalling 41 time points. These images were subsequently processed using a MATLAB script to register the 18 focal planes across the 41 time points.

Subsequent image analyses were performed using the FIJI distribution of ImageJ software. After re-stacking, bleach correction was applied using the histogram matching method.[109] To enhance the contrast between signal and background, maximum intensity and standard deviation Z projections of these bleach-corrected images were prepared and multiplied against each other. Using the TrackMate plugin,[110,111] foci and tracks were identified, screened through a threshold, and curated against false positives. Finally, to obtain the maximum intensity values of the foci in the original images before bleach correction, a copy of the TrackMate overlay data were saved and edited such that TrackMate redirected and reperformed the annotations on a maximum intensity Z projection of the re-stacked image without any other processing. At least 25 cells were monitored per genotype.

**Fluorescence microscopy—**Cell cultures were grown in YMD + all media at 30°C on a roller drum overnight, back-diluted to an OD $A_{600}$ of 0.2/mL, and returned to the roller drum for 4 h. 1 mLof each cell culture was then pelleted and resuspended in 30 μL of fresh YMD + all media, from which 3 μL was applied to a prepared 1.5% agarose YMD + all pad on top of a microscope slide and cover-slipped.

Images were acquired on a DeltaVision Personal DV system running Resolve3D softWoRx-Acquire 7.0.0 (Applied Precision), using a 40×1.35 NA oil-immersion objective (Olympus), with a pco.edge sCMOS camera (PCO). 5 μm image stacks were collected, with each Z-image being 0.2 μm apart, 2.5 μm above and below the plane of focus.

Image analyses were performed using the FIJI distribution of ImageJ software. To capture fluorescent foci in singular images, a two-dimensional maximum-intensity projection was generated for each collected z stack. The resulting images were then counted for foci, and for each genotype we counted at least 200 cells.

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### QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using R. Detailed results of the statistical analysis have been provided where appropriate in the figure legends.

For the quantitative mating assay in Figure 1C, three colonies of each genotype were analyzed independently, and the data are presented as the mean (+/− SD).

To assay silencing loss by fluorescence cytometry, each experiment in Figures 2B, 7B, and 7C was performed in duplicate or triplicate for each genotype and 10,000 cells were analyzed in each experiment. The data are presented as the mean of the mean frequency (+/− SEM) after appropriate gating and normalization using the *MATa1* enhancer/promoter. The details are in the figure legends. Statistical significance was determined using the Welch's 2-sample t test.

For Figures 4B and 4C, statistical significance was determined using the Fishers exact test. Statistical details of the results are provided in the figure legend.

For the MFM experiments in Figures 5B and 5C at least 25 cells were monitored per genotype and statistical analyses are provided in the figure legend.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Highlights

- Wu et al. show that Sir proteins silence only weak enhancers and promoters
- Silencing of strong regulatory elements requires increased recruitment of Sirs by silencers
- Sir proteins repress genes by altering transcription burst duration
- Chromatin remodelers function to counteract Sir-mediated repression

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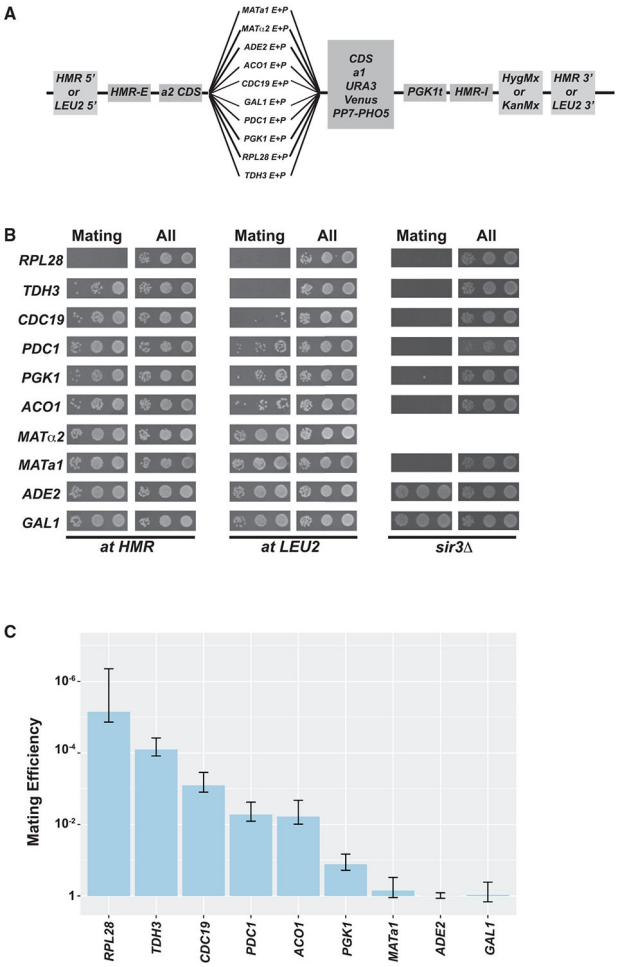


**Figure 1. Measuring silencing with the *MATa1* CDS by mating assays**

(A) Schematic representation of the silencing cassettes constructed from permutations of regulatory elements and reporter genes. Cassettes were integrated at the *LEU2* or *HMR* locus.

(B) Left: repression of wild-type HMR cassette at HMR. Strains contained the *MATa1* reporter under the control of different regulatory sequences, and 10-fold serial dilutions were spotted on appropriate plates. Each spotting experiment was done twice with these strains. Middle: repression of wild-type *HMR* at *LEU2*. Strains contained the *MATa1* reporter under the control of different regulatory sequences. Right: repression of wild-type *HMR* in *sir3*Δ strains.

(C) Quantitative mating of the wild-type *HMR* cassette located at *LEU2* as described previously. [105-107] The data are diploid colony-forming units/total cells and are mean values (±SD) from three independent experiments with these strains.

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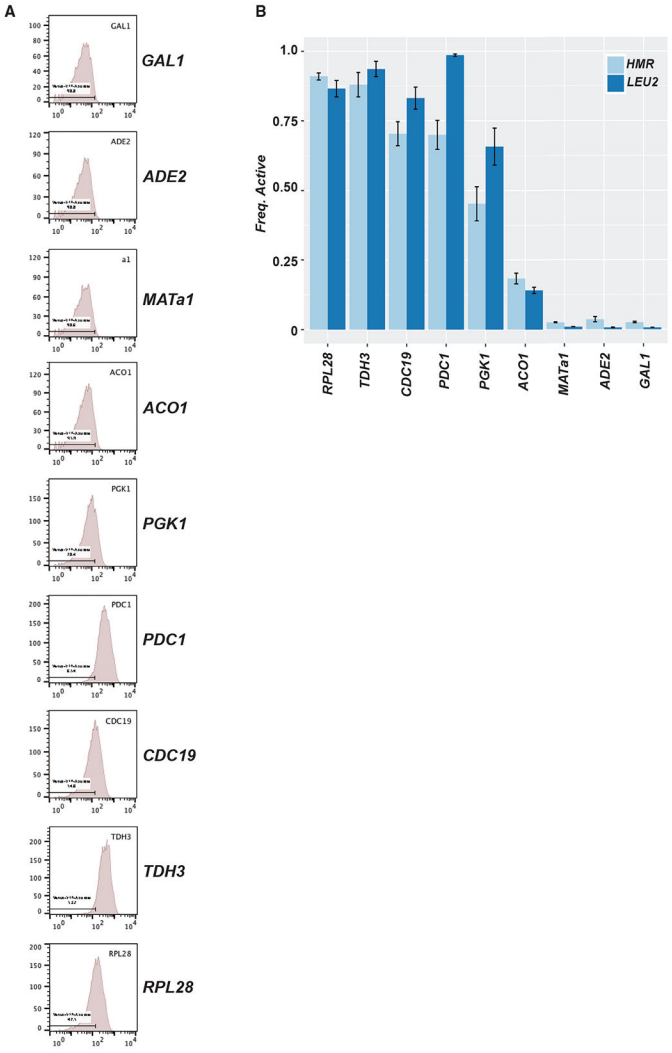


**Figure 2. Measuring silencing using the Venus fluorescent reporter**

(A) Different regulatory elements were linked to the Venus reporter gene, and the silenced cassette was integrated. Expression of the constructs was measured using a flow cytometer. Histograms of the fluorescence cytometry analysis were generated. The x axis corresponds to fluorescence levels and the y axis to the total cell counts. Three separate colonies were measured for each strain, and 10,000 cells were analyzed in each experiment.

(B) Repression of the *HMR* cassette located at *HMR* (light blue bars) or at *LEU2* (dark blue bars). Three separate colonies were measured for each strain. The frequency of cells that escaped silencing was determined as described in the results using the *MATa1* regulatory element as the reference control, and the data are presented as the mean frequency (±SEM). The p values were calculated using Welch's two-sample t test comparing expression at *HMR* versus *LEU2*: *RPL28* p = 0.2752, *TDH3* p = 0.3467, *CDC19* p = 0.09392, *PDC1* p = 0.03089, *PGK1* p = 0.08605, *ACO1* p = 0.1415, *MATa1* p = 0.003133, *ADE2* p = 0.0855, *GAL1* N/A.

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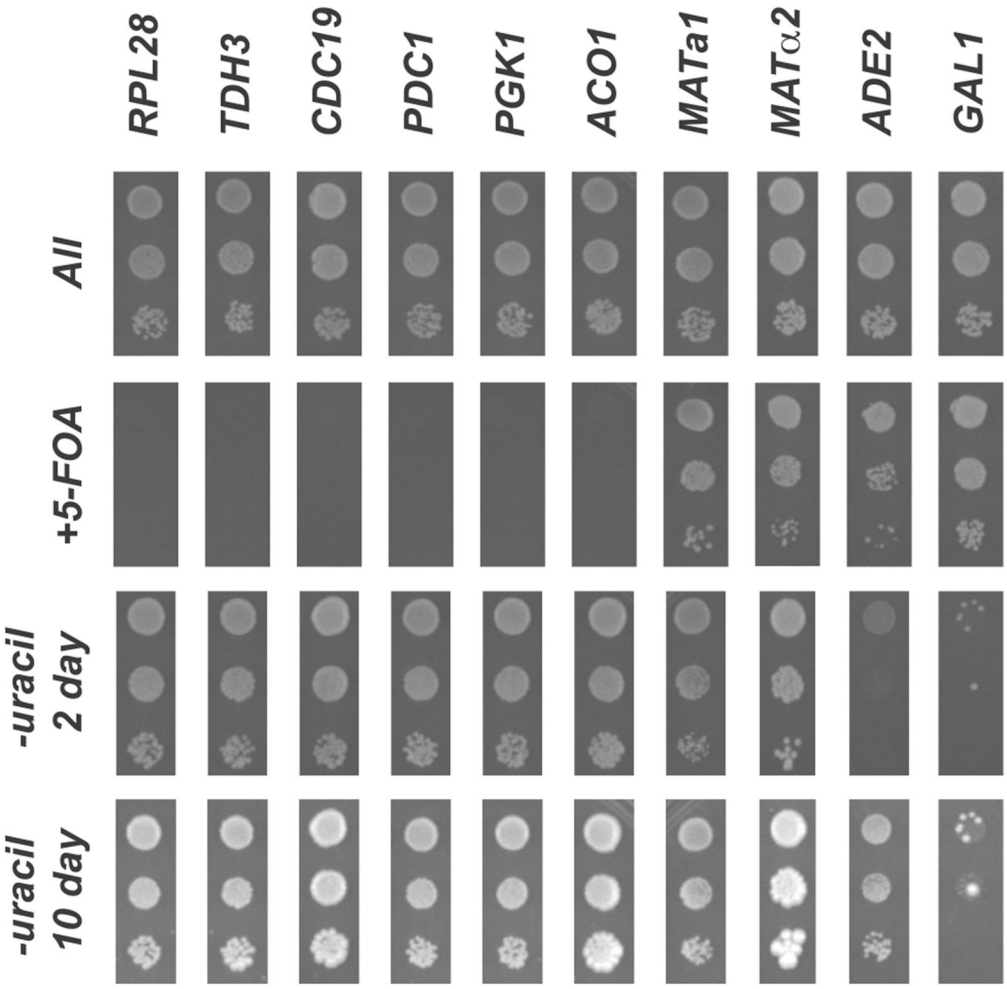


**Figure 3. Measuring silencing of the wild-type *HMR* cassette with the *URA3* CDS**

The *URA3* reporter was linked to various regulatory elements, and the *HMR* cassette was integrated at *LEU2*. Expression of the gene was monitored by growth on plates lacking uracil or containing 5-FOA. Each spotting experiment was done twice with these strains.

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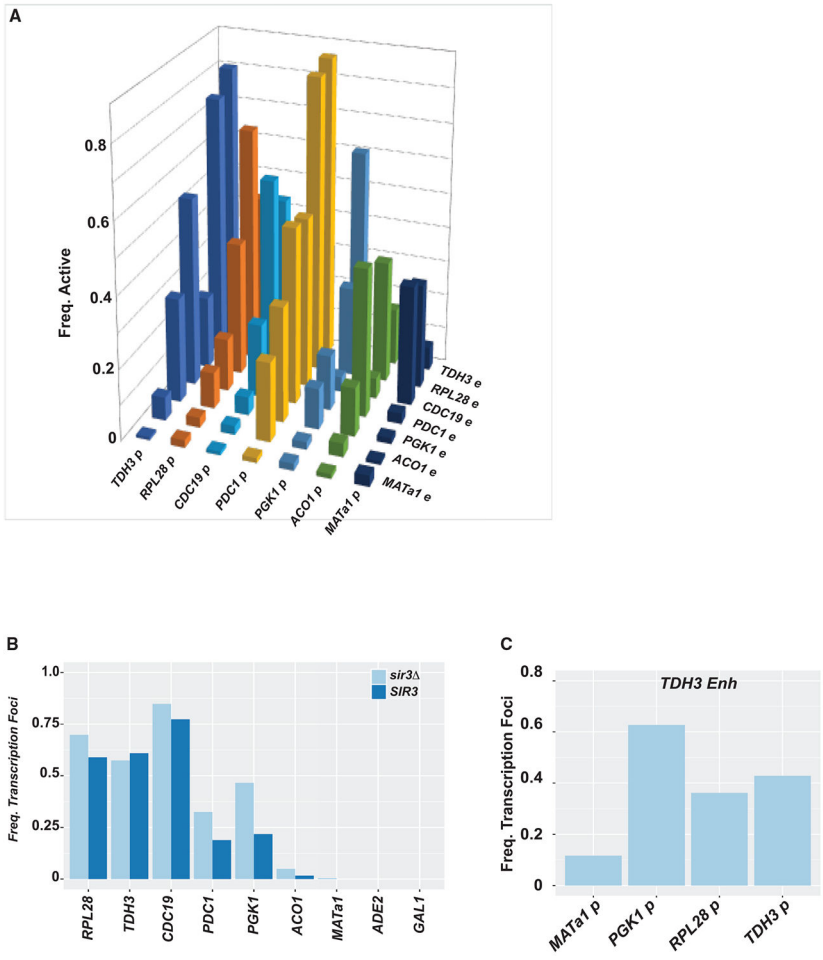


**Figure 4. The role of enhancers and core promoters in silencing**

(A) A 7 × 7 matrix of different combinations of UAS enhancers and core promoters with the Venus reporter gene was generated and integrated. Expression of these constructs was measured usinga flow cytometer. Two different sets of integrantswere analyzed between one ortwo times by flow cytometry as described for Figure 2B.

(B) Measurement of fluorescent mRNA synthesis using wide-field fluorescence microscopy. The reporter cassette (*PP7bs-PHO5*) linked to various regulatory elements was integrated at *HMR* in a strain constitutively expressing PP7-GFP. mRNA synthesis (GFP foci) was monitored using a wide-field fluorescence microscope. The number of fluorescent foci was quantified as a percentage of the total number of cells analyzed and plotted for wild-type cells as well as cells lacking Sir3. Each experiment was performed twice for each strain (two experiments across 2 days each for *SIR3* wild-type and *sir3*Δ strains). Results from the replicateswere pooled, leading to the analysis of between 359 and 569 cells for each genotype. Statistical significance was determine using Fisher's exact test: *RPL28* p = 0.0003, *TDH3* p = 0.25, *CDC19* p = 0.0031, *PDC1* p = $4.39 \times 10^{-7}$, *PGK1* p = $2.2 \times 10^{-16}$, *ACO1* p = 0.12.

(C) The *TDH3* enhancer was combined with four different core promoters (*MATa1*, *PGK1*, *RPL28*, and *TDH3*) and linked to the *PP7bs-PHO5* reporter and integrated at *HMR* in a strain constitutively expressing PP7-GFP. The number of fluorescent foci was quantified

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from at least 200 cells. Statistical analysis was performed as above comparing the *TDH3* promoter to the other promoters: *MATa1* $p = 2.2 \times 10^{-16}$, *PGK1* $p = 2.7 \times 10^{-6}$, *RPL28* p = 0.12.

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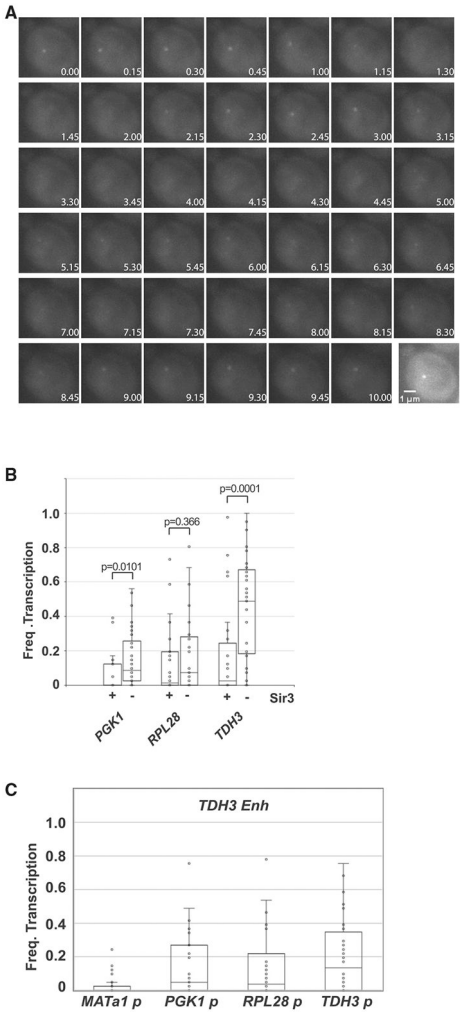


**Figure 5. Measurement of mRNA synthesis using a multi-focus fluorescence microscope**

(A) Ayeast cell undergoing silencing was imaged every 15 s across 18 different focal planes using a multi-focus fluorescence microscope. The appearance or disappearance of the focal spot was monitored over a 10-min period. The intensity of the dots was monitored and values above background were considered transcriptionally active. Multiple cells for each strain were monitored over multiple days (see the supplemental figures for the precise number of cells monitored for each strain).

(B) The length of time when a locus was transcriptionally active in each individual wild-type and *sir3*Δ cell was determined as a fraction of the total time and plotted as a boxplot. Statistical analysis comparing the wild-type strain with the *sir3*Δ strain was performed using Welch's two-sample t test: *PGK1* p = 0.01, *RPL28* p = 0.37, *TDH3* p = 0.0001.

(C) Effect of core promoter sequences on burst duration. The *TDH3* enhancer was combined with four different core promoters (*MATa1*, *PGK1*, *RPL28*, and *TDH3*) and linked to the *PP7bs-PHO5-GFP* reporter (see supplemental figures for the precise number of cells monitored for each strain) over different days. The length of time when a locus was active in each cell was determined as described above. Statistical analysis comparing the *TDH3*

promoter with the other promoters was performed using Welch's two-sample t test: *MATa1* $p = 2.5 \times 10^{-5}$, *PGK1* p = 0.2, *RPL28* p = 0.3.

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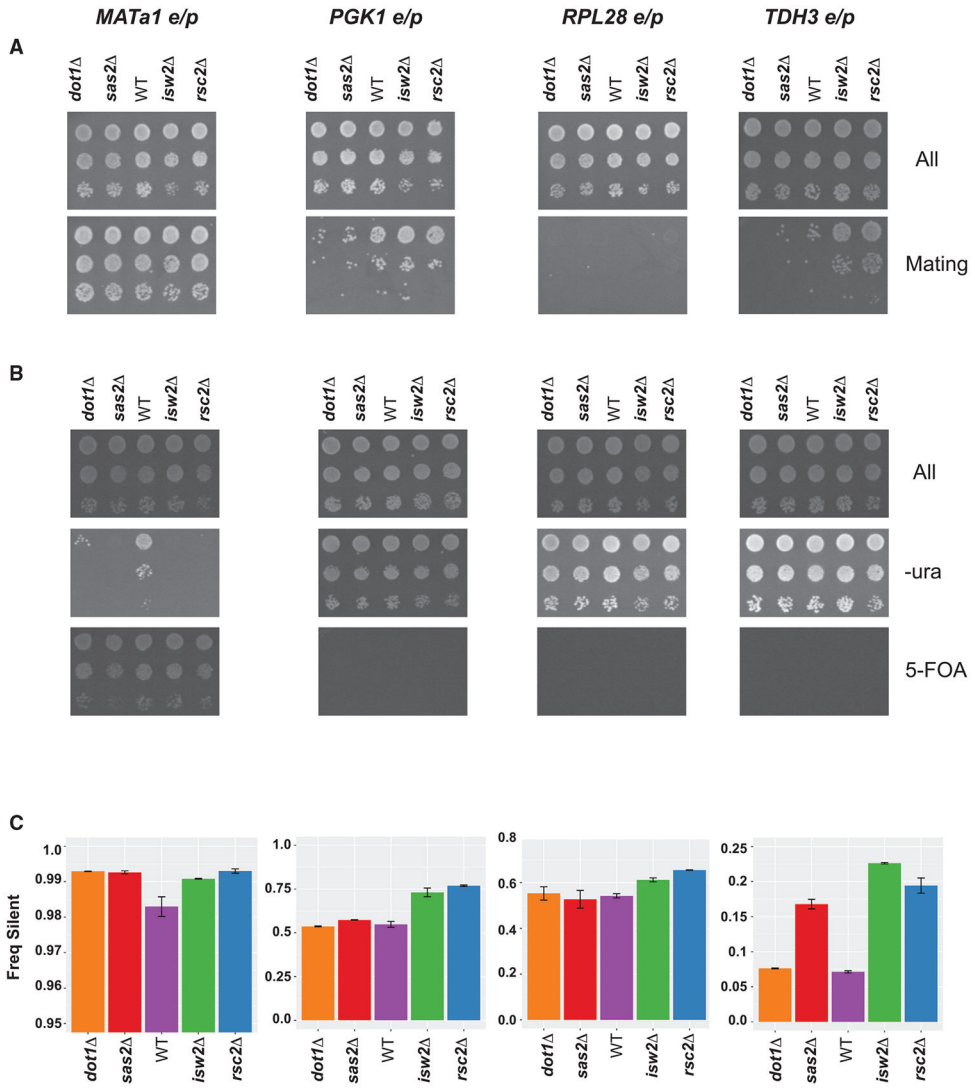


**Figure 6. Effects of mutants on repression of different regulatory elements**

(A) Repression of the wild-type *HMR* cassette monitored by mating assays in various mutants (sas2Δ, dot1Δ, isw2Δ, and rsc2Δ) as described in Figure 1.

(B) Repression of the wild-type *HMR* cassette with the *URA3* CDS in various mutants (sas2Δ, dot1Δ, isw2Δ, and rsc2Δ) as described in Figure 3.

(C) Fluorescence cytometry measurements of wild-type *HMR* with a Venus reporter in strains lacking Dot1, Sas2, Isw2, and Rsc2. Each measurement was performed with two colonies, and the mean and standard error are shown.

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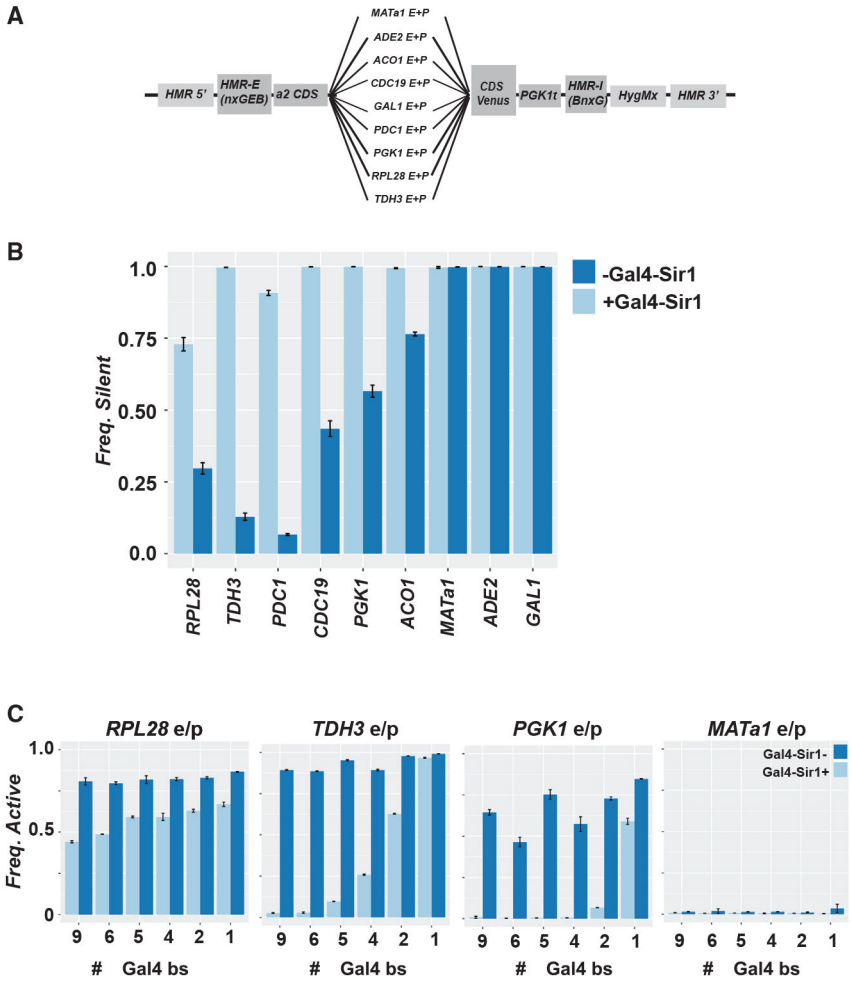


**Figure 7. Gal4-Sir1-mediated silencing of synthetic silencer-containing *HMR* cassettes**

(A) Schematic of the modified silenced locus.

(B) Repression of the *HMR* cassette containing four Gal4 binding sites at *HMR-E* and five binding sites at *HMR-I* was monitored by fluorescence cytometry using the Venus reporter gene at the *HMR* locus. The strains had Gal4-mRuby-Sir1 under the control of the *MET17* regulatory elements, and its expression was regulated by methionine. Three separate colonies were measured for each strain, here represented as the mean frequency (±SE).

(C) Repression of the *HMR* cassette containing variable numbers of Gal4 binding sites at *HMR-E* and *HMR-I* was monitored by fluorescence cytometry using the Venus reporter gene.

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## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Bacterial and virus strains | | |
| *E. coli* DH5α | Thermo Fisher Scientific | Cat#18265017 |
| Deposited data | | |
| Raw multi-focus microscopy images | This paper; Mendeley Data | https://doi.org/10.17632/35wh6dd4xc.1 |
| Raw wide-field images | This paper; Mendeley Data | https://doi.org/10.17632/fkrwmr58g5.1 |
| Variable Promoter raw multi-focus microscopy images | This paper; Mendeley Data | https://doi.org/10.17632/5m4hs68tpk.1 |
| Variable promoter raw wide-field images | This paper; Mendeley Data | https://doi.org/10.17632/h9wfssy253.1 |
| Experimental models: Organisms/strains | | |
| S. *cerevisiae:* Strain background: W303 | ATCC | ATCC 208353 |
| Software and algorithms | | |
| FlowJo | BD Life Sciences | RRID:SCR_008520 |
| MATLAB | The Mathworks Inc. | RRID:SCR_001622 |
| FIJI | Schindelin et al. [108] | RRID:SCR_002285 |
| R Project for Statistical Computing | R Core Team | RRID:SCR_001905 |
| softWoRx | Applied Precision | RRID:SCR_019157 |