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TESTING THE LIMITS OF TRANSCRIPTIONAL SILENCING IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Kenneth Wu

Testing the Limits of Transcriptional Silencing in *Saccharomyces cerevisiae*

DNA in eukaryotic organisms is complexed with histone and non-histone proteins to form chromatin. Gene activities are determined by chromatin states that are either permissive or restrictive to transcription. In the budding yeast *Saccharomyces cerevisiae*, chromatin states at the cryptic mating loci *HML* and *HMR* are mediated by silencer elements and the Sir proteins (Sir1, Sir2, Sir3, and Sir4). The Sir proteins bind unacetylated tails of the histones in chromatin and silence transcriptional activity by sterically hindering transcription factor access to chromatin. The silent state, once established, is transmitted stably through multiple cell divisions, but the factors contributing to the silent state are in constant flux, suggesting that the system can tolerate fluctuations in levels of these factors without functional consequences.

In the first part of this work, we sought to determine the levels of the individual factors necessary for silencing. We developed methods to measure the thresholds of individual proteins and modifications that lead to loss of the silent state. We show that silencing loss is not observed until more than half of the unacetylated histone complement at a silent locus is acetylated. For Sir proteins, our data suggest that silencing can tolerate significant reductions in the levels of Sir2 and Sir3 but cannot tolerate even a twofold reduction in Sir4 levels.

Transcriptional silencing functionally operates on enhancers and promoters of genes. In the second part of this work, we tested the susceptibility of various enhancer and core promoter elements to silencing at *HMR* using a panel of different enhancers and promoters of differing transcriptional strengths. Our data suggest that silencing of these elements occurs in a probabilistic manner dependent on properties of individual enhancers and core promoters. We find that constitutively active enhancers/promoters escape silencing to a large degree and the silencing machinery is only able to suppress transcription from moderately weak enhancers. However, our data also show that increased Sir1 binding to silencers can counteract the effect of strong enhancers/promoters. Using fluorescent reporters of nascent transcription, we show that the Sir proteins function in silencing by modulating the transcription frequency of enhancers. Altogether, this work suggests a model in which the Sir proteins function by reducing the probability that transcription factors bind to target sites at the enhancers of silenced domains, thereby affecting transcription frequency.

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CHAPTER 1

INTRODUCTION

Eukaryotic DNA is complexed with histones and non-histone proteins to form chromatin. The genome is organized into domains with distinct structures and function. Active genes reside in loosely packaged regions that are accessible to molecular probes, while transcriptionally silenced domains are highly condensed and inaccessible to molecular probes.

Regulatory elements such as enhancers, promoters and silencers are required for the proper regulation of genes during growth, development, and differentiation. Binding of sequence specific factors to the enhancers of genes leads to the recruitment of general transcription factors to the core promoter of genes and the induction of gene transcription. These proteins function in the context of chromatin. The chromatin structure functions to modulate gene regulation by the sequence specific transcription factors. Proper growth and differentiation of eukaryotic cells are dependent on the ability to establish, maintain, and inherit transcriptional activity states within chromatin domains through numerous cell divisions.

On silencing

Silencing is the process through which a stable transcriptionally inactive chromatin state is achieved. Silencing has three phases: establishment, maintenance, and inheritance. The cell signals for the assembly of any silent chromatin domain through establishment, keeps that domain continuously silenced through maintenance,

and reconstitutes the silent state after division through inheritance (Loo and Rine, 1995).

In the budding yeast *Saccharomyces cerevisiae*, silent chromatin can be found at the *HML* and *HMR* cryptic mating loci, as well as certain telomeric regions (Gartenberg and Smith, 2016). The transcriptionally inactive state is thought to be achieved by competition between silencing factors and transcription machinery to DNA (Chen and Widom, 2005).

At the *HM* loci, the silencers, the four Silent Information Regulator (Sir) proteins, and unacetylated histone H4K16 are necessary for the silent state. Silent telomeric domains do not require all such factors: only three of the four Sir proteins (Sir2, Sir3, and Sir4), as well as one of the silencer-binding proteins, Rap1, are necessary.

The silencers and silencer-binding proteins

The silencers contain DNA elements flanking the *HM* loci that recruit proteins that, while not individually wholly dedicated to silencing, altogether contribute to the silent chromatin state. Each silencer contains at least two of the three binding sites for the proteins Abf1, Rap1, and Orc1.

Abf1 is a transcription factor responsible for activating dozens of genes with diverse functions (Miyake et al., 2004). Rap1 is also a transcription factor capable of activating hundreds of targets (Lieb et al., 2001), including the mating type genes found at the active *MAT* and at the silenced *HM* loci (Kurtz and Shore, 1991). Of the

three silencer binding proteins, Rap1 is of particular interest, as silent telomeric regions contain repeats to which it binds (Berman et al., 1986). Finally, Orc1 is a subunit of the origin replication complex and regulates DNA replication at the origins of replication throughout the genome (Bell et al., 1995).

At *HML*, the presence of either one of the two flanking silencers is sufficient for the establishment of the silent state (Mahoney and Broach, 1989). In contrast, at *HMR*, only one of the two silencers, *HMR-I*, can accomplish this. (Berman *et al.*, 1986; Rivier et al., 1999). The individual binding sites within the silencers also exhibit some degree of redundancy: only two of the three binding sites at the *HMR-E* silencer are necessary for silencing (Brand et al., 1987).

Given the abundance and affinity of each of the silencer-binding proteins for other regions of the genome, and given their affinity for the Sir proteins, it is thought that their proximity in the silencers serves to nucleate the recruitment and spreading of the Sir proteins to compete against the binding of transcriptional machinery.

Although silencer elements can act in a bi-directional manner, the silent chromatin domains are counteracted by barrier elements: of these, the most characterized is the tRNA gene adjacent to *HMR* towards the telomere end, where the tRNA-specific RNA Pol III is necessary for barrier function (Donze et al., 1999; Donze and Kamakaka, 2001; Kamakaka and Thomas, 1990; Oki and Kamakaka, 2005). A similar role has been proposed for the *CHA1* gene near *HML* (Donze and

Kamakaka, 2001), and at Tbf1 and Reb1 transcription factor binding sites at the telomeres (Fourel et al., 1999).

The silent information regulators: *SIR1*

Sir1 has long been known to play a role in the establishment of silencing. When artificially tethered to *HM* loci lacking silencers, Sir1 protein can establish silencing, while tethered Sir2, Sir3, and Sir4 cannot (Chien et al., 1993). Furthermore, *SIR1* mutants result in two phenotypically metastable populations of cells for *HML* silencing. When tested for sensitivity to α mating factor, approximately 80% of *sir1Δ* cells were phenotypically de-repressed, while 20% maintained stable and mitotically heritable silencing, suggesting a role for *SIR1* in the inheritance of silencing (Pillus and Rine, 1989). A later study using fluorescent reporters confirmed this bimodal expression pattern in *SIR1* mutants, although the relative fractions were different and dependent on the silent locus being investigated (40% active at *HML* versus 90% active at *HMR*) (Xu et al., 2006). *SIR1* has also been found to play a role in the maintenance of the silent state: *sir1* mutants experience greater rates of transient silencing loss (Dodson and Rine, 2015). Thus, Sir1 is thought to play an important role in both the maintenance and the efficient inheritance of the silent state.

Sir1 has been shown to be recruited to the silencers of the *HM* loci through Orc1 binding (Chien *et al.*, 1993; Triolo and Sternglanz, 1996). It in turn appears to interact with Sir4, but not with the other Sir proteins (Triolo and Sternglanz, 1996).

The silent information regulators: *SIR2*

Sir2 is an NAD-dependent histone deacetylase necessary for silencing at *HM* loci and telomeres (Ghidelli et al., 2001; Imai et al., 2000). *S. cerevisiae* have four paralogs for *SIR2*, of which some can contribute to but are not necessary for the silent state: *HST1*, *HST2*, *HST3*, and *HST4*. Abrogating Sir2 activity results in a depletion of Sir complexes across the length of the *HM* loci, though they are present at the silencers, suggesting a role for Sir2 in the establishment and propagation of the repressed state along the silent domain (Rusche et al., 2002; Thurtle and Rine, 2014). Additionally, *SIR2* orthologs are found across a wide range of other organisms: *Staphylococcus aureus*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, mice, and humans (Braunstein et al., 1993).

Overexpression of *SIR2* results in a global hypoacetylation of histones (Braunstein *et al.*, 1993). Specifically, in budding yeast, Sir2 preferentially deacetylates lysine 16 of histone H4 (Imai *et al.*, 2000). Sir2 is allosterically stimulated for this activity when bound to Sir4 (Hsu et al., 2013; Tanny et al., 2004). 2013), without which its deacetylase activity is substantially reduced (Cubizolles et al., 2006). While the initial association of the Sir2/Sir4 complex to acetylated histone H4 lysine tails does not require enzymatic activity, it has been observed that this activity is necessary for association of the other Sir proteins at silent loci (Hoppe et al., 2002).

Sir2-mediated histone H4K16 deacetylase activity is primarily opposed by the Sas2 acetyltransferase, counteracting histone deacetylation and silent chromatin

assembly at other loci throughout the genome (Meijsing and Ehrenhofer-Murray, 2001; Suka et al., 2002).

The silent information regulators: *SIR3*

Sir3 is a paralog of Orc1, and associates with nucleosomes in a manner dependent on unmodified lysine 16 of the histone H4 tail and lysine 79 of the histone H3 globular domain (Bell *et al.*, 1995; Johnson et al., 1990; Onishi et al., 2007). The spreading of Sir3 along the silenced domain is dependent upon Sir2 mediated deacetylation of histones (Rusche *et al.*, 2002). Unacetylated H4K16 is critical: an acetyl mimic mutation at this site is sufficient to cause loss of the silenced state (Dion et al., 2005). Binding of Sir3 to nucleosomes restricts accessibility of various molecular probes to the underlying DNA sequences (Ghidelli *et al.*, 2001; Loo and Rine, 1994), suggesting a mechanism for silencing via steric hindrance.

Sir3 directly interacts with and is recruited to silenced chromatin domains by association with Rap1 (Moretti and Shore, 2001). Overexpression of *SIR3* results in its spread from silent telomeric domains without associated spreading of Sir2 or Sir4, suggesting its ability to contribute to both establishment and maintenance of silencing (Renauld et al., 1993; Strahl-Bolsinger et al., 1997).

The silent information regulators: *SIR4*

Sir4 serves as a scaffold in silencing due to its ability to directly interact with Sir1, Sir2 and Sir3, and is thus necessary for the establishment and maintenance of the silent state (Moazed et al., 1997). It is recruited to the silenced chromatin through

its association with silencer bound Rap1 (Moretti et al., 1994; Moretti and Shore, 2001). Yku70/80 is thought to facilitate this recruitment and contribute to organization of telomeres towards the nuclear periphery (Laroche et al., 1998; Tsukamoto et al., 1997).

Sir4 forms a hetrodimeric complex with Sir2 (Ghidelli *et al.*, 2001; Hoppe *et al.*, 2002; Moazed *et al.*, 1997) and is necessary for the recruitment of Sir2 to the silencers. It also independently interacts with Sir3 and is necessary for the spread of the silenced domain (Rusche *et al.*, 2002). Sir4 expression levels appear to play a role in the de novo establishment of silencing (Larin et al., 2015).

The assembly of silent chromatin

Given the observed interactions between Sir1 and Orc1, between Sir3 and Rap1, between Sir4 and Rap1, as well as the interactions between Sir4 with the other three Sir proteins, a model of silent chromatin assembly in budding yeast emerges. At the *HM* loci, the silencer-bound proteins Abf1, Rap1, and Orc1 recruit Sir1, which along with Rap1 facilities the association and recruitment of Sir2/3/4. At the telomeres, only Rap1 plays this role for Sir2/3/4 recruitment. Sir2 deacetylates H4K16 residues in nucleosomes adjacent to the silencers, which facilitates Sir3 binding, and the Sir2/3/4 complex thus populates throughout the local domain to prevent the association of transcriptional machinery (Gartenberg and Smith, 2016).

The higher-order structure of silent chromatin

As with other eukaryotes, budding yeast exhibit higher-order chromatin structure. Chromatin conformation analyses have shown that the silencers for a given *HM* locus are positioned to be close in proximity with each other, along with the other *HM* locus at the nuclear periphery. These interactions are controlled by the presence of Sir proteins, yKu70, and Esc1, homologous recombination repair proteins, and phosphorylated serine 129 of histone H2A (Kirkland and Kamakaka, 2013; Miele et al., 2009). At the telomeres, the silent domains are anchored by the activities of Sir4, yKu70, and Esc1. Rif1 is a protein that interacts with Rap1 and competes with Sir4 for binding to the telomeric Rap1. When *rif1* is deleted, Sir4 more freely associates near the nuclear membrane, coinciding with increased telomeric silencing (Taddei et al., 2004). Although these processes clearly involve factors that apparently overlap and contribute to the silent state, this anchorage to the nuclear periphery is not strictly necessary for silencing (Gartenberg et al., 2004).

Silent chromatin and DNA replication

Silencing establishment at *HMR*, though not *HML*, has an S-phase requirement. Interestingly, this requirement does not involve DNA replication (Kirchmaier and Rine, 2001). Since it is possible to establish silencing by utilizing an artificial silencer to tether Sir1 to *HMR*, it is unlikely that Orc1 or the binding site at the silencers is the primary contributor (Fox et al., 1997). The S-phase requirement appears to be dependent on the strength of the promoter being silenced (Ren et al., 2010) and has recently been shown to be due to the removal of transcription-favoring

histone modifications deposited onto promoter nucleosomes by Dot1 and Rtt109 (Goodnight and Rine, 2020).

Telomeric silencing is particularly sensitive to disruption by the overexpression of transcriptional activators following DNA replication (Aparicio and Gottschling, 1994). During DNA replication, nucleosomes are evicted upstream of the replication fork (Gruss et al., 1993), and redeposited downstream of the replication machinery (Gasser et al., 1996). Although the association between most of the H3/H4 tetramers from the evicted nucleosomes are not disrupted (Katan-Khaykovich and Struhl, 2011), they form only half of the complement in the resulting daughter cells. Although newly synthesized histone H4 are not acetylated on K16, the SAS-I complex, which contains Sas2, has interactions with the histone chaperones CAF-1 and Asf1, facilitating the acetylation of histone H4 as it is incorporated into the daughter chromatin (Meijsing and Ehrenhofer-Murray, 2001). Yet *CAF-1* and *ASF1* mutants have weakened silencing at the *HM* loci and telomeres (Zhang et al., 2000). In their review, Gartenberg and Smith offer a potential explanation: the resulting genome-wide hypoacetylation provides ample targets for promiscuous Sir2/3/4 association (Gartenberg and Smith, 2016).

Testing the limits of transcriptional silencing

Silencing is robust at the *HM* loci, but Dodson and Rine detected transient loss-of-silencing events by using a sensitive assay that measured Cre recombinase activity at these loci (Dodson and Rine, 2015). At *HML*, such events were observed 1

every 1000 divisions; at *HMR*, they were observed 0.7 every 1000 divisions. On the surface, the outcome of all this molecular activity appears simple: de-repression results in the inability for the budding yeast cell to mate. We know the system is robust and reliably reconstituted, yet at the same time, the dynamic properties of the system lead to the deconstruction of the silenced locus by opposing molecular forces in the cell. The degree to which the system can tolerate these opposing fluctuations is unknown, and to address this, the second chapter of my thesis was dedicated to developing a system to quantitatively alter H4K16 acetylation (through molecular mimics) and measuring silencing loss through fluorescence reporter. We also quantitatively compared the effects of Sir protein levels on silencing. Our results show that the system has a high buffering capacity, and that a large reduction in levels of histone modifications, as well as Sir4 protein, are required before the system is perturbed and the silent chromatin state is disrupted.

CHAPTER 2

Measuring the buffering capacity of gene silencing in *Saccharomyces cerevisiae*

The text and figures in this chapter are adapted from the following previously published material: Wu, K., Dhillon, N., Du, K., & Kamakaka, R. T. (2021). Measuring the buffering capacity of gene silencing in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(49), e2111841118.

INTRODUCTION

Multiple loci in yeast are transcriptionally silenced including the cryptic mating type loci *HML* and *HMR* on chromosome III as well as sub-telomeric sites (Gartenberg and Smith, 2016). At *HML* and *HMR*, DNA elements called silencers serve as binding sites for specific proteins, which in turn recruit the repressor proteins Sir1, Sir2, Sir3 and Sir4 (Chien *et al.*, 1993; Fox *et al.*, 1997; Hecht et al., 1996; Liu and Lustig, 1996). The histones at silent loci lack acetylation or methylation marks (O'Kane and Hyland, 2019) though they are enriched in phosphorylated histone H2A (Kirkland and Kamakaka, 2013; Kitada et al., 2011). The Sir2/Sir4 heterodimer deacetylates K9 in histone H3 and K16 in histone H4 thereby facilitating Sir3 binding to nucleosomes (Luo et al., 2002; Rusche *et al.*, 2002). Sir3, in turn, simultaneously interacts with and stabilizes the binding of the Sir2/Sir4 heterodimer with nucleosomes thus generating a feedback loop that aids in further binding and spreading of the Sir proteins across the silent chromosomal domain (Gartenberg and

Smith, 2016). Sir proteins in partnership with nucleosomes hinder the association and function of the transcription machinery with regulatory sequences thereby establishing the transcriptionally silent state at *HML* and *HMR.*

The levels of the Sir proteins are critical for stable gene silencing. Sir3p and Sir4p are dosage-dependent regulators of silencing (Le et al., 1997; Marshall et al., 1987). Increased dosage of Sir3p results in the increased spreading of the silent domain at telomeres and restoration of silencing in Sir1 mutants (Dhillon and Kamakaka, 2000; Renauld *et al.*, 1993; Strahl-Bolsinger *et al.*, 1997). Similarly, reducing Sir4 levels leads to inefficient establishment of gene silencing while moderately over-expressing Sir4 leads to a more rapid de novo establishment of silencing (Dhillon and Kamakaka, 2000; Larin *et al.*, 2015; Rine and Herskowitz, 1987; Sussel et al., 1993).

Besides the silencers and the Sir proteins, the post-translational modifications of the histones play a critical role in silencing. Studies utilizing various histone mutants have shown that a region of the histone H4 N-terminal tail from K16 to K20 is critical for silencing. In addition, a H4K16Q mutant (which is an acetyl mimic) results in a dramatic loss of silencing (Carmen et al., 2002; Hyland et al., 2005; Lin et al., 2008; Millar et al., 2004; Shahbazian and Grunstein, 2007; Yu et al., 2011) and Sir3 binding is dependent upon the deacetylation of this residue (Ehrentraut et al., 2011; Johnson et al., 1992; Johnson *et al.*, 1990; Onishi *et al.*, 2007; Wang et al., 2013). These data show that the absence of acetyl groups on K16 is crucial for silencing. However, it is currently unknown whether specific nucleosomes have to be

unacetylated for silencing or whether a majority of nucleosomes across the entire domain have to be unacetylated for silencing.

Once established, the silent state is stably maintained for several generations (Gottschling et al., 1990; Pillus and Rine, 1989; Sussel *et al.*, 1993). Occasional disruptions in silencing do occur but are rare and likely transient: One in a thousand cells stochastically lose silencing at *HML* while around seven in ten thousand cells stochastically lose silencing at *HMR*. It is presumed however, that the active state at these loci is short-lived before the silenced state is restored (Dodson and Rine, 2015).

Despite the high fidelity of the inheritance of the silent chromatin state, the individual components are not stably bound but in constant flux (Buck and Shore, 1995; Maillet et al., 1996; Marcand et al., 1996; Taddei et al., 2009). While the exchange of the core histones in chromatin is quite slow, except at specific regulatory elements (Dion et al., 2007; Misteli et al., 2000), the covalent modifications of the histones have half-lives of only a few minutes (Waterborg, 2001; 2002). While the presence of the Sir3 repressor is essential for silencing (Cheng et al., 1998; Miller and Nasmyth, 1984), analysis of heterochromatin and heterochromatic proteins indicates that repressor protein binding is also dynamic and is influenced by the acetylation and methylation state of the underlying chromatin (Buck and Shore, 1995; Cheng and Gartenberg, 2000; Cheutin et al., 2003; Festenstein et al., 2003; Maillet *et al.*, 1996; Marcand *et al.*, 1996; Taddei *et al.*, 2009). Thus, the overall picture is of a phenotypically stable silenced chromatin state being mediated by constituents that are in constant flux.

Adding further to the complexity of this molecular turmoil is an additional challenge that the cell must overcome to maintain silencing with high fidelity: DNA replication results in a near complete disruption of the chromatin state. Nucleosomes are unable to form on single-stranded DNA (Almouzni et al., 1990) and nucleosomal histones are evicted upstream of the replicating fork (Sogo et al., 1986) and redeposited downstream (Gasser *et al.*, 1996). During DNA replication, nucleosome positions and DNaseI hypersensitive sites (which are sites for binding of transcription factors) are disrupted (Lucchini et al., 2001; Solomon and Varshavsky, 1987; Vasseur et al., 2016) and following replication, the maturation of chromatin leads to the resetting of the original chromatin state (Annunziato and Seale, 1983; Bar-Ziv et al., 2016; Vasseur *et al.*, 2016). The vast majority of the H3/H4 parental tetramers are transferred intact but randomly onto one of the two daughter strands while the parental H2A/H2B dimers segregate randomly to the daughter strands (Annunziato, 2015; MacAlpine and Almouzni, 2013; Mello and Almouzni, 2001). Besides the replication mediated disruption of chromatin structure, the duplication of the DNA also results in the dilution of the parental histone complement by half. The twofold reduction in nucleosome number is restored by newly synthesized histones. Newly synthesized histones are decorated such that histone H4 is acetylated on K5 and K12 and histone H3 is acetylated on K9 and K56 (Benson et al., 2006; Ling et al., 1996; Masumoto et al., 2005; Sobel et al., 1995). The maturation of chromatin following replication involves the removal of these deposition specific modifications of the

histones, and the restoration of the modifications found in the mother cell (Bar-Ziv *et al.*, 2016).

The chromatin state that is disrupted during replication, creates a temporal window in the G2 phase of the cell cycle where silenced chromatin is more accessible to enzymatic probes (Aparicio and Gottschling, 1994; Cheutin *et al.*, 2003; Lau et al., 2002) and thus more prone to disruption. Counteracting this disruption are the silencer elements. Elimination of the silencers result in the inability of the silent state to reform following its disruption in S-phase (Cheng and Gartenberg, 2000). Furthermore, efficient inheritability of silencing requires the silencer bound proteins Rap1 and Sir1 (Pillus and Rine, 1989; Sussel *et al.*, 1993).

Besides the silencers, models have invoked a role for histone modification marks in the heritability of the silent state. *In silico* models (Mukhopadhyay and Sengupta, 2013; Sneppen and Dodd, 2012; 2015) suggest that stable inheritance of silencing involves parental modified nucleosomes helping in the templating and modification of nucleosomes containing newly synthesized histones. These models suggest that the efficient inheritance of silenced chromatin likely involves Sir protein binding to unacetylated parental nucleosomes followed by the deacetylation of spatially adjacent newly synthesized histones. The data have also led to a buffer model for the inheritance of the silent state (Huang et al., 2013) which suggests that the silent locus can tolerate significant fluctuations in Sir proteins and acetylation levels of the histones during replication. The occasionally acetylated nucleosome at the silent locus does not lead to a loss of silencing but silencing is lost when a

particular threshold of acetylation is breached. The level of tolerance in the system is unknown and experiments measuring this are currently lacking. To understand the quantitative relationships between H4K16 acetylation levels, Sir proteins and the stability of silencing, we developed assays to quantitatively alter H4K16 acetylation levels (using molecular mimics) and measure the effects of these changes on silencing. We concurrently used classical genetic methods to explore the effects of alterations in Sir protein levels on the stability of the silent state. Our data suggest that mere two-to-three-fold change in levels of histone marks and specific Sir proteins can affect the stability of the silent state of a large chromatin domain.

RESULTS

Histone acetylation is reduced over the silenced domain

We first characterized the chromatin state of the silenced locus in G1-arrested cells to determine the levels of various proteins and histone modifications at the silenced locus (Figure 1A). These data serve as a baseline control of the levels found in unperturbed silent cells. Using ChIP qPCR, we mapped the abundance of histone H3, Sir3, acetyl-histone H4K16 and acetyl-histone H3K56 at the silent *HMR* locus. A locus on chromosome 6R in an intergenic region between *YFR054c* and *IRC7* that had previously been shown to be nucleosomal and euchromatic was used as a control. For the ChIP qPCR analysis, all primer pairs were unique and had similar amplification efficiencies and did not generate any primer dimers. To quantify the

distribution, immunoprecipitated DNA and input DNA were quantified and equal amounts of input and immunoprecipitated DNA (~100pg) were then used for qPCR.

The silencers and the tRNA gene insulator adjacent to *HMR* are "nucleosomal depleted" and therefore we began our analysis by measuring the histone H3 distribution across the silent domain. The fold enrichment/depletion of histone H3 at various sites across *HMR* was compared to the control locus. This analysis showed that a site between the two silencers located within the silenced domain had a normal complement of histones as did a site in the euchromatic *GIT1* gene (Figure 1A). The silencers as well as the tDNA barrier were moderately "nucleosome-free" as expected, though the weaker than expected depletion of histone H3 could be due to the average size of the immunoprecipitated DNA (~300 bp) (Cole et al., 2012a; Cole et al., 2012b; Dhillon et al., 2009; Dion *et al.*, 2007; Oki and Kamakaka, 2005).

We next mapped Sir3 across the *HMR* domain. Sir3 was maximally present at the two silencers while its binding was reduced at the tDNA boundary of the silent domain and at a site within the silent domain which was consistent with previous observations (Thurtle and Rine, 2014; Valenzuela et al., 2008). This protein was completely absent from the euchromatic *GIT1* gene as well as at the control locus on chromosome 6R.

We next quantified the distribution of histone acetylation on H3K56 and H4K16 (Figure 1A) by ChIP. The IP was performed on the same crosslinked material as that used to map histone H3. Since the silencers and the *tDNA* barrier are depleted

of histones we normalized the distribution data for these histone modifications to histone H3 occupancy, thereby measuring the level of enrichment or depletion of these modifications on a per "nucleosome" basis compared to the control locus. On a per nucleosome basis compared to the control locus, H3K56 acetylation levels showed significant reduction across the entire silent domain and there was an around three-fold decrease in acetylation of H3K56 at *HMR* compared to chromosome 6R. There was an even more dramatic reduction in H4K16 acetylation at *HMR* compared to the control locus. The data show that on a "per nucleosome" basis, compared to the control locus, less than 10% of the histones were acetylated on H4K16 at *HMR*.

Design of the Cut and Flip system

We next wished to investigate the quantitative relationship between histone H4K16 acetylation and gene silencing. Previous work on histones have used one of two different sets of approaches. In one approach, the wild-type and mutant histone genes (with their own regulatory elements) are present on plasmids, and the mutant is compared to the wild-type strain after plasmid shuffle (Dai et al., 2008; Han et al., 1988; Kayne et al., 1988; Yu *et al.*, 2011). While a wealth of information has been garnered using this approach, this system is neither inducible nor tunable and so one is unable to observe the switch or study transition states. In addition, the histone genes are present on plasmids, which often fluctuate in copy number from cell to cell. In the second approach, the histone genes are under the control of a heterologous enhancer/promoter which can be induced (Dion *et al.*, 2007). With this approach expression of the histone gene is inducible and the gene can be expressed at varying

levels but expression occurs throughout the cell cycle in place of its normally restricted expression in the G1/S phase (Eriksson et al., 2012) and this is known to trigger cell cycle checkpoints (Gunjan and Verreault, 2003) and lead to dominant effects (Meeks-Wagner and Hartwell, 1986).

We therefore developed a system to overcome these issues. In *S. cerevisiae* there are two loci for histone H3 and H4: *HHT1-HHF1* and *HHT2-HHF2*. We constructed a strain lacking the *HHT1-HHF1* locus and where the wild-type histone *HHF2* locus was modified to accommodate two copies of the H4 coding sequence (Figure1B). R-recombinase recognition sites flanked the coding region of the wild-type H4 gene that had an HA tag at its N-terminus. Immediately downstream of the wild-type allele, we inserted a copy of an acetylation mimic mutant of the histone H4 gene (H4K16Q) fused to an N-terminal Myc tag. This H4K16Q allele lacked the *HHF2* UAS enhancer/promoter element and therefore was not transcribed. This altered strain also contained the R-recombinase under the control of the *GAL1* enhancer/promoter. The R-recombinase mediated flipping is a rapid and efficient method of creating a desired deletion (Li et al., 2001).

The experiment involved growth of yeast cells expressing the wild-type HA tagged H4 gene from its own UAS enhancer/promoter. Cells were arrested in G1 and the R-recombinase was induced by switching the carbon source to galactose. The recombinase induced recombination between the two R recognition sites flanking the wild-type H4 gene resulted in the flipping out (deletion) of the wild-type H4 copy thereby bringing the mutant H4K16Q gene in register with its native UAS enhancer/

promoter. Since the mutant H4 gene is brought under the control of its native UAS enhancer, the mutant protein is expressed only during the G1/S phase of the cell cycle and not over-produced and since the modified histone cassette is present at its native locus on chromosome 14, it does not suffer from changes in copy number.

Characterization of the Histone H4 Cut and Flip

MATa cells (*HML::URA3*p-GFP *GAL1*p-RecR::*LEU2 hhf1-hht1*Δ::KanMx *bar1*Δ::NatMx *HHF2*p-R-HA-*HHF2*-R-Myc-*hhf2K16Q*) were grown overnight in raffinose containing rich medium and arrested in the G1 phase of the cell cycle for 3 hours with alpha factor. We monitored arrest by microscopy as well as by flow cytometry (Figure 1C). Once cells had arrested in the G1 phase of the cell cycle, we shifted the cells to galactose-containing media to induce the R-recombinase. We ascertained that three to four hours of incubation in galactose were sufficient for maximal R-recombinase mediated switching of the *HHF2* alleles (data not shown). Cells were then released from the G1 arrest into dextrose containing media and aliquots of the cells were removed for further analysis at various time points.

Flow cytometry of the yeast cells showed that cells were arrested uniformly in G1. The analysis of these cells following their release from G1 arrest helped us identify the time for each S-phase and showed that the first S-phase occurred around 30 minutes after release (Figure 1C). The data also showed that most cells progressed through the second S-phase between 2 and 3 hours after their release, albeit with

reduced cell-cycle synchrony. The doubling time of this strain in YPD was also measured and was approximately 105 minutes.

We next monitored the switch of the wild-type to mutant *HHF2* alleles by protein blots using antibodies against the HA and Myc epitopes (Figure 1D). Protein extracts were prepared from approximately equal number of cells at each time point and the proteins were resolved on a 15% SDS-Polyacrylamide gel. The proteins after transfer to nitrocellulose membranes were probed with antibodies against HA, Myc or histone H2B. In G1 arrested cells, the predominant histone H4 protein was HA tagged wild-type protein. Following release, the levels of histone H4 containing the HA epitope reduced with a concomitant increase in the levels of mutant histone H4-Myc protein. We also monitored the levels of histone H2B as a control and as expected this protein remained relatively unchanged. The protein blots thus demonstrated that the switch cassette functioned as designed.

We then wished to determine if the switched histone H4K16Q mutant proteins were being incorporated into chromatin. Cells arrested in galactose as well as cells collected 2 and 4 hours after release from the G1 phase of the cell cycle were crosslinked with formaldehyde and the crosslinked chromatin was immunoprecipitated using anti-HA and anti-Myc antibodies (Figure 2A and 2B). Each experiment was performed with a minimum of two independently crosslinked samples and each sample was immunoprecipitated at least twice with the same antibody. The binding of the tagged histones at three different silent loci- *HML (GFP)*, *HMR (5'* of *HMR-E)* and telomere 6R, was monitored by qPCR. The data

showed that the levels of wild-type histone H4-HA bound to these loci decreased following release from alpha-factor arrest (Figure 2A), and the levels of mutant histone H4-Myc increased upon release (Figure 2B).

Having shown that following the switch, the mutant histone protein does become incorporated into silenced chromatin, we next investigated the effects of the switch in histones on silenced chromatin using qChIP with polyclonal antibodies against Sir3 (Figure 2C). In G1 arrested cells, Sir3 was bound to all three silenced loci- *HML*, *HMR* and *TEL6R*. Upon release from the G1 arrest, Sir3 levels reduced within 2h and there was very little Sir3 bound to these loci after 4h showing that incorporation of the mutant histone (H4K16Q) led to a loss of Sir3 binding and presumably the activation of the genes at these loci.

As a second measure of silencing loss, we measured mRNA levels of a GFP reporter present at *HML* using RT-qPCR (Figure 2D). We isolated mRNA from G1 arrested cells as well as from cells at 2- and 4-hours post-release and measured levels of GFP mRNA along with actin mRNA. In G1-arrested cells there was very little GFP mRNA compared to actin mRNA consistent with the locus being silenced. However, upon release from the arrest, we observed a large increase in GFP expression at the 2h time point which further increased at the 4h time point.

Fluorescence measurements of gene silencing

Molecular approaches often mask nuance and heterogeneity in data. While one can use mating ability to monitor silencing of the native genes at *HML* and *HMR*,

this assesses the silent state only in the G1 phase of the cell cycle and is challenging to monitor in single cells. A fluorescent protein reporter at these loci would circumvent these limitations. We therefore analyzed expression of GFP reporters inserted at *HML*, *HMR* and a telomere using fluorescence microscopy along with the cut and flip cassette. The GFP reporter we employed was a previously characterized, rapidly folding protein (folding/maturation time of ~20min) with a high turnover rate (half-life of ~35min, due to the presence of a *CLN2* PEST sequence) that localized to the nucleus (due to the presence of a nuclear localization signal) (Osborne et al., 2009; Osborne et al., 2011; Xu *et al.*, 2006). We integrated the GFP reporter under the control of either the *URA3* UAS enhancer/promoter or the alpha2 UAS enhancer/promoter at either the *HML* or *HMR* loci or *TEL7L*.

We first analyzed a set of yeast cells expressing either the wild-type H4 or H4K16Q mutant protein alone. These strains contained *HML* or *HMR* loci expressing a GFP reporter under the control of the *URA3* or alpha2 UAS enhancer and core promoter. We measured the GFP signal in cells in these strains using a fluorescent microscope (Figure 3A). In cells expressing only the wild-type histone H4 protein, we did not observe any GFP fluorescent signal from *HMR::URA3p-GFP, HMR::alpha2p-GFP, HML::URA3p-GFP* or *HML::alpha2p-GFP*. In cells expressing only the mutant H4K16Q protein, GFP fluorescence signal was robust and easily detected as predicted for this mutation (Johnson *et al.*, 1990; Lin *et al.*, 2008; Yu *et al.*, 2011). The absolute levels of detected fluorescence in the *H4*K16Q mutant varied both, with the silent locus and the UAS enhancer/promoter. At *HMR*, we consistently

saw higher GFP signal when it was under the control of the *URA3* UAS enhancer/promoter compared to the *alpha2* UAS enhancer/promoter and we saw a similar expression pattern at *HML*. Comparing *HMR* to *HML,* we observed greater derepression of the reporter at *HML* than *HMR,* as well as greater variation in expression of the reporter at *HML* compared to *HMR.* These data suggest that both UAS enhancer/promoter and silencer strength together influence expression levels of the genes at these silenced loci and are consistent with previous data (Motwani et al., 2012).

We also wished to confirm that the act of switching the histones did not perturb the silent state. We generated a cut and flip *HHF2* strain where the wild-type H4 could be switched to another wild-type H4 (*HHF2*p-R-HA-*HHF2*-R-Myc-*HHF2)*. Cells were arrested in G1, the cassette was switched and then cells were released into the cell cycle. GFP expression at *HML::URA3p-GFP* was then measured over time (Figure 3B). We did not observe any changes in GFP fluorescence upon switching of the histones; therefore, the histone switch in and of itself did not affect silencing.

To determine the quantitative relationship between H4K16Q levels at the silent loci and gene silencing, we employed strains where the wild-type H4 could be switched to a mutant H4K16Q. We arrested these cells in G1, switched the histone alleles using R-recombinase and then released these cells from the G1 arrest and monitored expression of GFP by fluorescence microscopy. At *HML,* when GFP was under control of the *URA3* UAS enhancer/promoter, measurable fluorescent signal was observed 2h after release from G1 arrest and reached maximal levels around 5h.

These data suggest that silencing was beginning to be lost during or soon after the second S-phase (Figure 3C).

When we measured GFP expression under the control of the alpha2 UAS enhancer/promoter at *HML*, measurable fluorescence was first observed around the 4h time point with maximal expression occurring around the 7h time point indicating that silencing was beginning to be lost in or after the third S-phase (Figure 3D).

We saw similar dynamics for the *HMR* locus. When the GFP reporter was under the control of the *URA3* UAS enhancer/promoter, we saw measurable GFP signal approximately 3h after the release while for the alpha2 UAS enhancer/promoter, GFP signal was first observed 4h after the release (Figure 3E and 3F).

We also analyzed silencing at telomere 7L. The GFP reporter under the control of the *URA3* UAS enhancer/promoter was inserted adjacent to *TEL7L*. Cells were arrested in G1, the histone allele was switched and GFP expression was measured after release. A measurable fluorescent signal was observed within 1h after release suggesting that ~50% replacement of wild-type H4 with H4K16Q was sufficient for weakening the silent state at this locus (Figure 3G).

It is possible that for *HML* and *HMR*, silencing in some cells begins to be lost at early time points but the increases in expression went undetected due to the limitations in the sensitivity of our fluorescent measurement set up. We nevertheless observed quantifiable loss-of-silencing at *TEL7L* at these early time points, showing

that the telomeres are more susceptible to changes in histone acetylation than the cryptic mating type loci and the inability to detect GFP signal from *HML* and *HMR* at early time points is not due to the time required for the maturation of the GFP fluorescent signal.

In this study we quantified silencing by measuring levels of GFP fluorescent signal in individual live yeast cells. The actual time when silencing is lost and transcription initiates from the silent locus will be different from the time when GFP fluorescent signal is detected by microscopy. The GFP mRNA is ~1000 bases long and with a yeast transcription elongation rate of 25 bases/second (Pelechano et al., 2010) would be transcribed within ~40 seconds. The yeast translation rate is 2.63 amino acids/second (Riba et al., 2019) and so GFP would be translated in ~2 minutes. The maturation time of the GFP protein used in this study is ~20 minutes (Osborne *et al.*, 2009; Osborne *et al.*, 2011; Xu *et al.*, 2006) and thus detection of the GFP fluorescent signal would be delayed ~23 minutes from the actual time of loss of silencing. Since we used one-hour time points for our fluorescence measurements, we do not believe that this offset prevents us from correlating our observations to cell cycle events.

Our results showed that at *HML* and *HMR*, silencing was not lost after the first S-phase but weakened during or after the second S-phase, when the wild-type H4 levels should have dropped to at least 25%. To confirm this result, we built a cut and flip *HHT2-HHF2* strain that contained the wild-type *HHT1-HHF1* alleles, thereby halving the fold-reduction of the wild-type H4 with each DNA replication event. In

this strain, the percent of chromatin-bound H4K16Q would approximately be 25% after the first S-phase, increase to 37.5% after the second S-phase and approach 50% after successive S-phases. We arrested this strain in G1, switched the *HHF2* allele from wild-type to H4K16Q, and monitored expression of the *URA3* UAS enhancer/promoter driven GFP reporter at *HML* (Figure 3H). In this strain, we did not observe expression of GFP after switching the *HHF2* alleles from wild-type to mutant suggesting that greater than 50% H4K16Q histones need to be incorporated at *HML* before a quantifiable GFP fluorescent signal can be observed.

Threshold of Sir proteins required for silencing

The model of Sir mediated silencing posits that a dynamic equilibrium between proteins involved in gene activation and gene silencing at a locus determines the transcriptional status of a gene (Gotta et al., 1997; Kirchmaier and Rine, 2001; Palladino et al., 1993; Renauld *et al.*, 1993) but the relative levels of Sir proteins necessary for silencing are not clear. As gene activation competes with gene silencing, silencing is likely to be less robust in strains with lower levels of the Sir proteins and switching from the silent to the active state should increase. Determining the amount of Sir proteins at which silencing is weakened/ lost would thus identify the threshold at which silencing domains become metastable and also identify the buffering capacity of silencing in a cell.

We decided to investigate the level of individual Sir proteins necessary for silencing. We used an approach where the silencing proteins were under the control

of their native UAS enhancers/promoters and lowered their absolute levels by analyzing silencing in haploid (*SIR+* or *sirΔ)*, diploid cells (*SIR+/SIR+* or *SIR+/sirΔ* or *sirΔ/sirΔ*) or triploid cells (*SIR+/SIR+/SIR+* or *SIR+/SIR+/sirΔ* or *SIR+/sirΔ/sirΔ* or *sirΔ/sirΔ/sirΔ*) carrying either three, two or a single copy of a *SIR* gene. Silencing was analyzed using a sensitive reporter system that was originally used to identify Sir mutants (Rine and Herskowitz, 1987). The system relies on the observation that a yeast strain with no mating information at the *MAT* locus (*mataΔ*) mates as an **a** cell, as long as the mating type information at *HMRα* is silent. However, unlike *MAT*a, *mataΔ* is recessive to *MATα*. Therefore, any loss of silencing at *HMRα* results in a phenotypic switch in the mating phenotype of this *mataΔ* strain from an a mating cell to an α mating cell.

For our experiments we generated strains that lacked functional gene information at *HML* (*hmlΔ::TRP1*) and *MAT* (*mataΔp*). These strains carried the *MATα* information under the control of a synthetic silencer at *HMR* (*HMRα*) (Gardner et al., 1999; Kamakaka and Rine, 1998). It should be noted that the diploid and triploid cells only contained a single *HMRα* locus. Thus, haploid cells were *HMRα,* diploid cells were *HMRα* /*hmrΔ::HIS3,* and triploids were *HMRα* /*hmrΔ::HIS3/hmrΔ::HIS3.* This ensured that the measurements of silencing were not influenced by varying numbers of the *HMRα* locus.

Silencing of these strains was monitored by growing these cells on minimal media plates containing mating-type tester lawns (Figure 4). Growth of cells on *MATa* tester lawn plates is an indication of loss of silencing from *HMRα*. Analysis of

strains with varying copies of the *SIR2* gene indicated that reduction in gene copies to ~33%, ~50%, ~67% compared to wild-type cells had no effect on silencing while reduction to 0% led to a complete loss of silencing. Similarly varying the gene copy number of *SIR3* to ~33%, ~50%, ~67% compared to wild-type cells had very subtle effects on silencing. In contrast, silencing was significantly lost when *SIR4* gene copy number was reduced to ~50% in a diploid cell and ~33% in a triploid cell but silencing was maintained when levels were lowered to ~67% in a triploid cell. These data suggest that Sir4 is a limiting component in gene silencing at *HMR*.

Sir2 is required to deacetylate histone H4 K16 while Sas2 is the histone acetyltransferase that competes with Sir2 in this process. We therefore monitored the effect of reducing Sas2 levels in haploid, diploid and triploid cells. Reductions in the gene copies of this acetyltransferase did not noticeably affect gene silencing at *HMR*.

The assumption underlying these experiments is that the changes in copy number of the genes is likely to concomitantly alter mRNA and protein levels in the cells. We first measured the protein levels of Sir3 in the different haploid, diploid and triploid cells. Equal numbers of logarithmically growing cells were lysed and the proteins in the total cell lysates were resolved on a 10% SDS-polyacrylamide gel. After transfer to a membrane, the membranes were probed with anti-Sir3 polyclonal antibodies. The data show that levels of Sir3 change with respect to gene copy number of *SIR3* (Figure 4B). However, it was difficult to quantify the reduction in protein levels in the different strains. We therefore decided to measure mRNA levels of *SIR3* in the different strains using RT-qPCR. We isolated RNA from cells and

measured levels of *SIR3* and actin mRNA. The data showed that relative to *ACT1*, the levels of *SIR3* mRNA change in parallel with changes in gene copy number (Figure 4C). Compared to a triploid cell with three copies of *SIR3* genes, the triploid cell with two copies of *SIR3* had reduced levels of *SIR3* mRNA which reduced even further in cells with just one copy of *SIR3* gene. Similar reductions were observed in a heterozygous diploid cell compared to a diploid with two copies of the *SIR3* gene.

DISCUSSION

The silencer and silencer bound proteins are necessary for efficient inheritance of the silent state (Cheng and Gartenberg, 2000; Pillus and Rine, 1989; Sussel *et al.*, 1993). The key role of the silencer bound proteins is to maintain a high concentration of Sir2, Sir3 and Sir4 proteins in the vicinity of the locus for the state to be re-established after its disruption during replication. It is likely that silencer strength influences the efficiency of inheritance since we consistently observe greater silencing mediated by the *HMR* silencers compared to the *HML* silencers which is in agreement with previous observations about silencer strengths (Motwani *et al.*, 2012; Shei and Broach, 1995).

In addition to the silencer, efficient inheritance of the silent state depends upon the nucleosomes remaining unacetylated. There are approximately 20 and 12 nucleosomes present at *HML* and *HMR* respectively (Ravindra et al., 1999; Weiss and Simpson, 1998). While it is possible that the deacetylation of a single key nucleosome is necessary for silencing, our data argue against this. We support a model where the

locus requires an aggregate level of acetylated nucleosomes for silencing to be lost. In this scenario, a domain would remain silent so long as the number of unacetylated nucleosomes are above a certain threshold. The silent locus can thus tolerate fluctuations in overall acetylation levels without functional consequence. The quantitative ChIP data normalized to histone H3 levels indicate that at *HMR*, ~5% to 10% of the nucleosomes are likely to be acetylated in wild-type cells compared to the control locus on chromosome 6R. The cut and flip experiments suggest that for *HML* and *HMR* to lose silencing, between 50% and 75% of the nucleosomes must acquire acetyl marks before the locus loses silencing. This difference highlights the buffering capacity of histone modification in gene silencing.

The bulk of the yeast nucleus is packaged into euchromatin and consistent with this is the observation that almost every histone H4 molecule is acetylated (Hecht et al., 1995; Kuo et al., 1998; Waterborg, 2001). The exception to this is the silent loci where histone H4 molecules are not acetylated. If one assumes for simplicity's sake that H4K16 acetylation is required for the spontaneous loss of silencing in yeast cells, then our data can be used to calculate the probability of a stochastically spontaneous acetylation of a nucleosome at the silent locus. Previous data have shown that in wild-type cells, silencing at *HML* is stochastically lost in one out of every 1000 cells with a similar value at *HMR* (Dodson and Rine, 2015). Based on our model, ~75% of the nucleosomes in that one cell would need to acquire H4K16 acetylation for the switch to occur. Therefore, at *HML*, for 15 out of the 20 nucleosomes (75%) to be simultaneously acetylated in that one cell, a single

nucleosome would need to have a ~1/1.6 (60%) probability of acquiring an acetyl group by chance ($1/(1.6)^{15}$= 1/1000). These numbers suggest that just a small reduction in the ability of acetyltransferases to acetylate a single nucleosome, when spread across a contiguous stretch of 15 to 20 nucleosomes, may be sufficient to generate a transcriptionally silent domain in the nucleus. This ability to silence would likely also be influenced by other factors such as the concentration of the Sir proteins, transcription activators, histone modifying enzymes as well as the positioning of nucleosomes over regulatory sequences and modifications of other histone residues (such as H3K56 and H3K79 and possibly H2AS129). Quantitative analysis of these factors should help generate a fuller understanding of gene silencing.

Silencing is a dynamic state and the key determinants for restoring the silent domain following its disruption during replication would be the relative local concentrations of transcription activators (and coactivators) and repressor (and corepressor) proteins at these loci (Aparicio and Gottschling, 1994; Donze *et al.*, 1999; Renauld *et al.*, 1993; Shei and Broach, 1995; Valenzuela et al., 2009). Our data identify one limiting component for silencing - Sir4. Deletion experiments in diploid and triploid cells showed that reducing copy numbers of the *SIR4* gene led to a significant loss of silencing while comparable reductions in *SIR3* or *SIR2* did not have similar effects. Since Sir2 is present in a complex with Sir4 (Ghidelli *et al.*, 2001; Moazed *et al.*, 1997) and Sir4 is necessary for the recruitment of Sir2 to silent loci (Hoppe *et al.*, 2002; Rusche *et al.*, 2002) our data would argue that reductions in level of Sir4 could lead to reductions in the levels of Sir2 at a silent domain leading to

concomitant increase in Sas2 mediated histone H4 K16 acetylation at the silent loci and a generation of a weakened silencing state. Mass spec measurements of Sir proteins indicate that Sir3 and Sir4 levels in the cell are equivalent (Ho et al., 2018) though protein immunoblots of wild-type asynchronously growing haploid cells suggest that Sir4 levers are reduced compared to Sir3 (data not shown) and in future precise controlled measurements of these proteins are likely necessary for a better understanding of the quantitative role of Sir4 in gene silencing.

Replication and acetylation

While silencing is mediated by proteins in constant flux, it is nevertheless stable and faithfully propagated through growth and cell division. There are likely many different factors that collectively lead to this high fidelity. The parental histones segregate randomly to the replicated daughter strands and in theory parental histones with active modifications (such as H4K16 acetyl) could ingress into the silenced domain and aid in the switch from silent to active state. However, while parental histones are evicted from the DNA during replication, they are re-deposited in close proximity to their original site, thereby reducing the probability of histones with active modifications being transferred to silenced chromatin (Jackson and Chalkley, 1985; Radman-Livaja et al., 2011b). Moreover, active chromatin is replicated early while silenced loci are replicated late (Friedman et al., 1995; Raghuraman et al., 2001) and this temporal separation would further reduce the likelihood that silent loci would become infiltrated by parental histones containing active chromatin marks such as acetylated histone H4. It is also highly unlikely that silent loci acquire H4K16

acetyl marks from newly synthesized histones, since newly synthesized histone H4 is acetylated on K12 and not K16 (Ai and Parthun, 2004; Sobel *et al.*, 1995). In addition, the presence of the silencers increases the local concentration of the Sir proteins compared to the global nuclear distribution of Sas2 acetyltransferase throughout the nucleus (Kimura et al., 2002; Suka *et al.*, 2002), thus reducing the probability of nucleosome acetylation and favoring the deacetylated state at silent loci. Lastly, the three-dimensional clustering of silent loci (Kirkland and Kamakaka, 2013; Maillet *et al.*, 1996) could create a pinball effect, trapping Sir proteins in the vicinity of the silent loci and increasing the effective local concentration of the Sir proteins at these loci. While Sir2 removes acetyl groups from nucleosomes that stochastically acquire the modifications because of the global presence of Sas2, the primary function of Sir4 is targeting Sir2 to the silent locus and preventing acetylation of the histones following their deposition onto newly replicated DNA. In opposition to these effects would be transcription which would result in the acetylation of histone H3 and H4 on K56 and K16 as well as the methylation of H3 on K79 (Goodnight and Rine, 2020; Norris and Boeke, 2010). Thus, a key function of the Sir proteins would be to preclude the formation of a transcription complex, possibly during or soon after S-phase by creating a chromatin state that is inhospitable to the formation of transcription complexes.

Binary versus analog silencing

If one assumes that transcription is a probabilistic event in individual cells, then the formation and maintenance of the silent state would be dependent upon the

relative levels of Sir proteins and transcription activators at a silent locus. Nucleosome occupancy over specific regulatory elements- either UAS enhancers or core promoters would affect the probability of gene activation and silencing. In addition, the aggregate level of histone modifications would affect the probability of a silent state being formed. Silencing has classically been shown to be an all-or-nothing phenomenon: a locus is either silent or active (Gottschling *et al.*, 1990; Pillus and Rine, 1989). An interesting observation from our studies is that during the loss of silencing at early time points we did not observe a digital "binary" response in the levels of GFP protein. When we measured the amount of GFP fluorescence in individual cells, we observed a continuum of values. This is consistent with recent observations measuring mRNA levels in partially silent cells (Dodson and Rine, 2015). These data suggest that at the level of mRNA and protein levels, there is no bimodal silencing phenotype and loss of silencing was not an all-or-nothing phenomenon. However, at the level of a specific phenotype, such as the ability of cells to mate, there must be a translation of the variable protein levels in individual cells into a binary choice for each cell- mating versus non-mating. Transcription is noisy and occurs in bursts. Partial silencing implies changes in either transcription burst frequency or burst size (Otto, 2019; Rodriguez and Larson, 2020; Wang et al., 2018). Burst size and frequency are affected by distinct DNA sequence elements. Burst frequency is regulated by UAS enhancers while burst size is affected by core promoters. Thus, in the context of partial silencing, changes in burst frequency or burst size would help identify the regulatory elements that are the targets of the

silencing machinery. Thus the observation of a partially silent state where there is variable expression in the levels of mRNA and protein in individual cells should in the future help illuminate the basic mechanism of silencing.

METHODS

Protein blots

Protein lysates were prepared and resolved on 10 or 15% SDS-polyacrylamide gel as described previously (Ghidelli *et al.*, 2001), except that glass beads were used to break open the cells. Monoclonal antibodies (HA.11 and 9E10) against the HA and Myc epitopes were from Covance while the anti- H2B antibodies were from Active Motif.

RT-qPCR

Total RNA was isolated from yeast cells as described (Schmitt et al., 1990). cDNA was prepared using the reverse transcription-qPCR kit (Luna RT-qPCR New England Biolabs).

Fluorescence activated cell sorting analysis

Cells were washed in 50mM Tris-HCL, pH7.5 and fixed in 70% ethanol for 1h at room temperature. Cells were then washed in 50mM Tris-HCL, pH7.5 and treated with 1mg/ml RNaseA at 37°C for 1h followed by ProteinaseK treatment (60µg/ml) at 55°C for 1h. Cells were washed and resuspended in phosphate-buffered

saline, filtered through a Nitex membrane and stained with Sytox Green stain. Flow cytometry was performed at the UCSC cytometry facility.

Fluorescence microscopy

Cells were grown exponentially in yeast peptone (YP) medium with 2% raffinose at 30°C to an OD_{600} of around 1. The culture was back-diluted to an OD_{600} of 0.125/mL in YP medium with 5 µM alpha-factor and 2% raffinose and incubated on a shaker at 30°C. After 3 hours, the cells were pelleted and transferred into yeast minimal (YM) medium with 5 µM alpha-factor, 2% galactose with appropriate amino acid supplements and incubated on a shaker at 30°C for 4 hours. Cells were pelleted, washed with medium lacking alpha-factor, and transferred into YM medium with 2% dextrose and amino acid supplements. Cells were grown on a shaker at 30°C and aliquots removed at appropriate times. After 7 h, the culture was diluted with fresh medium and allowed to grow for another 10 h at 30°C until the final time point.

For each time point, 1 mL of sample was removed and the cells were pelleted and resuspended in 20 µl YM 2% dextrose medium. 3 µl of the suspension was applied to a 1.5% agarose YMD pad on top of a microscope slide and cover-slipped. Images were acquired on a DeltaVision Personal DV system (Applied Precision), using a 40x 1.35 NA oil-immersion objective (Olympus), with a CoolSnap charge-coupled camera (Roper Scientific). 4 µm image stacks were collected, with each Z-image being 0.2 µm apart, 2 µm above and below the plane of focus. Image stacks

were taken for each time point and greater than 100 cells were captured across the fields-of-view.

Image analysis was performed using the FIJI distribution of ImageJ software. To measure fluorescence intensity per cell, a two-dimensional maximum-intensity projection was generated for each collected z-stack. A transmitted light image, taken at the center of each z-stack, was overlaid on top of the projection. The transmitted light image served as a guide to establish cell boundaries for maximum-intensity projections, such that maximum fluorescence intensity data could be collected per cell using the software's measuring tool. Data for approximately 100 cells per time point were collected, compiled into a spreadsheet, and graphed using R software with ggplot2 package.

Chromatin immunoprecipitation

Cells were grown in YPD media to an OD_{600nm} of 2.0 and then fixed with 1% formaldehyde for 10 min and then the cross linker was neutralized ensuring that around 10% of proteins were crosslinked to DNA. Cells were collected, resuspended in buffer and sonicated using the Bioruptor (Diagenode, Belgium) followed by a cup-horn (Branson, USA) sonicator to an average size of 300bp.

Immunoprecipitation reactions were performed with commercial antibodies to histone H3 Millipore, USA), Ac-K16 H4 (Millipore, USA), Ac-K56-H3 (Millipore, USA) or with polyclonal anti-Sir3 antibodies (Dhillon and Kamakaka, 2000; Dhillon *et al.*, 2009; Kirkland and Kamakaka, 2013; Oki and Kamakaka, 2005; Radman-

Livaja et al., 2011a) and immune complexes were collected with Protein G/A beads (Calbiochem, EMD Biosciences). Immunoprecipitated and input DNA were purified using Chelex 100 (Bio-Rad) (Nelson et al., 2006) and the amount of DNA was quantified using the Picogreeen dsDNA quantitation kit (Invitrogen, USA) and the PerkinElmer Viktor^3 Fluorescence Reader, prior to qPCR.

Equal amounts of IP DNA and input DNA were used for the qPCR reactions. Quantitative PCR reactions were carried out in a Rotor Gene 6000 with SYBR Green (Platinum SYBR Green qPCR SuperMix UDG, Invitrogen) and a three-step PCR program.

The fold difference between immunoprecipitated DNA (IP) and Input DNA for each qPCR amplified region were calculated as described (Litt et al., 2001), using the formula IP/Input=($2^{InputCt - IPCt}$). Each experiment involved at least two independent crosslinked samples with each sample immunoprecipitated twice with the same antibody.

Strains

Strain No.	Genotype
ROY7123	*MATa, ADE+, LYS+, his-, trp-, ura-, GAL1prm::RecR::LEU2, hhf1-hht1Δ::KanMX, bar1Δ::NatMX, R-5'UTR-HA-HHF2-3'UTR-R-5'UTR-MYC-hhf2(K16Q), hml-URA3prm::GFP*
ROY6452	*MATa, ADE+, lys?, his?, trp?, ura?, GAL1prm::RecR::LEU2, hhf1-hht1Δ::KanMX, bar1Δ::NatMX, R-5'UTR-HHF2-R- 5'UTR-hhf2(K16Q), hmr-URA3prm::GFP*

ROY7147	*MATa, ADE+, lys?, his?, trp?, ura?, GAL1prm::RecR::LEU2, hhf1-hht1Δ::KanMX, bar1Δ::NatMX, R- 5'UTR-hhf2(K16Q), hmr-URA3prm::GFP*
ROY7125	*MATa, ADE+, lys?, his?, trp?, ura-, Gal1prm::RecR::LEU2, hhf1-hht1Δ::KanMX, bar1Δ::NatMX, R-5'UTR-HHF2-3'UTR-R-5'UTR-hhf2(K1Q), hmr-ALPHA2prm::sfuGFP*
ROY7133	*MATa, ADE+, lys?, his?, trp?, ura-, Gal1prm::RecR::LEU2, hhf1-hht1Δ::KanMX, bar1Δ::NatMX, R-5'UTR-hhf2(K1Q), hmr-ALPHA2prm::sfuGFP*
ROY7111	*MATa, ADE+, LYS+, his-, trp-, ura-, GAL1prm::RecR::LEU2, hhf1-hht1Δ::KanMX, bar1Δ::NatMX, R-5'UTR-HHF2-3'UTR-R-5'UTR-hhf2(K16Q), hml-URA3prm::GFP*
ROY7136	*MATa, ADE+, LYS+, his-, trp-, ura-, GAL1prm::RecR::LEU2, hhf1-hht1Δ::KanMX, bar1Δ::NatMX, R-5'UTR-hhf2(K16Q), hml-URA3prm::GFP*
ROY6446	*MATa, ADE+, lys?, his?, trp?, ura?, GAL1prm::RecR::LEU2, hhf1-hht1Δ::KanMX, bar1Δ::NatMX, R-5'UTR-HHF2-R- 5'UTR-hhf2(K16Q), hml-ALPHA2prm::GFP*
ROY7145	*MATa, ADE+, lys?, his?, trp?, ura?, GAL1prm::RecR::LEU2, hhf1-hht1Δ::KanMX, bar1Δ::NatMX, 5'UTR-hhf2(K16Q), hml-ALPHA2prm::GFP*
ROY7116	*MATa, ADE+, LYS+, his-, trp-, ura-, GAL1prm::RecR::LEU2, hhf1-hht1Δ::KanMX, bar1Δ::NatMX, R-5'UTR-HHF2-3'UTR-R-5'UTR-HHF2, hml-URA3prm::GFP*
ROY7119	*MATa, ADE+, lys?, his?, trp?, ura-, GAL1prm::RecR::LEU2, hhf1-hht1Δ::KanMX, bar1Δ::NatMX, R-5'UTR-HHF2-3'UTR-R-5'UTR-hhf2(K16Q), TEL7L-URA3prm::GFP*
ROY6583	*MATa, ADE+, lys-, his?, trp?, ura?, GAL1prm::RecR::LEU2, bar1Δ::NatMX, R-5'UTR-HHF2-3'UTR-R-5'UTR-hhf2(K16Q), hml-URA3prm::GFP*
ROY6873	*mataΔp hmlΔTRP HMRssa ade2- lys2-*

ROY6878	*mataΔp hmlΔTRP HMRssa ade2- lys2- sir2ΔLEU*
ROY6880	*mataΔp hmlΔTRP HMRssa ade2+ lys2+ sir3ΔLEU*
ROY6882	*mataΔp hmlΔTRP HMRssa ade2- lys2+ sir4ΔLEU*
ROY6875	*mataΔp hmlΔTRP HMRssa ade2- lys2+ sas2ΔKan*
ROY7151	*mataΔp hmlΔTRP HMRssa ade2- lys2- sir2ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2- sir2ΔLEU*
ROY7152	*mataΔp hmlΔTRP HMRssa ade2- lys2- sir2ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2-*
ROY7153	*mataΔp hmlΔTRP HMRssa ade2- lys2-/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2-*
ROY7158	*mataΔp hmlΔTRP HMRssa ade2+ lys2+ sir3ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2+ sir3ΔLEU*
ROY7159	*mataΔp hmlΔTRP HMRssa ade2+ lys2- sir3ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2-*
ROY7163	*mataΔp hmlΔTRP HMRssa ade2- lys2+ sir4ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+ sir4ΔLEU*
ROY7164	*mataΔp hmlΔTRP HMRssa ade2- lys2- sir4ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2-*
ROY7168	*mataΔp hmlΔTRP HMRssa ade2- lys2+ sas2ΔKan/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+ sas2ΔKan*

ROY7169	*mataΔp hmlΔTRP HMRssa ade2- ly2s- sas2ΔKan/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2-*
ROY7154	*mataΔp hmlΔTRP HMRssa ade2- lys2- sir2ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 sir2ΔLEU ade2+ lys2-/ mataΔp hmlΔTRP hmrΔHIS3 sir2ΔLEU ade2+ lys2-*
ROY7155	*mataΔp hmlΔTRP HMRssa ade2- lys2- sir2ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 sir2ΔLEU ade2+ lys2-/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2-*
ROY7156	*mataΔp hmlΔTRP HMRssa ade2- lys2- sir2ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+*
ROY7157	*mataΔp hmlΔTRP HMRssa ade2- lys2-/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+*
ROY7160	*mataΔp hmlΔTRP HMRssa ade2+ lys2+ sir3ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2+ sir3ΔLEU/mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2+ sir3ΔLEU*
ROY7161	*mataΔp hmlΔTRP HMRssa ade2+ lys2- sir3ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2+ sir3ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2-*
ROY7162	*mataΔp hmlΔTRP HMRssa ade2+ lys2+ sir3ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+*
ROY7165	*mataΔp hmlΔTRP HMRssa ade2- lys2+ sir4ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+ sir4ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+ sir4ΔLEU*
ROY7166	*mataΔp hmlΔTRP HMRssa ade2- lys2- sir4ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+ sir4ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2-*
ROY7167	*mataΔp hmlΔTRP HMRssa ade2- lys2+ sir4ΔLEU/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+/ mataΔp hmlΔTRP hmrΔHIS3 ade2- lys2+*
ROY7170	*mata*D*p hmlΔTRP HMRssa ade- lys+ sas2DKan/ mataDp hmlΔTRP hmrΔHIS3 ade- lys+ sas2DKan/ mataDp hmlΔTRP hmrΔHIS3 ade- lys+ sas2DKan*

ROY7171	*mataΔp hmlΔTRP HMRssa ade2- lys2+ sas2ΔKan/mataΔp hmlΔTRP HMRssa ade2- lys2+ sas2ΔKa/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2-*
ROY7172	*mataΔp hmlΔTRP HMRssa ade2- lys2+ sas2ΔKan/ mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2- / mataΔp hmlΔTRP hmrΔHIS3 ade2+ lys2-*

Figure 1 (on next page). Characterization of *HMR* and the Cut and Flip system

(A) ChIP qPCR of various proteins in G1 arrested cells.

Histone H3, Sir3, H4K16 acetylation and H3K56 acetylation levels was measured across the HMR domain. Data is presented as the mean enrichment of IP/Input (as described in the materials and methods) for at least four IPs from two independent cross-links. Error bars are standard error from the mean. The data for H3K56 acetylation and H4K16 acetylation are presented as enrichment normalized to histone H3 enrichment in order to take into account variable levels of nucleosome occupancy.

(B) Schematic of the Histone H4 cut and flip cassette.

(C) G1 arrest and release fluorescence cytometry profiles of the Cut and Flip strain.

Ethanol fixed cells were stained with Sytox Green and analyzed by flow cytometry.

Panel 1: Fluorescence cytometry profile of asynchronously growing cells in raffinose containing medium. Panel 2: Fluorescence cytometry profile of cells arrested with alpha factor in galactose containing medium. Panels 3 to 6: Fluorescence cytometry profile of cells at the indicated times after release from alpha factor arrest into glucose containing media.

(D) Protein immunoblot analysis of cells arrested with alpha factor and released after switching of histone H4 alleles.

Yeast cells were grown overnight in raffinose containing rich medium, arrested with alpha factor and then transferred to galactose containing medium with alpha factor. Cells were released into YPD and aliquots of equivalent numbers of cells were removed at the specified times. Protein extracts were separated on a 15% SDS-polyacrylamide gel, transferred to membranes and probed with specific antibodies.

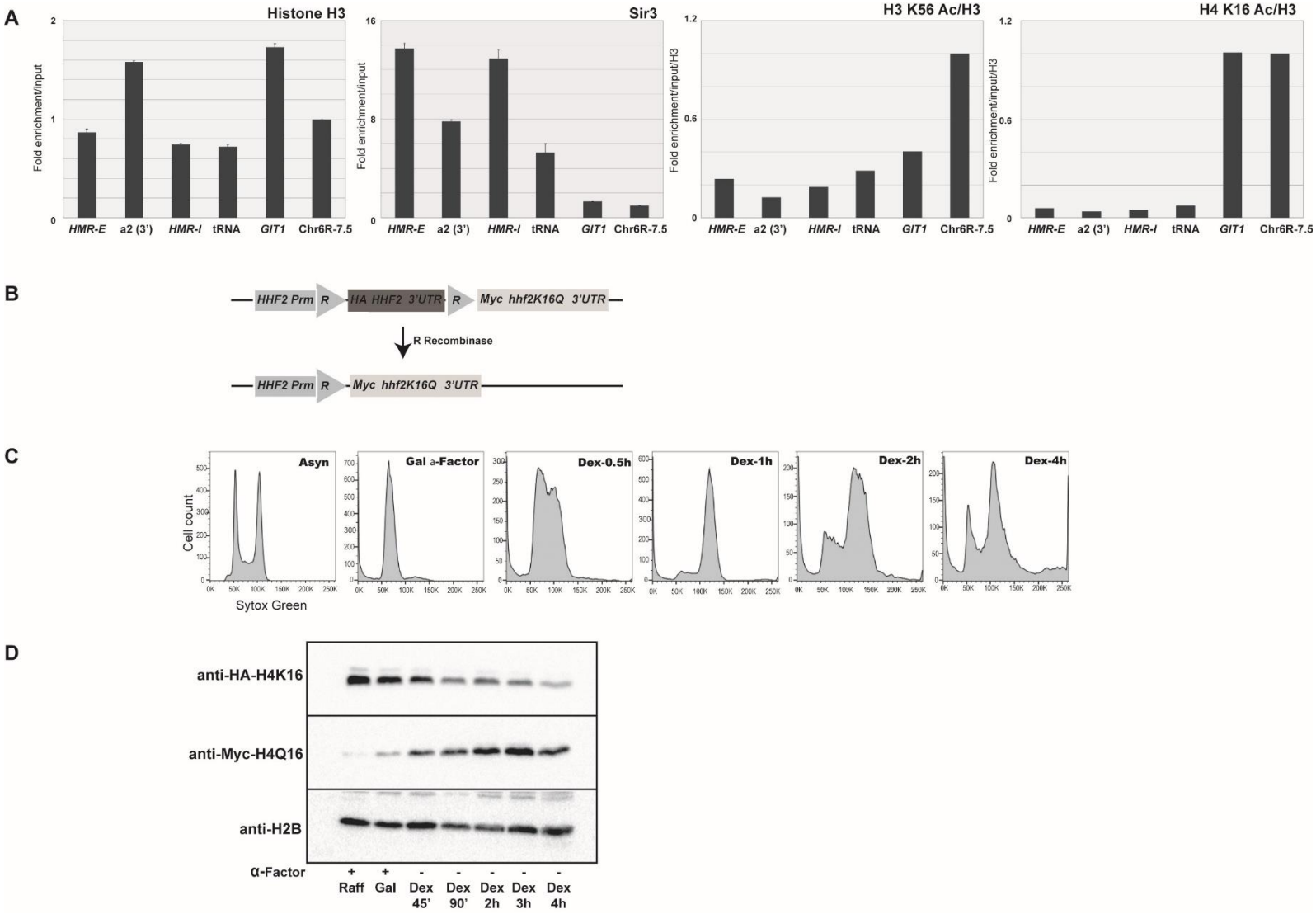

A
Histone H3
Sir3
H3 K56 Ac/H3
H4 K16 Ac/H3
Fold enrichment/input
Fold enrichment/input/H3
HMR-E
a2 (3')
HMR-I
tRNA
GIT1
Chr6R-7.5
B
HHF2 Prm
R
HA HHF2 3'UTR
Myc hhf2K16Q 3'UTR
R Recombinase
C
Asyn
Gal a-Factor
Dex-0.5h
Dex-1h
Dex-2h
Dex-4h
Cell count
Sytox Green
D
anti-HA-H4K16
anti-Myc-H4Q16
anti-H2B
α-Factor
Raff
Gal
Dex 45'
Dex 90'
Dex 2h
Dex 3h
Dex 4h

Figure 2 (on next page). Molecular analysis of silenced loci following Cut and Flip

(A) ChIP qPCR of unswitched histone H4 allele at silenced loci

The presence of wildtype HA-H4 was monitored by ChIP in unswitched (galactose containing medium with alpha factor) and 2h and 4h after switching of the histone H4 allele. The Y-axis represents the ratio of IP/Input DNA for each sample as described in the materials and methods. Error bars are standard error from the mean. The levels of the tagged proteins were mapped at three different loci- *HML (GFP)*, *HMR* (5' *HMR-E*) and *Chr6R (7.5)*.

(B) ChIP qPCR of the switched histone H4 allele at silenced loci

The presence of mutant Myc-H4 K16Q protein was monitored by ChIP in unswitched (galactose containing medium with alpha factor) and 2h and 4h after switching of the histone H4 allele. The Y-axis represents the ratio of IP/Input DNA for each sample as described in the materials and methods. Error bars are standard error from the mean. The levels of the tagged proteins were mapped at three different loci- *HML (GFP)*, *HMR* (5' *HMR-E*) and *Chr6R (7.5)*.

(C) ChIP qPCR measurement of Sir3 binding at silenced loci following switch of WT H4 to H4K16Q mutant

Sir3 binding at *HML (GFP)*, *HMR* (5' *HMR-E*) and *Chr6R (7.5)* was monitored using ChIP-qPCR in cells arrested with alpha factor and at 2h and 4h after switching the histone H4 allele and alpha factor release. Data is presented as the mean enrichment of IP/Input. Error bars are standard error from the mean.

(D) Measurement of mRNA expression of the GFP reporter at *HML* before and after switch of the histone H4 alleles.

Alpha factor arrested cells and cells released into rich medium were collected at 2h intervals and total RNA was extracted from these cells. GFP mRNA was quantitated by RT-qPCR and plotted as a function of time, normalized to *ACT1*.

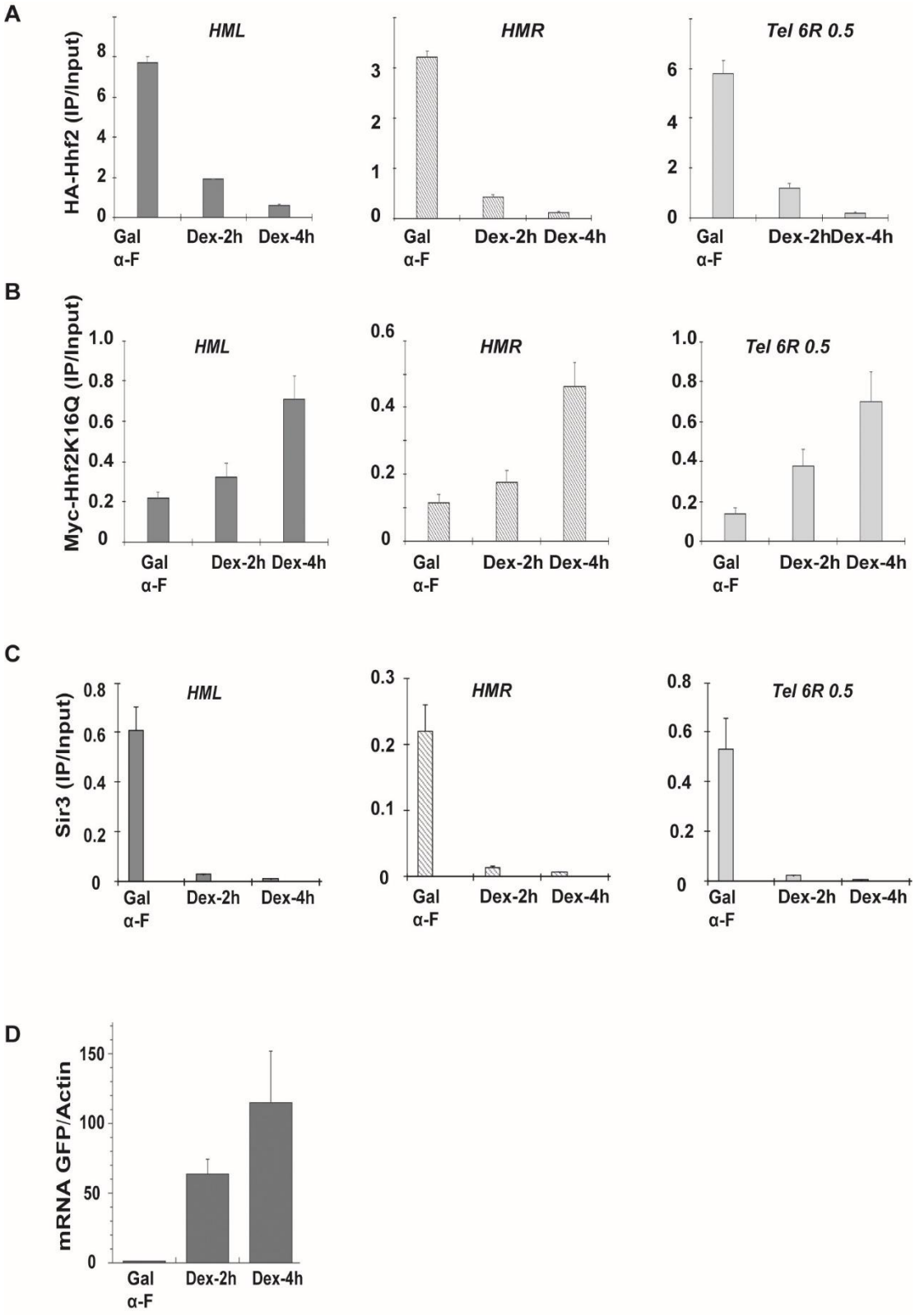

A
HA-Hhf2 (IP/Input)
HML
HMR
Tel 6R 0.5
Gal α-F
Dex-2h
Dex-4h
B
Myc-Hhf2K16Q (IP/Input)
HML
HMR
Tel 6R 0.5
Gal α-F
Dex-2h
Dex-4h
C
Sir3 (IP/Input)
HML
HMR
Tel 6R 0.5
Gal α-F
Dex-2h
Dex-4h
D
mRNA GFP/Actin
Gal α-F
Dex-2h
Dex-4h

Figure 3 (on next page). Fluorescent measurements of gene silencing

(A) Violin plots of GFP expression from silenced loci in strains expressing WT and mutant histone H4 K16Q alleles. Cells from a single colony containing the unflipped wild type cassette or a flipped cassette (confirmed by PCR) were grown in rich medium, imaged using a fluorescence microscope and the amount of fluorescence in each cell was quantitated and plotted as a box plot. For each sample, GFP fluorescence was measured in greater than 100 cells.

(B) Boxplots of GFP expression at of *HML::URA3p-GFP* following as a function of time after switching the histone H4 cassettes. The wild type histone HA-H4 cassette was switched to a wild type Myc-H4 cassette in G1 arrested cells and silencing at *HML::URA3p-GFP* was monitored in the cells after their release from the cell cycle arrest.

(C) GFP fluorescence was measured as a function of time in strains with modified *HML* containing GFP under the control of the *URA3* UAS enhancer/promoter. Cells were arrested in G1, the histone H4 cassette was switched from wild type H4 to mutant H4K16Q and cells were the released from the arrest.

(D) GFP fluorescence was measured as a function of time in strains with modified *HML* containing GFP under the control of the *a2* UAS enhancer/promoter. Cells were arrested in G1, the histone H4 cassette was switched from wild type H4 to mutant H4K16Q and cells were the released from the arrest.

(E) GFP fluorescence was measured as a function of time in strains with modified *HMR* containing GFP under the control of the *URA3* UAS enhancer/promoter. Cells were arrested in G1, the histone H4 cassette was switched from wild type H4 to mutant H4K16Q and cells were the released from the arrest.

(F) GFP fluorescence was measured as a function of time in strains with modified *HMR* containing GFP under the control of the *a2* UAS enhancer/promoter. Cells were arrested in G1, the histone H4 cassette was switched from wild type H4 to mutant H4K16Q and cells were the released from the arrest.

(G) Boxplots of GFP expression at the telomere following switching the histone cassette. GFP fluorescence measured as a function of time in strains with *TEL7L::URA3p-GFP*.

(H) GFP fluorescence was measured as a function of time in strains with *HML::URA3p-GFP* but also containing the wild type copy of the *HHT1-HHF1* locus.

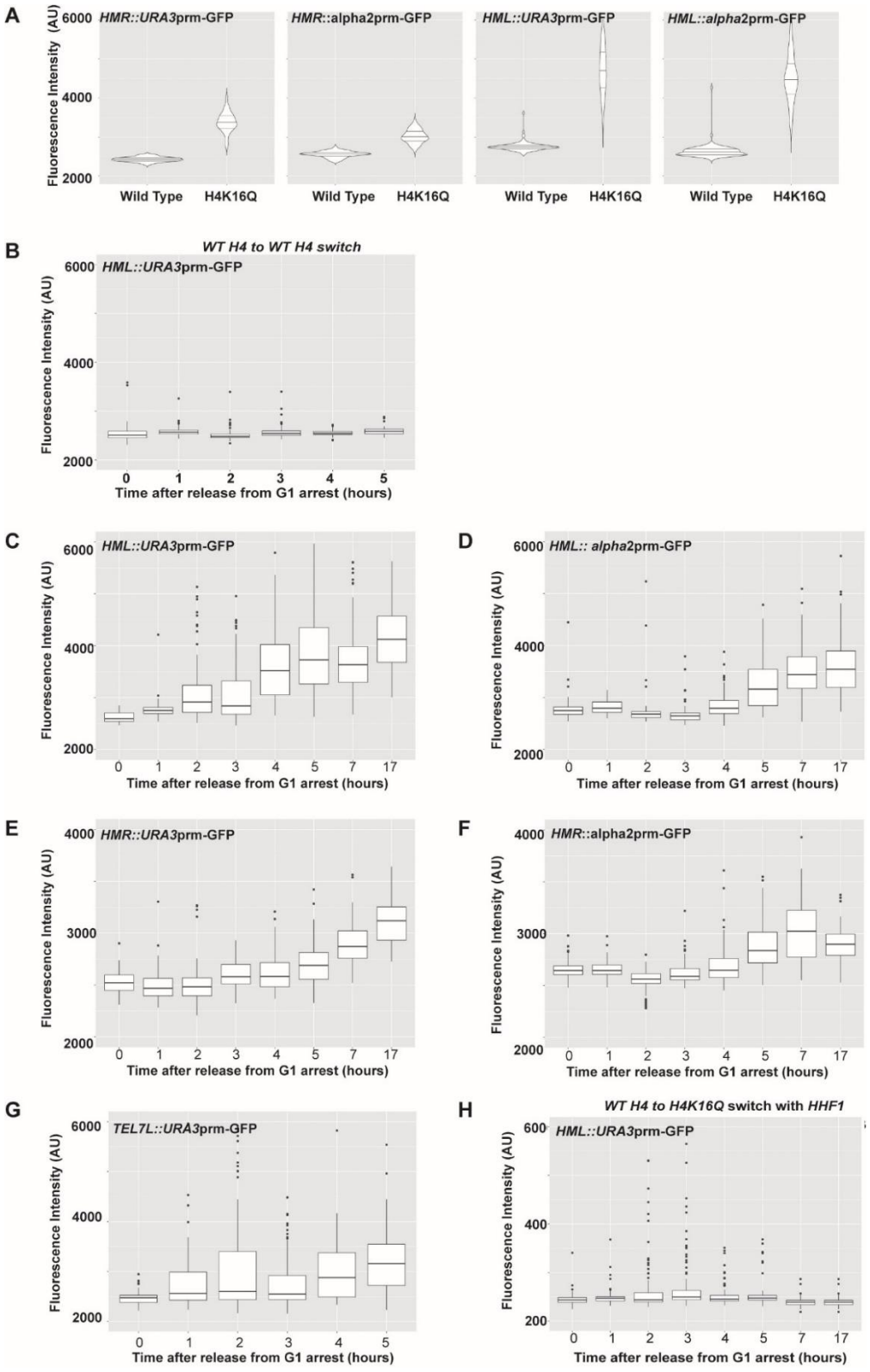

A
HMR::URA3prm-GFP
HMR::alpha2prm-GFP
HML::URA3prm-GFP
HML::alpha2prm-GFP
Fluorescence Intensity (AU)
Wild Type
H4K16Q
B
WT H4 to WT H4 switch
HML::URA3prm-GFP
Time after release from G1 arrest (hours)
C
HML::URA3prm-GFP
D
HML:: alpha2prm-GFP
E
HMR::URA3prm-GFP
F
HMR::alpha2prm-GFP
G
TEL7L::URA3prm-GFP
H
WT H4 to H4K16Q switch with HHF1
HML::URA3prm-GFP

Figure 4 (on next page). Effects of Sir gene dosage on silencing

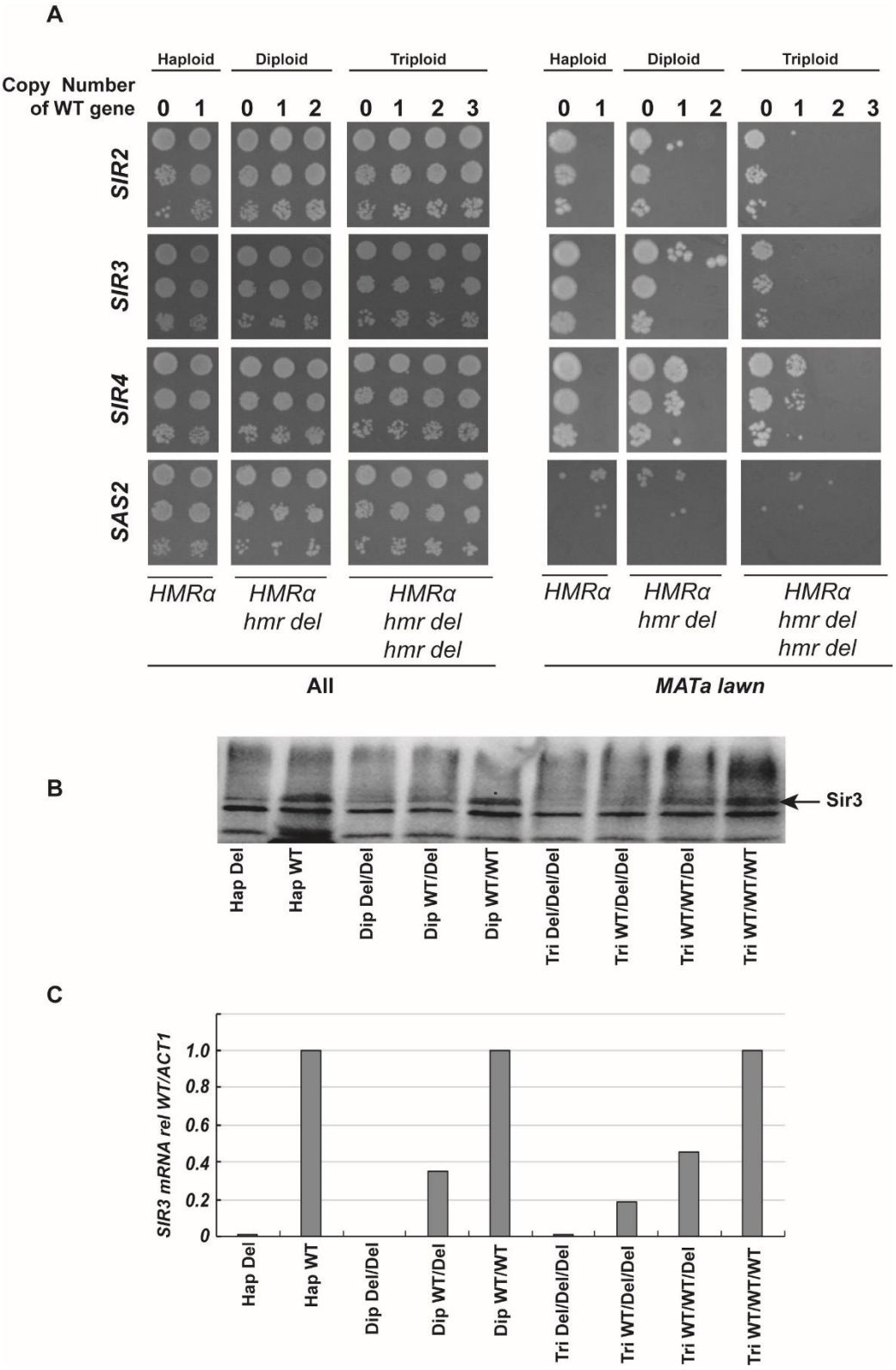
(A) Derepression of *HMRssα* was monitored in haploid, diploid and triploid cells containing variable gene copy numbers for the *SIR2, SIR3, SIR4* or *SAS2* genes. Loss of silencing resulted in a phenotypic switch in mating of the strain from a to α. Derepression of *HMRssα* was examined by plating 10- fold-serially diluted cells onto YMD media containing mating-type tester lawns.

(B) Protein immunoblot analysis of Sir3 levels in haploid, diploid and triploid cells containing variable copy numbers of the *SIR3* gene.

Equal numbers of yeast cells grown in YPD were harvested and total protein extracts were generated. Protein extracts were separated on a 10% SDS-polyacrylamide gel, transferred to membranes and probed with anti-Sir3 polyclonal antibodies.

(C) Measurement of mRNA expression of *SIR3* and *ACT1* in haploid, diploid and triploid cells containing variable copy numbers of the *SIR3* gene.

Equal numbers of yeast cells grown in YPD were harvested and total RNA was extracted from these cells. *SIR3* mRNA was quantitated by RT-qPCR, normalized to *ACT1* and plotted.


A
Haploid
Diploid
Triploid
Haploid
Diploid
Triploid
Copy Number of WT gene
0 1
0 1 2
0 1 2 3
0 1
0 1 2
0 1 2 3
SIR2
SIR3
SIR4
SAS2
HMRα
HMRα hmr del
HMRα hmr del hmr del
HMRα
HMRα hmr del
HMRα hmr del hmr del
All
MATa lawn
B
Sir3
Hap Del
Hap WT
Dip Del/Del
Dip WT/Del
Dip WT/WT
Tri Del/Del/Del
Tri WT/Del/Del
Tri WT/WT/Del
Tri WT/WT/WT
C
SIR3 mRNA rel WT/ACT1
1.0
0.8
0.6
0.4
0.2
0
Hap Del
Hap WT
Dip Del/Del
Dip WT/Del
Dip WT/WT
Tri Del/Del/Del
Tri WT/Del/Del
Tri WT/WT/Del
Tri WT/WT/WT

CHAPTER 3

Silencing can only suppress weak enhancers and promoters in *Saccharomyces cerevisiae*

INTRODUCTION

Variation in gene expression from cell-to-cell results in variation in phenotype across an otherwise genetically identical population. Classical studies on gene regulation usually measured average expression in populations of cells, masking differences that occur among individual cells. This variation, known as noise, is thought to arise from stochastic factors during the process of gene expression. Population heterogeneity of expression is thought to confer cells flexibility in responding to environmental conditions.

Noise can be expressed as the standard deviation divided by mean of gene expression squared, otherwise known as the coefficient of variation (CV^2) (Swain et al., 2002). This can be further delineated into intrinsic and extrinsic noise (Elowitz et al., 2002). Intrinsic noise can be determined by the properties of the gene and associated regulatory sequences, and the direct gene product. This would include enhancers, promoters, and silencers, as well as determinants of mRNA and protein decay. Extrinsic noise is determined by factors that are not a part of but rather interact with the gene or direct gene product, and this can be an effect of either their activity or concentration. In their work, Elowitz et al. pioneered a dual fluorescent reporter

system in Escherichia coli with which intrinsic and extrinsic noise values could be determined with single-cell measurements (Elowitz *et al.*, 2002).

Following the work of Elowitz et al., Raser and O'Shea recreated the dual fluorescent reporter system in the eukaryote S. cerevisiae and found that with the enhancers/promoters used in their study, extrinsic noise is the predominant contributor to total noise (Raser and O'Shea, 2004). Among the gene enhancers/promoters the authors tested, most retained constant intrinsic noise contributions irrespective of induced gene expression rate. However, a notable exception, *PHO5*, uniquely had higher intrinsic noise contribution at lower rates of induced gene expression. To explain this, they developed a model in which the enhancer/promoter had two distinct expression states, inactive and active, that were respectively restrictive and permissive of transcription. *PHO5* was known to have an enhancer/promoter chromatin remodeling transition step upstream and independent of transcription (Fascher et al., 1993). Infrequent transitions between active and inactive states; in other words, a bursting model of transcription, would recapitulate the high intrinsic noise found at lower expression states observed for *PHO5*.

Accordingly, Raser and O'Shea tested their bursting model by interfering with the regulatory factors of the upstream step. Mutating the upstream activating sequences (UAS) of the *PHO5* enhancer resulted in greater intrinsic noise contribution, as did mutating various chromatin remodelers. Conversely, when the TATA box of the promoter was mutated, the rate of transcription was lowered and resulted in a weaker intrinsic noise contribution. After Raser and O'Shea, Newman et

al. performed a quantitative proteomics study across a larger array of S. cerevisiae gene enhancers/promoters (Newman et al., 2006). Notably, they observed that under differing steady-state growth conditions, differences in protein levels are largely captured by mRNA levels, confirming that variations in gene expression mostly occur at the level of transcription. When grouping genes by ontology in their study, they also observed a marked influence of the TATA box, as well as regulation by chromatin remodelers and transcription factor, on noise.

Some genes are expressed in random uncorrelated events with constant probability over time, leading to a Poisson distribution of transcript amounts. Other genes are expressed in bursts, switching between periods of high transcription activity and longer periods of quiescence. Thus, highly active genes are likely to have a more uniform expression rate that is well-described by a Poisson distribution. At intermediate levels of expression, genes vary in their firing kinetics in a manner dependent on regulatory elements (Sanchez and Golding, 2013).

Zenklusen et al. combined single-cell transcript measurements with computational modeling and determined that in yeast, gene expression could fall under these two categories of expression (Zenklusen et al., 2008). Among the yeast genes they assayed, Zenklusen et al. found that constitutively active genes have Poissonian kinetics, whereas environmental-response genes exhibit bursting kinetics. Developments in fluorescently labeling nascent RNA transcripts allowed for a more direct observation of firing kinetics, lending credence to transcriptional bursting as a

basis for noise (Larson et al., 2011). Moreover, the approach allowed for the study of individual aspects of transcriptional bursting, such as frequency and size.

What is the molecular basis of this transcriptional bursting? Refinement of the model came with further analysis of *PHO5*. The regulatory sequences found at the enhancer of *PHO5* are known to occlude transcription factor binding (Mao et al., 2011). In their electron microscopy analysis of single *PHO5* molecules, Brown et al. observed that the active PHO5 promoter adopts multiple promoter nucleosome configurations (as opposed to two), suggesting that bursting is a consequence of the probabilistic transitions among promoter nucleosome states (Brown et al., 2013). Brown and Boeger later observed that these promoter nucleosome states arise solely from factors governing intrinsic noise (Brown and Boeger, 2014). These and other studies have demonstrated the nature and dynamics of gene activation in single cells.

Silencing is a complex but robust process to stably restrict gene expression in cells. It is primarily mediated by the binding of repressor proteins to specifically modified nucleosomes. For stable transcriptional silencing to occur, multiple mechanisms are believed to act in concert to reduce nucleosome mobility, reduce residence time for activator binding, occlude transcription factor binding sites, and disrupt the formation of the pre-initiation complex. However, few studies have explored the dynamics of gene silencing.

Xu et al. examined silencing dynamics by measuring fluorescence intensity at the silent *HML* and *HMR* in yeast, finding that transcriptional silencing states

between the silent loci are independent (Xu *et al.*, 2006). Moreover, they observed that by increasing the amount of a transcriptional activator for *URA3* called Ppr1, they could disrupt the silencing establishment and maintenance for a reporter driven by the *URA3* enhancer/promoter. Osborne and Rine expanded on the work Xu et al., using their fluorescence system to determine that chromatin-remodelers impacted the kinetics of silencing establishment (Osborne *et al.*, 2011).

Previously, we built combinatorial libraries of regulatory elements: enhancers, core promoters, 5' untranslated regions (5'UTRs), and 3' untranslated regions (3'UTRs) to delineate how these individual elements contribute to the extent and variation in gene expression (Dhillon et al., 2020). We now seek to further determine how regulatory elements involved in gene activation interact with regulatory elements that govern silent chromatin, specifically the silencer elements found at *HML* and *HMR*. In the wild-type state, the native *a* and *α* enhancers/promoters of the mating genes at these loci are robustly and stably silenced, such that they are rarely and only transiently expressed. However, ectopic insertion of heterologous gene promoters, such as those regulating the expression of *ADE2* (Sussel *et al.*, 1993) or *URA3* (Aparicio and Gottschling, 1994) exhibit bimodal states of expression, reflective of a proportion of cells where the gene is stably active and a proportion of cells where the gene is stably silent. Further studies suggest that these bimodal profiles of expression are susceptible to the integrity of the silencer elements (Sussel *et al.*, 1993), silencing proteins (Pillus and Rine, 1989), and transcription activators (Aparicio and Gottschling, 1994).

To further investigate the kinetics of gene silencing, we built a series of constructs and investigated 9 different enhancers and promoters that are associated with varying expression levels. These regulatory elements drove the expression of a variety of reporter genes to delineate the contribution of individual elements towards transcriptional activity at a silent locus. With the *a1* reporter, we quantitatively measure mating efficiency (and inversely, the frequency of silencing loss) during the G1 phase of the cell-cycle. With the *URA3* reporter, we determine the stability and heritability of the silent state over multiple cell cycles. Fluorescent reporters allow for a different view of silencing loss, and moreover provide us the opportunity to investigate variation in silencing amongst a population of cells when coupled with a dual color assay system. To characterize the individual contributions of enhancer versus promoter elements undergoing silencing and their contribution towards mean expression levels and variation, we build and interrogate silencing of a more complex 7 by 7 enhancer and promoter matrix driving fluorescent reporters. Finally, with the reporter system created by Larson et al., we measure transcription at the silenced loci.

RESULTS

General design of reporter constructs

Using Golden Gate Cloning, we assembled the different regulatory elements in a specific order such that each cassette contained a variable enhancer/promoter, the coding sequence of a given reporter gene, all flanked by the *HMR-E* and *HMR-I* silencers. A *KanMX* or *HygMX* selection marker lies outside the *HMR-I* silencer, and

the whole construct is integrated at either the left arm of chromosome III at *LEU2*, or at the endogenous *HMR* locus adjacent to the telomeric region on the right arm of chromosome III (*TEL3R*) (Figure 1).

Classical measurements of silencing

We used a classical approach to measure silencing. In a wild-type *MATα* yeast cell, the *a1* gene at the endogenous *HMR* locus is transcriptionally silenced. A *MATα* cell is capable of mating with a *MATa* cell in the G1 phase of the cell cycle, giving rise to diploid colonies that can grow on minimal medium plates. However, if the *a1* gene is not silenced, then the *MATα* cell is unable to mate and fails to form diploid colonies.

We built 9 different strains where the *a1* coding sequence at *HMR* was linked to various enhancers and promoters of variable strength. These constructs were then integrated at either *LEU2* or *HMR*. Our data show that strong enhancers/promoters, namely those of the *TDH3*, *PDC1*, *RPL28*, and *CDC19* genes resist silencing of the *a1* reporter, while weaker enhancers/promoters, such as those of *a1*, *ACO1* and *PGK1* were silenced to a greater extent (Figure 2A). In the absence of induction, the inducible *ADE2* and *GAL1* enhancers/promoters were unexpressed.

As a control, we measured silencing of these constructs in strains lacking *SIR3*. Silencing of the constitutive enhancers/promoters was completely lost in *sir3* mutants while the strains with the two inducible promoters were still able to mate as they were grown in non-inducing conditions (Figure 2B).

Our analysis also shows that silencing is dependent upon the location of the silenced domain. We observe greater silencing of these gene regulatory elements when the *HMR* cassette was integrated at the endogenous *HMR* locus near *TEL3R* than when integrated at the *LEU2* locus on the left arm of chromosome III (Figure 2C).

While the previous experiments provide a semi-quantitative view of gene silencing, quantitative mating assays offer more precise values. We took the strains described above, in which the various *HMR::a1* constructs were integrated at *LEU2*, and quantitatively measured mating frequency as a metric for silencing. Our quantitative mating assays reveal that phenotypic silencing is probabilistic in a manner dependent on enhancers/promoters (Figure 3A). Although the *a1* enhancer/promoter mating efficiency is very robust, it appears to be less so than that for uninduced *ADE2* and *GAL1* enhancer/promoter cassettes (Figure 3B). For the other regulatory elements, the previously observed lower mating efficiencies are quantitatively recaptured.

We next sought to determine how enhancers/promoters interact with silencing. To that end, with our 9 enhancers/promoters, we used a *URA3* reporter at *HMR* integrated at the *LEU2* locus and monitored cell growth on medium lacking uracil or containing 5-FOA. In this way, we could test the stability of silencing: expression of *URA3* allows cells to grow on medium lacking uracil, whereas repression of *URA3* over several generations allows cells to form colonies on medium containing 5-FOA, which is a hallmark of the stably silent state.

Our data show that while the *a1* enhancer/promoter was sufficiently silenced to permit cell growth on 5-FOA, it was also sufficient to drive growth on uracil auxotroph selection medium (Figure 4). This is likely due to the insertion of *HMR* construct at the *LEU2* locus on the left arm of chromosome III, as we did not observe this phenomenon when a similar assay was performed with a construct integrated at the *HMR* locus near *TEL3R* (Valenzuela et al., 2006). In contrast, the other constitutively expressing enhancers/promoters were unable to grow on 5-FOA plates at all. Altogether, the mating assay data indicate that while there can be transient repression of some enhancers/promoters (*ACO1* and *PGK1*) at *HMR*, the *URA3* reporter assays suggest that this repression is neither stable nor heritable.

Measurements of fluorescent gene expression at silent loci

A strength of fluorescent reporter assays is the ability to monitor single-cell expression levels across large populations of cells. One limitation of classical assays is that they typically offer a snapshot of activity within a certain time frame. The mating assays, for example, are dependent on the stage of the cell cycle, and transient expression outside of G1 would not be detected, as the cell is responsive to mating pheromone signaling only in G1. On the other hand, a fluorophore can be monitored in a continuous manner independent of cell states and is less subject to regulation by endogenous negative feedback loops.

We therefore built a series of constructs where a yellow fluorescent protein reporter (*Venus*) was placed under the control of the 9 enhancers/promoters flanked

by the native *HMR-E* and *HMR-I* silencers and integrated this cassette at either *LEU2* on the left arm of chromosome III or the native *HMR* locus near *TEL3R*. Using flow cytometry, we measured the expression of *Venus* coupled to these 9 enhancers/promoters. Although these genes were supposedly subject to silencing by the native *HMR* silencers, we observed robust reporter expression for constitutively active enhancers/promoters with the exception of *a1* (Figure 5). Moreover, the expression relationships seen for these regulatory elements while potentially being silenced were in line with what was observed for the same regulatory elements at an active locus in the absence of silencing (Dhillon *et al.*, 2020). These data suggest that the ability of a gene to be fully or partially silenced is a function of the strength of the regulatory element.

As a control, the same fluorescence analyses were performed on versions of these strains carrying a *sir3* deletion, in which we observed little change in fluorescence intensities compared to that of wild-type *SIR3* (Figure 5). As it is known that a *sir3* deletion causes complete silencing loss and lead to complete loss of mating, these results suggest that subtle changes in the silencing of *Venus* are not detectable by flow cytometry.

To test position-specific effects, we compared *Venus* expression across 9 enhancers/promoters in *HMR* constructs integrated at the endogenous *HMR* locus against those at *LEU2*. These data clearly show that silencing of enhancers/promoters depends upon the position on the chromosome, with the silencing of *Venus* being reduced when the *HMR::Venus* cassette was located at *LEU2* compared to *HMR*

(Figure 6). This shows that the location of a gene affects its expression, even when under the influence of the same silencer elements. Both the *LEU2* and *HMR* loci are located on chromosome 3, with *HMR* being closer to a telomeric ends that cluster together with *HML* and other heterochromatic loci. It has previously been suggested that this clustering facilitates a re-association of released repressor proteins, thereby increasing local repressor protein concentrations. *LEU2*, which is closer to the centromere away from other clustered heterochromatic loci, would thus not be as affected by such local silencing effects.

We then sought to determine whether the abundance of a silencing protein, Sir1, would have an observable effect on the various constructs that otherwise appear to escape silencing. Previous studies have shown that Sir1 can establish silencing when it is specifically targeted to a silencer via fusion to a Gal4 DNA binding domain, so long as the silencer itself contains binding sites for Gal4 (Chien *et al.*, 1993). We built strains where the *Venus* reporter is flanked by synthetic silencers containing binding sites for Gal4. The modified *HMR-E* silencer has 4 Gal4 binding sites, while the *HMR-I* silencer has 5. This silencer configuration has previously been shown to robustly silence genes when Gal4-Sir1 is present (Kirkland et al., 2015).

We created a set of strains in which *HMR::Venus* is driven by the 9 different enhancers/promoters but flanked by synthetic silencers containing these Gal4 binding sites. These strains did not have endogenous *SIR1*; rather, they had a *GAL4-SIR1* fusion gene regulated by a *MET17* promoter, which is repressed when methionine is present in the growth medium and expressed when methionine is absent. Induction of

Gal4-Sir1 was sufficient to reduce levels of *Venus* expression in all constitutive enhancer/promoter containing strains (Figure 7). Compared to other enhancers/promoters, the effects of the Gal4-Sir1 induction seemed to have more a more modest effect on the *RPL28* enhancer/promoter. Silencing with the Gal4 binding site containing silencers was greater than what was observed when these enhancers/promoters were under the control of the native endogenous *HMR-E* and *HMR-I* silencers (Figure 7). These data suggest that although sufficiently strong enhancers/promoters can escape silencing, increased dosage and/or direct recruitment of Sir1 to a silencer can overcome this effect to some extent.

Measurements of enhancer and promoter contributions to expression and noise

While our data indicate that silencing is sensitive to the strength of the enhancer/promoter, we were curious regarding the role of each element individually in the ability of a locus to be silenced. We were interested in knowing if there were specific core promoters or enhancers that were more or less susceptible to silencing. To that end, we used flow cytometry to analyze a matrix of 49 different *HMR* loci, where 7 enhancer and 7 promoter combinations were systematically built to drive the expression of a *Venus* reporter (Figure 8A).

As demonstrated in Figure 8A, the strong *TDH3* and *PDC1* enhancers were resistant to silencing, but despite this, the *a1* core promoter could strongly reduce gene expression from these strong enhancers. In contrast, the *RPL28* and *CDC19* enhancers, which were not as strong as *TDH3* or *PDC1* in the absence of silencing,

appear to better resist the silencing effects of the *a1* core promoter. Similarly, the *a1* enhancer also exhibited substantial silencing effects on gene expression that none of the other 8 tested promoters could overcome. Altogether, these data suggest that the contributions of the endogenous *a1* enhancer and promoter individually and substantially contribute to the silent state at *HMR*, and that *a1* promoter.

We next determined total noise by taking the coefficients of variation (CV) for gene expression across the 7 by 7 strain matrix. From this analysis, a general pattern emerges in which CV has an inverse relationship with expression strength (Figure 8B). Additionally, enhancers, but not promoters, appear to contribute more to CV.

We sought to determine the extent of the total noise that was due to intrinsic noise and the extent that was due to extrinsic noise. To perform this analysis, we turned to the previously described dual color reporter assay (Elowitz *et al.*, 2002). We built haploid yeast strains where the *HMR* cassettes, integrated at *LEU2*, contained the *Venus* reporter under the control of the 9 different enhancers/promoters. We also built a different set of haploid strains where the *HMR* cassettes, integrated at *LEU2*, contained the *mCherry* red fluorescent protein reporter under the control of the 9 different enhancers/promoters. Finally, to complete the dual color system, we mated the two strains to form diploids. We then measured expression levels of *HMR::Venus* and *HMR::mCherry* in the same cell using fluorescence cytometry. From these measurements, we calculated the intrinsic and extrinsic contributions of noise. The noise relationships from these analyses appear to corroborate our data on total noise from the 7 by 7 enhancer and promoter matrix. In all cases, the extrinsic noise of

expression across the silent loci was a relatively constant minor contribution, whereas the majority of total noise was intrinsic noise that was inversely proportional to enhancer/promoter strength (Figure 9).

Measurements of transcription

Monitoring expression of the fluorescent protein reporters undergoing transcriptional silencing lacks some of the limitations of classical approaches, but it is still not a direct measurement of transcription. Levels of fluorescent protein are still subject to additional layers of regulation: mRNA stability, translation efficiency, and protein stability all feed into final measurements, which thus are more reflective of a of history of gene expression rather than an ideal snapshot. To better directly visualize transcriptional silencing, we turned to the system developed by Larson et al., which takes advantage of a bacteriophage coat protein, PP7, fused to green fluorescent protein (PP7-GFP) (Larson *et al.*, 2011). An array of binding sites for PP7 are also added to the 5'UTR of a gene. As that gene is transcribed, the constitutively expressed PP7-GFP protein binds nascent transcripts in the nucleus and forms an observable fluorescent focus. Once transcription is complete, the nascent transcript is released from the nucleus and diffuses into the cytoplasm. Through fluorescence microscopy, this system thus allows for the visualization of nascent transcripts of a gene undergoing silencing by measurements of nuclear foci at a silenced locus. We built a set of strains where multiple binding sites for PP7-GFP were inserted into the 5'UTR of the *PHO5* gene. This cassette was placed under the control of the 9 different enhancers/promoters and integrated at *HMR* near *TEL3R*.

For our purposes, we generated these strains and determined the percentage of growing cells in which we detected foci as an analog for transcription frequency. The patterns that emerged roughly parallel previous observations of silencing loss and gene expression (Figure 10). Cells with weak enhancers/promoters such as *ACO1* and *PGK1*, which were partially silenced according to mating assays, had fewer transcription foci. In contrast, enhancers/promoters that robustly resisted silencing, such as *RPL28*, *TDH3*, and *PDC1* had a greater percentage of cells undergoing transcription. We also determined if the frequency of transcription foci changed when silencing was lost. To that end, we deleted *sir3* from this set of strains and observed increased frequency of transcription foci, particularly for the enhancers/promoters associated with intermediate levels of expression. We also measured the fluorescence intensities of the foci observed as an analog for burst size but did not observe any change in foci intensity in wild-type *SIR3* strains compared to *sir3*Δ strains (Figure 11). Altogether, these data suggest that silencing counteracts transcription by regulating transcription frequency, but not burst size.

DISCUSSION

Silencing is a probabilistic phenomenon

The outcomes of gene expression at silent loci are probabilistic, not deterministic. A current model for silencing is that it acts in a regional, sequence non-specific manner that renders it inaccessible to transcription machinery, but if so, we would expect discrete biphasic outcomes. With the fluorescence analysis we are not

able to observe discrete all-or-nothing patterns of silencing, but rather a continuum based on enhancer and promoter strength. By the quantitative mating assays, the *ADE2* and *GAL1* enhancers/promoters in non-inducing conditions resulted in higher probabilities of mating, and thus effective gene repression, than the endogenous *a1* promoter (*Figure 3*). Conversely, we could also detect differences in mating probabilities with enhancers/promoters stronger than *a1*. We saw a consistent pattern of silencing escape based on enhancer/promoter strength, with *a1*, *ADE2*, and *GAL1* being consistently silenced, the weaker constitutive enhancers/promoters *ACO1* and *PGK1* exhibiting intermediate levels of silencing, and strong constitutive enhancers/promoters *RPL28*, *TDH3*, *CDC19*, and *PDC1* exhibiting the highest levels of gene expression.

Silencing is dependent on enhancer and promoter strength

As *a1* is the only constitutive promoter that exhibits robust phenotypic silencing in our classical mating assays, and as the *a1* enhancer and the *a1* core promoter can individually repress gene expression at silent loci, our data suggest that the properties of the enhancer/promoter at the silent locus plays a vital role in phenotypic silencing. We assayed 8 other enhancers/promoters. 2 of these (*ADE2* and *GAL1*) are inducible promoters, analyzed under non-inducing conditions and they remained silent. The remaining 6 enhancers/promoters are constitutively expressed in glucose-containing media at varying strengths, though none were weak enough to be robustly silenced across our assays.

Although the effects of silencing appear weak against most constitutively active enhancers/promoters, we did observe increased silencing when we fused *GAL4* to *SIR1* and targeted this protein to a synthetic silencer. We note that the synthetic silencers in this system contain multiple consensus binding sites for Gal4, while Sir1 is recruited to the silenced domain via interactions with Orc1 at wild-type *HMR* silencers. We are thus unable to directly compare the kinetics of Orc1-Sir1 interactions with DNA versus that of Gal4-Sir1. Despite this limitation, it is apparent that sufficient *MET17* mediated induction of *GAL4-SIR1* expression can counteract silencing loss in a manner wild-type *SIR1* expression levels cannot. These data suggest that a limiting factor in silencing is the binding/stability of Sir1 to the silencers. Our data thus points towards gene activity at a silent locus as being an outcome of silencer, enhancer, and promoter properties.

Moreover, our 7 by 7 enhancer and promoter matrix analyses suggest that enhancer/promoter strength alone may not fully predict whether regulatory elements can escape silencing. Remarkably among the regulatory elements tested, while the *RPL28* and *CDC19* enhancers/promoters only drive intermediate levels of expression at a non-silenced locus, they also appear to resist silencing to the greatest extent. This suggests additional complexity, although the underlying reason for this phenomenon for the moment remains unclear.

When we delineate the individual contribution of enhancers and promoters to gene expression, we can outline some general patterns. As previously mentioned, the *a1* enhancer and promoter individually are more susceptible to gene silencing. The

TDH3 and *PDC1* core promoters appear to be the primary contributors to their expression strength. Coincidentally, though they do not appear to have the highest tested transcription frequencies, they did have among the highest burst sizes. Conversely, *RPL28* and *CDC19* appear to have strong enhancers but intermediate promoter strength and were associated with higher transcription frequencies but lower burst size (Figure 10). Transcriptional activity in a silent domain is thus as dependent on the properties of individual enhancers and promoters as they are on silencing factors. The underlying reason for this could be the affinities of these elements to bind various transcriptional activators, pre-initiation complex formation, as well as dynamics of nucleosome mobility and stability.

Sir proteins control gene expression by reducing burst frequency

While we did not observe dramatic effects of *sir* deletions across our tested enhancers and promoters, we did see effects of a *sir3* deletion on the frequency of cells undergoing transcription, but not burst intensity (Figure 9). When taken together with our observations of gene expression at *HMR* under Gal4-Sir1 induction (Fig. 7), our results point toward a possibility that the Sir proteins function by altering the ability of transcription factors to bind their cognate binding sites in gene enhancers at silenced domains, thus altering the probability that transcription occurs. We note that certain enhancers, such as *RPL28* and *CDC19*, appear to have the most profound effect on counteracting silencing compared to other enhancers such as *TDH3* and *PDC1*, which through further study, could shed light on the underlying mechanisms.

METHODS

Flow cytometry

Cells cultures were grown overnight in deep 96-well plates containing yeast minimal medium with 2% dextrose (YMD) at 30°C on a shaker, and back-diluted to an OD_{600} of around 0.2/mL. After 3 hours, the cells were strained through a NITEX membrane and transferred into a 96-well plate. Flow cytometry was performed using an Attune NxT flow cytometer (Thermo Fisher Scientific).

Fluorescence microscopy

Cells cultures were grown overnight in YMD at 30°C, and back-diluted to an OD_{600} of around 0.5/mL. 3 µl of the suspension was applied to a 1.5% agarose YMD pad on top of a microscope slide and cover-slipped. Images were acquired on a DeltaVision Personal DV system (Applied Precision), using a 40x 1.35 NA oil-immersion objective (Olympus), with a CoolSnap charge-coupled camera (Roper Scientific). 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 analysis was performed using the FIJI distribution of ImageJ software. To measure fluorescence intensity per cell, a two-dimensional maximum-intensity projection was generated for each collected Z-stack. For transcriptional frequency measurements, 100 cells were assayed for the presence or absence of fluorescent focus. For focus intensity analyses, measurements were taken using the Vale Lab Spot Intensity Analysis tool.

Software

Graphs were prepared using Microsoft Excel and R software with ggplot2 package.

Flow cytometry analyses were performed using FlowJo software (BD Life Sciences).

Strains

Strain No.	Genotype
E/P: enhancer/promoter	*a1*, *PDC1*, *PGK1*, *RPL28*, *TDH3*, *ACO1*, *CDC19*, *ADE2*, *GAL1*
ROY7300-7317	*MATα, ADE2+, lys2-, HMR-E/P-a1::KanMX* at *leu2*
ROY7480-7497	*MATα, ADE2+, lys2-, HMR-E/P-a1::KanMX* at *leu2, hmrΔURA3, sir3ΔLEU2*
ROY7230-7238	*MATα, ADE2+, lys2-, HMR-E/P-a1::HygMX* at *HMR*
ROY7240-7248	*MATa, ADE2+, lys2-, HMR-E/P-URA3::KanMX* at *leu2*
ROY7320-7339	*HMR::E/P-Venus::KanMX* at *leu2*
ROY7290-7298	*MATa, ADE2+, lys2-, HMR::E/P-mCherry::KanMX* at *leu2*
ROY7410-7418	diploid *HMR::E/P-Venus::KanMX* at *leu2* *HMR::E/P-mCherry::KanMX* at *leu2*

ROY7280-7288	*MATα, ADE2+, lys2-, HMR::E/P-Venus::KanMX* at *HMR*
ROY7460-7468	diploid *HMR::E/P-Venus::KanMX* at *HMR* *HMR::E/P-mCherry::KanMX* at *leu2*
ROY7520-7537	*ADE+, sir1ΔHIS3, HMR::E/P-Venus::KanMX* at *leu2*
ROY7540-7566	*ADE+, sir3ΔLEU2, HMR::E/P-Venus::KanMX* at *leu2*
ROY7419-7445	*HMR(4GEB)::E/P-Venus::HygMX at HMR, his3::MET17p-GAL4-SIR1::KanMX*
ROY7340-7357	*HMR-E/P-PP7-PHO5::HygMX at leu2, PP7-GFP::URA3*
ROY7360-7379	*ADE+, HMR-E/P-PP7-PHO5::HygMX at HMR, PP7-GFP::URA3*
ROY7500-7517	*ADE+, sir3ΔLEU2, HMR-E/P-PP7-PHO5::HygMX at HMR, PP7-GFP::URA3*

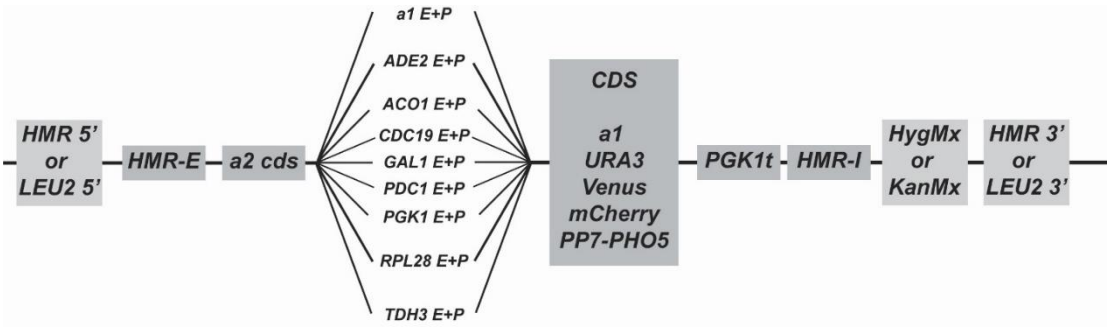


Figure 1. General scheme of construct design. Using Golden Gate Cloning, we assembled varying enhancer/promoter elements upstream of varying reporter gene coding sequences, followed by the *PGK1* terminator, all flanked by the *HMR-E* and *HMR-I* silencers. A selectable *HygMX* or *KanMX* marker lies outside of the silencer-flanked region, and homology arms lie at the end of the construct for integration at either *HMR* or *LEU2*.

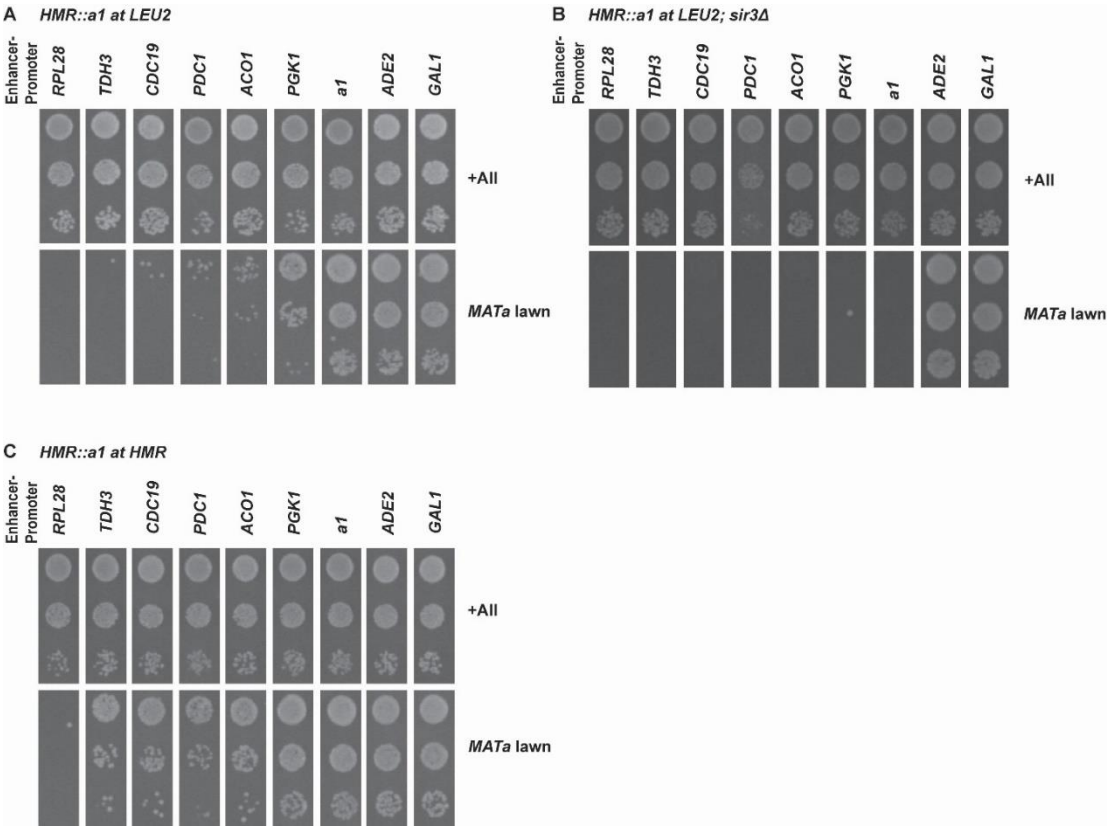


Figure 2. Enhancer/promoter properties affect silencing of *HMR::a1*. As a measure of silencing, activity of the *HMR::a1* reporter gene was determined by mating efficiencies by colony formation across 9 enhancer/promoters integrated (A) at *LEU2*, (B) at *LEU2* in *sir3Δ* strains, and (C) at *HMR*. Mating efficiencies varied depending on enhancer/promoter, with *a1* and the inducible promoters *ADE2* and *GAL1* remaining the most silent, and intermediate strength promoters *ACO1* and *PGK1* correspondingly exhibiting intermediate levels of mating, and thus silencing. The *sir3Δ* strains show that *ADE2* and *GAL1* maintain repression in noninducing conditions, and cassette integration at *HMR* shows stronger silencing when compared to integration at *LEU2*.

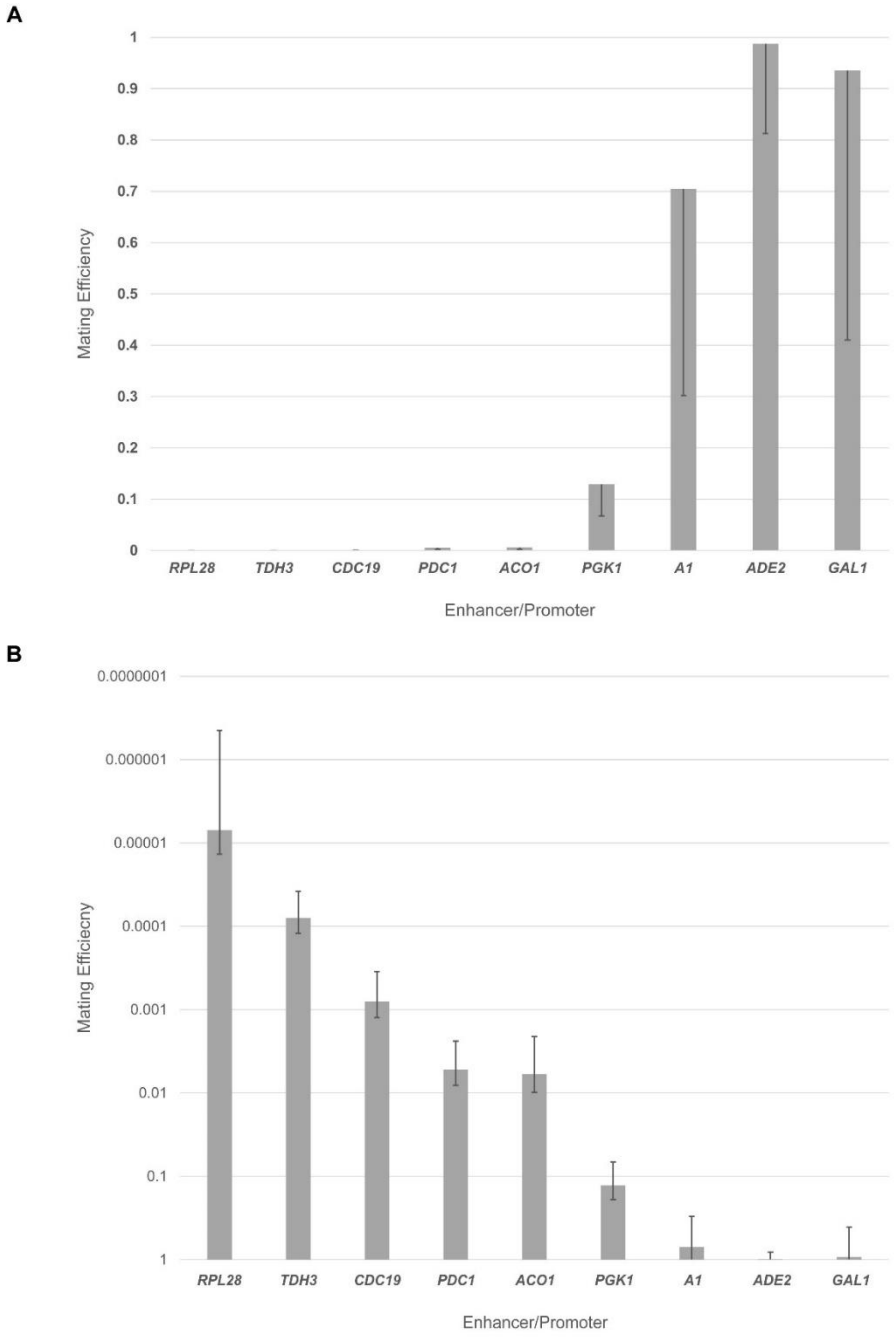


Figure 3. Quantitative mating assays capture patterns of silencing loss. Mean ±2 SD mating frequencies of *HMR::a1* strains across 9 enhancers/promoters were determined from assays performed in triplicate, represented in (A) linear scale and (B) inverse log10 scale.

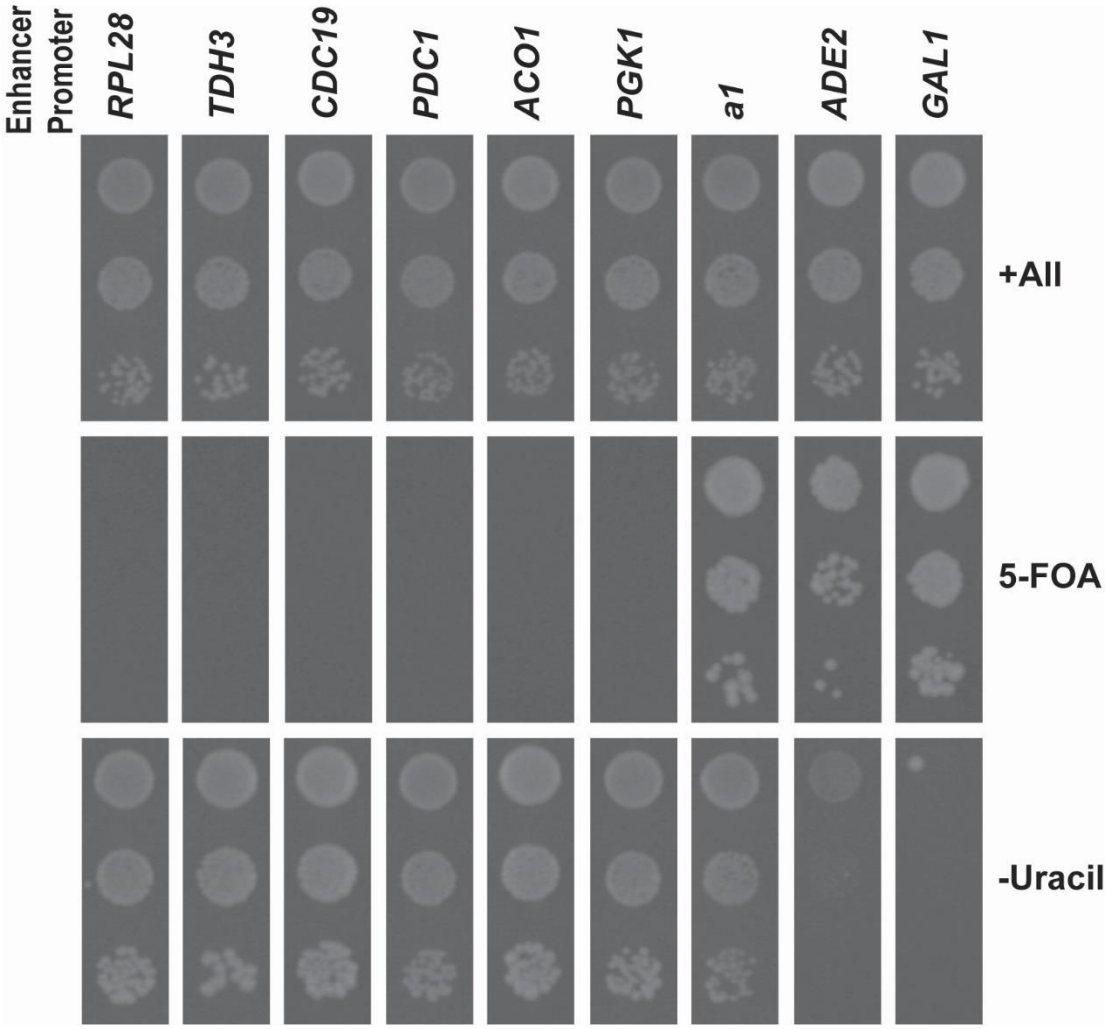


Figure 4. Silencing of *HMR::URA3* driven by constitutive enhancers/promoters is not stable or heritable. The growth pattern of colonies in negative 5-FOA selection show that expression of the *URA3* reporter by none of constitutively active enhancers/promoters can be stably silenced, with the exception of *a1*. Positive uracil auxotroph selection across the 9 enhancer/promoters suggests that all constitutively active enhancers/promoters can escape silencing of the *URA3* reporter to permit growth.

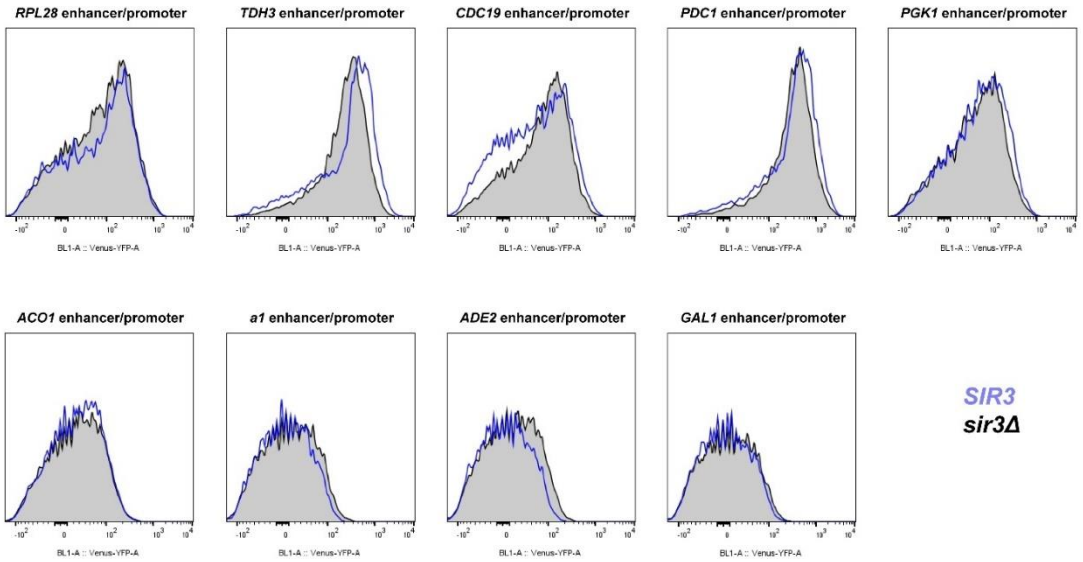


Figure 5. Enhancer/promoters affect *HMR::Venus* expression, but subtle effects are not captured. Flow cytometry was used to measure expression of *HMR::Venus* across 9 enhancers/promoters. As a control, the analysis was performed in the same set of strains carrying a *sir3* deletion. The fluorescence profiles of wild-type *SIR3* (outlined in blue) and *sir3Δ* (shaded in gray and outlined in black) cells show differences in expression levels by enhancer/promoter strength, but not between the presence versus absence of *SIR3*.

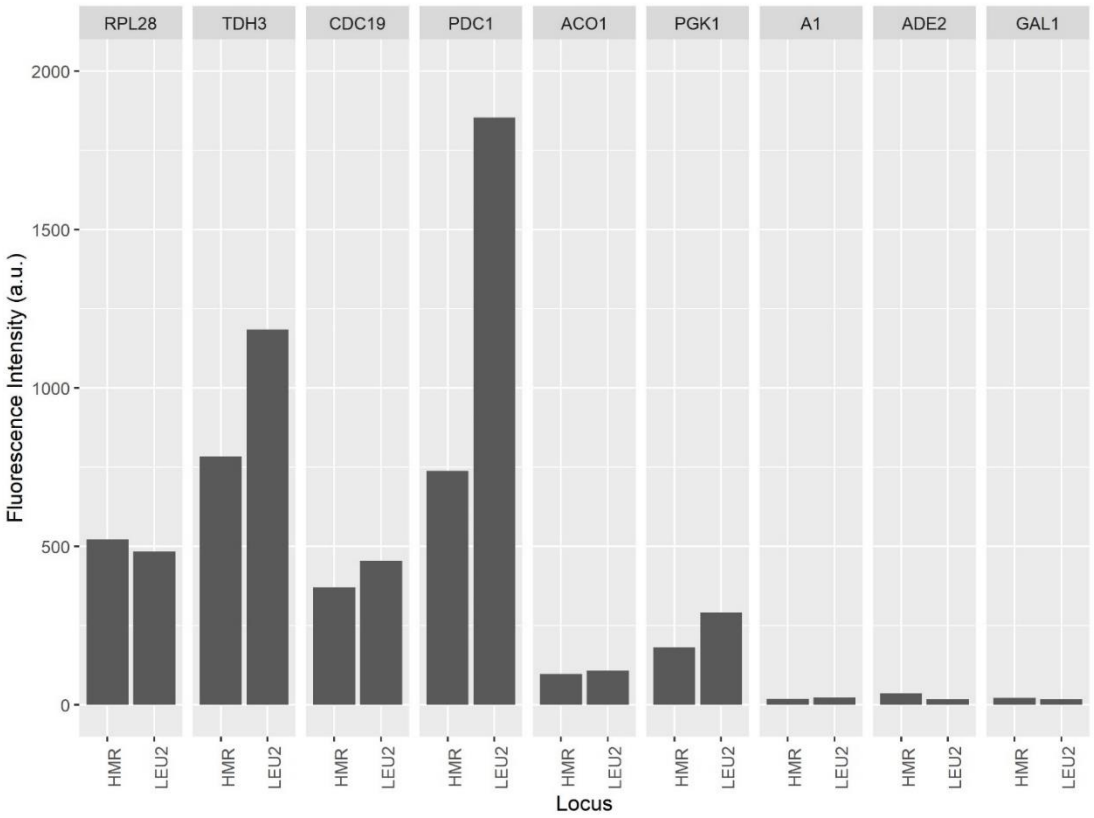


Figure 6. Position effects of *HMR::Venus* expression. Across 9 enhancers/promoters, mean *HMR::Venus* fluorescence intensities at *HMR* versus *LEU2* were determined by flow cytometry. Differences were especially pronounced for the strong enhancers/promoters *TDH3* and *PDC1*.

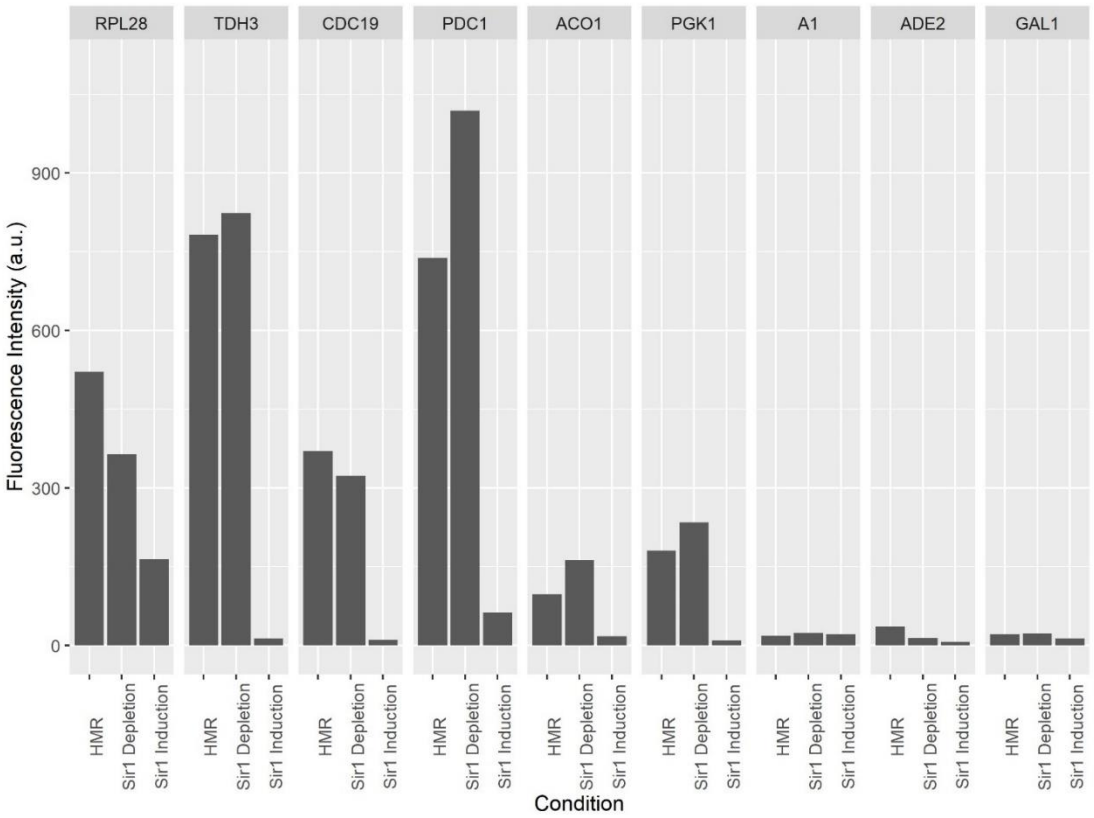


Figure 7. Sir1 abundance affects *HMR::Venus* expression. Across 9 enhancers/promoters, mean *HMR::Venus* fluorescence intensities were measured by flow cytometry in strains carrying wild-type versus synthetic silencers containing Gal4 binding sites for a Gal4-Sir1 driven by the *MET17* enhancer/promoter. A Sir1 depletion, induced by the presence of methionine, has moderate effects compared to wild-type *HMR*, but Sir1 induction through the absence of methionine appears to strongly resist enhancer/promoter mediated silencing loss.

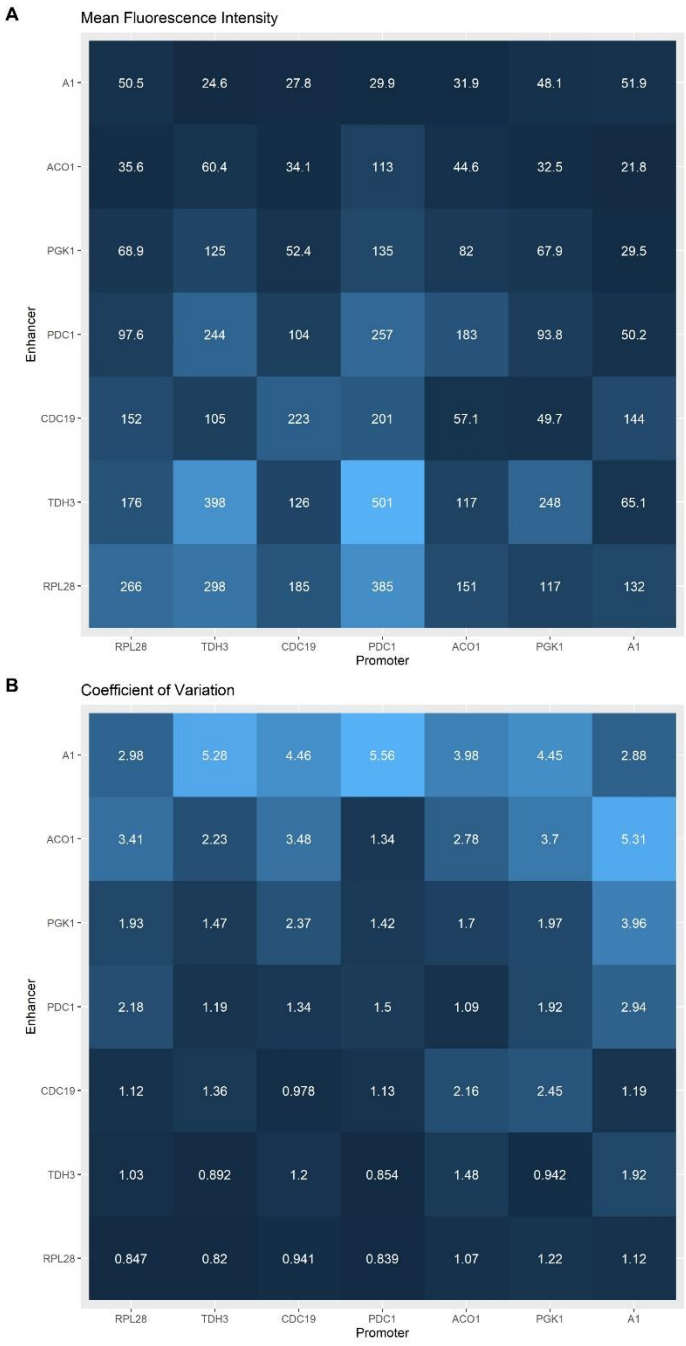


Figure 8. *HMR::Venus* activity in 7 by 7 enhancer and promoter matrix. As determined by flow cytometry, the heat maps of (A) means and (B) coefficients of variation of fluorescence intensity values suggest patterns in how individual regulatory elements can regulate the extent and variation of gene expression at a silent locus.

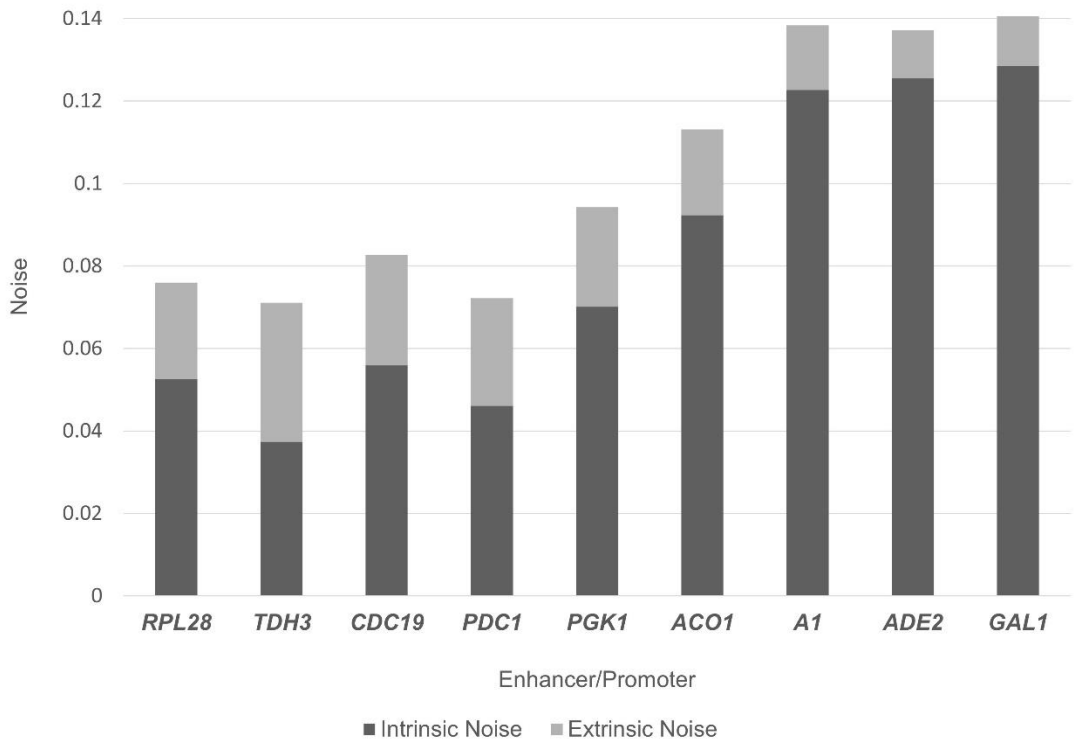


Figure 9. Intrinsic and extrinsic noise contributions as determined by dual color reporter assay. Higher total expression noise was observed with the *a1* enhancer/promoter along with the inducible *ADE2* and *GAL1* enhancers/promoters in non-activating conditions. In contrast, the strongest enhancer/promoters *TDH3* and *PDC1* appear to have the lowest relative noise. In all instances the extrinsic noise appears to form the minor but relatively constant contribution, while the intrinsic noise appears to constitute the major contribution.

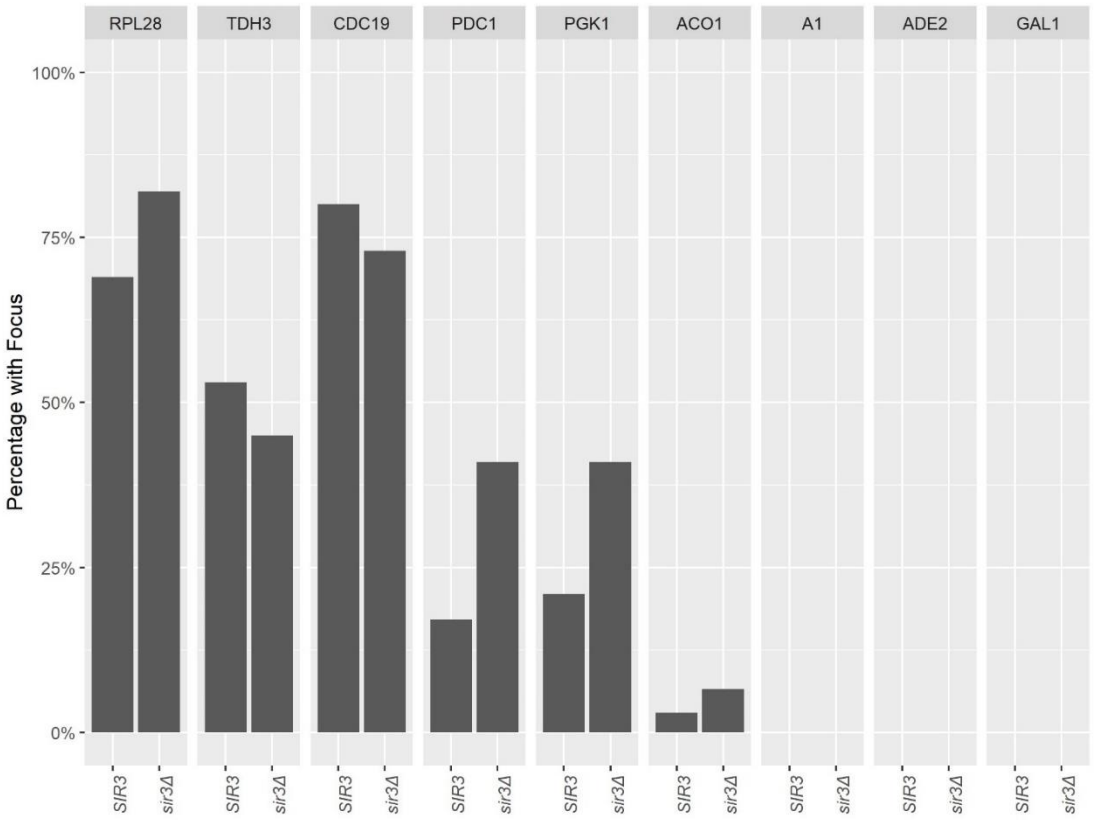


Figure 10. Transcription frequency at a silent locus is property of the enhancer/promoter as well as presence of Sir proteins. As an analog for transcription frequency, the frequency of PP7-GFP foci was measured across 9 assayed enhancer/promoters in *SIR3* and *sir3Δ* strains. Although *TDH3* and *PDC1* were the strongest enhancers/promoters for gene expression at active loci, it appears that the *RPL28* and *CDC19* promoters exhibited the highest transcription frequencies. No foci were observed for *a1*, *ADE2*, and *GAL1*. Without Sir3 expression, higher foci frequencies were observed in the intermediate strength enhancer/promoters *PDC1*, *PGK1*, and *ACO1*.

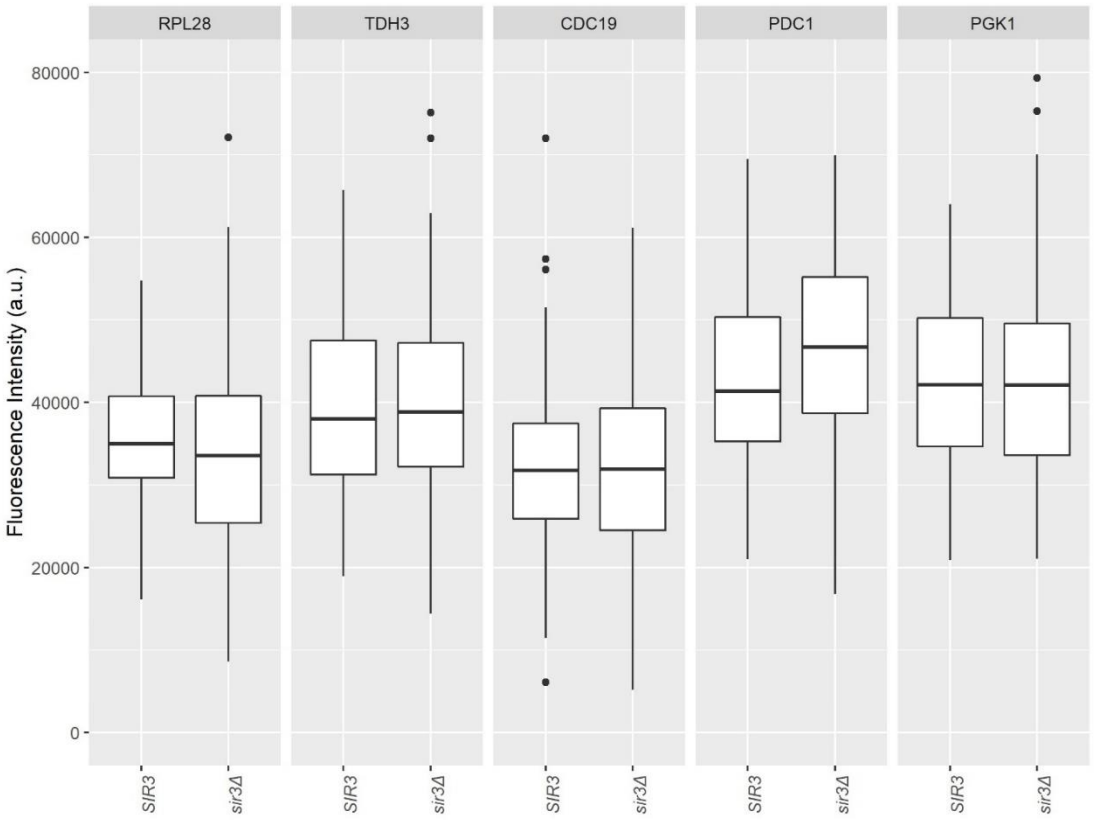


Figure 11. Distribution of transcriptional burst size across 9 assayed enhancer/promoters. Using focus intensity as an analog for burst size, there appear to be differences among tested enhancer/promoters, with *RPL28* and *CDC19* weaker than the rest. Deletion of *sir3* did not have any observable effect.

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