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

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Gene silencing in budding yeast is mediated by Sir protein binding to unacetylated nucleosomes to form a chromatin structure that inhibits transcription. Transcriptional silencing is characterized by the high-fidelity transmission of the silent state. Despite its relative stability, the constituent parts of the silent state are in constant flux, giving rise to a model that silent loci can tolerate such fluctuations without functional consequences. However, the level of tolerance is unknown, and we developed methods to measure the threshold of histone acetylation that causes the silent chromatin state to switch to the active state as well as to measure the levels of the enzymes and structural proteins necessary for silencing. We show that loss of silencing required 50 to 75% acetyl-mimic histones, though the precise levels were influenced by silencer strength and upstream activating sequence (UAS) enhancer/promoter strength. Measurements of repressor protein levels necessary for silencing showed that reducing *SIR4* gene dosage two- to threefold significantly weakened silencing, though reducing the gene copy numbers for Sir2 or Sir3 to the same extent did not significantly affect silencing suggesting that Sir4 was a limiting component in gene silencing. Calculations suggest that a mere twofold reduction in the ability of acetyltransferases to acetylate nucleosomes across a large array of nucleosomes may be sufficient to generate a transcriptionally silent domain.

silencing | Sir protein | histones | epigenetics | chromatin

Multiple loci in yeast are transcriptionally silenced, including the cryptic, mating-type loci *HML* and *HMR* on chromosome III as well as subtelomeric sites (1). 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 (2–5). The histones at silent loci lack acetylation or methylation marks (6), though they are enriched in phosphorylated histone H2A (7, 8). The Sir2/Sir4 heterodimer deacetylates K9 in histone H3 and K16 in histone H4, thereby facilitating Sir3 binding to nucleosomes (9, 10). 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 (1). 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 (11, 12). Increased dosage of Sir3p results in the increased spreading of the silent domain at telomeres and restoration of silencing in Sir1 mutants (13–15). Similarly, reducing Sir4 levels leads to inefficient establishment of gene silencing, while moderately overexpressing Sir4 leads to a more rapid de novo establishment of silencing (15–18).

Besides the silencers and the Sir proteins, the posttranslational 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 (19–24), and Sir3 binding is dependent upon the deacetylation of this residue (25–29). 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 (17, 30, 31). Occasional disruptions in silencing do occur but are rare and likely transient; one in a thousand cells stochastically lose silencing at *HML*, while ~7 in 10,000 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 (32).

Despite the high fidelity of the inheritance of the silent chromatin state, the individual components are not stably bound but in constant flux (33–36). While the exchange of the core histones in chromatin is quite slow, except at specific regulatory elements (37, 38), the covalent modifications of the histones have half-lives of only a few minutes (39, 40). While the presence of the Sir3 repressor is essential for silencing (41, 42), 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 (33–36, 43–45). Thus, the overall picture is of a phenotypically stable silenced chromatin state being mediated by constituents that are in constant flux.

Significance

Gene silencing, once established, is stably maintained for several generations. Despite the high fidelity of the inheritance of the silent state, individual components of silenced chromatin are in constant flux. Models suggest that silent loci can tolerate fluctuations in Sir proteins and histone acetylation levels, but the level of tolerance is unknown. To understand the quantitative relationships between H4K16 acetylation, Sir proteins, and silencing, we developed assays to quantitatively alter a H4K16 acetylation mimic allele and Sir protein levels and measure the effects of these changes on silencing. Our data suggest that a two- to threefold change in levels of histone marks and specific Sir proteins affects the stability of the silent state of a large chromatin domain.

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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 (46), and nucleosomal histones are evicted upstream of the replicating fork (47) and redeposited downstream (48). During DNA replication, nucleosome positions and DNaseI hypersensitive sites (which are sites for binding of transcription factors) are disrupted (49–51), and following replication, the maturation of chromatin leads to the resetting of the original chromatin state (51–53). 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 (54–56). 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 (57–60). 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 (53).

The chromatin state that is disrupted during replication creates a temporal window in the G2 phase of the cell cycle in which silenced chromatin is more accessible to enzymatic probes (44, 61, 62) and thus more prone to disruption. Counteracting this disruption are the silencer elements. Elimination of the silencers results in the inability of the silent state to reform following its disruption in S-phase (43). Furthermore, efficient inheritability of silencing requires the silencer-bound proteins Rap1 and Sir1 (17, 30).

Besides the silencers, models have invoked a role for histone modification marks in the heritability of the silent state. In silico models (63–65) 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 (66), 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 threefold change in the levels of histone marks and specific Sir proteins can affect the stability of the silent state of a large chromatin domain.

Materials and Methods

Protein Blots. Protein lysates were prepared and resolved on 10 or 15% SDS-polyacrylamide gel as described previously (67), except that glass beads were used to break open the cells. Monoclonal antibodies (HA.11 and 9E10) against the hemagglutinin (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 (68). Complementary DNA (cDNA) was prepared using the RT-qPCR kit (Luna RT-qPCR; New England Biolabs).

Fluorescence-Activated Cell Sorting Analysis. Cells were washed in 50 mM Tris·HCl, pH 7.5 and fixed in 70% ethanol for 1 h at room temperature. Cells were then washed in 50 mM Tris·HCl, pH 7.5 and treated with 1 mg/mL RNaseA at 37 °C for 1 h, followed by ProteinaseK treatment (60 μg/mL) at 55 °C for 1 h. 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 University of California Santa Cruz (UCSC) cytometry facility.

Fluorescence Microscopy. Cells were grown exponentially in yeast peptone (YP) medium with 2% raffinose at 30 °C to an optical density (OD_{600}) of ∼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 h, 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 h. 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. Then, 3 μL of the suspension was applied to a 1.5% agarose yeast minimal dextrose (YMD) pad on top of a microscope slide and cover slipped. Images were acquired on a DeltaVision Personal DV system (Applied Precision), using a 40× 1.35 numerical aperture (NA) oil-immersion objective (Olympus), with a CoolSnap charge-coupled camera (Roper Scientific). Then, 4-μm image stacks were collected, with each Z-image being 0.2 μm apart and 2 μm above and below the plane of focus. Image stacks were taken for each time point, and more 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 ∼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 yeast extract-peptone-dextrose (YPD) media to an OD_{600nm} of 2.0, then fixed with 1% formaldehyde for 10 min, and then the cross-linker was neutralized, ensuring that around 10% of proteins were cross-linked to DNA. Cells were collected, resuspended in buffer, and sonicated using the Bioruptor (Diagenode) followed by a cup–horn (Branson) sonicator to an average size of 300 base pair (bp).

Immunoprecipitation (IP) reactions were performed with commercial antibodies to histone H3 (Millipore), Ac-K16 H4 (Millipore), Ac-K56-H3 (Millipore), or with polyclonal anti-Sir3 antibodies (7, 15, 69–71), and immune complexes were collected with protein G/A beads (Calbiochem, EMD Biosciences). IP and input DNA were purified using Chelex 100 (Bio-Rad) (72), and the amount of DNA was quantified using the Picogreeen dsDNA quantitation kit (Invitrogen) and the PerkinElmer Viktor^3 Fluorescence Reader prior to qPCR.

Equal amounts of IP DNA and input DNA were used for the qPCR reactions. qPCR 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 IP DNA and input DNA for each qPCR amplified region were calculated as described (73), using the formula IP/Input = ($2^{InputCt \cdot IPCt}$). Each experiment involved at least two independent cross-linked samples, with each sample IP twice with the same antibody.

Strains and oligonucleotides used in this study are listed in Supplementary Table 1 and Table 2, respectively.

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 (Fig. 1*A*). These

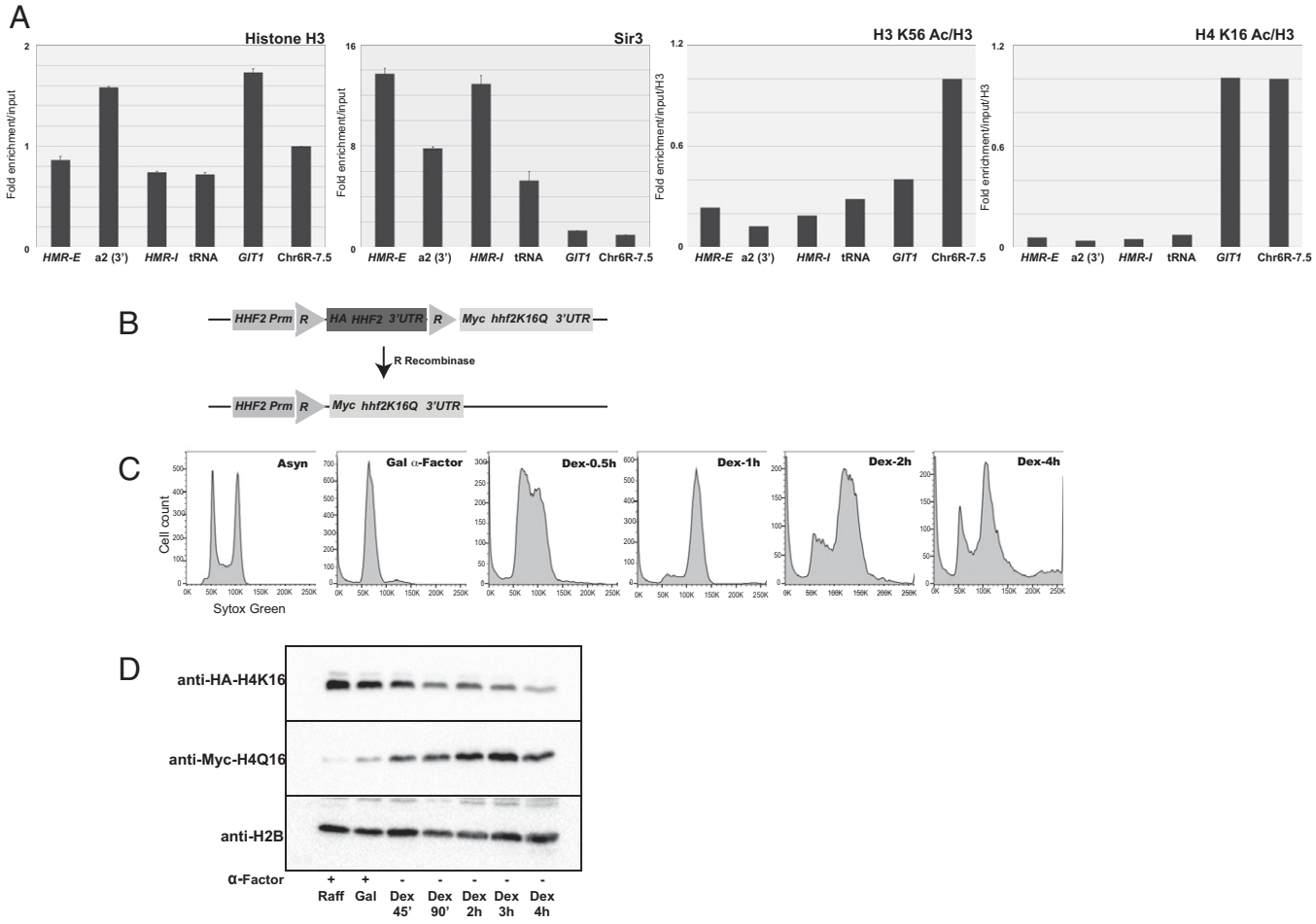

Fig. 1. Characterization of *HMR* and the cut and flip system. (*A*) ChIP qPCR of various proteins in G1-arrested cells. Histone H3, Sir3, H4K16 acetylation, and H3K56 acetylation levels were measured across the *HMR* domain. Data are presented as the mean enrichment of IP/input (as described in the *Materials and Methods*) for at least four IP DNAs from two independent cross-links. Error bars are SE 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 through 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. AU is arbitrary units; WT is wild-type cells.

data serve as a baseline control of the levels found in unperturbed silent cells. Using chromatin immunoprecipitation (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, had similar amplification efficiencies, and did not generate any primer dimers. To quantify the distribution, IP DNA and input DNA were quantified, and equal amounts of input and IP DNA (~100 pg) were then used for qPCR.

The silencers and the transfer RNA (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 (Fig. 1*A*). The silencers, as well as the tRNA gene (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 IP DNA (~300 bp) (38, 69, 70, 74, 75).

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 (76, 77). 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 (Fig. 1*A*) by ChIP. The IP was performed on the same cross-linked 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 with the control locus, H3K56 acetylation levels showed significant reduction across the entire silent domain, and there was an approximately threefold decrease in acetylation of H3K56 at *HMR* compared with chromosome 6R. There was an even more dramatic reduction in H4K16 acetylation at *HMR* compared with 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 (24, 78–80). 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 (38). 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 (81), and this is known to trigger cell cycle checkpoints (82) and lead to dominant effects (83).

We therefore developed a system to overcome these issues. In *Saccharomyces 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 for which the wild-type histone *HHF2* locus was modified to accommodate two copies of the H4 coding sequence (Fig. 1*B*). 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 (84).

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 (deleting) 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 overproduced, 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 h with alpha factor. We monitored arrest by microscopy as well as by flow cytometry (Fig. 1*C*). 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 3 to 4 h of incubation in galactose were sufficient for maximal R-recombinase–mediated switching of the *HHF2* alleles (*SI Appendix*, Fig. S1). 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 min after release (Fig. 1*C*). The data also showed that most cells progressed through the second S-phase between 2 and 3 h after their release, albeit with reduced cell cycle synchrony. The doubling time of this strain in YPD was also measured and was ∼105 min.

We next monitored the switch of the wild-type to mutant *HHF2* alleles by protein blots using antibodies against the HA and Myc epitopes (Fig. 1*D*). 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 whether the switched histone H4K16Q mutant proteins were being incorporated into chromatin. Cells arrested in galactose, as well as cells collected 2 and 4 h after release from the G1 phase of the cell cycle, were cross-linked with formaldehyde, and the cross-linked chromatin was IP using anti-HA and anti-Myc antibodies (Fig. 2 *A* and *B*). Each experiment was performed with a minimum of two independently cross-linked samples, and each sample was IP 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 (Fig. 2*A*), and the levels of mutant histone H4-Myc increased upon release (Fig. 2*B*).

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 (Fig. 2*C*). 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 2 h, and there was very little Sir3 bound to these loci after 4 h, 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 messenger RNA (mRNA) levels of a GFP reporter present at *HML* using RT-qPCR (Fig. 2*D*). We isolated mRNA from G1-arrested cells as well as from cells at 2 and 4 h postrelease and measured levels of GFP mRNA along with actin mRNA. In G1-arrested cells, there was very little GFP mRNA compared with actin mRNA, consistent with the locus being silenced. However, upon release from the arrest, we observed a large increase in GFP expression at the 2-h time point, which further increased at the 4-h time point.

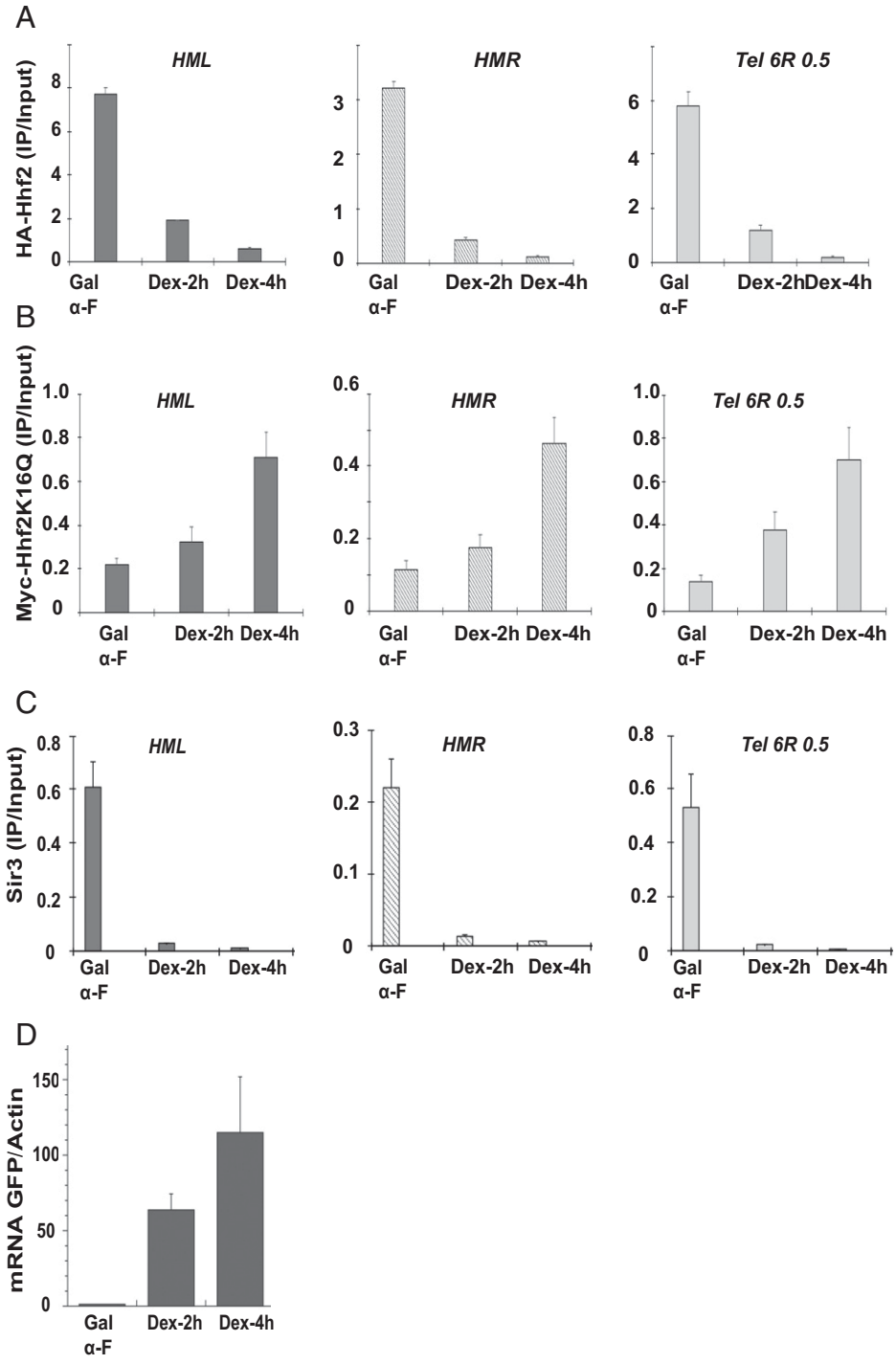

Fig. 2. Molecular analysis of silenced loci following cut and flip. (*A*) ChIP qPCR of unswitched histone H4 allele at silenced loci. The presence of wild-type HA-H4 was monitored by ChIP in unswitched (galactose-containing medium with alpha factor) and 2 and 4 h after switching of the histone H4 allele. The *y*-axis represents the ratio of IP/input DNA for each sample as described in the *Materials and Methods*. Error bars are SE 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 2 and 4 h 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 SE 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 2 and 4 h after switching the histone H4 allele and alpha factor release. Data are presented as the mean enrichment of IP/input. Error bars are SE 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 2-h 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*.

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 ∼20 min) with a high turnover rate (half-life of ∼35 min, due to the presence of a *CLN2* PEST sequence) that localized to the nucleus (because of the presence of a nuclear localization signal) (85–87). 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 (Fig. 3*A*). In cells expressing

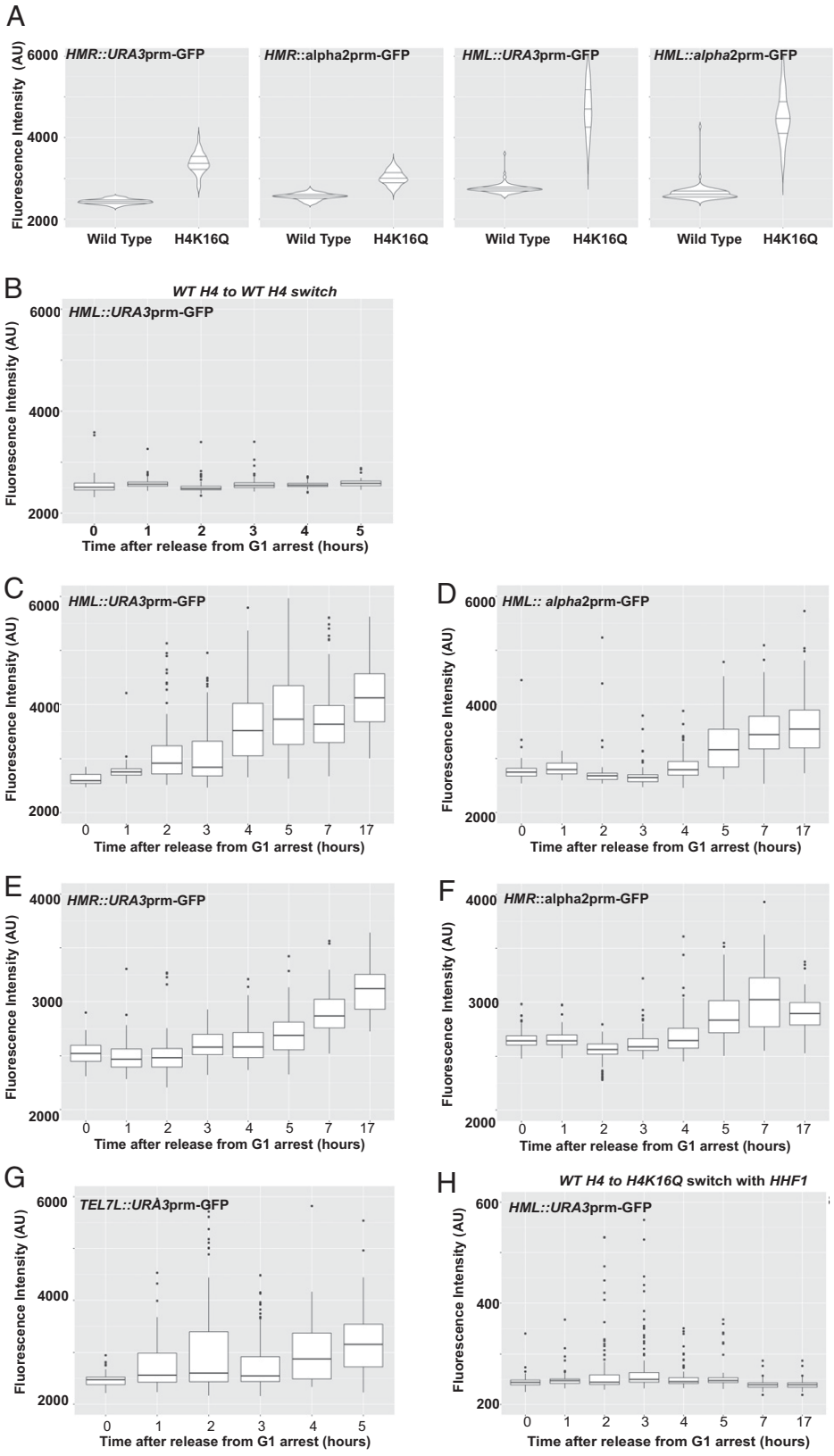

Fig. 3. Fluorescent measurements of gene silencing. (*A*) Violin plots of GFP expression from silenced loci in strains expressing WT and mutant histone H4 K16Q alleles. Cells from a single colony containing the unflipped wild-type cassette or a flipped cassette (confirmed by PCR) were grown in rich medium and 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 more than 100 cells. (*B*) Fluorescence measurements of GFP at *HML* following 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*) Boxplots of GFP expression at *HML::URA3p-GFP* as a function of time after switching the histone H4 cassettes. 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 released from the arrest. GFP fluorescence was measured as cells progressed through the cell cycle. (*D*) Boxplots of GFP expression at *HML::α2p-GFP* as a function of time after switching the histone H4 cassettes. GFP fluorescence was measured as a function of time in strains with modified *HML* containing GFP under the control of the *α2* UAS enhancer/promoter. Cells were arrested in G1, the histone H4 cassette was switched from wild-type H4 to mutant H4K16Q, and cells were released from the arrest. GFP fluorescence was measured as cells progressed through the cell cycle. (*E*) Boxplots of GFP expression at *HMR::URA3p-GFP* as a function of time after switching the histone H4 cassettes. 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 released from the arrest. GFP fluorescence was measured as cells progressed through the cell cycle. (*F*) Boxplots of GFP expression at *HMR::α2p-GFP* as a function of time after switching the histone H4 cassettes. GFP fluorescence was measured as a function of time in strains with modified *HMR* containing GFP under the control of the *α2* UAS enhancer/promoter. Cells were arrested in G1, the histone H4 cassette was switched from wild-type H4 to mutant H4K16Q, and cells were released from the arrest. GFP fluorescence was measured as cells progressed through the cell cycle. (*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.

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 (23, 24, 26). 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* with *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 (88).

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 in which 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 (Fig. 3*B*). 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 in which 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 2 h after release from G1 arrest and reached maximal levels around 5 h. These data suggest that silencing was beginning to be lost during or soon after the second S-phase (Fig. 3*C*).

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

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 ∼3 h after the release, while for the alpha2 UAS enhancer/promoter, GFP signal was first observed 4 h after the release (Fig. 3 *E* and *F*).

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 1 h after release, suggesting that ∼50% replacement of wild-type H4 with H4K16Q was sufficient for weakening the silent state at this locus (Fig. 3*G*).

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 because of the limitations in the sensitivity of our fluorescent measurement setup. 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 ∼1,000 bases long and, with a yeast transcription elongation rate of 25 bases/s (89), would be transcribed within ∼40 s. The yeast translation rate is 2.63 amino acids/s (90), and so GFP would be translated in ∼2 min. The maturation time of the GFP protein used in this study is ∼20 min (85–87), and thus, detection of the GFP fluorescent signal would be delayed ∼23 min from the actual time of loss of silencing. Since we used 1-h 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 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* (Fig. 3*H*). 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 (13, 91–93), 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 in which 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 (16). 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 *HMR*α is silent. However, unlike *MAT*a, *mataΔ* is recessive to *MAT*α. Therefore, any loss of silencing at *HMR*α 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 *MAT*α information under the control of a synthetic silencer at *HMR* (*HMR*α) (94, 95). It should be noted that the diploid and triploid cells only contained a single *HMR*α locus. Thus, haploid cells were *HMR*α,

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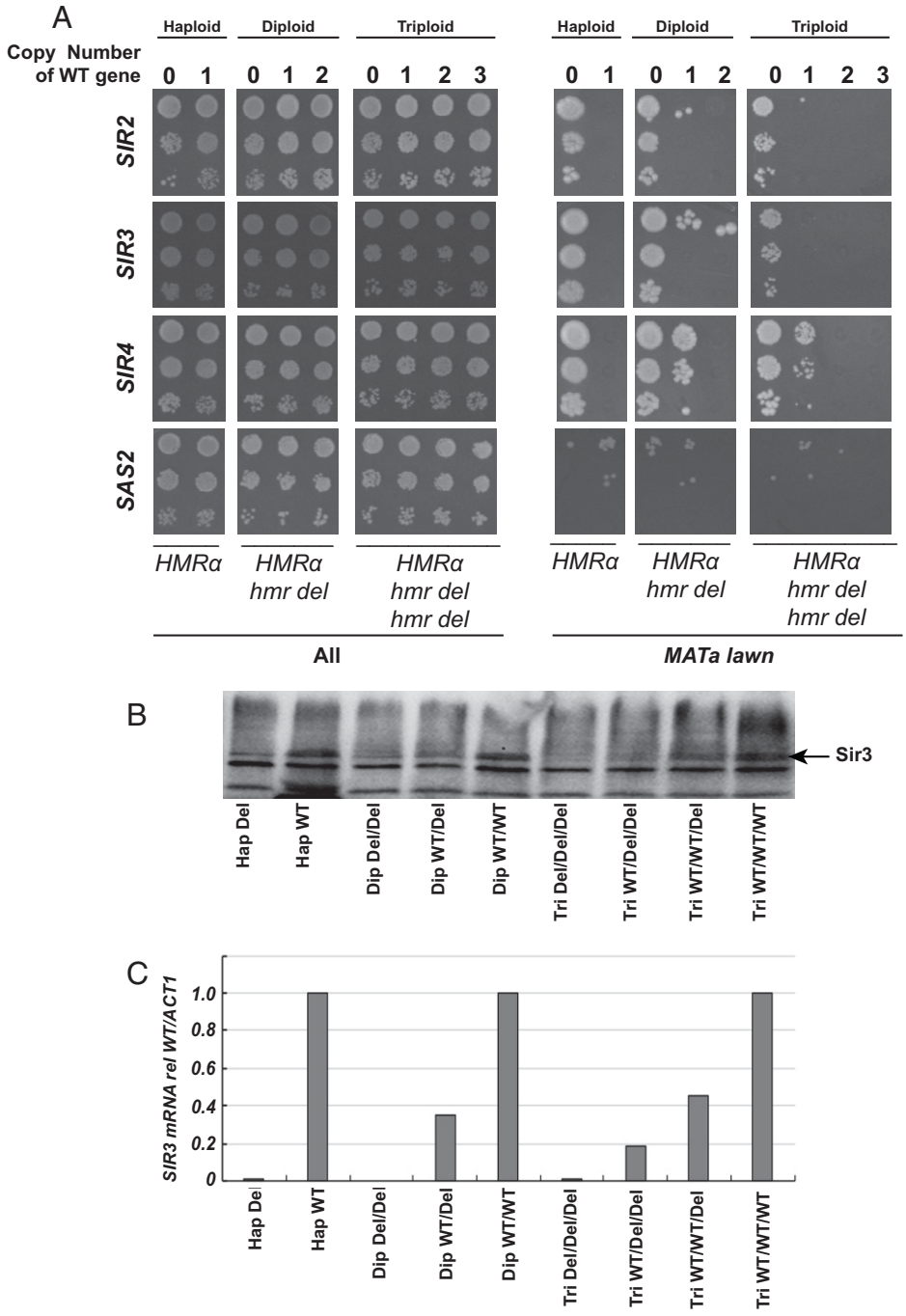

Fig. 4. Effects of Sir gene dosage on silencing. (*A*) Derepression of *HMRss*α was monitored in haploid, diploid, and triploid cells containing variable gene copy numbers for the *SIR2*, *SIR3*, *SIR4*, or *SAS2* genes. Loss of silencing resulted in a phenotypic switch in mating of the strain from a to α. Derepression of *HMRss*α 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 memb ranes, 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.

diploid cells were *HMR*α/*hmr*Δ::*HIS3*, and triploids were *HMR*α/*hmr*Δ::*HIS3*/*hmr*Δ::*HIS3*. This ensured that the measurements of silencing were not influenced by varying numbers of the *HMR*α locus.

Silencing of these strains was monitored by growing these cells on minimal media plates containing mating-type tester lawns (Fig. 4). The growth of cells on *MATa* tester lawn plates is an indication of loss of silencing from *HMR*α. Analysis of strains with varying copies of the *SIR2* gene indicated that reduction in gene copies to ∼33, ∼50, and ∼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, and ∼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 the gene copy number of *SIR3* (Fig. 4*B*). 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 (Fig. 4*C*). 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 the *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 (17, 30, 43). 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 (88, 96).

In addition to the silencer, efficient inheritance of the silent state depends upon the nucleosomes remaining unacetylated. There are ~20 and 12 nucleosomes present at *HML* and *HMR*, respectively (97, 98). 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 in which 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 (39, 99, 100). 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 1,000 cells with a similar value at *HMR* (32). 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 $[1/(1.6)^{15} = 1/1{,}000]$. 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, and 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 (13, 61, 96, 101, 102). 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 (67, 103) and Sir4 is necessary for the recruitment of Sir2 to silent loci (9, 104), 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 spectrometry measurements of Sir proteins indicate that Sir3 and Sir4 levels in the cell are equivalent (105), though protein immunoblots of wild-type asynchronously growing haploid cells suggest that Sir4 levels are reduced compared to Sir3 (*SI Appendix*, Fig. S2), and in the 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 the silent to the active state. However, while parental histones are evicted from the DNA during replication, they are redeposited in close proximity to their original site, thereby reducing the probability of histones with active modifications being transferred to silenced chromatin (106, 107). Moreover, active chromatin is replicated early while silenced loci are replicated late (108, 109), 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 (57, 110). 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 (111, 112), thus reducing the probability of nucleosome acetylation and favoring the deacetylated state at silent loci. Lastly, the three-dimensional clustering of silent loci (7, 35) 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 (113, 114). 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 (30, 31). 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 (32). 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 nonmating. Transcription is noisy and occurs in bursts. Partial silencing implies changes in either transcription burst frequency or burst size (115–117). 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 in which 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.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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