Impact of Homologous Recombination on Silent Chromatin in *Saccharomyces cerevisiae*

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ABSTRACT Specialized chromatin domains repress transcription of genes within them and present a barrier to many DNA–protein interactions. Silent chromatin in the budding yeast *Saccharomyces cerevisiae*, akin to heterochromatin of metazoans and plants, inhibits transcription of PolII- and PolIII-transcribed genes, yet somehow grants access to proteins necessary for DNA transactions like replication and homologous recombination. In this study, we adapted a novel assay to detect even transient changes in the dynamics of transcriptional silencing at *HML* after it served as a template for homologous recombination. Homologous recombination specifically targeted to *HML* via double-strand-break formation at a homologous locus often led to transient loss of transcriptional silencing at *HML*. Interestingly, many cells could template homology-directed repair at *HML* without an obligate loss of silencing, even in recombination events with extensive gene conversion tracts. In a population of cells that experienced silencing loss following recombination, transcription persisted for 2–3 hr after all double-strand breaks were repaired. mRNA levels from cells that experienced recombination-induced silencing loss did not approach the amount of mRNA seen in cells lacking transcriptional silencing. Thus, silencing loss at *HML* after homologous recombination was short-lived and limited.

KEYWORDS Sir2; HO; mating-type switching; double-strand break repair; Swi2; Dnl4

IN the budding yeast *Saccharomyces cerevisiae*, the genes at the cryptic mating type loci *HML* and *HMR* are silenced, allowing haploid cells to maintain their identities as either the **a** or **α** mating type. Yeast silent chromatin shares characteristics with heterochromatin of metazoans and plants, including hypoacetylation of histones (Braunstein *et al.* 1993; Suka *et al.* 2001), epigenetically inherited repression (Pillus and Rine 1989; Xu *et al.* 2006), and compact, higher-order chromatin structure (Bi and Broach 1997; Cheng *et al.* 1998; Weiss and Simpson 1998; Ravindra *et al.* 1999). The Silent Information Regulator (SIR) proteins Sir1–Sir4 establish silencing via recruitment to regulatory sites called silencers that flank both *HML* and *HMR*. Sir2, the founding member of the highly conserved sirtuin family of deacetylases, removes acetyl marks on histone H4 at position K16 and on histone H3 at positions K9

and K14 across the silenced domain (Imai *et al.* 2000; Landry *et al.* 2000; Smith *et al.* 2000). H4K16 deacetylation creates high-affinity binding sites for the Sir complex, comprising Sir2, Sir3, and Sir4, resulting in a chromatin domain that represses transcription of a variety of RNA PolII- and PolIII-transcribed genes [reviewed in Gartenberg and Smith (2016)].

Silent chromatin offers limited accessibility to many DNA-binding proteins (Gottschling 1992; Singh and Klar 1992; Loo and Rine 1994), yet must allow certain transactions like replication and homologous recombination to occur. In fact, homologous recombination within silent chromatin is a key aspect of yeast biology, as the process of mating-type switching depends upon it (Strathern *et al.* 1982; Kostriken *et al.* 1983). The mating type of haploid yeast is determined by the **a** or **α** allele present at the *MAT* locus. Unexpressed copies of the *MATa* and *MATα* genes reside within silent chromatin at *HML* and *HMR*. Mating-type switching initiates when the HO endonuclease creates a double-strand break at *MAT*, which is then repaired by homologous recombination templated from either *HML* or *HMR*. SIR proteins prevent access of HO to its recognition sequences at *HML* and *HMR*, ensuring that only *MAT* is available for cleavage. Recombination between *MAT*

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doi: <https://doi.org/10.1534/genetics.118.300704>

Manuscript received September 22, 2017; accepted for publication January 8, 2018; published Early Online January 16, 2018.

Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.118.300704/-/DC1.

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and one of the heterochromatic donor loci results in gene conversion of the sequences at *MAT* from either *HML* or *HMR*.

Mating-type switching in *S. cerevisiae* has provided a foundation for elucidating much of what is known about the repair of double-strand breaks by homologous recombination. Repair of the double-strand break at *MAT* begins with resection in the 5' to 3' direction on each side of the break (White and Haber 1990; Sun *et al.* 1991). The *Rad51* protein binds the exposed single strands and coordinates with other DNA-repair proteins, including *Rad54* and *Rad52*, to facilitate recognition of homologous sequences at either *HML* or *HMR* and carry out strand invasion of the donor locus. Repair of the double-strand break at *MAT* occurs through a type of recombination called synthesis-dependent strand annealing (SDSA), resulting in unidirectional transfer of genetic information from the donor locus, either *HML* or *HMR*, to *MAT* (Haber *et al.* 1980; Klar and Strathern 1984; Ira *et al.* 2006).

Considering the ability of silent chromatin to block DNA-protein interactions and the broad range of proteins needed to repair the cleaved *MAT* locus from the heterochromatic donors, one might expect an obligatory loss of silencing during mating-type switching to allow the recombination machinery access to the silenced template used for repair. To date, there has been no evidence that mating-type switching causes any loss of transcriptional silencing at either *HML* or *HMR*. However, traditional assays of silencing loss that rely on mating phenotypes have limited ability to reveal whether a donor locus becomes expressed as a result of a mating-type switch. In this study, we used a recently developed assay capable of detecting even transient disruptions of silencing (Dodson and Rine 2015) to investigate whether changes to silent chromatin dynamics at the *HML* locus result from its participation in homologous recombination.

Materials and Methods

All strains in this study (Table 1) were derived from strain JRY9628 (Dodson and Rine 2015), which is derived from W303 (R. Rothstein, Columbia University). Deletion of *HMR* was accomplished via one-step integration (Gueldener *et al.* 2002) of the *Kluyveromyces lactis* *LEU2* gene using the *hmrΔ::K.lac.LEU2* forward/reverse primers and confirmed with sequencing. *pGAL10:HO* (Herskowitz and Jensen 1991) was integrated at the *LEU2* locus with the *leu2Δ::pGAL10:HO* forward/reverse primers.

To create the *SNP-INC* at *HML::cre*, site-directed mutagenesis was performed on the *HML::cre* sequence from JRY9628 using the *HML-INC* forward/reverse primers. To construct the pseudo-*MAT* locus, two rounds of site-directed mutagenesis were performed on the *HML::cre* sequence from JRY9628 using the *SNP-R* forward/reverse primers and the *SNP-L* forward/reverse primers. The pseudo-*MAT* locus was inserted between the *YHL045W* and *YHL044W* open reading frames on the left arm of ChrVIII, replacing the sequences between base-pairs 13,192 and 13,237, using the *ChrVIII::pseudo-MAT* forward/reverse primers. For strain JRY10818, the *HML::cre* sequence with

SNP-INC was inserted at the same location on ChrVIII with the *ChrVIII::pseudo-MAT* forward/reverse primers.

To remove the endogenous *HO* recognition sequence from *HML::cre* in strain JRY10819, Gibson assembly (Gibson *et al.* 2009) was performed with two overlapping fragments of *HML::cre* from JRY9628 using primers $\Delta HO-5'$ and $\Delta HO-3'$ in a way that deleted base pairs 356–475 of the $\alpha 1$ sequence, leaving $\alpha 1$ with base pairs 1–355;476–528 intact.

To insert the *HO* recognition sequence into the *K. lactis* *URA3* sequence, a three-step Gibson assembly was performed that inserted base pairs 377–465 of the $\alpha 1$ sequence between base pairs 200 and 201 of the *K. lactis* *URA3* sequence using primers *K.lac.URA3-HO-5'* and *K.lac.URA3-HO-3'*. The *K.lacURA3-HO* construct was then inserted at the same position on the left arm of ChrVIII as *pseudo-MAT* in JRY10817 with the *ChrVIII::K.lac.URA3-HO* forward/reverse primers.

To insert the *K. lactis* *URA3* sequence containing the *HO* recognition sequence with *SNP-INC* and *SNP-R* onto ChrIX, a three-step Gibson assembly was performed that inserted base pairs 377–465 of the $\alpha 1$ sequence containing *SNP-INC* and *SNP-R* between base pairs 200 and 201 of the *K. lactis* *URA3* sequence using primers *K.lac.URA3-HO-5'* and *K.lac.URA3-HO-3'*. This construct was then inserted on the right arm of ChrIX in between base pairs 428,440 and 428,441 with the *ChrIX::K.lac.URA3-HO-INC-SNPR* forward/reverse primers.

To insert the *swi2Δ10R* allele into the *SWI2* locus, a sequence to create a guide RNA to target cleavage of *SWI2* by Cas9 was first cloned into the BsmBI sites of pYTK050 (Lee *et al.* 2015) using the *SWI2*-sgRNA forward/reverse primers. The full guide RNA sequence was excised from this plasmid and inserted into the BsaI sites of pWCD2257 [described in Figure S5B of Lee *et al.* (2015)], which contains the Cas9 sequence on a yeast *CEN/ARS* plasmid, thus creating pJR3417. pJR3417 was then transformed into JRY10817 along with a portion of *swi2Δ10R* from CP1413 (Manning and Peterson 2014) that was PCR-amplified using the *swi2Δ10R* forward/reverse primers to create strain JRY10820. Successful replacement of *SWI2* by *swi2Δ10R* was confirmed with sequencing.

All genomic positions described are from the S288C reference genome version R64-2-1, which can be accessed at http://downloads.yeastgenome.org/sequence/S288C_reference/genome_releases/.

Galactose induction of double-strand breaks

Strains were grown at 30° in liquid Complete Supplement Mixture (CSM)-Trp (Sunrise Science Products, San Diego, CA) with raffinose (2% w/v) as a carbon source and containing G418 to select for red cells, then diluted into liquid CSM-Trp medium with raffinose and allowed to grow to an A600 of 0.2–0.4. Cultures were then split and received either galactose or raffinose to a final concentration of 2%. For experiments with a glucose recovery, cultures were centrifuged at 4000 rpm for 15 min, then resuspended in CSM-Trp medium containing glucose (2% w/v) and allowed to grow. For each

Table 1 Yeast strains

Name	Genotype
JRY10817	<i>matΔ::NatMX, lys2, ChrVIII:13192-13237::pseudo-MAT, leu2Δ::pGAL10:HO, HMLα1-INC:α2Δ::cre, hmrΔ::K.I.LEU2, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:KanMX:loxP:yEGFP:tADH1, can1-100, his3-11</i>
JRY10818	<i>matΔ::NatMX, lys2, ChrVIII:13192-13237::pseudo-MAT-INC, leu2Δ::pGAL10:HO, HMLα1-INC:α2Δ::cre, hmrΔ::K.I.LEU2, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:KanMX:loxP:yEGFP:tADH1, can1-100, his3-11</i>
JRY10819	<i>matΔ::NatMX, lys2, ChrVIII:13192-13237::K.I.URA3-HO, ChrIX:428440::K.I.URA3-HO-INC, leu2Δ::pGAL10:HO, HMLα1ΔHO:α2Δ::cre, hmrΔ::K.I.LEU2, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:KanMX:loxP:yEGFP:tADH1, can1-100, his3-11</i>
JRY10820	<i>matΔ::NatMX, lys2, swi2Δ::swi2Δ10R, ChrVIII:13192-13237::pseudo-MAT, leu2Δ::pGAL10:HO, HMLα1-INC:α2Δ::cre, hmrΔ::K.I.LEU2, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:KanMX:loxP:yEGFP:tADH1, can1-100, his3-11</i>
JRY10821	<i>matΔ::NatMX, dnl4Δ::LYS2, ChrVIII:13192-13237::pseudo-MAT, leu2Δ::pGAL10:HO, HMLα1-INC:α2Δ::cre, hmrΔ::K.I.LEU2, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:KanMX:loxP:yEGFP:tADH1, can1-100, his3-11</i>
JRY10822	<i>matΔ::NatMX, lys2, sir3Δ::K.I.URA3, HMLα2Δ::CRE, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:KanMX:loxP:yEGFP:tADH1, can1-100, his3-11, leu2-3,112</i>
JRY10823	<i>matΔ::NatMX, lys2, ura3Δ::pGPD:loxP:yEmRFP;tCYC1:KanMX:loxP:yEGFP:tADH1, can1-100, his3-11, leu2-3,112 ; pJR2538</i>

time point, a sample of culture was diluted in CSM-Trp with glucose and plated onto CSM-Trp glucose plates.

Colony imaging and silencing-loss analysis

Colonies were grown for 5–7 days on CSM-Trp glucose plates and then imaged with a Zeiss Axio Zoom.V16 microscope equipped with ZEN software (Zeiss, Jena, Germany), a Zeiss AxioCam MRm camera, and a PlanApo Z 0.5× objective. Sectoring patterns were scored manually. Colonies that were at least one-quarter green were counted as “early silencing-loss” events. Red colonies with wild-type levels of sectoring were scored as colonies that “maintained silencing.” Colonies that were petite or with morphologies suggestive of reciprocal crossover leading to a dicentric chromosome were omitted from analysis.

DNA blots

DNA hybridization blots were performed as previously described, with only minor changes (Southern 2006). Probes were radiolabeled by random priming with ³²P-αdCTP using either the Amersham Rediprime II Random Primer Labeling System (GE Healthcare) or the Amersham Megaprime DNA Labeling System (GE Healthcare). Membranes were exposed 16–72 hr with a Storage Phosphor Screen (GE Healthcare) and imaged with a Typhoon Trio (GE Healthcare). To determine the ratio of pseudo-MAT molecules cut, the intensity of each pseudo-MAT band was quantified using the Gel Analysis function of ImageJ software (National Institutes of Health, Bethesda, MD). Ratios were calculated by dividing the sum of intensities of the two cut pseudo-MAT bands by the sum of intensities of the cut and uncut pseudo-MAT bands.

For blots probing the pseudo-MAT and *HML::cre* loci (Figure 1C, Figure 5C, and Figure 6B), DNA was digested with *Xba*I and *Pac*I. The probe comprised a 505 bp sequence centered around the HO recognition sequence that was amplified from the *HML::cre* sequence of JRY10817 using the *HO-α1* probe forward/reverse primers.

For blots probing the *URA3-HO* sequences (Figure 3B), DNA was digested with *Pac*I and *Pvu*II. The probe comprised a 501 bp sequence surrounding the HO recognition sequence that was

amplified from the *K. lactis URA3-HO* sequence of JRY10819 using the *K.lac.URA3-HO* probe forward/reverse primers.

RNA preparation and quantitative RT-PCR

RNA was extracted from cells using the hot-acid-phenol method (Kebaara *et al.* 2012). 10 μg of RNA was digested with DNase I (Invitrogen, Carlsbad, CA) then purified with phenol-chloroform extraction followed by precipitation with 100% ethanol and 0.3 M sodium acetate (pH 5.2). Complementary DNA was synthesized from 2 μg of DNase-treated RNA with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and oligo(dT) primers. Quantitative PCR of cDNA was executed to detect the 3' end of the *cre* transcript specific to *HML::cre* with the *cre-3'-RTPCR* forward/reverse primers using the Thermo Scientific DyNAmo HS SYBR Green qPCR Kit (Fisher Scientific, Chicago, IL) and a Mx3000P machine (Stratagene, acquired by Agilent Technologies, Santa Clara, CA). Expression levels were normalized first to *ACT1* mRNA, which was measured using the *ACT1-RTPCR* forward/reverse primers, and then to *cre* mRNA levels from strain JRY10822. Samples were analyzed in technical triplicate.

Calculating gene conversion frequencies

For colony genotyping, colonies were resuspended in 200 μl TE buffer and 100 μl of glass beads. 200 μl of 25:24:1 phenol:chloroform:isoamyl alcohol was added, and tubes were subjected to two rounds of 20-sec bead-beating using a Fast-Prep-24 (MP Biomedicals, Santa Ana, CA). Tubes were centrifuged for 10 min at 15,000 rpm, and 1 μl of the aqueous phase was removed for PCR. PCR amplification of the pseudo-MAT locus on ChrVIII was carried out using the *pseudo-MAT-amplify* forward/reverse primers and Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA). A PCR cleanup reaction was performed using Agencourt AMPure XP reagent and the Beckman NX automated liquid handler by the University of California at Berkeley DNA Sequencing Facility, and PCR products were sequenced with primers *pseudo-MAT-seq-A* and *pseudo-MAT-seq-B*. Sequencing traces were analyzed using SnapGene software (from GSL Biotech; available at snapgene.com). For

Figure 1 (A) Diagram of the CRASH (Cre-Reported Altered States of Heterochromatin) assay used to measure transcriptional silencing stability at *HML*. (B) Schematic for directing homologous recombination between *HML::cre* and pseudo-MAT in strain JRY10817. The region between the dashed lines represents the 2.4 kb sequence of *HML::cre* that was inserted onto the left arm of chromosome VIII. The first 844 bp of the *cre* open reading frame were included, omitting the protein's active-site sequences. The sequence on chromosome VIII in green indicates that it is transcribed whereas the homologous sequences in black at *HML::cre* sequence are silenced. The open triangles represent three SNPs that distinguish the homologous regions: two SNPs on chromosome VIII on either side of the site of the HO-induced double-strand break, SNP-L and SNP-R, and one SNP within *HML* that destroys the HO recognition sequence, SNP-INC. SNP-L is located 45 bp into the *cre* open reading frame, 721 bp from the site of HO cleavage, and SNP-R is at the 20th base pair of the Z region, ~16 bp away from the site of the double-strand break. The arrow indicates the site of HO cleavage. (C) DNA hybridization blot of double-strand-break kinetics in JRY10817. A blot evaluating *HML::cre* and pseudo-MAT showed double-strand-break induction at pseudo-MAT after HO induction for the times shown. Here and in subsequent figures, the ratio of cut/total pseudo-MAT band intensities were calculated as described in Materials and Methods.

each of the three differentiating SNPs, sequencing traces that showed peaks of both the original pseudo-MAT sequence and the sequence at *HML::cre* were scored as partial gene conversions and those that showed only the *HML::cre* sequence were scored as full gene conversions. Multiple negative control samples in the sequencing process with no template DNA failed to produce PCR amplicons for sequencing, indicating that the presence of both alleles within a single colony reflected heterogeneity of genotype within the colony rather than cross-contamination of sequencing samples.

Measuring half-life of *cre* mRNA

Strain JRY10823, bearing the plasmid pJR2538 with *pGAL::cre* (Table 2), was grown to saturation in CSM-His + galactose medium, then diluted to an OD of 0.25 in the same medium and grown to an OD of 0.5. At this point, a preinduction sample was removed. The culture was centrifuged for 10 min at 4000 rpm and resuspended in CSM-His with glucose to turn off *cre* expression. A time 0 sample was immedi-

ately taken, and samples were taken every 10 min for the first 60 min and then every 15 min for the next 60 min. RNA extraction and quantitative RT-PCR were performed as described above.

Data availability

All strains and plasmids are available upon request. Supplemental Material, Table S1 in File S1 contains the oligonucleotide sequences used for strain construction.

Results

Design of a pseudo-MAT locus to test stability of gene silencing at a heterochromatic donor locus

To ask whether silencing stability is affected by homologous recombination within the silenced domain, we created a strain that allowed temporal control of homology-directed repair of a double-strand break at a custom-designed locus using *HML* as a donor. To monitor silencing stability, we employed an assay

Table 2 Plasmids used in this study

Name	Description	Source
pJR2538	<i>pGAL1::cre HIS3 ampR</i> <i>CEN/ARS</i>	Goldstein <i>et al.</i> 1999

previously developed by our lab called the Cre-Reported Altered States of Heterochromatin, or CRASH, assay (Dodson and Rine 2015). In this assay, the *cre* recombinase gene replaces the native $\alpha 2$ sequence at *HML*. Loss of silencing at *HML::cre* leads to the production of Cre protein, which then acts on a reporter cassette with loxP sites positioned around *RFP* and *GFP* genes, such that Cre-mediated recombination creates a permanent and heritable switch in fluorescence from red to green and a loss in resistance to the drug G418 (Figure 1A).

During mating-type switching, a double-strand break at *MAT* results in transposition of the sequence from the *HML* donor locus to the *MAT* locus, where it is then expressed. Hence, transposition of *cre* from *HML* to *MAT* would cause all cells to switch from *RFP* expression to *GFP* expression, rendering the CRASH assay ineffective. To induce a double-strand break that would be repaired by recombination targeted to *HML::cre* yet circumvent this problem, we constructed a pseudo-*MAT* locus on the left arm of ChrVIII (Figure 1B). This pseudo-*MAT* locus consisted of a 2.4 kb region of *HML::cre*, omitting the silencers as well as the 3' end of the *cre* open reading frame, which encodes the protein's active site required for Cre function. The lack of these sequences and flanking homology guaranteed that repair of the double-strand break at the pseudo-*MAT* locus could not restore a functional *cre* gene at that locus. The endogenous recognition sequence of the *HO* endonuclease found within the $\alpha 1$ open reading frame served as a target for double-strand break induction at the pseudo-*MAT* locus. The *HO* gene under control of the *GAL10* promoter allowed temporal regulation of double-strand break induction by addition of galactose to the growth medium. Under physiological conditions of *HO* expression, Sir proteins prevent access of *HO* to its recognition sequence at *HML*, but *HO* overexpression can lead to low levels of *HML* cleavage (Connolly *et al.* 1988; K. Sieverman 2017). To ensure that *HO* caused double-strand break formation only at the pseudo-*MAT* locus and not at *HML::cre* itself, we mutated the $\alpha 1$ sequence at *HML::cre* to contain an SNP that eliminates *HO*'s ability to cleave it, historically known as *MAT α -inc* (Nickoloff *et al.* 1990) but called SNP-INC here. To further distinguish the homologous sequences at *HML::cre* and pseudo-*MAT*, we added two SNPs to the pseudo-*MAT* locus on either side of the site of *HO* cleavage: one 721 bp away from the site of *HO* cleavage, within the *cre* sequence, called SNP-L, and one 16 bp to the right of the *HO* recognition sequence, called SNP-R. We also deleted the *MAT* and *HMR* loci in this strain to prevent cleavage by *HO* at its recognition sequence within them.

The pseudo-*MAT* locus was inserted 13 kbp from the end of the left arm of ChrVIII. We chose this location to encourage efficient recombination between *HML::cre* and pseudo-*MAT*

based upon two considerations: recombination efficiency correlates with contact frequency, as measured by high-throughput chromosome conformation capture (Hi-C) (Lee *et al.* 2016), and with similarity in chromosomal arm length (Agmon *et al.* 2013). Mating-type switching involves only nonreciprocal recombination. To allow us to focus exclusively on the outcomes of nonreciprocal events, the orientation of *HML::cre* and pseudo-*MAT* was such that reciprocal recombination via cross-over would create a dicentric chromosomal fusion. Such events can be recognized by the slow growth and abnormal colony morphology resulting from various resolutions of the dicentric chromosome. Focusing our analysis on colonies with typical morphology allowed us to evaluate only nonreciprocal recombination events that would not lead to *cre* expression from the pseudo-*MAT* locus. Upon induction of *HO* in this strain (JRY10817), double-strand breaks were evident within 30 min, with breaks persisting but reduced after 4 hr of *HO* expression (Figure 1C).

Homology-directed repair decreased the stability of silencing at the donor locus

Induction of *HO* and subsequent plating on solid medium lacking galactose yielded colonies in which at least one-quarter of cells had switched from *RFP* to *GFP* expression, indicating a silencing-loss event early in colony growth. Moreover, there was a clear trend of increasing silencing-loss events with increasing duration of *HO* induction (Figure 2A). A subculture incubated in noninducing (raffinose) medium yielded red colonies, indicating that without exposure to galactose, the stability of silencing at *HML::cre* was unaffected.

To determine whether the loss in silencing stability upon *HO* induction resulted from the presence of *HO* itself rather than from the resulting double-strand break, we created a strain identical to the assay strain but also harboring SNP-INC at pseudo-*MAT* (JRY10818), thus lacking any recognition sequence for *HO* to cleave. In this strain, induction of *HO* resulted in no discernable increased likelihood of silencing loss (Figure 2B). Hence, destabilization of silencing at *HML::cre* depended on double-strand-break formation at the pseudo-*MAT* locus, rather than growth in galactose medium or some yet unrecognized ability of *HO* to influence silencing loss.

To investigate whether recombination involving *HML::cre* itself was required for the increase in silencing-loss events or whether repair of a double-strand break anywhere in the genome would cause silencing instability, we induced *HO* cleavage at a locus whose template for homology-directed repair was not *HML::cre*. Specifically, an *HO* recognition sequence was engineered into a *URA3* gene at the same position on ChrVIII as pseudo-*MAT*, with an uncleavable version of the same *URA3* construct inserted on ChrIX (Figure 3A). As expected, *HO* induction in this strain (JRY10819) resulted in double-strand-break formation on ChrVIII with cleavage levels similar to those seen at pseudo-*MAT* (Figure 3B). Even in colonies grown from cells with the longest induction times, there was no increase in silencing loss at *HML::cre* (Figure

Figure 2 Silencing loss after *HO* induction by galactose. (A) Increased likelihood of silencing loss after *HO* induction and double-strand breaks at pseudo-MAT in JRY10817. (B) JRY10818, which harbors the SNP-INC allele at both *HML::cre* and pseudo-MAT, did not show increased likelihood of silencing loss after *HO* induction. As a negative control in both experiments, part of the culture was maintained in raffinose. Here and in subsequent figures, early silencing-loss events were defined as colonies that were at least one-quarter green, indicating a silencing-loss event by the four-cell stage of the colony. A minimum of 300 colonies (A) or 175 colonies (B) was analyzed for each time point. For each time point in (A), the means and SD of three independent biological replicates are shown.

3C). Thus, the silencing instability induced at *HML::cre* after cleavage of ChrVIII required a double-strand break in a sequence homologous to *HML::cre*.

Gene conversion of SNPs at pseudo-MAT was diagnostic of recombination with *HML::cre*

In mating-type switching, homologous recombination via SDSA results in gene conversion of the double-strand break recipient sequence to that of its donor locus. We analyzed the DNA sequence at pseudo-MAT for evidence of gene conversion of the three SNPs differentiating it from *HML::cre* in colonies grown on solid medium lacking galactose after *HO* induction. After a 1-hr *HO* induction, 42% of the resultant colonies showed evidence of gene conversion at the pseudo-MAT locus of at least one of the three SNPs (Figure 4A). The fraction of the population that experienced gene conversion exceeded the fraction that lost silencing, as discussed below.

The SNP-INC was most likely to gene convert: ~41% of colonies showed appearance of the SNP-INC sequence in the pseudo-MAT locus after 1 hr of *HO* induction. SNP-L and SNP-R were less likely to gene convert: ~24.6% of colonies showed gene conversion at SNP-L and 26.2% at SNP-R after 1 hr of induction. Many colonies showed only partial gene conversion of any given SNP, meaning that both the original pseudo-MAT and the *HML::cre* sequences were detected at the pseudo-MAT locus within the DNA from a single colony. We discuss this genotypic heterogeneity further below.

Recombination could occur without silencing loss

Inducing recombination between pseudo-MAT and *HML::cre* led to a population of cells that experienced a greater likelihood of silencing loss, yet many cells gave rise to red colonies that had maintained silencing after 4 hr of *HO* expression. The DNA blot (Figure 1C) suggested that not all recipient loci had been cleaved at any time point, but that analysis could

not distinguish between molecules that had been cut and repaired and molecules that had never received a double-strand break. Additionally, 34% of colonies showed no evidence of gene conversion after 4 hr of *HO* induction (data not shown). Therefore, it was possible that colonies that maintained silencing arose from cells that did not experience a double-strand break and subsequent recombination between pseudo-MAT and *HML::cre*. Alternatively, some cells may have managed to execute homology-directed repair from *HML::cre* with no effect on silencing stability.

To distinguish these two possibilities, we analyzed gene conversion frequencies from colonies that were either red or at least one-quarter green to ask whether gene conversion was evident in both populations. After a 1-hr *HO* induction, ~84% of colonies that lost silencing early in colony growth exhibited gene conversion at pseudo-MAT (Figure 4B), demonstrating a strong correlation between recombination with *HML::cre* and silencing instability. Interestingly, ~30% of red colonies, which maintained silencing through multiple rounds of cell division, also showed evidence of gene conversion (Figure 4C). Therefore, homologous recombination between pseudo-MAT and *HML::cre* did not lead to an obligatory loss of silencing.

Conceivably, silencing might be maintained only in cells with short conversion tracts, while those with extensive gene conversion may lose silencing stability. However, gene conversion into the *cre* sequence at SNP-L, 721 bp from the *HO* cleavage site was possible without subsequent transcriptional activation of the *cre* gene at *HML*, as evidenced by the ~13% of colonies that maintained silencing yet experienced gene conversion at SNP-L (Figure 4C).

Kinetics and extent of silencing loss

To investigate how quickly silencing loss at *HML::cre* appeared after *HO* induction and how long it persisted after

Figure 3 Recombination elsewhere in the genome did not increase likelihood of silencing loss. (A) Schematic for directing homologous recombination between ChrVIII and ChrIX in strain JRY10819. An 89 bp sequence of the Yα-Z1 regions spanning the HO recognition sequence from *MATα* was inserted into the *K. lactis* *URA3* gene, which was placed at the same place on ChrVIII where the pseudo-*MAT* locus resided. The same sequence was copied onto ChrIX with the SNP-INC substitution that prevented HO from cleaving ChrIX. Additionally, the HO recognition sequence was deleted from *HML::cre*, thus directing all HO-mediated double-strand breaks to ChrVIII and inducing homology-directed repair off of ChrIX. The arrow indicates the site of HO cleavage. (B) DNA hybridization blot of double-strand break induction kinetics in JRY10819. A blot evaluating the *URA3-HO-INC* on ChrVIII and *URA3-HO-INC* on ChrIX showed double-strand-break induction at *URA3-HO* after *HO* induction for the times shown. A band indicative of a reciprocal crossover between ChrVIII and ChrIX appeared after 3 hr of *HO* induction. (C) HO cleavage leading to recombination events between ChrVIII and ChrIX did not increase the likelihood of silencing loss at *HML::cre*. As a negative control, part of the culture was maintained in raffinose. A minimum of 400 colonies was analyzed for each time point.

homology-directed repair, we monitored mRNA levels of the *cre* transcript specific to *HML::cre* before, during, and after a 1-hr *HO* induction. We calculated the half-life of *cre* mRNA to be ~3 min after glucose shutoff of a *cre* gene being driven by the *pGAL1* promoter (Figure 5A). Thus, measurements of *cre* mRNA by quantitative RT-PCR closely reflected the real-time transcriptional output of cells. Prior to double-strand break induction, *cre* mRNA levels were ~9000-fold lower than those in a *sir3Δ* strain lacking transcriptional silencing at *HML::cre* (Figure 5B). After a 1-hr induction, *cre* mRNA levels increased ~10-fold. *Cre* expression peaked at nearly 100-fold higher than pre-double-strand break induction 2 hr after quenching *HO* expression, at which time all double-strand breaks at pseudo-*MAT* appeared to be repaired (Figure 5D). Five hours after quenching *HO*, *cre* mRNA levels had returned to ~10-fold higher levels than before double-strand break induction (Figure 5B), suggesting that many cells that had lost silencing had restored it by this time and were no longer producing *cre* transcripts. In strain JRY10818 harboring the SNP-INC at pseudo-*MAT*, expressing *HO* did not cause an

increase in *cre* transcript levels, indicating that the increase in *cre* transcripts was specific to cultures experiencing the double-strand break at the pseudo-*MAT* locus (Figure 5C).

To estimate the extent of silencing loss resulting from homologous recombination, we compared the number of colonies that experienced silencing loss before, during, and after a 1-hr *HO* induction and compared these values to the *cre* mRNA levels measured by quantitative RT-PCR. Prior to *HO* induction, ~2% of cells gave rise to colonies with an early silencing-loss event (Figure 5E). After a 1-hr *HO* induction, ~23% of resultant colonies experienced an early silencing-loss event. The percentage of colonies with silencing loss remained similar after a 2-hr quench of *HO* expression with glucose, suggesting that no additional cells lost silencing during that time. The highest levels of *cre* expression were seen at the 2-hr quench time, at which point they were ~1% that of a *sir3Δ* population (Figure 5B). Assuming that the population of cells that experienced silencing loss reestablished silencing with similar kinetics, we inferred that 23% of the population expressed *cre* at a maximum of 1% of full

Figure 4 Gene conversion frequencies at pseudo-MAT after a 1-hr *HO* induction in (A) all colonies, (B) colonies that experience an early silencing-loss event, and (C) colonies that maintained silencing. For each of the three SNPs distinguishing pseudo-MAT and *HML::cre*, the percentage of colonies that showed evidence of gene conversion is shown. Gene conversion events were scored as full gene conversion events if we detected only the *HML::cre* sequence and partial events if we detected both the *HML::cre* and pseudo-MAT sequences within a single colony. The means and SD of three independent biological replicates are shown. A minimum of 142 colonies was analyzed for each replicate of the overall population shown in (A).

derepression, as measured from mRNA from the entire population. Therefore, the cells that lost silencing after homologous recombination within *HML::cre* expressed *cre* at much lower levels per cell than did fully derepressed cells.

A special allele of *SWI2* influenced homologous recombination frequency and likelihood of silencing loss after recombination

Previous studies indicate that the presence of Sir3, one of the structural components of silent chromatin, prevents an early step of homologous recombination (Sinha *et al.* 2009). The nucleosome remodeling activity of the SWI/SNF complex is required *in vitro* for successful synapsis between Rad51-coated filaments and Sir3-bound nucleosomal donors. *SWI2* encodes the ATPase of the SWI/SNF complex and interacts directly with Sir3 *in vitro* (Manning and Peterson 2014). An allele of *SWI2*, *swi2Δ10R*, lacks sequences necessary for Swi2's interaction with Sir3 and eliminates the ability of the SWI/SNF complex to facilitate pairing between Rad51-coated filaments and Sir3-coated nucleosomal donors *in vitro* (Manning and Peterson 2014). Although *swi2Δ10R* is not reported to inhibit recombination between *MAT* and *HMR* *in vivo* (Manning and Peterson 2014), we hypothesized that the stability of silenced chromatin during recombination may be affected in this mutant.

We repeated the *HO* induction time course in a *swi2Δ10R* mutant (JRY10820) that was otherwise isogenic with our assay strain. The fraction of colonies with an early silencing-loss event was not significantly different after *HO* induction in the *swi2Δ10R* mutant relative to the *SWI2* parent strain (*P* value > 0.05 for 1-hr induction and 2-hr induction when analyzed with an unpaired *t*-test) (Figure 6A), suggesting no increased likelihood of silencing loss in the *swi2Δ10R* mutant after double-strand break formation at pseudo-MAT. Furthermore, there was no obvious difference in

the kinetics of break induction and repair in the *swi2Δ10R* mutant relative to a wild-type culture (Figure 6B vs. Figure 1C and Figure 5D).

The overall recombination frequency between pseudo-MAT and *HML::cre* was slightly reduced in the *swi2Δ10R* mutant after 1-hr of *HO* induction. 44.8% of wild-type colonies showed evidence of a gene conversion event, either full or partial, for at least one of the three SNPs, (Figure 4A), while 33.2% of *swi2Δ10R* mutant colonies experienced a gene conversion event anywhere (Figure 6C) (*P*-value = 0.0036, unpaired *t*-test). Thus, the overall frequency of gene conversion events was somewhat greater in strains with a wild-type *SWI2* allele. Gene conversion frequencies were reduced in *swi2Δ10R* colonies that both maintained silencing (*P*-value = 0.0026, unpaired *t*-test) and experienced early silencing loss (*P*-value = 0.0095, unpaired *t*-test) (Figure 6, D and E), although the reduction was more pronounced in the population that maintained silencing. Hence, the mutant *swi2Δ10R* allele slightly reduced the ability of the cleaved pseudo-MAT locus to participate in homologous recombination, and successful recombination events occurred more frequently in colonies that experienced an early silencing-loss event.

In addition to the lower overall frequency of gene conversion events, the *swi2Δ10R* allele slightly increased the likelihood of silencing loss at *HML::cre* after homologous recombination. Wild-type colonies and *swi2Δ10R* colonies that did not show evidence of gene conversion experienced early silencing-loss events at frequencies that were not significantly different (*P*-value > 0.05, unpaired *t*-test) (Figure 7A vs. Figure 7C). In colonies that experienced a gene conversion event, full or partial, for at least one of the three SNPs, those that were *swi2Δ10R* mutants were more likely to lose silencing than their wild-type counterparts (*P*-value = 0.0174, unpaired *t*-test) (Figure 7B vs. Figure 7D). Thus, homologous recombination led to silencing loss more

Figure 5 (A) Kinetics of *cre* mRNA stability after glucose-mediated shutoff of *pGAL1::cre* in pJR2538. (B and C) Quantitative RT-PCR quantification of *cre* mRNA before and after a 1-hr *HO* induction and subsequent quench with glucose in (B) a strain with a cleavable pseudo-MAT locus (JRY10817) and (C) a strain without a cleavable pseudo-MAT locus (JRY10818). *Cre* mRNA levels were normalized to *ACT1* mRNA levels and all data points are relative to *cre* mRNA levels of a *sir3Δ* strain (JRY10822), which lacks transcriptional silencing. Data represent the means and SD of three technical replicates. (D) DNA hybridization blot of double-strand-break formation and repair kinetics. A blot evaluating *HML::cre* and pseudo-MAT showed double-strand break induction at pseudo-MAT after a 1-hr *HO* induction and subsequent quench with glucose. (E) Percentage of colonies that were all green, at least half green but not all green, or at least one-quarter green but not half green during the quantitative RT-PCR time course. Data shown are the means of two independent biological replicates.

frequently at *HML::cre* in the presence of the *swi2Δ10R* mutant allele.

Investigating causes of genotypic heterogeneity within colonies

As mentioned earlier, we observed some partial gene conversion events at the pseudo-MAT locus, whereby both the original pseudo-MAT and *HML::cre* sequences were detected within a single colony after *HO* induction. This genetic heterogeneity suggested that not all cells within the colony experienced the same molecular outcome at the pseudo-MAT

locus after double-strand break induction. Double-strand breaks can be repaired independently of homologous recombination by nonhomologous end joining (NHEJ), and breaks made by *HO* often lack a sequence scar when repaired by NHEJ (Kramer *et al.* 1994; Moore and Haber 1996; Haber 2012). We hypothesized that the genotypic heterogeneity observed within colonies might be a result of postreplication breaks in which one chromatid of ChrVIII underwent homology-directed repair at pseudo-MAT while the other was nonhomologously end joined, leading to two different repair outcomes and thus two genotypes within the colony. To test

Figure 6 (A) As in wild type, *HO* induction increased likelihood of silencing loss in a *swi2Δ10R* mutant (JRY10820). Early silencing-loss events were defined as above. As a negative control, part of the culture was maintained in raffinose. A minimum of 460 colonies was analyzed for each time point. For all time points other than 3 hr in galactose and 4 hr in galactose, the means and SD of three independent biological replicates are shown. For the 3- and 4-hr galactose time points, the means of two independent biological replicates are shown. (B) A DNA hybridization blot evaluating *HML::cre* and pseudo-MAT showed double-strand break induction at pseudo-MAT after *HO* induction in *swi2Δ10R* (JRY10820) for the times shown. (C–E) Gene conversion frequencies in *swi2Δ10R* at pseudo-MAT after 1-hr *HO* induction in (C) all colonies, (D) colonies that experienced an early silencing-loss event, and (E) colonies that maintained silencing. For each of the three SNPs distinguishing pseudo-MAT and *HML::cre*, the percentage of colonies that showed evidence of gene conversion is shown. Gene conversion events were scored as in previous figures. The means and SD of three independent biological replicates are shown. For data points with overlapping error bars, the error bar color matches its reference data. A minimum of 125 colonies was analyzed for each replicate of the overall population shown in (A).

this, we deleted the DNA ligase IV gene, *DNL4*, which is required for NHEJ (Wilson *et al.* 1997), in the parental strain used for all of the experiments above. In this mutant (JRY10821), we did not observe a difference in likelihood of silencing loss upon *HO* induction after a 1-hr *HO* induction (P -value > 0.05, unpaired *t*-test, data not shown). The overall gene conversion frequencies after a 1-hr *HO* induction were not significantly different between wild type and *dnl4Δ* (P -value > 0.05 for gene conversion anywhere, unpaired *t*-test) (Figure 8A). Furthermore, the frequency of partial gene conversions was not significantly different in the *dnl4Δ* mutant (P -value > 0.05 for partial gene conversion anywhere, unpaired *t*-test) (Figure 8A). Thus, repair through NHEJ was not a major contribution to removal of double-strand breaks and did not account for the genotypic heterogeneity within colonies.

Yeast cells sometimes fail to separate fully after completing a round of cell division. To determine whether the genotypic heterogeneity within colonies might have resulted from incomplete separation of single cells at the time of plating, leading to colonies with more than one original founder cell, we used a micromanipulator to separate single cells after 1 hr of *HO* induction onto solid medium, ensuring that the resultant colonies were progeny of one cell. Sequencing results from these colonies showed that the appearance of partial gene conversion events persisted, yet were reduced relative to colonies plating by standard glass-bead-spreading (P -value = 0.0174 for partial gene conversion events anywhere, unpaired *t*-test) (Figure 8B). Thus, although incomplete separation after cell division contributed to the genetic heterogeneity seen within colonies, differences in sequence at the pseudo-MAT locus existed between founder cells and their progeny.

Figure 7 Frequencies of early silencing-loss events after a 1-hr *HO* induction in wild-type colonies (JRY10817) that (A) did not exhibit gene conversion and (B) exhibited at least partial gene conversion, and *swi2Δ10R* colonies (JRY10820) that (C) did not exhibit gene conversion and (D) exhibited at least partial gene conversion at each of the three SNPs distinguishing pseudo-*MAT* and *HML::cre*. Partial gene conversion events were scored as above. The means and SD of three independent biological replicates are shown in each panel.

To further investigate the genetic heterogeneity observed within colonies, we analyzed the sequence at pseudo-*MAT* after a 1-hr *HO* induction in colonies from pedigrees of mother cells and their first two daughter cells. We collected sequencing data from 27 complete pedigrees, 25 of which showed no evidence of gene conversion in colonies arising from the mother or her first two daughters.

In the first complete pedigree with a gene conversion event, all three resultant colonies maintained silencing. The mother cell showed no gene conversion at SNP-L and full gene conversion at both SNP-INC and SNP-R. The first daughter showed no gene conversion at SNP-L or SNP-R, but full gene conversion at SNP-INC. The second daughter's genotype matched that of the mother. Hence, the first cell division gave rise to one cell that did not experience gene conversion at SNP-R and one that did.

In the second complete pedigree with a gene conversion event, the mother and her first two daughters showed full gene conversion at all three SNPs. Interestingly, the mother cell gave rise to a colony that maintained silencing, the first daughter gave rise to a colony that was one-quarter green, and the second daughter gave rise to a colony that was all green.

Thus, a single gene conversion event that occurred in the mother cell prior to the first division affected the likelihood of silencing loss in later cell divisions.

We observed one incomplete pedigree comprising a mother cell and only her first daughter cell, in which both resultant colonies were all green. The colony arising from the mother cell showed full gene conversion at all three SNPs, while the colony from the first daughter showed full gene conversion at SNP-L and SNP-INC but partial gene conversion at SNP-R. Hence, genotypic heterogeneity at pseudo-*MAT* could manifest after the first cell division.

Discussion

In this study, we developed an assay to test the consequences of homologous recombination within silent chromatin on the stability of gene silencing. We used the *HO* endonuclease gene under control of the *GAL10* promoter to temporally control double-strand-break induction at pseudo-*MAT*, a locus that shared homology with *HML::cre*. We found that double-strand break repair templated from *HML::cre* increased the frequency of silencing loss events, although recombination

Figure 8 Gene conversion frequencies at pseudo-MAT after a 1-hr *HO* induction in (A) a *dnl4Δ* mutant (JRY10821) and (B) wild-type cells (JRY10817) that were micromanipulated to ensure only one founder cell per colony. The means and SD of three independent biological replicates are represented in each panel. For data points with overlapping error bars, the error bar color matches its reference data.

was possible without disruptions to silencing even in repair events with extensive gene conversion tracts. Recombination-induced expression from *HML::cre* was rapid and lasted on the order of hours before silencing was reestablished. Double-strand-break induction and silencing-loss outcomes were not slightly affected in a *swi2Δ10R* mutant background. Founder cells and their progeny did not always share the same sequence at the pseudo-MAT locus after gene conversion, leading to genetic heterogeneity within some colonies.

Silencing loss often, but not always accompanied homologous recombination

After induction of *HO*, many cells gave rise to colonies that had lost silencing in at least one cell by the four-cell stage of growth, resulting in colonies that were at least one-quarter green. This increase in silencing loss events was specific to strains in which a double-strand break was repaired from *HML::cre*, as homologous recombination not involving *HML::cre* did not change the likelihood that it would lose silencing.

In approximately one-third of colonies that did not lose silencing after 1 hr of *HO* induction, gene conversion occurred at the pseudo-MAT locus. Thus, homologous recombination targeted to *HML::cre* led to distinct outcomes where cells either did or did not lose silencing after homology-directed repair.

Why homologous recombination led to silencing loss in some cells but not others remains an interesting puzzle. It was unlikely that the extent of gene conversion tract determined whether silencing was lost, as gene conversion of the distant SNP-L occurred in ~13% of colonies that did not lose silencing. In mating-type switching, homology between the double-strand break at *MAT* and the donor locus occurs only on one side of the double-strand break, ensuring that repair events proceed via a type of homologous recombination known as SDSA (Haber *et al.* 1980; Klar and Strathern 1984; Ira *et al.* 2006). However, multiple mechanisms of homologous recombination can repair a double-strand break [reviewed in Pâques and Haber (1997)]. Perhaps in our assay strain, recombination events between pseudo-MAT and *HML::cre* could be repaired by different mechanisms of homologous recombination, some of which led to silencing loss and some of which did not. Mating-type switching is restricted to the end of G1 (Strathern and

Herskowitz 1979), whereas our experiments were conducted in cycling cells. Conceivably, the timing of either the double-strand break or the recombination event itself could influence whether or not the *HML::cre* donor locus lost silencing. For example, the sirtuin deacetylase *Hst3* is a cell cycle-regulated protein important for maintaining silencing stability at *HML* (Dodson and Rine 2015) and is degraded after S-phase but before anaphase (Delgosaie *et al.* 2014). Finally, it is also possible that the likelihood of silencing loss after recombination reflected stochasticity in the access of the transcription machinery to *HML::cre*.

Silencing loss was transient and limited

We monitored the kinetics and levels of transcripts from *HML::cre* before, during, and after *HO* induction by quantitative RT-PCR. Transcription at *HML::cre* was highest after completion of homologous recombination, perhaps because the presence of repair proteins prevented access of the transcription machinery to the donor locus until recombination was complete. In the cells that lost silencing, transcription at *HML::cre* resulting from homologous recombination likely did not approach the full level of derepression. It is unclear whether the green cells contributing to the colonies that were at least one-quarter green but not all green had lost silencing at the time of plating, or whether those silencing-loss events occurred postplating. It is likely that both of these scenarios are possible, either due to incomplete separation of red and green cells or due to a delay in silencing loss after repair. In the latter scenario, the all-green colonies alone would contribute to the pool of mRNA seen by quantitative RT-PCR within the first few hours. Even so, the increase in all-green colonies seen after *HO* induction to 5–10% of the population would have given rise to only 1% of *cre* mRNA amounts expected with full derepression, further supporting the conclusion that silencing loss did not approach full derepression.

Cre mRNA levels had decreased substantially 5 hr after quenching *HO* expression, but were still ninefold higher than before the induction. A longer time course would presumably show transcriptional silencing returning to its preinduction levels. Conceivably, some cells within the population may have undergone reciprocal recombination, giving rise to a

functional *cre* gene that might not be fully silenced at the pseudo-MAT locus on the resulting dicentric chromosome. Although colonies resulting from such events were excluded from our plating-based analyses, the samples collected for quantitative RT-PCR analysis could include a small percentage of cells expressing *cre* from such a restored pseudo-MAT locus.

Interactions between *Swi2* and *Sir3* influenced silencing stability during homologous recombination

Silent chromatin prevents access of many DNA binding proteins to the sequences within it, and previous studies established a role for ATP-dependent nucleosome remodelers in successful recombination between the *MAT* locus and its heterochromatic donors (Chai *et al.* 2005; Sinha *et al.* 2009; Tsukuda *et al.* 2009). *In vitro*, the SWI/SNF complex is necessary to allow pairing between Rad51-coated filaments and Sir3-coated nucleosomes in an assay that simulates recombination with silent chromatin (Sinha *et al.* 2009). Physical interactions between Sir3 and the ATPase subunit of the SWI/SNF complex, Swi2 (Manning and Peterson 2014), are necessary for successful *in vitro* synapsis between Rad51 filaments and Sir3-coated nucleosomal donors in that assay. An allele of *SWI2*, *swi2Δ10R*, which abolishes the interaction between Sir3 and SWI/SNF, prevents *in vitro* pairing of Rad51 filaments with Sir3-bound nucleosomes.

In our assay, the *swi2Δ10R* mutant slightly reduced the recombination frequency between pseudo-MAT and *HML::cre*, and recombination events were slightly more likely to lead to an early silencing-loss event. This suggests a minor role for the interaction between Swi2 and Sir3 in both promoting homologous recombination within *HML::cre* and preventing silencing loss after recombination. Because the effects seen in the *swi2Δ10R* mutant were not severe, perhaps Sir3 is successfully removed from nucleosomes at *HML* *in vivo* by another nucleosome remodeling complex. Alternatively, Sir3 removal from nucleosomes may not be critical for homologous recombination between the pseudo-MAT locus and *HML::cre*, similar to the *in vivo* findings (Manning and Peterson 2014).

Double-strand-break induction kinetics

Kinetic studies of HO-mediated cleavage of the endogenous *MAT* locus, where it is possible to distinguish the uncut *MATα* and repaired *MATα*, report that full cleavage is achieved after ~30 min of HO induction (Hicks *et al.* 2011), yet at no time did we see complete cleavage of the pseudo-MAT locus, and the percentage of cleaved pseudo-MAT molecules never exceeded 40% throughout the time course. In our study, DNA blot analysis could not distinguish molecules that had been cleaved and repaired from those that were never cleaved due to the sequence identity between the initial and repaired pseudo-MAT products. Thus, it was not clear whether double-strand break induction at our pseudo-MAT locus was slower than at the endogenous *MAT* locus, or whether cleavage kinetics were similar to those previously

reported. It was also possible that the cleaved pseudo-MAT molecules seen at later time points reflect molecules that had been repaired by recombination but without gene conversion, leaving the HO recognition sequence intact for further rounds of cleavage. We would expect that after an adequate time of HO expression, all pseudo-MAT molecules would show gene conversion to the uncleavable SNP-INC sequence found at the donor *HML::cre* locus.

Genetic and phenotypic heterogeneity within colonies

Silencing loss and recombination events were assayed in colonies grown after HO induction, representing a temporal difference in the occurrence of these events and their detection. Interestingly, many colonies displayed genotypic and phenotypic heterogeneity regarding the repaired sequence at pseudo-MAT and the silencing loss from *HML::cre*.

Approximately half of gene conversion events at pseudo-MAT after a 1-hr HO induction were partial gene conversion events, in which both the original pseudo-MAT and the donor *HML::cre* sequences were detected in a single colony. Some of this genetic heterogeneity was likely due to the propensity of cells to remain attached after rounds of cell division, as micromanipulating single cells onto solid medium after HO induction to ensure a single founder cell reduced the frequency of colonies with partial gene conversion events. However, some of the separated cells still gave rise to colonies with partial gene conversion events, thus requiring other explanations. One possible explanation could be that recombination in the founder cell led to a mismatch at pseudo-MAT that was not resolved prior to replication, causing both alleles to be propagated in the colony. Two pedigrees that we observed support this model. In one of our complete pedigrees, the mother cell showed full gene conversion at SNP-R while the daughter cell showed no gene conversion at SNP-R, suggesting that repair leading to a mismatch happened prior to replication, and each cell inherited a chromosome with the opposite genotype. In our incomplete pedigree, the appearance of a partial gene conversion only in the daughter but not the mother suggests that the double-strand break and repair happened postreplication, and the chromosome inherited by the daughter contained a mismatch. Additionally, the partial gene conversion events may have resulted from differences in the way a founder cell experienced HO-mediated double-strand breaks or subsequent repair from its progeny. Perhaps the HO protein was asymmetrically distributed to either the mother or daughter cell, which could result in one cell experiencing a double-strand break but not the other. In this scenario, the genetic heterogeneity within a colony would result from different events in the first cells that form the colony rather than a single event that leads to two molecular outcomes. Although we did not observe an outcome in our pedigree analysis that supports this model, we cannot rule out that possibility.

As with the genetic heterogeneity seen within a colony, micromanipulating single cells onto solid medium after HO induction revealed some colonies that were at least one-quarter

green but not completely green, reflecting silencing-loss events that occurred after the first cell division (Figure S1 in File S1). Our pedigree analysis showing gene conversion prior to the first cell division but silencing loss in the second and third cell divisions suggests that it is possible that homologous recombination with pseudo-MAT affected chromatin at the *HML::cre* locus in a way that did not manifest silencing loss immediately, resulting in a delay in *cre* transcription by one or two cell divisions. Perhaps the chromatin at *HML::cre* after homologous recombination was stable enough to immediately maintain silencing in some cells, but changes to epigenetic marks or another factor that influences silencing stability were not restored to their pre-recombination status. Despite these nuances, there was a clear causal link between double-strand-break repair template from *HML::cre* and subsequent silencing loss.

Implications beyond *S. cerevisiae*

In this study, homologous recombination targeted to *HML* led to an increased likelihood of transcriptional silencing loss in many, but not all, cells. The silencing loss that did occur at *HML* after homologous recombination was transient and limited, which likely resulted from the efficiency and rapid kinetics with which yeast cells can establish silencing *de novo* when all necessary components are available (Osborne *et al.* 2009). In organisms or genetic backgrounds that cannot rapidly reestablish transcriptional silencing within heterochromatic loci, homologous recombination might alter chromatin structures in a way that has long-lasting effects on the transcriptional output of such loci. Conceivably, other structures of chromatin may also be vulnerable to disruption by homologous recombination. This possibility may become evident with the increase in frequency of Cas9-mediated genome editing studies.

Acknowledgments

We thank Ryan Janke and Martin Kupiec for invaluable insights into the design of multiple experiments in this study. We thank Anne Dodson for thoughtful input and strain contribution, including construction of strain JRY10822. We thank Craig Peterson for providing the *swi2Δ10R* construct, and the Deuber laboratory at University of California at Berkeley for the plasmid enabling Cas9-mediated strain construction. We thank Oskar Hallatschek for use of the Zeiss Axio Zoom.V16, Carlos Bustamante for use of the Typhoon Trio imager, and the University of California at Berkeley DNA Sequencing Facility for preparation of samples for sequencing. We thank Doug Koshland, Elçin Ünal, Dana Carroll, and Kathleen Ryan for helpful discussion. Finally, we thank members of our laboratory, Aisha Ellahi, Melanie Kaiser, Gavin Schlissel, Davis Goodnight, and Daniel Saxton, for thoughtful discussion and technical guidance. This work was supported by a grant from the National Institutes of Health (GM31105), and by a National Institutes of Health National Research Service Award Trainee appointment on grant number T32 HM 007232.

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Communicating editor: A. Mitchell