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Cell-cycle regulation of silent chromatin in *Saccharomyces cerevisiae*

by

Davis W Goodnight

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Jasper Rine, Chair
Professor Barbara Meyer
Professor Xavier Darzacq
Professor Kathleen Ryan

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Abstract

Cell-cycle regulation of silent chromatin in *Saccharomyces cerevisiae*

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Davis W Goodnight

Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor Jasper Rine, Chair

In eukaryotes, the physical packaging of DNA into chromatin plays a major role in modulating gene expression. In the budding yeast *Saccharomyces cerevisiae*, silent chromatin, a repressive chromatin structure analogous to the heterochromatin found in animals, is required for cell mating-type identity. Silent chromatin forms at the cryptic mating type loci *HML* and *HMR* and depends on the interactions between the Silent Information Regulator SIR proteins and nucleosomes. A longstanding model for how Sir proteins build a repressive chromatin structure posits a stepwise process of histone tail deacetylation by Sir2, followed by recruitment of Sir3 and Sir4 to those deacetylated tails. However, this model is incomplete, as it does not account for the observed dependence of silencing establishment on progression through S phase of the cell cycle, which has been known for decades, but remained unexplained when I began these studies. I worked to resolve this mystery first by characterizing the establishment of silencing using modern molecular techniques and then by performing genetic analysis to identify the molecular determinants of silencing establishment.

Using the inducible allele *SIR3-EBD*, I demonstrated that silencing establishment has similar cell-cycle requirements at *HML* and *HMR*, which corrected an incorrect claim in the literature. This finding simplified the possible models that could explain an S-phase dependence for silencing establishment, as the models did not have to account for locus-specific effects. Using chromatin immunoprecipitation followed by sequencing (ChIP-seq), I confirmed the earlier finding that Sir protein recruitment and spread across *HML* and *HMR* does not require cell-cycle progression. However, I also showed that S phase promotes Sir protein binding beyond the level that can be obtained without cell-cycle progression. Using single-molecule RNA fluorescence in situ hybridization (smRNA-FISH) to study the establishment of silencing, I found that transcriptional repression occurs gradually in individual cells, not via discrete transitions between the expressed and repressed states. Thus, the model for silencing must account for intermediate levels of repression occurring during establishment. I found that cells lacking *DOT1, SAS2*, or

RTT109, which code for anti-silencing histone modifying enzymes, could partially establish silencing without S phase passage. Dot1 was particularly interesting, because the anti-silencing mark it deposits, methylation of H3K79, lacks a known demethylase, and thus the mark can only be removed through histone turnover, which occurs mainly during S phase. Consistent with this expectation, I found that Sir proteins were unable to deplete H3K79 methylation from *HML* and *HMR* without passage through S phase. Together, these results suggest that removal of methylation from H3K79 is a major cell-cycle-dependent step in the establishment of silent chromatin.

While silencing establishment relies on incorporation of new silencing-competent histones during S phase, a long-standing hypothesis in the field of heterochromatin is that the epigenetic inheritance of the silent state depends on inheritance of modified parental histones during S phase. Recent work from the Rine lab has demonstrated that parental histones are locally redeposited after DNA replication at the non-Sir-bound *GAL10* locus. Furthermore, mutations in the replisome components *DPB3* and *MCM2* severely reduce local histone inheritance at *GAL10,* but do not cause complete loss of epigenetic memory at silent loci, calling into question the model that local histone inheritance is required for epigenetic inheritance. A major open question is whether the mechanisms of histone position memory at *GAL10* function similarly at the silent locus *HML*.

I found that histones are locally inherited at *HML* in wild-type cells and that this inheritance seems to be weakened in *sir* mutant cells. In the course of these experiments, I observed a synthetic sickness that occurs when cells lack both the histone inheritance factor Dpb3 and the histone deacetylases Hst3 and/or Hst4, which remove a histone modification that marks newly-synthesized histones. A genetic screen for suppressors of this synthetic phenotype yielded mutations in many proteasome subunits, suggesting that aberrant degradation of some protein, potentially H3 itself, is responsible for the sickness in these cells. The work on both local histone inheritance at *HML* and the interaction between *DPB3* and *HST3/4* is ongoing.

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Acknowledgments

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Chapter 1:

Introduction to silencing and nucleosome dynamics in *Saccharomyces cerevisiae*

The trillions of cells in a human all harbor essentially identical genetic codes in their DNA. That is to say, the many different types of cells found throughout the body all have the same set of inherited "instructions" to perform the disparate functions needed to sustain life. The organism must therefore ensure that only the appropriate information is decoded in any given cell type, such that each cell performs its proper function—transmitting action potentials in the case of neurons, secreting insulin in the case of pancreatic beta cells, etc.—but not the functions of other cell types. The mechanisms by which cells decode specific information to drive function constitute the field of "gene regulation."

Gene regulation occurs at many levels, but the most fundamental node of control is transcription. In general, if a protein-coding gene is transcribed by RNA polymerase II, the transcript will subsequently be translated by the ribosome and give rise to a protein product. In eukaryotes, transcriptional regulation depends both on DNA-binding transcription factors which bind target sequences and function to promote or repress transcription of a given gene, and the broader chromatin context in which a gene is located, including particular histone modifications and nucleosome-binding proteins. In some eukaryotes, transcriptional regulation is even further complicated by the presence of RNA interference (RNAi)-driven chromatin regulation and DNA methylation, which add additional levels of control to gene expression. Evolution will make use of any strategy of regulation that increases the fitness of the organism, guided only by the utility that allowed its selection. Molecular biologists have made great strides to understand gene regulation via reductionism. We hope that in studying the constituent pieces of a dizzying biological puzzle, the sum of knowledge about the parts leads us closer to an understanding of the whole. Studying transcriptional regulation in the budding yeast *Saccharomyces cerevisiae* is an explicitly reductionist approach: yeast cells are able to regulate their genomes dynamically using a markedly simpler toolkit than that of animals. The work presented in this dissertation is in service of that aim: to understand the mechanisms of gene regulation mechanisms in budding yeast, and in so doing gain insight into how the process works across eukaryotes.

1.1 Silencing in *Saccharomyces cerevisiae*

Approximately one billion years ago, a single cell underwent a division and produced two daughter cells. The descendants of one of those cells, after many more divisions, eventually gave rise to all animals, including humans, while the other cell's descendants gave rise to all fungi, including the budding yeast *Saccharomyces cerevisiae*. This last common ancestor of animals and fungi, had already evolved all of the necessary features for eukaryotic life. This unity at the foundation of eukaryotic life allows us to use *S. cerevisiae* as a model organism to better understand the biology of humans. Our human-centric worldview leads us to imagine that the ancestral cell was simple, probably not unlike a modern yeast cell, and that the emergence of multicellularity and eventually humanity was accompanied by the evolution of complex new proteins to perform "higher" functions. To some degree this is true: there are certainly molecules and processes observed in specialized cells of plants and animals that are found nowhere else in

biology. But this common perception of evolution is at its core incorrect: evolution is not a march from simplicity toward complexity. The budding yeast has undergone just as much evolutionary change since the bifurcation point as we have. One clear illustration of this principle is evident when we compare the repressive chromatin structures formed in humans and in *Saccharomyces*.

Silent chromatin in *Saccharomyces cerevisiae* is functionally analogous to heterochromatin, a form of chromatin originally defined in other eukaryotes by morphological criteria. Heterochromatin and silent chromatin are both compact chromatin structures that reduce DNA accessibility to protein binding and thus antagonize transcription. In many eukaryotes, most notably animals, heterochromatin occurs in two flavors. "Constitutive" heterochromatin forms over domains of repetitive DNA and represses transcription and recombination for the life of the organism (Elgin and Reuter, 2013). In contrast, "facultative" heterochromatin forms over dynamically regulated blocs of genes to repress transcription only during particular stages of development (Simon and Kingston, 2009). These forms of heterochromatin are defined by specific histone modifications, as well as the enzymes that deposit those modifications, and proteins that are recruited by the modifications. Interestingly, homologs of many of the key proteins involved in constitutive and facultative heterochromatin—namely the methyltransferases Su(var)3-9 and E(z), and the histone-binding heterochromatin protein 1 (HP1)—are inferred to have been present in the last common ancestor of yeast and metazoans (Talbert et al., 2019). Also present in this ancestral cell was the machinery for RNA interference (RNAi) and DNA methylation, both of which play major roles in heterochromatin-mediated gene repression in diverse organisms (Iyer et al., 2011; Martienssen and Moazed, 2015). Thus, the last common ancestor of budding yeast and humans likely had a form of repressive chromatin that was quite similar at the molecular level to the heterochromatin seen today in humans and many other modern animals and plants. However, in that lineage that gave rise to *Saccharomyces*, something striking happened: the ancestral genes for DNA methylation, RNAi, constitutive heterochromatin, and facultative heterochromatin were all lost. In their place, the budding yeast built a new molecular system for accomplishing much the same task: silent chromatin.

Why would a cell constitutively repress any genetic information? In the case of *S. cerevisiae*, repression of the mating type loci *HML* and *HMR* is necessary for cells to mate (Herskowitz et al., 1992). Yeast have both a sexual and a nonsexual life. In the sexual life cycle, there are two mating types, **a** and α[1]. Haploid **a** cells mate with haploid α cells, and the resulting diploid **a**/α cells are sterile, but can undergo meiosis to produce haploid progeny. Mating types, analogous to biological

[1] Why were the yeast mating types named **a** and α, two letters that appear nearly identical in many typefaces and require switching between Greek and English keyboards to type? We can thank two of the heroes of yeast genetics, Gertrude and Carl Lindegren, for this frustrating nomenclature, but to my knowledge, no evidence exists as to why these names were chosen. In the two decades after Winge's 1935 description of the yeast life cycle, various naming conventions were used for mating types, including + and – in the Lindegrens' first paper on the subject in May 1943 (Lindegren and Lindegren, 1943a). This is the same naming system they had used to describe *Neurospora* mating types, which they had studied before turning to yeast. Within half a year, though, they had begun referring to the two yeast mating types "provisionally" as **a** and α, and the rest is history (Lindegren and Lindegren, 1943b). They may have been influenced by George Beadle's *Neurospora* nomenclature, which used the letters A and a to refer to the mating types. My best guess, purely conjecture, is that by late 1943 it was clear to the Lindegrens that (1) using + and – to refer to mating-type alleles across different organisms would eventually lead to confusion and that (2) using a convention like Beadle's A/a could also create confusion, since capital/lowercase distinctions were already in use to distinguish dominant/recessive or wild-type/mutant alleles. A pair of letters that appear similar to each other and are of the same case, like **a** and α, perhaps connote the symmetry and distinguishability of mating types.

sexes in multicellular organisms, are determined by whether the **a** or α master regulator genes are present at the *MAT* locus of chromosome III. Some wild strains of yeast can change mating types after cell division via the generation of a DNA double-strand break at the *MAT* locus and homology-directed repair from either the *HMLα* or *HMRa* locus (Haber, 2012; Strathern et al., 1982). Thus, in addition to a functional copy of the mating type information at *MAT*, cells must have extra copies of the mating type information elsewhere. If the master regulator genes encoded at *HML* or *HMR* were to be expressed, the cell would gain the **a**/α diploid gene expression pattern and thus be sterile. These extra copies of the mating information are therefore stably repressed, and this repression does not depend on the promoter sequence, since, e.g., *HMRa* and *MATa* share the same promoter but have different expression properties. Rather, the repression of *HML* and *HMR* is a property of the locus where the sequence resides.

The locus-specific repression of *HML* and *HMR* is mediated by the products of the Silent Information Regulator genes *SIR1-4*. Several genetic screens identified mutant alleles of these four genes through defects in mating, but their specific role in silencing was demonstrated in a genetic screen that searched for mutants capable of de-repressing *HML* and *HMR* (Rine and Herskowitz, 1987). Deletion of *SIR2, SIR3,* or *SIR4* leads to complete loss of silencing at *HML* and *HMR*, whereas deletion of *SIR1* causes a bistable phenotype wherein silent loci are either fully repressed or fully de-repressed in individual cells, with rare switches between the two epigenetic states (Pillus and Rine, 1989). The mechanisms by which the four proteins encoded by the *SIR* genes contribute to silencing has been the subject of intense study for over four decades. In addition to their effects at *HML* and *HMR,* Sir2/3/4 also drive a form of silencing at telomeres, and Sir2 also participates in a separate silencing complex at ribosomal DNA. These forms of silencing have been reviewed elsewhere (Gartenberg and Smith, 2016), and the work in this dissertation will focus on silencing at *HML* and *HMR*.

Sir proteins are recruited to regulatory sites termed silencers, which flank the *HML* and *HMR* loci. These sequences were identified by deletion analysis of plasmid-borne copies of *HML* and *HMR* (Abraham et al., 1984; Feldman et al., 1984). Two features of the silencers were quickly noticed: (1) some DNA sequence elements were found in multiple silencers and (2) the silencers all possessed autonomous replication (ARS) activity, meaning they could initiate DNA replication on a plasmid. Subsequent work explained the common sequence elements by identifying DNA-binding proteins that bound multiple silencers: all four silencers are bound by the Origin Recognition Complex (ORC), and each silencer is also bound by Repressor-activator protein Rap1 and/or ARS-binding factor Abf1 (Bell et al., 1993; Buchman et al., 1988; Foss et al., 1993; Kimmerly et al., 1988). Rap1 recruits both Sir3 and Sir4 to silencers via protein-protein interactions with the Rap1 C-terminal domain (Chen et al., 2011; Luo et al., 2002; Moretti et al., 1994; Moretti and Shore, 2001). Meanwhile, ORC recruits Sir1 to silencers via protein-protein interactions with the Orc1 subunit (Triolo and Sternglanz, 1996). In the case of Abf1, despite common claims in the literature that Abf1 recruits a Sir protein via a protein-protein interaction, to the best of my knowledge, this result has never been published[2]. It is possible that in addition to their roles in recruiting Sir proteins, silencer-binding factors also help create a silencing-conducive chromatin environment by depleting nucleosomes from silencers: in a systematic study of 104

[2] In looking backwards through references in search of a datum showing that Abf1 is involved in recruiting Sir proteins to *HMR* or *HML*, I arrive at the following from Gasser and Cockell, 2001: "Abf1p appears to act by direct interaction with Sir3p, to help nucleate mating type repression (P. Moretti and D. Shore, personal communication)"

yeast transcription factors, Rap1, Abf1, and Orc1 stood out as three of the proteins most capable of depleting nucleosomes from their vicinity (Yan et al., 2018). The capacity of nucleosome-depleted regions to position flanking nucleosomes and, in so doing, modulate silencing has been recently appreciated by work from Daniel Saxton's work (Saxton and Rine, 2020). The evolutionary co-option of these three proteins in silencing is notable: all three have essential functions outside of silencing, Rap1 and Abf1 as transcriptional activators and ORC as the initiator of DNA replication. The relationship between these proteins' silencing functions and other essential functions has been the focus of much research, including the open question of how Abf1 and Rap1 can serve as both activators and repressors depending on the context, and whether ORC's role in DNA replication and silencing are connected (described below in Section 1.2).

The mechanism of Sir protein recruitment to silencers via sequence-specific adaptor proteins is conceptually simple, but does not explain how Sir proteins repress transcription. The *HML* and *HMR* silencers are over a kilobase away from the promoters that they regulate, and thus any model for silencing requires an explanation for how Sir proteins can act at a distance to repress transcription. Sir-mediated repression was shown early to induce locus-wide alterations to the chromatin at *HML* and *HMR* (Gottschling, 1992; Loo and Rine, 1994; Nasmyth, 1982; Singh and Klar, 1992), which contributed to the idea that Sir proteins bind across the silent loci, not just at silencers (Hecht et al., 1995a). Chromatin immunoprecipitation (ChIP) studies of silencing revealed that Sir2, Sir3, and Sir4 bind across the silent domains, while Sir1 associates only with the silencers (Hecht et al., 1996; Hoppe et al., 2002; Rusché et al., 2002; Strahl-Bolsinger et al., 1997). The spread of Sir2/3/4 from the silencers is dependent on a complex network of protein-protein interactions among the Sir proteins (thoroughly reviewed in Gartenberg and Smith, 2016). As a central example, Sir4 binds to itself (Chang et al., 2003; Murphy et al., 2003), to Sir1 (Bose et al., 2004; Triolo and Sternglanz, 1996), to Sir3 (Chang et al., 2003; Liou et al., 2005; Moazed et al., 1997), and, as a stable heterodimer, to Sir2 (Hsu et al., 2013; Moazed et al., 1997). A key role for the histone H4 tail in silencing, and specifically for an interaction between Sir3 and deacetylated lysine residues of the H4 tail was demonstrated first genetically (Johnson et al., 1990; Kayne et al., 1988; Megee et al., 1990; Park and Szostak, 1990) and later via biochemistry that confirmed that Sir3 and Sir4 have high affinity for deacetylated histone tails (Carmen et al., 2002; Hecht et al., 1995a; Liou et al., 2005). These observations, coupled with the seminal discovery that Sir2 is an NAD-dependent histone deacetylase (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000) form the basis for our understanding of how Sir proteins "spread" across a locus (Hoppe et al., 2002; Rusché et al., 2002). Sir protein recruitment to silencers is thought to allow Sir2 to deacetylate adjacent or nearby nucleosomes at H4K16, which in turn favors binding of Sir3 and Sir4 to the deacetylated nucleosome. Because Sir4 and Sir2 form a complex, the binding of Sir4 to the deacetylated histone tail allows Sir2 to further deacetylate subsequent histones along the chromatin array.

While deacetylation of H4K16 is regarded as the defining histone modification associated with silencing, other histone-modifying enzymes have also been shown to regulate silent chromatin. Some of these associations are tenuous and might reflect indirect effects, given the elaborate crosstalk between different chromatin marks (Talbert and Henikoff, 2021). Generally, convincing claims of a direct connection between a given histone modification and silencing require *in vitro* data in addition to genetics, because mutant analysis changes the global pool of histones, making it nearly impossible to attribute phenotypes specifically to defects in the chromatin at the silent

loci. One histone modification that has *bona fide* effects on silencing is methylation of H3K79, which is deposited by the methyltransferase Disruptor of telomeric silencing, Dot1 (Singer et al., 1998; Van Leeuwen et al., 2002). Structural and biochemical studies have converged on a model wherein Dot1 and Sir proteins mutually antagonize each other by directly competing for shared binding surfaces on the nucleosome and via their respective histone modifications (Altaf et al., 2007; Armache et al., 2011; Valencia-Sánchez et al., 2021). Indeed, in a beautiful convergence of genetics and structural biology, the very point mutations in *SIR3* that suppressed the silencing phenotypes of H4 tail mutants, were identified decades later to lie at the interface that binds to unmethylated H3K79 (Armache et al., 2011). While H4K16 deacetylation is thought to be an essential step in the spread of Sir proteins across *HML* and *HMR*, whether other histone modifications need to be added or removed to facilitate Sir protein spread is an open question.

The nucleation and spread model provides a framework for understanding silencing, but there are major missing pieces in a complete description of the molecular mechanisms of *SIR*-mediated gene repression. One fascinating mystery, which forms the basis for the work described in Chapter 2 of this dissertation, is how the cell cycle regulates silencing establishment (further discussed in Section 1.2). Another is that, at a fundamental level, we cannot explain exactly how Sir protein binding represses transcription. This latter question can be divided into two parts, both of which are open areas of research: (1) which specific silencing-dependent molecular changes determine whether repression occurs? and (2) which steps in transcription are inhibited by silencing?

To the first question, it seems likely that both the binding of Sir proteins and the Sir2-dependent deacetylation of H4K16 are necessary for silencing, although the interdependence of those two processes makes it difficult to study one without the other. The combination of Sir protein binding and histone tail deacetylation might drive repression *per se*, but there is also evidence that Sir3 dimers form bivalent interactions with adjacent nucleosomes, driving a compaction of chromatin structure (Behrouzi et al., 2016; Swygert et al., 2018). In addition, multiple lines of evidence suggest that silent chromatin forms a higher-order chromatin structure. Most convincingly, DNA supercoiling assays show that *HMR* and *HML* have *SIR*-dependent changes in supercoiling that cannot be explained by the effects of transcription or histone acetylation (Bi and Broach, 1997; Cheng and Gartenberg, 2000). The supercoiling change in silent chromatin could be due to increased nucleosome density, but micrococcal nuclease mapping reveals that silencing leads to better positioning of nucleosomes, but does not change the total number or location of nucleosomes (Nasmyth, 1982; Thurtle and Rine, 2014; Weiss and Simpson, 1999). To date, no studies have conclusively determined whether changes in higher-order chromatin structure are required for gene repression, or are simply correlated with it. There is ample precedent for overzealous interpretations of correlations leading the silencing field astray. One notable example is that despite significant cell biology work on the localization of silent chromatin to the nuclear periphery, this subnuclear localization is not required for silencing (Gartenberg et al., 2004).

To the second question, much work has gone into identifying the specific steps of transcription that are inhibited by silencing, but the results have been contradictory. Nasmyth showed early that several DNase I hypersensitive sites at *HML* and *HMR* are regulated by the *SIR* genes, including, notably, one that fell at the site of HO endonuclease activity, which could explain *HML* and *HMR*'s resistance to HO cutting (Nasmyth, 1982). Subsequently, it was shown that silenced chromatin is generally resistant to DNA methylases and endonucleases, which formed the basis for the "steric

occlusion" model of silencing, wherein Sir proteins function to prevent access of DNA-binding proteins to their targets (Gottschling, 1992; Loo and Rine, 1994; Singh and Klar, 1992). However, one of those initial studies also found that reduced methylase accessibility was a general feature of repressed loci (i.e., uninduced *GAL10*, *PHO5*, etc.), and not specific to silent chromatin, which could suggest that such accessibility reductions are not due specifically to Sir-mediated occlusion (Singh and Klar, 1992). A major limitation of the occlusion model is that silent chromatin is clearly not completely refractory to all DNA transactions, such as replication or homologous recombination. Further counterevidence to the occlusion model was provided by ChIP studies that measured the effects of silencing on the recruitment of transcription machinery to chromatin (Chen and Widom, 2005; Gao and Gross, 2008). While these two studies reached different conclusions as to the specific step in transcription that was inhibited—respectively PolII binding itself (Chen and Widom, 2005) or transcription elongation (Gao and Gross, 2008)—they both demonstrated that some activating proteins could access silent chromatin, meaning that silencing does not prevent transcription by broadly preventing all DNA-protein interactions. The inconsistency of the previous results, not to mention the drastic technological improvements that have occurred in the intervening years, makes this question an appealing target for future work.

Silent chromatin has long been known to have dynamic properties (Cheng and Gartenberg, 2000), and the field would be well-served by developing models for repression that account for our current view of the highly dynamic nature of transcription (Liu and Tjian, 2018). In particular, there has been scant investigation into the dynamics of Sir protein association with chromatin. One speculative framework for thinking about silencing is in terms of the ON and OFF rates with which Sir proteins and transcription-favoring proteins associate with chromatin. Silencing and transcription could mutually inhibit each other by modulating the ON/OFF rates for protein-chromatin interactions, via histone modifications and direct competition for binding surfaces on chromatin.

1.2 Regulation of silencing establishment by the cell cycle

So far in describing silencing, I have invoked the idea of a stepwise process of silent chromatin "establishment" whereby Sir proteins are recruited to silencers, spread across *HML* and *HMR,* and repress transcription. For most of the studies referenced heretofore, the idea of "establishment" was an abstraction, in that the experiments were actually steady-state measurements of the phenotypes of mutant cells or biochemical interactions that differed between non-silenced and silenced loci. This abstraction is partially because in wild-type cells, silencing is constitutive, save for rare, transient losses of silencing (Dodson and Rine, 2015). Thus "silencing establishment" does not really *happen* in the same sense that heterochromatin formation *happens* in animals, wherein at one stage of development heterochromatin doesn't exist, and then at some later stage it does. In contrast, for a wild-type yeast cell, we should really think of silencing as a process of constantly reinforcing an already-established repressive domain. We can however, force cells to undergo silencing establishment, through the use mutant conditions that either destabilize silencing in a way that causes losses and gains of silencing to occur (e.g., *sir1*Δ) or allow for inducible silencing (e.g., temperature-sensitive *sir* alleles). The latter strategy has proved especially fruitful in allowing for precise experimental control over silencing establishment, which allows us to study whether the model of silencing presented above is consistent with the actual dynamic process of building a silent chromatin domain.

The discovery of ARS sequences in the silencers of *HML* and *HMR* inspired Miller & Nasmyth to study the relationship between DNA replication and silencing (Miller and Nasmyth, 1984). Strikingly, they showed that cells arrested in G1 of the cell cycle were unable to repress *HMLa* or *HMRa* when switched from the non-permissive temperature to the permissive temperature for the temperature-sensitive *sir3-8* allele. Furthermore, they showed that when cells passed from G1 to early S phase they were similarly unable to repress transcription, but that when cells were allowed to pass from G1 to early M, repression was possible. They concluded that DNA replication of *HMR* was somehow necessary for silencing to be established at that locus. The early ideas for how DNA replication might regulate silencing establishment revolved around replication creating a transiently opened or otherwise different property at a locus, and thus altering the binding properties of some transcriptional activator or repressor.

The following decade was a heyday for studies of eukaryotic DNA replication initiation, enabled in part by the development of 2D gel electrophoresis methods to directly study origins on plasmids and in chromosomes (Brewer and Fangman, 1987; Huberman et al., 1988) and the identification of ORC as the ARS-binding eukaryotic DNA replication initiator (Bell and Stillman, 1992). In line with the known role of ARS sequences in silencer activity, it was quickly shown that in addition to promoting DNA replication, ORC also had a key role in transcriptional silencing (Foss et al., 1993; Micklem et al., 1993). But explanations for how DNA replication could drive silencing establishment were still lacking, and the ORC findings prompted particularly fanciful ideas related to origin firing and replication fork passage leading to spread of Sir proteins from silencers. The discovery that ORC binds to Sir1 provided a hypothesis that cell-cycle-regulated ORC-Sir1 interactions could drive silencing establishment (Triolo and Sternglanz, 1996). However, the same study showed that ORC was dispensable for silencing if Sir1 was artificially recruited to silencers, weakening the connection between silencing and DNA replication. The possibility that ORC was involved in conferring the DNA replication requirement for silencing establishment was conclusively rejected in a study showing that when ORC binding sites were completely removed from *HMR* and a synthetic Gal4-Sir1 fusion protein was used to nucleate Sir proteins, silencing still occurred and indeed still required S phase for establishment (Fox et al., 1997). Contemporaneous genetic experiments demonstrated that the role of ORC in promoting silencing was separable from its role in promoting replication initiation (Dillin and Rine, 1997). Thus, the hunt continued for how DNA replication might promote silencing establishment, without ORC as a key player.

Back-to-back papers published in 2001 completely broke the paradigm of the field of silencing establishment by demonstrating that Miller & Nasmyth's presumed DNA replication requirement did not in fact require DNA replication (Kirchmaier and Rine, 2001; Li et al., 2001)[3]. In both studies, *HMR* was excised from the chromosome using site-specific recombination and the resulting DNA episomes, which lacked origins of replication, were assayed for silencing establishment. Surprisingly, silencing establishment could occur on these non-replicating episomes to the same extent as it could on the chromosome, and this establishment still required

[3] Ann Kirchmaier's paper will always be one of my favorites for its succinctness and elegance, which are reflected in its title, "DNA Replication-Independent Silencing in *S. cerevisiae*." I would like to officially record here that my own paper on the subject, "S-phase-independent silencing establishment in *Saccharomyces cerevisiae*," was named in homage to it.

progression through S phase. Thus, DNA replication of *HMR* was not required for silencing establishment. Instead, some other S-phase-dependent process was.

Around the same time that DNA replication was shown not to be essential for silencing establishment, the involvement of various replication-coupled chromatin assembly proteins on silencing was demonstrated (Enomoto and Berman, 1998; Tyler et al., 1999; Zhang et al., 2000). Dissecting the roles of these players, including the processivity clamp PCNA and the histone chaperones Asf1 and CAF-I, in silencing has proven difficult, given the subtle phenotypes of mutations in these genes and overlapping molecular roles of the proteins in chromatin assembly (thoroughly reviewed in Young and Kirchmaier, 2012). By and large, the phenotypes of chromatin assembly mutants have been studied outside the specific context of silencing establishment, except for one study that found PCNA mutants to have mild defects in cell-cycle-dependent silencing establishment (Miller et al., 2010). It seems likely that the subtle silencing phenotypes observed in cells mutant for chromatin assembly genes are due to the mis-regulation of specific histone modifications associated with newly-incorporated histones, and in at least some cases, the defect lies not in incorrectly incorporating silencing-refractory histones at silent loci, but rather in incorrectly incorporating silencing-competent histones throughout the genome (Miller et al., 2010, 2008; Young and Kirchmaier, 2012).

The finding that silencing establishment does not require DNA replication led to a surge of work on the topic, aiming to identify what non-replication S-phase process drives gene repression. Two follow-up studies looked at the potential role for steps beyond S phase in silencing establishment and made some fascinating observations, but reached different conclusions (Kirchmaier and Rine, 2006; Lau et al., 2002). Lau and colleagues concluded that both S-phase-dependent and M-phase-dependent processes drove silencing establishment, and, furthermore, identified the M-phase process as the dissolution of sister chromatid cohesion. In contrast, Kirchmaier and Rine found that S phase is the sole cell-cycle window required for robust gene repression. The differences in the way silencing was induced—the temperature-sensitive *sir3-8* allele for the Bell lab and the inducible fusion protein Gal4-Sir1 for the Rine lab—could explain the different results. In the Gal4-Sir1 induction strain *HMR-I* was deleted and the ORC binding site at *HMR-E* was replaced four Gal4 binding sites, thus removing ORC entirely from *HMR*. This could lead to artificially precocious silencing establishment by driving super-physiological recruitment of Sir1 to the four Gal4 sites or by removing an unidentified negative role of ORC for silencing. Meanwhile, the design of the *sir3-8* experiments did not allow any time for Sir3 to be re-synthesized before passage through S phase, and thus could have driven sub-physiological recruitment of Sir3 before the critical S phase window for silencing establishment. Kirchmaier & Rine conceded that cohesin could indeed have a negative effect on silencing establishment, but that in the case where Sir proteins are recruited before S phase, and thus before cohesin loading, this negative effect might be avoided. This possibility has yet to be rigorously tested (further discussed in Chapter 2).

Above and beyond its discussion of whether there are silencing establishment steps beyond S phase, the 2006 Kirchmaier & Rine study revealed a host of new data about how silencing establishment is limited by the cell cycle. One peculiar finding that remains unexplained is that when *HMR* is on an episome, it displays a precocious silencing phenotype, with the episomal form able to repress *HMR* by early S phase, while the chromosomal form requires passage through S

phase[4]. In light of the work described in Chapter 2, this could reflect a difference in histone turnover between chromosomal and episomal DNA, but that possibility has not been tested. The most striking finding of the study was that both Sir protein nucleation at silencers and spread across *HMR* could occur without cell-cycle progression. Critically, this spread of Sir proteins was associated with modest but incomplete deacetylation of histone tails. Thus, one possible mechanistic explanation of the S-phase step in silencing establishment was that a threshold level of histone deacetylation was not reached until after S phase. Alternatively, some unidentified S-phase-dependent step in silencing establishment may occur downstream of Sir protein binding to *HMR*. Another study analyzed the kinetics of silencing-relevant histone modifications during the course of silencing establishment and the effects of removing those modifications on Sir protein recruitment (Katan-Khaykovich and Struhl, 2005). The study showed that in cycling cells, histone deacetylation precedes depletion of the anti-silencing H3K79 methylation. Furthermore, while induction of silencing could drive deacetylation of histones on a replicating or non-replicating *HMR*, in agreement with the Kirchmaier result, depletion of Dot1-deposited H3K79 methylation could occur robustly only on replicating *HMR*. Together, these results strongly hinted that cell-cycle-dependent changes to histone modifications could be limiting in silencing establishment, but the work did not actually include any cell-cycle-controlled experiments, which limited the authors' ability to make the strongest versions of their claims.

The studies described so far all investigated silencing establishment at *HMR*. This focus is because strains in which wild-type *HMLα* is de-repressed lose sensitivity to α factor, and thus cannot be easily arrested in G1. Two groups were undaunted by this limitation and attempted to investigate silencing establishment at *HML*, and both reported the surprising finding that *HML* and *HMR* have fundamentally different cell-cycle requirements for silencing establishment (Lazarus and Holmes, 2011; Ren et al., 2010). In both cases, cells were arrested with hydroxyurea or nocodazole to obviate the need for α factor responsiveness, and in both cases, the authors claimed that *HML* was less dependent on cell-cycle progression for silencing establishment than *HMR*. Ren and colleagues attributed the difference between *HML* and *HMR* to *HML* having a weaker promoter than *HMR*, and thus being more amenable to silencing than *HMR*. In contrast, the Holmes group found that a tRNA gene adjacent to *HMR* conferred the cell cycle requirement for establishment. These two studies would seem to force a reappraisal of the mechanisms of silencing, and lead us to believe that the differences between *HML* and *HMR* are more profound than we had previously appreciated. However, both papers are subject to considerable technical limitations. In both cases, the authors failed to adequately control for the known negative autoregulation of *HMLα* via the a1/α2 transcriptional repressor (Siliciano and Tatchell, 1986). In addition, the reliance on non-quantitative RT-PCR by Lazarus & Holmes led to considerable experiment-to-experiment variability in the reported data, which could also have affected the major conclusions of the paper. Given the uncertainty regarding the conclusions of these two papers, it was important to consider whether the *HML* vs. *HMR* dichotomy held up to analysis using more modern, well-controlled assays (see Chapter 2 for a more thorough discussion).

[4] All cell-cycle arrest strategies have their own quirks that demand careful analysis and good controls. Hydroxyurea "arrests" at "early S phase" are particularly fraught, because hydroxyurea, an inhibitor of ribonucleotide reductase, does not actually *arrest* cells, but rather dramatically slows the progression of S phase via activation of multiple checkpoints (Alvino et al., 2007).

All of the work above led the field of silencing establishment to be somewhat impenetrable at the point when I began working on it in 2016. By accepting *prima facie* the arguments from the described studies, the unidentified S-phase-dependent step in silencing establishment should come downstream of Sir protein binding, act at *HMR* but not at *HML*, be alleviated in absence of the tRNA adjacent to *HMR*, and precede a separate cohesin-regulated step in silencing establishment. Theorizing about what possible mechanism could align with such disparate data proved dizzying. In Chapter 2, I will show that making progress in this field required first re-assessing some of these prior results, which resulted in a new view of silencing establishment that is at once simpler and fully supported by the data.

1.3 Histone dynamics and epigenetic inheritance

While Chapter 2 of this dissertation is concerned with the means by which the cell cycle promotes changes in chromatin state, in Chapter 3 I present work on how chromatin state might be preserved through the cell cycle. In the introduction to that chapter, I discuss the particular context of that work, but here I offer a brief perspective on the field of epigenetic inheritance of chromatin. The field of epigenetics, like the broader field of gene regulation, has its origin in the question of how an organism with only one set of genetic information can give rise to a variety of cell types with disparate forms and functions. Epigenetics specifically is concerned with the inheritance of gene regulation through cell division and even through generations.

The conflation of the terms "epigenetics" and "chromatin" is pervasive and pernicious (Deans and Maggert, 2015; Henikoff and Greally, 2016). One source of this issue is that "epigenetics" has come to have two related meanings, one strict and one broad. One articulation of the strict definition is that "an epigenetic trait is a *stably heritable* phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Berger *et al.*, 2009; emphasis added). The broader definition encompasses all mechanisms "that impart temporal and spatial control on the activities" of genes (Holliday, 1990). The key distinction falls in the heritability of a given gene regulatory mechanism. This may appear to be a minor distinction, but I believe that the dual use of "epigenetics" to refer to both the inheritance of an expression state and the sum of all forms of gene regulation has led many, including those in the field, to the unsupported conclusion that mechanisms of gene regulation are by and large heritable[5]. The corollary of this, given that chromatin modifications are a major contributor to gene expression, is that histone modifications are presumed *a priori* to be subject to mechanisms of inheritance, which is not generally true[6]. The modification cycle for most histone marks in most contexts is probably more like the following: (1) a sequence specific factor or factor associated with some DNA transaction—replication, transcription, damage, etc.—recruits an enzyme that deposits a histone modification; (2) effector proteins are recruited by the mark to perform some function; and (3) after the inciting recruitment factor is removed, the mark is eventually lost through histone turnover or enzymatic removal. In this context, the *information* associated with gene regulation lies not with the modified histone,

[5] Philosophers of language discuss the hypothesis of "linguistic relativity" or Whorfianism, which posits that the language we use to describe an object has a major role in our understanding of the object itself. I cannot vouch for the overall validity of Whorfianism, but it seems quite true in looking at the use of the term "epigenetics."

[6] This perspective is likely also influenced by overzealous analogy to DNA methylation, which is epigenetically propagated based on the activities of the hemi-methylation-specific DNA methylases in prokaryotes and the so-called "maintenance" methylases in eukaryotes.

but with the inciting modifier, such that after cell division, the chromatin state will only be maintained if the inciting modifier is present in the daughter cell.

Notwithstanding all of the above caveats, some chromatin-mediated gene regulation does indeed display epigenetic characteristics. In fact, the *sir1*Δ phenotype in *S. cerevisiae* (Pillus and Rine, 1989) is a model chromatin-mediated epigenetic phenomenon, and active research is ongoing as to the molecular nature of the epigenetic memory in *sir1*Δ cells (Saxton and Rine, 2019 and unpublished). Even in the case of chromatin-mediated epigenetics, the direct role of the histone modifications in carrying epigenetic information has not been well supported in most cases. Indeed, for a histone modification to be truly epigenetic, two strict preconditions must be met. First, a single protein module must contain both a "reader" domain that recognizes a modification and a "writer" enzymatic domain that deposits the same modification on nearby histones, such that after DNA replication, the modified histones that were passed from one generation to the next can also form a template for propagation of the state. This condition is met by the enzymes that deposit H3K9 methylation in fission yeast and humans, and the propagation of this mark can occur epigenetically (Audergon et al., 2015; Ragunathan et al., 2015). The second condition is that the genomic location of a modified histone before DNA replication is remembered after DNA replication. Otherwise, even if there were a self-templating read-write process that could spread old modification information to new histones, the old information would be dispersed in the genome, not retained at the particular locus where it served to modulate gene expression. Comparatively little study has gone into this process, although Gavin Schlissel found while in the lab that in yeast, histones do remember their locations after DNA replication to some degree (Schlissel and Rine, 2019). The work presented in Chapter 3 aims to expand on that finding, with the specific question of whether chromatin modifications modulate the local inheritance of histone molecules.

Chapter 2:

S-phase-independent silencing establishment in *Saccharomyces cerevisiae*[1]

2.1 Abstract

The establishment of silent chromatin, a heterochromatin-like structure at *HML* and *HMR* in *Saccharomyces cerevisiae*, depends on progression through S phase of the cell cycle, but the molecular nature of this requirement has remained elusive despite intensive study. Using high-resolution chromatin immunoprecipitation and single-molecule RNA analysis, we found that silencing establishment proceeded via gradual repression of transcription in individual cells over several cell cycles, and that the cell-cycle-regulated step was downstream of Sir protein recruitment. In contrast to prior results, *HML* and *HMR* had identical cell-cycle requirements for silencing establishment, with no apparent contribution from a tRNA gene adjacent to *HMR*. We identified the cause of the S-phase requirement for silencing establishment: removal of transcription-favoring histone modifications deposited by Dot1, Sas2, and Rtt109. These results revealed that silencing establishment was absolutely dependent on the cell-cycle-regulated interplay between euchromatic and heterochromatic histone modifications.

2.2 Introduction

Inheritance of gene expression state often accompanies the inheritance of genetic content during cell division. Indeed, the eukaryotic replication fork plays host to the enzymes needed to replicate DNA as well as intricate machinery that reassembles chromatin in the wake of replication. However, during development, cell division is also coupled to the rewiring of gene expression patterns that lead to the generation of new cell types. An understanding of chromatin and epigenetics requires an understanding of the mechanisms by which cells can both faithfully transmit chromatin state through cell division, and subvert that inheritance to establish new cell types. The silent chromatin controlling mating-type identity in *Saccharomyces cerevisiae* offers a tractable context for exploring how cell-cycle-regulated chromatin dynamics lead to the establishment of new expression states.

The maintenance of the correct mating type in *Saccharomyces* relies on both the expression of the **a** or α mating-type genes at the *MAT* locus and the heterochromatin-mediated silencing of copies of those same genes at *HML* and *HMR* (Herskowitz, 1989). Silencing is dependent on the Silent Information Regulator genes, *SIR1-4*, whose study has led to an understanding of how silencing is achieved (Gartenberg and Smith, 2016; Rine and Herskowitz, 1987). *HML* and *HMR* are flanked by DNA sequences termed silencers, which recruit the DNA-binding proteins Rap1, Abf1, and ORC. These in turn recruit the Sir proteins via protein-protein interactions. Sir protein recruitment to silencers is followed by the spread of Sir proteins across the multi-kilobase loci by iterative cycles of deacetylation of the tails of histones H3 and H4 by Sir2 and binding of Sir3 and Sir4 to those deacetylated histone tails (Hecht et al., 1995b; Hoppe et al., 2002; Rusché et al., 2002).

Despite decades of work, a longstanding puzzle remains at the heart of the mechanism of silencing: cells must pass through S phase to establish silencing, but the identity of the elusive cell-

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cycle-dependent component is unknown (reviewed in Young and Kirchmaier, 2012). Cells with a temperature-sensitive *sir3-8* allele arrested in G1 cannot repress *HMRa1* when switched from the non-permissive temperature to the permissive temperature, but can when allowed to progress through the cell cycle (Miller and Nasmyth, 1984). DNA replication *per se* is not required for silencing establishment. Excised DNA circles bearing *HMR* but no origin of replication, can be silenced if allowed to pass through S phase (Kirchmaier and Rine, 2001; Li et al., 2001). Thus, some feature of S-phase, but not DNA replication itself, is crucial for silencing establishment.

Interestingly, low-resolution chromatin immunoprecipitation (ChIP) studies showed that Sir protein recruitment to *HMR* can occur with or without cell-cycle progression, suggesting that Sir protein binding and silencing are not inextricably linked (Kirchmaier and Rine, 2006). If Sir proteins can bind to a locus but not silence it, then other molecular changes must be required to create silencing-competent chromatin. In cycling cells undergoing silencing establishment, removal of histone modifications associated with active transcription occurs over several cell cycles (Katan-Khaykovich and Struhl, 2005). Furthermore, deletion of genes encoding enzymes that deposit euchromatic histone marks modulates the speed of silencing establishment in cycling cells (Katan-Khaykovich and Struhl, 2005; Osborne et al., 2009), suggesting that removal of these marks is a key step in building heterochromatin. It is unknown whether the removal of euchromatic marks is related to the S-phase requirement for silencing establishment.

To better understand how chromatin transitions from the active to repressed state are choreographed, we developed an estradiol-regulated Sir3 fusion protein which, combined with high-resolution ChIP and RNA measurements, allowed precise experimental analysis of silencing establishment with single-cell resolution. We characterized the molecular changes that occur during silencing establishment and identified the genetic drivers of the S-phase requirement for silencing establishment.

2.3 Materials and Methods

Yeast strains

Strains used in this study are listed in **Tabe 2.1**. All strains were derived from the W303 background using standard genetic techniques (Dunham et al., 2015; Gietz and Schiestl, 2007). Deletions were generated using one-step replacement with marker cassettes (Goldstein and McCusker, 1999; Gueldener, 2002). The *sir3-8* allele was introduced by a cross to the strain Y3451 (Xu et al., 2006). The tRNA gene *tT(AGU)C* was seamlessly deleted using the "delitto perfetto" technique as described previously (Storici and Resnick, 2006). The *MCD1-AID* strain was generated by first inserting *O.s.TIR1* at the *HIS3* locus by transforming cells with PmeI-digested pTIR2 (Eng et al., 2014). Then, *3xV5-AID2:KanMX* was amplified from pVG497 (a gift from Vincent Guacci and Douglas Koshland) with primers that included homology to *MCD1*, followed by transformation. The mutant allele *HML** was synthesized as a DNA gene block (Integrated DNA Technologies) and integrated using CRISPR-Cas9 technology as previously described (Brothers and Rine, 2019). The *EBD* sequence was amplified by PCR from *cre-EBD78* in the strain UCC5181 (Lindstrom and Gottschling, 2009) with primers that included homology to *SIR3,* then transformed using CRISPR-Cas9. Mutations of *HHT1* and *HHT2* were generated using CRISPR-Cas9, with oligonucleotide repair templates. For all mutant analyses, at least two independent transformants or meiotic segregants were tested.

Culture growth and cell-cycle manipulations

All experiments were performed on cells growing in yeast extract peptone + 2% dextrose (YPD) at 37°C, which led to more switch-like behavior for *SIR3-EBD* than growth at 30°C. For biological replicates, independent cultures were started from the same strain or from two isogenic strains. For steady-state measurements, cells were grown overnight in YPD, then diluted and grown in fresh YPD to a density of ~2-8 x 10^6 cells/mL. For cell-cycle control experiments, cells were grown overnight in YPD, followed by dilution and growth in fresh YPD for ≥ 3 hours until cultures reached a density of ~2 x 10^6 cells/mL. Then, α factor (synthesized by Elim Biopharmaceuticals; Hayward, CA) was added to a final concentration of 10 nM and the cultures were incubated for ~2 hours until >90% of cells appeared unbudded. For *rtt109*Δ strains, this incubation was ~3 hours. For experiments with prolonged α-factor arrests, additional α factor was added every ~2 hours to maintain the arrest. To release cells from α-factor arrest, protease from *Streptomyces griseus* (Sigma-Aldrich P5147; St. Louis, MO) was added to the media at a final concentration of 0.1 mg/mL. To re-arrest cells at G2/M, nocodazole (Sigma-Aldrich M1404) was added to a final concentration of 15 μg/mL. For *SIR3-EBD* induction, **β**-estradiol (Sigma-Aldrich E8875) was added to a final concentration of 50 μM from a 10 mM stock in ethanol. For Mcd1-AID depletion, 3-indoleacetic acid (auxin; Sigma-Aldrich I2886) was added to a final concentration of 750 μM from a 1 M DMSO stock. For *sir3-8* temperature shifts, cells were grown continuously at 37°C, then shifted to 24°C for the length of the experiment.

RNA extraction and RT-qPCR

For each sample, at least ~1 x 10^7 cells were collected by centrifugation and RNA was purified using an RNeasy Mini Kit (Qiagen 74104; Hilden, Germany), including on-column DNase digestion (Cat No. 79254), according to manufacturer's recommendations. 2 μg of RNA was reverse transcribed using SuperScript III reverse transcriptase (Thermo Fisher Scientific 18080044; Waltham, MA) and an "anchored" oligo-dT primer (an equimolar mixture of primers with the sequence $T_{20}VN$, where V represents any non-T base). qPCR was performed using the DyNAmo HS SYBR Green qPCR kit (Thermo Fisher Scientific F410L), including a Uracil-DNA Glycosylase (Thermo Fisher Scientific EN0362) treatment, and samples were run using an Agilent Mx3000P thermocycler. Oligonucleotides used for qPCR are listed in **Table 2.2**. cDNA abundance was calculated using a standard curve and normalized to the reference gene *ALG9*. Each reaction was performed in triplicate, and a matched non-reverse-transcribed sample was included for each sample.

Chromatin immunoprecipitation and sequencing

For MNase ChIP experiments, ~5 x 10^8 cells were crosslinked in 1% formaldehyde at room temperature for 60 minutes (**Figure 2.7A, 2.7B**) or 15 minutes (all other figures). Following a 5-minute quench in 300 mM glycine, cells were washed twice in ice-cold TBS and twice in ice-cold FA lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl, 1 mM EDTA, 1% Triton, 0.1% sodium deoxycholate) + 0.1% SDS + protease inhibitors (cOmplete EDTA-free protease inhibitor cocktail, Sigma-Aldrich 11873580001). Cell pellets were then either flash frozen or lysed. For lysis, cell pellets were resuspended in 1 mL FA lysis buffer without EDTA + 0.1% SDS and ~500 μL 0.5-mm zirconia/Silica beads (BioSpec Products; Bartlesville, OK) were added. Cells were lysed using

a FastPrep-24 5G (MP Biomedicals; Irvine, CA) with 6.0 m/s beating for 20 seconds followed by 2 minutes on ice, repeated 4 times total. Lysate was transferred to a new microcentrifuge tube, then spun at 4°C for 15 minutes at 17,000 rcf. The pellet was resuspended in 1 mL FA lysis buffer without EDTA + 0.1% SDS + 2 mM $CaCl_2$. The samples were incubated at 37°C for 5 minutes, followed by addition of 4 U MNase (Worthington Biochemical LS004798; Lakewood, NJ) per 1 x 10^7 cells. Samples were placed on an end-over-end rotator at 37°C for 20 minutes, followed by addition of 1.25 mM EDTA to quench the digestion. Samples were spun at 4°C for 10 minutes at 17,000 rcf, and the supernatant containing fragmented chromatin was saved.

For sonication ChIP experiments (**Figure 2.9** and **Figure 2.13** only), fragmentation was performed as above, except following lysis and spin-down, the pellet was resuspended in ~500 µL FA Lysis Buffer + 0.1 % SDS, split between two tubes and sonicated using a Bioruptor Pico (Diagenode Inc.; Denville, NJ) for 15 cycles of 30 seconds ON followed by 30 seconds OFF. Following sonication, samples were spun at 4°C for 10 minutes at 17,000 RCF and supernatants were re-pooled.

The fragmented chromatin was split, saving 50 µL as the input sample, and using the remaining ~900 µL for immunoprecipitation. IP for Sir4-13xMyc was performed using 50 µL Pierce Anti-c-myc magnetic beads (Thermo Fisher Scientific 88843) per sample. Beads were equilibrated by washing 5x in FA Lysis buffer + 0.1% SDS + 0.05% Tween, then resuspended in in 50 µL per sample of FA Lysis buffer + 0.1% SDS + 0.05% Tween. For Sir3-EBD, IP was performed using 25 µL of rabbit polyclonal anti-ERα (sc-8002; Santa Cruz Biotechnology, Inc.; Dallas, TX). For H3K79me3, IP was performed using 5 µL of rabbit polyclonal anti-H3K79me3 (C15410068; Diagenode). All immunoprecipitations were performed in an end-over-end rotator at 4°C overnight in the presence of 0.5 mg/mL BSA (NEB B9000S; Ipswich, MA). For the Sir3-EBD and H3K79me3 IPs, samples were incubated for 1 hour at 4°C with 50 µL Dynabeads Protein A magnetic beads (Thermo Fisher Scientific 10002D), equilibrated as described above for Anti-c-myc beads. The following washes were performed, placing each sample on an end-over-end rotator for ~5 minutes between each: 2x washes with FA Lysis + 0.1% SDS + 0.05% Tween; 2x washes with Wash Buffer #1 (FA Lysis buffer + 0.25 M NaCl + 0.1% SDS + 0.05% Tween); 2x washes with Wash Buffer #2 (10 mM Tris, pH 8; 0.25 M LiCl; 0.5% NP-40; 0.5% sodium deoxycholate; 1 mM EDTA + 0.1% SDS + 0.05% Tween); and 1x wash with TE + 0.05% Tween. Samples were eluted by adding 100 µL TE + 1% SDS to the beads and incubating at 65°C for 10 minutes with gentle shaking. The eluate was saved, and the elution was repeated for a total eluate volume of 200 µL. Input samples were brought to a total volume of 200 µL with TE + 1% SDS. IP and input samples were incubated with 10 µL of 800 U/mL Proteinase K (NEB P8107S) at 37°C for 2 hours, followed by overnight incubation at 65°C to reverse crosslinking.

DNA was purified using a QIAquick PCR purification kit (Qiagen 28104). Libraries were prepared for high-throughput sequencing using the Ultra II DNA Library Prep kit (NEB E7645L). For IP samples, the entire purified sample was used in the library prep reaction. For the input samples, 10 ng were used. Samples were multiplexed and paired-end sequencing was performed using either a MiniSeq or NovaSeq 6000 (Illumina; San Diego, CA).

Sequencing reads were aligned using Bowtie2 (Langmead and Salzberg, 2012) to a reference genome derived from SacCer3, modified to include the mutant *HML** and *mat*Δ. Reads were normalized to the non-heterochromatic genome-wide median (i.e., to the genome-wide median excluding rDNA, subtelomeric regions, and all of chromosome III). For the MNase ChIP-seq experiments, only mononucleosome-sized fragments (130-180 bp) were mapped. Analysis was performed using custom Python scripts and displayed using IGV (Thorvaldsdóttir et al., 2013).

For coverage calculations in **Figure 2.13**, read coverage for each gene was calculated using the bedcov function of SAMtools (Li et al., 2009), and then normalized to the length of each gene and mean coverage for all genes. Scatter plots were generated using ggplot2 (Wickham, 2016). All raw and processed sequencing data were deposited at the GEO under the accession number GSE150737.

Single-molecule RNA fluorescence in situ hybridization

For smRNA-FISH experiments, cells were grown and cell-cycle arrests were performed as described above. Preparation of samples for imaging was as previously described (Chen et al., 2018). The only modification from that protocol was the concentration of zymolyase used: for cycling cells, 5 µL of 1.25 mg/mL zymolyase-100T (VWR IC320932; Radnor, PA) was used during the spheroplasting; for arrested cells, 5 µL of 2.5 mg/mL zymolyase-100T was used. Probes were synthesized by Stellaris (Biosearch Technologies; Novato, CA). Probes for *cre* and *KAP104* were previously described (Dodson and Rine, 2015). The sequences the newly-designed probes for *a1* are listed in **Table 2.3**. Probes for *cre* and *a1* were coupled to Quasar 670 dye, while probes for *KAP104* were coupled to CAL Fluor Red 590 dye. Probes for *cre* and *KAP104* were used at a final concentration of 100 nM. Probes for *a1* were used at a final concentration of 25 nM.

Imaging was performed on an Axio Observer Z1 inverted microscope (Zeiss; Oberkochen, Germany) equipped with a Plan-Apochromat 63x oil-immersion objective (Zeiss), pE-300 ultra illumination system (CoolLED; Andover, UK), MS-2000 XYZ automated stage (Applied Scientific Instrumentation; Eugene, OR), and 95B sCMOS camera (Teledyne Photometrics; Tucson, AZ). The following filter sets were used: for Quasar 670, Cy5 Narrow Excitation (Chroma Cat No. 49009; Bellows Falls, VT); for CAL Fluor Red 590, filter set 43 HE (Zeiss); for DAPI, multiband 405/488/594 filter set (Semrock Part No. LF405/488/594-A-000; Rochester, NY). The microscope was controlled using Micro-manager software (Edelstein et al., 2014). Z-stack images were taken with a total height of 10 µm and a step size of 0.2 µm. Manual cell outlining and automatic spot detection was performed using FISH-quant (Mueller et al., 2013) and data was plotted using ggplot2 (Wickham, 2016). Representative images were generated using FIJI (Schindelin et al., 2012).

Protein extraction and immunoblotting

Protein extraction and immunoblotting were performed as previously described (Brothers and Rine, 2019). The primary antibodies used were 1:20,000 Rabbit anti-Hexokinase (Rockland #100-4159; Limerick, PA) and 1:2,500 Mouse anti-V5 (Thermo Fisher Scientific R960-25). The secondary antibodies used were 1:20,000 IRDye 800CW Goat anti-Mouse (Li-Cor; Lincoln, NE) and 1:20,000 IRDye 680RD Goat anti-Rabbit (Li-Cor).

Flow cytometry

For every cell-cycle experiment, an aliquot of cells was taken for flow-cytometry analysis of DNA content to monitor cell-cycle stage. Sample preparation and flow cytometry was performed as described previously (Schlissel and Rine, 2019).

2.4 Results

2.4.1 S phase as a critical window for silencing establishment

Previous studies of silencing establishment have used a variety of strategies to controllably induce silencing establishment, each with their own strengths and weaknesses (see, e.g., Miller and Nasmyth, 1984; Kirchmaier and Rine, 2001; Li, Cheng and Gartenberg, 2001; Lazarus and Holmes, 2011). We sought a new tool to induce silencing that would allow preservation of the structure of the silencers at *HML* and *HMR* and minimally perturb cell physiology upon induction. To do this, we fused the coding sequence of the estrogen binding domain (*EBD*) of the mammalian estrogen receptor α to *SIR3*, making *SIR3*'s function estradiol-dependent (**Figure 2.1A**; Lindstrom and Gottschling, 2009; Picard, 1994). Estradiol addition frees the EBD from sequestration by Hsp90, and hence the induction is rapid because it does not require new transcription or translation (McIsaac et al., 2011). *SIR3-EBD* strains grown without estradiol failed to repress *HMR*, mimicking the *sir3*Δ phenotype, while those grown with estradiol repressed *HMR* to a similar degree as wild-type *SIR3* strains (**Figure 2.1B**).

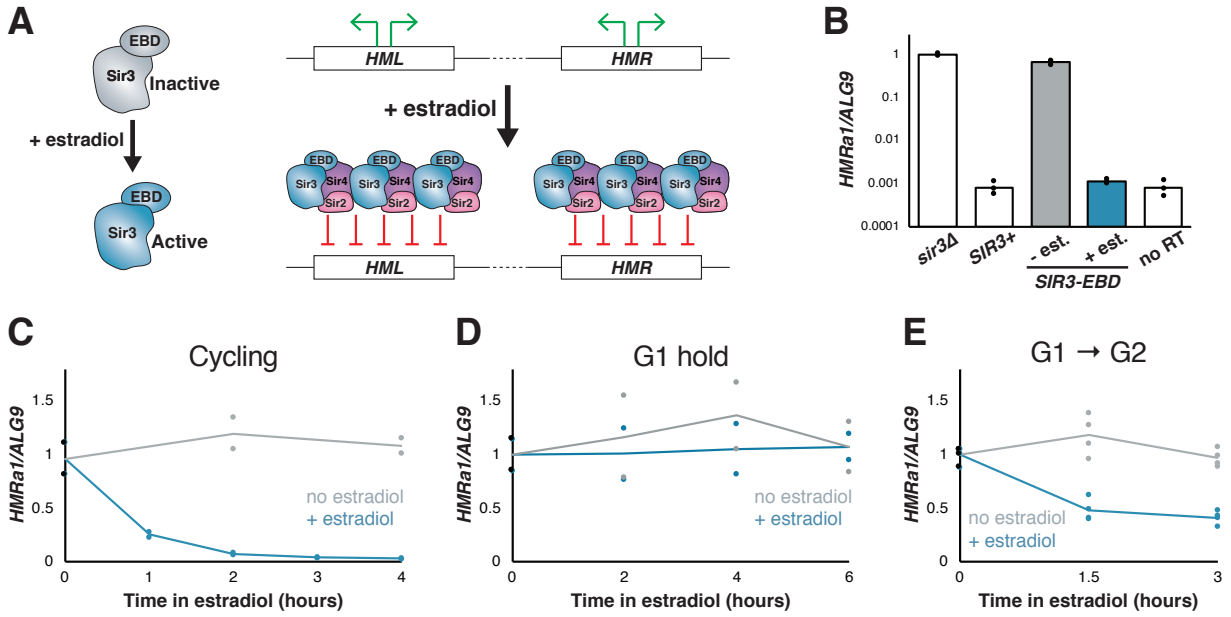

Figure 2.1: Silencing establishment using *SIR3-EBD* required S-phase progression. (A) Schematic for *SIR3-EBD* activation. When estradiol is absent, *SIR3-EBD* is kept inactive and *HML* and *HMR* are expressed. Upon addition of estradiol, *SIR-EBD* is activated and *HML* and *HMR* are repressed. **(B)** RT-qPCR of mRNA from *sir3*Δ (JRY12168), *SIR3*$^{+}$ (JRY12171), and *SIR3-EBD* (JRY12170) cells grown with ethanol (solvent control) or estradiol (N=3 for each condition). The *HMRa1*$^{\text{no RT}}$/*ALG9* value for *SIR3*$^{+}$ cells established that *SIR3*$^{+}$ cells and *SIR3-EBD* cells grown with estradiol silenced *HMRa1* to essentially the limit of detection. **(C)** *SIR3-EBD* cultures (JRY12169, JRY12170) were grown to mid-log phase, then split and grown in medium with either estradiol or ethanol added. Silencing was monitored by RT-qPCR in a time course after estradiol addition. t=0 represents the point of estradiol addition for this and subsequent experiments. **(D)** *SIR3-EBD* cultures (JRY12169, JRY12170) were arrested in G1 with α factor, then split, with either ethanol or estradiol added. The arrest was maintained for 6 hours, and silencing was assayed by RT-qPCR throughout. **(E)** *SIR3-EBD* cultures (JRY12169, JRY12170; 2 replicates of each genotype) were arrested in G1 with α factor, then split and released to G2/M by addition of protease and nocodazole in the presence of either ethanol or estradiol. In this and all subsequent figures, dots represent biological replicates, and the bars/lines represent the averages of biological replicates.

To test whether the *SIR3-EBD* allele retained the requirement for cell-cycle progression to repress *HMR*, estradiol was added to cells that were either cycling or arrested in G1 by α factor. In cycling cells, silencing establishment of *HMR* occurred gradually over several hours (**Figure 2.1C**). However, in cells arrested in G1, estradiol led to no measurable repression of *HMR*, even after many hours (**Figure 2.1D**). Thus, silencing of *HMR* could not occur without progression through the cell cycle, in agreement with prior results using other conditional alleles.

Prior work indicated that S phase is a critical window during which cells may undergo partial silencing establishment (Kirchmaier and Rine, 2001; Lau et al., 2002; Miller and Nasmyth, 1984). Consistent with this, when we arrested cells in G1, then induced *SIR3-EBD* and allowed them to proceed through S phase and re-arrested them at G2/M, *HMR* was repressed ~60% from its starting levels (**Figure 2.1E**). Crucially, the extent of this partial repression was stable over many hours in these G2/M-arrested cells. Thus, a repression-permissive window or event occurred between G1 and the beginning of mitosis that allowed partial silencing establishment, and further repression was not possible while arrested at G2/M. Indeed, after 3 hours in estradiol, cycling cells were repressed >20-fold from their starting value, compared to only ~3-fold for cells arrested after a single S phase (compare **Figures 2.1C** and **2.1E**). This requirement for multiple cell cycles to occur before full gene repression was consistent with prior studies of silencing establishment both in cell populations and at the single-cell level (Katan-Khaykovich and Struhl, 2005; Osborne et al., 2009).

Having established the validity of the *SIR3-EBD* fusion as a tool for studying silencing, we revisited two mutants that have been reported to bypass cell-cycle requirements for silencing establishment. In one study, depletion of the cohesin subunit Mcd1/Scc1 allowed for increased silencing in G2/M-stalled cells (Lau, Blitzblau & Bell 2002). In another study, deletion of a tRNA gene adjacent to *HMR*, termed *tT(AGU)C*, which is known to bind cohesin, was found to allow partial silencing establishment at *HMR* without S-phase progression (Lazarus and Holmes 2011). Using *SIR3-EBD* in combination with an auxin-inducible degron (AID)-tagged Mcd1, we found no effect of depleting cohesin or deleting the tRNA gene in regulating silencing establishment (**Figure 2.2**). Thus, at least for strains using *SIR3-EBD*, the genetic basis for the cell-cycle requirement for silencing establishment at *HMR* was unknown. Possible explanations for the discrepancies between our results and earlier reports are discussed below.

Our finding that *tT(AGU)C* did not regulate silencing establishment led us to reconsider the broader claim that *HMR* is distinct from *HML* in its requirement of S phase passage for silencing establishment (Lazarus and Holmes, 2011; Ren et al., 2010). Earlier silencing establishment assays at *HML* were complicated by the strong silencing-independent repression of *HMLα1* and *HMLα2* by the **a**1/α2 repressor (Herskowitz, 1989; Siliciano and Tatchell, 1986): when both **a** and α information are expressed in the same cell, the **a**1 and α2 proteins form a transcriptional repressor whose targets include the *HMLα* promoter. Unless strains are carefully designed, assays conventional measures of silencing will also inadvertently measure this silencing-independent repression. To circumvent this limitation, we constructed an allele of *HML* with nonsense mutations in both *α1* and *α2*, so that the α1 and α2 proteins were never made, even when *HML* was de-repressed. This modification also allowed us to use α factor to arrest cells while studying silencing establishment at *HML*, which was not possible before because expression of either the α1 or α2 protein renders cells insensitive to α factor. We also introduced additional single nucleotide polymorphisms into the regions of *HML* that are homologous to *HMR*, to allow unambiguous assignment of high-throughput sequencing reads to the two loci (see below). We refer to the mutant locus as *HML** and the mutant alleles as *hmlα1** and hml*α2** hereafter.

When cells with *HML** and *SIR3-EBD*, were arrested in G1 and then treated with estradiol, they were unable to silence *hmlα1** or *hmlα2** while kept in G1 (**Figure 2.3A**, **2.3B**). Interestingly, expression of *hmlα1** and hml*α2** increased markedly over the course of the α-factor arrest. This α-factor-dependent hyper-activation was observed even in *sir3*Δ cells in which no silencing occurs (**Figure 2.3E**, **2.3F**). We identified two previously-unreported binding sites for Ste12, the transcription factor activated by mating pheromone, in the bidirectional *α1*/*α2* promoter, which explains the increased expression when cells are exposed to α factor (**Figure 2.3G**; Dolan et al., 1989). Both *hmlα1** and *hmlα2** decreased in expression following release from G1 to G2/M (**Figure 2.3C**, **2.3D**), suggesting that S phase was required for partial silencing establishment at *HML*. Notably, the fold change in expression that followed a single passage through S phase was not identical among *HMRa1*, *hmlα1**, and *hmlα2**. Thus, some S-phase-dependent process was important for silencing both *HML* and *HMR*, but the effects of that process varied in magnitude between these two loci.

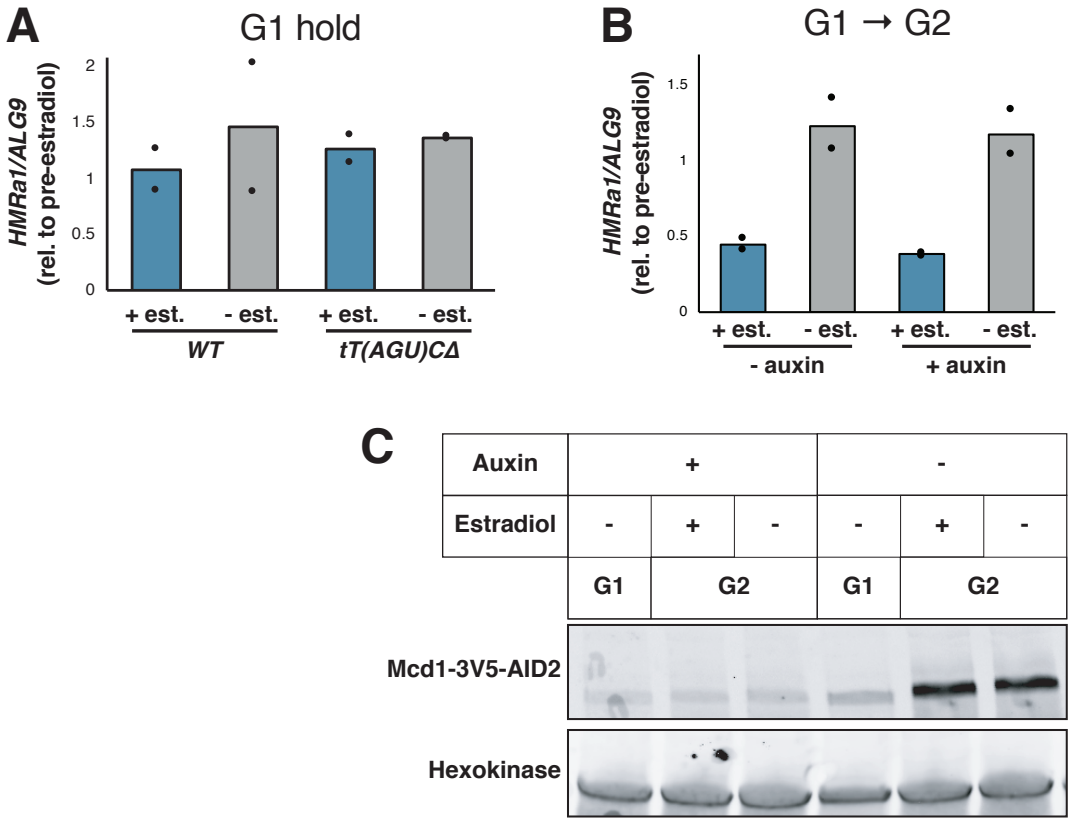

Figure 2.2: Effects of cohesin depletion and *tT(AGU)C* deletion on silencing establishment. (A) *SIR3-EBD* strains (JRY12269, JRY12270) and *SIR3-EBD* strains with seamless deletion of *tT(AGU)C* (JRY12267; JRY12268) were arrested in G1 with α factor, then split, with half the culture receiving estradiol and half receiving ethanol. Samples were collected after 3 hours for RT-qPCR, with each sample normalized to its own pre-estradiol value. **(B)** Cells with *MCD1-AID* (JRY12560, JRY12561) were arrested in G1 with α factor, then split, with half receiving auxin and the other half receiving DMSO (solvent control). After 30 minutes, each culture was further split, with half receiving estradiol and the other half ethanol. All cultures were released to G2/M by addition of protease and nocodazole. Cells were collected after 3 hours for RT-qPCR, with each sample normalized to its own pre-estradiol value. **(C)** Immunoblot analysis showing Mcd1-AID depletion for experiment described in (B).

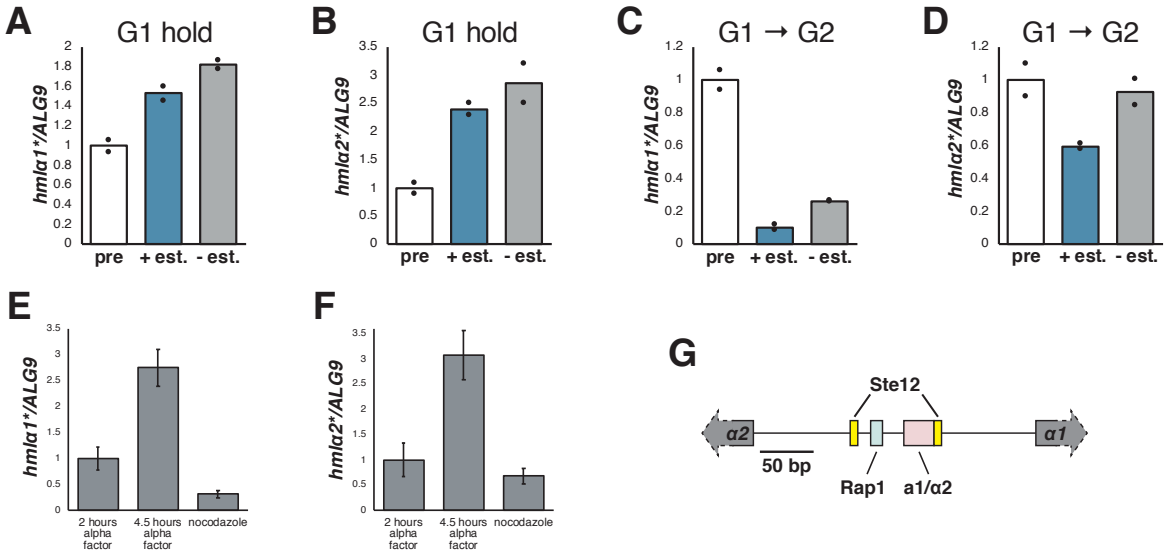

Figure 2.3: Silencing establishment required S phase at *HML. (A)** Cells with *SIR3-EBD* (JRY12169) were arrested in G1 with α factor, then split, with one sub-culture receiving estradiol and the other receiving ethanol. Cells were collected after 6 hours in estradiol and analyzed by RT-qPCR for *hmlα1**. **(B)** RT-qPCR for *hmlα2** in the same cells described in (A). **(C)** Cells were arrested in G1 with α factor, then split and released to G2/M with protease and nocodazole, with one sub-culture receiving estradiol and the other receiving ethanol. Cells were collected after 3 hours in estradiol and analyzed by RT-qPCR for *hmlα1**. The pre-estradiol samples for this experiment were the same as those described in (A). **(D)** RT-qPCR for *hmlα2** in the same cells described in (C). **(E)** Cells lacking *SIR3* (JRY11966) were arrested in α factor for 2 hours, then split, with half staying in α factor and the other being released to G2/M by addition of protease and nocodazole. Cells were analyzed by RT-qPCR for *hmlα1*,* with error bars representing standard deviation among technical replicates (N=1 biological sample). **(F)** RT-qPCR for *hmlα2** in the cells described in (E). **(G)** Map of the *α1/α2* promoter, showing newly-identified Ste12 motifs (TGAAACA) along with previously-identified binding sites for Rap1 and a1/α2.

2.4.2 Silencing establishment occurred through a partially repressive intermediate

We were interested in the partial silencing observed in cells that transited through a single S phase after *SIR3-EBD* induction, in which transcription of *HMRa1* was down ~60% (see **Figure 2.1E**). This appearance of a stable intermediate level of silencing could reflect either of two distinct phenomena at the single-cell level (**Figure 2.4A**). One possibility was that cells have a ~60% chance of establishing stable heterochromatin during the first S phase after *SIR3-EBD* induction and a ~40% chance of failing to do so. This possibility would resonate with the behavior of certain mutants, e.g. *sir1*Δ, wherein silent loci can exist in one of two epigenetic states: stably repressed or stably de-repressed, with rare transitions between the two (Pillus and Rine, 1989; Xu et al., 2006). Alternatively, every cell might reach a partially repressive chromatin state at *HMR* during the first S phase during silencing establishment.

To distinguish between these possibilities, we used single-molecule RNA fluorescence in-situ hybridization (smRNA-FISH) to quantify the expression of *HMRa1*during the establishment process. If silencing establishment proceeded via individual cells transitioning between the discrete "ON" and "OFF" states during S phase, we would expect an accumulation of cells with zero transcripts during the establishment of silencing, with no change in the average number of transcripts in those cells still expressing *HMRa1*. However, if silencing establishment proceeded

via partially repressive intermediates in individual cells, we would expect a shift downward in the mean number of transcripts per cell (**Figure 2.4A**). In both cases, cells with zero transcripts would accumulate over time.

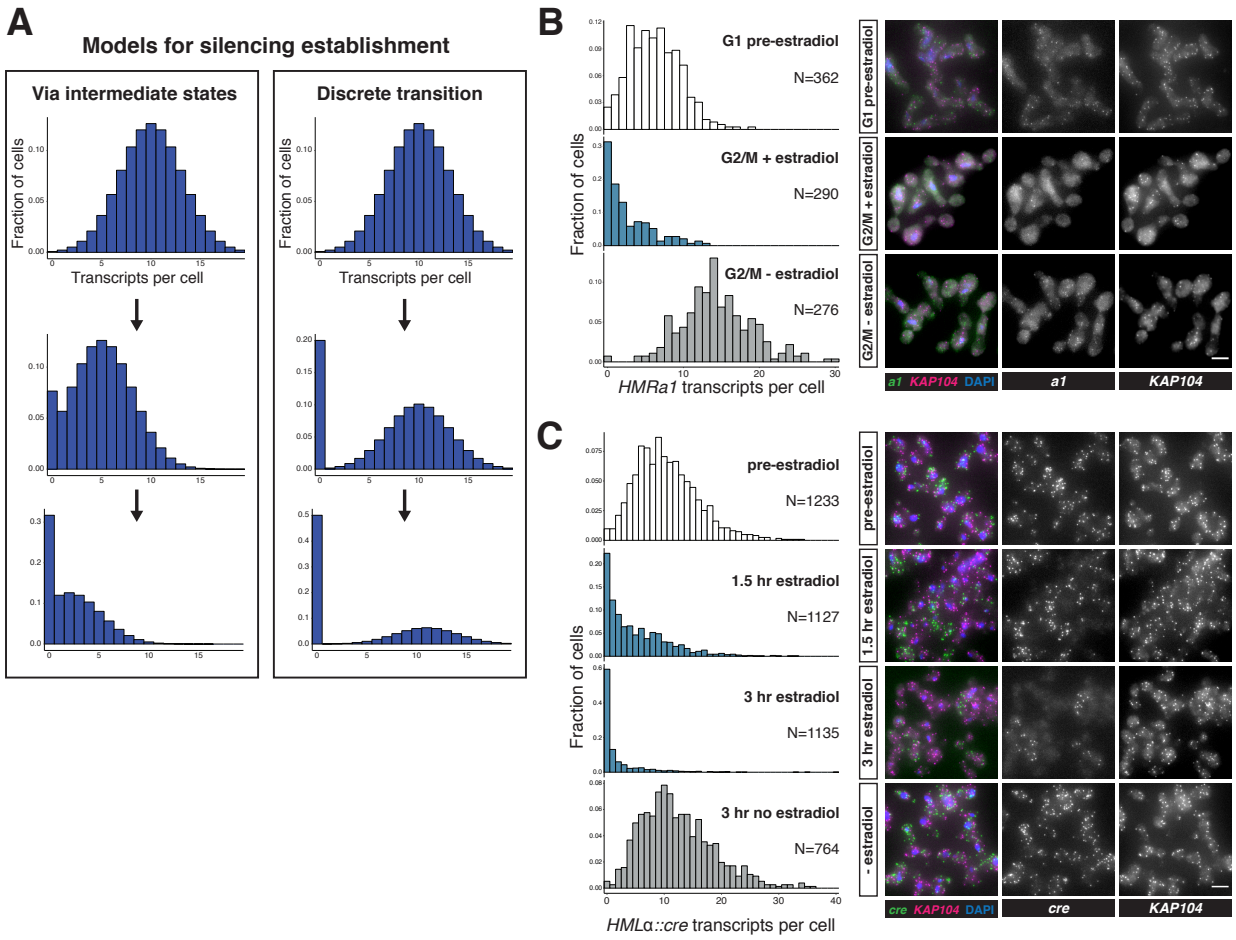

Figure 2.4: Silencing establishment proceeded via gradual repression in individual cells. (A) Potential models for silencing establishment. Before silencing establishment (top), mRNA transcripts are present as a distribution around a mean. If silencing establishment occurred via intermediate states (left), the mean number of transcripts per cell would decrease over time, with complete silencing, i.e., zero transcripts per cell, occurring as the probability distribution shifted toward the y axis. If silencing establishment occurred via discrete transitions (right), an increasing fraction of cells would have zero transcripts over time, but the distribution of cells with >0 transcripts would retain the same shape. **(B)** smRNA-FISH for *HMRa1* during silencing establishment after 1 S phase. A *SIR3-EBD* culture (JRY11762) was arrested in G1 with α factor ("G1 pre-estradiol"), then split and released to G2/M by addition of protease and nocodazole in the presence of either estradiol or ethanol. Samples were collected 2 hours after estradiol addition. **(C)** smRNA-FISH for *HMLα::cre* during silencing establishment in cycling cells. A *SIR3-EBD* strain bearing the *HMLα::cre* reporter (JRY12514) was grown to mid-log phase ("pre-estradiol"), then the culture was split in two, with one sub-culture receiving estradiol and the other receiving ethanol. Samples were collected for smRNA-FISH at t=1.5 hours and t=3 hours after estradiol addition. For both (B) and (C), the images displayed are representative maximum-intensity Z-projections. The data shown in (B) and (C) each represent one of two replicate experiments, for which the other replicate is shown in Figure 2.5 and Figure 2.6, respectively. Scale bars = 5 μm.

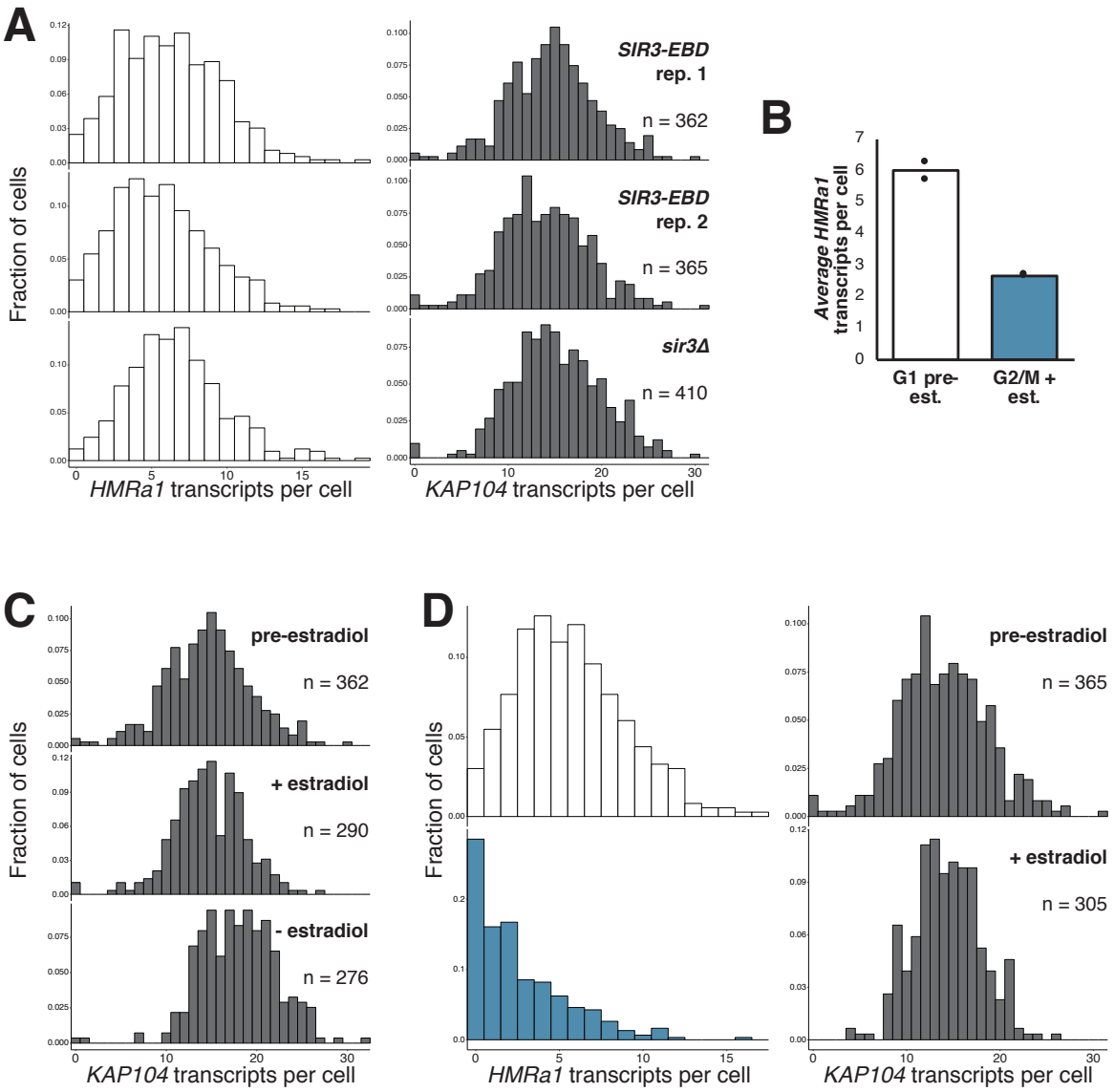

Figure 2.5: Gradual silencing establishment at *HMR*. (A) Quantification of *HMRa1* and *KAP104* transcripts in G1-arrested *SIR3-EBD* (JRY11762, JRY11763) and *sir3*Δ (JRY11966) cells. The *SIR3-EBD* data are the same as displayed in Figure 2.4 and Figure 2.5D. **(B)** Average number of *HMRa1* transcripts per cell before and after silencing establishment, quantified from data shown in Figure 2.4B and Figure 2.5D. Compare changes in mRNA levels to values from bulk measurement in Figure 2.1E. **(C)** Quantification of *KAP104* transcripts per cell for experiment described in Figure 2.4B. **(D)** Replicate experiment to that shown in Figure 2.4B with isogenic cells (JRY11763).

As expected, *SIR3-EBD* cells arrested in G1 without estradiol had similar numbers of *HMRa1* transcripts as *sir3*Δ cells (**Figure 2.5A**). When we added estradiol and allowed the cells to go through S phase to G2/M, the decrease in transcript number in the population of cells analyzed closely mirrored the results we obtained using RT-qPCR, confirming that our single-molecule analysis was consistent with bulk measurements (**Figure 2.5B**, compare to **Figure 2.1E**).

This decrease occurred via a reduction in the average number of transcripts per cell, and not simply by an increase in the number of cells with zero transcripts (**Figure 2.4B**, **Figure 2.5C, 2.5D**). Thus, individual cells undergoing silencing establishment at *HMR* formed partially repressive heterochromatin after a single S phase. To determine whether the stepwise repression seen at *HMR* was a general feature of silencing establishment or if it was particular to *HMR* and/or the cell-cycle conditions tested, we performed an analogous experiment using the *HMLα::cre* allele that has been previously characterized by smRNA-FISH (Dodson and Rine, 2015). In this experiment, we analyzed the number of *cre* transcripts per cell over time following induction of *SIR3-EBD*, without any cell-cycle perturbations. Silencing establishment at *HML* also occurred via partially repressive intermediate states (**Figure 2.4C**, **Figure 2.6A, 2.6B**). The degree of repression observed by smRNA-FISH was quantitatively similar to the measurement of the same gene by RT-qPCR (**Figure 2.6C, 2.6D**). Together, these results suggested that silencing establishment proceeded via the gradual repression of genes by the Sir proteins, and that there were specific windows of the cell cycle during which transcriptional tune-down could occur.

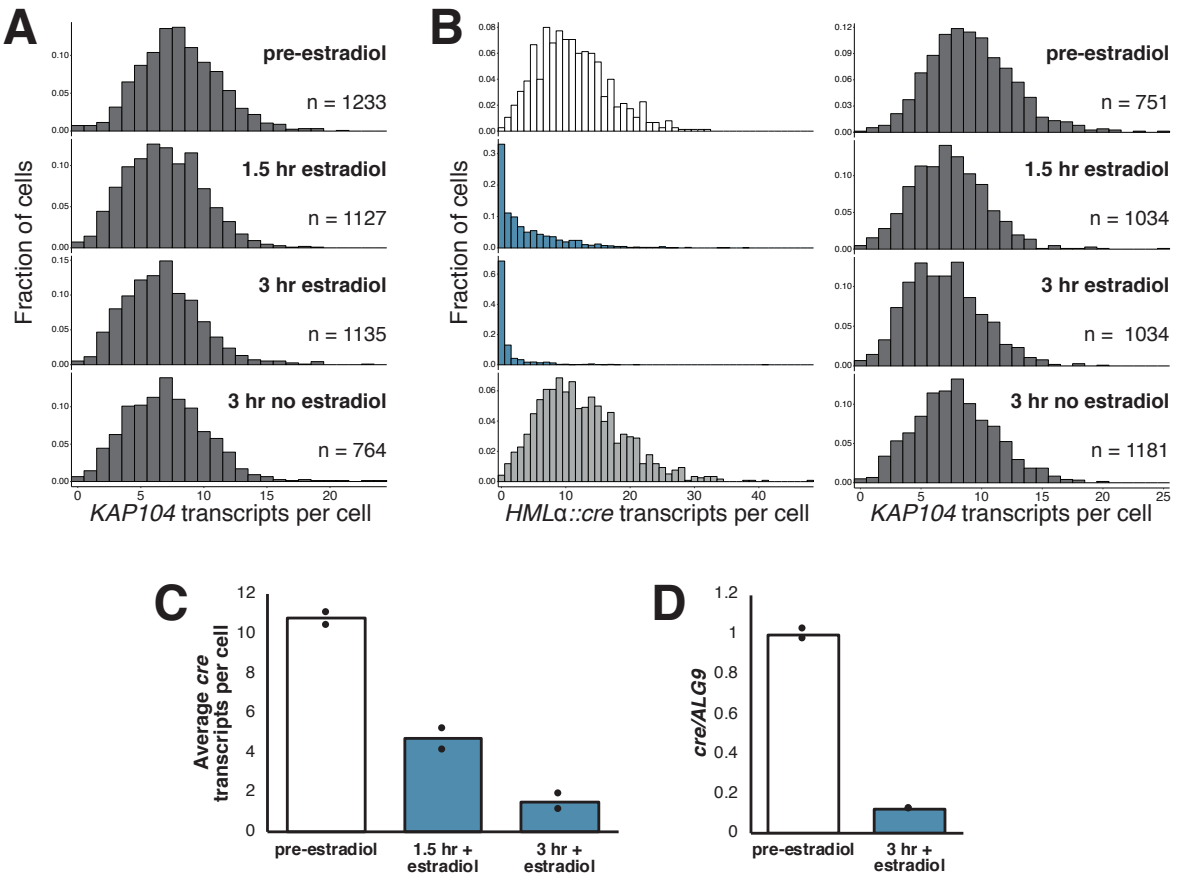

Figure 2.6: Gradual silencing establishment at *HMLα::cre*. (A) Quantification of *KAP104* transcripts per cell for experiment described in Figure 2.4C. **(B)** Replicate experiment to that shown in Figure 2.4B with isogenic cells (JRY12513). **(C)** Average number of *cre* transcripts per cell before and during silencing establishment, quantified from data shown in Figure 2.4C and Figure 2.6B. **(D)** Bulk measurement RT-qPCR for *cre* during silencing establishment in an analogous experiment to that described in Figure 2.4C.

2.4.3 Extensive Sir protein binding could occur without gene repression

The gradual silencing establishment described above might be achieved via increased Sir protein recruitment to *HML* and *HMR* during each passage through a specific cell-cycle window. Alternatively, Sir protein recruitment might be independent of the cell cycle, in which case passage through the cell cycle would favor repression via a step occurring after Sir protein recruitment. To test whether Sir protein recruitment was limited in the cell cycle, we performed ChIP-seq on myc-tagged Sir4 during silencing establishment. The tagged Sir4-myc is functional for silencing (**Figure 2.1B**), and its localization at *HML* and *HMR* is indistinguishable from Sir2-myc and Sir3-myc in wild-type cells (Thurtle and Rine, 2014).

We developed a protocol for ChIP-seq using MNase-digested chromatin, which resulted in increased signal-to-noise over standard sonication-based ChIP-seq (**Figure 2.7, Figure 2.8, Figure 2.9**). One limitation of this approach is that MNase can digest non-nucleosomal DNA, and thus the silencers at *HML** and *HMR* and the tRNA gene adjacent to *HMR*, which are not nucleosome-bound, are under-recovered relative to sonication ChIP (**Figure 2.9**).

As expected, ChIP-seq on cells with wild-type *SIR3* revealed strong binding of Sir4-myc throughout *HML** and *HMR* (**Figure 2.7A & 2.7B**). *SIR3-EBD* cells grown with estradiol gave profiles that were indistinguishable in both the strength of binding and the location of binding. In *sir3*Δ cells and *SIR3-EBD* cells grown without estradiol, some Sir4-myc binding across *HML** and *HMR,* though severely reduced, was still evident (**Figure 2.7A & 2.7B**). This weak binding was observed in multiple replicates with different crosslinking times and was not observed in cells with untagged Sir4 (**Figure 2.8A**). We also performed ChIP-seq for Sir3-EBD using an antibody to the estrogen receptor, and found that its binding was strongly dependent on the presence of estradiol, and its binding pattern was indistinguishable from Sir4-myc (**Figure 2.10**). The apparent weak Sir3-EBD signal at *HML** and *HMR* in the absence of estradiol did not drive the weak Sir4-myc binding described above, as the same Sir4-myc binding was observed in *sir3*Δ cells as in *SIR3-EBD* cells grown without estradiol.

ChIP-seq data from *SIR3-EBD* cells arrested in G1 without estradiol revealed the same weak enrichment of Sir4-Myc at *HML** and *HMR* that we observed in cycling cells (**Figure 2.7** & **Figure 2.8C**). However, upon addition of estradiol in cells kept in G1, we saw a strong increase in Sir4-myc binding across the loci (**Figure 2.7C** & **Figure 2.8C**). The increase in Sir4-myc binding to *HMR* was not associated with any change in expression of *HMRa1*, which remained completely de-repressed (**Figure 2.8B**). Hence, Sir protein binding across *HML** and *HMR* was not sufficient to lead to gene silencing. When cells were allowed to pass from G1 to G2/M, the resulting partial silencing was correlated with an increase in Sir4-myc binding at *HML** and *HMR* (**Figure 2.7C** & **Figure 2.8C**). Thus, Sir proteins binding throughout *HML** and *HMR* in absence of cell-cycle progression achieved no repression, and some S-phase-dependent process promoted further binding and partial repression. Together, these data revealed the existence of cell-cycle-regulated steps beyond Sir binding required to bring about silencing.

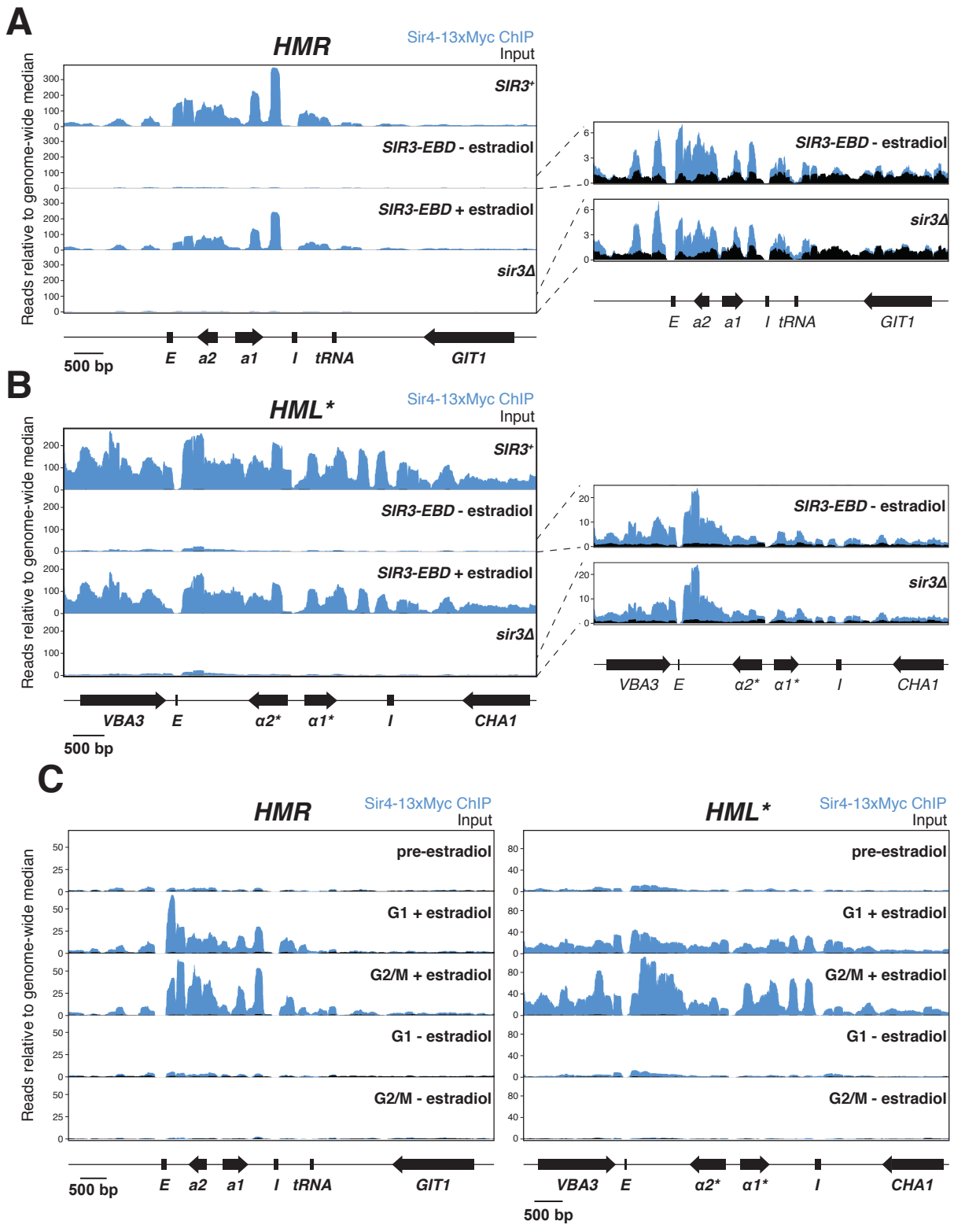

Figure 2.7: Sir protein binding and silencing were separable phenomena. *(continued on next page)*

Figure 2.7 ***(continued from previous page)*** All panels show Sir4-13xMyc ChIP-seq signal in blue and input in black. Read counts were normalized to the non-heterochromatin genome-wide median. IP and input values are plotted on the same scale. **(A)** Left, ChIP-seq for Sir4-13xMyc at *HMR* in strains with *SIR3*$^{+}$ (JRY12172), *sir3*Δ (JRY12168), and *SIR3-EBD* (JRY12170) grown with or without estradiol and fixed for 60 minutes in formaldehyde. Right, same data as the left panel for *sir3*Δ and *SIR3-EBD* without estradiol, enlarged to show IP levels above input. **(B)** Same as (A), but showing data from *HML**. **(C)** ChIP-seq for Sir4-13xMyc during silencing establishment at *HMR* (left) and *HML** (right). Cultures of *SIR3-EBD* cells (JRY12169) were arrested in G1 with α factor ("pre-estradiol"), then split four ways. Two sub-cultures were maintained in G1 in medium with estradiol or ethanol ("G1 + estradiol" and "G1 - estradiol"). The other two sub-cultures were released to G2/M by addition of protease and nocodazole; and received either estradiol or ethanol ("G2/M + estradiol" and "G2/M - estradiol"). After 3 hours in medium with estradiol or ethanol, cultures were fixed in formaldehyde for 15 minutes and collected for ChIP-seq. Data shown represent one of two replicates, with the other shown in Figure 2.8.

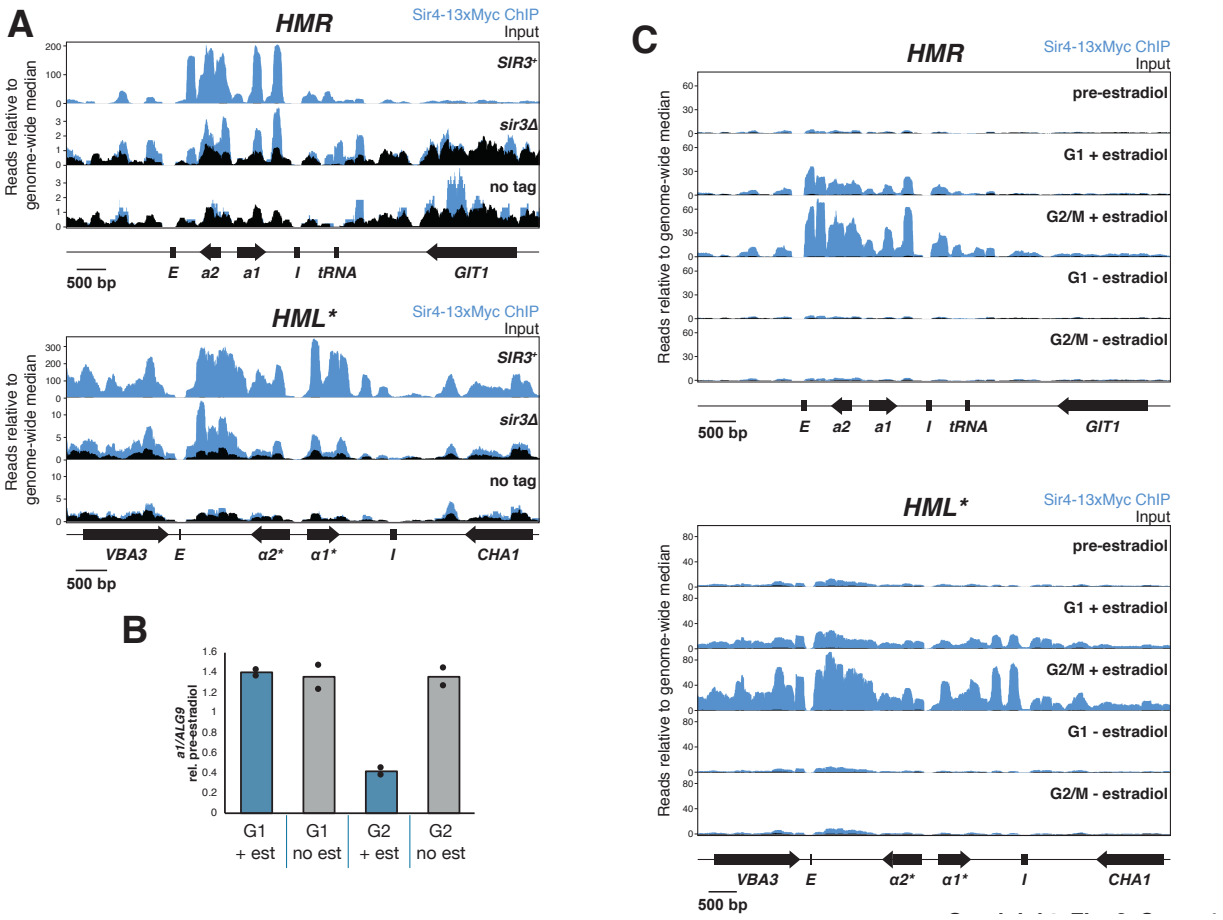

Figure 2.8: Silencing establishment ChIP-seq. All ChIP-seq panels show Sir4-13xMyc in blue and input in black. Read counts were normalized to the non-heterochromatin genome-wide median. IP and input values are plotted on the same scale. **(A)** ChIP-seq for Sir4-13xMyc at *HMR* (top) and *HML** (bottom) in strains with *SIR3*$^{+}$ (JRY12171) and *sir3*Δ (JRY12167). Also shown is an equivalent experiment in cells with untagged Sir4 (JRY12269). Cells were grown to mid-log phase and fixed for 15 minutes in formaldehyde. **(B)** RT-qPCR analysis of silencing establishment at *HMRa1* from samples that were used for ChIP-seq described in Figure 2.7C and 2.8C. Values are scaled to the pre-estradiol value for each sample. **(C)** Replicate experiment to that described in Figure 2.7C, using an isogenic strain (JRY12170).

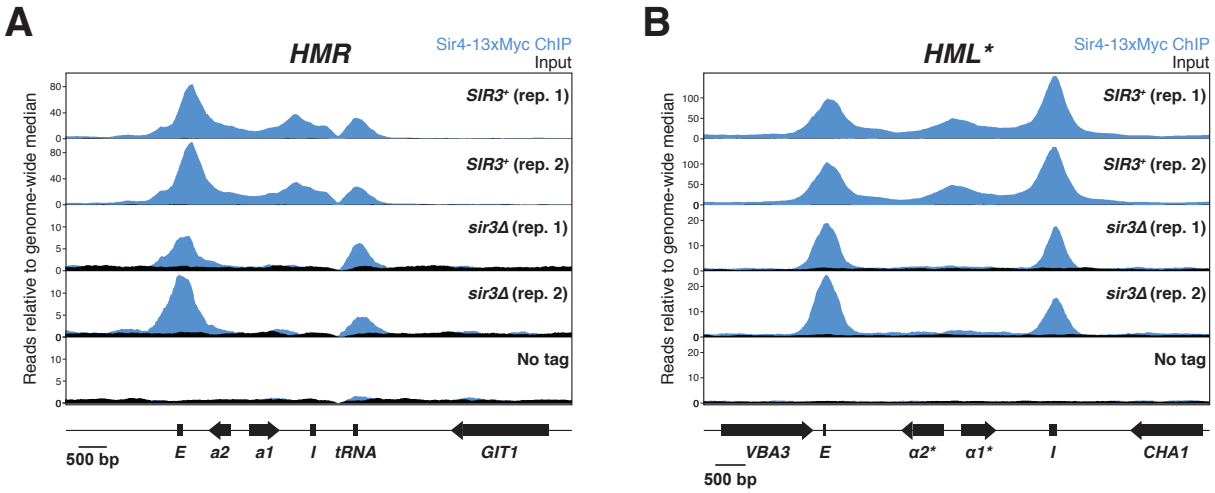

Figure 2.9: ChIP-seq with sonicated chromatin. All panels show Sir4-13xMyc in blue and input in black. Read counts were normalized to the non-heterochromatin genome-wide median. IP and input values are plotted on the same scale. Cells with *SIR3*$^{+}$ (JRY12171, JRY12172), *sir3*Δ (JRY12167, JRY12168), and a no-tag control (JRY12169) were grown to mid-log phase and fixed for 15 minutes in formaldehyde. **(A)** ChIP-seq for Sir4-13xMyc at *HMR*. **(B)** ChIP-seq for Sir4-13xMyc at *HML**.

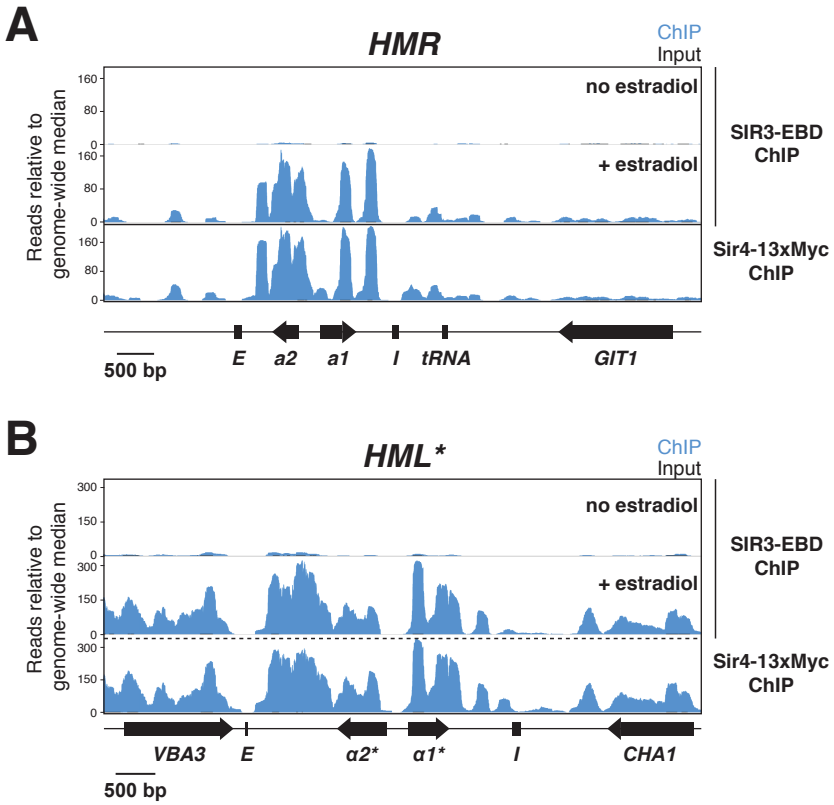

Figure 2.10: ChIP-seq for Sir3-EBD. All panels show ChIP-seq signal in blue and input in black. Read counts were normalized to the non-heterochromatin genome-wide median. IP and input values are plotted on the same scale. Cells with *SIR3-EBD* (JRY12170) were grown to mid-log phase with or without estradiol, then fixed for 15 minutes in formaldehyde. Also shown is Sir4-13xMyc ChIP signal from Figure 2.8A. **(A)** Sir3-EBD and Sir4-13xMyc ChIP-seq at *HMR*. **(B)** Sir3-EBD and Sir4-13xMyc ChIP-seq at *HML**.

2.4.4 Removal of H3K79 methylation was a critical cell-cycle-regulated step in silencing establishment

Given that silencing at *HML* and *HMR* was established only during a discrete window of the cell cycle, the key issue was to identify what molecular event(s) occurred during this window and why it/they were limited in the cell cycle. A mutant that could establish silencing while arrested in G1 would potentially identify that molecular event.

The histone methyltransferase Dot1 has several characteristics that suggest it might act as an antagonist of silencing establishment. Dot1 methylates histone H3 on lysine 79 (H3K79), which interferes with Sir3 binding to nucleosomes (Altaf et al., 2007; Armache et al., 2011; Van Leeuwen et al., 2002; Yang et al., 2008). Dot1 is unique among yeast histone methyltransferases in lacking a counteracting demethylase that removes H3K79 methylation. Thus, removal of H3K79 methylation can be achieved only through turnover of the histones that bear it, such as occurs during S phase, when new histones are incorporated that lack H3K79 methylation (De Vos et al., 2011). Indeed, *dot1*Δ *SIR3-EBD* cells arrested in G1 robustly repressed *HMRa1*, *hmlα1**, and *hmlα2** upon addition of estradiol (**Figure 2.11**, **Figure 2.12A, 2.12B**). This phenotype was not limited to *SIR3-EBD* strains. Strains bearing the temperature-sensitive *sir3-8* allele and *dot1*Δ could also establish silencing in G1 when shifted from the non-permissive temperature to the permissive temperature (**Figure 2.11E**). Thus, removal of H3K79me from *HML* and *HMR* was one crucial S-phase-specific step during silencing establishment.

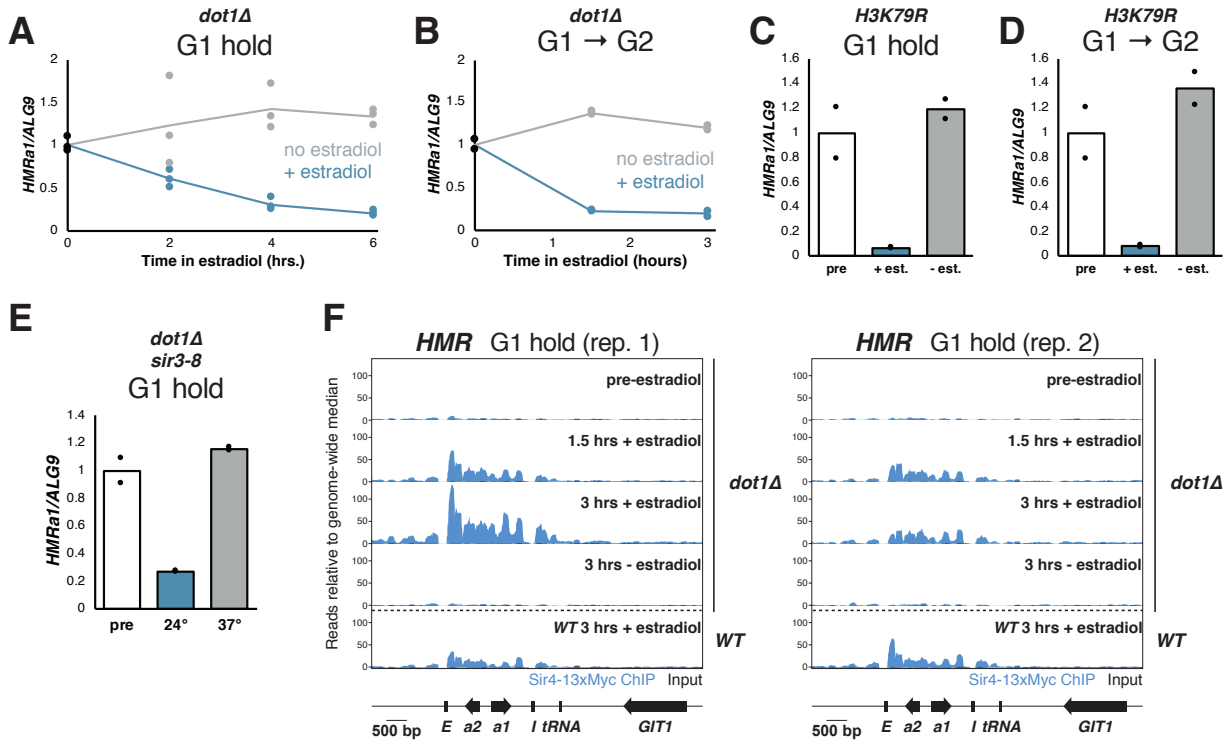

Figure 2.11: Cells without H3K79 methylation established silencing without cell-cycle progression. (A) Cultures of *dot1*Δ cells (JRY12443, JRY12445) were arrested in G1 with α factor, then split, with half receiving ethanol and the other half receiving estradiol. Silencing was monitored by RT-qPCR over time after estradiol addition. **(B)** *dot1*Δ mutants were arrested in G1 with α factor, then released to G2/M *(continued on next page)*

Figure 2.11 ***(continued from previous page)*** by addition of protease and nocodazole, and either ethanol or estradiol. Silencing was monitored by RT-qPCR over time after estradiol addition. **(B)** *dot1*Δ mutants were arrested in G1 with α factor, then released to G2/M by addition of protease and nocodazole, and either ethanol or estradiol. Silencing was monitored by RT-qPCR over time after estradiol addition. **(C)** Cultures of cells in which lysine 79 was mutated to arginine in both *HHT1* and *HHT2* in two isogenic strains (*H3K79R*; JRY12851, JRY12852) were arrested in G1 with α factor, then split, with one sub-culture receiving ethanol and the other receiving estradiol. Silencing was assayed by RT-qPCR after 6 hours in ethanol. **(D)** *H3K79R* cells were arrested in G1 with α factor, then released to nocodazole with protease and nocodazole and either estradiol or ethanol. Silencing was assayed by RT-qPCR after 3 hours in estradiol. The pre-estradiol sample for this experiment was the same culture used in (C). **(E)** Cultures of *dot1*Δ *sir3-8* cells (JRY12859, JRY12890) were grown at the non-permissive temperature for *sir3-8* (37°C) and arrested in G1 with α factor, then split, with half shifted to the permissive temperature (24°C) and other half staying at the non-permissive temperature. Silencing was assayed by RT-qPCR after 6 hours. **(F)** Cultures of *dot1*Δ cells (JRY12443, JRY12444) were arrested in G1 with α factor ("pre-estradiol"), then split, with half the culture receiving ethanol, and the other half receiving estradiol. After 1.5 hours and after 3 hours, samples were fixed for 15 minutes in formaldehyde and collected for ChIP. Sir4-13xMyc ChIP-seq signal is in blue and input in black, each normalized to the non-heterochromatin genome-wide median and plotted on the same scale. Also displayed are two replicates of wild-type G1 cells after 3 hours in estradiol from Figures 2.7C and Figure 2.8C.

To test whether the *dot1*Δ phenotype was due specifically to methylation at H3K79, both copies of histone H3 were mutated to encode arginine at position 79 (*H3K79R*), a mimic for the non-methylated state. This mutant also allowed for robust silencing establishment in G1, in fact, to a stronger degree than *dot1*Δ (**Figure 2.11C**). Strains with H3K79 mutated to leucine (*H3K79L*) or methionine (*H3K79M*) failed to establish silencing even after passage through S phase (**Figure 2.12C, 2.12D**), confirming the importance of the positive charge on H3K79 in silencing. Notably, even though G1-arrested *H3K79R* cells could strongly repress *HMRa1* (~15-fold), this was still incomplete relative to fully silenced cells, which repressed *HMRa1* >1000-fold (see **Figure 2.1B**). Thus, either increased time or cell cycle progression promoted silencing establishment even in absence of H3K79 methylation.

In addition to promoting S-phase-independent silencing establishment, *dot1*Δ and *H3K79R* cells that passed from G1 to G2/M also repressed *HMRa1* more robustly than did wild-type cells transiting the same cell-cycle window (**Figure 2.11B & 2.11D**, compare to **Figure 2.1E**). S-phase passage markedly increased the speed of silencing establishment in *dot1*Δ cells, though the ultimate degree of repression was similar whether cells passed through S phase or stayed in G1 (compare **2.11A & 2.11B**). Thus, some feature of S phase still promoted silencing establishment in cells lacking H3K79 methylation.

To understand how H3K79 methylation prevented silencing establishment, we performed ChIP-seq for Sir4-myc in *dot1*Δ cells undergoing silencing establishment in the absence of cell-cycle progression. G1-arrested *dot1*Δ cells already displayed clear partial silencing establishment after 1.5 hours in estradiol (**Figure 2.12E**). However, the level of Sir4-myc recruitment to *HML** and *HMR* at this early time point was similar to the recruitment observed in wild-type cells, in which no gene repression had occurred after 3 hours (**Figure 2.11F** & **Figure 2.12F**). Therefore, even though *dot1*Δ and wild-type cells had indistinguishable levels of Sir binding, that binding gave rise to different transcriptional effects. Thus, removal of H3K79me did not regulate silencing establishment by controlling Sir protein binding.

We next tested explicitly whether H3K79 methylation depletion can occur during a G1 arrest and during passage through S phase. As noted previously, in wild-type cells, H3K79 trimethylation (H3K79me3) is almost completely absent from *HML** and *HMR*, but in *sir3*Δ mutants, H3K79me3 is present at both loci (**Figure 2.13A, 2.13B**). When *SIR3-EBD* was induced

in cells arrested in G1, no change in H3K79me3 was observed (**Figure 2.13**), even though robust Sir protein recruitment could occur (**Figure 2.7C**). Following S phase, though, H3K79me3 was partially depleted from *HMR* and from *hmlα1**, but not from *hmlα2** (**Figure 2.13**). Thus, Sir protein binding in G1 was insufficient to change H3K79me3 levels at *HML* and *HMR*. The first depletion of this mark occurred concomitantly with S phase.

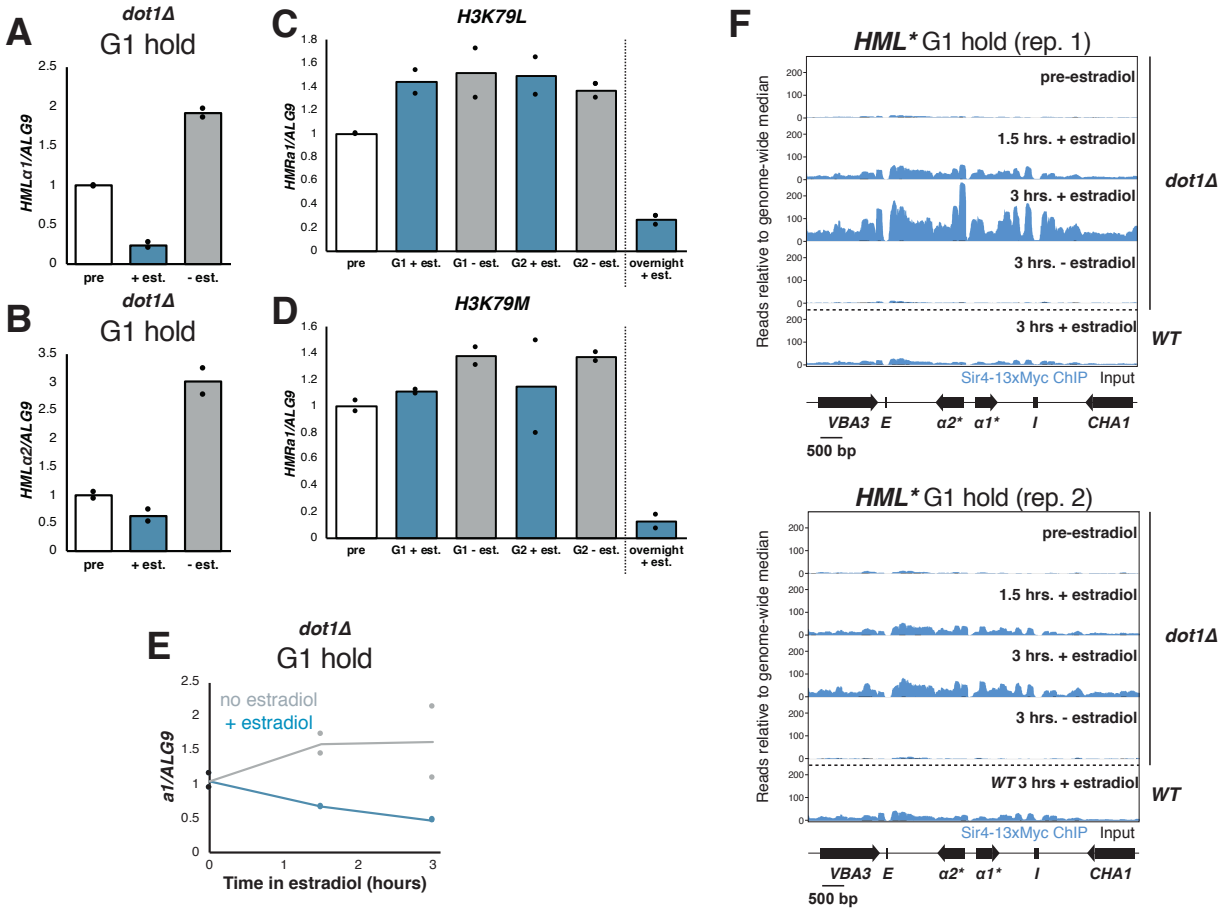

Figure 2.12: Silencing establishment in *dot1*Δ cells. (A) *dot1*Δ cells (JRY12443) were arrested in G1 with α factor, then split, with one sub-culture receiving estradiol and the other receiving ethanol. Cells were collected after 6 hours in estradiol and RT-qPCR was performed for *hmlα1**. The data in this plot are also shown in Figure 2.14 for comparison with other mutants. **(B)** RT-qPCR for *hmlα2** in the cells described in (A). **(C)** Cells with lysine 79 of H3 mutated to leucine in both *HHT1* and *HHT2* in two isogenic strains (*H3K79L;* JRY12854, JRY12855) were arrested in G1 with α factor, then split four ways. Two cultures were kept in G1, with one receiving estradiol and the other ethanol. The other two cultures were released to G2/M by addition of protease and nocodazole, and either estradiol or ethanol. Cells were collected after 3 hours for the G2/M samples and after 6 hours for the G1 samples, and RT-qPCR was performed for *HMRa1*. Also shown is a sample grown overnight in medium with estradiol. **(D)** Cells with lysine 79 of H3 mutated to methionine in both *HHT1* and *HHT2* in two isogenic strains (*H3K79M*; JRY12857, JRY12858) were subjected to the same experiment described in (C). **(E)** RT-qPCR analysis of silencing establishment at *HMRa1* from samples that were used for ChIP-seq shown in Figure 2.11F and Figure 2.12F. **(F)** Sir4-13xMyc binding at *HML** from the same samples displayed in Figure 2.11F. Sir4-13xMyc is in blue and input in black, plotted on the same scale. Read counts were normalized to the non-heterochromatin genome-wide median.

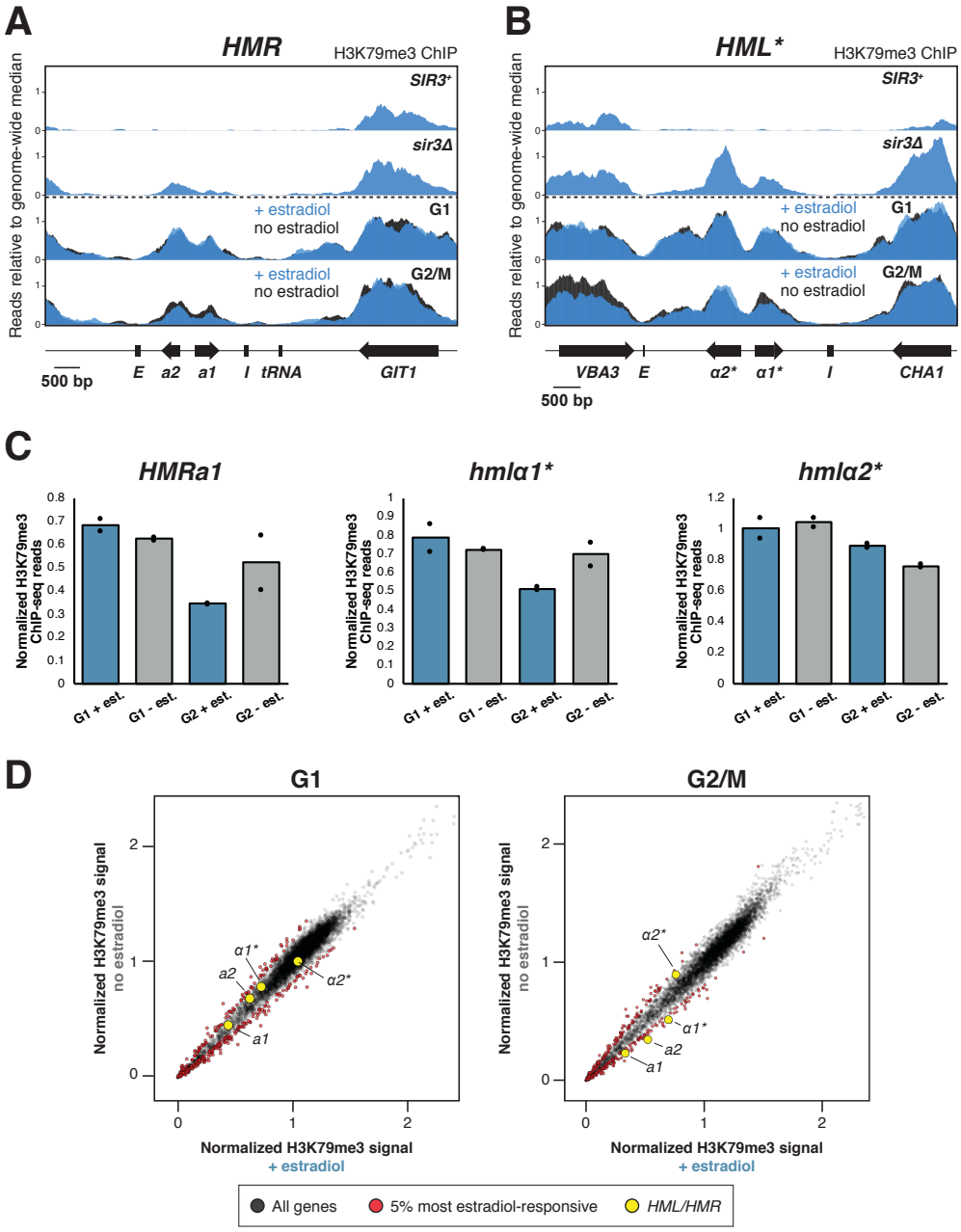

Figure 2.13: H3K79 trimethylation dynamics during silencing establishment. *SIR3-EBD* cultures (JRY12169, JRY12170) were arrested in G1 with α factor, then split four ways. Two cultures were kept in G1, with one receiving estradiol (blue) and the other ethanol (black). The other two cultures were released to G2/M by addition of protease and nocodazole, and either estradiol (blue) or ethanol (black). Cells were collected after 3 hours and subjected to ChIP-seq for H3K79me3. **(A)** ChIP-seq for H3K79me3 at *HMR*. For the top two panels, cultures of *SIR3*$^{+}$ (JRY12171) and *sir3*Δ (JRY12167) cells were grown to mid-log phase and subjected to ChIP-seq for H3K79me3. All data are plotted on the same scales. For the G1 and G2/M samples, the plotted value is the average of two biological replicates. **(B)** ChIP-seq for H3K79me3 at *HML**. **(C)** Total ChIP-seq coverage was calculated for each gene and scaled to the length of the gene and the genome-wide mean coverage. Displayed is the normalized H3K79me3 signal for *HMRa1, hmlα1**, and *hmlα2**. **(D)** Normalized H3K79me3 signal for all genes after estradiol or ethanol addition, in G1 (left) and in G2/M (right). The plotted values represent the average of two biological replicates. The 5% most estradiol-responsive genes are shaded in red and the genes at *HML* and *HMR* are enlarged and shaded in yellow.

2.4.5 *SAS2* and *RTT109* contributed to limiting silencing establishment to S phase

The crucial role of H3K79 methylation removal in silencing establishment led us to consider other chromatin modifications that might regulate silencing establishment. Two histone acetyltransferases, Sas2 and Rtt109, were especially interesting given the S-phase dynamics of the marks they deposit and their known relevance to silencing. Sas2, the catalytic component of the SAS-I complex, acetylates H4K16 during S phase (Kimura et al., 2002; Meijsing and Ehrenhofer-Murray, 2001; Reiter et al., 2015; Suka et al., 2002). The removal of H4K16 acetylation by Sir2 is the central histone modification associated with silencing (Imai et al., 2000; Johnson et al., 1990; Landry et al., 2000; Park and Szostak, 1990). Rtt109 acetylates newly-incorporated histone H3 at lysines 9 and 56 during S phase, and this acetylation is largely removed by Hst3 and Hst4 by the time of mitosis (Adkins et al., 2007; Celic et al., 2006; Driscoll et al., 2007; Fillingham et al., 2008; Schneider et al., 2006). Mutations in *SAS2* and *RTT109* have both been shown to have subtle silencing phenotypes (Imai et al., 2000; Miller et al., 2008).

Interestingly, both *sas2*Δ and *rtt109*Δ mutations led to partial repression of *HMR* upon *SIR3-EBD* induction in cells arrested in G1 (**Figure 2.14A**). The magnitude of this effect was weaker than in *dot1*Δ cells but highly significant. Cells without *RTT109* grew slowly and were less sensitive to α factor than wild-type cells, so we cannot exclude the possibility that a population of *rtt109*Δ cells passed through S phase during the experiment, contributing to the observed phenotype **(Figure 2.15).** When combined with *dot1*Δ, both *sas2*Δ and *rtt109*Δ led to a further increase in silencing establishment. Thus, *SAS2* and *RTT109* impeded silencing establishment by a different mechanism than *DOT1*. Silencing establishment in cells lacking both *SAS2* and *RTT109* was not significantly different from that of the single mutants. Strikingly, triple mutant *sas2*Δ *rtt109*Δ *dot1*Δ strains established silencing no better than single-mutant *dot1*Δ cells. Interestingly, the G1 phenotypes we observed at *HMR* for *dot1*Δ, *sas2*Δ, and *rtt109*Δ single mutant cells were largely similar at *hmlα1** (**Figure 2.14B**). Altogether, these findings demonstrate that *SAS2*, *RTT109*, and *DOT1* inhibit silencing establishment outside of S phase.

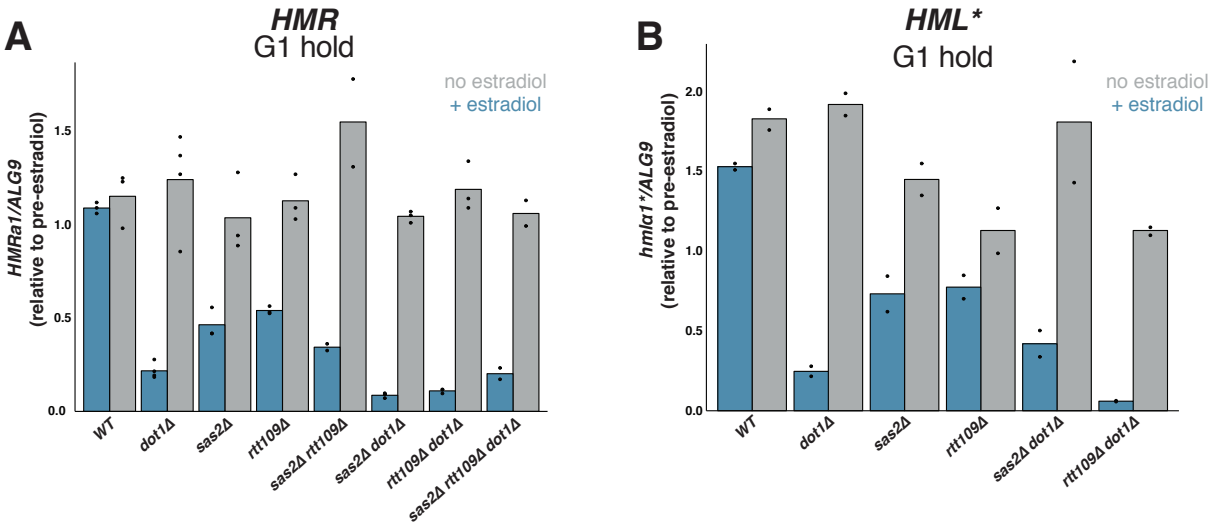

Figure 2.14: Effects of *SAS2* and *RTT109* on silencing establishment in G1. *(continued on next page)*

Figure 2.14 ***(continued from previous page)*** For all strains, cultures were arrested in G1 with α factor, then split, with one sub-culture receiving estradiol and the other receiving ethanol. Silencing was assayed by RT-qPCR 6 hours after additions. Each sample was normalized to its own pre-estradiol value. The following strains were used. WT: JRY12169; *dot1*Δ: JRY12443, JRY12445; *sas2*Δ: JRY12615, JRY12616; *rtt109*Δ: JRY12689, JRY12690; *sas2*Δ *rtt109*Δ: JRY12765, JRY12766; *sas2*Δ *dot1*Δ: JRY12618, JRY12619; *rtt109*Δ *dot1*Δ: JRY12691, JRY12692; *sas2*Δ *rtt109*Δ *dot1*Δ: JRY12767, JRY12768. **(A)** Silencing establishment of *HMRa1* by RT-qPCR. The level of repression observed in each mutant was significantly greater than in wild type (Two-tailed T-test; $p < 0.005$ for each pair-wise comparison). The level of repression observed in *sas2*Δ *dot1*Δ and *rtt109*Δ *dot1*Δ double mutants was significantly greater than in the *dot1*Δ single mutant ($p < 0.01$ for each pair-wise comparison), but there was no significant difference between the values from the *dot1*Δ single mutant and the triple mutant *sas2*Δ *rtt109*Δ *dot1*Δ ($p = 0.70$). **(B)** Silencing establishment of *hmlα1** by RT-qPCR in a subset of mutant strains. The level of repression for each mutant was significantly greater than in wild type ($p < 0.05$ for each pair-wise comparison). The level of repression observed in the *rtt109*Δ *dot1*Δ double mutant was significantly greater than in the *dot1*Δ single mutant ($p = 0.028$), but there was no significant difference between the values from *sas2*Δ *dot1*Δ double mutant and the *dot1*Δ single mutant ($p = 0.19$).

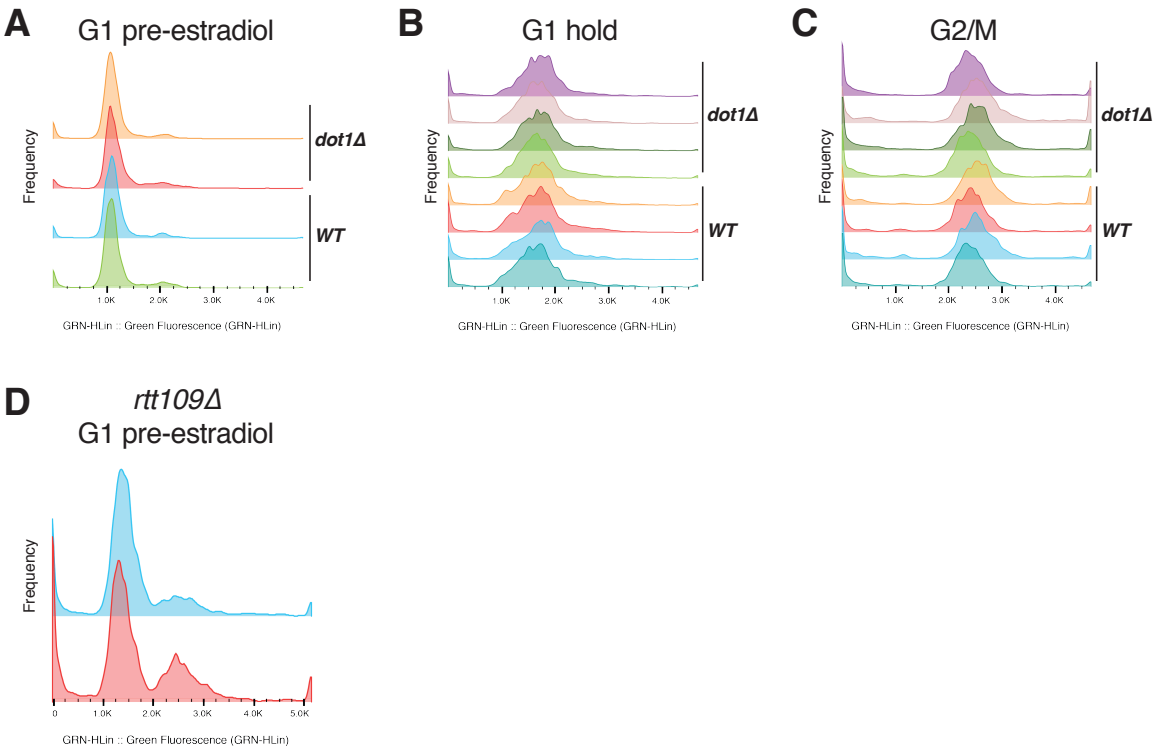

Figure 2.15 : Representative flow cytometry profiles. (A) Wild-type (JRY12169) and *dot1*Δ (JRY12443) cells arrested in G1 with α factor for ~2 hours. **(B)** Wild-type and *dot1*Δ cells kept in G1 for 6 hours, as in, e.g., Figure 2.1D. **(C)** Wild-type and *dot1*Δ cells after 3 hours in G2/M, as in, e.g., Figure 2.1E. **(D)** Cells lacking *RTT109* (JRY12689, JRY12691) arrested in G1 with α factor for ~3 hours did not arrest uniformly.

2.5 Discussion

In this study, we identified why silencing establishment requires cell cycle progression. These results highlighted the value of studying the dynamics of silencing both in populations of cells and at the single cell level. By monitoring changes in chromatin and changes in expression simultaneously, we documented effects that were elusive at steady state, but critical for a mechanistic understanding of the process. We found that the cell-cycle-dependent removal of euchromatic marks was a major driver of a cell's ability to establish stable heterochromatin. Interpretation of our results required critical reassessment of some earlier results.

2.5.1 Silencing establishment occurred by tuning down transcription in individual cells after Sir proteins were bound

The classic model for silencing establishment involves two steps: nucleation of Sir proteins at the silencers, followed by spreading of Sir proteins from silencers via the stepwise deacetylation of nucleosomes by Sir2 and subsequent binding of Sir3 and Sir4 to deacetylated positions of H3 and H4 tails (Hecht et al., 1995b; Hoppe et al., 2002; Rusche et al., 2003; Rusché et al., 2002). In the classic model, individual Sir proteins are recruited to the silencers, but the spread across the locus is dependent on all three proteins Sir2/3/4, with both continuous Sir protein binding and histone deacetylation being required for gene repression (Johnson et al., 2009; Yang and Kirchmaier, 2006). The binding of a Sir2/3/4 complex to internal nucleosomes at *HML* and *HMR* is thought to drive gene repression at least partly through sterically preventing other proteins from accessing the underlying DNA (Loo and Rine, 1994; Steakley and Rine, 2015). Repression may also rely on inhibition of specific steps in transcription downstream of activator binding (Chen and Widom, 2005; Gao and Gross, 2008; Johnson et al., 2013). A puzzling observation is that, qualitatively, the nucleation and spread of Sir2, Sir3, and Sir4 to *HML* and *HMR* appears to be cell-cycle-independent, even though the silencing activity of these proteins is clearly dependent on cell cycle progression (Kirchmaier and Rine, 2006).

Surprisingly, we found that silencing establishment led to increased Sir4 binding both at silencers and across the silent loci, beginning from a low-level distributed binding that was present even in the absence of Sir3. The weak Sir4 binding across *HML* and *HMR* in *sir3*Δ cells suggested that the full Sir2/3/4 complex was not necessary for the distributed binding of Sir4. Rather, Sir3 appeared to stabilize or otherwise enhance Sir4-nucleosome interactions. Further high-resolution ChIP-seq studies will be needed to determine whether Sir2, Sir3, and Sir4, which localize indistinguishably in wild-type cells (Thurtle and Rine, 2014), behave similarly in absence of the full complex, and during silencing establishment.

Upon induction of *SIR3-EBD*, G1-arrested wild-type cells could robustly recruit Sir4 to *HML* and *HMR* without causing any gene repression at these loci. Passage through S phase led to increased Sir binding and partial silencing establishment. However, in G1-arrested *dot1*Δ cells, in which Sir4 binding patterns were indistinguishable from G1-arrested wild-type cells, induction of *SIR3-EBD* caused partial silencing establishment. Together, these observations indicate that a key regulated step in building heterochromatin occurred after the major silencing factors were already present at the locus. Two interpretations were compatible with our data. First, the non-repressive Sir4 binding observed in G1 and the repressive Sir4 binding observed in G2/M could differ in some parameter that is not apparent in crosslinking ChIP experiments, such as differences in the on and off rates for Sir4 binding to nucleosomes. Second, Sir binding could be unable to drive

transcriptional changes until competing euchromatic marks on chromatin are relieved. Consistent with the latter interpretation, we found that Sir protein binding could lead to changes in H3K79 trimethylation only after S phase. In addition, a prior study of telomeric silencing found that while Sir protein binding was detectable at both repressed and de-repressed telomeres, euchromatic marks, including H3K79me, were found only at de-repressed telomeres, and that, in vitro, H3K79me could disturb silencing without changing Sir protein binding (Kitada et al., 2012).

Our smRNA-FISH results showed that silencing establishment proceeds via the gradual tune-down of transcription in individual cells, and that this tune-down occurs over multiple cell cycles. Interestingly, the fraction of cells with zero transcripts after a single S phase (~30%, see **Figure 2.4B**), is similar to the fraction of cells that established phenotypic silencing after a single division in a previous study (Osborne et al., 2009). While these results are not directly comparable, as the previous study assayed silencing at *HML* and used a different induction strategy, one possibility is that phenotypic silencing only arises when transcript number falls to zero in a given cell. This result conflicts with a prior study of silencing establishment in single cells using a fluorescent reporter at *HML*. That study concluded that silencing establishment proceeded via discrete transitions from the "ON" to the "OFF" state (Xu et al., 2006). However, because that study relied on qualitative assessment of fluorescence intensity in individual cells, it may not have been possible to ascertain intermediate states. Indeed, our data illustrate an inherent limitation of qualitative measurements of single-cell parameters: in the smRNA-FISH images in **Figure 2.4**, a striking feature is the dichotomy between cells with no transcripts and those with some transcripts. That observation might lead to the conclusion that silencing establishment is caused by the complete shutdown of transcription stochastically in some cells. However, as illustrated by **Figure 2.4A**, that dichotomy is expected from both an "all-or-nothing" model and a "gradual transition" model. It is only through the quantitative analysis that we could see the gradual decrease in transcription in individual cells.

Whether silencing acts through steric occlusion or through a more specific inhibition of some component necessary for transcription, it is difficult to explain how any intermediate in the assembly of a static heterochromatin structure could drive partial repression. The simplest explanation for how partially repressive chromatin could form would be that silencing machinery and transcriptional machinery both come on and off the chromatin, and the establishment of silencing involves a change in the relative rates of those two processes. In that case, histone modifications could be crucial in shifting the balance.

2.5.2 Euchromatic histone mark removal was a key cell-cycle-regulated step in silencing establishment

Removal of Dot1-deposited methylation of H3K79 was a critical step in silencing establishment. This finding was consistent with earlier studies of cycling cells, which found that *dot1*Δ cells established silencing more quickly than wild-type cells (Katan-Khaykovich and Struhl, 2005; Osborne et al., 2009). Indeed, Katan-Khaykovich and Struhl proposed a model for silencing establishment in which Sir protein binding and histone deacetylation occur rapidly, followed by slow removal of methylation over several cell cycles, which is consistent with our findings. Removal of H3K79 methylation appears to be the primary reason why cells need to progress through S phase to establish silencing. Dot1 is thought to reduce the Sir3 BAH domain's affinity for the nucleosome core by methylating H3K79 (Martino et al., 2009; Ng et al., 2002a; Onishi et al., 2007). In addition to Dot1 and Sir3 both binding the nucleosome core at H3K79, they also

compete for binding to the H4 tail, and deacetylation of the tail by Sir2 is thought to favor Sir3 binding at the expense of Dot1(Altaf et al., 2007). Thus, through modifications at H4K16 and H3K79, transcription and silencing mutually antagonize each other. In a cell in G1, even if Sir2/3/4 are able to displace Dot1 by deacetylating H4K16, H3K79me will remain until histones are turned over, which seems to explain the S-phase requirement for silencing establishment.

Silencing establishment at *HMR* does not require replication of the locus, as shown by the ability of excised episomes bearing *HMR* but no replication origin to establish silencing in an S-phase-dependent manner (Kirchmaier and Rine, 2001; Li et al., 2001). This finding presented a major mystery: what S-phase-specific process other than replication fork passage drives silencing establishment? Our results suggest that an influx of H3 molecules lacking methylation at K79 could be the solution. Replication-independent histone exchange can occur throughout the cell cycle (Dion et al., 2007; Rufiange et al., 2007; Schlissel and Rine, 2019), which means that a replicating or non-replicating copy of *HMR* can incorporate histone molecules from the nuclear pool. Outside of S phase, ~90% of all H3 in the nucleus is methylated at K79 (Van Leeuwen et al., 2002), so histone exchange would likely lead to incorporation of the silencing-refractory methylated form. However, during S phase, a large quantity of newly-synthesized non-methylated H3 is present. Therefore, histone incorporation during S phase through either replication-coupled chromatin assembly or replication-independent histone would lead to incorporation of many H3 molecules that are not methylated at K79. This might explain why silencing establishment can occur at *HMR*, whether it is replicated or not, and why that establishment depends on S phase.

We found that *H3K79R* mutants, which mimicked the non-methylated state of H3K79, also established silencing in G1-arrested cells and did so even more strongly than *dot1*Δ mutants. A simple explanation for this difference in impact of the two mutations could be that Sir3 binds more strongly to arginine than lysine at position 79. Alternatively, Dot1 has been shown to have several methyltransferase-independent functions, and it was possible that one of these functions acted to promote silencing. In particular, Dot1 has recently been shown to possess histone chaperone activity that is independent of its ability to methylate histones (Lee et al., 2018). In addition, Dot1 has the methyltransferase-independent ability to stimulate ubiquitination of histone H2B (van Welsem et al., 2018). The latter result is particularly interesting, because H2B ubiquitination is itself required for both H3K79 methylation (Briggs et al., 2002; Ng et al., 2002b) and H3K4 methylation (Dover et al., 2002; Sun and Allis, 2002). Conflicting reports have pointed to a role of H3K4 methylation in silencing (Fingerman et al., 2005; Mueller et al., 2006; Santos-Rosa et al., 2004). Thus, it is possible that in a *dot1*Δ mutant, the removal of H3K79me *per se* promotes silencing, but an indirect effect through H2Bub and/or H3K4me partially counteracts the H3K79me effect.

The histone acetyltransferases Sas2 and Rtt109 also had roles in limiting silencing establishment to S phase. Individually, *sas2*Δ and *rtt109*Δ mutations led to partial silencing establishment in G1-arrested cells, and each of these effects was additive with a *dot1*Δ mutation. Acetylation of H4K16 by Sas2, like methylation of H3K79 by Dot1, is critical in distinguishing euchromatin and heterochromatin. Interestingly, in a previous study, while *dot1*Δ sped silencing establishment at *HML*, *sas2*Δ delayed silencing establishment by that assay (Osborne et al., 2009). The single-cell α-factor response assay used in that study required cells to fully repress *HML* to gain the **a** mating type identity, whereas our assay used more direct measures of changes in transcription at *HML* and *HMR*. Thus, one explanation consistent with both results is that *sas2*Δ cells begin silencing more readily than wild-type cells, but take more cell cycles to reach full repression. This could be the result of the competing effects of the *sas2*Δ mutation: hypoacetylation

of histones at *HML* and *HMR* might increase Sir protein recruitment, while the global pool of hypoacetylated histones can also titrate Sir proteins away from *HML* and *HMR*.

The ability of *rtt109*Δ cells to drive partial silencing establishment in G1-arrested cells was surprising. Like Sas2, Rtt109 binds to Asf1 and acetylates newly-synthesized histones (Driscoll et al., 2007), but H3K56 acetylation is removed after S phase by the sirtuins Hst3 and Hst4 (Celic et al., 2006). The residual H3K56ac present outside of S phase is due to transcription-coupled histone turnover, which incorporates new histones marked with H3K56ac (Rufiange et al., 2007). A negative role for H3K56 acetylation in silencing has been observed, although this has not been well-characterized (Dodson and Rine, 2015; Miller et al., 2008). One simple model is that H3K56ac favors transcription, and thus impedes silencing establishment. However, given genome-wide acetylation and deacetylation of H3K56, indirect effects cannot be excluded.

2.5.3 Silencing establishment occurred via similar mechanisms at different loci

The mechanism of repression at the two silent mating type loci, *HML* and *HMR*, is generally assumed to be quite similar, but there are mutations that cause effects only at one of the two loci, and others that cause divergent phenotypes between the two loci (Park and Szostak, 1990; Ehrenhofer-Murray, Rivier and Rine, 1997; Yan and Rine, unpublished). Earlier studies concluded that cell-cycle requirements for silencing establishment differed at *HML* and *HMR* (Ren, Wang and Sternglanz, 2010; Lazarus and Holmes, 2011) In contrast, in both wild-type cells and the mutant conditions we tested, both loci behaved similarly. A major innovation that distinguished our studies from the prior studies was our use of a mutant *HML* that allowed unambiguous study of its expression by ensuring that α1 and α2 proteins would not be made. This strategy removed the strong repressive effect that the **a**1/α2 repressor has on transcription from the *HML* promoter, which was a confounding influence in earlier experimental designs that could have led to apparent cell-cycle independent silencing of *HML*. In the course of this work, we found that the *HML* promoter is subject to hyperactivation by α factor, which further complicates studies of silencing establishment at the locus. More work is clearly needed to fully understand how silencing establishment is regulated at *HML*.

We did note one distinction between *HML* and* HMR. After a single S phase, H3K79 trimethylation was depleted from *HMR* and *hmlα1**, but not from *hmlα2**. Given that H3K79 methylation is a major regulator of silencing establishment, this could explain the observation that *hmlα2** was also the gene whose silencing was weakest after a single S phase of silencing establishment. The interrelation between promoter strength, transcription-coupled histone modification, and silencing remains a fascinating topic for future study.

This fundamental similarity between *HML* and *HMR* in silencing establishment was further evidenced by the lack of an effect of the tRNA gene adjacent to *HMR,* or the tRNA gene's binding partner, cohesin, loss of either of which were reported to allow early silencing establishment in previous studies (Lau et al., 2002; Lazarus and Holmes, 2011). The reason behind the differences between our results and those of the previous studies is not clear. We did observe subtle silencing-independent fluctuations in *HMRa1* expression through the cell cycle, which may have confounded earlier results that relied on non-quantitative RT-PCR assays (data not shown). We cannot exclude the possibility that differences between *SIR3-EBD* and earlier inducible alleles contributed to the different results, as temperature, metabolism, and hormone addition could each affect silencing or the cell cycle in unappreciated ways. Lazarus & Holmes's use of the galactose promoter to drive *SIR3* expression would alter Sir3 concentration and the stoichiometry of the SIR complex, both of which would be expected to be important parameters in regulating silencing establishment.

2.5.4 Do the contributions of *DOT1*, *SAS2*, and *RTT109* completely resolve the cell-cycle requirement for silencing establishment?

In *dot1*Δ mutants, S phase still dramatically accelerated silencing establishment, indicating that some feature of S phase beyond H3K79me removal was important in those cells. In addition, we found no case in which silencing establishment in G1-arrested cells matched the degree of silencing observed after overnight growth in estradiol. However, the ~90% repression observed in, e.g., G1-arrested *H3K79R* cells should be sufficient to completely turn off transcription at *HML* and *HMR* in the majority of cells (see **Figure 2.4**). The quantitative gap in the level of silencing seen at steady state and that which is achieved in the experiments reported here could reflect a requirement for further cell-cycle steps or more time to complete silencing establishment. Others have identified a cell-cycle window between G2/M and G1 that contributes to silencing establishment (Lau et al., 2002), and none of our data were inconsistent with that result. Identifying mutant conditions in which G1-arrested cells and cycling cells establish silencing at an equal rate will be required before the cell-cycle regulated establishment of silencing is fully understood. In addition, future studies should address whether the antagonistic effects of euchromatic histone modifications on silencing establishment can be counteracted by increasing SIR complex concentration.

Together, our data suggest that silencing establishment cannot proceed without removal of histone modifications that favor transcription. In this view, at any stage of the cell cycle, Sir proteins can bind to *HML* and *HMR*. Passage through S phase leads to incorporation of new histones, which, crucially, lack H3K79 methylation. This decrease of H3K79me by half leads to both further Sir binding and decreased transcription. However, one cell cycle is not sufficient to fully deplete activating marks, and successive passages through S phase complete the process of silencing establishment.

Table 2.1: Yeast strains used in this study: All strains listed were generated for this study and derived from the W303 background. Unless otherwise noted, all strains are *ADE2; can1-100; leu2-3,112; ura3-1; lys2-; TRP1; matΔ::HygMX; bar1Δ::loxP:k.l.LEU2:loxP*

Strain	Genotype
JRY11664	*hmlΔ::HML*; sir3-8*
JRY11762	*hmlΔ::HML*; SIR3-EBD78*
JRY11763	*hmlΔ::HML*; SIR3-EBD78*
JRY11966	*hmlΔ::HML*; sir3Δ::k.l.URA3*
JRY12167	*hmlΔ::HML*; sir3Δ::k.l.URA3; SIR4-13xMyc::KanMX*
JRY12168	*hmlΔ::HML*; sir3Δ::k.l.URA3; SIR4-13xMyc::KanMX*
JRY12169	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX*
JRY12170	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX*
JRY12171	*hmlΔ::HML*; SIR4-13xMyc::KanMX*
JRY12172	*hmlΔ::HML*; SIR4-13xMyc::KanMX*
JRY12267	*hmlΔ::HML*; SIR3-EBD78; tT(AGU)CΔ*
JRY12268	*hmlΔ::HML*; SIR3-EBD78; tT(AGU)CΔ*
JRY12269	*hmlΔ::HML*; SIR3-EBD78*
JRY12270	*hmlΔ::HML*; SIR3-EBD78*
JRY12443	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; dot1Δ::HisMX*
JRY12444	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; dot1Δ::HisMX*
JRY12445	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; dot1Δ::HisMX*
JRY12513	*bar1Δ::loxP hmlα2Δ::CRE-hmlα1Δ::k.l.LEU2_ORF; SIR3-EBD78*
JRY12514	*bar1Δ::loxP hmlα2Δ::CRE-hmlα1Δ::k.l.LEU2_ORF; SIR3-EBD78*
JRY12560	*hmlΔ::HML*; SIR3-EBD78; his3Δ::C.G.HIS3:O.s.TIR1; MCD1-3V5-AID2:KanMX*
JRY12561	*hmlΔ::HML*; SIR3-EBD78; his3Δ::C.G.HIS3:O.s.TIR1; MCD1-3V5-AID2:KanMX*
JRY12615	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; sas2Δ::k.l.URA3*
JRY12616	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; sas2Δ::k.l.URA3*
JRY12618	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; dot1Δ::HisMX; sas2Δ::k.l.URA3*
JRY12619	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; dot1Δ::HisMX; sas2Δ::k.l.URA3*
JRY12689	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; rtt109Δ::k.l.URA3*
JRY12690	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; rtt109Δ::k.l.URA3*
JRY12691	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; dot1Δ::HisMX; rtt109Δ::k.l.URA3*
JRY12692	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; dot1Δ::HisMX; rtt109Δ::k.l.URA3*
JRY12765	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; rtt109Δ::NatMX; sas2Δ::k.l.URA3*
JRY12766	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; rtt109Δ::NatMX; sas2Δ::k.l.URA3*
JRY12767	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; dot1Δ::HisMX rtt109Δ::NatMX; sas2Δ::k.l.URA3*
JRY12768	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; dot1Δ::HisMX rtt109Δ::NatMX; sas2Δ::k.l.URA3*
JRY12851	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; hht1K79R; hht2K79R*
JRY12852	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; hht1K79R; hht2K79R*
JRY12854	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; hht1K79L; hht2K79L*
JRY12855	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; hht1K79L; hht2K79L*
JRY12857	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; hht1K79M; hht2K79M*
JRY12858	*hmlΔ::HML*; SIR3-EBD78; SIR4-13xMyc::KanMX; hht1K79M; hht2K79M*
JRY12859	*hmlΔ::HML*; sir3-8; dot1Δ::HisMX*
JRY12890	*hmlΔ::HML*; sir3-8; dot1Δ::HisMX*

Table 2.2: Oligonucleotides used for RT-qPCR

Name	Sequence
a1 Forward	GGCGGAAAACATAAACAGAAC
a1 Reverse	GGGTGATATTGATGATTTTCCC
α1 Forward	TCACAGGATAGCGTCTGGAA
α1 Reverse	TCAGCGAGCAGAGAAGACAA
α2 Forward	TCCACAAATCACAGATGAGT
α2 Reverse	GTTGGCCCTAGATAAGAATCC
cre Forward	CGTACTGACGGTGGGAGAAT
cre Reverse	CCCGGCAAAACAGGTAGTTA
ALG9 Forward	CGTTGCCATGTTGTTGTATG
ALG9 Reverse	GCCAGCCTAGTATACTAGCC

Table 2.3: Newly designed probes used for smRNA-FISH.

***a1* Probe sequences**
CTACAAATATCATCCATGTTGT
AGAGTTCTGTTTATGTTTTCCG
TCTCAGTACCTAGAATGTTAAA
AGTATTGAGATTGATTTCATCA
ACTTTAGTCAAATTACTTTCCA
TTTTGTGTAATGTATGTTGCTC
GGTATATTTCTAACCTATTGTT
GCTCTTCTCTTTTTTAATGTGG
TGATATTGATGATTTTCCCTTT
TTCTAAAAATGCCCGTGCTTGG
CTTTGCTTTCTTCTAAAAACCT
TCTTCTTTTTCCTTGGAATTAA
TAATGCCACATTTCTTTGCAAC
CCAAACTCTTACTTGAAGTGGA
GATCTCATACGTTTATTTATGA

Chapter 3:

Toward a context-specific understanding of histone inheritance

3.1 Abstract

A major open question in epigenetics is whether inheritance of modified histones contributes to the inheritance of epigenetic chromatin states. One precondition of any model that includes histones as memory carriers is that histones remember their position through DNA replication. Our lab has recently shown this to be the case at one locus, *GAL10*, but we do not yet know whether robust local inheritance of histones occurs genome-wide. Here we show that local histone inheritance also occurs at the silent *HML* locus, and that the locus displays reduced local histone inheritance when de-repressed. In the course of this work, we also found that cells lacking the histone inheritance factor *DPB3* are hypersensitive to the sirtuin inhibitor nicotinamide. This sensitivity is due to inhibition of the H3K56 deacetylases Hst3 and Hst4. We isolated suppressors of the *dpb3*Δ nicotinamide sensitivity and found that most suppressors had mutations in genes related to proteasome-mediated degradation. We speculate that degradation of H3 itself may be involved in the phenotype, and discuss ongoing work to further characterize the genetic interaction between *DPB3,* a factor involved in retention of parental histones, and *HST3/4*, factors involved in the proper labeling of new histones.

3.2 Introduction

Decoding the grammar of histone modifications and their effects on nuclear processes including transcription has been a major focus in the field of gene regulation. That histones are covalently modified by methylation and acetylation has been known for almost sixty years (Murray, 1964; Phillips, 1963). It was quickly discovered that these modifications could regulate transcription *in vitro* (Allfrey et al., 1964). Evidence for the *in vivo* role for histone modifications in regulating gene expression was elusive for decades after these initial discoveries. Pioneering genetic studies of silencing in budding yeast provided the first evidence for direct functional roles of histone modifications in gene regulation *in vivo* (Clark-Adams et al., 1988; Johnson et al., 1992, 1990; Kayne et al., 1988; Megee et al., 1990; Park and Szostak, 1990). These studies established that changes in histone genes caused changes in gene expression, and identified dynamically acetylated residues on the tail of histone H4 that were dispensable for normal growth, but absolutely required for silencing. An explosion of biochemical studies of histone-modifying enzymes began in the late 1990s following the Allis group's purification of the first histone acetyltransferase (Brownell et al., 1996). Such work is ongoing, but considerable progress has been made on understanding how given histone modifications are deposited, what proteins are recruited by the modifications, and how the modifications impact processes such as transcription (Talbert and Henikoff, 2021).

A similarly feverish line of inquiry has looked into the potential for these modifications to serve as information carriers. The logic of storing information in histones is that, assuming there exists a sufficiently robust mechanisms to pass histones from the pre-replication DNA strand to the post-replication daughter strands, the informative modifications on a given nucleosome could establish a means for stably propagating gene expression patterns from mother cell to daughter

cell. Indeed, in some specific contexts, this propagation of state is observed (Audergon et al., 2015; Gaydos et al., 2014; Ragunathan et al., 2015). Generally, though, absent specific evidence about their heritability, histone marks can be thought of in the same terms as any post-translational protein modification: they provide a way to modulate activity or change binding properties of a protein, but do not necessarily confer any emergent properties with regard to inheritance.

The model that histones might pass information through replication demands, at a minimum, that a given histone molecule resides at approximately the same genomic location before and after DNA replication. Historically, this precondition has been difficult to test directly *in vivo*, because it requires the ability to know the location of a given histone molecule before and after DNA replication. So while experiments that label old and new histones (Dion et al., 2007; Radman-Livaja et al., 2011) can assay histone turnover rate at different genomic locations, they can only distinguish histones by age, not by location history. Similarly, assays that investigate histone modifications immediately after DNA replication have shown evidence that a given locus maintains similar modification states before and after replication (Reverón-Gómez et al., 2018), but this analysis cannot formally distinguish between local inheritance and rapid modification of new histones at that locus.

Recent work from our lab developed a technique to directly track parental histones through DNA replication in *Saccharomyces cerevisiae* (Schlissel and Rine, 2019). In this study, all histone H3 molecules in the cell were tagged with the biotin acceptor AviTag peptide, which can be biotinylated by the bacterial biotin ligase BirA (Beckett et al., 1999; Fairhead and Howarth, 2015). BirA was fused to the DNA-binding protein TetR and recruited to a specific locus, in this case *GAL10*, by inserting the operator sequence *tetO* at that locus (**Figure 3.1**). It was found that splitting BirA into N-terminal and C-terminal halves, and thus limiting enzymatic activity to only when the two halves co-localized at a *tetO* binding site, increased signal-to-noise dramatically. By biotinylating histones at one locus and then stopping labeling by addition of doxycycline, which blocks TetR-*tetO* binding, one can directly investigate where the histones that were bound to *GAL10* before replication localize after DNA replication. Schlissel & Rine found that when *GAL10* was transcriptionally inactive, histones were exquisitely retained at the same locus after DNA replication. When *GAL10* transcription was induced in arrested cells, histones were locally retained initially, but were lost from the locus over several hours, suggesting that transcription increases nucleosome turnover directly or via recruitment of some other histone-displacing factor.

The factors that mediate parental histone inheritance at the replication fork are only beginning to be understood. Studies that label old histones and track them through replication have identified several factors that are important for parental histone inheritance and resolved which factors contribute to inheritance on the leading versus the lagging strands. In mammals and yeast, Mcm2, a component of the replicative MCM2-7 helicase, transmits old histones to the lagging strand (Gan et al., 2018; Petryk et al., 2018). In yeast, two nonessential subunits of the leading strand DNA polymerase ε, Dpb3 and Dpb4, facilitate transfer of parental histones to the leading strand (Yu et al., 2018). Deletion of *DPB3* or mutation of the histone-binding domain of *MCM2* lead to loss of local histone inheritance at *GAL10* (Schlissel and Rine, 2019). These same mutations cause partial silencing defects, but do not completely eliminate epigenetic heritability of the silent state in *sir1*Δ cells (Saxton and Rine, 2019). Therefore, either sufficient histone inheritance occurs in the mutants to propagate the silent state or memory of silencing is carried by something other

than histones. Other components of the replisome, namely Ctf4 and the primase DNA Pol α, have been identified for their role in lagging strand histone inheritance (Gan et al., 2018), but the question of how histone chaperones and/or other components of the chromatin assembly machinery might distinguish parental from newly-synthesized histones remains an open question. In addition, another group working in mouse embryonic stem cells reported different locus-specific histone inheritance properties between active and repressed loci (Escobar et al., 2019). We aimed to study whether the local histone inheritance properties of *GAL10* were true elsewhere in the genome, and whether silent chromatin displays specific histone inheritance properties.

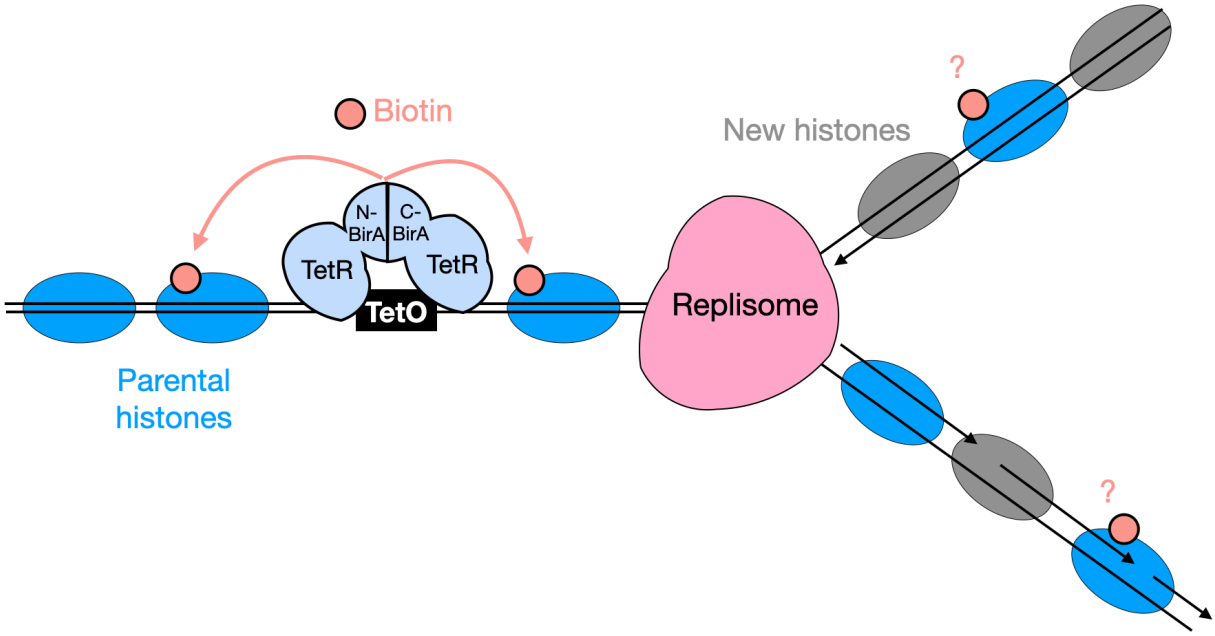

Figure 3.1: TetR-BirA labels histones at a specific locus. TetR binds to TetO as a dimer and recruits the two halves of split BirA to the locus. When co-localized, the two halves of split BirA form a functional biotin ligase which biotinylates adjacent histones on the C-terminal H3 AviTag. After replication, parental histones are tracked via biotin-specific pulldown with streptavidin or an anti-biotin antibody.

3.3 Materials and Methods

Yeast strains and growth conditions

Strains used in this study (**Table 3.3**) were derived from the histone-labeling strains described previously, which were built in the W303 background (Schlissel and Rine, 2019). Strains were transformed and crossed using standard genetic techniques, including yeast-optimized CRISPR-Cas9 gene editing with gene block repair templates, as described previously (Brothers and Rine, 2019; Dunham et al., 2015; Gietz and Schiestl, 2007). The *HML* locus in these strains includes the following modifications: *α2* is replaced with *yEmRFP*, *α1* has an early nonsense mutation, and the two Ste12 binding sites in the promoter (Goodnight and Rine, 2020) are scrambled to prevent Ste12 binding. Various strains were generated using CRISPR-Cas9 to insert *tetO* sites at various positions in *HML* and *GAL10*. All of these modified loci were confirmed by Sanger sequencing.

Cells were grown and cell-cycle manipulations were performed as described in Chapter 2, except that all cells were grown at 30°C. For histone tracking experiments, doxycycline (Sigma D9891) was added to a final concentration of 0.1 mg/mL from a freshly-prepared stock of 10 mg/mL doxycycline. In cell-cycle-controlled experiments, cells were kept in doxycycline for 30 minutes before release to the next phase of the cell cycle.

For assaying nicotinamide sensitivity, standard media plates were supplemented with either 25 mM or 5 mM nicotinamide (Sigma N3376) as noted in the text. For dilution plating assays, cells were first resuspended to OD ~ 1 (~1 x 10^7 cells/mL) in sterile water, from which 10-fold serial dilutions were performed in a 96-well plate. From each well, 5 µL of cell suspension was plated.

Chromatin affinity purification and chromatin immunoprecipitation

For each pulldown, ~2-5 x 10^8 cells were crosslinked in 1% formaldehyde for 15 minutes at room temperature, then quenched with 300 mM glycine for 5 minutes. Cells were washed twice with ~10 mL TBS and once with 1 mL FA lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl, 1 mM EDTA, 1% Triton, 0.1% sodium deoxycholate) + 0.1% SDS + cOmplete EDTA-free protease inhibitor (Sigma-Aldrich 11873580001). Cell pellets were resuspended in FA lysis buffer + 0.1% SDS + protease inhibitor and lysed using a FastPrep-24 5G (MP Biomedicals) with 6.0 m/s beating for 40 seconds followed by 2 minutes on ice, repeated 4 times total. The lysate was transferred to a 15-mL Bioruptor tube and ~200 µL of sonication beads were added (Diagenode C01020031). Sonication was performed in a Bioruptor Pico (Diagenode) with 10 cycles of 30 seconds ON followed by 30 seconds OFF. The sonicated lysate was transferred to a new 1.5-mL tube and spun down at 4°C for 15 minutes at 17,000 RCF. The supernatant, now the soluble chromatin, was transferred to a new tube, and 10% was saved for the input.

For streptavidin pulldowns, 20 µL per sample of Dynabeads MyOne Streptavidin C1 beads (Thermo Fisher 65002) was washed 3 times in FA lysis buffer + 0.1% SDS, then added to the soluble chromatin along with 5 µL of 20 mg/mL BSA (NEB B9000S). The mixture was incubated, rotating, overnight at 4°C. The beads were washed twice with Wash Buffer (10 mM Tris pH 8, 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA) and 3 times with TE + 3% SDS, with ~5 minutes of rotating between each wash. To elute the samples, 75 µL FA lysis buffer + 0.1% SDS + 5 µL of 10 mg/mL RNase A was added and the sample was incubated at 65°C with shaking. After 1 hour, 5 µL of 20 mg/mL proteinase K was added, and the sample was incubated for another hour at 65°C with shaking, for a total of 2 hours at 65°C. The DNA was purified using either a PCR purification kit (Qiagen) or SPRI clean-up beads (UC Berkeley DNA Sequencing Facility).

For anti-biotin immunoprecipitations, 2 µL of anti-biotin antibody (Abcam ab53494) was added to each soluble chromatin sample along with 5 µL of 20 mg/mL BSA. This mixture was incubated overnight at 4°C while rotating. Then 25 µL of Protein A Dynabeades (Invitrogen 10002D) were washed 3 times in FA lysis buffer + 0.1% SDS and then added to the samples and incubated for 1 hour at 4°C. The samples were then washed with the following buffers, incubating at room temperature for ~5 minutes on an end-over-end rotator between each wash: 2x washes in FA lysis buffer + 0.1% SDS; 2x washes in Wash buffer #1 (FA lysis buffer + 0.25 M NaCl + 0.1% SDS); 2x washes in Wash Buffer #2 (10 mM Tris pH 8, 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA); 1 wash in TE. Samples were eluted and DNA was purified as described above for streptavidin pulldown, except in TE + 1% SDS.

For anti-V5 immunoprecipitations, 40 µL of anti-V5 agarose beads (Sigma A7345) was washed 3x in FA lysis buffer + 0.1% SDS, then added along with 5 µL 20 mg/mL BSA and incubated overnight rotating at 4°C. The washes and elution were as described above for anti-biotin immunoprecipitations.

Samples were analyzed either by qPCR or sequencing as described in Chapter 2.

Nicotinamide sensitivity suppressor screen and mutation identification

Strains JRY13506 (*MATa)* and JRY13532 (*MATα*) were streaked to YPD to isolate clonal colonies. From each parent strain, 24 colonies were resuspended in water and ~10^5 cells were spread onto a YPD plate supplemented with 25 mM nicotinamide (NAM). Plates were incubated at 30°C for 2-3 days until clear fast-growing colonies had appeared from the slow-growing lawn. Putative suppressors were re-streaked to YPD + 25 mM NAM to confirm the NAM insensitivity and isolate clonal populations to save and analyze. From both parent strains, 47 independent suppressors (23 *MATa* and 24 *MATα*) were isolated.

DNA was isolated from each suppressor strain using the YeaStar Genomic DNA kit per manufacturer's recommendations (Zymo Research D2002) and diluting final eluate into 100 µL TE. The DNA solution was sheared in 0.65-mL Bioruptor tubes using the Pico Bioruptor with 13 cycles of 30 s ON/30 s OFF. Sheared DNA was prepared for sequencing using the NEB Ultra II library prep kit (NEB E7645L) and sequenced on an Illumina MiniSeq.

Sequencing reads were aligned to the SacCer3 genome using Bowtie2 (Langmead and Salzberg, 2012) and converted to sorted BAM files using SAMtools (Li et al., 2009). Variants were called using the SAMtools command *mpileup* piped to the BCFtools command *call*. Variants that were identified in multiple samples, and were thus unlikely to be novel causative mutations, were excluded using the BCFtools command *isec* with the option "-n-1". Variant *.vcf* files were filtered using the BCFtools command *filter* to exclude variants for which either (1) reference-mapping reads were present >20% as frequently as variant reads or (2) the variant quality score *QUAL* was <50. The merged list of variants was uploaded to the Variant Effect Predictor (McLaren et al., 2016), and the resulting list of variant effects was filtered for those that would alter a protein coding sequence, and sorted for whether the effect was likely to be deleterious using the SIFT metric included in the VEP analysis (Kumar et al., 2009).

3.4 Results

3.4.1 Assessing chromatin effects on histone inheritance

To measure the effect of silencing on histone inheritance, we constructed strains with the same TetR-split BirA fusion as described previously (Schlissel and Rine, 2019), but with *tetO* sequence inserted at the silent locus *HML*. The strains also included the *hmlα2*Δ*::yEmRFP* allele (Saxton and Rine, 2019), which can be used to monitor silencing, as well as the *hmlα1** allele (Goodnight and Rine, 2020), which allows for α-factor sensitivity. We reasoned that histone dynamics may vary as a function of position when the histones are located near genetic features such as promoters and gene bodies (Dion et al., 2007; Kassem et al., 2020). Thus, we generated a trio of strains with *tetO* located at different features of *HML*, with the assumption that while transcription-dependent effects could vary among the strains, silencing-dependent effects would be observed in all strains (**Figure 3.2**).

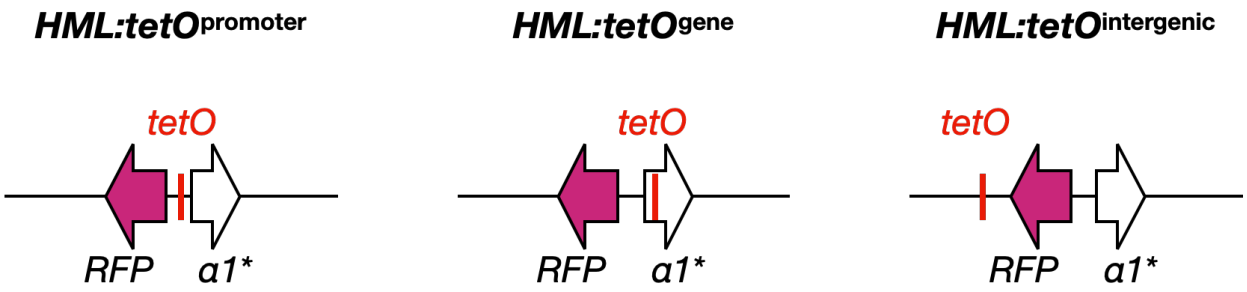

Figure 3.2: *HML:tetO* strains. In all strains, the two Ste12 binding sites at the *HML* promoter were deleted. In the *HML:tetO*^{promoter} strain, the *tetO* sequence replaced one of the two Ste12 binding sites. All *tetO* sequences were placed in linker or nucleosome-depleted regions.

To assess whether histone inheritance was similar at *HML* to that observed at *GAL10* previously, we performed analogous experiments to those from the *GAL10* study (Schlissel and Rine, 2019). Cells were grown with the TetR-split BirA fusion proteins constitutively expressed, thus labeling the H3 molecules at *HML:tetO* with biotin. Then, cells were arrested in G1 with α factor and doxycycline was added to stop labeling. Cells were either maintained in G1 or released to G2/M (**Figure 3.3**). By measuring histone biotinylation using streptavidin pulldown followed by qPCR in these two conditions, we could distinguish between replication-independent histone turnover and replication-dependent histone turnover.

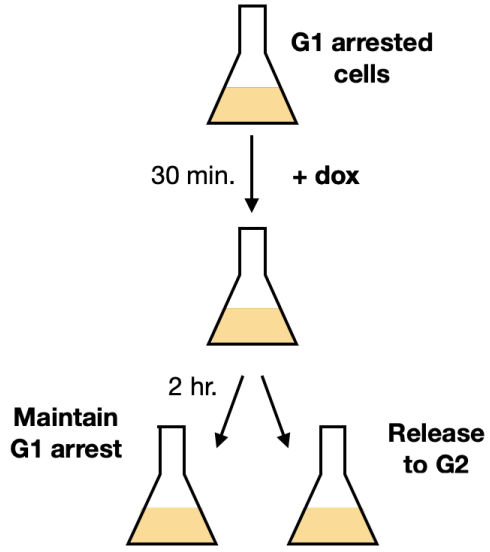

Figure 3.3: Design of H3 biotinylation experiments. As described in Materials & Methods, cells were arrested in G1 and either maintained in G1 or released to G2/M after addition of doxycycline.

In initial experiments comparing the *HML:tetO* strains, the strength of labeling above background varied from strain to strain, depending on the location of *tetO* within *HML* (**Figure 3.4**, "pre" samples). After labeling was turned off by addition of doxycycline, the strains with promoter-located *tetO* and intergenic *tetO* appeared to have higher replication-dependent histone turnover than the gene body *tetO* strain. However, we noticed an unexpected feature of the pulldown efficiencies in these experiments: cells arrested for long periods in α factor or nocodazole

displayed markedly lower recovery after pulldown than cycling cells or cells arrested for a short time (**Figure 3.5**). Input DNA quantity scaled as expected across samples—i.e., approximately identical for all G1-arrested samples and ~2x higher for cells that had been through S phase, indicating that the efficiency of cell lysis or DNA purification were not affected by the arrest conditions (**Figure 3.5**, left). However, after streptavidin pulldown, the quantity of recovered DNA was far higher in samples that were not subjected to long arrests (**Figure 3.5**, right). The simplest explanation for this observation is that some compound present in the cell lysate from cells that had experienced extended arrests outcompetes DNA for access to the streptavidin-coated beads. Condition-dependent differences in histone pulldown efficiency should not affect the reported data if the change in pulldown efficiency is the same at *HML:tetO* and the control non-biotinylated locus to which it is compared—*ALG9* in these experiments—but we have not yet demonstrated that this is the case. One piece of evidence suggesting that the quality of the data might be negatively affected by these normalization concerns is seen in the *HML:tetO*^{gene} experiment in **Figure 3.4**: the biotinylation signal is >50% of its starting value after DNA replication, which would run counter to the strong expectation that parental histones should be diluted at least by half during S phase. Altogether, for the data presented in **Figure 3.4**, the subtle differences in histone inheritance observed across conditions may be limited by a combination of relatively low signal-to-noise and unpredictable background signal. These limitations led us to consider modifications to the experimental protocol that could alleviate those concerns.

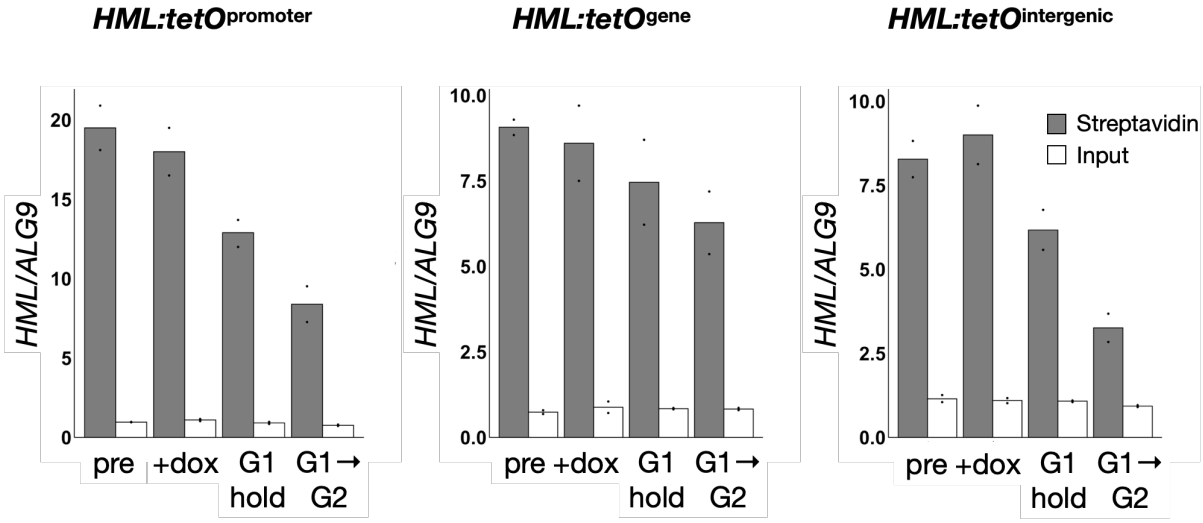

Figure 3.4: Replication-dependent and replication-independent histone turnover at *HML:tetO*. Cells from strains JRY13483 (*HML:tetO*^{promoter}), JRY13474 (*HML:tetO*^{gene}), and JRY13485 (*HML:tetO*^{intergenic}), were arrested in α factor for 2.5 hours ("pre" sample). Doxycycline was added for 30 minutes ("+dox"). The culture was then split, and cells were either kept in G1 or released to G2/M for 2 hours. At each time point, cells were fixed and collected for streptavidin pulldown. Dots represent biological replicates and bars represent the average of biological replicates.

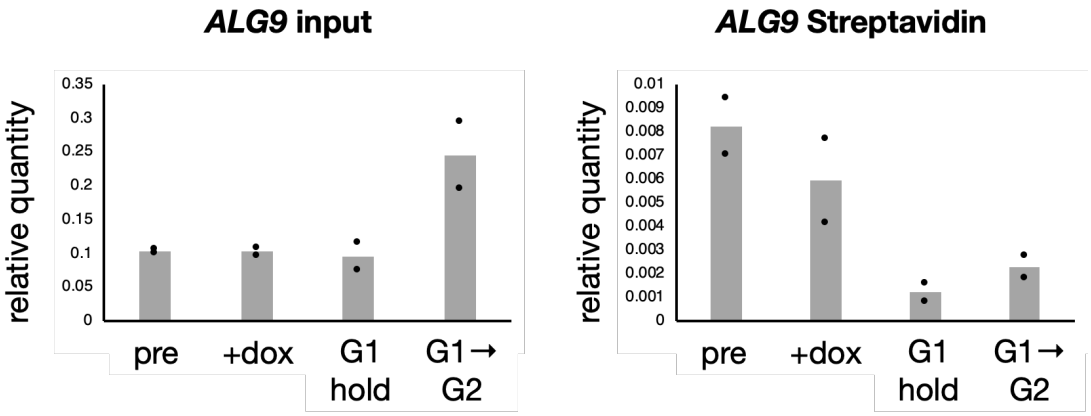

Figure 3.5: Streptavidin pulldown efficiency is reduced in long arrests. The plotted data are from the same experiment as presented in Figure 3.4, using the strain JRY13483 (*HML:tetO*^{promoter}) as a representative example. Plotted are the raw values for *ALG9* quantity for input samples (left) and streptavidin pulldown samples (right). Levels of *ALG9*, an arbitrary non-biotinylated locus, are shown, because they represent the background level of signal to which *HML* is normalized in the experiments in Figure 3.4.

As one attempt to increase signal, we performed analogous experiments to those described above, but using an anti-biotin antibody instead of streptavidin to pull down on biotinylated histones. In an attempt to reduce the differences in pulldown efficiency across conditions, we also shortened the length of the post-doxycycline incubation to only 75 minutes from 3 hours, just enough time for all cells to pass through S phase. While this approach did lead to a considerably higher signal-to-noise ratio, it did not change the pulldown efficiency reduction in G2/M-arrested cells (data not shown). With the increased signal, though, we were able to begin investigating the effect of silencing on histone inheritance (**Figure 3.6**). In wild-type cells, parental histones at *HML* strongly remember their position, to a similar degree as that observed at *GAL10* (Schlissel and Rine, 2019). Interestingly, when we compared a *SIR* strain to a *sir* mutant strain by qPCR, the degree of replication-coupled parental histone loss, as measured at the intergenic operator, increased in the *sir* mutant (**Figure 3.6A**). The amount of labeling was also reduced at the onset of the experiment in *sir* mutants (compare "pre" samples). We performed ChIP-seq on these samples to identify whether histone labeling or retention was being fully captured by the qPCR result. The sequencing results recapitulated the qPCR finding that *sir* mutant cells lost parental histones more dramatically than did *SIR* cells (**Figure 3.6B**). However, the sequencing did not show any apparent *SIR*-dependent difference in pre-doxycycline labeling efficacy. This could be due to a slightly altered labeling radius in the *SIR* cells compared to *sir* mutant cells (**Figure 3.6C**). The peaks of biotinylation are offset by ~50-100 bp from each other, which could cause an apparent under-recovery of one or the other by qPCR, based on the exact location of the primers.

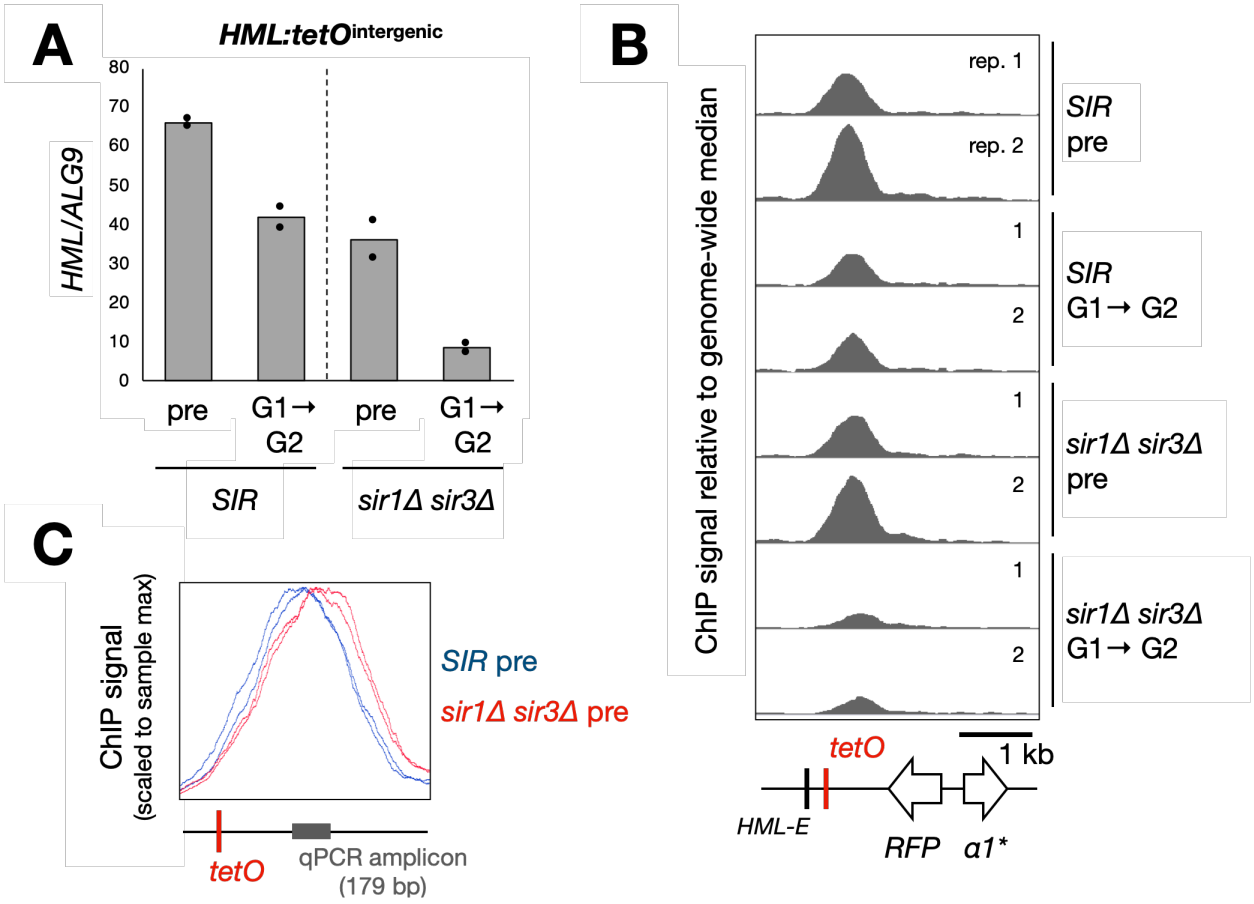

Figure 3.6: Loss of silencing reduces replication-coupled histone inheritance. Cells with wild-type *SIR* alleles (JRY13544) or *sir1*Δ *sir3*Δ cells (JRY13541) were arrested in α factor for 2.5 hours ("pre" sample). Doxycycline was added for 30 minutes, followed by release to G2/M for 75 minutes (105 minutes total after doxycycline addition). Cells were fixed and processed for anti-biotin immunoprecipitation, followed by qPCR (A) or sequencing (B, C). Data in (B) are scaled to genome-wide median value, while data in (C) are scaled to the sample maximum value, to more easily see the shape of the labeling.

3.4.2 Nicotinamide sensitivity in absence of histone inheritance factor *DPB3*

Cells lacking the DNA polymerase ε component Dpb3 or with a histone-binding mutation in the replicative helicase component Mcm2 display reduced parental histone inheritance, with the double mutants having an additive effect (Petryk et al., 2018; Schlissel and Rine, 2019; Yu et al., 2018). In the course of our work studying the intersection of parental histone inheritance and silencing, we discovered that double mutant *dpb3*Δ *mcm2-3A* cells displayed a hypersensitivity when treated with the sirtuin inhibitor nicotinamide (NAM; **Figure 3.7**). Deleting *SIR1* and *SIR3* in a *dpb3*Δ *mcm2-3A* strain did not cause a growth defect, and thus the NAM sensitivity was not due to a failure of silencing. Through back-crossing the double mutant strain, we observed that *dpb3*Δ was largely responsible for the NAM sensitivity (**Figure 3.8**, top). On its own, *mcm2-3A* caused no NAM sensitivity, and the double mutant *dpb3*Δ *mcm2-3A* was very slightly sicker than the single *dpb3*Δ mutant. Even though *mcm2-3A* does not contribute strongly to the mutant phenotype, given that we had begun our analysis with the *dpb3*Δ *mcm2-3A* double mutant we continued to use that mutant as our parent strain for subsequent analyses.

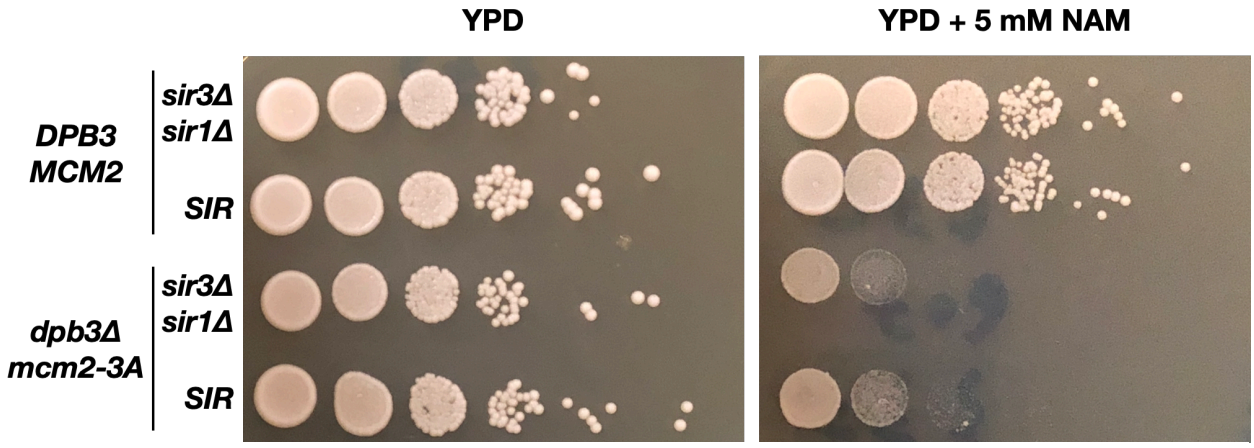

Figure 3.7: Nicotinamide sensitivity of *dpb3*Δ *mcm2-3A* cells. The following strains were grown for 2 days on YPD or YPD + 5 mM NAM: *DPB3 MCM2 SIR1 SIR3* (JRY13483); *DPB3 MCM2 sir1*Δ *sir3*Δ (JRY13168); *dpb3*Δ *mcm2-3A SIR1 SIR3* (JRY13481); *dpb3 mcm2-3A sir1*Δ *sir3*Δ (JRY13170).

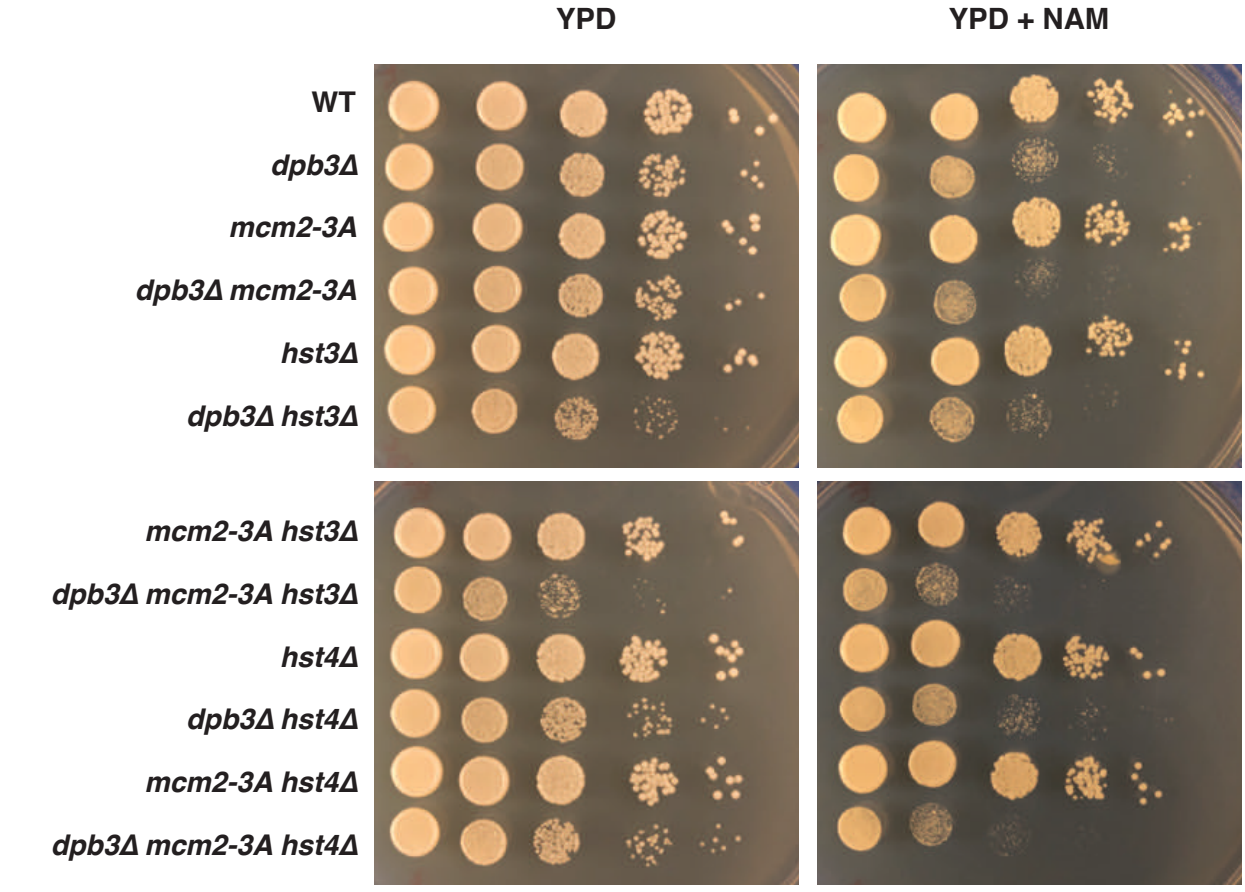

Figure 3.8: Nicotinamide sensitivity is due to inhibition of *HST3* and *HST4* and does not depend on *MCM2*. The following strains were grown for 2 days on YPD or YPD + 5 mM NAM: WT (JRY13508); *dpb3*Δ (JRY13509); *mcm2-3A* (JRY13510); *dpb3*Δ *mcm2-3A* (JRY13511); *hst3*Δ (JRY13512); *dpb3*Δ *hst3*Δ (JRY13513); *mcm2-3A hst3*Δ (JRY13514); *dpb3*Δ *mcm2-3A hst3*Δ (JRY13515); *hst4*Δ (JRY13516); *dpb3*Δ *hst4*Δ (JRY13517); *mcm2-3A hst4*Δ (JRY13518); *dpb3*Δ *mcm2-3A hst4*Δ (JRY13519). All strains are *MATα.*

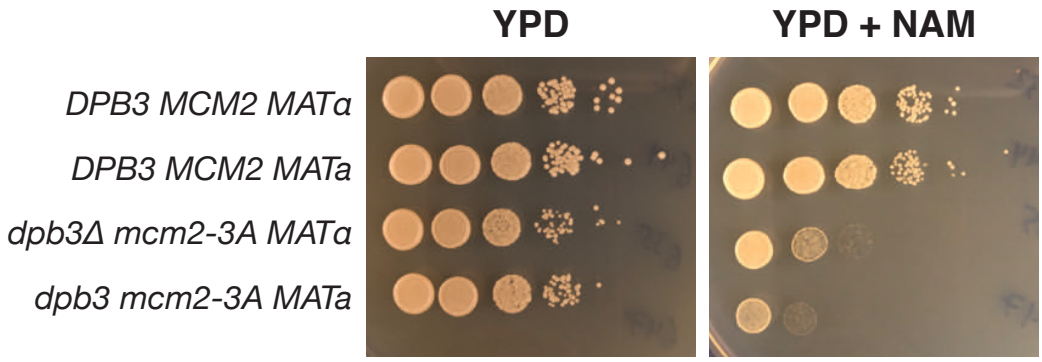

Figure 3.9: *MATa dpb3*Δ cells are more sensitive to nicotinamide than *MATα dpb3*Δ cells. The following strains were grown for 2 days on YPD or YPD + 5 mM NAM: *DPB3 MCM2 MATα* (JRY13508); *DPB3 MCM2 MATa* (JRY13520); *dpb3*Δ *mcm2-3A MATα* (JRY13511); *dpb3*Δ *mcm2-3A MATa* (JRY13523).

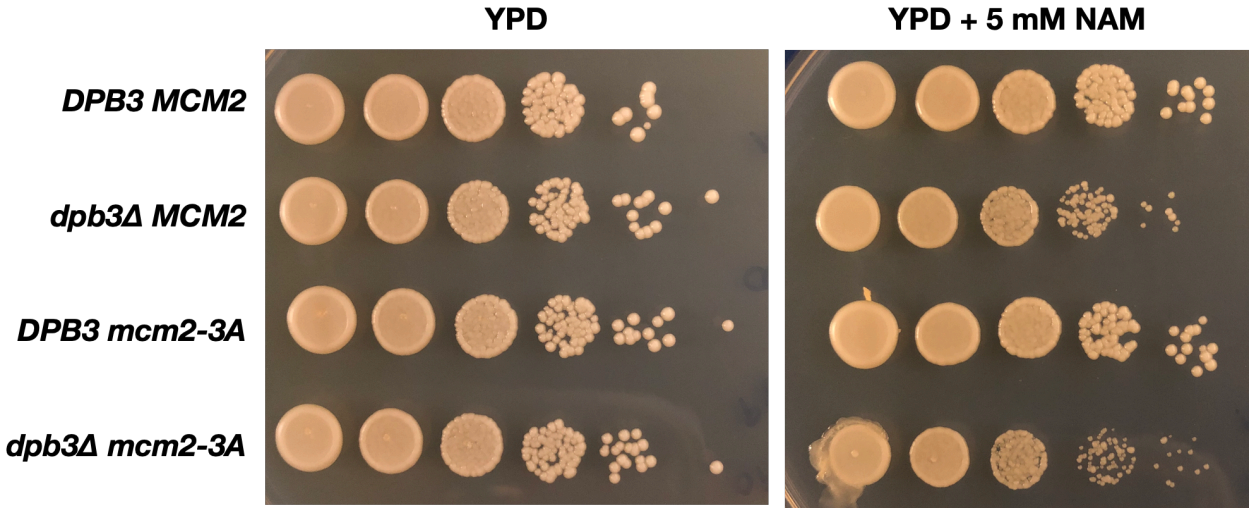

Figure 3.10: Previously-described *dpb3*Δ strains were less sensitive to nicotinamide. The following strains were grown for 2 days on YPD or YPD + 5 mM NAM: *DPB3 MCM2* (JRY11471); *dpb3*Δ *MCM2* (JRY11550); *DPB3 mcm2-3A* (JRY11589); *dpb3*Δ *mcm2-3A* (JRY11590). Note that the *DPB3 MCM2* strain is a CRASH assay strain, while the mutants are all FLAME assay strains (Saxton and Rine, 2019).

In addition to inhibiting Sir2, nicotinamide also inhibits the other sirtuins, two of which, Hst3 and Hst4, are known to have a role in replication-coupled histone dynamics. Newly-synthesized histones are acetylated by Rtt109 on H3K56, which is thought to facilitate incorporation of new histones into DNA during replication (Driscoll et al., 2007; Li et al., 2008). After S phase, H3K56 acetylation is removed by Hst3 and Hst4, and failure to deacetylate H3K56 leads to growth defects that are due to replication stress (Celic et al., 2008, 2006; Maas et al., 2006). Thus, we were intrigued by the possibility that the nicotinamide sensitivity in *dpb3*Δ *mcm2-3A* cells, which have defects in parental histone inheritance, might be due to defects in the removal of new histone marks. Indeed, the *dpb3*Δ *mcm2-3A* strains grew poorly when combined with *hst3*Δ

or *hst4*Δ, and each grew worse when plated on NAM, indicating that both *HST3* and *HST4* contribute to the phenotype (**Figure 3.8**). Deletion of *RTT109*, which codes for the H3K56 acetyltransferase, suppressed the *dpb3*Δ *mcm2-3A* NAM sensitivity (**Table 3.1** and below), further confirming that the phenotype was due to defects in removal of H3K56ac by Hst3 and Hst4. From the same cross that generated the *hst3*Δ and *hst4*Δ mutants, we noticed that *MATa dpb3*Δ cells were considerably more sensitive to NAM than *MATα* cells (**Figure 3.9**). In these strains, because *HML* does not encode any functional α proteins, *MATa* cells retain their **a** gene expression pattern on NAM, while *MATα* cells on NAM de-repress *HMRa* and become pseudo-diploid by expressing both **a** and α genes.

The strong nicotinamide sensitivity observed in *dpb3*Δ cells was counter to expectations based on previous work on the effects of *DPB3* on silencing (Daniel Saxton, personal communication). Indeed, when we analyzed a previously-generated *dpb3*Δ strain (Saxton and Rine, 2019), it displayed nicotinamide sensitivity, but of far weaker magnitude than the sensitivity of the histone tracking strains (**Figure 3.10**). The histone tracking strains have a number of modifications relative to the strains used by Saxton & Rine (see **Table 3.3**), the most likely of which to cause increased *dpb3*Δ NAM sensitivity is the AviTag on histone H3. Even though the *dpb3*Δ NAM sensitivity is enhanced by some feature of the strain's genetic background, the enhanced sensitivity is still specific to the combination of inhibited Hst3/Hst4 and *dpb3*Δ. Thus, we treated the histone tracking *dpb3*Δ strain as a sensitized background in which to query the *dpb3*Δ NAM sensitivity.

Several suppressors of *hst3*Δ *hst4*Δ synthetic sickness have been previously identified (Celic et al., 2008, 2006; Collins et al., 2007; Driscoll et al., 2007). These suppressors fall into three classes: (1) mutations that decrease H3K56 acetylation, (2) mutations that decrease alternative clamp loading and favor PCNA loading, and (3) mutations in components of the Rtt101-Mms1-Mms22 E3 ligase, which is thought to act downstream of H3K56ac in several replication-coupled processes and may be directly stimulated by H3K56ac (Collins et al., 2007; Han et al., 2013). We tested these previously identified *hst3*Δ *hst4*Δ suppressors for their ability to suppress *dpb3*Δ NAM sensitivity (**Table 3.1**). Strikingly, the mutations that affect H3K56ac itself or the downstream Rtt101-Mms1-Mms22 E3 ligase all suppressed the NAM sensitivity observed in *dpb3*Δ *mcm2-3A* cells. In contrast, none of the mutants involved in clamp loading had any affect. Thus, in absence of Dpb3, hyperacetylation of H3K56 acetylation inhibits cell growth via a mechanism involving the Rtt101-Mms1-Mms22 E3 ligase, but not via aberrant DNA clamp loading.

To further understand the source of the *dpb3*Δ nicotinamide sensitivity, we performed a genetic screen to identify suppressors of the sensitivity. Spontaneous suppressors emerged frequently when *dpb3*Δ *mcm2-3A* cells were plated on 25 mM NAM, which is consistent with previous reports that both *dpb3*Δ cells and *hst3*Δ *hst4*Δ cells have mutator phenotypes (Aksenova et al., 2010; Kadyrova et al., 2013). We isolated and analyzed 47 independently derived spontaneous suppressors from the *dpb3*Δ *mcm2-3A* histone tracking strains JRY13506 (*MATa*) and JRY13532 (*MATα*). We sequenced these 47 suppressors and identified high-confidence protein-coding changes. Because we have not yet followed the linkage of phenotype to genotype through a cross, each coding change can presently only be described as a putative hit. The results of the screen are presented in **Table 3.2**. Almost all suppressor strains had at least two protein-

coding changes, so while we can tentatively ascribe suppressor phenotypes to some individual mutations, further analysis is needed to confirm this. Many of suppressor strains (29 out of 47) had mutant alleles of a proteasome component, with mutations in many individual subunits across both the 20S core particle and the 19S regulatory particle (Finley et al., 2016). The broad spectrum of proteasome mutations indicates that the suppressive phenotype is likely due to a general inhibition of proteasome activity. In addition to proteasome subunits, we also identified several mutations in genes with known roles in protein degradation, including the proteasome chaperone Poc4, the E2 ubiquitin ligase Rad6, the ubiquitin protease Ubp14, and the SUMO-targeted ubiquitin ligase Uls1. Together, these data suggest that proteasome-dependent degradation of some target(s) is required for the sensitivity of *dpb3*Δ cells to nicotinamide.

Mutant Class	Mutation	Strain Number	MAT	Growth on YPD	Growth on NAM
dpb3Δ mcm2-3A parent strains		JRY13532	α	+++	–
		JRY13506	*a*	+++	–
1	***asf1*Δ**	JRY13612	α	+++	+
		JRY13599	*a*	+++	+
	***rtt109*Δ**	JRY13539	α	++	++
		JRY13537	*a*	++	++
2	***dcc1*Δ**	JRY13608	α	+++	–
		JRY13595	*a*	+	–
	***rad24*Δ**	JRY13609	α	+++	–
		JRY13596	*a*	+++	–
	***ctf8*Δ**	JRY13610	α	+	–
		JRY13597	*a*	+	–
	***ctf4*Δ**	JRY13619	α	+++	–
		JRY13606	*a*	+++	–
	***mec3*Δ**	JRY13613	α	+++	–
		JRY13600	*a*	+++	–
	***ctf18*Δ**	JRY13615	α	+	–
		JRY13602	*a*	+	–
	***elg1*Δ**	JRY13616	α	+++	–
		JRY13603	*a*	+++	–
	***rad17*Δ**	JRY13617	α	+++	–
		JRY13604	*a*	+++	–
	***ddc1*Δ**	JRY13618	α	+++	–
		JRY13605	*a*	+++	–
3	***mms22*Δ**	JRY13614	α	+++	+/++
		JRY13601	*a*	+++	+/++
	***mms1*Δ**	JRY13620	α	+++	+/++
		JRY13607	*a*	+++	+/++
	***rtt101*Δ**	JRY13611	α	+++	++
		JRY13598	*a*	+++	+

Table 3.1: Effects of previously identified *hst3*Δ *hst4*Δ suppressors on *dpb3*Δ nicotinamide sensitivity. All strains were *dpb3*Δ *mcm2-3A*. The indicated strains were grown on YPD and YPD + 25 mM NAM to test for nicotinamide sensitivity. The level of growth was assessed qualitatively, with +++ indicating equivalent growth to wild-type cells on YPD, ++ indicating slightly weaker growth, + indicating weak but significant growth, and – indicating no or extremely weak growth. Mutations that suppress the *dpb3*Δ NAM sensitivity are noted in green. Mutants in Class 1 are the genes that are required for H3K56 acetylation (Celic et al., 2006; Driscoll et al., 2007). Mutants in Class 2 are those that suppress *hst3*Δ *hst4*Δ mutations by inhibiting alternate clamp loading or increasing PCNA loading (Celic et al., 2008). Mutants in Class 3 are components of an E3 ligase that acts downstream of H3K56 acetylation (Collins et al., 2007; Han et al., 2013).

Mutant identifier	Protein coding changes	Mutant identifier	Protein coding changes
A1	***PUP3***, *JHD2, NUP133, GPR1*	A5	*SOK2, NOP8, POL3,* ***PRE3***
A2†††	***DCR2,*** ***RPN6***†	A6	*NUP57,* ***MAM1****, PRE10*
A3	***POC4****, RPD3*	A7	*YLR255C, VHS2,* ***NIS1****,* ***RPT3***
B1	*CLC1, YKR032W,* ***PRE2***	B5	***PRE2***
B2	*GPR1, MRPL1,* ***PUP1***	B6	***RPN2***
B3	*IPT1,* ***ULS1***	B7	***NIS1***
C1	*VPS8,* ***TFA2***	C5	***YGR114C****, MEH1, YBR225W, SAP190, NIS1, TIF4632,* ***RPT6,*** ***VPS13, YGR115C****
C2	*VPS8, RPF1,* ***HPF1,*** ***PRE8***	C6	*YPR114W,* ***CBS2, QRI7,*** ***PRE7***
D1	*PLM2,* ***PRE4***	C7	*ZRG8,* ***RPN5****
D2	***CWC15****	D5	*YBR225W, GPX2, RSN1,* ***RTT101***
D3	*ECM22,* ***UBP14****	D6†††	*SKI3,* ***PUP1***
E1	***CSF1, SAS3***	D7	***SRP1, RAD6***
E2	***YNL205C****,* ***PRE4***	E5	*YLR255C,* ***RPN5****
E3†††	*ESP1, KIN2*	E6	*YBR225W, NUP57, PUF3, RAD50,* ***SKI3,*** ***PRE4***
F1	***SLX1****,* ***RPT3***	E7	*MDM20,* ***PUP3***
F2	*NGR1, SAS10, ENT1, ECM22,* ***UCC1****, PRE4*	F5	No protein-coding changes identified††
F3	*YCR087W, ASN2*	F6	*NSR1, YGR160W,* ***DOT1,*** ***RPT2***
G1	No protein-coding changes identified	F7	*MTC4,* ***YNL205C****,* ***RPT5***
G2	***RTC1****,* ***KIP1****	G5	***BUD4****, NSR1, YGR160W, IMD4, GDB1,* ***PRE7***
G3	***PRE4***	G6	*YBR225W, PUF3,* ***EGH1, NNK1***
H1	*SAS10, RKM3, CAF4, SPO1, DPM1,* ***SEM1****	G7	*YLR255C, ASG1, ECM22,* ***PUP3***
H2	*NOP8, UBP14*	H5	*NMD2,* ***RPP0, PSD1,*** ***PRE2***
H3	***RTC1****	H6	*YBL070C, SAS10,* ***ROM2, RNH1,*** ***YNL205C****,* ***PRE6***
		H7	***YGR114C****,* ***YGR115C****, IRC20,* ***RPN2****

Table 3.2: Mutants identified in *dpb3*Δ NAM suppressor screen. The left and right columns of mutants were isolated, respectively, from the *MATa* parent strain JRY13506 and the *MATα* parent strain JRY13532. Amino acid changes flagged as deleterious by SIFT are marked in **bold**. Nonsense mutations are in addition marked with an asterisk (*). Genes encoding proteasome subunits are colored green.

† The mutation in *RPN6* in mutant A2 is at the stop codon, and generates a 7-residue readthrough.

†† No coding mutation was identified in mutant F5, but a point mutation was identified in the TATA box upstream of *RPN6*.

††† Mutants A2, E3, and D6 display dominant NAM resistance when crossed back to the opposite mating type parent strain

3.4 Discussion and Future Directions

The project described in this chapter is a work in progress, and thus the discussion will address both tentative conclusions and ongoing work.

3.4.1 Silencing may regulate histone dynamics

I showed that in wild-type cells, parental histone inheritance is robust in silent chromatin. Furthermore, I showed preliminary evidence that silencing at *HML* may be important for the inheritance of parental histones through DNA replication. This would seem to be consistent with the increase in histone turnover observed at *GAL10* when the locus was transcribed (Schlissel and Rine, 2019). A critical issue still to be resolved is whether the reduced histone inheritance is due to transcription itself or a failure of a silencing-specific histone inheritance pathway. The existence of an epigenetic silencing phenotype in some mutants suggests that there are mechanisms in place to ensure the faithful transmission of the silent state (Pillus and Rine, 1989). However the model that the silent state is specifically mediated by inheritance of histones has been refuted (Saxton and Rine, 2019). Daniel Saxton's work on epigenetic inheritance and Gavin Schlissel's work on parental histone inheritance converged with the finding that *dpb3*Δ *mcm2-3A* cells had essentially no local parental histone inheritance, but retained the ability to propagate epigenetic chromatin states. This suggested that histones could not be the carriers of epigenetic memory. However, Gavin's histone inheritance experiments were somewhat limited by the difficulties in performing cell-cycle arrests in *dpb3*Δ *mcm2-3A* strains, and he was forced to infer histone retention from mixed populations of cells, some of which were appropriately arrested and some of which were not. In my own preliminary experiments with similar strains, I have observed that while *dpb3*Δ *mcm2-3A* cells do indeed have severely reduced local histone retention at both *GAL10* and *HML*, but the inheritance is not completely eliminated. Thus, it is formally possible that in *dpb3*Δ *mcm2-3A* cells, the reduced but not eliminated local inheritance of modified parental histones was sufficient to give rise to the epigenetic memory Daniel observed in those strains. If inheritance of parental histones were important for the inheritance of the silent state, the machinery that retains parental histones at *GAL10* would seem to be sufficient to propagate silencing, assuming that same machinery functions during replication at *HML*. That the same inheritance mechanisms function at both *GAL10* and *HML* remains to be demonstrated, and it is possible that a different mechanism of parental histone transfer exists at silent chromatin.

At the *HML:tetO*$^{\text{intergenic}}$ locus where histone inheritance was measured, the *tetO* site was ~800 base pairs away from the *hmlα2*Δ*::yEmRFP* gene, which would suggest that the silencing effect on histone inheritance is mediated by an effect on the chromatin at the locus, rather than transcription *per se.* However, the *tetO* site is at the 3' end of pseudogene *YCL068C*, which is weakly transcribed in *sir* mutant cells (Ellahi et al., 2015), so direct effects mediated by the transcription of that gene cannot be excluded. Further experiments are in progress to study this distinction, including an attempt to completely remove transcription of *YCL068C* by mutating its putative promoter sequence. The increase in histone turnover at transcribed *GAL10* is replication-independent, and thus determining whether silencing effects at *HML* are replication-dependent or -independent could determine whether the effects are analogous to the *GAL10* transcription effects or distinct. If transcription were directly evicting nucleosomes, then the strength of transcription

would be expected to correlate with the rate of histone loss, so comparing the histone inheritance at *HML:tetO*^{intergenic}, in which the *tetO* overlaps with a very weakly transcribed pseudogene and *HML:tetO*^{gene}, in which the *tetO* is in the much more highly transcribed gene, α*1*, would provide a test for the directness of a transcription effect.

The possible confounding effect of arrest-specific changes in pulldown efficiency means that we must interpret the comparisons between different conditions carefully. However, even if comparing the ratio of biotinylation between arrests, e.g., G1 vs. G2/M, may be fraught, comparing the relative loss in different mutants in the same arrest condition, e.g. *sir3*Δ G2/M vs. *SIR3* G2/M, should be more robust. To avoid the issue completely, we can perform non-cell-cycle-regulated experiments, which can measure the kinetics of histone loss, but are limited because they cannot distinguish between replication-dependent and replication-independent effects.

3.4.2 A genetic interaction between *DPB3* and *HST3/4*

We reported an uncharacterized synthetic sickness that depends on loss of a protein involved in parental histone inheritance, Dpb3, and the enzymes that remove a defining mark from newly synthesized histones, Hst3 and Hst4. A recent study identified *dpb3*Δ as causing synthetic sickness in a *hst3*Δ *hst4*Δ background, and *dpb3*Δ was also identified in a screen for nicotinamide-sensitive mutants, but neither of these results was further characterized (Choy et al., 2016; Gershon and Kupiec, 2021). We are interested in dissecting whether this phenotype is due to errors specifically related to these proteins' roles mediating histone dynamics. In absence of Hst3 and Hst4 activity, histone H3 is hyperacetylated at K56, which is usually a mark of new histones (Celic et al., 2006), while in absence of Dpb3, old histone inheritance to the leading strand is reduced (Yu et al., 2018). Nicotinamide sensitivity in *dpb3*Δ cells could suggest that H3K56 acetylation reduces the residual leading-strand histone inheritance mechanisms in *dpb3*Δ cells. Alternatively, H3K56 acetylation may cause defects in lagging strand parental histone inheritance that cause synthetic defects when combined with the leading strand reduction caused by removal of *DPB3*. It is unclear how the Dpb3, Mcm2, and other components of the parental histone inheritance machinery specifically recognize their substrates, and given that H3K56 acetylation is a mark for new histones, the removal of that mark may be important for labeling a histone as "parental" for inheritance.

The nicotinamide sensitivity we observed in *dpb3*Δ cells was more severe in *MATa* cells than *MATα* cells. When nicotinamide is added to these strains, Sir2 is inhibited, causing *MATα* cells to adopt the **a**/α diploid gene expression pattern, while *MATa* cells retain the **a** expression pattern. There are few gene expression differences between haploids and diploids, with most relating to mating regulation (Ellahi et al., 2015; Galgoczy et al., 2004). One notable exception is that non-homologous end-joining is repressed in diploids, where homologous recombination (HR) is favored (Åström et al., 1999; Lee et al., 1999). One speculative explanation for increased homologous recombination suppressing *dpb3*Δ NAM sensitivity is via the role of H3K56 acetylation in promoting the activity of the cullin Rtt101-Mms1-Mms22 E3 ligase (Han et al., 2013). Rtt101-Mms1-Mms22 is thought to mediate replication restart at stalled replication forks by promoting HR-mediated repair between newly-replicated strands (Alabert et al., 2009; Buser et al., 2016; Duro et al., 2008; McGlynn and Lloyd, 2002). Thus, in nicotinamide, increased H3K56 acetylation could decrease the activity of Rtt101-Mms1-Mms22, and thus inhibit HR-

mediated restart of stalled forks. The pseudodiploid-specific increase in homologous recombination activity could partially alleviate the sickness associated with stalled replication forks.

Our screen for suppressors of *dpb3*Δ nicotinamide sensitivity yielded many mutants in proteasome subunits. We reasoned that because *dpb3*Δ H3-Avi strains are more sensitive to nicotinamide than *dpb3*Δ strains with wild-type H3, degradation of H3 itself might be driving the sensitivity. The Rtt101-Mms1-Mms22 E3 ligase complex has been shown to ubiquitinate histone H3 in an H3K56ac-dependent manner (Han et al., 2013). Furthermore, deletion of *RTT101, MMS1,* or *MMS22* suppresses the sickness of *hst3*Δ *hst4*Δ strains (Collins et al., 2007). Thus, ubiquitin-mediated degradation of H3 could increase in NAM-treated cells and drive a synthetic sickness with *dpb3*Δ. Deletion of *RTT101* does not affect the half-life of H3, which was taken as evidence that Rtt101-dependent ubiquitination of H3 does not target H3 for proteasomal degradation (Han et al., 2013). However, bulk half-life measurements might not be affected if Rtt101-dependent proteasomal degradation of H3 were occurring, but only on a subset of histones. Thus, it is possible that H3 acetylated at K56 is subject to Rtt101-Mms1-Mms22-dependent degradation. Our use of the H3-Avi sensitized background for the screen meant that if H3 degradation were involved in the phenotype, it could be explained by one of two mechanisms: (1) The combination of *dpb3*Δ and nicotinamide leads to increased proteasome-dependent degradation of H3, which slows growth, and the AviTag further destabilizes H3, enhancing the phenotype; or (2) the AviTag on H3 causes elevated proteasome-dependent degradation of H3, which sensitizes the cells to some other proteasome-independent defect caused by the combination of *dpb3*Δ and nicotinamide. If the first possibility were true, meaning that the proteasome is a *bona fide* mediator of *dpb3*Δ NAM sensitivity, we would expect the identified proteasome mutants to suppress the *dpb3*Δ NAM sensitivity in strains with wild-type H3. In addition, H3 degradation would be expected to increase in absence of *DPB3* or upon NAM treatment. If the second possibility were true, we would only see such effects in H3-Avi strains. We are currently investigating whether the AviTag, nicotinamide, and removal of *DPB3* affect H3 levels. In addition, we are continuing to investigate all mutants, including for whether they suppress the nicotinamide sensitivity of a *dpb3*Δ strain with a wild-type H3.

Of the identified suppressors of *hst3*Δ nicotinamide sensitivity that do not appear to have mutations in proteasome subunits or other protein degradation factors, several are worthy of comment. *SAS3*, which codes for the catalytic subunit of the NuA3 complex (John et al., 2000), *TFA2*, which codes for a component of the TFIIE core transcription factor (Feaver et al., 1994), and *CWC15*, which codes for a spliceosome component (Ohi et al., 2002) are involved in transcription and pre-mRNA processing, and mutations in those genes might suppress sensitivity by reducing expression of another gene. Two frameshift variants of *RTC1* were identified. Rtc1 is a positive regulator of TORC1 activity as a component of the SEACAT complex, which is analogous to the mammalian GATOR complex (Algret et al., 2014). Thus, mutation of *RTC1* may reduce TORC1 activity, leading to many changes to cell metabolism. In addition, one strain had a mutation in the gene that codes for the Nnk1 kinase, which physically and functionally interacts with TORC1 (Breitkreutz et al., 2010). TORC1 signaling leads to increased H3K56 acetylation, at least partly via effects on Hst3 and Hst4 protein levels and localization (Chen et al., 2012; Workman et al., 2016), and thus a decrease in TORC1 signaling could suppress nicotinamide sensitivity by decreasing H3K56ac. Finally, one of only three dominant suppressors had a mutation

in *ESP1*, the gene for yeast separase, with the mutation located in the Pds1-binding domain (Ciosk et al., 1998; Jensen et al., 2001). The DNA damage checkpoint inhibits Esp1 by stabilizing its repressor Pds1, and *hst3*Δ *hst4*Δ cells are known to have chronic DNA damage checkpoint activation (Celic et al., 2008; Yam et al., 2021). Overexpression of *ESP1* can lead to bypass of DNA damage checkpoint arrest (Tinker-Kulbetg and Morgan, 1999). Thus, one possibility is that the *dpb3*Δ NAM sensitivity is due to hyperactivation of the DNA damage checkpoint and the mutant *ESP1* allele alleviates the checkpoint inhibition. Further analysis is needed, including, critically, experiments to identify whether the *dpb3*Δ NAM sensitivity suppression is indeed due to these mutations.

Table 3.3: Yeast strains used in this study: Unless otherwise noted, all strains are *ADE2; can1-100; leu2-3,112; ura3-1; lys2-; trp1-1; HHT1-1xAvi; HHT2-1xAvi; hoΔ::CDC37p-TetR-BirA(1-213); bar1Δ::CDC37p-TetR-BirA(214-320).* All strains were generated for this study by transforming parent strains from Schlissel and Rine, 2019. The final four strains are from Saxton and Rine, 2019, and the full genotype is displayed.

Strain	Genotype
JRY13506	*MATa; GAL10:tetO^{gene}; HML:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A*
JRY13532	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}; dpb3Δ::KanMX; mcm2-3A*
JRY13483	*MATa; GAL10:tetO^{promoter}; HML:tetO^{promoter}*
JRY13474	*MATa; GAL10:tetO^{gene}; HML:tetO^{gene}*
JRY13485	*MATa; GAL10:tetO^{gene}; HML:tetO^{intergenic}*
JRY13544	*matΔ::loxP-KanMX-loxP; GAL10:tetO^{gene}; HML:tetO^{intergenic}*
JRY13541	*matΔ::loxP-KanMX-loxP; GAL10:tetO^{gene}; HML:tetO^{intergenic}; sir1Δ::LEU2; sir3Δ::NatMX*
JRY13168	*MATa; GAL10:tetO^{promoter}; HML:tetO^{promoter}; sir1Δ::LEU2; sir3Δ::NatMX*
JRY13481	*MATa; GAL10:tetO^{promoter}; HML:tetO^{promoter}; dpb3Δ::HIS3MX; mcm2-3A*
JRY13170	*MATa; GAL10:tetO^{promoter}; HML:tetO^{promoter}; dpb3Δ::HIS3MX; mcm2-3A; sir1Δ::LEU2; sir3Δ::NatMX*
JRY13508	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}*
JRY13509	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}; dp3Δ::HisMX*
JRY13510	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}; mcm2-3A*
JRY13511	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}; dpb3Δ::HisMX; mcm2-3A*
JRY13512	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}; hst3Δ::NatMX*
JRY13513	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}; dpb3Δ::HisMX; hst3Δ::NatMX*
JRY13514	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}; mcm2-3A; hst3Δ::NatMX*
JRY13515	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}; dpb3Δ::HisMX; mcm2-3A; hst3Δ::NatMX*
JRY13516	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}; hst4::NatMX*
JRY13517	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}; dpb3Δ::HisMX; hst4Δ::NatMX*
JRY13518	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}; mcm2-3A; hst4Δ::NatMX*
JRY13519	*MATα; GAL10:tetO^{gene}; HML:tetO^{gene}; dpb3Δ::HisMX; mcm2-3A; hst4Δ::NatMX*
JRY13520	*MATa; GAL10:tetO^{gene}; HML:tetO^{gene}*
JRY13523	*MATa; GAL10:tetO^{gene}; HML:tetO^{gene}; dpb3Δ::HisMX; mcm2-3A*
JRY13595	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; dcc1Δ::NatMX*
JRY13596	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; rad2Δ::NatMX*
JRY13597	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; ctf8Δ::NatMX*
JRY13598	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; rtt101Δ::NatMX*
JRY13599	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; asf1Δ::NatMX*
JRY13600	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; mec3Δ::NatMX*
JRY13601	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; mms22Δ::NatMX*
JRY13602	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; ctf18Δ::NatMX*
JRY13603	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; elg1Δ::NatMX*
JRY13604	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; rad17Δ::NatMX*
JRY13605	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; ddc1Δ::NatMX*
JRY13606	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; ctf4Δ::NatMX*
JRY13607	*MATa; GAL10:tetO^{gene}; dpb3Δ::HygMX; mcm2-3A; HML:tetO^{gene}; mms1Δ::NatMX*
JRY13608	*MATα; GAL10:tetO^{gene}; dpb3Δ::KanMX; mcm2-3A; HML:tetO^{gene}; dcc1Δ::NatMX*
JRY13609	*MATα; GAL10:tetO^{gene}; dpb3Δ::KanMX; mcm2-3A; HML:tetO^{gene}; rad24Δ::NatMX*

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JRY13610	*MATα; GAL10:tetO^{gene}; dpb3Δ::KanMX; mcm2-3A; HML:tetO^{gene}; ctf8Δ::NatMX*
JRY13611	*MATα; GAL10:tetO^{gene}; dpb3Δ::KanMX; mcm2-3A; HML:tetO^{gene}; rtt101Δ::NatMX*
JRY11471	*ADE2, lys2, TRP1, hmlΔ, matΔ::NatMX, HMRα, hmrα2Δ::CRE (N14), ura3Δ::loxP:yEmRFP:tCYC1:KanMX:loxP:yEGFP:tADH1, can1-100, his3-11,15, leu2-3,112*
JRY11550	*ADE2, lys2, sir1Δ::LEU2, dpb3Δ, hmlΔ::NatMX, matΔ::KanMX, HMRα, hmrα2Δ::yEGFP, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1*
JRY11589	*ADE2, lys2, sir1Δ::LEU2, mcm2-3A, hmlΔ::NatMX, matΔ::KanMX, HMRα, hmrα2Δ::yEGFP, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1*
JRY11590	*ADE2, lys2, sir1Δ::LEU2, dpb3Δ, mcm2-3A, hmlΔ::NatMX, matΔ::KanMX, HMRα, hmrα2Δ::yEGFP, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1*

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Appendix: Additional studies of silencing[1]

Before becoming a scientist, I assumed that research progress was basically linear: one experiment gives a result that suggests a new hypothesis, which is then tested in the next experiment that suggests the next hypothesis, so on and so on, until a complete publication-worthy set of data accumulates. In the course of graduate school, I've learned how woefully incorrect this popular image of science is. The direct line that I had imagined is actually more like a subway system of interweaving and unpredictable research directions. To belabor the metaphor, much of my time in graduate school was spent taking random trains in uncertain directions, transferring when the signs look promising, and frantically backtracking when they did not. The work described earlier in the dissertation constitute the story I tell of my journey, which emphasizes the successes, the creative leaps, and the conclusive results. The final pages here concern a few parts of the journey that didn't fit into the main narrative of the work described earlier, but that might provide food for thought for future researchers in the lab or in the field. All of these results should be viewed as preliminary, since they lack the thorough controls and follow-up work that would be needed to draw firm conclusions.

A1: Using endonuclease accessibility to measure silent chromatin conformation

Early in graduate school, I was fascinated by the idea that silent chromatin might adopt a higher-order chromatin structure (Bi and Broach, 1997; Valenzuela et al., 2008), and that this structure might contribute to the gene repression observed at *HML* and *HMR*. I joined the lab soon after Debbie Thurtle published a study that investigated *HML* and *HMR* by ChIP-seq (Thurtle and Rine, 2014), and one of her findings was that when Sir proteins or H3 was immunoprecipitated from cross-linked chromatin, certain regions of *HML* and *HMR* were under-recovered. This was taken as evidence that silent chromatin might be forming a conformation *in vivo* that buried the core of the locus to the extent that, when cross-linked with formaldehyde, pockets of chromatin were protected from antibody binding. This result was especially intriguing in light of Ann Kirchmaier's finding that Sir protein binding across *HMR* was not sufficient to drive gene repression (Kirchmaier and Rine, 2006). I thought that maybe Sir protein binding could occur irrespective of cell-cycle stage, but that S phase promoted the formation of the repressive "superstructure[2]."

The idea of a repressive "superstructure" is built on the "occlusion" model of silencing (Loo and Rine, 1994), but makes a prediction that goes beyond the occlusion model: some sub-regions within *HML* and *HMR* should be *more* inaccessible to protein binding than others. I wanted to test that model. I imagined that by assaying endonuclease accessibility across *HML* or *HMR,* much as Stephen Loo had done, that I might find such regions of relative inaccessibility. In designing the assay, I made two key changes to Loo's method. First, I induced expression of the endonuclease *in vivo*, rather than adding nuclease to purified nuclei. I hoped that this would avoid

[1] The references in this appendix are listed in the main bibliography.

[2] This was the initial inspiration for the ChIP-seq experiments presented in Chapter 2. We anticipated that if superstructure formation were required for the formation of the silent state, in G1 arrested cells undergoing silencing establishment, we might see essentially uniform Sir protein binding across *HML* and *HMR*. Then, once the putative S phase superstructure formation occurred, we thought we might see preferential loss of Sir ChIP signal at certain regions of the locus.

any alteration to chromatin structure that might occur during the nucleus purification protocol. Second, rather than targeting nucleases to the endogenous restriction sites in silent chromatin, I introduced cut sites for the homing endonuclease I-SceI. By using the same cut site at different locations across *HML*, I thought I would avoid the variability inherent in using different restriction enzymes that target different sites. In pilot experiments, I assayed I-SceI accessibility and cutting by Southern blot. If the project had moved forward, my plan was to design a high-throughput sequencing assay to measure DNA accessibility for dozens or even of hundreds of pooled strains, and create a high resolution "accessibility map" of *HML*.

The first tests of I-SceI activity *in vivo* revealed the major limitation of this approach. With a test set of four I-SceI sites, two of which were in linkers and two of which were in nucleosomes, it was apparent that placement of an I-SceI site in a nucleosome severely reduced I-SceI's cutting efficiency (**Figure A1**). If I could assay accessibility only at linker DNA, my hypothetical "accessibility map" of *HML* would have, at best, ~150 base pair resolution. Furthermore, because much of *HML* has poorly-positioned nucleosomes, the actual resolution might be even lower than that. It's possible that with further optimization, I could have increased the cutting at nucleosomal DNA, but these results came around the same time I was preparing for my qualifying exam, which shifted my focus more conclusively toward studying the cell cycle. The idea that Thurtle and Rine's ChIP-seq under-recovery and Bi and Broach's negative supercoiling both reflect the same silent chromatin "superstructure" remains a possibility. However, the ChIP-seq results presented in Chapter 2 do not show any strong changes in Sir protein localization upon transition from the unsilenced G1 form to the silenced G2/M form, suggesting that a change in superstructure might not be the causative change in S phase that drives silencing establishment.

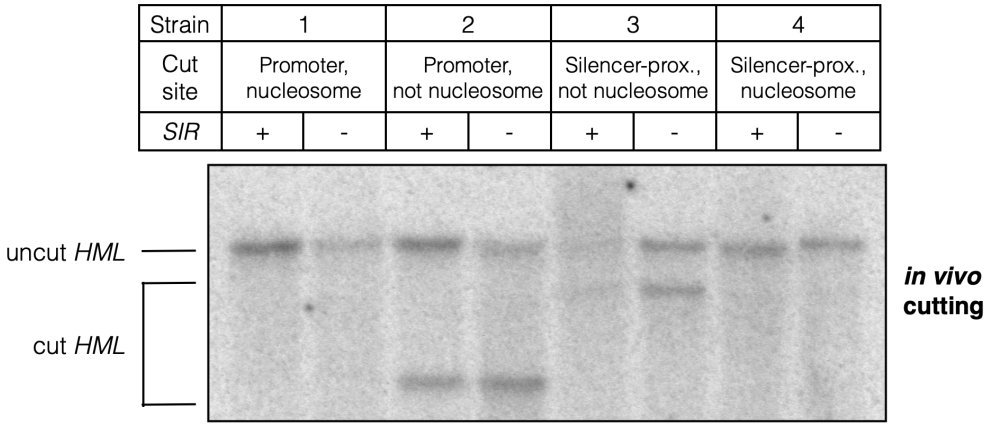

Figure A1: Nucleosomes limit I-SceI accessibility. Strains with I-SceI cut sites introduced at four different positions across *HML* were pre-grown in lactate-containing media, followed by I-SceI induction by addition of 2% galactose for 2 hours. DNA was isolated by phenol:chloroform extraction, followed by ethanol precipitation. 5 µg of DNA was digested overnight with PstI-HF, then run on an agarose gel and transferred to a nylon membrane and incubated with a radiolabeled *HML* probe.

A2: Genome-wide data on silencing establishment and the cell cycle

When they were in the lab, Aisha Ellahi and Debbie Thurtle performed the first RNA-seq study of the genome-wide targets of silencing (Ellahi et al., 2015). To do this, they compared RNA

levels in wild-type, *sir2*Δ, *sir3*Δ and *sir4*Δ cells, reasoning that genes whose expression changed in all three mutants were those with silencing-dependent changes in gene expression. They found that very few subtelomeric genes were targets of silencing, in agreement with Debbie's earlier ChIP-seq result that Sir proteins bind to subtelomeres only at discrete loci near telomeric repeats and X elements (Thurtle and Rine, 2014). Given my finding that *HML* and *HMR* both required S phase for silencing establishment, I was interested in whether the *SIR*-repressed genes outside *HML* and *HMR* have a similar requirement.

I performed an experiment analogous to those described in Chapter 2, wherein I arrested *SIR3-EBD* cells in G1 and then added estradiol to turn on silencing and either kept the cells arrested in G1 or allowed them to pass to G2/M. At each collection point, I collected cells and performed RNA-seq. I focused my analysis on the set of genes Aisha and Debbie identified as *SIR*-regulated. When Sir3-EBD was induced and cells were held in G1, 18 genes had significant >2-fold changes in expression (**Figure A2a**). Only one of these genes, *HSP12*, was previously identified as *SIR*-regulated (Ellahi et al., 2015), but the direction of the regulation was opposite—*SIR*-repressed in the earlier study, but *SIR*-activated here. For 16 out of 18 genes, Sir3-EBD induction increased expression, suggesting that the expression change was not due to a direct silencing effect. The only significant GO terms enriched in this set of genes were glucose-6-phosphate metabolism (3 out of 18 genes) and hydrogen peroxide catabolism (2 out of 18 genes). Investigation of the SGD description of these genes' functions revealed various metabolic processes, many of which appear stress-regulated. Thus, I think the most likely explanation is that the addition of estradiol causes a minor stress response, independent of silencing. To confirm this, I would need to perform a control where estradiol was added to a strain lacking Sir3-EBD or with an EBD not fused to Sir3. That none of Aisha and Debbie's *SIR*-regulated genes are repressible without passage through S phase suggests that cell-cycle progression is a general requirement for silencing establishment.

After cells passed through S phase, 8 known *SIR*-regulated genes were significantly repressed (**Figure A2b**, orange points). Three of those genes were *HMRa1*, *HMRa2*, and *HMLα1,* as expected based on the results described in Chapter 2 and earlier studies[3]. The remaining 5 genes are all located directly adjacent to *HML* and *HMR*. Thus, the silencing establishment that does occur after a single S phase is only in direct proximity to *HML* and *HMR*. The remaining subtelomeric genes that are subject to silencing (16 in Aisha and Debbie's data) must therefore require further time or cell-cycle steps in order to repress transcription. Four additional genes appeared to be *SIR*-repressed in this experiment, but did not appear in Aisha and Debbie's earlier analysis—*MNC1, SCR1, RRT15*, and *YGR161W-B*. Of the four genes, two are from repetitive sequences—*RRT15* is homologous to the rDNA and *YGR161W-B* is a Ty transposon—and one, *MNC1,* is a very short gene (201 bp), suggesting that the apparent repression of these three may be artifactual. The final gene, *SCR1*, encodes the RNA component of the signal recognition particle and overlaps *ARS519*. Recent studies have suggested a role for Sir proteins at euchromatic origins (Hoggard et al., 2018), and thus it is formally possible that *SCR1* is directly regulated by the Sir proteins. However, Aisha and Debbie's analysis did not show *SCR1* to be *SIR*-regulated, and thus

[3] The expression change of *HMLα2* upon induction of Sir3-EBD was statistically significant, but did not meet the 2-fold change in FPKM threshold I set for this analysis. This is in strong agreement with the RT-qPCR data presented in Figure 2.3D, which showed that *hmlα2** had only a ~30% reduction in transcription upon induction of Sir3-EBD and passage of a single S phase.

one would have to explain how *SCR1* could be repressed by Sir3 during silencing establishment, but not at steady state.

I did not push these results any farther. Partially, this was because the conclusions were self-contained: (1) establishment of silencing requires S phase at both the canonical *HML/HMR* loci and all other known *SIR*-repressed loci, and (2) silencing establishment can occur after a single S phase only at *HML* and *HMR*. The latter point suggests that the silencing is strongest at *HML* and *HMR*, which is consistent with the finding that the few *bona fide SIR*-regulated subtelomeric genes are quite lowly expressed, even in absence of Sir proteins (Ellahi et al., 2015).

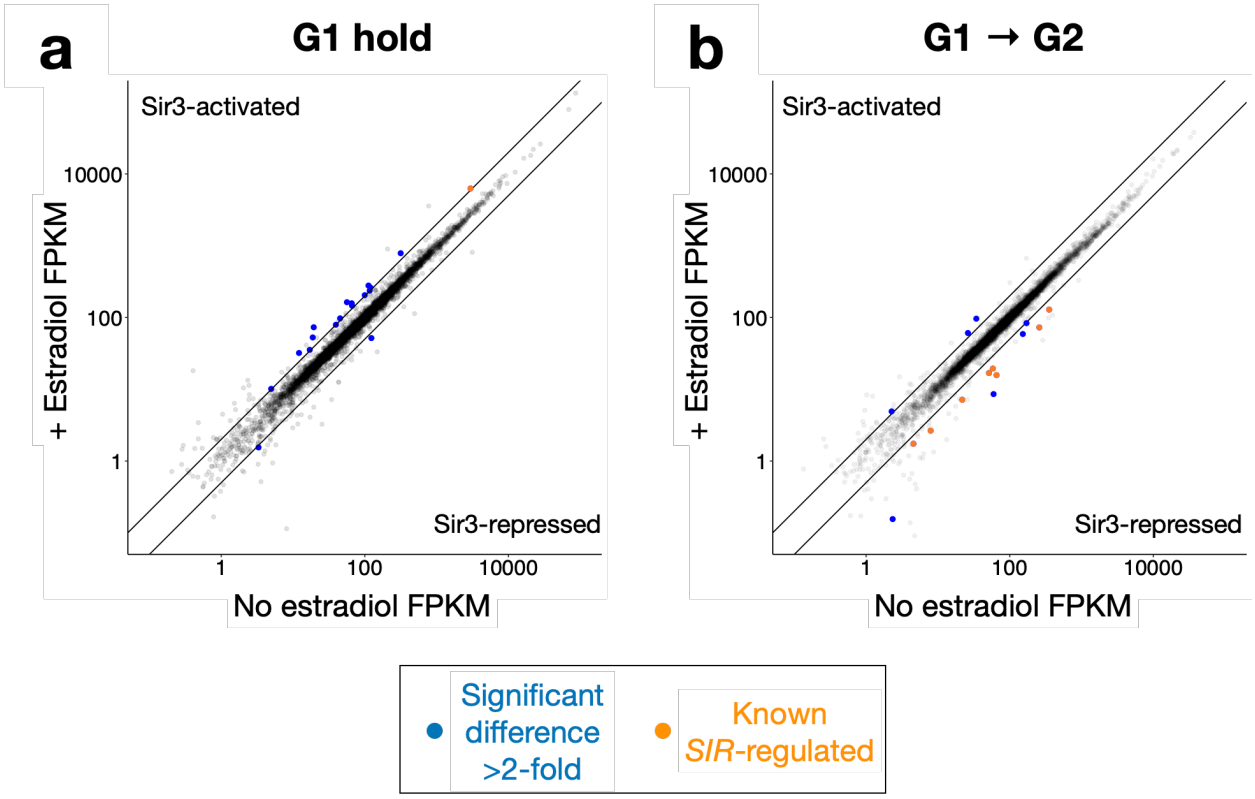

Figure A2: Silencing establishment at subtelomeric genes requires at least 1 S phase. *SIR3-EBD* strains were arrested in G1 with α factor. Then, the culture was split and cells were either maintained in α factor for 3 hours (**a**) or released to nocodazole for 3 hours (**b**) in the presence of estradiol to induce Sir3-EBD or presence of solvent only. The experiment was performed in duplicate, and the average values are plotted here. Each gene is replicated by a dot, with dots above the diagonal reflecting genes with higher expression when Sir3-EBD was induced and. The diagonal lines on each plot represent two-fold changes in expression between the +estradiol and –estradiol condition.

A3: Cell-cycle requirements for silencing establishment using *SIR4-EBD*

Before I began using *SIR3-EBD* to induce silencing establishment, I spent over a year doing so with the *SIR4-EBD* allele. This was purely by chance: I started constructing strains with *SIR3-EBD* and *SIR4-EBD* simultaneously, and the *SIR4-EBD* strains were ready first. At the time, I figured that the two induction strategies would be similar, i.e., that the same cell-cycle

requirements would exist when turning on silencing using *SIR3-EBD* or *SIR4-EBD.* In hindsight, this may or may not be true.

To begin at the end, I eventually stopped using *SIR4-EBD* for a simple reason: I realized, after doing a control experiment that I should have done far earlier, that *SIR4-EBD* did not display the exquisite switch-like behavior that I needed to study the transition between the silencing ON and OFF states. The level of *HMRa1* transcription in *SIR4-EBD* cells grown without estradiol was ~2-fold lower than *sir3*Δ mutant cells (data not shown). Thus, I reasoned, if I used those strains to study silencing establishment, I would actually be studying the transition from a partially silenced state to a fully silenced state, which was not desirable. This led me to switch to the *SIR3-EBD* work described in Chapter 2.

However, before abandoning *SIR4-EBD* entirely, I found by happenstance that when the *SIR4-EBD* allele was present in the same cell as the *SIR3-13xMyc* allele, full de-repression of *HMR* occurred *could* occur in absence of estradiol. Thus, while *SIR4-EBD* on its own had some activity even in absence of estradiol, in combination with the apparently slightly hypormophic *SIR3-13xMyc*, this activity was insufficient to cause partial silencing. So, by using a *SIR4-EBD SIR3-13xMyc* strain, I could actually study silencing establishment from the de-repressed state. Interestingly, when I did perform silencing establishment assays on *SIR4-EBD SIR3-13xMyc* cells, the cell-cycle requirements were dramatically different from those described in Chapter 2 for *SIR3-EBD* cells (**Figure A3**). Rather than partially establishing silencing after a single S phase, these cells seemed to require two S-phase passages to repress *HMR* (**Figure A3a**) and to recruit Sir3-myc to *HMR* (**Figure A3b**). Note that both experiments displayed represent a single biological replicate, and occurred while I was still optimizing my techniques for both RT-qPCR and ChIP-seq, and thus should be treated carefully. The most liberal interpretation of these results was that induction of *SIR3* and induction of *SIR4* led to fundamentally different silencing establishment processes. This would be consistent with the earlier finding that Sir4 is required to recruit Sir3 to silencers, but not *vice versa* (Rusché et al., 2002). Thus, one possibility was that Sir4 recruitment to silencers required a single S phase, and that the subsequent recruitment and spread of Sir3 required a second S phase. A more conservative interpretation of the result would be that the hypomorphic *SIR3-13xMyc* allele led to an artifactually delayed silencing establishment process.

Ultimately, I decided that the quirkiness of these strains—i.e., the need for a hypomorphic *SIR3* allele to counteract a hyperactive *SIR4* allele—would not be conducive to publication-quality results. But I still think there's a reasonable possibility that beginning silencing establishment from a *sir4*$^{-}$ state, wherein the silent loci are completely devoid of Sir proteins, might reveal different cell-cycle requirements than beginning it from a *sir3*$^{-}$ state, wherein Sir2/4 are already bound to silencers. The use of better-behaved conditional *SIR4* alleles would be necessary to test this possibility.

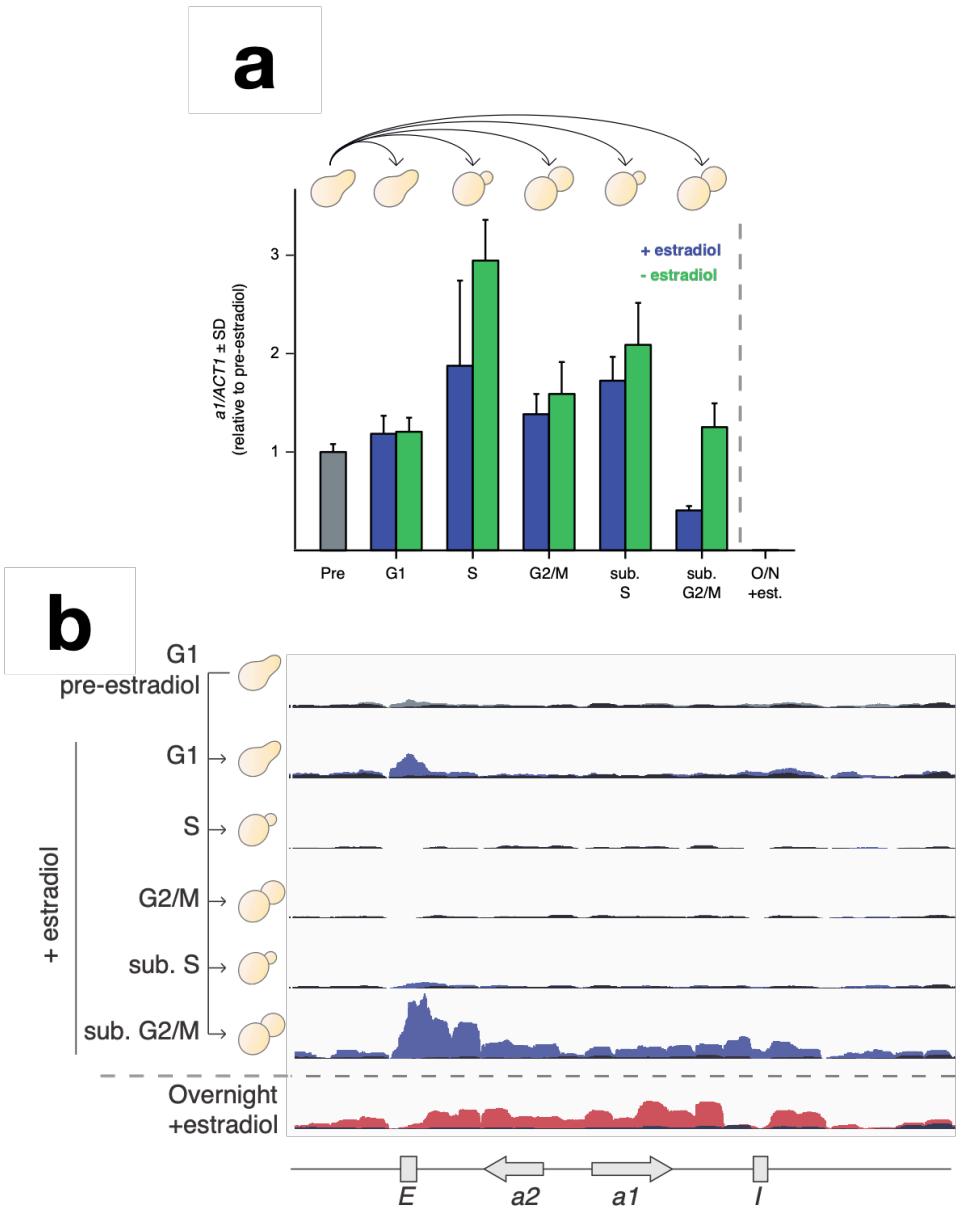

Figure A3: *SIR4-EBD SIR3-13xMyc* strains require two S phase passages to establish silencing. Cells with *SIR4-EBD and SIR3-13xMyc* were arrested in α factor, then either estradiol or solvent only was added and the cultures were split into sub-cultures that were either maintained in G1 or released and re-arrested at S phase with hydroxyurea or G2/M with nocodazole. For the "subsequent S" and "subsequent G2/M," cells were released and allowed to freely cycle until the next G1, then re-arrested with hydroxyurea or nocodazole. At each time-point, cells were collected for either RT-qPCR (**a**) or ChIP-seq (**b**). In (**a**), error bars represent the standard deviation of technical replicates for a single biological replicate. In (**b**), the displayed signal is normalized to the genome-wide median and plotted on the same scale across all samples. Also plotted, in black, is the input value for each sample.