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S phase inheritance of transcriptional silencing: The role of Sir1 and Asf1

Author
Chu, Leslie Elaine

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S phase inheritance of transcriptional silencing: The role of Sir1 and Asf1

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Leslie Elaine Chu

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Shirley C. Chu

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S phase Inheritance of Transcriptional Silencing:

The Role of Sir1 and Asf1

Leslie E. Chu

To preserve the transcriptional program of dividing cells, silent and active chromatin domains must not only be continuously maintained but also faithfully inherited. Cell cycle progression, however, poses significant potential challenges to the inheritance of silent chromatin states. The chromatin structure undergoes major structural alterations in S and M phase and these alterations likely affect the higher order heterochromatin chromatin structure. In a cell cycle, however, changes in defined transcriptional programs rarely occur. Thus, if DNA replication and Mitosis disrupt silent chromatin, then there must be factors that restore silencing immediately following its disruption.

In the budding yeast, *Saccharomyces cerevisiae,* I studied how transcriptionally silent states are inherited. I identified for the first time two proteins required for the inheritance of silencing. I demonstrated that these two proteins, Sir1, a silencer associated protein, and Asf1, a nucleosome deposition factor, are S phase specific inheritance factors. Using $sir1^{td}$ and $asf1^{td}$ conditional alleles, I also demonstrated that the S phase loss of silencing correlates with DNA replication of the silent locus, suggesting that DNA replication is the S phase event that disrupts transcriptional silencing. Finally, I showed that the core silencing protein, Sir3, remains associated with

HMLalpha for at least three generations after silencing is lost. This indicates that Sir3 may template the inheritance of silent chromatin following S phase.

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CHAPTER 1

Introduction

Epigenetic inheritance of gene expression regulates important aspects of cell differentiation, development and physiology [1]. These epigenetic states are stable and heritable suggesting that they must be duplicated and propagated during each cell cycle [2-4]. A failure to propagate epigenetic states can drastically change gene expression programs, resulting in a variety of diseases [1, 5].

Cell cycle progression, however, poses significant potential challenges to the inheritance of transcriptionally silent chromatin states [6, 7]. The chromatin structure undergoes major structural alterations in S and M phase and these alterations likely affect the higher order silent chromatin structure. During S phase, DNA replication disrupts chromatin at the nucleosome level [8, 9]. Since nucleosomes are the foundation for all chromatin, it is assumed that silent chromatin is similarly disrupted by DNA replication [6]. Mitotic chromosome condensation also results in massive chromatin restructuring and this restructuring can disrupt transcriptionally silent chromatin [7, 10].

In a cell cycle, changes in defined transcriptional programs rarely occur [11]. Thus, if DNA replication and Mitosis disrupt silent chromatin, then there must be factors required to restore silencing immediately following its disruption. This thesis focuses on identifying the cell cycle event that disrupts silent chromatin, the factors required to restore silencing following its disruption and the silent chromatin marks that template the inheritance of silent chromatin.

Chromatin Characteristics

Eukaryotic cells package their DNA into a nucleoprotein complex called chromatin. The fundamental unit of chromatin is the nucleosome which is composed of

147 base pairs of DNA wrapped around an octamer of core histones [6, 12]. The histone octamer is made of two of each type of histone: H2A, H2B, H3 and H4 and each nucleosome is linked to the next nucleosome by small segments of linker DNA [6, 12]. In most organisms, the binding of histone H1 to linker DNA sequences between nucleosomes leads to even further chromatin compaction [6, 12].

Changes to this highly organized chromatin structure by various mechanisms, such as post-translational modification of histone tails (PTM), ATP-dependent chromatin remodeling and the replacement of "conventional" histones with histone variants, regulates gene expression [13]. Some post-translational modifications such as acetylation and phosphorylation are reversible and dynamic and often induce gene expression [14, 15]. Other modifications such as methylation are more stable and are involved in the long term maintenance of the expression status of regions of the genome [12, 15]. Furthermore, ATP-dependent chromatin remodeling regulates transcriptional activity in regions of the genome by permitting chromatin fluidity [13]. It has also been shown that the incorporation of histone variants into nucleosomes alters nucleosome charge and structure, thus regulating transcriptional programs in the genome [12, 13].

Gene expression programs are constrained in large measure by the segregation of the genome into euchromatin and heterochromatin [16, 17]. Euchromatin corresponds to actively transcribed regions of the genome that decondense during interphase and replicate early in S phase [16, 17]. The regulatory sequences in euchromatin regions are also accessible to nucleases [18] and contain hyperacetylated H3 and H4 histone tails [16, 17]. In contrast, heterochromatin is transcriptionally silent, highly condensed and late replicating [16, 17, 19].

Heterochromatin Structure

Heterochromatin is defined and distinguished from euchromatin by several key epigenetic characteristics. First, the histone tails in heterochromatin are hypoacetylated and hypermethylated [12, 14, 19]. Of special interest is the hypoacetylation of H4 lysine 16 and hypermethylation of H3 lysine 9 [12, 14, 17]. These specific lysine residues are binding sites for heterochromatic proteins, the second key heterochromatin characteristic. SIR2 and HP1 are heterochromatin proteins that recognize histone H4 lysine 16 and histone H3 lysine 9, respectively [19-22]. Upon interacting with the histones, SIR2 and HP1 recruit additional heterochromatin proteins to form the higher order heterochromatin structure [21, 23]. Finally, heterochromatin differs from euchromatin in its core nucleosome composition. While euchromatin is composed of nucleosomes containing the histone variant H2A.Z, heterochromatin is composed of nucleosomes containing conventional histone H2A [13, 24].

Heterochromatic Regions In Saccharomyces cerevisiae

Genetic studies have identified several regions in the *Saccharomyces cerevisiae* genome that are transcriptionally silenced. These regions are the rRNA-encoding DNA, the telomeres and the two silent mating type loci (*HMLalpha* and *HMRa*) [25]. We focus our studies on the two silent mating type loci *HMLalpha* and *HMRa*.

Cell type in budding yeast is determined by the genetic information present at the *MAT* locus on chromosome III [26, 27]. Haploid cells express either “a” or “alpha” information from the *MAT* locus [26, 27]. *HMLalpha* and *HMRa* also contain intact

copies of the *MATalpha* and *MATa* genes, respectively [26, 28, 29]. By serving as repositories of "a" or "alpha" information for gene conversion of the *MAT* locus, these *HM* loci allow haploid cells to switch their mating type [26, 28]. However, to ensure that only the *MAT* locus specifies the mating type of the cell, the *HM* loci must be kept transcriptionally silent [25, 28].

Heterochromatin Chromatin Components

Transcriptional silencing of *HMLalpha* and *HMRa* is regulated by both cis-acting silencer elements and trans-acting factors [28, 29]. *HMLalpha* and *HMRa* are flanked by pairs of silencer elements, *HML-E/HML-I* [18, 28, 30] and *HMR-E/HMR-I* [18, 28, 31], respectively. These silencers contain binding sites for at least two of three DNA binding proteins, ORC, Rap1 and Abf1 [28, 32-35]. At other loci in the *S. cerevisiae* genome, Rap1 and Abf1 [32-34, 36] are two of the most common transcription activators while ORC is the origin recognition complex, essential for initiating DNA replication [37]. At the *HM* loci, however, ORC, Rap1 and Abf1 only function to nucleate the formation of heterochromatin [35, 38-41].

During the nucleation of silencing, ORC recruits Sir1 [38, 42-45], which then, in combination with Rap1 and Abf1 [39-41], facilitates the loading of a core Sir2-4 complex [29, 46-48]. Once this initial Sir2-4 complex is recruited to the silencer, cooperative interactions enable the recruitment and spreading of additional Sir2-4 complexes throughout the silent locus [29, 46, 48].

Recent studies showed that the deacetylation of histone H4 lysine 16 by Sir2 is critical for SIR complex assembly and silencing [21, 49, 50]. This deacetylation is

coupled to NAD hydrolysis, resulting in the synthesis of a metabolite, *O*-acetyl-ADP-ribose (AAR) [21, 51]. While the deacetylation of histone H4 lysine 16 creates a high affinity-binding site for the SIR complex on histone H4, AAR induces a structural rearrangement in the Sir complex [21]. This structural rearrangement causes the binding of multiple copies of Sir3 to Sir2/Sir4, resulting in polymerization of the SIR complex along the chromatin fiber [21].

Establishment, Maintenance and Inheritance of silent chromatin

Three aspects of transcriptional silencing are often studied: Establishment, Maintenance and Inheritance. Establishment is when non-silent chromatin, euchromatin, is transformed into silent chromatin, heterochromatin. Maintenance is the continuous preservation of heterochromatin at all points in the cell cycle. Inheritance is the restoration and propagation of heterochromatin following progression through the cell cycle [25, 28, 52].

In *S. cerevisiae*, the concepts of establishment, maintenance and inheritance of transcriptional silencing were first illuminated by the phenotype of *sir1* mutants. Pillus and Rine [53] demonstrated that in a population of genetically identical *MATa sir1* cells, 20% of the cells were transcriptionally silent at *HMLalpha* while 80% of the cells were transcriptionally active at *HMLalpha*. Interestingly, both silent and active states were stably inherited for at least 10 generations. Several conclusions were obtained from this study. First, since both states are stable for multiple generations, they are both heritable. Second, since *HMLalpha* is expressed in 80% of *sir1* cells, and these cells rarely establish silencing, Sir1 must promote the establishment of the silent state. Finally, since 20% of

sir1 cells and their descendants retain the silent state, Sir1 is not required to maintain or inherit the silent state.

Maintenance of Silent Chromatin

Since the maintenance of silent chromatin refers to the continual preservation of the silent state, a failure to maintain transcriptional silencing is characterized by rapid switching from silent chromatin to active chromatin [25, 52]. The three core structural components of heterochromatin, Sir2, Sir3 and Sir4 are the most well studied maintenance proteins [54, 55]. Consistent with the characteristics of a maintenance protein, conditional inactivation of Sir2, Sir3 or Sir4 conditional alleles result in immediate and complete loss of silencing [11, 56-58].

Establishment of Silent Chromatin

Recently, there has been tremendous interest in understanding how silent chromatin states are established. Using conditional *sir3*^{ts} alleles, studies show that de novo silent chromatin formation occurs in three discrete steps. First, the Sir proteins are recruited to the silencers [29, 46, 48, 56]. Then, the Sir proteins spread throughout the target locus [29, 46, 48]. Finally, progression through S phase [11] and M phase [47, 56], but not DNA replication [59, 60], modify the Sir-protein complexes to generate a transcriptionally silent chromatin structure.

Epigenetic Inheritance of Silent Chromatin

Conceptually, the process of establishment and inheritance of a chromatin states differ substantially. To establish a chromatin state, all the components of the new structure must be recruited to the locus de novo [25, 52]. In contrast, when a structure is inherited, both sister chromatids could inherit a partial structure, from the parent, consisting of correctly modified histones and heterochromatin proteins [25, 52, 54]. Given the right affinities and circumstances, these partial structures could then template the formation of the complete heterochromatin structure through both cooperative interactions and modifications.

Several studies have focused on identifying the cell cycle events and factors required for the inheritance of silencing. Using site-specific recombination to conditionally excise *HML-E,* the Broach lab showed that silencers, though not required to maintain silencing, are required for the inheritance of *HMLalpha* silencing [61, 62]. Further experiments, by Holmes et.al., suggested that *HML-E* is required for the inheritance of silencing following progression through Mitosis but not S phase [63]. In the S phase experiment, however, *HML-E* was only excised for 1 hour and I have shown that complete *HML-E* excision requires at least 2.5 hours (data not shown). Thus, Holmes' S phase experiment is inconclusive, since *HML-E* excision is incomplete. Though this study demonstrates a key role for Mitosis and cis-acting silencers in regulating the inheritance of heterochromatin, it fails to elucidate how S phase affects the inheritance of heterochromatin.

Transcriptional Silencing During The Cell Cycle

In the course of each cell cycle, the genome is faithfully duplicated and divided between two daughter cells. To stably propagate defined gene expression patterns, cells must not only replicate their DNA, but they must also duplicate the epigenetic chromatin structure [4, 6, 64, 65]. Once duplicated, the epigenetic chromatin structure must then be segregated to the daughter cells [7].

However, DNA replication and Mitosis, the cell cycle events that duplicate and segregate chromatin, completely disrupt the chromatin structure [10, 66]. This fact has generated several long-standing questions. First, how is transcriptional silencing affected with progression through the cell cycle? Second, if silencing is disrupted, what cell cycle events disrupt silencing? Third, what factors restore the silent state immediately following its disruption? Though progress has been made in understanding how Mitosis affects heterochromatin, little is known about how DNA replication affects heterochromatin.

In Mitosis, the chromatin structure is completely reorganized. Chromosomes condense during Prophase, resulting in genome-wide nucleosome rearrangement [10]. Since nucleosomes are the foundation of heterochromatin, chromosome condensation likely disrupts the higher order heterochromatin structure. Supporting this hypothesis, Holmes et.al., demonstrated that progression through Mitosis, in the absence of silencers, disrupts *HMLalpha* transcriptional silencing [63]. This result shows that cell cycle events disrupt heterochromatin and inheritance factors, in this case silencers, restore silencing immediately following its disruption.

DNA replication also presents a major challenge to the inheritance of silent chromatin. First, the higher order structure of silent chromatin must be unraveled in order for the replication machinery to access the underlying DNA [6, 67, 68]. Second, nucleosomes are partially dismantled then reassembled during DNA replication, resulting in a nucleosome bare region of 400 to 600 base pairs immediately behind the replication fork [8, 67, 68]. Third, although nucleosomes are readily inherited from parent to daughter DNA, this inheritance provides each daughter chromosome with only half the necessary complement of nucleosomes [69, 70]. These observations support the hypotheses that chromosome replication disrupts silent chromatin and that the inheritance of transcriptional silencing requires mechanisms to restore the silent chromatin state following replication.

S phase Inheritance Of Transcriptional Silencing

Since no disruption of transcriptional silencing has been detected during S phase, one must hypothesize that the disruption is extremely transient and the inheritance of silent chromatin is tightly coupled to the disruptive event. Several models explaining how silent chromatin is inherited, during S phase, have been proposed.

The first model suggests a mechanism by which silent chromatin templates its own reformation. In this model, the inheritance of the silent chromatin state requires propagation of a silent chromatin "mark" [6, 25, 64]. During DNA replication, preexisting nucleosomes from the parental genome are recycled and deposited onto the newly generated daughter strands [69, 70]. Specifically, the parental H3-H4 tetramers are randomly distributed to the two daughter strands. Thus, any stable histone

modification or epigenetic mark associated with the H3-H4 tetramers would be transferred from one generation to the next, serving as a silent chromatin "mark".

In this model, Sir proteins could remain bound to the H3-H4 tetramer during replication. The parental Sir2 protein could then deacetylate histone H4 lysine 16 in newly deposited nucleosomes [71, 72]. This deacetylation reaction would then promote the binding of Sir2-4 complexes to the newly deacetylated histones [21]. Furthermore, the cooperative interactions between the parental bound Sir proteins and silencer binding proteins could direct the unbound Sir complexes to deacetylated histones in silent chromatin rather than deacetylated histones elsewhere in the genome. Consistent with this hypothesis, silencers are required for the inheritance of silent chromatin.

A second way in which chromatin states could be inherited is through coupling chromatin assembly and nucleosome modification to DNA replication [4, 6, 25, 64, 73]. For instance, Sas2, a histone acetyltransferase that specifically acetylates H4 lysine 16 [74-78], could be recruited to DNA through Cac1, Asf1 and PCNA [76, 79-81]. Such a link would lead to newly synthesized DNA that is primed for Sir2 deacetylation of H4 lysine 16 [82]. This deacetylation could then stimulate Sir2-4 complex spreading, throughout the locus.

Finally, it is possible that the replication coupled mechanism and the molecular memory mechanism, described above, act concurrently in the inheritance of silencing. In this case, the replication coupled mechanism could recruit Sas2 to acetylate H4 lysine 16 [74-76, 78]. Parental Sir2 molecules, the molecular memory mechanism, could then deacetylate the "charged" H4 lysine 16 [72, 82, 83]. This deacetylation could specifically target Sir2-4 complexes to the chromatin and restore the silent chromatin structure [21].

Dissertation Overview

In this dissertation I will describe our efforts to understand how transcriptional silencing is inherited following progression through S phase. In Chapter 2, I demonstrate that Sir1, a silencer binding protein, and Asf1, a nucleosome deposition factor, are S phase specific inheritance factors. Using conditional alleles of $sir1^{td}$ and $asf1^{td}$, I also demonstrate that the loss of silencing correlates with DNA replication of the silent locus, suggesting that DNA replication is the S phase event that disrupts transcriptional silencing. In Chapter 3, I demonstrate that the core silencing protein, Sir3, remains associated with *HMLalpha* for at least three generations after silencing is lost. This is the first evidence that silent chromatin marks remain associated with chromatin following a failure to inherit silencing.

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CHAPTER 2

A silencer-associated protein and a histone chaperone are redundantly required for the inheritance but not the maintenance of a silent domain

Submitted for publication.

To preserve the transcriptional program of dividing cells, silent and active chromatin domains must not only be continuously maintained but also faithfully inherited during chromosome replication and segregation. However, proteins required for inheritance but dispensable for maintenance of these domains have not been described. Here we use conditional alleles to show that the ORC-associated silencer protein Sir1 and the ubiquitous histone chaperone Asf1 are redundantly required for S phase inheritance of silencing in *S. cerevisiae*, but are not essential for its maintenance. Inheritance appears to involve an S-phase dependent disruption of silencing followed by an active restoration involving these proteins. Thus, events occurring at both the nucleation site of silencing as well as on chromatin, play roles in epigenetic inheritance of a silent domain.

INTRODUCTION

Gene expression programs are constrained in large measure by the segregation of a genome into transcriptionally active euchromatin and transcriptionally silent heterochromatin [1]. These epigenetic chromatin states are thought to regulate important aspects of cell differentiation, development and physiology [2]. A key feature distinguishing these states from more malleable transcriptional programs is their heritability [3, 4]. Such heritability indicates that epigenetic chromatin states must be duplicated and propagated during each cell cycle [5-7]. Disruption of these states can drastically alter gene expression programs, and in some cases is thought to result in diseases such as Prader-Willi Syndrome, Angelman Syndrome, Beckwith-Wiedemann Syndrome and several cancers [8]. Little is known, however, about how heterochromatin

and euchromatin are faithfully inherited during cell cycle events such as replication and segregation, when chromatin undergoes major structural changes.

The inheritance of heterochromatin can be distinguished from two other aspects of heterochromatin metabolism, its establishment and maintenance [3]. The establishment of heterochromatin involves the *de novo* formation of heterochromatin from euchromatin, whereas maintenance and inheritance involves the preservation of heterochromatin once it is established. Heterochromatin maintenance and inheritance can be distinguished by their requirement during the cell cycle. Functions that maintain heterochromatin integrity are continually required independent of cell cycle progression. Functions that specifically promote heterochromatin inheritance are only needed during cell cycle progression to counter the challenges presumably imposed by replication and segregation.

These distinct functions in heterochromatin metabolism have been best dissected at the silent mating type loci in the budding yeast *Saccharomyces cerevisiae* [9-11]. Budding yeast mating type is specified by the *MAT* locus, which can either harbor an *a* or α mating type allele [12]. The silent mating type loci *HML*α and *HMRa* store transcriptionally silent copies of these alleles so that they can be used to gene convert the *MAT* locus to the opposite mating type [12]. Both loci are incorporated into a heterochromatin-like structure whose formation requires flanking DNA sequence elements, termed silencers (*HML-E* and *HML-I* for *HML*α, and *HMR-E* and *HMR-I* for *HMRa*) and several chromatin bound proteins [13-15]. The silencers are thought to establish silencing by providing binding sites for at least two of three DNA binding proteins, ORC, Rap1 and Abf1 [16-18]. ORC recruits the silencing protein Sir1, which

together with Rap1 and/or Abf1 initiate the loading of the Sir2-4 complex along the length of the silenced locus [19-21]. In addition to this recruitment, passage through both S [22] and M phase [23] are somehow required to establish full transcriptional silencing, although DNA replication is not essential [24, 25]. Sir2-4, which constitute the core of this budding yeast heterochromatin [3], are needed to maintain as well as establish the heterochromatin [22, 26, 27]. Like other eukaryotes, both histone modifications and histone variants distinguish budding yeast heterochromatin from euchromatin, but their precise role in establishment, maintenance, and inheritance have not been teased apart [3, 28-30].

The inheritance of transcriptional silencing during cell cycle progression is the least understood aspect of silencing at the silent mating type loci. S phase, in particular, is expected to impose two challenges to this inheritance. First, because passage of the replication fork disrupts and reorganizes chromatin at the nucleosome level, it has been assumed that higher order heterochromatin structure is also disrupted [31, 32]. Second, each daughter DNA molecule only acquires half the heterochromatin components that were used to enforce transcriptional silencing in the parent [33]. These challenges suggest that the inheritance of transcriptional silencing during S phase involves the disruption then active restoration of heterochromatin structure. Moreover, it has been speculated that such restoration could be templated by molecular features, or marks, of heterochromatin that are bequeathed to the daughter molecules. Nonetheless, there is no direct evidence that S phase disrupts heterochromatin. Nor is anything known about the extent of this disruption, the mechanism of restoration, and whether there are "inheritance factors" specialized to carry out this restoration.

In this study, we present a system that allows us to dissect the S phase inheritance of transcriptional silencing at *HMLα*. This system was made possible by our discovery that Sir1 and the histone chaperone Asf1 are redundantly required for this S phase inheritance. In their absence, S phase does indeed disrupt transcriptional silencing, providing an opportunity to molecularly characterize the disrupted heterochromatin state. These studies also indicate that the restoration of heterochromatin structure after S phase is an active process involving proteins that function at the silencer and during de novo nucleosome assembly.

MATERIALS AND METHODS

Strain and plasmid construction. Strains (Table 5-1) and plasmids (Table 5-2) were constructed as described in Chapter 5 Materials and Methods.

Total RNA extraction. Total RNA was prepared from yeast cells using an Acid-Phenol extraction. 10ml of an OD=1.0 culture was harvested by centrifugation (3 min at 3,000 rpm) and frozen in liquid nitrogen. Cells were resuspended in 500µl of TES buffer (10mM Tris-Cl pH7.5, 10mM EDTA, 0.5%SDS) and 500µl of Acid Phenol (pH 4.3). To extract the RNA from the cells, the mixture was incubated for one hour at 65˚C with periodic vortexing, placed on ice for 5 min and then microcentrifuged at top speed for 5 minutes at 4˚C. The supernatant was transferred to Phase Lock Tubes containing chloroform (500µl), shaken ten times and microcentrifuged for 5 min at top speed. The

supernatant was transferred to a tube containing Acid Phenol (500µl), vortexed vigorously for 10 seconds and microcentrifuged for 5 min at top speed. The aqueous phase was transferred to a new Phase Lock Tube and 500µl of chloroform was added, shaken ten times and microcentrifuged for 5 min at top speed. The chloroform extraction was repeated and then the aqueous phase was transferred to a new tube and mixed with 40µl of 3M sodium acetate (pH 5.3) and 1ml of 100% ethanol. RNA was pelleted by microcentrifugation at top speed for 15 min. The pellet was washed by vortexing in 70% ethanol. After drying, pellets were resuspended in 100-150µl of water and stored at -80˚C.

cDNA preparation. cDNA was generated using the Stratagene First Strand cDNA synthesis protocol. 10ug of total RNA, 500nM OJL1569 and 500nM OJL1578 were mixed in a 20µl RT reaction to synthesize HMLalpha2 and Act1 cDNA respectively. Following the cDNA synthesis, the RNA was hydrolyzed by incubating the reaction at 65˚C for 10 minutes with 14.2µl of AHS (1ml water, 320µl NaOH, 100µl 0.5M EDTA). The hydrolysis reaction was neutralized by adding 66µl of NPS (5.3ml water, 320µl 1M HCl, 1ml 3M NaOAc). The cDNA was then precipitated by microcentrifugation for 30 min in 250µl of 100% ethanol. The pellet was washed by vortexing in 70% ethanol and after drying, pellets were resuspended in 20µl of water.

RT-PCR. RT-PCR was performed using the Stratagene MX3000P System and results were analyzed using the accompanying software. PCR was performed in a reaction containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1mM $MgCl_2$, 2.5mM each dNTP and

Taq polymerase. Cycling parameters were 94˚C for 5 min and then 40 cycles of 94˚C for 30 sec, 52˚C for 30 sec, 72˚C for 1 min and 75˚C for 30 sec, followed by a final incubation of 72˚C for 5 min. We used 1/5 of the cDNA in reactions to detect *HMLalpha2* and 1/25 of the cDNA in reactions to detect *ACT1*. HMLalpha2 message was detected using 50nM OJL678 and 50nM OJL1681 while Act1 message was detected using 50nM OJL1577 and 50nM OJL1578. A standard curve for each primer set was generated using ten-fold serial dilutions of yeast genomic DNA, ranging from 10ng/μl to 10^{-2} ng/μl. For each sample the HMLalpha2 and Act1 cDNA concentration was determined and then the HMLalpha2 cDNA/Act1 cDNA ratio was calculated. These ratios were scaled in each experiment so that the wildtype W303 HMLalpha2 cDNA/Act1 cDNA ratio was 1.

Urea protein extract. Denatured protein samples were prepared using a urea lysis protocol. 10ml of an OD=1.0 culture was harvested by centrifugation for 3 min at 3,000 rpm. Pelleted cells were resuspended in 500μl of water and transferred to a screw cap tube (USP #MCTS-806). Cells were pelleted by microcentrifugation at top speed for 1 min, frozen in liquid nitrogen and thawed on ice. Once thawed, the cells were resuspended in 200μl of Urea buffer (20mM Tris pH 7.4, 7M Urea, 2M Thiourea, 4% CHAPS and 1% DTT). 200μl of 0.5mm glass beads were added to the tube and the cells were then lysed by two rounds of 1 min bead beating. Beads were separated from the extract by poking a hole in the bottom of the screw cap tube (using a 22 gauge needle), placing the screw cap tube into a new eppendorf tube and spinning the stacked tubes in a microfuge on a setting of soft 6,000 rpm for 30 sec. The urea protein extract was

incubated at 25˚C for 30 min on a nutator and then spun in a microfuge at full speed for 10 min at 4˚C. The supernatant was transferred to a new tube and the concentration was determined using the Bradford assay.

Western blot analysis. Western Blot analysis was used to monitor HA-Sir1, HA-Asf1, Asf1 and Pgk1 protein levels. For the anti-HA and Pgk1 western blots, 35μg of each urea protein sample was run on a 7.5% SDS gel and blotted to a nitrocellulose membrane. Membranes were stained with Ponceau S for 1 min, imaged to monitor protein loading and then rinsed with water. The membranes were incubated in 50ml of blocking buffer (10% Carnation dry milk, 20mM Tris pH 7.5, 0.5M NaCl, 0.1% Triton X-100) for 60 min at room temperature and then incubated for 60 min in 40ml of antibody buffer (2% Carnation dry milk, 20mM Tris pH 7.5, 0.5M NaCl, 0.1% Triton X-100) containing either: An anti-HA antibody (Convance MMS-101R mouse anti-HA 16B12) at a 1:1000 dilution or the anti-Pgk1 antibody (Santa Cruz mouse anti-Pgk1 sc-48342) at a dilution of 1:2500. Membranes were quickly rinsed twice with 50ml of the wash buffer (20mM Tris pH 7.5, 0.5M NaCl, 0.1% Triton X-100) and then washed twice, 10 min per wash, with 50ml of the wash buffer. After washing, membranes were incubated for 30 min in 40ml of antibody buffer containing a 1:2000 dilution of sheep anti-mouse HRP secondary antibody (Amersham NA931V). Membranes were rinsed twice with 50ml of wash buffer and then washed three times, 10 min per wash. Western blots were developed using Pierce SuperSignal West Dura Extended Duration Substrate and exposed to Amersham Hyperfilm MP. For the Asf1 and HA-Sir1 western blots in Figure 2, a total of 100μg of protein was run in each lane. The protein concentration at the top of each lane refers to

the amount of either Asf1 or HA-Sir1 protein extract loaded in that lane. For lanes where the Asf1 and HA-Sir1 protein amount is less than 100μg, either asf1 null or sir1 null extract is added to bring the total protein level to 100μg. The HA-Sir1 blot was treated according to the above HA-Sir1 protocol whereas the Asf1 samples were run on a 12% SDS gel and blotted to a nitrocellulose membrane. The Asf1 membrane was then stained with Ponceau S for 1 min, imaged and rinsed. Following the staining, the membrane was incubated for 60 min in milk buffer (1xPBS, 0.1% Tween-20 and 5% Carnation dry milk) and subsequently incubated for 2hr in milk buffer containing a 1:10,000 dilution of an anti-Asf1 rabbit polyclonal (gift from P. Kaufman). The membrane was washed 5 times, 5 min per wash, in 1xPBS and 0.1% Tween-20 and then incubated in milk buffer containing a 1:10,000 dilution of donkey anti-rabbit HRP (Amersham NA934). Following the secondary antibody incubation, the membrane was washed 5 times, 5 min per wash and imaged according to the anti-HA and Pgk1 protocol (see above).

Chromatin immunoprecipitation assay. ChIP assays were performed as described previously [34]with a monoclonal anti-HA antibody (Convance MMS-101R mouse anti-HA 16B12). Cells were fixed in 1% formaldehyde for 2hr followed by sample processing described in Sharp et al. 2003 [34]. RT-PCR analysis, detailed above, was performed on 3% of the precipitated DNA and 1.5% of the input DNA. The DNA regions of interest, *HML-E, HML-I, MAT, ACT1* and *BUD3* were analyzed using the respective primer sets listed in Chapter 5 Table 3. A standard curve for each primer set was generated using ten-fold serial dilutions of yeast genomic DNA, ranging from 10ng/μl to 10^{-2} ng/μl. Using the corresponding primer standard curve, we calculated

starting DNA concentrations (Initial Concentrations) for each sample. "Initial Concentrations" for *HML-E, HML-I, MAT* and *ACT1* were normalized for loading error by dividing by the "Initial Concentration" for *BUD3*. Normalized precipitated DNA values were then divided by normalized input DNA values to determine the relative Sir1 recovery. These values were then scaled so that the *sir1*Δ values were equal to 1. This scaled value is the "Relative Sir1 recovery."

Flow cytometry. Cells were fixed and stained with 1μM Sytox Green (Molecular Probes, Eugene, OR) as previously described [35].

RESULTS

Sir1 and Asf1 are required for *HMLalpha* silencing. Previous studies showed that deletion mutants of either *SIR1* or *ASF1* alone resulted in partial transcriptional silencing defects, while deletions of both proteins resulted in complete loss of *HMLalpha* silencing [36]. Since Asf1 is a histone chaperone [37-39] and Sir1 is a silencer-associated protein required for the establishment of silencing [19, 40, 41], we hypothesize that they may have a role in the inheritance of silencing. To quantitatively and comparatively assess the loss of silencing in *sir1 asf1* cells, we measured HMLalpha2 steady state mRNA levels, which has a short half-life (<5 minutes) [42, 43], making the steady state levels an accurate reflection of nascent transcript levels. Quantitative PCR for HMLalpha2 showed that mutations in either *sir1* or *asf1* alone had weak silencing defects, while *sir1*

asf1 double mutants had severe silencing defects, as severe as a *sir3*Δ (Fig. 5-1). These results confirm that Sir1 and Asf1 together, play a critical role in *HMLalpha* silencing.

***4xHA-sir1^{td}* and *4xHA-asf1^{td}* conditional allele characterization.** Existing reagents were inadequate to determine if Sir1 and Asf1 are required for the establishment, maintenance, or inheritance of *HMLalpha* silencing. Consequently, we created *4xHA-sir1^{td}* (*sir1^{td}*) and *4xHA-asf1^{td}* (*asf1^{td}*) conditional alleles to determine what aspect of *HMLalpha* silencing Sir1 and Asf1 are required for. To distinguish between establishment, maintenance and inheritance, our conditional alleles needed to be fully functional at permissive conditions, fully defective at restrictive conditions and rapidly degraded following a shift from permissive to restrictive conditions. We predict that if these proteins were involved in the maintenance of silent chromatin, then silencing would be lost immediately following Sir1^{td} and Asf1^{td} degradation. However, if Sir1 and Asf1 were required for the inheritance but not the maintenance of silent chromatin, then silencing would only be lost following progression through the cell cycle.

sir1^{td} and *asf1^{td}* conditional alleles were created using a modified version of the ts-degron system [42, 43]. In this modified degron system, the genes of interested, *SIR1* and *ASF1*, were N-terminally fused to a DNA segment encoding a methionine repressible promoter, a ts-degron system (Ubiquitin-R-DHFR-1xHA tag) and three additional HA tags. Protein levels were further regulated by replacing the endogenous *UBR1,* an E3 ubiquitin ligase that targets the ts-degron for degradation, with an inducible *pGAL-UBR1*. Thus, Sir1 and Asf1 degrons were expected to be expressed when grown in permissive

conditions, dextrose media lacking methionine, and degraded when grown in restrictive conditions, galactose media containing methionine.

We monitored protein levels and HMLalpha mRNA levels to characterize the *sir1*^{td} and *asf1*^{td} alleles. Under permissive conditions, both Sir1^{td} and Asf1^{td} proteins levels were sufficient for complete silencing of *HMLalpha* while under restrictive conditions both proteins were sufficiently degraded for fully defective *HMLalpha* silencing (Fig. 5-2A & B). When grown under restrictive conditions, "undetectable" Asf1^{td} protein levels were determined to be 4 fold below the endogenous Asf1 protein level (Fig. 5-2A, right panel) while "undetectable" Sir1^{td} protein association with *HML-E* and *HML-I* was determined to be 6 fold below the permissive association level (Fig. 5-2C).

Kinetics of Sir1^{td} and Asf1^{td} protein degradation and HMLalpha2 mRNA expression. We then analyzed the kinetics of Sir1^{td} and Asf1^{td} degradation to determine if silencing was rapidly lost following a shift from permissive to restrictive conditions. *sir1*^{td} and *asf1*^{td} cells were grown under asynchronous permissive conditions and at time 0 they were shifted to restrictive conditions (Fig. 5-3A). Two hours after shifting to restrictive conditions Sir1^{td} protein was undetectable by western blotting and three hours after shifting to restrictive conditions Asf1^{td} protein was undetectable by western blotting (Fig. 5-3C). Furthermore, complete loss of *HMLalpha* silencing occurred in 9 hours, 2 doublings, after both Sir1 and Asf1 degron proteins were undetectable by western blot (Fig. 5-3B, C & D). This loss of *HMLalpha* silencing, in the absence of Sir1 and Asf1,

indicates that both proteins are required for either the maintenance or inheritance of *HMLalpha* transcriptional silencing, consistent with previous observations [36].

Sir1 and Asf1 are not required for the maintenance of silencing in G1. We next tested if Sir1 and Asf1 are required for the maintenance of *HMLalpha* silencing in cells arrested in G1 phase. If Sir1 and Asf1 are required for the maintenance of silent chromatin, then silencing would be immediately lost following $Sir1^{td}$ and $Asf1^{td}$ degradation. However, if Sir1 and Asf1 are required for the inheritance but not the maintenance of silent chromatin, then silencing would only be lost following progression through the cell cycle. To demonstrate that silencing can be lost at a fixed point in the cell cycle, following deactivation of a maintenance protein, we grew *sir3-8^{ts} hmr*Δ cells under permissive conditions, arrested them in G1 with alpha factor and once the cells were fully arrested, we then added 0.2M hydroxyurea, a compound that blocks DNA synthesis by inhibiting ribonucleotide reductase. While held in the G1/0.2M hydroxyurea arrest, cells were shifted to restrictive conditions for 1 hour and samples were then harvested every hour for 7 hours (Fig. 2-1A). We observed that the addition of hydroxyurea prevents the *sir3-8^{ts} hmr*Δ cells from progressing through S phase (Fig. 2-1B, *sir3-8^{ts} hmr*Δ flow cytometry), since *sir3-8^{ts} hmr*Δ cells immediately loose silencing when shifted to restrictive conditions, making them unresponsive to alpha factor (Fig. 2-1B, *sir3-8^{ts} hmr*Δ budding index). We also observed that *HMLalpha* silencing is completely lost by the 0 hour time point, demonstrating that silencing is rapidly lost at a fixed point in a cell cycle, following inactivation of a transcriptional silencing maintenance protein (Fig. 2-1E).

We then determined if Sir1 and Asf1 are required for the maintenance of silencing by arresting *sir1*^{td} *asf1*^{td} cells in G1 with alpha factor and either keeping them at permissive conditions or shifting them to restrictive conditions. Cells were held under these conditions until both proteins were undetectable by western blot and Sir1^{td} association with *HML-E* and *HML-I* was equal to its association after 48 hours under restrictive conditions (Fig. 2-1C & 2-1D, 0 vs 48 hours). 0.2M hydroxyurea was then added to each G1 arrested culture and samples were harvested every hour for 7 hours (Fig. 2-1A). Flow cytometry, monitoring DNA content, and budding index, monitoring progression past "START", confirmed that all degron strains remained G1 arrested for the entire experiment (Fig. 2-1B).

Under permissive conditions, all four strains remained silenced at the *HMLalpha* locus (Fig. 2-1E). Upon shifting to restrictive conditions both *sir1*^{td}*ASF1* and *sir1*^{td} *asf1*^{td} strains showed slight silencing defects. However, at the 7 hour time point, these silencing defects only measured 10% of the *sir3*^{ts} *hmr*Δ silencing defect (Fig. 2-1E). The low level and slow kinetics of *HMLalpha* loss of silencing, in the absence of Sir1 and Asf1, indicate that these proteins are not essential for maintaining silencing in G1.

Sir1 and Asf1 are not required for the maintenance of silencing in Mitosis. We also tested if Sir1 and Asf1 are required for maintenance in M phase. To obtain a tight mitotic arrest, we replaced the endogenous *CDC20* with a repressible *pMET-cdc20*. *sir1*^{td} *asf1*^{td} *pMET-cdc20* cells were synchronized in G1 with alpha factor and then released into a permissive Mitotic arrest containing nocodozole, a microtubule depolymerizing agent, and methionine, to repress *pMET-cdc20* transcription. Once arrested in Mitosis, cultures

were shifted to restrictive conditions for 3 hours to degrade Sir1^{td} and Asf1^{td} proteins (data not shown). Cells were held in the Mitotic arrest for 7 hours, as monitored by budding index (Fig. 5-4B), and samples were harvested every hour (Fig. 5-4A). Similar to the G1 results, there was no significant loss of silencing, only a 15% defect, compared to the *sir3^{ts} hmr*Δ strain, after 7 hours (Fig. 5-4C).

Sir1 and Asf1 are required for the inheritance of silencing. We reasoned that if Sir1 and Asf1 are not required for the maintenance of *HMLalpha* silencing, then they might be required for its inheritance following cell cycle progression. To test this hypothesis, we first determined if Sir1 and Asf1 are required for *HMLalpha* silencing in cells synchronously released from G1. We synchronized *sir1^{td} asf1^{td}* cells in G1 with alpha factor, shifted them to restrictive conditions to degrade both Sir1^{td} and Asf1^{td} proteins (Fig. 5-5C) and then released the cells from the G1 arrest into restrictive log phase growth conditions (Fig. 5-5A). We monitored HMLalpha2 mRNA expression and observed that once the *sir1^{td} asf1^{td}* cells were released from G1, silencing was rapidly lost (Fig. 5-5D). This rapid loss occurred between 1-2 hours after release, when the majority of *sir1^{td} asf1^{td}* cells completed S phase (Fig. 5-5B, right panel). After 7 hours, the *sir1^{td} asf1^{td}* cells were fully derepressed at *HMLalpha*. Since Sir1 and Asf1 are not required for the maintenance of silencing in arrested cells, but are required for silencing in cycling cells, we conclude that Sir1 and Asf1 are required for the inheritance of *HMLalpha* silencing.

Progression through one cell cycle disrupts *HMLalpha* silencing. Since our previous finding demonstrates that progression through multiple cell cycles, in the absence of Sir1 and Asf1, disrupts *HMLalpha* silencing, we sought to determine if progression through a single cell cycle also disrupts silencing. *sir1*^{td}*asf1*^{td} cells were synchronized in G1 with alpha factor and then shifted to restrictive conditions to degrade Sir1^{td} and Asf1^{td} proteins (Fig. 5-6). Cells were then released from the G1 arrest into a restrictive Mitotic arrest, to synchronize the cells for hydroxyurea addition and then released into a alpha factor/0.2M hydroxyurea arrest (Fig. 2-2A). 2 hours after release from the Mitotic arrest, the majority of cells had progressed from Mitosis into the next G1 (Fig. 2-2C, left panel) and silencing was completely lost by the 5 hour time point (Fig. 2-2D, "G1 to G1"). This result demonstrates that progression through one cell cycle, in the absence of Sir1 and Asf1, is sufficient to disrupt *HMLalpha* silencing.

Sir1 and Asf1 are not required for inheriting *HMLalpha* silencing through Mitosis. Since progression through one cell cycle, in the absence of Sir1 and Asf1, perturbed *HMLalpha* transcriptional silencing, we sought to determine if progression through S phase or M phase alone was sufficient to disrupt silencing. To test if Mitosis disrupts *HMLalpha* silencing, we arrested *sir1*^{td}*asf1*^{td} cells in a permissive Mitotic arrest and then shifted the culture to restrictive conditions to degrade both Sir1^{td} and Asf1^{td} proteins (Fig. 5-6). Cells were then released from the Mitotic arrest into a G1 arrest, consisting of alpha factor and 0.2M hydroxyurea (Fig. 2-2B). As determined by budding index and flow cytometry, two hours after release from the Mitotic arrest, the majority of cells had progressed from Mitosis into G1 (Fig. 2-2C, right panel). In the cells that had progressed

through Mitosis, without Sir1 or Asf1, *HMLalpha* silencing was only slightly derepressed, 15% of the maximum level (Fig. 2-2D). This result demonstrates that, in the absence of Sir1 and Asf1, progression through Mitosis is not sufficient to disrupt *HMLalpha* silencing.

Sir1 and Asf1 are required for inheriting *HMLalpha* silencing through S phase. Since the transition through Mitosis is not sufficient to disrupt *HMLalpha* silencing, we sought to determine if the transition through S phase is sufficient. Cells were synchronized in G1 with alpha factor and shifted to restrictive conditions to degrade both $Sir1^{td}$ and $Asf1^{td}$ proteins (Fig. 5-7). In the absence of both proteins, cells were released from G1 into Mitosis (Fig. 2-3A). Using flow cytometry and budding index to monitor cell cycle position we observed that 96% of cells completed S phase 2 hours after release from G1 (Fig. 3-3B). We also observe that at this 2 hour time point, the loss of silencing was 25% of the maximum level and 6 hours after release silencing was fully disrupted (Fig. 2-3C). These results demonstrate that progression through S phase, in the absence of Sir1 and Asf1, is sufficient to disrupt *HMLalpha* silencing.

S phase is required to disrupt *HMLalpha* silencing. We then tested if progression through S phase is required to disrupt *HMLalpha* silencing. $sir1^{td}$ $asf1^{td}$ cells were synchronized in G1 with alpha factor and shifted to restrictive conditions to degrade both $Sir1^{td}$ and $Asf1^{td}$ proteins (data not shown). Cells were then released for 4 hours into 0.2M hydroxyurea, an early S phase arrest (Fig. 2-4B, 0 to 4 hours). At this point, *HMLalpha* silencing was only slightly deregulated, 10% of the maximum level (Fig. 2-

4C), demonstrating that progression through early S phase was not sufficient to disrupt silencing. After 4 hours in 0.2M hydroxyurea, the cells were released into a Mitotic arrest (Fig. 2-4A) and 1 hour after this release, the 5 hour time point, *HMLalpha* was completely derepressed (Fig. 2-4C). This rapid loss of silencing tightly correlated with the completion of S phase, 2C DNA content (Fig. 2-4B, 5 hours), demonstrating that S phase is required to disrupt *HMLalpha* silencing.

DISCUSSION

The work presented here paves the way for understanding how silent chromatin states are inherited during S phase. We identify Sir1 and Asf1 as two proteins that together are required for the inheritance but not maintenance of silencing during S phase. We demonstrate that S phase does indeed disrupt transcriptionally silent heterochromatin and uncover an active mechanism for restoring this silent chromatin state. Finally, we establish a powerful system for molecular analysis of the disruption and restoration of an epigenetic chromatin state during S phase.

An important question we are now poised to address is whether DNA replication through heterochromatin is required to disrupt heterochromatin during S phase. The sensitivity of this disruption to hydroxyurea, which blocks the bulk of DNA replication, is consistent with such a hypothesis. Importantly, *HML*α, which replicates late in S phase, would not have replicated in the amount of hydroxyurea that we used (R. Morreale, personal communication). Nonetheless, experiments to specifically block replication at HMLα are under way to address this question directly.

A second key question we can now experimentally address is what is the molecular composition of heterochromatin following the S phase disruption. Our preliminary results indicate that Sir2 and Sir3 proteins are present on disrupted heterochromatin following S phase (L. Chu, unpublished data). More extensive work, however, will be needed to determine whether these or other proteins are actually inherited from parental chromatin. Similar studies can also examine the fate of histone modifications and variant characteristics of parental heterochromatin. Such analyses will identify what molecular features of heterochromatin are in position to act as epigenetic chromatin marks.

Our results also demonstrate for the first time that Sir1 is required for inheritance through S phase. This result differs from *sir1Δ* studies which show that Sir1 is required for the establishment but not the maintenance or inheritance of silencing [9]. It is likely, however, that the *sir1Δ* study failed to observe a role for Sir1 in the inheritance of silencing because of the redundant Asf1 inheritance function.

Asf1 may facilitate the inheritance of silencing through its nucleosome deposition activity [38, 44-47] and its ability to recruit other silencing regulators, such as Sas2 [36, 48-50]. In this model, Asf1, which associates with the replication machinery [46, 51], could recruit Sas2, a histone acetyltransferase, to the newly replicated daughter chromosomes. Sas2 could then acetylate the newly deposited histone H4 lysine 16 [36, 48, 50, 52-54]. Parental Sir2 molecules, bound to the parental H3-H4 tetramers, could then deacetylate the newly acetylated H4 lysine 16 [55]. As previously described, this deacetylation would then induce a structural rearrangement in the Sir complex followed by the binding of multiple copies of Sir3 to Sir2/Sir4 [55]. Polymerization of the SIR

complex along the chromatin fiber would finally generate a transcriptionally silent heterochromatin structure.

Finally, we have generated a powerful tool to dissect the inheritance process. We show that our conditional alleles separate the inheritance process into two fundamental steps: (1) S phase dependent disruption followed by (2) active restoration. Consequently, this system will not only enable us to determine what S phase event perturbs silent chromatin, but will also allow us to analyze the inheritance mechanism.

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Figure 2-1. Sir1 and Asf1 are not required for the G1 maintenance of *HMLalpha* silencing. (A) Experimental strategy. s*ir1*^{td} (YJL5880), asf1*1*^{td} (YJL5801) and s*ir1*^{td} asf1*1*^{td} (YJL5824) cells were grown in log phase permissive conditions (SDC-MET), synchronized in G1 with alpha factor, shifted to fresh media containing either permissive or restrictive (YEPgal + 2mM MET) conditions and held for 3 hours. After the 3 hour incubation, 0.2M hydroxyurea was added to the G1 arrested cultures. Samples were harvested every hour for 7 hours (0 through 7 hour time points). s*ir3*^{ts} *hmr* (YJL6078) cells were grown in log phase YEPD permissive conditions (23˚C), synchronized in G1 with alpha factor, shifted to fresh media at either permissive (23˚C) or restrictive (37˚C) temperatures, and held for 1 hour. After the 1 hour incubation, hydroxyurea was added to the culture to maintain a tight G1 arrest. As described above, samples were harvested every hour for 7 hours while held in G1. (B) Cell cycle position. Sytox staining followed by flow cytometry was used to monitor DNA content. Budding index was used to monitor the G1 arrest and progression past "Start". (C) Western blot. Protein extracts were processed for each time point and 35µg of each extract was resolved on a 7.5% SDS gel. Membranes were probed with anti-HA and anti-Pgk1 (loading control) antibodies. (D) anti-HA ChIP assay to monitor Sir1p association. Samples were harvest for each time point and processed as described in Materials and Methods. (E) *HMLalpha2* expression. RNA was isolated and subjected to RT-PCR followed by quantitative PCR. The HMLalpha2/ACT1 ratio, determined by quantitative PCR, was adjusted so that the W*T* ratio was 1.0. In each case, the standard error was calculated based on three experiments.

Figure 2-1

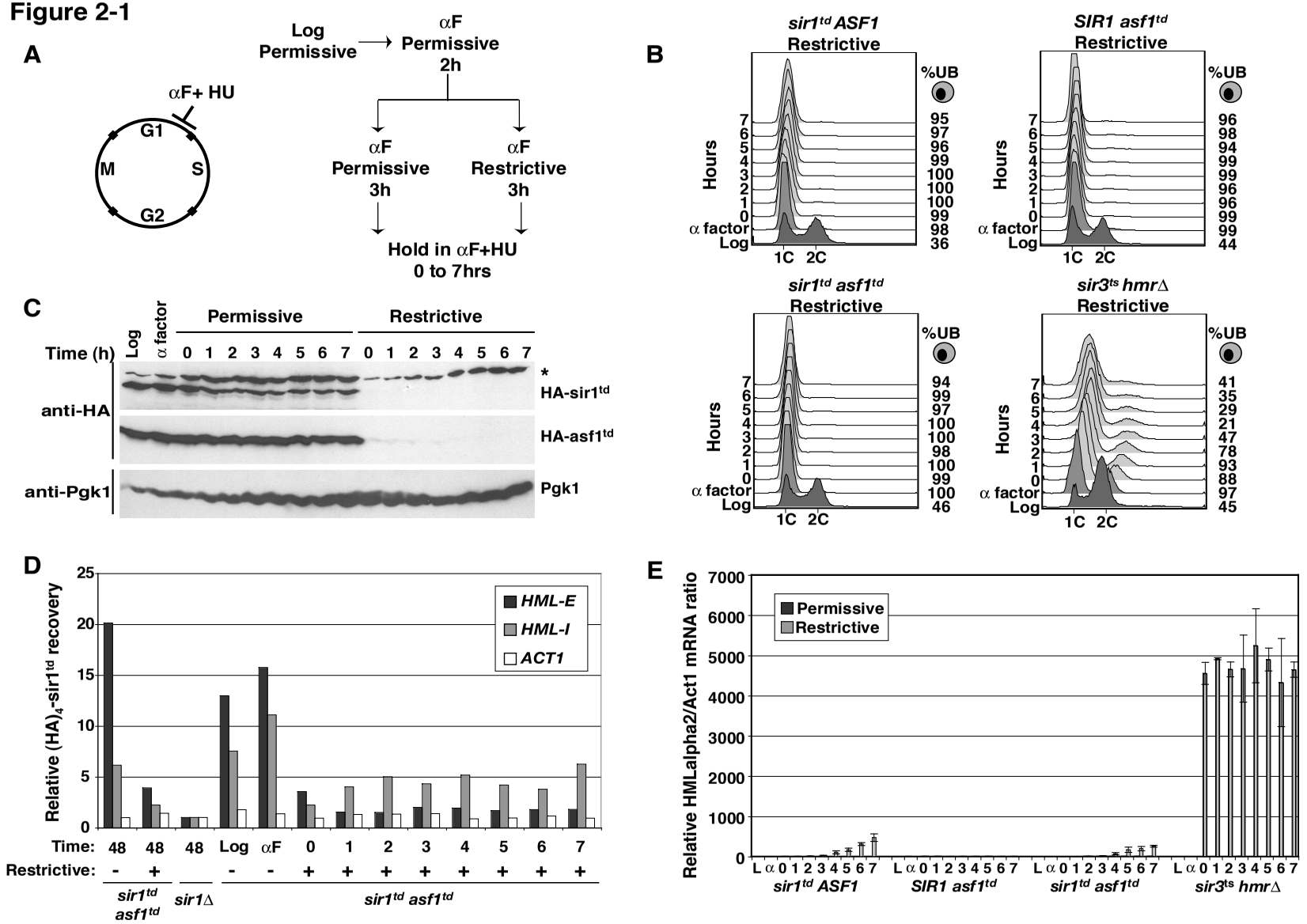
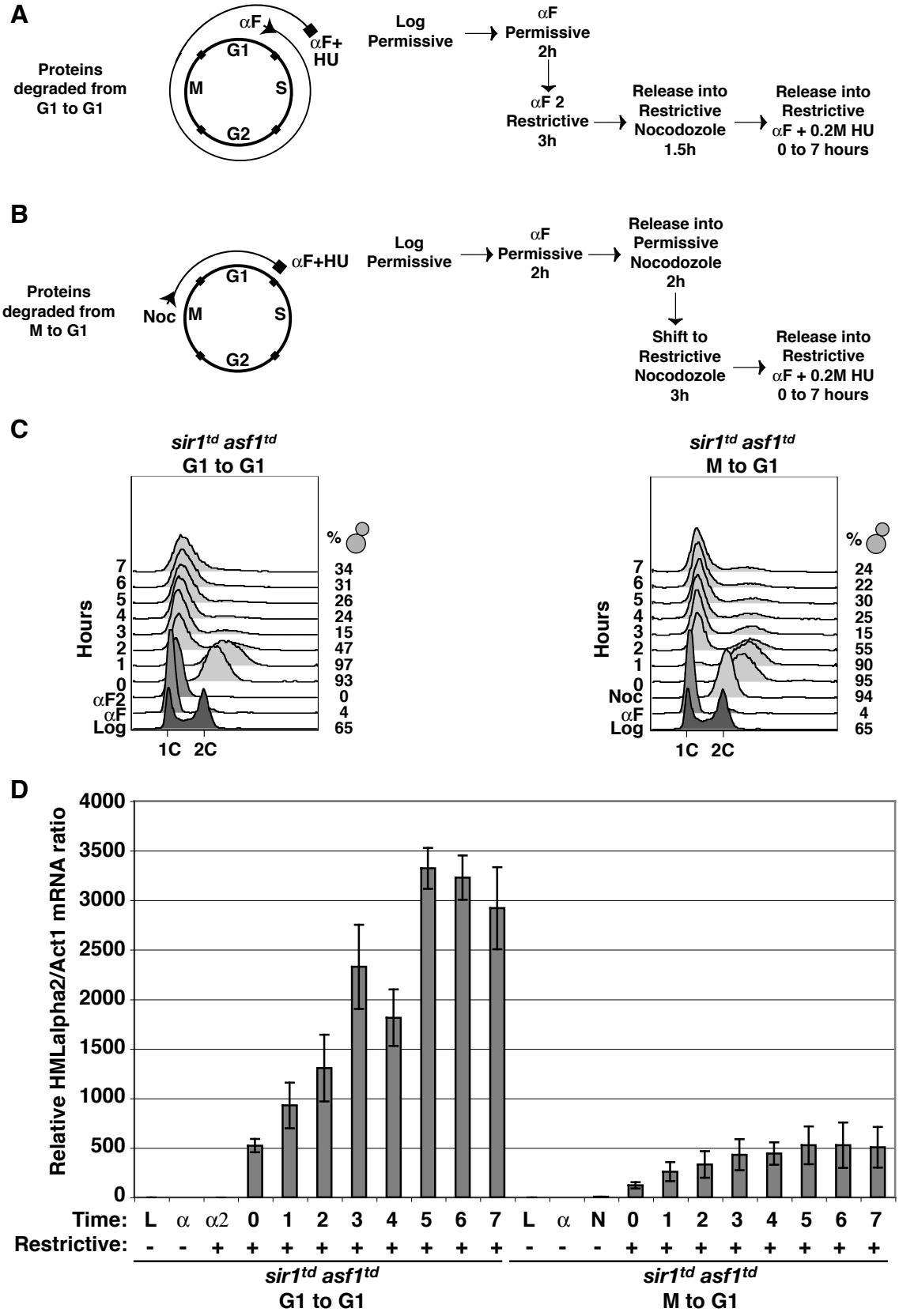

A
αF+ HU
G1
S
G2
M
Log Permissive
αF Permissive 2h
αF Permissive 3h
αF Restrictive 3h
Hold in αF+HU 0 to 7hrs
B
sir1td ASF1 Restrictive
SIR1 asf1td Restrictive
sir1td asf1td Restrictive
sir3ts hmrΔ Restrictive
%UB
Hours
α factor
Log
1C 2C
C
Permissive
Restrictive
Time (h)
Log
α factor
anti-HA
anti-Pgk1
HA-sir1td
HA-asf1td
Pgk1
D
Relative (HA)4-sir1td recovery
HML-E
HML-I
ACT1
Time:
Restrictive:
sir1td asf1td
sir1Δ
sir1td asf1td
E
Relative HMLalpha2/Act1 mRNA ratio
Permissive
Restrictive
sir1td ASF1
SIR1 asf1td
sir1td asf1td
sir3ts hmrΔ

Figure 2-2. Progression through mitosis, in the absence of Sir1 and Asf1, is not sufficient to disrupt *HMLalpha* silencing. (A) Experimental strategy to determine if progression through one cell cycle, without Sir1 or Asf1, disrupts *HMLalpha* silencing. s*ir1*^{td} asf*1*^{td} cells were grown in log phase permissive conditions, synchronized in G1 with alpha factor and shifted for 3 hours into a restrictive G1 arrest. Cells were released from the G1 arrest into a restrictive Metaphase arrest. Once 100% of the cells were Metaphase arrested, they were released into a 7 hour restrictive G1/early S phase arrest. Time points were harvested every hour during the 7 hour release. (B) Experimental strategy to determine if progression from Mitosis to G1, without Sir1 or Asf1, disrupts *HMLalpha* silencing. s*ir1*^{td} asf*1*^{td} (YJL5824) cells were grown in log phase permissive conditions (SDC-MET), pre-synchronized in G1 with alpha factor and released into a Metaphase arrest (using nocodozole). While maintaining the Metaphase arrest, cells were shifted to restrictive conditions (YEPgal + 2mM MET) and after 3 hours they were released into a G1/early S phase arrest (alpha factor + 0.2M hydroxurea). Time points were harvested every hour during the 7 hour release. (C) Cell cycle position. DNA content was monitored by flow cytometry and the G1 arrest, progression past "Start" and the G2 arrest were monitored by budding index. (D) *HMLalpha2* expression. RNA was isolated, processed and analyzed as described in Figure 2-1.

Figure 2-2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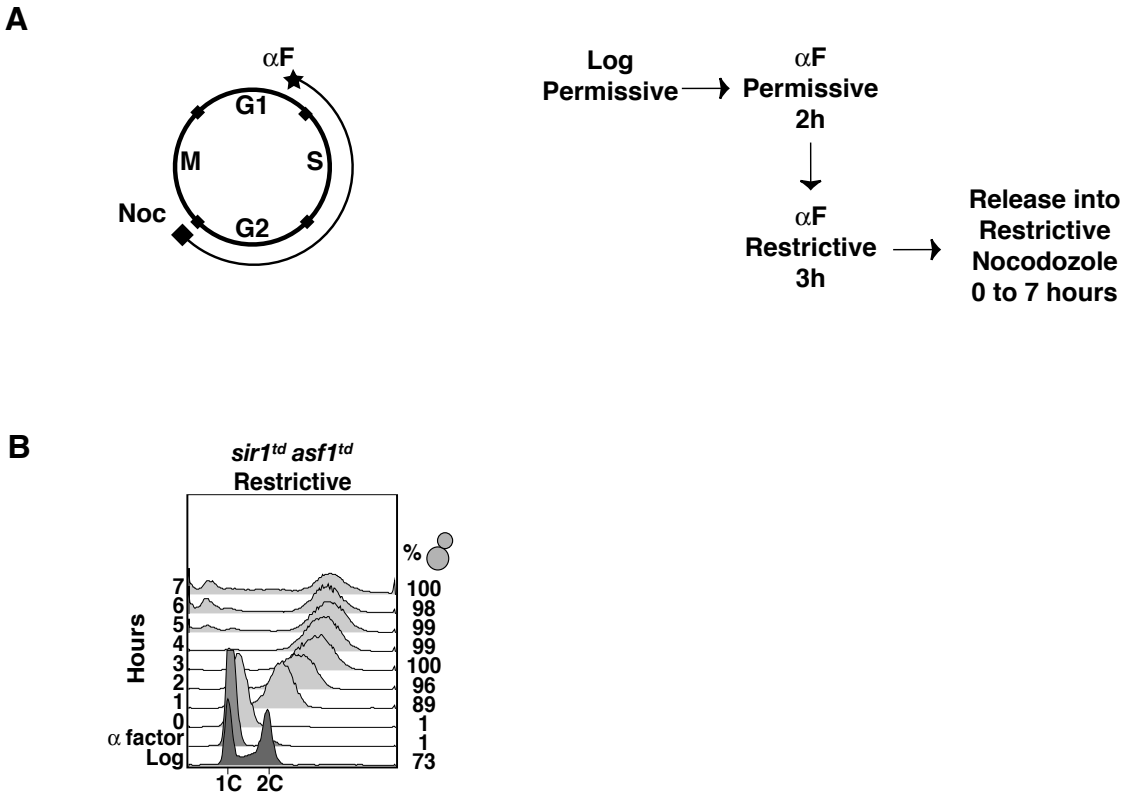
Proteins degraded from G1 to G1
αF
αF+ HU
G1
M
S
G2
Log Permissive
αF Permissive 2h
αF 2 Restrictive 3h
Release into Restrictive Nocodozole 1.5h
Release into Restrictive αF + 0.2M HU 0 to 7 hours
B
Proteins degraded from M to G1
αF+HU
Noc
Log Permissive
αF Permissive 2h
Release into Permissive Nocodozole 2h
Shift to Restrictive Nocodozole 3h
Release into Restrictive αF + 0.2M HU 0 to 7 hours
C
sir1td asf1td
G1 to G1
sir1td asf1td
M to G1
Hours
%
7 34
6 31
5 26
4 24
3 15
2 47
1 97
0 93
αF2 0
αF 4
Log 65
1C 2C
Noc 94
24
22
30
25
15
55
90
95
D
Relative HMLalpha2/Act1 mRNA ratio
4000
3500
3000
2500
2000
1500
1000
500
0
Time: L α α2 0 1 2 3 4 5 6 7 L α N 0 1 2 3 4 5 6 7
Restrictive: - - + + + + + + + + - - - + + + + + + + +
sir1td asf1td
G1 to G1
sir1td asf1td
M to G1

Figure 2-3. Progression through S phase, in the absence of Sir1 and Asf1, is sufficient to disrupt *HMLalpha* silencing. (A) Experimental strategy. s*ir1*^{td} asf*1*^{td} (YJL5824) cells were grown in log phase permissive conditions (SDC-MET), synchronized in G1 with alpha factor and shifted to restrictive conditions (YEPgal + 2mM MET) for 3 hours while maintaining the G1 arrest. Cells were then released for 7 hours into restrictive media containing nocodozole (arrests cells in Metaphase) and time points were harvested every hour. (B) Cell cycle position. DNA content for each time point was measured using Sytox staining of the DNA followed by flow cytometry. Budding index was used to monitor the G1 arrest, progression past "Start" and the mitotic arrest. (C) *HMLalpha2* expression. RNA was isolated from samples taken at the indicated time points and analyzed as described in Figure 2-1.

Figure 2-3


A
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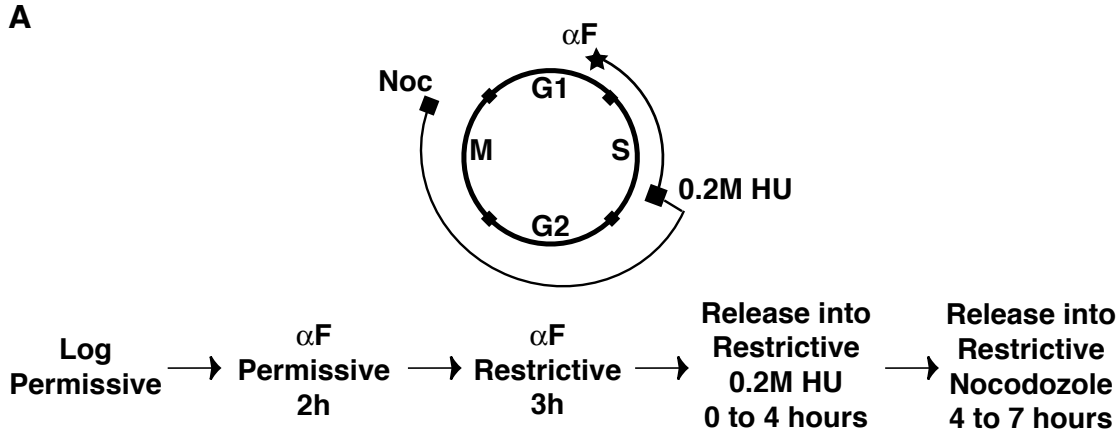
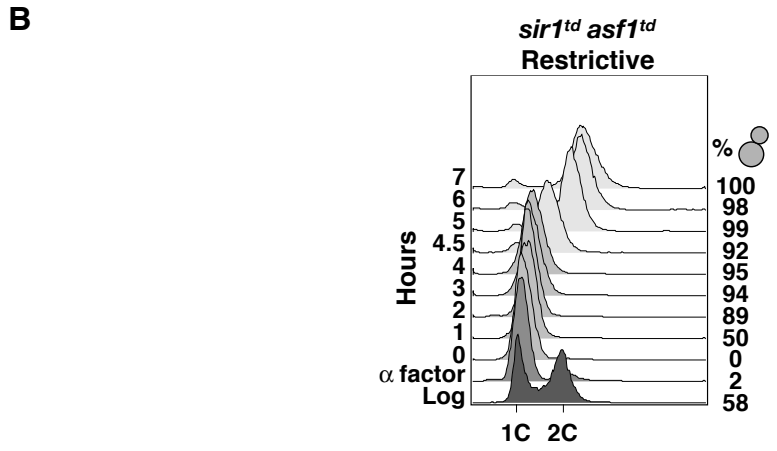
G1
M
S
Noc
G2
Log
Permissive
αF
Permissive
2h
αF
Restrictive
3h
Release into
Restrictive
Nocodozole
0 to 7 hours
B
sir1td asf1td
Restrictive
%
Hours
7 100
6 98
5 99
4 99
3 100
2 96
1 89
0 1
α factor 1
Log 73
1C 2C
C
Relative HMLalpha2/Act1 mRNA ratio
5000
4000
3000
2000
1000
0
Log
alpha factor
0
1
2
3
4
5
6
7
Hours after release into restrictive conditions

Figure 2-4. Progression through S phase, in the absence of Sir1 and Asf1, is required to disrupt *HMLalpha* silencing. (A) Experimental strategy. s*ir1*^{td} asf*1*^{td} (YJL5824) cells were grown in log phase permissive conditions (SDC-MET), synchronized in G1 with alpha factor and shifted to restrictive conditions (YEPgal + 2mM MET) for 3 hours while maintaining the G1 arrest. Cells were then released for 4 hours into 0.2M hydroxurea, an early S phase arrest (HU, 0 through 4 hour time points). After 4 hours in HU, cells were released from the early S phase arrest into nocodozole, a Metaphase arrest (Noc, 4.5 through 7 hour time points). Samples were harvested through out the experiment at the indicated time points. (B) Cell cycle position. DNA content was measured by flow cytometry. Budding index was used to monitor the G1 arrest, progression passed "Start", and the Metaphase arrest. (C) HMLalpha2 expression. RNA was isolated from samples taken at the indicated time points and analyzed as described in Figure 2-1. The "Block" row describes the cell cycle inhibitor used in each time point (alpha factor, hydroxyurea and nocodozole).

Figure 2-4


A
αF
Noc
G1
M
S
0.2M HU
G2
Log Permissive
αF Permissive 2h
αF Restrictive 3h
Release into Restrictive 0.2M HU 0 to 4 hours
Release into Restrictive Nocodozole 4 to 7 hours


B
sir1td asf1td
Restrictive
%
Hours
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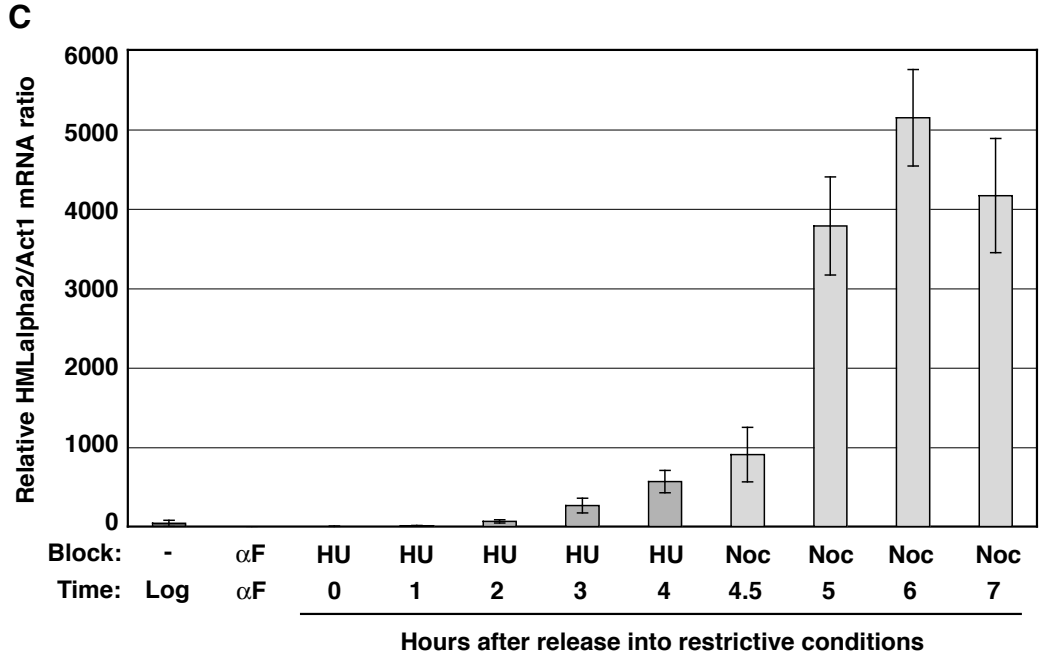
6 98
5 99
4.5 92
4 95
3 94
2 89
1 50
0 0
α factor 2
Log 58
1C 2C


C
Relative HMLalpha2/Act1 mRNA ratio
6000
5000
4000
3000
2000
1000
0
Block: - αF HU HU HU HU HU Noc Noc Noc Noc
Time: Log αF 0 1 2 3 4 4.5 5 6 7
Hours after release into restrictive conditions

CHAPTER 3

Analysis of the chromatin structure following a failure to inherit silencing

Prior to this study, it was unclear how transcriptionally silent states are faithfully inherited from one generation to the next. In Chapter 2 we demonstrate, for the first time, that the inheritance of transcriptional silencing involves an S-phase dependent disruption of silencing followed by Sir1/Asf1 mediated restoration. The mechanism of Sir1/Asf1 inheritance, however, remains to be determined. To study the inheritance mechanism, we analyze the chromatin structure of *HMLalpha* immediately following a failure to inherit silencing. We found that the heterochromatin protein, Sir3, associates with *HMLalpha* for at least three generations after silencing is lost. These results suggest that Sir3 serves as a "molecular memory marker" that can template the inheritance of silencing. We also observe that euchromatin features, Htz1 and acetylated-H4, associate with *HMLalpha* before silencing is lost, suggesting that a heterochromatin to euchromatin transformation induces a loss of silencing. Thus, following a failure to inherit silencing, *HMLalpha* has a dynamic chromatin structure that is transcriptionally active, yet ready to be silenced by Sir1 and Asf1.

INTRODUCTION

During each cell cycle, the genome is faithfully duplicated and divided between two daughter cells. Furthermore, the mother cell must not only replicate its DNA, but it must also duplicate the chromatin structure [1-4]. This duplication of the chromatin structure, epigenetic inheritance, is essential for proper gene expression in subsequent generations [2, 4]. Though studies have attempted to understand the mechanism of epigenetic inheritance, little is known about the inheritance process. It is hypothesized however,

that epigenetic inheritance mechanisms act concurrently with DNA replication during S phase [1-3, 5, 6].

These mechanisms likely act concurrently with DNA replication since replication presents a major challenge to the inheritance of silent chromatin. First, the higher order structure of silent chromatin must be unraveled in order for the replication machinery to access the underlying DNA [7, 8]. Second, nucleosomes are partially dismantled then reassembled during DNA replication, resulting in a nucleosome bare region of 400 to 600 base pairs immediately behind the replication fork [7-9]. Third, although nucleosomes are readily inherited from parent to daughter DNA, this inheritance provides each daughter chromosome with only half the necessary complement of nucleosomes [10, 11]. These observations support the hypotheses that chromosomal replication disrupts silent chromatin and that the inheritance of transcriptional silencing requires mechanisms to restore the silent chromatin state following replication. Because no disruption of transcriptional silencing has been detected during S phase, one must also hypothesize that the disruption is extremely transient because the inheritance of silent chromatin is tightly coupled to the act of DNA replication.

If DNA replication disrupts the silent chromatin structure, it is likely that some aspect of this silent state is retained on the newly replicated daughter molecules. Retention of such a silent chromatin "mark" would provide the biological memory needed to restore each daughter segment to the same chromatin state as its parent [2, 3, 12]. The inheritance of parental nucleosomes provides several ways in which such a mark could be faithfully transmitted. As parental nucleosomes are replicated, their histone octamers are disassembled into H3-H4 tetramers and H2A-H2B dimers and then

reassembled on the daughter DNA [10, 11]. Because each replicated chromosomal segment bequeaths its parental H3-H4 tetramers directly to its daughter segments, the tetramer is a potential vehicle for transmitting a silent chromatin mark. For example, the inheritance of tetramers with hypoacetylated H3 and H4 or persistent association with the Sir2-4 complex could prompt the restoration of the silent chromatin state in daughter chromosomes. Presumably an important step in this restoration will be propagation of the silent chromatin mark to all the newly synthesized non-parental histones that are also incorporated in daughter chromosomes. In contrast, if the inheritance of silencing is blocked, the silent chromatin mark may disappear on the daughter chromosomes.

Two key issues for understanding the inheritance of chromatin states during DNA replication are: (1) What provides the molecular memory of each chromatin state in the daughter DNA and (2) based on this memory, what factors are required to restore the appropriate state immediately after DNA replication?

In Chapter 2 we identified, for the first time, two S phase specific inheritance proteins, Sir1 and Asf1. We showed that Sir1 and Asf1 are required to restore silencing immediately following bulk DNA replication. By identifying these factors, we have also generated an important tool to dissect the inheritance process. Our $sir1^{td}$ $asf1^{td}$ conditional alleles separate the inheritance process into two fundamental steps: (1) S phase dependent disruption followed by (2) active restoration. This separation will allow us to analyze the chromatin structure immediately following the S phase disruption. The newly disrupted heterochromatin structure will likely contain the silent chromatin marks, molecular memory, required for inheritance.

In this study, we analyze the chromatin structure of *HMLalpha*, immediately following a failure to inherit silencing. We show that the euchromatin factors, Htz1 and acetylated-H4, associate with *HMLalpha* before silencing is lost. Though Htz1's association with *HMLalpha is* not required for the loss of silencing, it does mark *HMLalpha's* transformation from heterochromatin to euchromatin. We also demonstrate, for the first time, that Sir3, a core silencing protein, remains associated with *HMLalpha* for at least three generations after transcriptional silencing is lost. The persistence of Sir3 at *HMLalpha* suggests that Sir3 is an epigenetic chromatin "mark" that can template the restoration of silent chromatin. These results suggest that immediately following a failure to inherit silencing, euchromatin and heterochromatin proteins associate in a chromatin structure that is transcriptionally active, yet primed to become silenced.

MATERIALS AND METHODS

Strains. All strains used in this study are listed in Table 3-1 and congenic to W303. YJL5444 and YJL5447 were generated by sporulating YJL5432 (*MAT@/mat::NatMX4 SIR1/sir1::ADE2 cac1::LEU2/CAC1 asf1::his5+/ASF1 trp1-1::{Galp-UBR1, TRP1}/trp1-1::{Galp-UBR1, TRP1} bar1::HISG/BAR1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11/his3-11 ade2-1/ade2-1 can1-100/CAN1 {Mata, Ura3}*). YJL6675 was generated by disrupting the endogenous *HTZ1* locus in YJL5447 with a KanMX4 PCR fragment. YJL5347 was generated by sporulating YJL4944 (*mat::NatMX4/MAT@ SIR1/sir1::{pCUP1-ts degron-Sir1, URA3} CAC1/cac1::LEU2 ASF1/asf1::his5+ cdc28-as1/CDC28 ura3-1/ura3-1 trp1-1/trp1-1::{Galp-UBR1, TRP1} leu2-3,112/leu2-3,112*

his3-11/his3-11 ade2-1/ade2-1 can1-100/CAN1 bar1::HIS G/bar1::KanMX6). To generate YJL5783, the endogenous *UBR1* in YJL5347 (mat::NatMX4 trp1-1::{Galp-UBR1, TRP1} bar1::HISG ura3-1 leu2-3,112 his3-11 ade2-1 can1-100) was replaced by a ubr::LEU2 PCR product. To generate YJL5801, the endogenous *ASF1* in YJL5783 was replaced by pLC132 (BsiWI digested) using one-step gene disruption. The enodgenous *SIR1* in YJL5801 was then replaced by pLC125 (NheI digested) using one-step gene disruption to generate YJL5824. YJL6671 was then generated by disrupting the endogenous *HTZ1* locus in YJL5824 with a KanMX4 PCR product. YJL6403 was generated by disrupting the endogenous *SIR3* and *HMRa* in YJL5237 (*mat::NatMX4 SIR1 CAC1 ASF1 mat::NatMX4 CDC28 trp1-1 bar1::HISG ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*) with KanMX6 and URA3MX PCR products, respectively. YJL6667 was generated by disrupting the endogenous *HTZ1* locus in YJL5444 with a KanMX4 PCR product.

Standard genetic techniques were used to manipulate yeast strains [13] and standard protocols were used for DNA manipulation [14]. All deletions and replacements were confirmed by PCR and by mutant phenotype analysis. All primer sequences used in this study are available upon request. Bacterial strain DH5α was used for DNA amplification.

Plasmids. All plasmids used in this study are listed in Table 3-2. Plasmid pLC124 is a modified version of the ts-degron plasmid pPW66R described in [27]. pLC124 consists of a SacII to Not1 ts-degron fragment (pCUP1-R-Ubiquitin-DHFR-1xHA), a Not1 to Not1 3xHA fragment, a Not1/HindIII to XhoI 175bp N-terminal Sir1 fragment and a

pRS306 vector backbone. pLC125 is derived from pLC124. The pCUP1 in pLC124 was replaced with a SacII to EcoR1 pMET fragment to generate pLC125. Similarly, pLC132 consists of a SacII to EcoR1 pMET fragment, an EcoR1 to Not1 ts-degron fragment (R-Ubiquitin-DHFR-1xHA), a Not1 to Not1 3xHA fragment, a Not1/HindIII to Xho1 205bp N-terminal Asf1 fragment and a pRS402 backbone.

Total RNA extraction. Total RNA was prepared from yeast cells using an Acid-Phenol extraction. 10ml of an OD=1.0 culture was harvested by centrifugation (3 min at 3,000 rpm) and frozen in liquid nitrogen. Cells were resuspended in 500µl of TES buffer (10mM Tris-Cl pH7.5, 10mM EDTA, 0.5%SDS) and 500µl of Acid Phenol (pH 4.3). To extract the RNA from the cells, the mixture was incubated for one hour at 65˚C with periodic vortexing, placed on ice for 5 min and then microcentrifuged at top speed for 5 minutes at 4˚C. The supernatant was transferred to Phase Lock Tubes containing chloroform (500µl), shaken ten times and microcentrifuged for 5 min at top speed. The supernatant was transferred to a tube containing Acid Phenol (500µl), vortexed vigorously for 10 seconds and microcentrifuged for 5 min at top speed. The aqueous phase was transferred to a new Phase Lock Tube and 500µl of chloroform was added, shaken ten times and microcentrifuged for 5 min at top speed. The chloroform extraction was repeated and then the aqueous phase was transferred to a new tube and mixed with 40µl of 3M sodium acetate (pH 5.3) and 1ml of 100% ethanol. RNA was pelleted by microcentrifugation at top speed for 15 min. The pellet was washed by vortexing in 70% ethanol. After drying, pellets were resuspended in 100-150µl of water and stored at -80˚C.

cDNA preparation. cDNA was generated using the Stratagene First Strand cDNA synthesis protocol. 10ug of total RNA, 500nM OJL1569 and 500nM OJL1578 were mixed in a 20µl RT reaction to synthesize HMLalpha2 and Act1 cDNA respectively. Following the cDNA synthesis, the RNA was hydrolyzed by incubating the reaction at 65˚C for 10 minutes with 14.2µl of AHS (1ml water, 320µl NaOH, 100µl 0.5M EDTA). The hydrolysis reaction was neutralized by adding 66µl of NPS (5.3ml water, 320µl 1M HCl, 1ml 3M NaOAc). The cDNA was then precipitated by microcentrifugation for 30 min in 250µl of 100% ethanol. The pellet was washed by vortexing in 70% ethanol and after drying, pellets were resuspended in 20µl of water.

RT-PCR. RT-PCR was performed using the Stratagene MX3000P System and results were analyzed using the accompanying software. PCR was performed in a reaction containing 10mM Tris-HCl (pH 8.3), 50mM KCl, 1mM $MgCl_2$, 2.5mM each dNTP and Taq polymerase. Cycling parameters were 94˚C for 5 min and then 40 cycles of 94˚C for 30 sec, 52˚C for 30 sec, 72˚C for 1 min and 75˚C for 30 sec, followed by a final incubation of 72˚C for 5 min. We used 1/5 of the cDNA in reactions to detect *HMLalpha2* and 1/25 of the cDNA in reactions to detect *ACT1*. HMLalpha2 message was detected using 50nM OJL678 and 50nM OJL1681 while Act1 message was detected using 50nM OJL1577 and 50nM OJL1578. A standard curve for each primer set was generated using ten-fold serial dilutions of yeast genomic DNA, ranging from 10ng/µl to 10^{-2} ng/µl. For each sample the HMLalpha2 and Act1 cDNA concentration was determined and then the HMLalpha2 cDNA/Act1 cDNA ratio was calculated. These

ratios were scaled in each experiment so that the wildtype W303 HMLalpha2 cDNA/Act1 cDNA ratio was 1.

Chromatin immunoprecipitation assay. ChIP assays were performed as described previously [28] with: polyclonal anti-Sir3 antibody (gift from Madhani Lab), polyclonal anti-Htz1 antibody (gift from Madhani Lab), or polyclonal anti-tetra-acetylated H4 (gift from Madhani Lab). Cells were fixed in 1% formaldehyde for 1hr followed by sample processing described in Sharp et al. 2001. RT-PCR analysis, detailed above, was performed on 3% of the precipitated DNA and 1.5% of the input DNA. The DNA regions of interest, HML-BE, *HML-E, HMLalpha2, HML-I, ACT1* and *BUD3* were analyzed using the respective primer sets listed in Table 3-3. A standard curve for each primer set was generated using ten-fold serial dilutions of yeast genomic DNA, ranging from 10ng/μl to 10^{-2} ng/μl. Using the corresponding primer standard curve, we calculated starting DNA concentrations (Initial Concentrations) for each sample. "Initial Concentrations" for HML-BE, *HML-E, HMLalpha2, HML-I* and *ACT1* were normalized for loading error by dividing by the "Initial Concentration" for *BUD3*. Normalized precipitated DNA values were then divided by normalized input DNA values to determine the relative recovery.

RESULTS

Characterization of the *HMLalpha* chromatin structure in permissive and restrictive conditions. In Chapter 2, we showed that Sir1 and Asf1 were required to restore silencing following progression through S phase. It remains to be determined, however, how Sir1 and Asf1 are targeted to the perturbed silent locus. One possible targeting mechanism may require the retention of some aspect of the silent state on the newly replicated daughter molecules. Retention of such a silent chromatin "mark" would provide the biological memory needed to not only target Sir1 and Asf1, but also template the restoration of each daughter locus to the same chromatin state as the parent.

To determine if a chromatin mark is retained following a failure to inherit silencing, we analyzed the chromatin structure of *HMLalpha* at four regions: HML-BE, HML-E, HMLalpha2 and HML-I (Fig. 3-1). We characterized the chromatin structure of *HMLalpha* in $sir1^{td}$ $asf1^{td}$ cells when silencing was inherited and disrupted. To perform this analysis, we grew $sir1^{td}$ $asf1^{td}$ cells under permissive and restrictive log phase conditions for 48 hours. We then used chromatin immunoprecipitation to monitor Sir3 (a core silencing protein), Htz1 (a euchromatin H2.A variant) and poly-acetylated-H4 (a euchromatin characteristic) association throughout *HMLalpha*. In cells grown under permissive conditions, we observed that Sir3 maximally associated at all regions of *HMLalpha* (Fig. 3-2A, orange) while Htz1 was excluded from the locus (Fig. 3-2B, orange). In contrast, cells grown under restrictive conditions had Htz1 associated throughout *HMLalpha* (Fig. 3-2A, yellow) while Sir3 was excluded from the locus (Fig. 3-2B, yellow). Thus, in permissive conditions when transcriptional silencing is intact,

HMLalpha exists in a heterochromatin state and in restrictive conditions when silencing is lost, *HMLalpha* exists as euchromatin.

We also analyzed the chromatin structure of *HMLalpha* in *WT* and *sir3::KanMX6* cells to ensure that the chromatin structure of *HMLalpha* in permissive and restrictively grown $sir1^{td}$ $asf1^{td}$ cells is similar to that of *WT* and *sir3* cells, respectively. We observe that $sir1^{td}$ $asf1^{td}$ cells, grown under permissive conditions, had similar Sir3 and Htz1 chromatin association profiles to *WT* cells (Fig. 3-2A&B). We also found that $sir1^{td}$ $asf1^{td}$ cells, grown under restrictive conditions, had chromatin association profiles identical to a *sir3* strain's profile (Fig. 3-2A&B). Thus, under permissive conditions, $sir1^{td}$ $asf1^{td}$ cells behave like *WT* cells and under restrictive conditions, they behave like *sir3* cells.

Cell cycle progression does not affect *HMLalpha's* heterochromatic structure. We sought to determine if Sir3, Htz1 and acetylated-H4 association with *HMLalpha* is affected by progression through the cell cycle. To study this, $sir1^{td}$ $asf1^{td}$ cells were grown in log phase permissive conditions, synchronized in G1 with alpha factor and held in the G1 arrest for 3 hours. Cells were then released from the G1 arrest into permissive log phase conditions and samples were harvested at 0, 1, 3, 5 and 7 hours after release (Fig. 3-3A). Chromatin immunoprecipitation showed that Sir3, Htz1 and acetylated-H4 association, at all *HMLalpha* regions, remained constant and equal to *WT* levels throughout the cell cycle (Fig. 3-3 B, C & D). Thus Sir3, Htz1 and acetylated-H4 association with *HMLalpha* is not regulated by cell cycle progression.

Sir3, Htz1 and acetylated-H4 associate with *HMLalpha* when silencing is lost. Silent chromatin features that remain associated with *HMLalpha* following a loss of silencing, potentially serve as a memory mechanism for the silent chromatin state. In contrast, euchromatin features that associate with *HMLalpha* when silencing is lost, potentially disrupt the heterochromatin structure. We monitored Sir3, Htz1 and acetylated-H4 association to determine which silent and active chromatin marks associated with *HMLalpha* when silencing was lost. In the previous section, we showed that Sir3 association with *HMLalpha* remained constant, throughout the cell cycle while Htz1 and acetylated-H4 were excluded from *HMLalpha,* when silencing was inherited. Thus, any change in Sir3, Htz1 and acetylated-H4 association with *HMLalpha,* when silencing was lost, resulted from a failure to inherit silencing and was not cell cycle induced variation.

To analyze the chromatin structure immediately following a failure to inherit silencing, we grew $sir1^{td}$ $asf1^{td}$ cells in log phase permissive conditions, synchronized the cells in G1 with alpha factor and shifted the cells for 3 hours to restrictive conditions. After holding the cells for 3 hours in restrictive conditions, both Sir1 and Asf1 degron proteins were undetectable by Western Blot (Chapter 5, Fig. 5-5C). In the absence of both proteins, cells were released from the G1 arrest into restrictive log phase conditions (Fig. 3-4A).

We monitored HMLalpha2 mRNA expression and observed that two hours after release from G1, the majority of $sir1^{td}$ $asf1^{td}$ cells completed S phase and had a 2C DNA content (Chapter 5, Fig. 5-5B, right panel). This completion of S phase correlated with a 70% loss of silencing (Chapter 5, Fig. 5-5D). Though most $sir1^{td}$ $asf1^{td}$ cells lost silencing 2 hours after release from G1, Sir3 remained associated with *HMLalpha* for the

duration of the experiment (Fig. 3-4B). Consistent with previous studies [15], these results demonstrated the Sir3 association with *HMLalpha* was not sufficient to generate a heterochromatin structure. Likely, higher order remodeling of the Sir2-4 complex is required to create a transcriptionally silent chromatin structure. This persistence of Sir3 at *HMLalpha* after a failure to inherit silencing also suggests that Sir3 is an epigenetic chromatin "mark" that can template the restoration of silent chromatin.

We also observed that Htz1 associated with HML-E, HMLalpha and HML-I (Fig. 3-4C) 1 hour after release from the G1 arrest while acetylated-H4 associated with *HMLalpha* (Fig. 3-4D) 3 hours after release. These results demonstrated that Htz1 and acetylated-H4 associated with *HMLalpha* before a loss in transcriptional silencing was detected, suggesting that the association of Htz1 and acetylated-H4 with *HMLalpha* causes the heterochromatin to euchromatin transformation. Thus, Htz1 and acetylated-H4's association with *HMLalpha* potentially induces the loss of *HMLalpha* silencing.

Htz1 is not sufficient to disrupt *HMLalpha* silencing. In Chapter 2, we showed that progression through S phase, in the absence of Sir1 and Asf1, disrupted the inheritance of *HMLalpha* silencing. Consequently, we wanted to determine how progression through S phase affects Sir3, Htz1 and acetylated-H4 association with *HMLalpha*. We grew $sir1^{td}$ $asf1^{td}$ cells in log phase permissive conditions, synchronized them in G1 with alpha factor and held the cells for 3 hours in a permissive G1 arrest. The cells were then released from the G1 arrest into a Mitotic arrest (Fig. 3-5A). Consistent with previous results, quantitative PCR showed that *HMLalpha* transcriptional silencing remained intact during the experiment (Fig. 3-5B). Furthermore, chromatin immunoprecipitation showed that

Sir3 association, at all *HMLalpha* regions, remained constant and equal to *WT* levels during S phase (Fig. 3-5C). Surprisingly, we also observed that Htz1 became associated with *HMLalpha* 3 hours after release from the G1 arrest (Fig. 3-5D). This result demonstrated that progression through S phase perturbed the heterochromatin structure. However, since there was no detectable loss of silencing (Fig. 3-5B), the chromatin disruption was likely minor. These results also suggest that Htz1's association with *HMLalpha*, though not sufficient to disrupt silencing, may be required as an initial step to transform heterochromatin into euchromatin.

Htz1 is not required to disrupt *HMLalpha* silencing. We sought to determine if Htz1's association with *HMLalpha* is required as an initial step in the loss of transcriptional silencing. We grew *WT, htz1::KanMX4, sir1::ADE2 asf1::his5+* and *sir1::ADE2 asf1::his5+ htz1::KanMX4* cells in log phase conditions for 48 hours. Using quantitative PCR, HMLalpha2 expression was monitored for each strain and normalized to Act1 transcript levels. If Htz1's association with *HMLalpha* was required for the loss of silencing, then *sir1::ADE2 asf1::his5+ htz1::KanMX4* cells would have a reduced silencing defect compared to the defect in *sir1::ADE2 asf1::his5+* cells. Our results showed, however, that *sir1::ADE2 asf1::his5+ htz1::KanMX4* and *sir1::ADE2 asf1::his5+* cells had equivalent silencing defects (Fig. 3-6). Similarly, restrictively grown $sir1^{td}$ $asf1^{td}$ and $sir1^{td}$ $asf1^{td}$ *htz1::KanMX4* strains had severe silencing defects (Fig. 3-6). These results demonstrate that Htz1's association with *HMLalpha,* following a failure to inherit silencing, only marks the transformation of heterochromatin to euchromatin and is not required for the loss of silencing.

DISCUSSION

Heterochromatin disassembly is a gradual process, providing time for silent chromatin restoration. The dynamics of heterochromatin inheritance through S phase and the role of various histone modifications in templating the restoration of silent chromatin following its disruption are not well understood. It is widely believed, however, that silent chromatin marks are retained on newly disrupted heterochromatin, providing a molecular memory for the restoration of silencing [1-3, 5, 6]. Here, we use the Sir1/Asf1 inheritance system described in Chapter 2, to determine if euchromatin and heterochromatin marks provide a molecular memory for each chromatin state. To monitor the chromatin structure following a failure to inherit silencing, we use chromatin immunoprecipitation to follow Sir3, Htz1 and acetylated-H4 association with *HMLalpha*.

Our results show that heterochromatin proteins associate with *HMLalpha* for at least 3 generations after silencing is lost. Specifically, Sir3 associates with *HMLalpha* for more than 5 hours following a failure to inherit silencing. Consistent with previously published results [15], our findings demonstrate that Sir protein association is not sufficient to generated a transcriptionally silent heterochromatin structure. However, the persistence of heterochromatin features after silencing is lost suggests that a silent chromatin molecular memory mechanism exists and this mechanism is important in restoring silencing.

Sir1 and Asf1 restore the silent chromatin structure following is disruption. Our results show that heterochromatin marks persist at *HMLalpha* many generations after silencing is lost and these marks may act as a molecular memory mechanism that templates the restoration of heterochromatin. It is possible that Sir1 and Asf1 recognize these silent chromatin marks and use them, to restore silencing after progression through S phase.

Asf1 is a master chromatin regulator. Two of its key activities are: (1) H3/H4 tetramer deposition onto chromatin throughout the cell cycle [16-20] and (2) recruitment of Sas2 [21-24], a H4 lysine16 histone acetyltransferase, to chromatin. Asf1's H3/H4 deposition activity and ability to recruit Sas2, may be involved in the inheritance of silencing. We propose that while depositing H3/H4 tetramers onto chromatin, Asf1 interacts with chromatin regions that fail to inherit silencing and also recruits Sas2 to these newly disrupted heterochromatin regions. Sas2 could then acetylate histone H4 lysine16 in any newly deposited daughter nucleosome [21, 24, 25]. Sir2, retained on parent nucleosomes, could then deacetylate the acetylated histone H4 lysine 16 to generate an *O*-acetyl-ADP-ribose metabolite (AAR) [26]. This deacetylation of histone H4 lysine 16 creates a high affinity-binding site for the Sir2-4 complex, while AAR induces a structural rearrangement in the complex that causes the binding of multiple copies of Sir2, Sir3 and Sir4 to chromatin [26]. Thus, the AAR-induced structural change in the SIR complex is ultimately required to form the higher order heterochromatin structure.

Chromatin dynamics and the loss of transcriptional silencing. Various euchromatin marks have been implicated in inhibiting the binding of silencing proteins. Our studies reveal, however, that Htz1, a euchromatin mark is not required nor is it sufficient to disrupt HMLalpha silencing. Specifically, we demonstrate that Htz1 and Sir3 simultaneously associate with transcriptionally silent regions, during S phase. Our results demonstrate that the binding of a single euchromatin mark does not inhibit the binding of silencing proteins. It remains to be determined, however, if the association of multiple euchromatin marks disrupt transcriptional silencing.

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Figure 3-1. Chromatin immunoprecipitation was used to analyze the chromatin structure of *HMLalpha,* in cells progressing through the cell cycle. To accurately assess the chromatin structure of *HMLalpha*, four regions in the locus were analyzed: HML-BE, HML-E, HMLalpha2 and HML-I (highlighted in red).

Figure 3-1

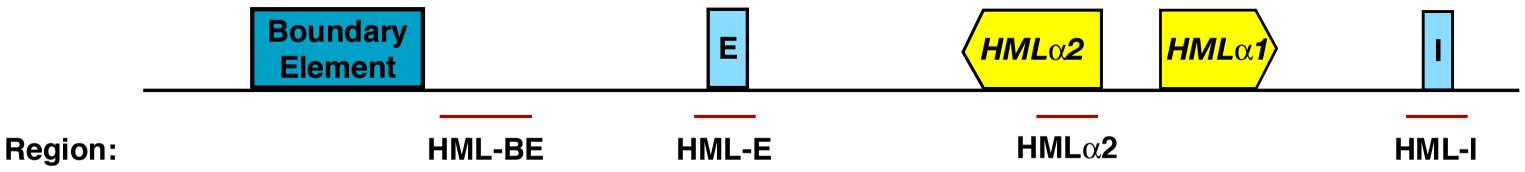
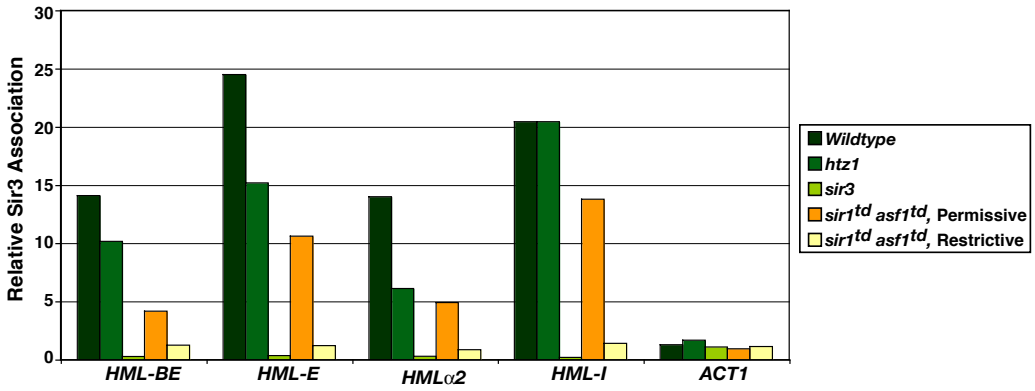

Boundary Element
E
HMLα2
HMLα1
I
Region:
HML-BE
HML-E
HMLα2
HML-I

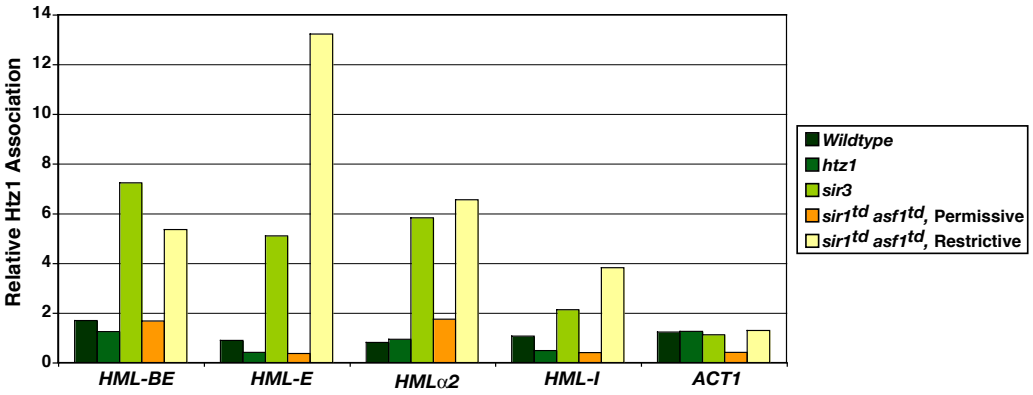
Figure 3-2. Log phase association of Sir3, Htz1 and acetylated H4 with *HMLalpha*. *WT* (YJL5444), *htz1* (YJL6667) and *sir3* (YJL6403) cells were grown under log phase conditions for 48 hours. Similarly, $sir1^{td}$ $asf1^{td}$ (YJL5824) cells were grown under permissive (SDC-MET) or restrictive (YEPgal + 2mM MET) conditions for 48 hours. Cells were then harvested and chromatin containing extracts were prepared from cells treated with formaldehyde for 1 hour. These extracts were immunoprecipitated with (A) anti-Sir3 antibodies, (B) anti-Htz1 antibodies, or (C) anti-poly-acetylated H4 antibodies. DNA was amplified by quantitative PCR using primers specific to HML-BE, HML-E, HMLalpha2, HML-I, ACT1 (euchromatin region) and BUD3 (normalization control). These primers were also used to amplify DNA isolated from extracts before immunoprecipitation (input). The relative recoveries are determined by calculating the normalized IP value/normalized input value ratio and normalizing that ratio with respect to the BUD3 ratio.

Figure 3-2

A Sir3 Antibody



B Htz1 Antibody



C Acetylated H4 Antibody

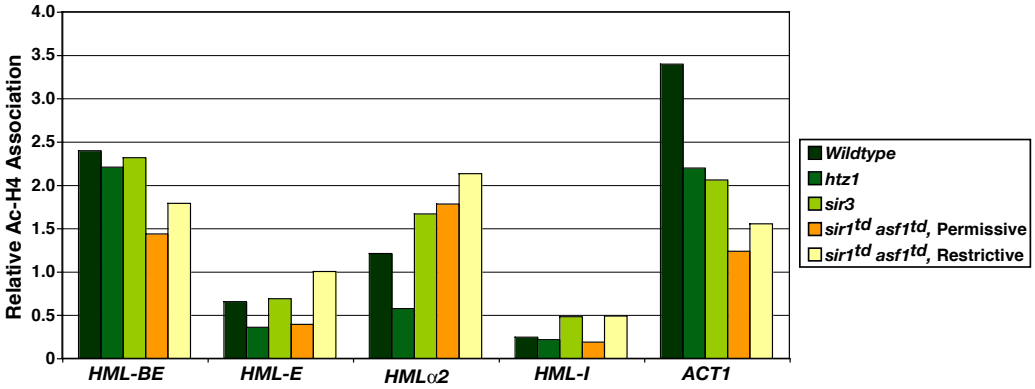


Figure 3-3. Sir3 and hypoacetylated H4 continuously associate with *HMLalpha,* in *sir1*^{td} *asf1*^{td}cells progressing through a permissive cell cycle. (A) *sir1*^{td}*asf1*^{td} cells (YJL5824) cells were grown in log phase permissive conditions (SDC-MET), synchronized in G1 with alpha factor and held in permissive conditions for 3 hours while maintaining the G1 arrest. Cells were then released into permissive log phase conditions for 7 hours and time points were harvested at 0, 1, 3, 5, and 7 hours after release. Chromatin immunoprecipitation was used to analyze Sir3 (B), Htz1 (C) and acetylated H4 (D) association with *HMLalpha*. *WT* (YJL5444), *sir3* (YJL6403) and *htz1* (YJL6667) cells were grown in log phase YEPD conditions to monitor proteins levels under: endogenous conditions (*WT)*, when silencing is perturbed (*sir3*), and when Htz1 is perturbed. All samples were processed and analyzed as described in Figure 3-2.

Figure 3-3

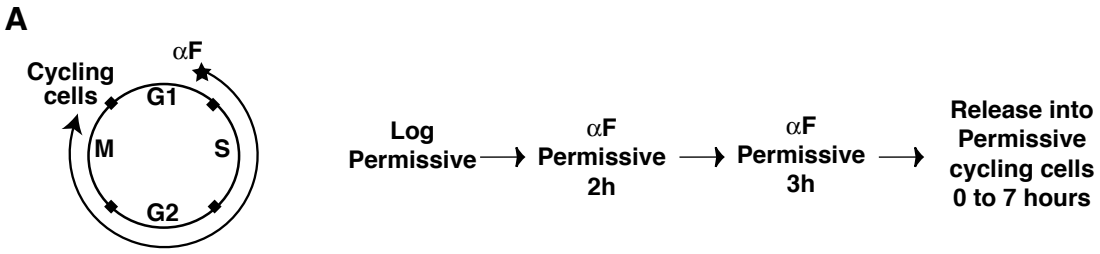
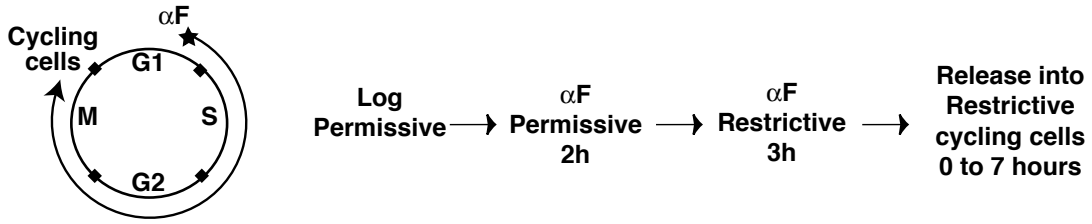

A
αF
Cycling cells
G1
S
G2
M
Log Permissive
αF Permissive 2h
αF Permissive 3h
Release into Permissive cycling cells 0 to 7 hours
B Sir3 Antibody
HML-BE
HML-E
HMLalpha
HML-I
ACT1
Sir3 Association
L α 0 1 3 5 7 WT sir3 htz1
C Htz1 Antibody
Htz1 Association
D Ac-H4 Antibody
AcH4 Association

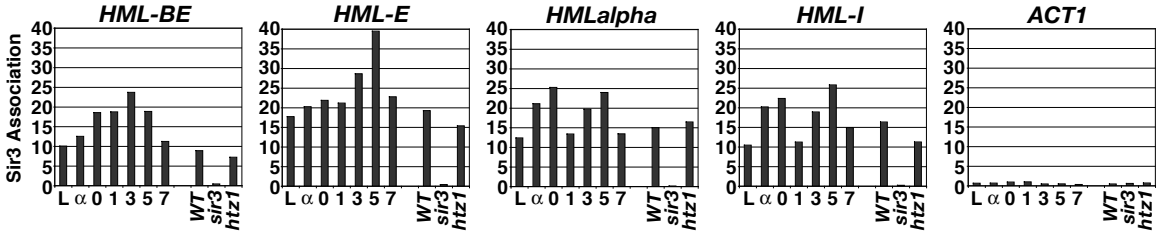
Figure 3-4. Sir3, Htz1 and acetylated H4 associate with *HMLalpha* in *sir1*^{td} *asf1*^{td} cells progressing through a restrictive cell cycle. (A) *sir1*^{td} *asf1*^{td} cells (YJL5824) cells were grown in log phase permissive conditions (SDC-MET), synchronized in G1 with alpha factor and shifted to restrictive conditions for 3 hours while maintaining the G1 arrest. Cells were then released into restrictive log phase conditions for 7 hours and time points were harvested at 0, 1, 3, 5, and 7 hours after release. Chromatin immunoprecipitation was used to analyze Sir3 (B), Htz1 (C) and acetylated H4 (D) association with *HMLalpha*. *WT* (YJL5444), *sir3* (YJL6403) and *htz1* (YJL6667) cells were grown in log phase YEPD conditions to monitor proteins levels under: endogenous conditions (*WT)*, when silencing is perturbed (*sir3*), and when Htz1 is perturbed. All samples were processed and analyzed as described in Figure 3-2.

Figure 3-4

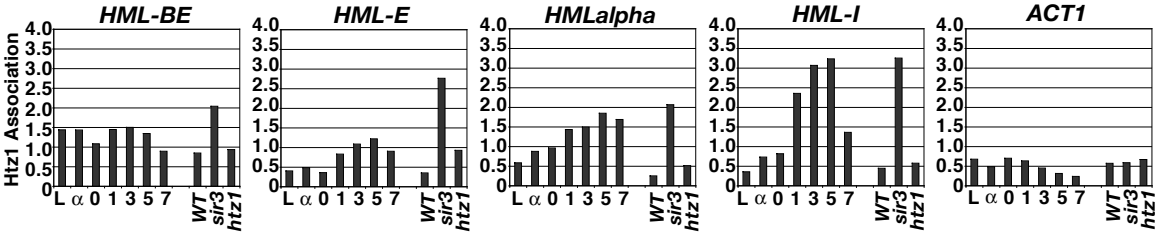
A



B Sir3 Antibody



C Htz1 Antibody



D Ac-H4 Antibody

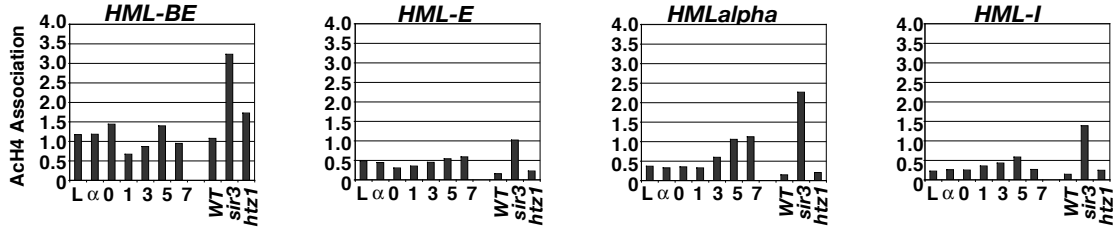


Figure 3-5. Htz1 association with *HMLalpha* is not sufficient to disrupt silencing. (A) *sir1*^{td} *asf1*^{td} cells (YJL5824) cells were grown in log phase permissive conditions (SDC-MET), synchronized in G1 with alpha factor and held in permissive conditions for 3 hours while maintaining the G1 arrest. Cells were then released into a permissive mitotic arrest, using nocodozole, for 7 hours and time points were harvested at 0, 1, 3, 5, and 7 hours after release. (B) Quantitative PCR to monitor *HMLalpha* silencing. RNA was isolated and subjected to both RT-PCR and quantitative PCR. cDNA was amplified with primers to both *HMLalpha2* and *ACT1* (control). The HMLalpha2/ACT1 ratio was determined by quantitative PCR and expressed relative to the *WT* sample (*WT* ratio is set to 1.0). Chromatin immunoprecipitation was used to analyze Sir3 (C) and Htz1 (D) association with *HMLalpha*. *WT* (YJL5444), *sir3* (YJL6403) and *htz1* (YJL6667) cells were grown in log phase YEPD conditions to monitor proteins levels under: endogenous conditions (*WT)*, when silencing is perturbed (*sir3*), and when Htz1 is perturbed. All samples were processed and analyzed as described in Figure 3-2.

Figure 3-5

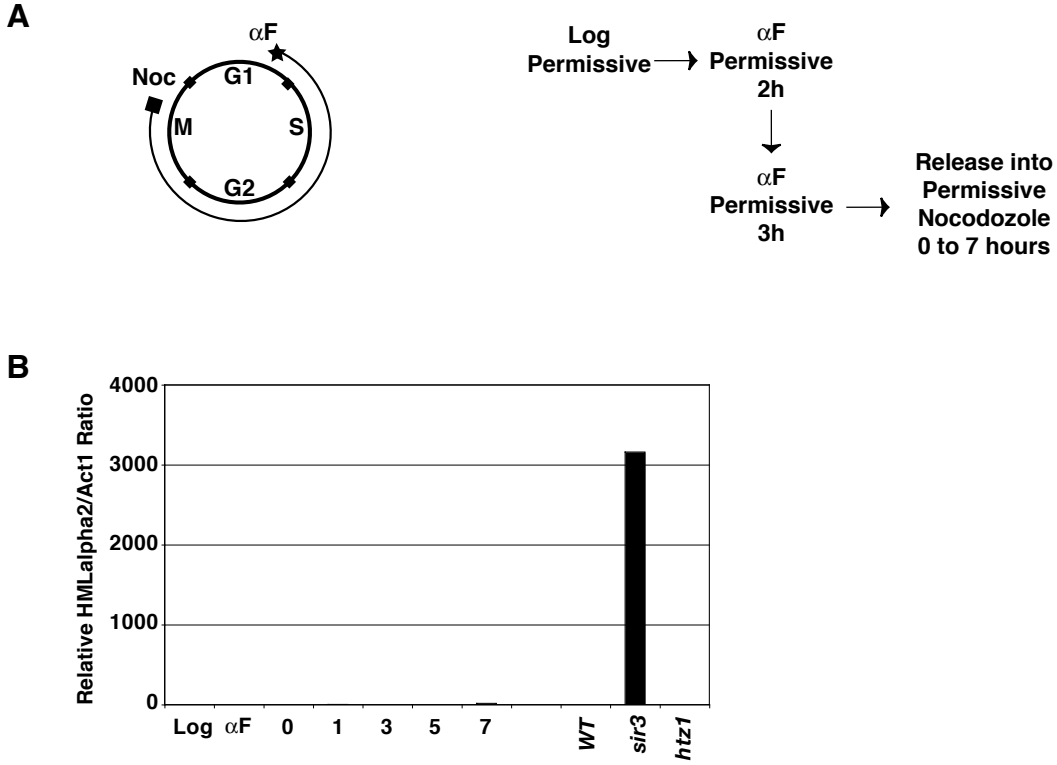

A
αF
Noc
G1
M
S
G2
Log
Permissive
αF
Permissive
2h
αF
Permissive
3h
Release into
Permissive
Nocodozole
0 to 7 hours
B
Relative HMLalpha2/Act1 Ratio
4000
3000
2000
1000
0
Log αF 0 1 3 5 7 WT sir3 htz1
C Sir3 Antibody
HML-BE
HML-E
HMLalpha
HML-I
ACT1
Sir3 Association
L α 0 1 3 5 7 WT sir3 htz1
D Htz1 Antibody
Htz1 Association

Figure 3-6. In the absence of Sir1 and Asf1, Htz1 is not required to perturb the inheritance of *HMLalpha* silencing. *WT* (YJL5444), *htz1* (YJL6667), *sir1 asf1* (YJL5447) and *sir1 asf1 htz1* (YJL6675) cells were grown under log phase YEPD conditions for 48 hours. Similarly, $sir1^{td}asf1^{td}$(YJL5824) and $sir1^{td}asf1^{td}htz1$ (YJL6671) cells were grown in log phase permissive (P: SDC-MET) and restrictive (R: YEPGal + 2mM MET) conditions for 48 hours. RNA was isolated and subjected to both RT-PCR and quantitative PCR. cDNA was amplified with primers to both *HMLalpha2* and *ACT1* (control). The HMLalpha2/ACT1 ratio was determined by quantitative PCR and expressed relative to the *WT* sample (*WT* ratio is set to 1.0).

Figure 3-6

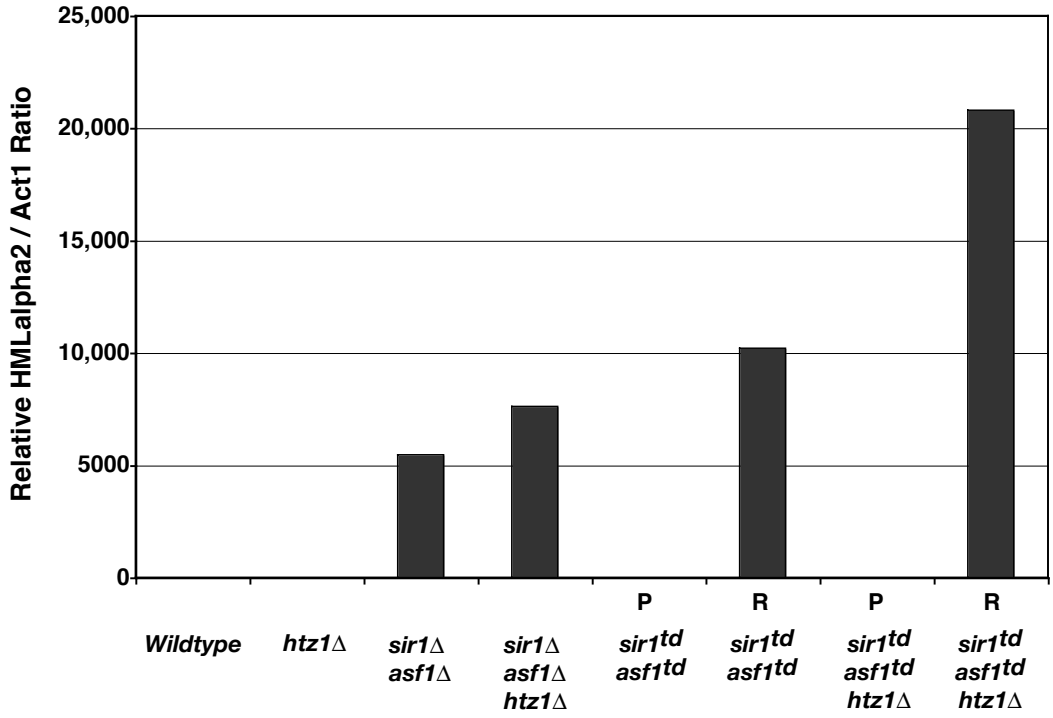

Relative HMLalpha2 / Act1 Ratio
25,000
20,000
15,000
10,000
5000
0
P
R
P
R
Wildtype
htz1Δ
sir1Δ asf1Δ
sir1Δ asf1Δ htz1Δ
sir1td asf1td
sir1td asf1td
sir1td asf1td htz1Δ
sir1td asf1td htz1Δ

Table 3-1. Strains used in this study

Strains	Genotype	Source
	Congenic to W303	
YJL5444	*mat::NatMX4 ade2-1 leu2-3,112 his3-11 trp1-1::{pGal-UBR1, TRP1} ura3-1*	This study
YJL5447	*mat::NatMX4 sir1::ADE2 asf1::his5+ ade2-1 leu2-3,112 his3-11 trp1-1::{pGal-UBR1, TRP1} ura3-1*	This study
YJL5824	*mat::NatMX4 trp1-1::{Galp-UBR1, TRP1} bar1::HISG ubr1::LEU2 asf1::{pMET-td-ASF1,ADE2} sir1::{pMET-td-SIR1,URA3} ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*	This study
YJL6403	*mat::NatMX4 sir3::KanMX6 hmr::URA3MX trp1-1 bar1::HISG ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*	This study
YJL6667	*mat::NatMX4 htz1::KanMX4 ade2-1 leu2-3,112 his3-11 trp1-1::{pGal-UBR1, TRP1} ura3-1*	This study
YJL6671	*mat::NatMX4 trp1-1::{Galp-UBR1, TRP1} bar1::HISG ubr1::LEU2 asf1::{pMET-td-ASF1,ADE2} sir1::{pMET-td-SIR1,URA3} htz1::KanMX4 ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*	This study
YJL6675	*mat::NatMX4 sir1::ADE2 asf1::his5+ htz1::KanMX4 ade2-1 leu2-3,112 his3-11 trp1-1::{pGal-UBR1, TRP1} ura3-1*	This study

Table 3-2. Plasmids used in this study

Plasmid	Description	Source
pKI1458	pRS304 pGAL-UBR1	This lab
pLC124	pRS306 pCUP1-4xHA-sir1^{td}	This study
pLC125	pRS306 pMET-4xHA-sir1^{td}	This study
pLC129	pRS316 MATa Cen, Ars	This study
pLC132	pRS402 pMET-4xHA-asf1^{td}	This study

Table 3-3. Primers used in this study

Primer	Purpose	Conc	Sequence
OJL1569	*HMLalpha2* cDNA	500nM	5'-GATAAACTGGTATTCTTCATTAG-3'
OJL1578	*ACT1* cDNA/ antisense QPCR	500nM 50nM	5'-AAGCCAAGATAGAACCACCAATCCA-3'
OJL1577	*ACT1* sense	50nM	5'-TCACTATTGGTAACGAAAGATTCAG-3'
OJL1678	*HMLalpha2* antisense	50nM	5'-CAGTAATGGTAGTAGTGAGTTG-3'
OJL1681	*HMLalpha2* sense	50nM	5'-CCCATTAAAGACCTTTTAAATCCAC-3'
OJL1723	*HML-E* sense	60nM	5-'GGTGTATCGCAATGGAATG-3'
OJL1724	*HML-E* antisense	60nM	5'-GAAAAAATGTAGGTTGAATTTGG-3'
OJL1725	*HML-I* sense	60nM	5-'CGATGCTTATTGTGCTTTG-3'
OJL1726	*HML-I* antisense	60nM	5'-TAGTGTGCCCAGCTTTTATGTC-3'
OJL1836	*HML-BE* sense	50nM	5'-CTTCTTTGGTTTTGCCCTCTG-3'
OJL1837	*HML-BE* antisense	50nM	5'-AATCACACCACTTGCAATGG-3'
OJL1904	*BUD3* sense	50nM	5'-ATAACATCGTAATCTTAGACGTCTT-3'
OJL1905	*BUD3* antisense	50nM	5'-GCCCTATGTTCGTCTGTTGAAGGGT-3'

CHAPTER 4

Conclusion

How eukaryotic cells stably propagate defined gene expression patterns from one generation to the next has been a long-standing question. In recent years, it has been shown that the inheritance of gene expression patterns requires not only DNA replication, but also duplication of the epigenetic chromatin structure [1-4]. When I began the work in this dissertation, it was unclear how progression through S phase affects the silent chromatin structure. Though it was hypothesized that DNA replication disrupts heterochromatin, multiple studies did not detect a loss of silencing following DNA replication of the silent locus [5]. These results suggested that if DNA replication disrupts silent chromatin, then the inheritance mechanism must be tightly coupled to the disruptive process. Chapters 2 and 5, submitted for publication, focus on identifying both the cell cycle events that perturb silent chromatin and the factors required to restore silencing following the disruptive event. Chapter 3 then attempts to identify the inheritance mechanism and demonstrates that a silent chromatin mark likely templates the inheritance of silent chromatin. Finally, Chapter 3 also demonstrates that a single euchromatin factor is not sufficient to perturb transcriptional silencing.

What Proteins Are Required For The Inheritance Of Silent Chromatin?

In Chapters 2 and 5, we demonstrate that Sir1 and Asf1 are required for the inheritance but not the maintenance of silent chromatin. Prior to this study, proteins required only for the inheritance of silencing had not been identified. Using *sir1*^{td} and *asf1*^{td} conditional alleles, described in Appendix I, we show that Sir1 and Asf1 are not required to maintain silencing in G1 or Mitosis. However, in cycling cells, we show that Sir1 and Asf1 are required for silencing, demonstrating that they are essential for

inheritance. This is the first identification and characterization of proteins required for the inheritance and not the maintenance of transcriptional silencing.

What Cell Cycle Events Disrupt Silent Chromatin?

Another major conclusion from the work presented in Chapters 2 and 5 was that an S phase event, possibly DNA replication, disrupts silent chromatin. Previous work from the Holmes lab suggests that progression through M phase and not S phase, in the absence of silencers, disrupts silencing [6]. This led many in the field to assume that Mitosis is the predominant cell cycle event that disrupts transcriptional silencing while S phase does not perturb the inheritance of silencing. However, in Chapters 2 and 5, we demonstrate that progression through S phase but not M phase, in the absence of Sir1 and Asf1, disrupts transcriptional silencing. In particular, we show that while progression through early and mid S phase do not affect silencing, late S phase and completion of DNA replication are required to disrupt silencing, in the absence of Sir1 and Asf1.

At this point, we have not conclusively shown that DNA replication is the S phase event that disrupts silencing. However, since *HMLalpha* is one of the latest replicating regions in the *Saccharomyces cerevisiae* genome [7], one would predict that if DNA replication disrupts silencing, then a loss of silencing would only occur at the end of S phase. Consistent with this hypothesis, we demonstrate that there is a tight temporal correlation between the completion of DNA replication and the loss of silencing. Also supporting the hypothesis that DNA replication is the S phase event that disrupts silent chromatin, studies have shown that nucleosomes, the heterochromatin foundation, are dismantled and then reassembled during DNA replication [8, 9].

Though we have demonstrated that Sir1 and Asf1 are required to restore silencing following progression through S phase, we acknowledge that there likely exist other proteins required for the S phase and M phase inheritance of silencing. This hypothesis is supported by reports that silencers are required for the M phase inheritance of silencing [6]. Furthermore, to identify M phase inheritance proteins, conditional alleles for all silencer- associated proteins should be generated. Our system, described in Appendix I, can be used to generate conditional alleles of the candidate proteins.

What Is The Mechanism Of Silent Chromatin Inheritance?

In Chapter 3, we demonstrate that heterochromatin proteins remain associated with *HMLalpha* long after silencing is disrupted. A long standing model for the mechanism of silent chromatin inheritance suggests that silent chromatin features are retained on chromatin after silencing is disrupted [1, 3, 10]. These retained silent chromatin features could then serve as a molecular memory mechanism to template the restoration of silent chromatin. In Chapter 3, we analyzed the chromatin structure of *HMLalpha* following a failure to inherit silencing. We found that the heterochromatin protein, Sir3, remains associated with *HMLalpha* for at least 3 generations after silencing is perturbed. According to the model described above, this silent chromatin feature can potentially act as a molecular memory mechanism to template the reformation of silent chromatin.

To determine if the retained Sir3 molecules template the restoration of silent chromatin, one could reactivate $sir1^{td}$ and $asf1^{td}$ in two conditions: (1) immediately following a failure to inherit silencing, when Sir proteins are still associated with

HMLalpha and (2) several days after silencing is lost, when there are no Sir proteins associated with *HMLalpha*. This experiment would address whether or not Sir3 association with *HMLalpha* is required for Sir1/Asf1 mediated restoration of silencing.

Our observations, in Chapter 3, support the model that inheritance of silencing requires both a replication coupled mechanism and a molecular memory mechanism. In this model, Asf1, a nucleosome deposition factor associated with the replication machinery [11-15], could recruit Sas2 to the newly replicated daughter chromosomes [16-19]. Sas2 could then acetylate the newly deposited histone H4 lysine 16 [16, 20-22]. Parental Sir2 molecules, bound to the parental H3-H4 tetramers, could then deacetylate the newly acetylated H4 lysine 16 [23, 24]. This deacetylation would target Sir2-4 complexes to chromatin, resulting in the restoration of the silent chromatin structure.

Do Euchromatin Factors Disrupt Transcriptional Silencing?

In Chapter 3, we also show that the association of a single euchromatin factor in a heterochromatin locus is not required nor is it sufficient to disrupt transcriptional silencing. Numerous euchromatin marks have been implicated in inhibiting the binding of silencing proteins, resulting in the disruption of the silent chromatin structure [25]. In Chapter 3, however, we demonstrate that the association of Htz1, a euchromatin histone variant, is not sufficient to disrupt *HMLalpha* silencing. Specifically, we show that the incorporation of Htz1 throughout *HMLalpha* does not perturb transcriptional silencing. We also show that the failure to inherit silencing occurs in the absence of Htz1, indicating that Htz1 is not required for the S phase silencing disruption. Furthermore, though we demonstrate that a single euchromatin factor is not required nor is it sufficient to disrupt

silencing, it is possible that the association of multiple euchromatin factors in a heterochromatin region would perturb silencing.

Conclusion

The work presented in this dissertation is the first step in understanding how silent chromatin states are inherited through S phase. I have identified two proteins required for the restoration of silencing following progression through S phase. I have demonstrated that silent chromatin marks are retained on chromatin, when silencing is lost, potentially serving as a mechanism to template the restoration of silent chromatin. Finally, I have shown that an S phase event, possibly DNA replication, is sufficient to disrupt transcriptional silencing.

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CHAPTER 5

Supplemental Data for Chapter 2

MATERIALS AND METHODS

Plasmids. All plasmids are described in Table 5-2. Plasmid pLC124 is a modified version of the ts-degron plasmid pPW66R described in [3]. pLC124 consists of a SacII to Not1 ts-degron fragment (pCUP1-R-Ubiquitin-DHFR-1xHA), a Not1 to Not1 3xHA fragment, a Not1/HindIII to XhoI 175bp N-terminal Sir1 fragment and a pRS306 vector backbone. pLC125 is derived from pLC124. The pCUP1 in pLC124 was replaced with a SacII to EcoR1 pMET fragment to generate pLC125. Similarly, pLC132 consists of a SacII to EcoR1 pMET fragment, an EcoR1 to Not1 ts-degron fragment (R-Ubiquitin-DHFR-1xHA), a Not1 to Not1 3xHA fragment, a Not1/HindIII to Xho1 205bp N-terminal Asf1 fragment and a pRS402 backbone. pLC129 consists of a SacII to ClaI 4300bp *MATa* locus fragment inserted into pRS316.

Strains. All strains are described in Table 5-1 and are congenic to W303. YJL5444, YJL5447, YJL5471 and YJL5473 were generated by sporulating YJL5432 (*MAT@/mat::NatMX4 SIR1/sir1::ADE2 cac1::LEU2/CAC1 asf1::his5+/ASF1 trp1-1::{Galp-UBR1, TRP1}/trp1-1::{Galp-UBR1, TRP1} bar1::HISG/BAR1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11/his3-11 ade2-1/ade2-1 can1-100/CAN1 {Mata, Ura3}*). YJL5347 was generated by sporulating YJL4944 (*mat::NatMX4/MAT@ SIR1/sir1::{pCUP1-ts degron-Sir1, URA3} CAC1/cac1::LEU2 ASF1/asf1::his5+ cdc28-as1/CDC28 ura3-1/ura3-1 trp1-1/trp1-1::{Galp-UBR1, TRP1} leu2-3,112/leu2-3,112 his3-11/his3-11 ade2-1/ade2-1 can1-100/CAN1 bar1::HIS G/bar1::KanMX6*). To generate YJL5783, the endogenous *UBR1* in YJL5347 was replaced by a ubr::LEU2 PCR

product. To generate YJL5801, the endogenous *ASF1* in YJL5783 was replaced by pLC132 (BsiWI digested) using one-step gene disruption. The enodgenous *SIR1* in YJL5801 was then replaced by pLC125 (NheI digested) using one-step gene disruption to generate YJL5824. YJL5938 was then generated by replacing the endogenous *CDC20* with pVN180 (MscI digested) in YJL5824. To generate YJL5880, the endogenous *SIR1* in YJL5783 was replaced by pLC125 (NheI digested) using one-step gene disruption. YJL6377 was generated by disrupting the endogenous *MAT* locus in CFY1463 (*MATa ade2-1 leu2-3,112 his3-11 trp1-1 can1-100 HMR-SSa SIR1-3xHA,KanR*) with a NatMX4 PCR product. YJL6403 was generated by disrupting the endogenous *SIR3* locus and *HMRa* locus in YJL5237 (*mat::NatMX4 SIR1 CAC1 ASF1 mat::NatMX4 CDC28 trp1-1 bar1::HISG ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*) with KanMX6 and URA3MX PCR products, respectively. YJL6078 was generated by disrupting the endogenous *MAT* locus in JRY2334 (*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*), replacing the endogenous *SIR3* with a sir3-8 allele using loop-in/loop-out of pLP1184 (ClaI digested), disrupting the endogenous *BAR1* locus with a KanMX6 PCR product and disrupting the endogenous *HMRa* locus with a URA3MX PCR product.

Standard genetic techniques were used to manipulate yeast strains [1] and standard protocols were used for DNA manipulation [2]. All deletions and replacements were confirmed by PCR and by mutant phenotype analysis. All primer sequences used in this study are available upon request. Bacterial strain DH5α was used for DNA amplification.

1. Fink, C.G.a.G.R., *Guide to Yeast Genetics and Molecular Biology*. Methods in Enzymology, ed. C.G.a.G.R. Fink. Vol. 194. 1991, San Diego: Academic Press.
2. Russell, J.S.a.D., *Molecular cloning: a laboratory manual, 3rd Edition*. 3 ed. Vol. 1. 2001, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
3. Dohmen, R.J., P. Wu, and A. Varshavsky, *Heat-inducible degron: a method for constructing temperature-sensitive mutants.* Science, 1994. **263**(5151): p. 1273-6.

Figure 5-1. Sir1 and Asf1 cooperate to ensure full silencing at *HMLalpha*. *WT* (YJL5444, *sir1* (YJL5471), *asf1* (YJL5473), *sir1 asf1* (YJL5447) and *sir3 hmr* (YJL6403) strains were grown to log phase in YEPD. RNA was isolated and subjected to both RT-PCR and quantitative PCR. cDNA was amplified with primers to both *HMLalpha2* and *ACT1* (control). The HMLalpha2/ACT1 ratio was determined by quantitative PCR and expressed relative to the *WT* sample (*WT* ratio is set to 1.0). In each case, the standard error was calculated based on three experiments.

Figure 5-1

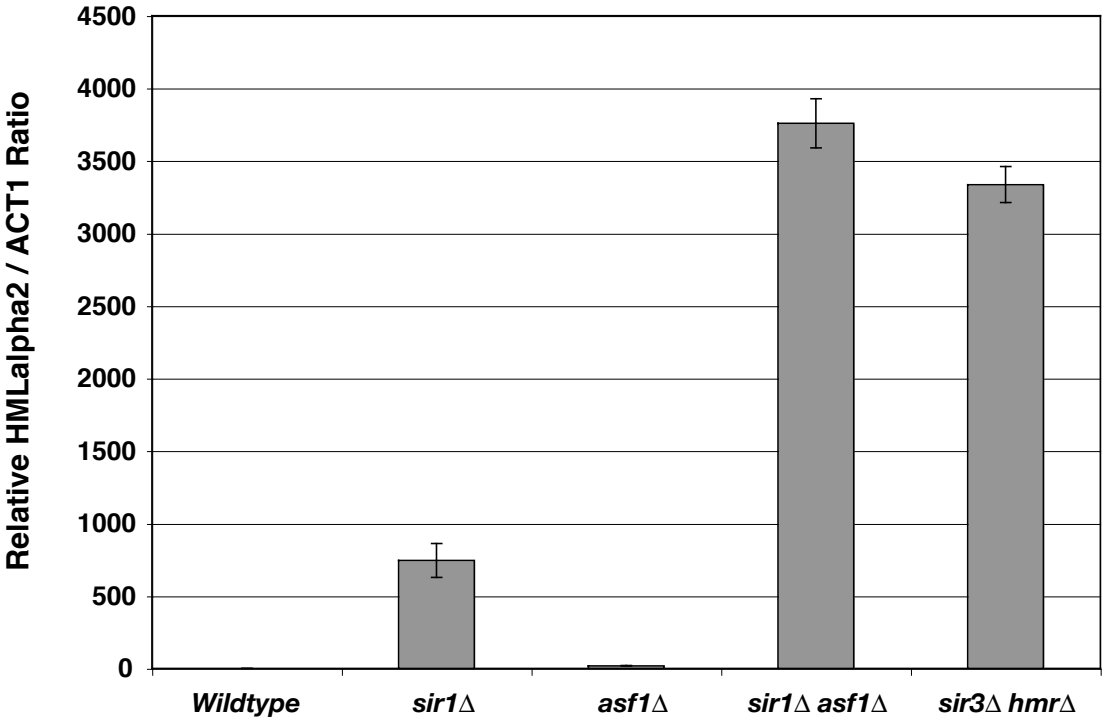
Relative HMLalpha2 / ACT1 Ratio
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Wildtype
sir1Δ
asf1Δ
sir1Δ asf1Δ
sir3Δ hmrΔ

Figure 5-2. *sir1*^{td} and *asf1*^{td} conditional alleles are fully functional under permissive conditions and completely defective under restrictive conditions. (A) Western blots comparing endogenous and conditional degron protein levels. Left panel: Log phase *SIR1-3xHA* (YJL6377) and *sir1* (YJL5471) cells were grown in YEPD conditions for 48 hours while *4xHA-sir1*^{td} (YJL5880) cells were grown in permissive (SDC-MET) and restrictive (YEPgal + 2mM MET) conditions. The indicated amounts of protein extract were resolved on a 7.5% SDS gel and probed with anti-HA and anti-Pgk1 (loading control) antibodies. Right panel: Log phase *ASF1* (YJL5783) and *asf1* (YJL5473) cells were grown in YEPD while *4xHA-asf1*^{td} (YJL5801) cells were grown in permissive and restrictive conditions for 48 hours. Protein extracts were resolved on a 12% SDS gel and probed with anti-Asf1 and anti-Pgk1 (loading control) antibodies. (B) *HMLalpha2* expression. *sir1*^{td}, *asf1*^{td} and *sir1*^{td} *asf1*^{td} (YJL5824) strains were grown in permissive (P) and restrictive (R) conditions for 48 hours while *WT* (YJL5444), *sir3 hmr* (YJL6403), *sir1* (YJL5471), *asf1*(YJL5473) *and sir1 asf1* (YJL5447) cells were grown in YEPD. RNA was isolated from the samples and analyzed as described in Materials and Methods. (C) anti-HA ChIP assay to monitor Sir1p association. *4xHA-sir1*^{td} cells were grown under permissive and restrictive conditions while *sir1* and *SIR1-3xHA* cells were grown in YEPD. Chromatin containing extracts were prepared as described in Materials and Methods. In each case, the standard error was calculated based on three experiments.

Figure 5-2

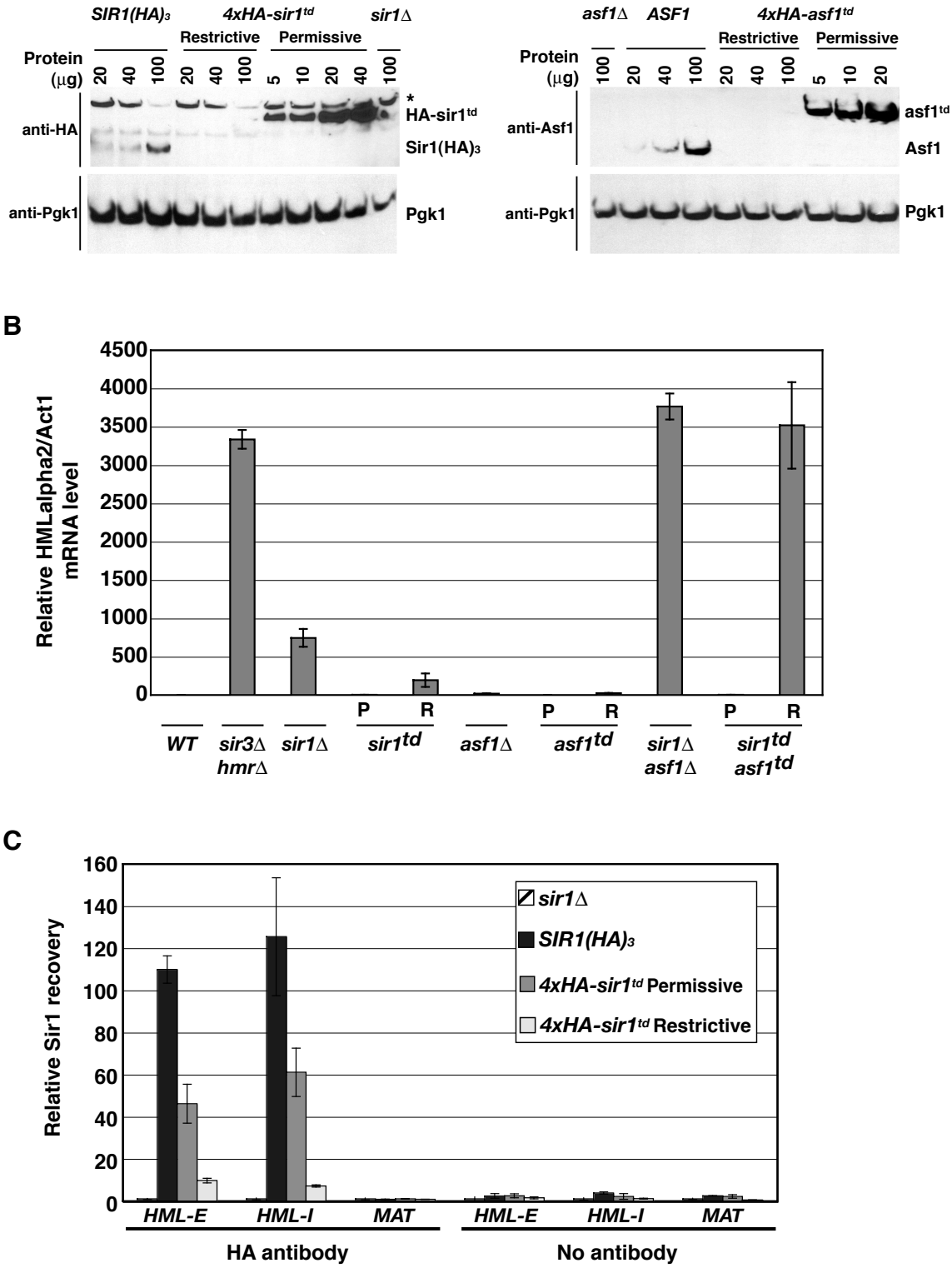
A
SIR1(HA)3
4xHA-sir1td
sir1Δ
Restrictive
Permissive
Protein (μg)
20 40 100 20 40 100 5 10 20 40 100
anti-HA
* HA-sir1td
Sir1(HA)3
anti-Pgk1
Pgk1
asf1Δ
ASF1
4xHA-asf1td
Restrictive
Permissive
Protein (μg)
100 20 40 100 20 40 100 5 10 20
anti-Asf1
asf1td
Asf1
anti-Pgk1
Pgk1
B
Relative HMLalpha2/Act1 mRNA level
4500
4000
3500
3000
2500
2000
1500
1000
500
0
P R P R P R
WT
sir3Δ hmrΔ
sir1Δ
sir1td
asf1Δ
asf1td
sir1Δ asf1Δ
sir1td asf1td
C
Relative Sir1 recovery
160
140
120
100
80
60
40
20
0
sir1Δ
SIR1(HA)3
4xHA-sir1td Permissive
4xHA-sir1td Restrictive
HML-E
HML-I
MAT
HML-E
HML-I
MAT
HA antibody
No antibody

Figure 5-3. Inactivation of both *sir1*^{td} and *asf1*^{td} leads to complete loss of *HMLalpha* silencing within two doublings after protein depletion. (A) Experimental strategy. s*ir1*^{td} (YJL5880), *asf1*^{td} (YJL5801) and s*ir1*^{td} asf*1*^{td} (YJL5824) cells were grown in log phase permissive conditions (SDC-MET) and at t=0 cells were shifted to log phase permissive and log phase restrictive (YEPgal + 2mM MET) conditions. Log phase cells were grown in permissive and restrictive conditions for 18 hours and samples were harvested at the indicated time points. (B) Cell doubling analysis. At the indicated time points, 3µl hemocytometer readings we taken to determine the number of cells in each culture. The starred time points highlight a doubling in cell number and the time between each star is the time required to complete 1 cell cycle. In each case, the standard error is calculated from three experiments. (C) Western blot. Protein extracts were processed for each time point and 35µg of each extract was resolved on a 7.5% SDS gel. Membranes were probed with anti-HA and anti-Pgk1 (loading control) antibodies. (D) *HMLalpha2* expression. RNA was isolated and subjected to RT-PCR followed by quantitative PCR. Samples were then analyzed as described in Chapter 2 Materials and Methods.

Figure 5-3

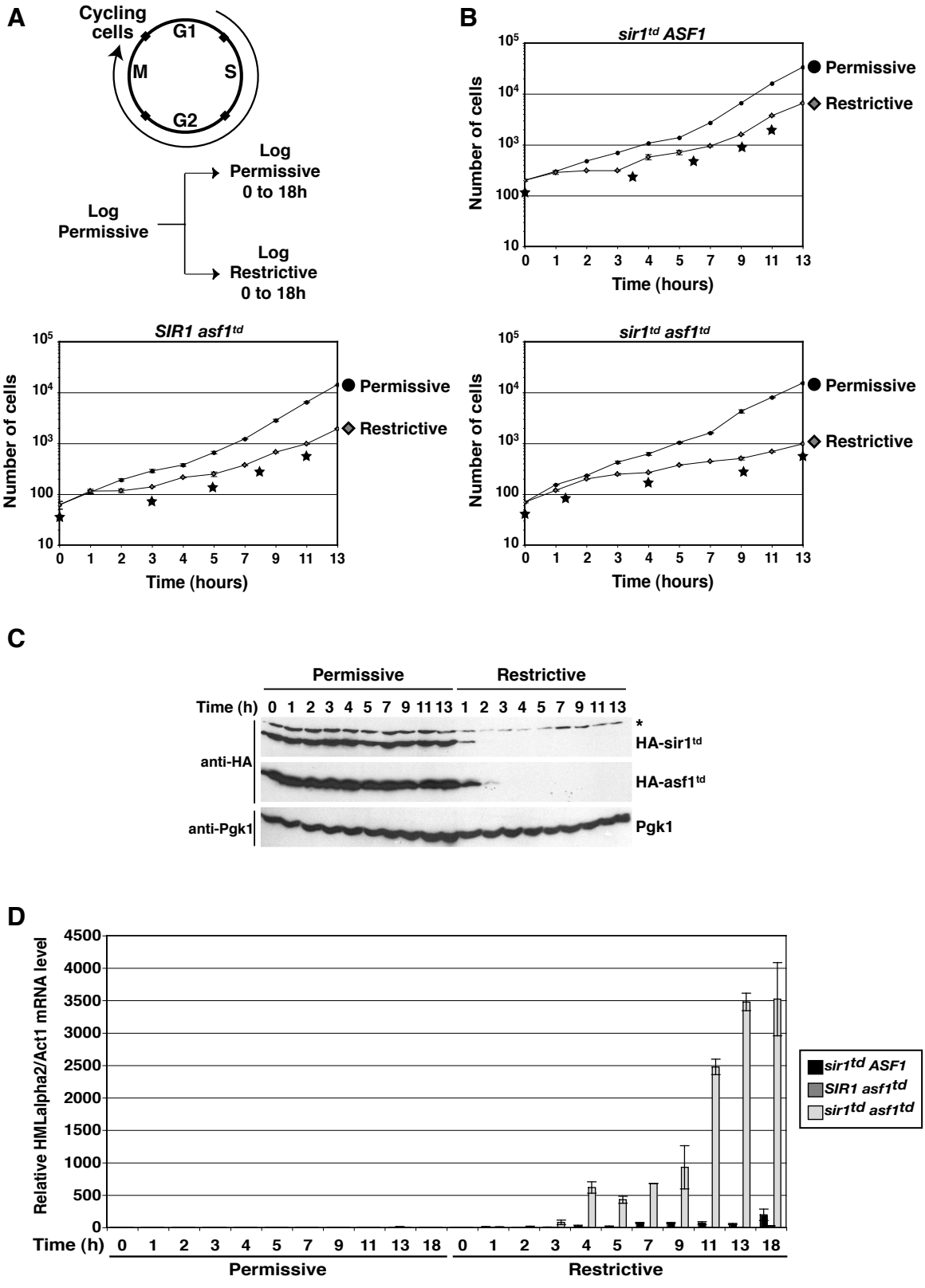
A
Cycling cells
G1
S
G2
M
Log Permissive
Log Permissive 0 to 18h
Log Restrictive 0 to 18h
B
sir1td ASF1
Number of cells
Time (hours)
Permissive
Restrictive
SIR1 asf1td
sir1td asf1td
C
Permissive
Restrictive
Time (h) 0 1 2 3 4 5 7 9 11 13 1 2 3 4 5 7 9 11 13
anti-HA
HA-sir1td
HA-asf1td
anti-Pgk1
Pgk1
D
Relative HMLalpha2/Act1 mRNA level
Time (h) 0 1 2 3 4 5 7 9 11 13 18 0 1 2 3 4 5 7 9 11 13 18
Permissive
Restrictive
sir1td ASF1
SIR1 asf1td
sir1td asf1td

Figure 5-4. Sir1 and Asf1 are not required for the G2 maintenance of *HMLalpha* silencing. (A) Experimental strategy. s*ir1*^{td} asf1*1*^{td} *pMET-cdc20* (YJL5938) cells were grown in log phase permissive conditions (SDC-MET), presynchronized in G1 with alpha factor and released into permissive Metaphase arrest conditions (SERaff + 2mM Met +Nocodozole). Once 100% of the cells were Metaphase arrested, they were shifted to restrictive (YEPgal + 2mM MET + Nocodozole) conditions for 3 hours. After the 3 hour incubation, samples were harvested every hour for 7 hours (0 through 7 hour time points) while held in the Metaphase arrest. Log phase s*ir3*^{ts} *hmr* (YJL6078) cells were grown in YEPD permissive conditions (23˚C), presynchronized in G1 with alpha factor and released into permissive Metaphase arrest conditions (23˚C + Nocodozole). Once 100% of the cells were Metaphase arrested, they were shifted to restrictive temperatures (37˚C + Nocodozole) for 1 hour. As described above, samples were harvested every hour for 7 hours while held in the Metaphase arrest. (B) Cell cycle position. Budding index for each time point was used to monitor the G1 arrest, progression past "Start" and the Metaphase arrest. (C) *HMLalpha2* expression. RNA was isolated and subjected to both RT-PCR and quantitative PCR. Samples were analyzed as described in Chapter 2 Materials and Methods. In each case, the standard error was calculated based on three experiments.

Figure 5-4

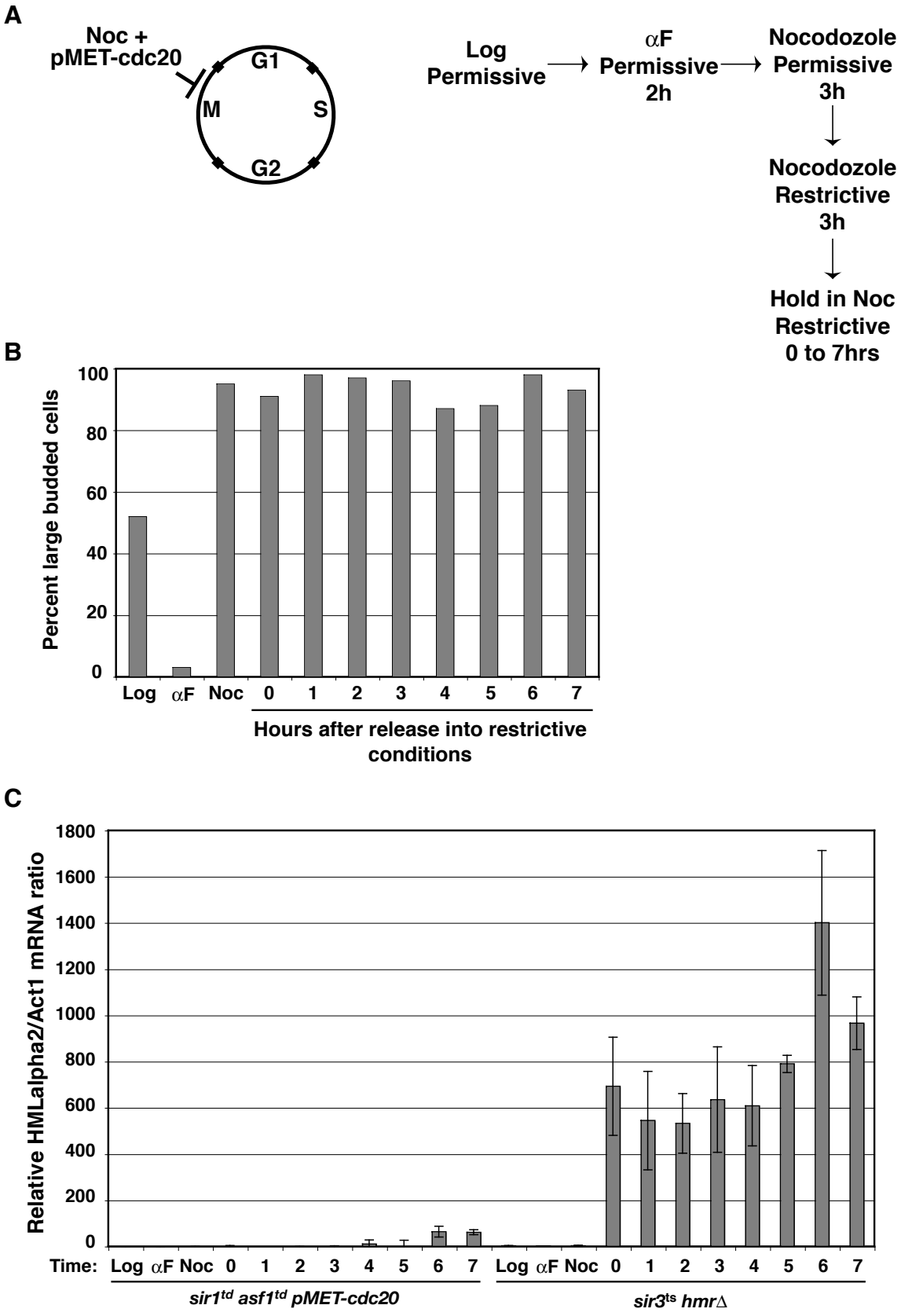
A
Noc +
pMET-cdc20
G1
S
G2
M
Log
Permissive
αF
Permissive
2h
Nocodozole
Permissive
3h
Nocodozole
Restrictive
3h
Hold in Noc
Restrictive
0 to 7hrs
B
Percent large budded cells
100
80
60
40
20
0
Log
αF
Noc
0
1
2
3
4
5
6
7
Hours after release into restrictive conditions
C
Relative HMLalpha2/Act1 mRNA ratio
1800
1600
1400
1200
1000
800
600
400
200
0
Time:
Log
αF
Noc
0
1
2
3
4
5
6
7
sir1td asf1td pMET-cdc20
sir3ts hmrΔ

Figure 5-5. Sir1 and Asf1 are required for the inheritance of *HMLalpha* silencing. (A) Experimental strategy. s*ir1*^{td} (YJL5880), *asf1*^{td} (YJL5801) and s*ir1*^{td} asf*1*^{td} (YJL5824) cells were grown in log phase permissive conditions (SDC-MET), synchronized in G1 with alpha factor and shifted to restrictive conditions (YEPgal + 2mM MET) for 3 hours while maintaining the G1 arrest. Cells were then released into restrictive log phase conditions for 7 hours and time points were harvested every hour during the 7 hour release. (B) Cell cycle position. DNA content for each time point was measured using Sytox staining followed by flow cytometry. Budding index was used to monitor the point when cells passed "Start" and their cell cycle distribution. (C) Western blot. Protein extracts were processed for each time point and 35µg of each extract was resolved on a 7.5% SDS gel. Membranes were probed with anti-HA and anti-Pgk1 (loading control) antibodies. (D) *HMLalpha2* expression. RNA was isolated from samples taken at the indicated time points and analyzed as described in Chapter 2 Materials and Methods.

Figure 5-5

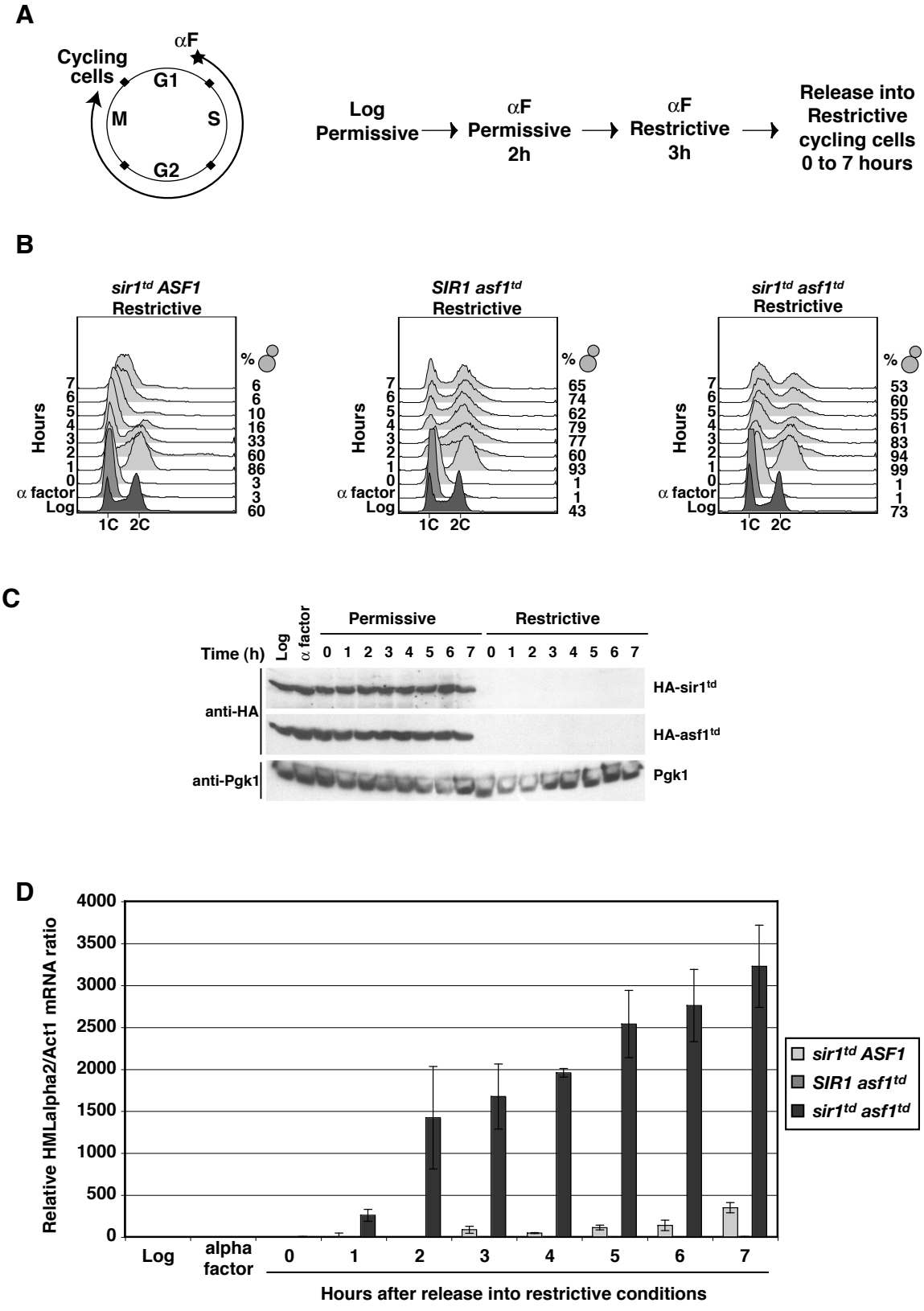
A
αF
Cycling cells
G1
S
G2
M
Log Permissive
αF Permissive 2h
αF Restrictive 3h
Release into Restrictive cycling cells 0 to 7 hours
B
sir1td ASF1 Restrictive
SIR1 asf1td Restrictive
sir1td asf1td Restrictive
Hours
α factor Log
1C 2C
%
6 6 10 16 33 60 86 3 3 60
65 74 62 79 77 60 93 1 1 43
53 60 55 61 83 94 99 1 1 73
C
Time (h)
Log
α factor
Permissive
Restrictive
0 1 2 3 4 5 6 7 0 1 2 3 4 5 6 7
anti-HA
anti-Pgk1
HA-sir1td
HA-asf1td
Pgk1
D
Relative HMLalpha2/Act1 mRNA ratio
4000
3500
3000
2500
2000
1500
1000
500
0
Log
alpha factor
0 1 2 3 4 5 6 7
Hours after release into restrictive conditions
sir1td ASF1
SIR1 asf1td
sir1td asf1td

Figure 5-6. Western Blot for Figure 2-2. Protein extracts were processed for each time point and 35µg of each extract was resolved on a 7.5% SDS gel. Membranes were probed with anti-HA and anti-Pgk1 (loading control) antibodies.

Figure 5-6

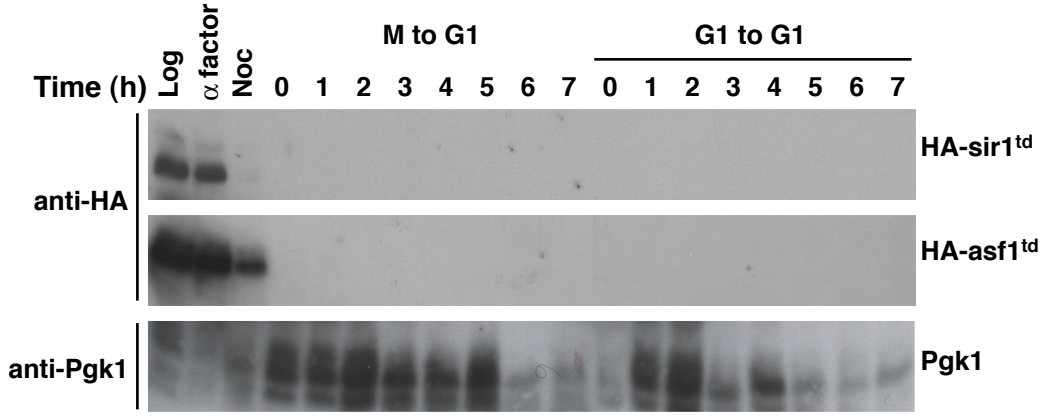
M to G1
G1 to G1
Time (h)
Log
α factor
Noc
0 1 2 3 4 5 6 7 0 1 2 3 4 5 6 7
anti-HA
HA-sir1td
HA-asf1td
anti-Pgk1
Pgk1

Figure 5-7. Western blot for Figure 2-3. Protein extracts were processed for each time point and 35µg of each extract was resolved on a 7.5% SDS gel. Membranes were probed with anti-HA and anti-Pgk1 (loading control) antibodies.

Figure 5-7

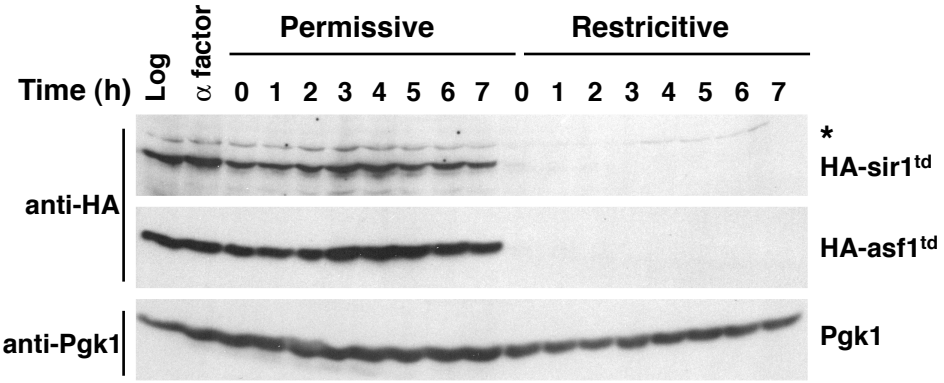
Permissive
Restricitive
Time (h)
Log
α factor
0 1 2 3 4 5 6 7 0 1 2 3 4 5 6 7
anti-HA
anti-Pgk1
*
HA-sir1td
HA-asf1td
Pgk1

Table 5-1. Strains used in this study

Strains	Genotype	Source
	Congenic to W303	
CFY1463	*MATalpha ade2-1 leu2-3,112 his3-11 trp1-1 can1-100 HMR-SSa SIR1-3xHA,KanR*	C. Fox
JRY2334	*CAC1 ASF1 ade2-1 leu2-3,112 his3-11 trp1-1 ura3-1*	R. Kamakaka
YJL4944	*mat::NatMX4/MAT@ SIR1/sir1::{pCUP1-ts degron-Sir1, URA3} CAC1/cac1::LEU2 ASF1/asf1::his5+ cdc28-as1/CDC28 ura3-1/ura3-1 trp1-1/trp1-1::{Galp-UBR1, TRP1} leu2-3,112/leu2-3,112 his3-11/his3-11 ade2-1/ade2-1 can1-100/CAN1 bar1::HIS G/bar1::KanMX6*	This study
YJL5237	*mat::NatMX4 SIR1 CAC1 ASF1 mat::NatMX4 CDC28 trp1-1 bar1::HISG ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*	This study
YJL5347	*SIR1 CAC1 ASF1 mat::NatMX4 CDC28 trp1-1::{Galp-UBR1, TRP1} bar1::HISG ura3-1 leu2-3,112 his3-11 ade2-1 can1-100* **(spore from YJL4944)**	This study
YJL5432	*MAT@/mat::NatMX4 SIR1/sir1::ADE2 cac1::LEU2/CAC1 asf1::his5+/ASF1 trp1-1::{Galp-UBR1, TRP1}/trp1-1::{Galp-UBR1, TRP1} bar1::HISG/BAR1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11/his3-11 ade2-1/ade2-1 can1-100/CAN1 {Mata, Ura3}*	This study

YJL5444	*mat::NatMX4 ade2-1 leu2-3,112 his3-11 trp1-1::{pGal-UBR1, TRP1} ura3-1*	This study
YJL5447	*mat::NatMX4 sir1::ADE2 asf1::his5+ ade2-1 leu2-3,112 his3-11 trp1-1::{pGal-UBR1, TRP1} ura3-1*	This study
YJL5471	*mat::NatMX4 sir1::ADE2 ade2-1 leu2-3,112 his3-11 trp1-1::{pGal-UBR1,TRP1} bar1::hisG ura3-1*	This study
YJL5473	*mat::NatMX4 asf1::his5+ ade2-1 leu2-3,112 his3-11 trp1-1::{pGal-UBR1,TRP1} bar1::hisG ura3-1*	This study
YJL5783	*mat::NatMX4 CDC28 trp1-1::{Galp-UBR1, TRP1} bar1::HISG ubr1::LEU2 ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*	This study
YJL5801	*mat::NatMX4 CDC28 trp1-1::{Galp-UBR1, TRP1} bar1::HISG ubr1::LEU2 asf1::{pMET-td-ASF1,ADE2} ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*	This study
YJL5824	*mat::NatMX4 CDC28 trp1-1::{Galp-UBR1, TRP1} bar1::HISG ubr1::LEU2 asf1::{pMET-td-ASF1,ADE2} sir1::{pMET-td-SIR1,URA3} ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*	This study
YJL5880	*mat::NatMX4 CDC28 trp1-1::{Galp-UBR1, TRP1} bar1::HISG ubr1::LEU2 sir1::{pMET-td-4xHA-sir1,URA3} ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*	This study

YJL5938	*mat::NatMX4 CDC28 trp1-1::{Galp-UBR1, TRP1} bar1::HISG ubr1::LEU2 asf1::{pMET-td-ASF1,ADE2} sir1::{pMET-td-SIR1,URA3} cdc20::{pMET-cdc20, KANMX6} ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*	This study
YJL6078	*mat::NatMX4 sir3-8 bar1::KanMX6 hmr::URA3 ade2-1 leu2-3,112 his3-11 trp1-1 ura3-1*	This study
YJL6377	*mat::NatNX4 ade2-1 leu2-3,112 his3-11 trp1-1 can1-100 HMR-SSa SIR1-3xHA,KanR*	This study
YJL6403	*mat::NatMX4 sir3::KanMX6 hmr::URA3MX trp1-1 bar1::HISG ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*	This study

Table 5-2. Plasmids used in this study

Plasmid	Description	Source
pKI1458	pRS304 pGAL-UBR1	This lab
pLC124	pRS306 pCUP1-4xHA-sir1^{td}	This study
pLC125	pRS306 pMET-4xHA-sir1^{td}	This study
pLC129	pRS316 MATa Cen, Ars	This study
pLC132	pRS402 pMET-4xHA-asf1^{td}	This study
pLP1184	sir3-8	L. Pillus
pVN180	pRS400 pMET-CDC20	This lab

Table 5-3. Primers used in this study

Primer	Purpose	Conc	Sequence
OJL1569	*HMLalpha2* cDNA	500nM	(5'-GATAAACTGGTATTCTTCATTAG-3')
OJL1578	*ACT1* cDNA/ antisense QPCR	500nM 50nM	(5'-AAGCCAAGATAGAACCACCAATCCA-3')
OJL1577	*ACT1* sense	50nM	(5'-TCACTATTGGTAACGAAAGATTCAG-3')
OJL1678	*HMLalpha2* antisense	50nM	(5'-CAGTAATGGTAGTAGTGAGTTG-3')
OJL1681	*HMLalpha2* sense	50nM	(5'-CCCATTAAAGACCTTTTAAATCCAC-3')
OJL1723	*HML-E* sense	60nM	(5-'GGTGTATCGCAATGGAATG-3')
OJL1724	*HML-E* antisense	60nM	(5'-GAAAAAATGTAGGTTGAATTTGG-3')
OJL1725	*HML-I* sense	60nM	(5-'CGATGCTTATTGTGCTTTG-3')
OJL1726	*HML-I* antisense	60nM	(5'-TAGTGTGCCCAGCTTTTATGTC-3')
OJL1727	*MAT* sense	50nM	(5'-CACCGCACAATTCATCATTTGCGT-3')
OJL1728	*MAT* antisense	50nM	(5-'CTGGGTAGAGTCTTATTGGCAAGA-3')
OJL1904	*BUD3* sense	50nM	(5'-ATAACATCGTAATCTTAGACGTCTT-3')
OJL1905	*BUD3* antisense	50nM	(5'-GCCCTATGTTCGTCTGTTGAAGGGT-3')

APPENDIX I

Optimizing the ts-degron system

INTRODUCTION

Three aspects of transcriptional silencing are often studied: Establishment, Maintenance and Inheritance. Establishment is when non-silent chromatin, euchromatin, is transformed into silent chromatin, heterochromatin. Maintenance is the continuous preservation of heterochromatin at all points in the cell cycle. Inheritance is the restoration and propagation of heterochromatin following progression through the cell cycle [1, 2]. To determine if a protein is required for the establishment, maintenance, or inheritance of silencing, conditional mutants of that protein must be generated and studied.

Several studies have used conditional *sir3*^{ts} mutants to show that progression through S phase [3] and M phase [4] but not DNA replication [5, 6] are required for the establishment of silencing. These studies use conditional alleles to understand the mechanism of establishing silencing. Thus, conditional alleles can be used to identify a protein's execution point and mechanism of action.

The "ts-degron" system, created in the Varshavsky laboratory [7, 8], can be used to generate conditional alleles from any gene of interest (GOI). These conditional alleles are created by fusing the 5' end of the GOI to the "ts-degron" construct. The 5' end of the "ts-degron" is composed of a Ubiquitin residue that when cleaved, reveals an arginine. This N-terminal arginine destabilizes the fusion protein, via the N-end rule [9]. A modified Dihydrofolate Reductates (DHFR) region, on the 3' side of the arginine, unfolds at higher temperatures, thus exposing lysines to ubiquitination. An inducible *UBR1*, the E3 ubiquitin ligase [10], is also introduced into the "ts-degron" containing

cell. This inducible *UBR1* conditionally ubiquitinates lysines, resulting in conditional destruction of the protein [10].

In this study, we describe the process of modifying Varshavsky's "ts-degron" construct to generate a more efficient conditional allele system. We show that our system generates a conditional protein that is fully functional under permissive conditions and fully defective under restrictive conditions. We also show that our conditional alleles are degraded rapidly, following a shift from permissive to restrictive conditions. Lastly, our degron system works with numerous proteins.

MATERIALS AND METHODS

Strains. All strains used in this study are listed in Table AI-1. To generate YJL4888, the endogenous *SIR1* in YJL4526 (*MATa asf1:: his5+ ade2-1 leu2-3,112 his3-11 trp1-1::{pGal-UBR1, TRP1} ura3-1*) was replaced by pLC125 (digested with NheI) using one-step gene disruption. The endogenous *BAR1* in YJL4888 was then disrupted with a KanMX6 PCR product, generating YJL5709. YJL5720 was generated by disrupting the endogenous *UBR1* in YJL5709 with a LEU2 PCR product. YJL5292 was generated by sporulating YJL5111 (*SIR1/sir1::{pCup1-td-SIR1, URA3} CAC1/cac1::LEU2 ASF1/asf1::his5+ mat::NatMX4/Mat@ cdc28-as1/CDC28 ura3-1/ura3-1 trp1-1/trp1-1::{Galp-UBR1, TRP1} leu2-3,112/leu2-3,112 his3-11/his3-11 ade2-1/ade2-1 can1-100/CAN1 bar1::HIS G/bar1::KanMX6 {Mata, ADE2})*.

Plasmids. Plasmid pLC124 is a modified version of the ts-degron plasmid pPW66R described in [7]. pLC124 consists of a SacII to Not1 ts-degron fragment (pCup1-R-

Ubiquitin-DHFR-1xHA), a Not1 to Not1 3xHA fragment, a Not1/HindIII to XhoI 175bp N-terminal Sir1 fragment and a pRS306 vector backbone. pLC125 is derived for pLC124. The pCup1 in pLC124 was replaced with a SacII to EcoR1 pMet3 fragment to generate pLC125.

Total RNA extraction. Total RNA was prepared from yeast cells using an Acid-Phenol extraction. 10ml of an OD=1.0 culture was harvested by centrifugation (3 min at 3,000 rpm) and frozen in liquid nitrogen. Cells were resuspended in 500µl of TES buffer (10mM Tris-Cl pH7.5, 10mM EDTA, 0.5%SDS) and 500µl of Acid Phenol (pH 4.3). To extract the RNA from the cells, the mixture was incubated for one hour at 65˚C with periodic vortexing, placed on ice for 5 min and then microcentrifuged at top speed for 5 minutes at 4˚C. The supernatant was transferred to Phase Lock Tubes containing chloroform (500µl), shaken ten times and microcentrifuged for 5 min at top speed. The supernatant was transferred to a tube containing Acid Phenol (500µl), vortexed vigorously for 10 seconds and microcentrifuged for 5 min at top speed. The aqueous phase was transferred to a new Phase Lock Tube and 500µl of chloroform was added, shaken ten times and microcentrifuged for 5 min at top speed. The chloroform extraction was repeated and then the aqueous phase was transferred to a new tube and mixed with 40µl of 3M sodium acetate (pH 5.3) and 1ml of 100% ethanol. RNA was pelleted by microcentrifugation at top speed for 15 min. The pellet was washed by vortexing in 70% ethanol. After drying, pellets were resuspended in 100-150µl of water and stored at -80˚C.

Northern Blot. 20µg of total RNA was run on a 1.5% formaldehyde gel (2g agarose, 113ml H_20, 13ml 10xMOPS, 4ml 37% formaldehyde), in 1xMOPS, at 70V for 3 hours. The gel was washed in water for 60 minutes and then set up for transfer to a Genescreen-Plus membrane. RNA was transferred from the gel to the membrane for at least 12 hours in 10XSSC (1.5M NaCl, 0.1M Sodium Citrate pH7). The membrane was rinsed for 5 minutes in 10xSSC, air dried for 15 minutes on Whatman paper, crosslinked using an auto setting of 12µJ, and then baked at 80˚C under 20Hg of pressure for 2 hours. Start membrane "Prehyb" by incubating the membrane with 7ml pre-warmed Express Hyb Solution (Clontech: 636832) for at least 40 minutes. Add the probe (see below) to the pre-hybed tube and incubate at 68˚C for 3 hours. Pour off the excess liquid, rinse the tube and the blot with 150ml Wash1 (2xSSC, 0.05%SDS) and then pour off this rinse. Wash the blot 2 times in Wash 1, 10 minutes per wash at RT. Transfer the blot to Wash2, pre-heated to 50˚C and incubate at 50˚C for 15 minutes. Shake off the excess liquid and image the blot.

Northern Blot probe. Use OJL1567 (5'- TACCCATTAAAGACCTTTTAAATCC-3') and OJL1569 (5'- GATAAACTGGTATTCTTCATTAG-3') to generate a DNA stock for the HMLalpha2 probe. Dilute the DNA stock to 5ng/ul and combine 5µl of this 5ng/ul stock (25ng DNA) with 5µl OJL1569. Heat the mix at 95˚C for 5 minutes, cool to RT, spin and add: 4µl dTTP, 4µl dGTP, 4µl dCTP, 5µl 10xReaction Buffer, 2µl Enzyme and 16µl water (Amersham: RPN1605). Spin the mixture, add 5µl dATP-alphaP^{32}, incubate at 37˚C for 10 minutes, add 5µl of 0.2M EDTA and spin quickly at 3,000rpm. Add to a pre-packed (3,000rpm, 1 minute) micro-spin column (Amersham: 27-5120-01) and elute

by spinning at 3,000rpm for 2 minutes. Heat the probe at 95˚C for 10 minutes and ice for at least 15 minutes.

Urea protein extraction. Denatured protein samples were prepared using a urea lysis protocol. 10ml of an OD=1.0 culture was harvested by centrifugation for 3 min at 3,000 rpm. Pelleted cells were resuspended in 500µl of water and transferred to a screw cap tube (USP #MCTS-806). Cells were pelleted by microcentrifugation at top speed for 1 min, frozen in liquid nitrogen and thawed on ice. Once thawed, the cells were resuspended in 200µl of Urea buffer (20mM Tris pH 7.4, 7M Urea, 2M Thiourea, 4% CHAPS and 1% DTT). 200µl of 0.5mm glass beads were added to the tube and the cells were then lysed by two rounds of 1 min bead beating. Beads were separated from the extract by poking a hole in the bottom of the screw cap tube (using a 22 gauge needle), placing the screw cap tube into a new eppendorf tube and spinning the stacked tubes in a microfuge on a setting of soft 6,000 rpm for 30 sec. The urea protein extract was incubated at 25˚C for 30 min on a nutator and then spun in a microfuge at full speed for 10 min at 4˚C. The supernatant was transferred to a new tube and the concentration was determined using the Bradford assay.

Western blot analysis. Western Blot analysis was used to monitor HA-Sir1 protein levels. 35µg of each urea protein sample was run on a 7.5% SDS gel and blotted to a nitrocellulose membrane. Membranes were stained with Ponceau S for 1 min, imaged to monitor protein loading and then rinsed with water. The membranes were incubated in 50ml of blocking buffer (10% Carnation dry milk, 20mM Tris pH 7.5, 0.5M NaCl, 0.1%

Triton X-100) for 60 min at room temperature and then incubated for 60 min in 40ml of antibody buffer (2% Carnation dry milk, 20mM Tris pH 7.5, 0.5M NaCl, 0.1% Triton X-100). The antibody buffer contained anti-HA antibody (Convance MMS-101R mouse anti-HA 16B12) at a 1:1000 dilution. Membranes were quickly rinsed twice with 50ml of the wash buffer (20mM Tris pH 7.5, 0.5M NaCl, 0.1% Triton X-100) and then washed twice, 10 min per wash, with 50ml of the wash buffer. After washing, membranes were incubated for 30 min in 40ml of antibody buffer containing a 1:2000 dilution of sheep anti-mouse HRP secondary antibody (Amersham NA931V). Membranes were rinsed twice with 50ml of wash buffer and then washed three times, 10 min per wash. Western blots were developed using Pierce SuperSignal West Dura Extended Duration Substrate and exposed to Amersham Hyperfilm MP.

RESULTS

pCup1-Sir1 degron protein is inefficiently degraded. Prior to this body of work, proteins that promote the inheritance of silencing had not been identified. Several observations suggested, however, that the histone deposition factors, CAF-1 (Cac1, Cac2 and Cac3) and Asf1 participated in this process along with Sir1 [11]. First, the restoration of silencing after passage of a replication fork can not occur without the assembly of new nucleosomes [12-15]. Furthermore, CAF-1 and Asf1 nucleate nucleosome assembly by depositing the histone H3-H4 tetramer onto naked DNA [16, 17] and are believed to target newly replicated DNA by interacting with PCNA [18, 19], the processivity factor for replicative polymerases. Second, triple mutants of *cac1*, *asf1*,

and *pol30* (PCNA) displayed *HMR*a silencing defects using a sensitized HMR reporter [16, 17], establishing a role for CAF-1 and Asf1 in silencing. Third, although neither *sir1* nor *cac1 asf1* strains had a major silencing defect, the triple mutant and the *sir1 asf1* double mutant were completely defective for silencing [11], suggesting that Sir1 could provide a parallel mechanism of inheritance. To test whether Sir1 and Asf1 work together to ensure the inheritance of silencing at *HMLalpha*, we generated $sir1^{td}$ and $asf1^{td}$ conditional alleles.

Engineering good $sir1^{td}$ *and* $asf1^{td}$ conditional alleles was a multi-stage process. We began with a construct containing Varshavsky's "ts-degron" system (Fig. AI-1), regulated by the Cup1 promoter and fused to *SIR1* (Fig. AI-1B, line 1). During Stage 1, we generated cells containing the *pCup1-*$sir1^{td}$ construct, the endogenous *UBR1* and an exogenous galactose inducible *UBR1* (Fig. AI-1B). In this system, the Sir1 degron protein was constitutively expressed from the Cup1 promoter and degraded by a combination of constitutive endogenous *UBR1* and galactose inducible *UBR1*. As an initial step characterization step, we monitored the kinetics of Sir1 degron protein degradation. *pCup1-*$sir1^{td}$ cells (YJL5292) were grown at 25˚C in log phase permissive conditions. At time 0, cells were shifted to log phase restrictive conditions and grown for 20 hours (Fig. AI-2A). Immunoblotting using anti-HA antibodies showed that Sir1 degron protein levels were stable for 5 hours after shifting to restrictive conditions (Fig. AI-2B). The slow kinetics of pCup1-Sir1 degron protein degradation suggested several possibilities: (1) the Sir1 degron protein was inefficiently ubiquitinated, (2) Sir1 protein levels were high, requiring 5 hours to degrade the protein, or (3) the galactose induction of *UBR1* was slow. Thus, modifications to the ts-degron system were needed to generate

a conditional allele that was rapidly degraded following a shift from permissive to restrictive conditions.

pMet3-Sir1 degron protein is highly unstable. Our experiments, described above, demonstrated that the pCup1-Sir1 degron protein was inefficiently degraded after shifting the cells to restrictive conditions. Consequently, we refined our control over the Sir1 degron protein level by replacing the constitutive Cup1 promoter with a repressible Met3 promoter (Fig. AI-1C). During Stage 2, we generated cells containing the *pMet3-sir1*^{td} construct, the endogenous *UBR1* and the exogenous galactose inducible *UBR1* (Fig. AI-1C). In this strain, the addition of methionine repressed expression of pMet3-Sir1 degron protein while galactose induced additional Ubr1 protein production. We sought to determine if the pMet3-Sir1 degron protein was fully functional under permissive conditions and fully defective under restrictive conditions. We grew the *pMet3-sir1*^{td} containing cells (YJL4888) under four log phase conditions for 48 hours: (1) Dextrose without Methionine at 25˚C, (2) Dextrose without Methionine at 30˚C, (3) Galactose with Methionine at 30˚C, and (4) Galactose with Methionine at 33˚C. Immunoblotting using anti-HA antibodies showed that the Sir1 degron protein was only present in cells grown in Dextrose without Methionine at 25˚C (Fig. AI-3A). All other conditions were sufficient to degrade pMet3-Sir1 degron protein to levels undetectable by immunoblot analysis (Fig. AI-3A). Furthermore, quantitative PCR to monitor HMLalpha2 mRNA levels, showed that *HMLalpha* had lost silencing in all conditions (Fig. AI-3B). These results demonstrate that the pMet3-Sir1 degron protein is not fully functional, capable of full silencing, under permissive conditions.

The Sir1 degron protein is degraded by the endogenous *UBR1.* We showed that our Stage 2 degron construct was highly unstable under permissive conditions, Dextrose without Methionine. Under these permissive conditions, pMet-Sir1 degron protein should be expressed and pGal-UBR1 protein should not be expressed. Thus, one would expect Sir1 degron protein levels to remain constant and silencing to remain intact under permissive conditions. We observed, however, that Sir1 degron protein was degraded in permissively grown cells, suggesting that the endogenous *UBR1's* constitutive activity degraded Sir1. To test this hypothesis, we monitored the Sir1 degron protein's half life in *pMet3-sir1^{td} pGal-UBR1* cells containing the endogenous *UBR1,* YJL5709, and in cells where the endogenous *UBR1* was disrupted (Fig. AI-1D, YJL5720).

pMet3-sir1^{td} pGal-UBR1 UBR1 (YJL5709) and *pMet3-sir1^{td} pGal-UBR1 ubr1::LEU2* (YJL5720) cells were grown at 30˚C in log phase permissive conditions, Dextrose without Methionine. At time 0, the cells were shifted to semi-permissive log phase conditions, Dextrose with Methionine, to shut-off Sir1 degron expression. Samples were harvested to monitor Sir1 degron protein levels in the presence and absence of the endogenous *UBR1* (Fig. AI-4A). Immunoblot analysis showed that the Sir1 degron protein was highly unstable in cells containing the endogenous *UBR1* (Fig. AI-4B). In cells containing the endogenous *UBR1,* Sir1 degron protein levels were immediately undetectable by immunoblot (Fig. AI-4B). However, in cells lacking the endogenous *UBR1* (YJL5720), Sir1 degron protein persisted for 5 hours after shutting off Sir1 expression (Fig. A1-4C). These results demonstrate that the endogenous *UBR1* significantly contributed to the Sir1 degron protein's instability.

The *pMet3-sir1^{td} pGal-UBR1* system is a good conditional allele system, in the absence of the endogenous *UBR1*. We sought to determine if our conditional alleles were fully functional at permissive conditions, fully defective at restrictive conditions and rapidly degraded after shifting from permissive to restrictive conditions. To address these questions, we grew cells containing our system, *pMet3-sir1^{td} pGal-UBR1 ubr::LEU2* (YJL5720), at 25˚C in log phase permissive conditions and then shifted the cells to restrictive log phase conditions for 18 hours (Fig. AI-5A). We used immunoblot analysis with anti-HA antibodies to monitor Sir1 degron protein levels. We observed that Sir1 degron protein levels were constant under permissive conditions (Fig. AI-5B, Dex - Met) and were undetectable 2 hours after shifting to restrictive conditions (Fig. AI-5B). We also found that *HMLalpha* remained transcriptionally silent, equal to wild type silencing levels, in cells grown under permissive conditions (Fig. AI-5C). Furthermore, *HMLalpha* silencing was rapidly lost after shifting cells to restrictive conditions (Fig. AI-5C). The initial loss of silencing occurred 3 hours after shifting cells to restrictive conditions (Fig. AI-5C), 1 hour after Sir1 degron protein levels were undetectable by immunoblot (Fig. AI-5B&C). These results demonstrate that our *pMet3-sir1^{td} pGal-UBR1 ubr::LEU2* conditional allele system is fully functional for silencing in permissive conditions, fully defective for silencing in restrictive conditions and rapidly degraded after shifting from permissive to restrictive conditions.

CONCLUSIONS

We developed a system that allows us to generate good conditional alleles of any protein in the *Saccharomyces cerevisiae* genome. We used the Varshavsky laboratory's "ts-degron" technology as the foundation for our system. Two rounds of optimization to the Varshavshy laboratory's ts-degron system were required to generate a system where the conditional alleles were fully functional under permissive conditions, fully defective under restrictive conditions, and rapidly degraded following a shift from permissive to restrictive conditions.

Initially, we attempted to generate a *SIR1* conditional allele using the Varshavsky laboratory's ts-degron technology [7, 8]. We discovered, however, that their system was unable to rapidly degrade the Cup1 expressed Sir1 degron protein. Consequently, we modified Varshavsky's degron construct by replacing the Cup1 promoter with a repressible Met3 promoter. We then discovered that regulating both the degron protein expression and degradation resulted in a highly unstable $sir1^{td}$ conditional allele. Further experiments revealed that the degron's instability was caused by the endogenous *UBR1*. Consequently, we performed a second modification round to Varshavsky's ts-degron system and disrupted the endogenous *UBR1*. We then demonstrated that our modified degron system could be used to generate good conditional alleles.

We show that the $sir1^{td}$ conditional alleles generated from our modified degron system behave like wild type alleles, able to provide robust silencing when grown under permissive conditions. Furthermore, when grow under restrictive conditions, our $sir1^{td}$ conditional alleles behave like null alleles, fully defective for silencing. Finally, using

our *sir1*^{td} alleles, we show that silencing is rapidly lost after shifting cells from permissive to restrictive conditions. Thus, we generated a highly refined conditional allele system that can be used to generate conditional alleles of any *S. cerevisiae* gene.

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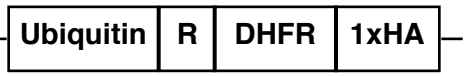
Figure AI-1. Ts-degron construction process. (A) The core ts-degron unit consisted of a ubiquitin residue, a destabilizing arginine "R", a Dihydrofolate Reductase region (DHFR), and a single HA tag. (B) During stage 1 YJL5292 was generated. This strain contained *SIR1* fused to the ts-degron system. Expression of this fusion protein was regulated by the Cup1 promoter and a galactose inducible *UBR1*. (C) During stage 2, JL4888 and YJL5709 were generated. These strains contained *SIR1* fused to the ts-degron system. Expression of this fusion protein was regulated by the repressible Met3 promoter and a galactose inducible *UBR1*. (D) During stage 3, YJL5720 was generated. Similar to the above strains, this strain contained *SIR1* fused to the ts-degron system and the expression of this fusion protein was regulated by the repressible Met3 promoter and a galactose inducible *UBR1*. In this strain, however, the endogenous *UBR1* gene was disrupted, making the galactose inducible *UBR1* the sole source of Ubr1 protein.

Figure AI-1

A ts-degron: 

B	**Stage 1: YJL5292**	**Chromosome Location**
	sir1::{Cup1 promoter - ts degron - SIR1 ORF}	**XI**
	trp1-1::{Gal1 promoter - UBR1 ORF}	**IV**
	Ubr1 promoter - UBR1 ORF	**VII**

C	**Stage 2: YJL4888 & YJL5709**	
	sir1::{Met3 promoter - ts degron - SIR1 ORF}	**XI**
	trp1-1::{Gal1 promoter - UBR1 ORF}	**IV**
	Ubr1 promoter - UBR1 ORF	**VII**

D	**Stage 3: YJL5720**	
	sir1::{Met3 promoter - ts degron - SIR1 ORF}	**XI**
	trp1-1::{Gal1 promoter - UBR1 ORF}	**IV**
	ubr1::LEU2	**VII**

Figure AI-2. In restrictive conditions, pCup1-td-Sir1 protein levels are stable. (A) Experimental design. *pCup1-td-SIR1, pGal-UBR1,* and *UBR1* (YJL5292) containing cells were grown at 25˚C in log phase permissive conditions (SRaffinose-UT + 1μM $CuSO_4$ + 0.05% Dextrose), for 16 hours. Cells were then shifted to log phase restrictive conditions (33˚C, YepGal + 4mM MET) and grown for 20 hours. Samples were harvested at 0, 0.5, 1, 2, 5 and 20 hours after the shift to restrictive conditions. (B) Western Blot. Protein extracts were processed for each time point and 35μg of each extract was resolved on a 7.5% SDS gel. Membranes were Ponceau stained, to monitor loading, and probed with anti-HA antibodies, to monitor Sir1 degron protein levels.

Figure AI-2

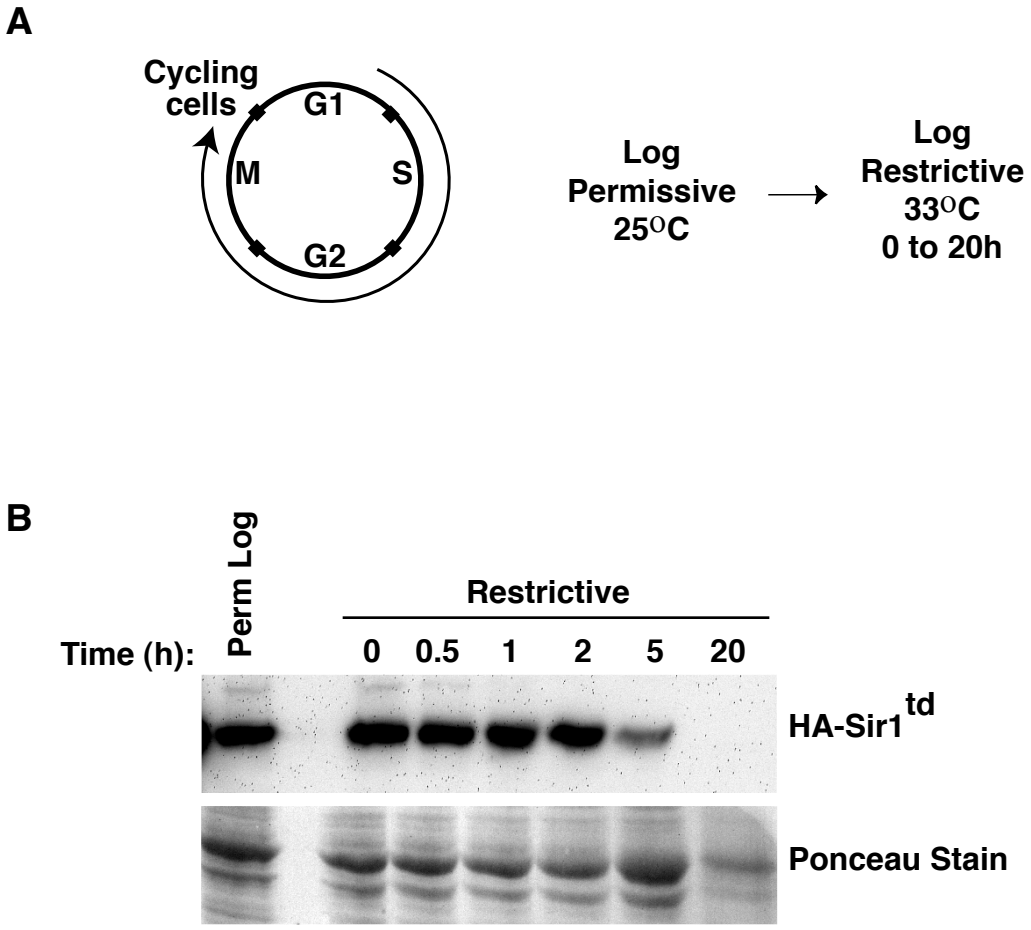
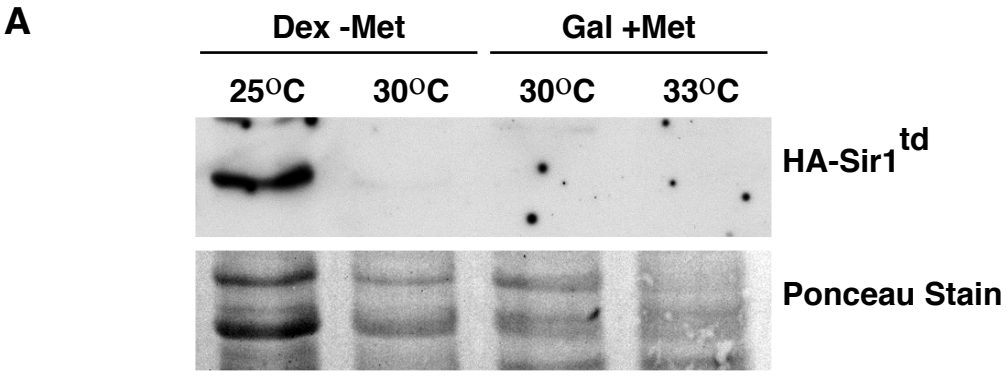

A
Cycling cells
G1
S
G2
M
Log Permissive 25°C
Log Restrictive 33°C 0 to 20h
B
Perm Log
Restrictive
Time (h):
0
0.5
1
2
5
20
HA-Sir1td
Ponceau Stain

Figure AI-3. Under permissive conditions, pMet3-td-Sir1 protein is unstable. *pMet3-td-SIR1, pGal-UBR1,* and *UBR1* (YJL4888) cells were grown under four log phase conditions: (1) Dextrose/No Methionine/25˚C; (2) Dextrose/No Methionine/30˚C; (3) Galactose/Methionine/30˚C; and (4) Galactose/Methionine/33˚C. (A) Immunoblot analysis. Protein extracts were processed for each sample and 35µg of each extract was resolved on a 7.5% SDS gel. Membranes were Ponceau stained, to monitor loading, and probed with anti-HA antibodies, to monitor Sir1 degron protein levels. (B) Northern blot for HMLalpha2 expression. 20µg of total RNA was run for each sample on a 1.5% formaldehyde gel. Gels were processed as described in Appendix I Materials and Methods. To monitor HMLalpha2 mRNA levels, blots were probed using an alpha-P^{32}-dATP labeled probe.

Figure AI-3


A
Dex -Met
Gal +Met
25°C
30°C
30°C
33°C
HA-Sir1td
Ponceau Stain

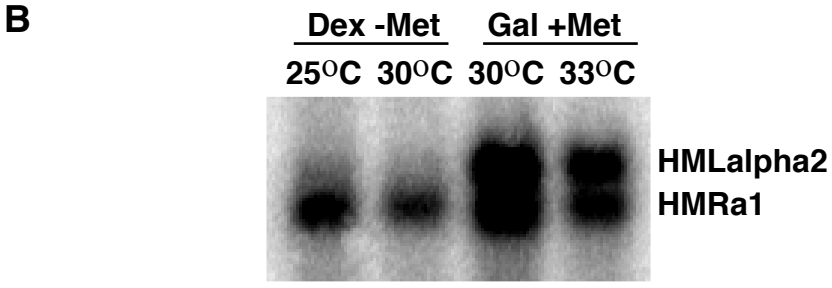
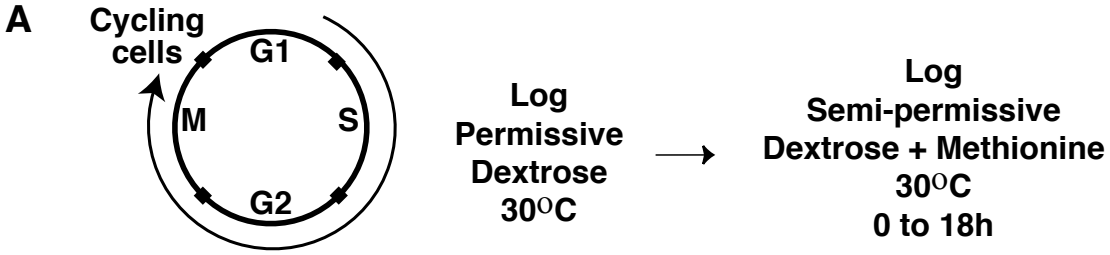
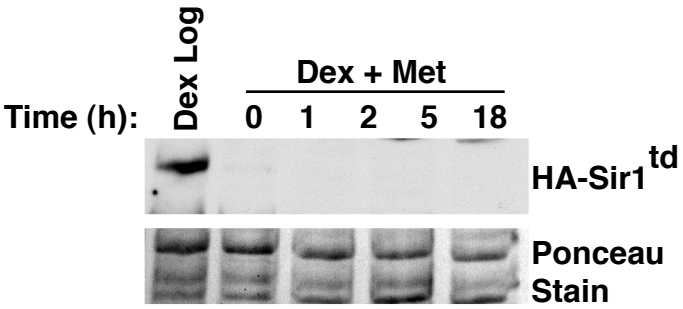

B
Dex -Met
Gal +Met
25°C
30°C
30°C
33°C
HMLalpha2
HMRa1

Figure AI-4. In permissive conditions, the endogenous *UBR1* degrades pMet3-td-Sir1 protein. (A) Experimental design. *pMet3-td-SIR1, pGal-UBR1, UBR1* (YJL5292) containing cells and *pMet3-td-SIR1, pGal-UBR1, ubr1::LEU2* (YJL5720) containing cells were grown overnight at 30˚C in log phase permissive conditions (SDC). Cells were then shifted to log phase semi-permissive conditions (30˚C, SDC + 4mM MET) and grown for 18 hours. Samples were harvested at 0, 1, 2, 5 and 18 hours after the shift to semi-permissive conditions. (B) YJL5709 Western Blot. Protein extracts were processed for each time point and 35µg of each extract was resolved on a 7.5% SDS gel. Membranes were Ponceau stained, to monitor loading, and probed with anti-HA antibodies, to monitor Sir1 degron protein levels. (C) YJL5720 Western Blot. Samples were processed a described above.

Figure AI-4


A
Cycling cells
G1
S
G2
M
Log
Permissive
Dextrose
30°C
Log
Semi-permissive
Dextrose + Methionine
30°C
0 to 18h


B
YJL5709: sir1::{pMet-td-SIR1} + trp1-1::{pGal1-UBR1} + pUbr1-UBR1
Dex Log
Dex + Met
Time (h):
0 1 2 5 18
HA-Sir1td
Ponceau Stain

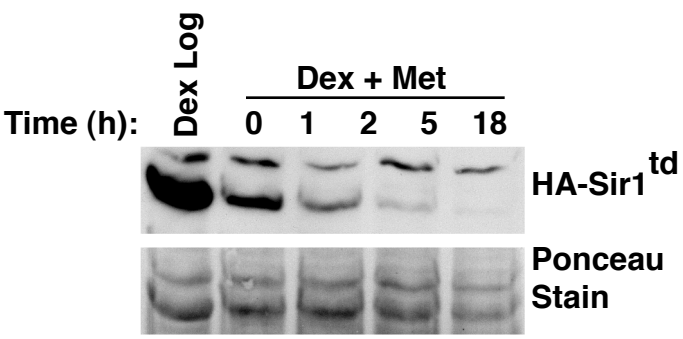

C
YJL5720: sir1::{pMet-td-SIR1} + trp1-1::{pGal1-UBR1} + ubr1::LEU2
Dex Log
Dex + Met
Time (h):
0 1 2 5 18
HA-Sir1td
Ponceau Stain

Figure AI-5. *pMet3-td-YFG, pGal-UBR1, ubr1::LEU2* is a good conditional allele system. (A) Experimental design. *pMet3-td-SIR1, pGal-UBR1, ubr1::LEU2* (YJL5720) containing cells were grown overnight at 25˚C in log phase permissive conditions (SRaffinose-MUT + 0.05% Dextrose). Cells were then shifted to log phase restrictive conditions (30˚C, YepGal + 4mM MET) and grown for 18 hours. Samples were harvested at 1, 2, 3, 4, 5.5 and 18 hours after the shift to restrictive conditions. (B) Western Blot. Protein extracts were processed for each time point and 35µg of each extract was resolved on a 7.5% SDS gel. Membranes were Ponceau stained, to monitor loading, and probed with anti-HA antibodies, to monitor Sir1 degron protein levels. (C) Northern Blot for HMLalpha2 mRNA. 20µg of total RNA from each sample was run on a 1.5% Formaldehyde gel. Gels were then processed as described in Appendix I Materials and Methods: Northern Blot Analysis.

Figure AI-5

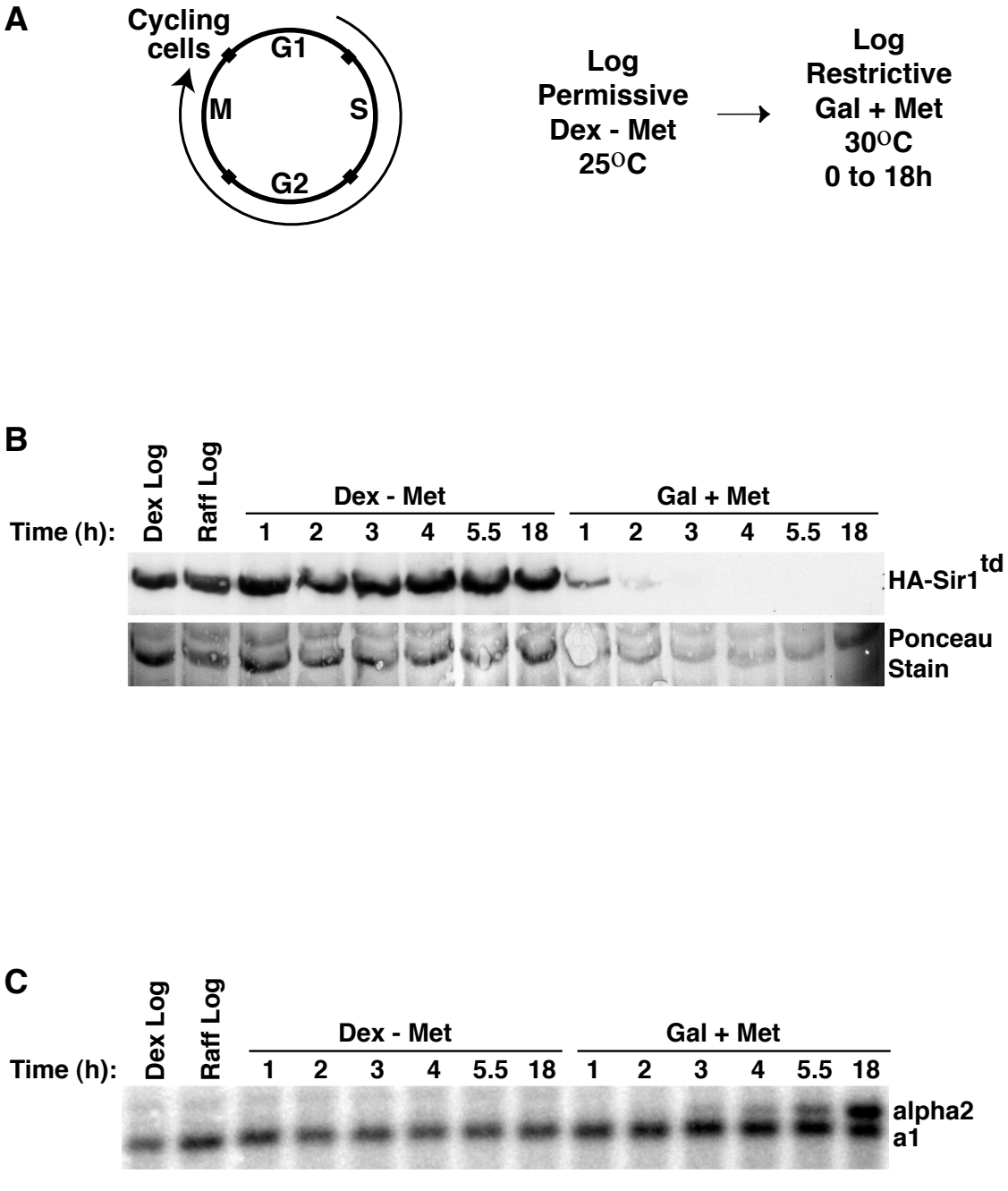

A
Cycling cells
G1
S
G2
M
Log Permissive Dex - Met 25°C
Log Restrictive Gal + Met 30°C 0 to 18h
B
Dex Log
Raff Log
Dex - Met
Gal + Met
Time (h):
1 2 3 4 5.5 18 1 2 3 4 5.5 18
HA-Sir1td
Ponceau Stain
C
Dex Log
Raff Log
Dex - Met
Gal + Met
Time (h):
1 2 3 4 5.5 18 1 2 3 4 5.5 18
alpha2
a1

Table AI-1. Strains used in this study

Strains	Genotype	Source
	Congenic to W303	
YJL4888	*MATa SIR1::{pMet-td-3xHA-SIR1, URA3} asf1::his5+ trp1-1::{pGal-UBR1, TRP1} ura3-1 ade2-1 leu2-3,112 his3-11*	This study
YJL5292	*mat::NatMX4 sir1::{pCup1-td-3xHA-SIR1, URA3} asf1::his5+ trp1-1::{Galp-UBR1, TRP1} bar1::HISG ura3-1 leu2-3,112 his3-11 ade2-1 can1-100*	This study
YJL5709	*MATa SIR1::{pMet-td-3xHA-SIR1, URA3} asf1::his5+ trp1-1::{pGal-UBR1, TRP1} ade2-1 leu2-3,112 his3-11 ura3-1 bar1::KanMX6*	This study
YJL5720	*MATa SIR1::{pMet-td-3xHA-SIR1, URA3} asf1::his5+ trp1-1::{pGal-UBR1, TRP1} ade2-1 leu2-3,112 his3-11 ura3-1 bar1::KanMX6 ubr1::LEU2*	This study

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Kristin E Chu _____ 5/1/07 _____

Author Signature Date