

AD \_\_\_\_\_

Award Number: W81XWH-04-1-0416

TITLE: Molecular Analysis of the Inheritance of Transcriptional Silencing

PRINCIPAL INVESTIGATOR: Leslie E. Chu, Ph.D.

CONTRACTING ORGANIZATION: University of California San Francisco  
San Francisco, CA 94158

REPORT DATE: April 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |             |                                  |                            | Form Approved<br>OMB No. 0704-0188            |                                           |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|----------------------------------|----------------------------|-----------------------------------------------|-------------------------------------------|
| Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.                                                                                                                                                                                                                                                                                                            |             |                                  |                            |                                               |                                           |
| 1. REPORT DATE<br>01-04-2007                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |             | 2. REPORT TYPE<br>Annual Summary |                            | 3. DATES COVERED<br>26 Mar 2004 – 25 Mar 2007 |                                           |
| 4. TITLE AND SUBTITLE<br><br>Molecular Analysis of the Inheritance of Transcriptional Silencing                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |             |                                  |                            | 5a. CONTRACT NUMBER                           |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |             |                                  |                            | 5b. GRANT NUMBER<br>W81XWH-04-1-0416          |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |             |                                  |                            | 5c. PROGRAM ELEMENT NUMBER                    |                                           |
| 6. AUTHOR(S)<br><br>Leslie E. Chu, Ph.D.<br><br>Email: <a href="mailto:leslie.chu@ucsf.edu">leslie.chu@ucsf.edu</a>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |             |                                  |                            | 5d. PROJECT NUMBER                            |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |             |                                  |                            | 5e. TASK NUMBER                               |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |             |                                  |                            | 5f. WORK UNIT NUMBER                          |                                           |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br><br>University of California San Francisco<br>San Francisco, CA 94158                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |             |                                  |                            | 8. PERFORMING ORGANIZATION REPORT NUMBER      |                                           |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |             |                                  |                            | 10. SPONSOR/MONITOR'S ACRONYM(S)              |                                           |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |             |                                  |                            | 11. SPONSOR/MONITOR'S REPORT NUMBER(S)        |                                           |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br>Approved for Public Release; Distribution Unlimited                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |             |                                  |                            |                                               |                                           |
| 13. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |             |                                  |                            |                                               |                                           |
| 14. ABSTRACT<br>Silent chromatin domains prevent transcription by packaging DNA into a higher order heterochromatin structure that is inaccessible to the transcriptional machinery. To preserve the transcriptional program in dividing cells, silent and active chromatin domains must be faithfully inherited during chromosome replication and segregation. A failure to propagate set transcriptional programs can drastically change gene expression programs, resulting in a variety of diseases and cancers. In this study, we use the budding yeast, <i>Saccharomyces cerevisiae</i> , to understand how proliferating cells inherit defined chromatin states. We identified, for the first time, two proteins, Sir1 and Asf1, specifically required for the inheritance of transcriptional silencing. We also generated conditional alleles of these inheritance proteins and show that our conditional alleles separate the inheritance process into two fundamental steps: S phase dependent disruption followed by active restoration. These fundamental findings allow us to further dissect and understand the process of epigenetic inheritance, enabling us to devise novel treatments against cancers arising from aberrant gene silencing. |             |                                  |                            |                                               |                                           |
| 15. SUBJECT TERMS<br>Inheritance, Transcriptional Silencing, Heterochromatin, Cell Cycle, Sir1, Asf1, DNA replication                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |             |                                  |                            |                                               |                                           |
| 16. SECURITY CLASSIFICATION OF:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |             |                                  | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES                           | 19a. NAME OF RESPONSIBLE PERSON           |
| a. REPORT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | b. ABSTRACT | c. THIS PAGE                     |                            |                                               | USAMRMC                                   |
| U                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | U           | U                                | UU                         | 39                                            | 19b. TELEPHONE NUMBER (include area code) |

## Table of Contents

|                                          |           |
|------------------------------------------|-----------|
| <b>Introduction.....</b>                 | <b>4</b>  |
| <b>Body.....</b>                         | <b>6</b>  |
| <b>Key Research Accomplishments.....</b> | <b>16</b> |
| <b>Reportable Outcomes.....</b>          | <b>17</b> |
| <b>Conclusions.....</b>                  | <b>18</b> |
| <b>References.....</b>                   | <b>19</b> |
| <b>Supporting Data.....</b>              | <b>20</b> |

## 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 and cancers [1, 5].

Carcinogenesis often results from inappropriate activation of transcriptionally silent genes and inappropriate silencing of transcriptionally active genes. In some cases, the transcriptional silencing of a gene, such as the p16 tumor suppressor gene, can contribute to uncontrolled proliferation [6] while the activation of genes can confer deadly metastatic potential to otherwise curable tumors. Thus, understanding how transcriptional programs are stably inherited is critical in identifying the etiology of cancers.

Cell cycle progression, also known as cell division, poses significant potential challenges to the inheritance of transcriptionally silent chromatin states [7, 8]. 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 [9, 10]. Since nucleosomes are the foundation for all chromatin, it is assumed that silent chromatin is similarly disrupted by DNA replication [7]. Furthermore, mitotic chromosome condensation results in massive chromatin restructuring and this restructuring potentially disrupts transcriptionally silent chromatin [8, 11].

In a cell cycle, changes in set transcriptional programs rarely occur [12]. Thus, if DNA replication and Mitosis disrupt silent chromatin, then factors that restore silencing immediately following its disruption must exist. Using the budding yeast, *Saccharomyces cerevisiae*, I analyze the molecular mechanisms that both disrupt and restore set transcriptional programs. I first identify two factors required for the inheritance of transcriptional silencing. I then determine when in the cell cycle transcriptional silencing is disrupted in the absence of these factors. Finally, I analyze the silent chromatin structure immediately following a failure to inherit silencing. By determining which cell cycle event(s) disrupt silencing and how silencing



is restored following its disruption, we can begin to devise novel treatments against cancers arising from aberrant gene silencing.

## BODY

### STATEMENT OF WORK SUMMARY:

#### **Task 1. Determine if DNA replication through a transcriptionally silent region disrupts its silent state.**

Using conditional alleles of *sir1<sup>td</sup>* and *asf1<sup>td</sup>* generated in Task 3, I show that Sir1 and Asf1 are sufficient to restore *HMLalpha* transcriptional silencing with progression through S phase. However, when DNA replication is blocked silencing remains intact in the absence of Sir1p and Asf1p. These findings are consistent with the model that DNA replication disrupts transcriptional silencing and Sir1p and Asf1p are required for the inheritance of transcriptional silencing following DNA replication.

#### **Task 2. Analyze the chromatin structure of the *HMLalpha* locus when silencing is not inherited.**

Using conditional alleles of *sir1<sup>td</sup>* and *asf1<sup>td</sup>* described in Task 3, I show that Sir3p, a core silencing protein, remains associated with *HMLalpha* when silencing is not inherited. I also show that Htz1p and acetylated histone H4, euchromatin promoting factors, become enriched at *HMLalpha* when silencing is not inherited. These findings suggest that the *HMLalpha* chromatin structure becomes a dynamic heterochromatin/euchromatin structure that is transcriptionally active, yet ready to be silenced by Sir1p and Asf1p.

#### **Task 3. Determine if CAF-I (composed of 3 subunits Cac1, Cac2, and Cac3), Asf1, and Sir1 are required for the inheritance of transcriptional silencing.**

Conditional alleles of *Sir1<sup>td</sup>* and *Asf1<sup>td</sup>* were generated to determine if these factors cooperate in the inheritance of silencing at *HMLalpha*. Using these conditional mutants, I show that Sir1p and Asf1p are required for the inheritance of *HMLalpha* transcriptional silencing.

**TASKS:****Task 1. Determine if DNA replication through a transcriptionally silent region disrupts the silent state.**

DNA 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. 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 [13]. 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 [14] [15]. 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.

**Task 1A. Determine when in the cell cycle transcriptional silencing is lost.**

- **Is progression through one cell cycle sufficient to disrupt *HMLalpha* transcriptional silencing?**

In Task 3E I demonstrated that progression through multiple cell cycles, in the absence of Sir1 and Asf1, disrupts *HMLalpha* transcriptional silencing. Consequently, I sought to determine if progression through one cell cycle is sufficient to disrupt silencing. *sir1<sup>td</sup> asf1<sup>td</sup>* cells were synchronized in G1 with alpha factor and then shifted to restrictive conditions to degrade Sir1 and Asf1 proteins. Cells were then release from a restrictive G1 arrest into a Mitotic arrest, to synchronize the cells for hydroxyurea (HU) addition, and then released into an alpha factor/0.2M HU arrest (Fig. 1A). 2 hours after release from the Mitotic arrest, the majority of the cells had progressed from Mitosis into the next G1 (Fig. 1C, left panel) and silencing was fully perturbed 5 hours after release (Fig.

1D, G1 to G1 experiment). This result demonstrates that progression through one cell cycle, in the absence of Sir1 and Asf1, is sufficient to disrupt *HMLalpha* silencing.

- **Is progression through Mitosis sufficient to disrupt *HMLalpha* transcriptional silencing?**

Since progression through one cell cycle is sufficient to disrupt *HMLalpha* silencing, I sought to determine if progression through S phase or Mitosis alone is sufficient to perturb silencing. During Mitosis the chromatin structure is completely reorganized. Chromosomes condense during Prophase, resulting in genome-wide nucleosome rearrangement. Since nucleosomes are the foundation of heterochromatin, chromosome condensation likely disrupts the higher order heterochromatin structure.

To determine if Sir1 and Asf1 restore transcriptional silencing following progression through Mitosis, we synchronized *sir1<sup>td</sup> asf1<sup>td</sup>* conditional cells in a Mitotic arrest, using Nocodazole, and then shifted the culture to restrictive conditions to degrade both Sir1<sup>td</sup> and Asf1<sup>td</sup> proteins. Once both Sir1<sup>td</sup> and Asf1<sup>td</sup> proteins were degraded, cells were released from the Mitotic arrest into a G1 arrest (Fig. 1B). As determined by budding index, monitoring cell division, and flow cytometry, monitoring DNA content, the majority of cells progressed from Mitosis into the next G1, 2 hours after release from the mitotic arrest (Fig. 1C, right panel). In the cells that progressed through Mitosis without Sir1 and Asf1, *HMLalpha* silencing was slightly perturbed, 15% of the maximum level (Fig. 1D, M to G1 experiment). This result demonstrates that in the absence of Sir1 and Asf1, progression through Mitosis is not sufficient to disrupt *HMLalpha* silencing.

- **Is progression through S phase sufficient to disrupt *HMLalpha* transcriptional silencing?**

As an initial step to determine if DNA replication disrupts the silent chromatin structure, I examined whether progression through S phase is sufficient to disrupt transcriptional silencing. *sir1<sup>td</sup> asf1<sup>td</sup>* conditional cells were synchronized in G1 with alpha factor and shifted to restrictive conditions to degrade both Sir1<sup>td</sup> and Asf1<sup>td</sup> proteins. In the absence of both proteins, cells were released from G1 into Mitosis (Fig. 2A). 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. 2B). We also observe that at this 2 hour time point the loss of silencing was 25% of the maximum level and by 6 hours, silencing was fully disrupted (Fig. 2C). These results demonstrate that progression through S phase is sufficient to disrupt transcriptional silencing in the absence of Sir1 and Asf1.

**Task 1B and 1C. Determine if DNA replication is required for a loss of silencing in *sir1<sup>td</sup>* *asf1<sup>td</sup>* cells.**

In Task 1A I demonstrated that progression through S phase is sufficient to disrupt *HMLalpha* silencing. Since DNA replication is a major S phase event, I wanted to determine if DNA replication is required to disrupt silencing. *sir1<sup>td</sup> asf1<sup>td</sup>* cells were synchronized in G1 using alpha factor and then shifted to restrictive conditions to degrade both Sir1<sup>td</sup> and Asf1<sup>td</sup> proteins. Cells were then release for 4 hours into 0.2M hydroxyurea (HU), an early S phase arrest (Fig. 3A, 0 to 4 hours). At this point, *HMLalpha* silencing was only slightly deregulated, 10% of the maximum level (Fig. 3C, 0 to 4 hours), demonstrating that progression through early S phase is not sufficient to disrupt *HMLalpha* silencing. After 4 hours in 0.2M HU, the cells were released through S phase into a Mitotic arrest (Fig. 3A, 4 to 7 hours). One hour after release from the 0.2M HU arrest, the 5 hour time point, *HMLalpha* was fully desilenced (Fig. 3C, 5 hours). This rapid loss of silencing tightly correlated with the completion of S phase, 2C DNA content (Fig. 3B). Since DNA replication of *HMLalpha* is a late S phase event, this result strongly suggests that replication perturbs *HMLalpha* silencing.

**Task 2. Analyze the chromatin structure of the HML locus when silencing is not inherited.**

Prior to this study, it was unclear how transcriptionally silent states are inherited from one generation to the next. In Task 1 I demonstrate, for the first time, that the inheritance of transcriptional silencing involves an S-phase dependent disruption followed by Sir1/Asf1 mediated restoration. The mechanism of Sir1/Asf1 inheritance, however, remained to be determined. To study the inheritance mechanism I analyzed the chromatin structure of

*HMLalpha* following a failure to inherit silencing. 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. I monitored Sir3, Htz1 and acetylated-H4 association to determine which silent and active chromatin marks associate with *HMLalpha*, following a loss of silencing.

**Task 2A and 2B. Use chromatin immunoprecipitation (ChIP) to analyze Sir3 association with *HMLalpha*.**

To analyze the chromatin structure immediately following a failure to inherit silencing, I grew *sir1<sup>td</sup> asf1<sup>td</sup>* cells in log phase permissive conditions, synchronized the cells in G1 with alpha factor, and then shifted the cells for 3 hours to restrictive conditions until both Sir1 and Asf1 proteins were undetectable. In the absence of both proteins, cells were released from the G1 arrest into restrictive log phase conditions (Fig. 4A). Using flow cytometry I observed that two hours after release from G1, the majority of *sir1<sup>td</sup> asf1<sup>td</sup>* cells completed S phase and had a 2C DNA content (Fig. 10B). This completion of S phase correlated with a 70% loss of silencing (Fig. 10B). Though most *sir1<sup>td</sup> asf1<sup>td</sup>* cells lost silencing 2 hours after release from G1, Sir3 remained associated with *HMLalpha* for the duration of the experiment (Fig. 4B). Consistent with previous studies[16], these results demonstrate that Sir3 association with *HMLalpha* is 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.

**Task 2C. Use chromatin immunoprecipitation (ChIP) to analyze Htz1 and acetylated Histone H4 association with *HMLalpha*.**

Using the same experimental protocol, described in Task 2A and 2B, I analyzed Htz1 and acetylated Histone H4 association with *HMLalpha*. Htz1 is a H2.A histone variant found

exclusively in euchromatic regions while Histone H4 is highly acetylated in euchromatin and hypoacetylated in heterochromatin regions.

I observed that Htz1 associated with HML-E, HMLalpha and HML-I (Fig. 4C) 1 hour after release from the G1 arrest while acetylated-H4 associated with *HMLalpha* (Fig. 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* caused the heterochromatin to euchromatin transformation. Thus, Htz1 and acetylated-H4's association with *HMLalpha* potentially induced the loss of *HMLalpha* silencing.

#### **Task 2D. Determine if Htz1 association with *HMLalpha* is required to disrupt silencing.**

Given the results from Task 2C, I sought to determine if Htz1's association with *HMLalpha* was required as an initial step in the loss of transcriptional silencing. I 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. 5). Similarly, restrictively grown *sir1<sup>td</sup> asf1<sup>td</sup>* and *sir1<sup>td</sup> asf1<sup>td</sup> htz1::KanMX4* strains had severe silencing defects (Fig. 5). These results demonstrated that Htz1's association with *HMLalpha*, following a failure to inherit silencing, only marked the transformation of heterochromatin to euchromatin and was not required for the loss of silencing.

#### **Task 3. Determine if CAF-I, Asf1 and Sir1 are required for the inheritance of transcriptional silencing.**

Prior to this study, proteins that promote the inheritance of silencing had not been identified. However, several observations suggested that the histone deposition factors, CAF-I (composed

of Cac1, Cac2 and Cac3) and Asf1 participate in this process along with Sir1 [17]. First, the restoration of silencing after passage of a replication fork cannot occur without the assembly of new nucleosomes. CAF-1 and Asf1 nucleate nucleosome assembly by depositing the histone H3-H4 tetramer onto naked DNA [18] and are believed to target newly replicated DNA by interacting with PCNA [19], the processivity factor for replicative polymerases. Second, triple mutants of *cac1*, *asf1*, and *pol30* (PCNA) display *HMRa* silencing defects using a sensitized *HMRa* reporter [19], establishing a role for CAF-1 and Asf1 in silencing. This role may not be essential, however, since I have shown that *HMRa* silencing, as directly analyzed by Northern analysis, is virtually intact in *cac1 asf1* double mutants. Thus, if CAF-1 and Asf1 have a role in the inheritance of silencing, another mechanism for promoting inheritance must be working in parallel.

Further investigation suggested that Sir1, a protein thought to be required for only the establishment of silencing could provide this parallel mechanism of inheritance. *sir1* stains, like the *asf1 cac1* strains do not have a major silencing defect (Fig. 6). However, the triple mutant, *sir1 cac1 asf1*, is completely defective for silencing and the *sir1 asf1* double mutant is severely defective for silencing (Fig. 6) [17]. Since the *sir1 asf1* mutant has a severe silencing defect and a faster doubling time than the *sir1 cac1 asf1* mutant, I decided to focus my studies on Sir1 and Asf1.

### **Task 3A and 3B. Generate temperature sensitive alleles of SIR1 and ASF1.**

To determine if Sir1 and Asf1 are required for the inheritance of transcriptional silencing, I generated conditional alleles of *SIR1* and *ASF1* (*sir1<sup>td</sup>* and *asf1<sup>td</sup>*). These conditional alleles were created using the ts-degtron system [20]. Ts-degtron proteins are degraded by a three step process. First, transcription of the protein is regulated by an inducible promoter that can be shut-off. Second, the protein is destabilized by the N-terminal arginine (R) which targets the protein for degradation by the N-End-Rule [20]. Third, induction of the ubiquitin ligase, Ubr1, allows for ubiquitination of the ts-degtron protein which results in targeting of the protein to the proteasome for degradation. Using this ts-degtron method I was able to generate extremely effective conditional alleles of *SIR1* and *ASF1*. *sir1<sup>td</sup> asf1<sup>td</sup>* cells were grown under log phase



conditions at either permissive or restrictive conditions for 48 hours. At permissive conditions, the *sir1<sup>td</sup> asf1<sup>td</sup>* strain is fully functional for *HMLalpha* silencing (Fig. 7b) and at restrictive conditions it is fully defective for silencing (Fig. 7b). When grown under restrictive conditions, "undetectable" Asf1<sup>td</sup> protein levels were determined to be 4-fold below endogenous Asf1 protein levels (Fig. 7A, right panel) while "undetectable" Sir1<sup>td</sup> protein association with *HML-E* and *HML-I* was determined to be 6-fold below the permissive association level (Fig. 7A, left panel, and C).

**Task 3C. Determine if Sir1 and Asf1 are required for either the maintenance or inheritance of transcriptional silencing.**

Sir1 is a silencing protein thought only to have a role in the de novo generation of silent chromatin, the establishment of silencing [21]. If Sir1 is only required for the establishment and not the maintenance or inheritance of silencing, then regions of transcriptionally silent DNA would remain silent, in the absence of Sir1. Using the *sir1<sup>td</sup> asf1<sup>td</sup>* system, I studied if Sir1 in combination with Asf1 is required for the maintenance or inheritance of silencing. *sir1<sup>td</sup> asf1<sup>td</sup>* cells were grown under asynchronous permissive conditions and at time 0 they were shifted to restrictive conditions (Fig. 8A). Two hours after shifting to restrictive conditions, Sir1<sup>td</sup> protein was undetectable by western blotting and three hours after shifting to restrictive conditions Asf1<sup>td</sup> protein was undetectable (Fig. 8C). Furthermore, complete loss of *HMLalpha* silencing occurred 9 hours, 2 doublings, after both Sir1 and Asf1 degon proteins were undetectable (Fig. 8B, C & D). This loss of *HMLalpha* silencing, in the absence of Sir1 and Asf1, demonstrates that both proteins are required for either the maintenance or inheritance of silencing.

**Task 3D. Determine if Sir1 and Asf1 are required for the maintenance of transcriptional silencing.**

Synchronized cell cycle experiments must be used to determine if a protein is required for the maintenance or inheritance of silencing. Proteins required for the maintenance of silencing are continuously required for silencing, even when cells are arrested at a fixed point in the cell cycle. However, proteins required for the inheritance of silencing are only required to restore silencing

when it is perturbed by a cell cycle event. Consequently, proteins required for the inheritance of silencing are needed only in cells progressing through the cell cycle.

To determine if Sir1 and Asf1 are required for the maintenance of silencing, *sir1<sup>td</sup> asf1<sup>td</sup>* cells were grown under permissive conditions, arrested in G1, and then shifted to restrictive conditions. Cells were held in the G1 arrest for the duration of the experiment (Fig. 9A). The initial time point of the experiment,  $t=0$ , was determined as the time when both *sir1<sup>td</sup>* and *asf1<sup>td</sup>* proteins were first undetectable by western blot and chromatin immunoprecipitation (Fig. 9C and D). Flow cytometry, monitoring DNA content, and budding index, monitoring progression past "START", confirmed that the strains remained G1 arrested for the entire experiment (Fig. 9B). Furthermore, quantitative PCR showed that the strains remained silent for the duration of the 7 hour experiment (Fig. 9E). This result suggests that Sir1 and Asf1 are not required for the maintenance of silencing since.

As a positive control for this experiment, I show that *HMLalpha* silencing is fully defective in a *sir3<sup>ts</sup> hmr* strain (Fig. 9E). Sir3 is a hallmark maintenance protein, which is continuously required for transcriptional silencing. Thus, Sir1 and Asf1 are not required for the maintenance of transcriptional silencing.

### **Task 3E. Determine if Sir1 and Asf1 are required for the inheritance of transcriptional silencing.**

Since Sir1 and Asf1 are not required for the maintenance of silencing, I performed an experiment to determine if they are required for the inheritance of silencing. Similar to the previous experiment, *sir1<sup>td</sup> asf1<sup>td</sup>* cells were grown under permissive conditions, arrested in G1 and then shifted to restrictive conditions to degrade both Sir1<sup>td</sup> and Asf1<sup>td</sup> proteins. At  $t=0$ , when Sir1 and Asf1 proteins were undetectable by western blot (Fig. 10C) the cells were released from the G1 arrest into restrictive log phase conditions (Fig. 10A). We monitored *HMLalpha2* mRNA expression and observed that once the cells were released from the G1 arrest silencing was rapidly lost (Fig. 10D). This rapid loss occurred 1-2 hours after release, when the majority of cells completed S phase (Fig. 10B). After 7 hours, the cells were fully derepressed at

*HMLalpha*. This result suggests that Sir1 and Asf1 are required for the restoration of silencing in cells progressing through the cell cycle.

### Key Research Accomplishments

- I have generated extremely effective conditional alleles of Sir1 and Asf1.
- Contrary to previous findings, Sir1 functions in the inheritance of silencing and not just the establishment of silencing.
- Sir1 and Asf1 are not required for the maintenance of transcriptional silencing.
- Sir1 and Asf1 are required for the inheritance of transcriptional silencing.
- An S phase event disrupts the silent chromatin structure and Sir1 and Asf1 are required to restore silencing after that event.
- Progression through Mitosis is not sufficient to disrupt silencing in the absence of Sir1 and Asf1.
- Loss of *HMLalpha* silencing correlates with DNA replication of *HMLalpha* suggesting that DNA replication is the S phase event that disrupts silencing.
- Heterochromatin (Sir3) and euchromatin (Htz1 and acetylated H4) factors associate with *HMLalpha* following a failure to inherit silencing.

## Reportable Outcomes

### **Presentations:**

Mechanism for the inheritance of transcriptional silencing in budding yeast. Research in Progress Seminar. University of California San Francisco. March 2005.

Epigenetic inheritance of transcriptionally silent chromatin. Abcam Chromatin Structure and Function Conference. Dominican Republic. December 2006.

### **Publications:**

A silencer-associated protein and a histone chaperone are redundantly required for the inheritance but not the maintenance of a silent domain. PNAS. June 2007. Submitted for publication.

## Conclusions

A key question in today's scientific world is: How are epigenetic states stably propagated for many generations? Prior to this study, very little was known about the inheritance of these epigenetic states. Using conditional alleles I show that Sir1 and Asf1 cooperate in the inheritance of transcriptionally silenced chromatin states. I also demonstrate that progression through S phase, in the absence of Sir1 and Asf1, disrupts transcriptional silencing. Finally, I show that silent chromatin components remain associated with *HMLalpha* long after silencing is lost, thus serving as a "molecular memory" to template the restoration of heterochromatin.

The identification of these inheritance proteins and elucidating how they function to restore silencing has far reaching affects in understanding the etiology of cancers. Many cancers arise from changes in cellular transcriptional programs. Genes that are transcriptionally silent become activated, and genes that are normally expressed become transcriptionally silenced. How and when these transcriptional alterations occur, remains to be determined. One can hypothesize, however, that cancers resulting from silent transcriptional programs becoming active, stem from a failure to inherit the silent chromatin state. Conversely, cancers resulting from active transcriptional programs becoming silent, stem from inappropriate transcriptional silencing. Knowing that Sir1 and Asf1 are required for the faithful propagation of silent chromatin following DNA replication will enable us to design therapies to treat diseases arising from inappropriate gene silencing and activation.

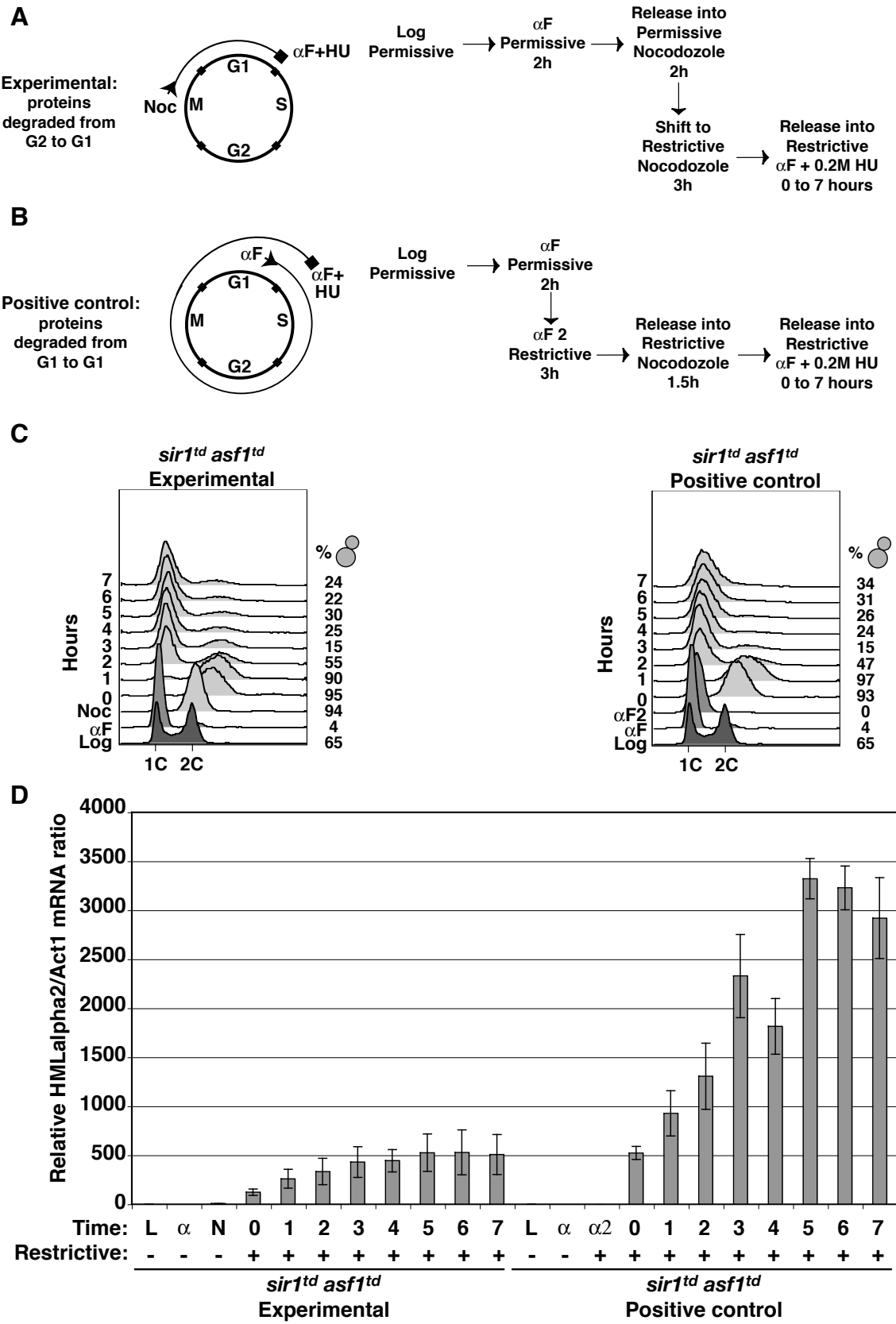
## REFERENCES

1. Waggoner, D., *Mechanisms of disease: epigenesis*. Semin Pediatr Neurol, 2007. **14**(1): p. 7-14.
2. Bailis, J.M. and S.L. Forsburg, *It's all in the timing: linking S phase to chromatin structure and chromosome dynamics*. Cell Cycle, 2003. **2**(4): p. 303-6.
3. McNairn, A.J. and D.M. Gilbert, *Epigenomic replication: linking epigenetics to DNA replication*. Bioessays, 2003. **25**(7): p. 647-56.
4. Wallace, J.A. and T.L. Orr-Weaver, *Replication of heterochromatin: insights into mechanisms of epigenetic inheritance*. Chromosoma, 2005. **114**(6): p. 389-402.
5. Horsthemke, B., *Epimutations in human disease*. Curr Top Microbiol Immunol, 2006. **310**: p. 45-59.
6. Holst, C.R., et al., *Methylation of p16(INK4a) promoters occurs in vivo in histologically normal human mammary epithelia*. Cancer Res, 2003. **63**(7): p. 1596-601.
7. Ehrenhofer-Murray, A.E., *Chromatin dynamics at DNA replication, transcription and repair*. Eur J Biochem, 2004. **271**(12): p. 2335-49.
8. Belmont, A.S., *Mitotic chromosome structure and condensation*. Curr Opin Cell Biol, 2006. **18**(6): p. 632-8.
9. Gruss, C., et al., *Disruption of the nucleosomes at the replication fork*. Embo J, 1993. **12**(12): p. 4533-45.
10. Gruss, C. and J.M. Sogo, *Chromatin replication*. Bioessays, 1992. **14**(1): p. 1-8.
11. Komura, J. and T. Ono, *Disappearance of nucleosome positioning in mitotic chromatin in vivo*. J Biol Chem, 2005. **280**(15): p. 14530-5.
12. Miller, A.M. and K.A. Nasmyth, *Role of DNA replication in the repression of silent mating type loci in yeast*. Nature, 1984. **312**(5991): p. 247-51.
13. Gasser, R., T. Koller, and J.M. Sogo, *The stability of nucleosomes at the replication fork*. J Mol Biol, 1996. **258**(2): p. 224-39.
14. Gruss, C., et al., *Disruption of the nucleosomes at the replication fork*. Embo J, 1993. **12**(12): p. 4533-45.
15. Lucchini, R., R.E. Wellinger, and J.M. Sogo, *Nucleosome positioning at the replication fork*. Embo J, 2001. **20**(24): p. 7294-302.
16. Kirchmaier, A.L. and J. Rine, *DNA replication-independent silencing in S. cerevisiae*. Science, 2001. **291**(5504): p. 646-50.
17. Ehrenhofer-Murray, A.E., D.H. Rivier, and J. Rine, *The role of Sas2, an acetyltransferase homologue of Saccharomyces cerevisiae, in silencing and ORC function*. Genetics, 1997. **145**(4): p. 923-34.
18. Tyler, J.K., et al., *The RCAF complex mediates chromatin assembly during DNA replication and repair*. Nature, 1999. **402**(6761): p. 555-60.
19. Zhang, Z., K. Shibahara, and B. Stillman, *PCNA connects DNA replication to epigenetic inheritance in yeast*. Nature, 2000. **408**(6809): p. 221-5.
20. Levy, F., J.A. Johnston, and A. Varshavsky, *Analysis of a conditional degradation signal in yeast and mammalian cells*. Eur J Biochem, 1999. **259**(1-2): p. 244-52.
21. Pillus, L. and J. Rine, *Epigenetic inheritance of transcriptional states in S. cerevisiae*. Cell, 1989. **59**(4): p. 637-47.

**Figure 1.** 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. *sir1<sup>td</sup> asf1<sup>td</sup>* 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. *sir1<sup>td</sup> asf1<sup>td</sup>* (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 nocodazole). 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 and subjected to RT-PCR followed by quantitative PCR. The *HMLalpha2*/ACT1 ratio, determined by quantitative PCR, was adjusted so that the WT ratio was 1.0. In each case, the standard error was calculated based on three experiments.

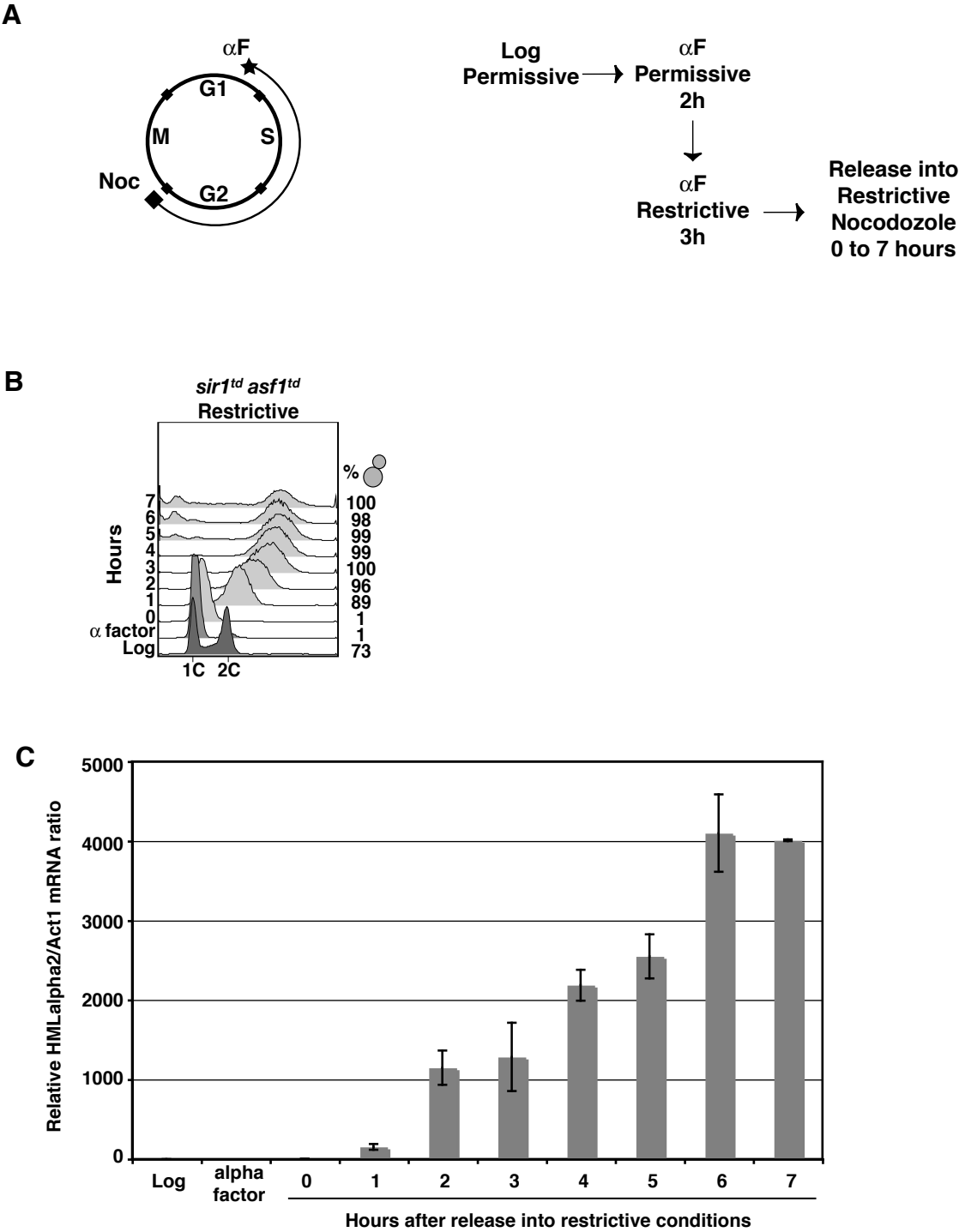


**Figure 1**



**Figure 2.** Progression through S phase, in the absence of Sir1 and Asf1, is sufficient to disrupt *HMLalpha* silencing. (A) Experimental strategy. *sir1<sup>td</sup> asf1<sup>td</sup>* (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 nocodazole (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 and subjected to RT-PCR followed by quantitative PCR. The *HMLalpha2*/ACT1 ratio, determined by quantitative PCR, was adjusted so that the WT ratio was 1.0. In each case, the standard error was calculated based on three experiments.

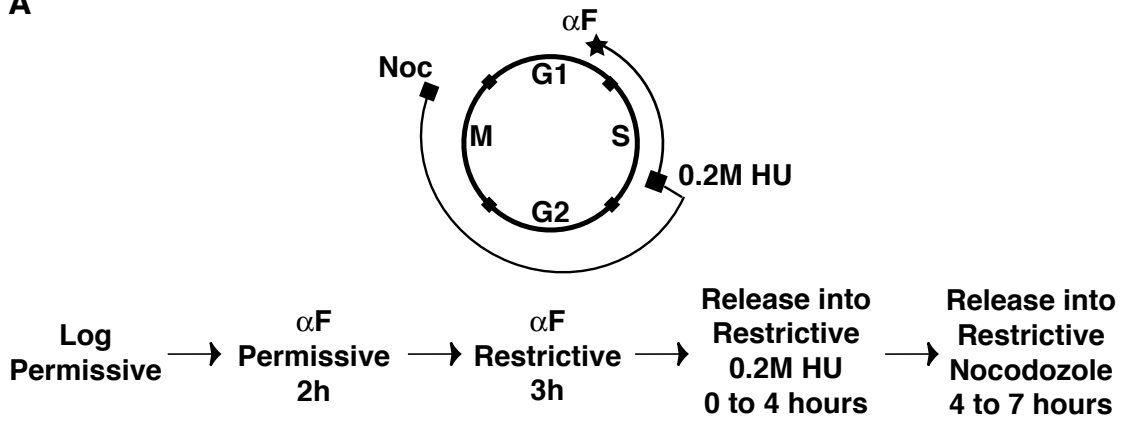
**Figure 2**



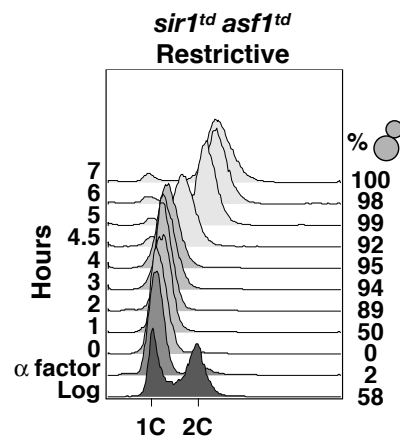
**Figure 3.** Loss of *HMLalpha* silencing correlates with the completion of S phase. (A) Experimental strategy. *sir1<sup>td</sup> asf1<sup>td</sup>* (YJL5824) cells were grown in log phase permissive conditions (SDC-MET), synchronized in G1 with alpha factor and shifted to restrictive conditions (YEPgal + 2mM MET) for 3 hours while maintaining the G1 arrest. Cells were then released for 4 hours into 0.2M hydroxyurea, 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 nocodazole, 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 and subjected to RT-PCR followed by quantitative PCR. The *HMLalpha2*/*ACT1* ratio, determined by quantitative PCR, was adjusted so that the *WT* ratio was 1.0. In each case, the standard error was calculated based on three experiments. The “Block” row describes the cell cycle inhibitor used in each time point (alpha factor, hydroxyurea and nocodazole).

Figure 3

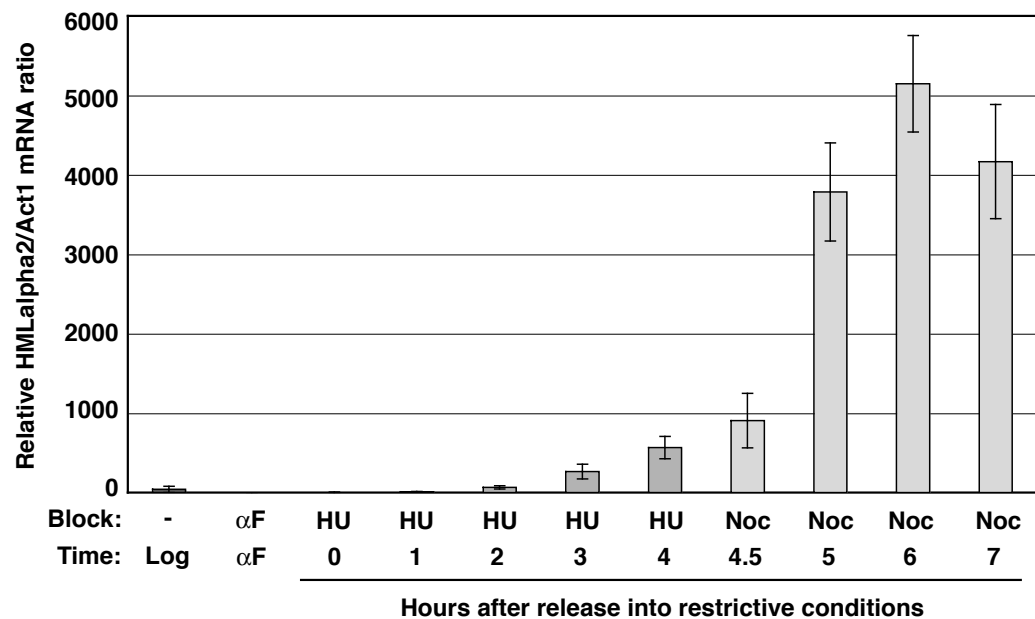
A



B

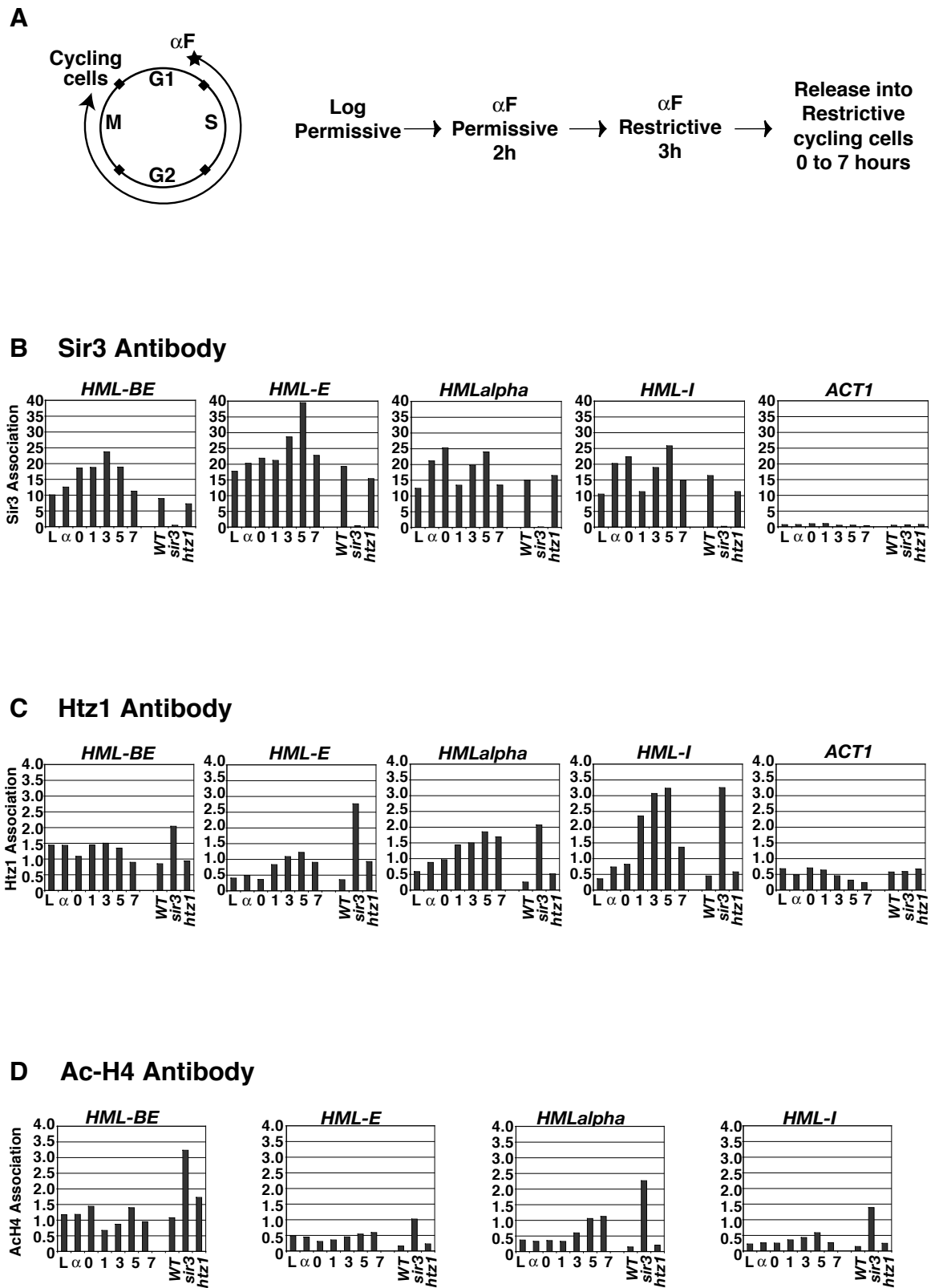


C



**Figure 4.** Sir3, Htz1 and acetylated H4 associate with *HMLalpha* in *sir1<sup>td</sup> asf1<sup>td</sup>* cells progressing through a restrictive cell cycle. (A) *sir1<sup>td</sup> asf1<sup>td</sup>* 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.

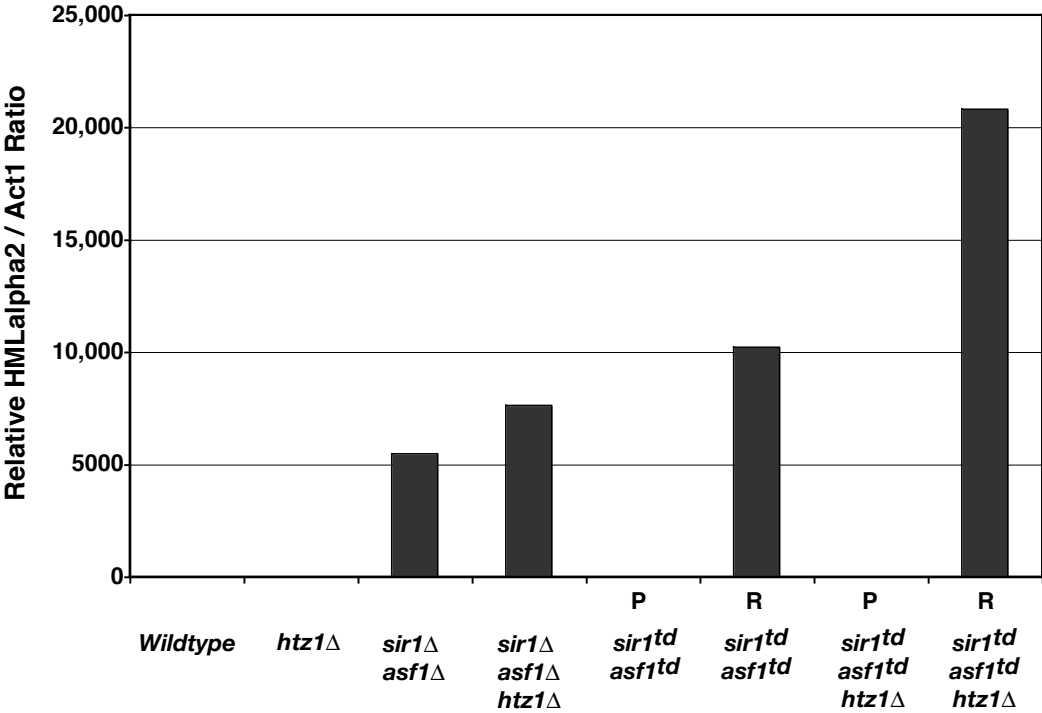
Figure 4



**Figure 5.** 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<sup>td</sup>asf1<sup>td</sup>* (YJL5824) and *sir1<sup>td</sup>asf1<sup>td</sup>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).

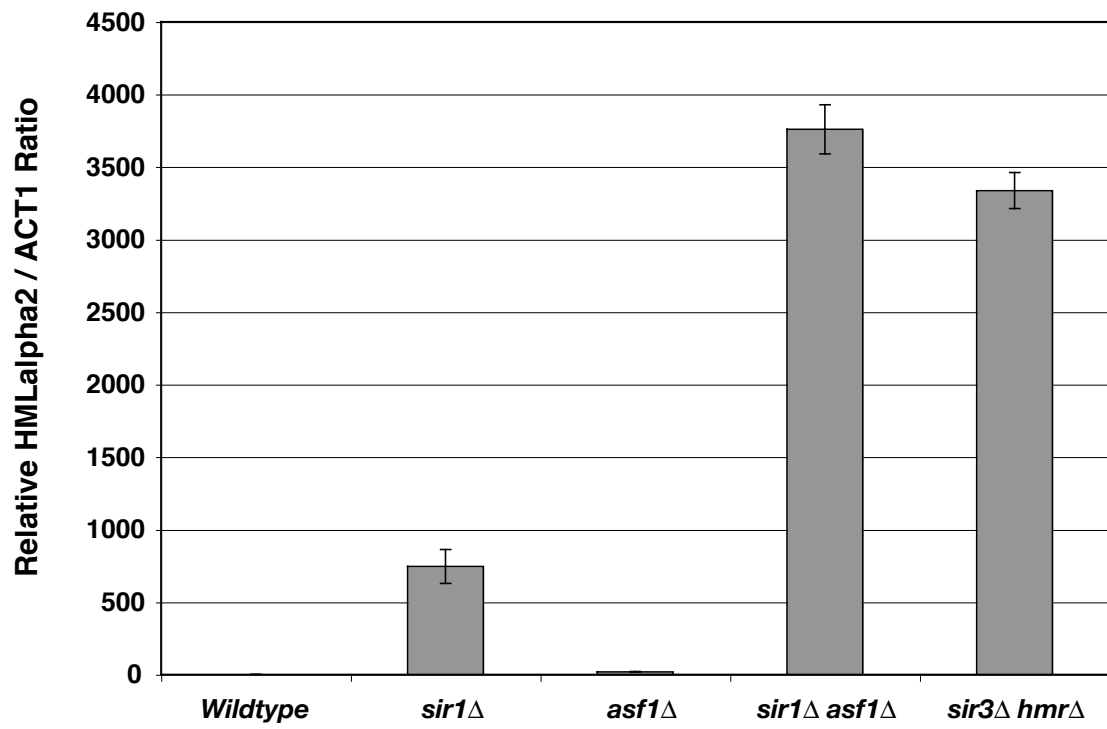


**Figure 5**



**Figure 6.** 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.

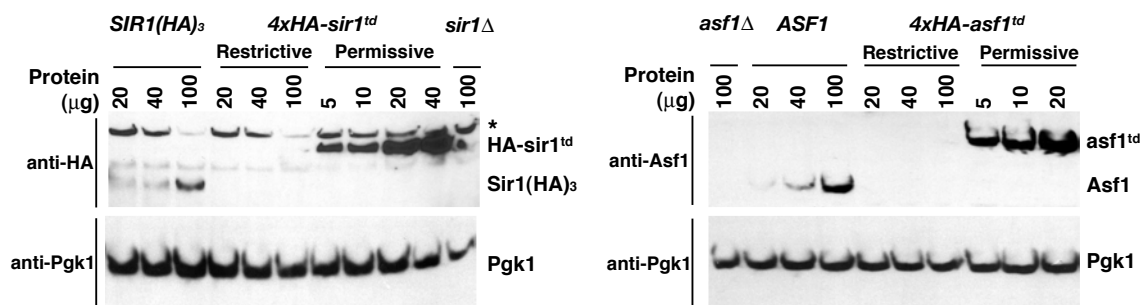
**Figure 6**



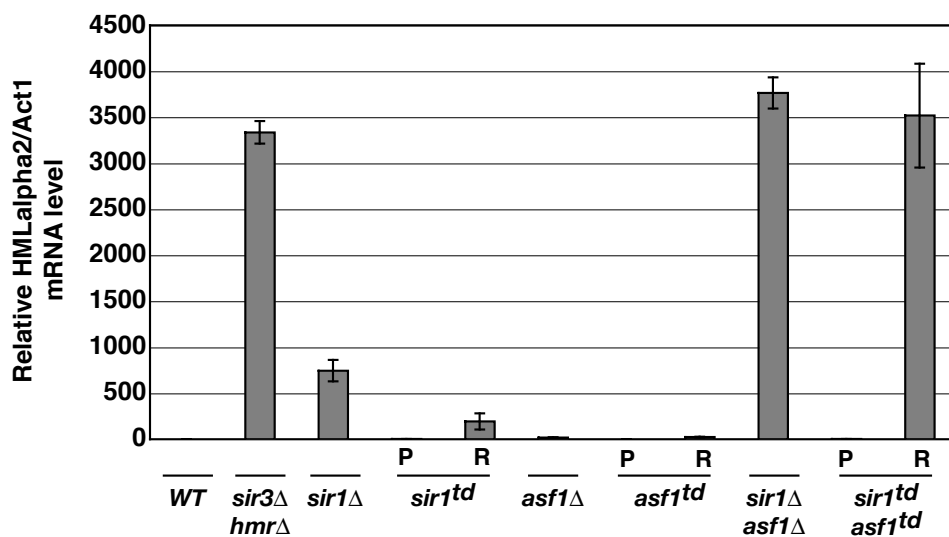
**Figure 7.** *sir1<sup>td</sup>* and *asf1<sup>td</sup>* conditional alleles are fully functional under permissive conditions and completely defective under restrictive conditions. (A) Western blots comparing endogenous and conditional degron protein levels. Left panel: Log phase *SIR1-3xHA* (YJL6377) and *sir1* (YJL5471) cells were grown in YEPD conditions for 48 hours while *4xHA-sir1<sup>td</sup>* (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<sup>td</sup>* (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<sup>td</sup>*, *asf1<sup>td</sup>* and *sir1<sup>td</sup> asf1<sup>td</sup>* (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 and subjected to RT-PCR followed by quantitative PCR. The *HMLalpha2/ACT1* ratio, determined by quantitative PCR, was adjusted so that the *WT* ratio was 1.0. In each case, the standard error was calculated based on three experiments. (C) anti-HA ChIP assay to monitor Sir1p association. *4xHA-sir1<sup>td</sup>* cells were grown under permissive and restrictive conditions while *sir1* and *SIR1-3xHA* cells were grown in YEPD. Chromatin containing extracts were prepared and in each case, the standard error was calculated based on three experiments.

**Figure 7**

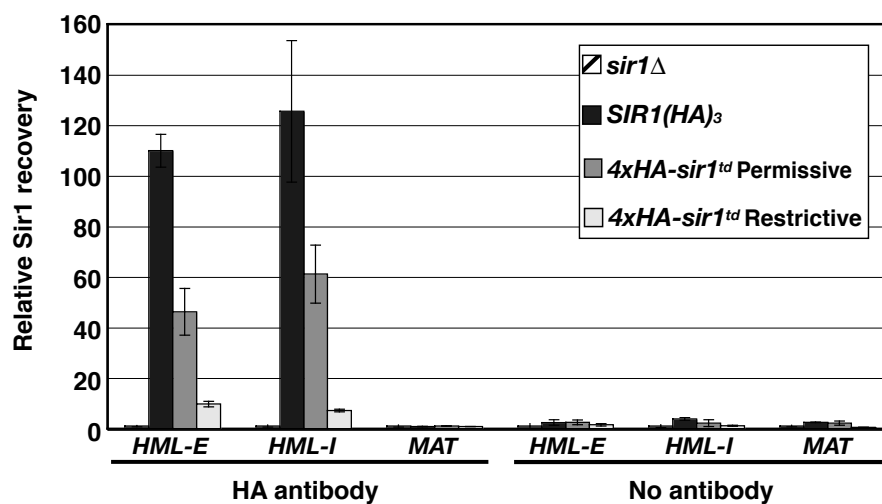
**A**



**B**

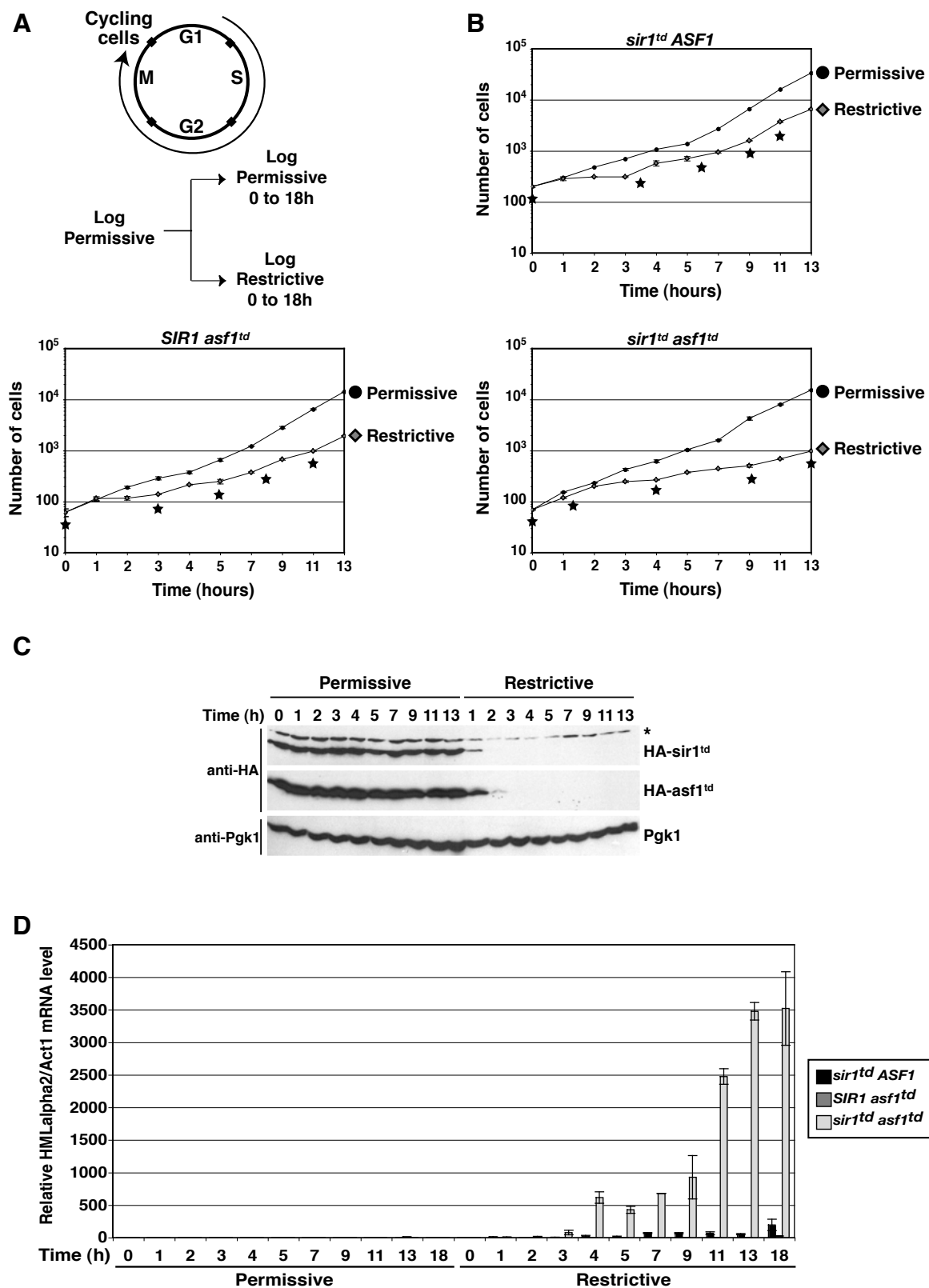


**C**



**Figure 8.** Inactivation of both *sir1<sup>td</sup>* and *asf1<sup>td</sup>* leads to complete loss of *HMLalpha* silencing within two doublings after protein depletion. (A) Experimental strategy. *sir1<sup>td</sup>* (YJL5880), *asf1<sup>td</sup>* (YJL5801) and *sir1<sup>td</sup> asf1<sup>td</sup>* (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 were 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.

**Figure 8**

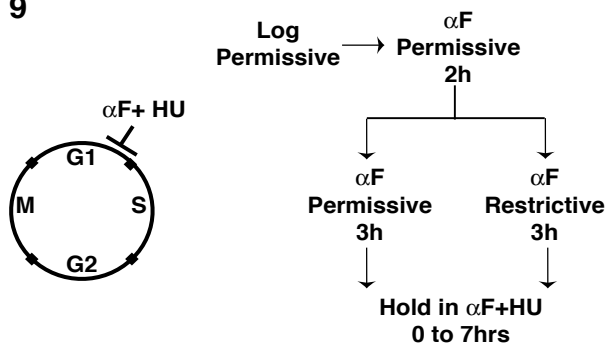


**Figure 9.** Sir1 and Asf1 are not required for the G1 maintenance of *HMLalpha* silencing. (A) Experimental strategy. *sir1<sup>td</sup>* (YJL5880), *asf11<sup>td</sup>* (YJL5801) and *sir1<sup>td</sup> asf11<sup>td</sup>* (YJL5824) cells were grown in log phase permissive conditions (SDC-MET), synchronized 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). *sir3<sup>ts</sup> 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. (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 WT ratio was 1.0. In each case, the standard error was calculated based on three experiments.

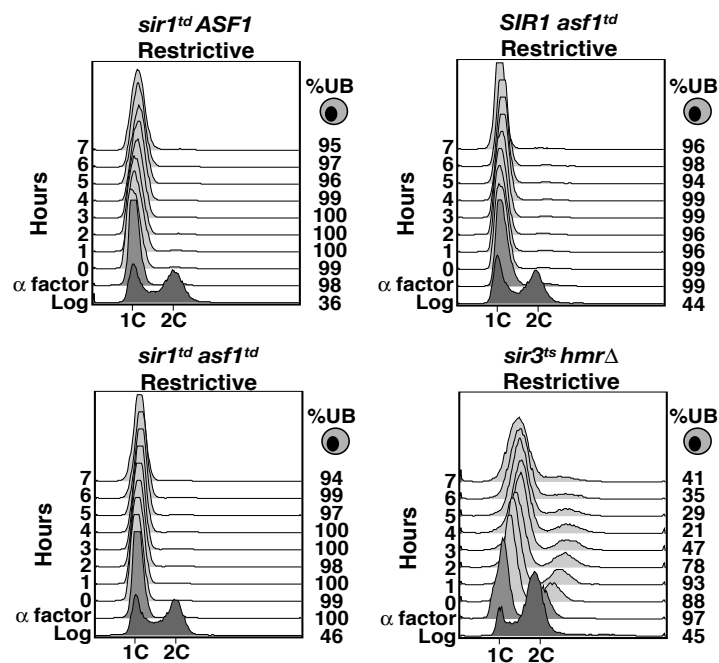


**Figure 9**

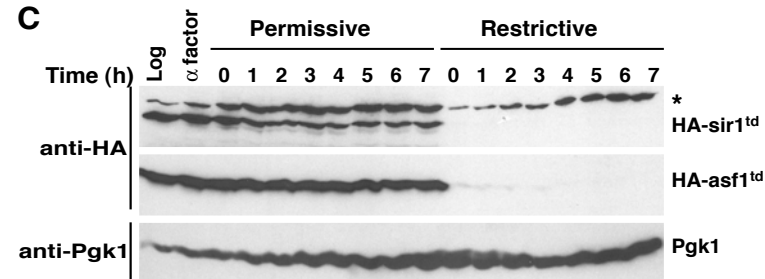
**A**



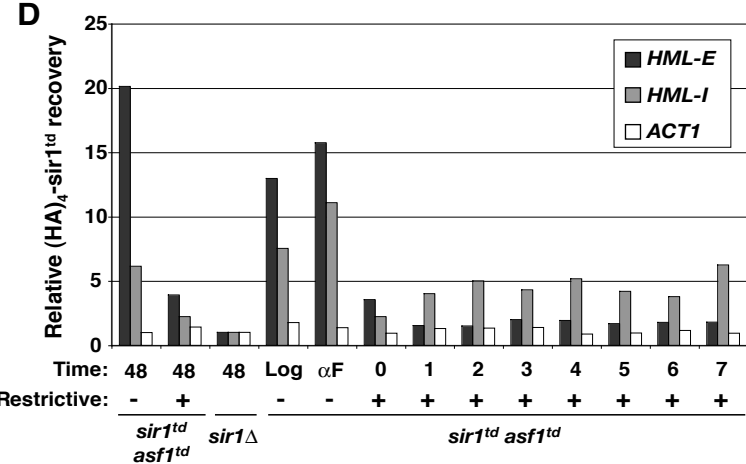
**B**



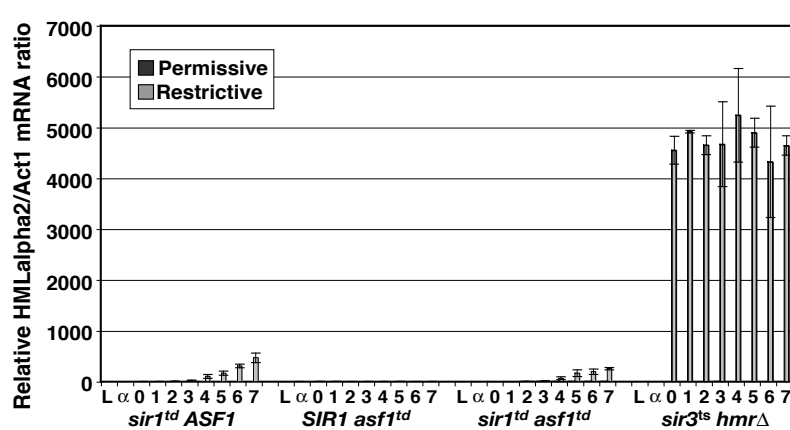
**C**



**D**



**E**



**Figure 10.** Sir1 and Asf1 are required for the inheritance of *HMLalpha* silencing. (A)

Experimental strategy. *sir1<sup>td</sup>* (YJL5880), *asf1<sup>td</sup>* (YJL5801) and *sir1<sup>td</sup> asf1<sup>td</sup>* (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 and subjected to RT-PCR followed by quantitative PCR. The *HMLalpha2*/ACT1 ratio, determined by quantitative PCR, was adjusted so that the WT ratio was 1.0. In each case, the standard error was calculated based on three experiments.

Figure 10

