Conversion of a Replication Origin to a Silencer through a Pathway Shared by a Forkhead Transcription Factor and an S Phase Cyclin

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Silencing of the mating-type locus *HMR* in *Saccharomyces cerevisiae* requires DNA elements called silencers. To establish *HMR* silencing, the origin recognition complex binds the *HMR-E* silencer and recruits the silent information regulator (Sir)1 protein. Sir1 in turn helps establish silencing by stabilizing binding of the other Sir proteins, Sir2–4. However, silencing is semistable even in *sir1Δ* cells, indicating that *SIR1*-independent establishment mechanisms exist. Furthermore, the requirement for *SIR1* in silencing a sensitized version of *HMR* can be bypassed by high-copy expression of *FKH1* (*FKH1^{hc}*), a conserved forkhead transcription factor, or by deletion of the S phase cyclin *CLB5* (*clb5Δ*). *FKH1^{hc}* caused only a modest increase in Fkh1 levels but effectively reestablished Sir2–4 chromatin at *HMR* as determined by Sir3-directed chromatin immunoprecipitation. In addition, *FKH1^{hc}* prolonged the cell cycle in a manner distinct from deletion of its close paralogue *FKH2*, and it created a cell cycle phenotype more reminiscent to that caused by a *clb5Δ*. Unexpectedly, and in contrast to *SIR1*, both *FKH1^{hc}* and *clb5Δ* established silencing at *HMR* using the replication origins, *ARS1* or *ARSH4*, as complete substitutes for *HMR-E* (*HMRΔE::ARS*). *HMRΔE::ARS1* was a robust origin in *CLB5* cells. However, initiation by *HMRΔE::ARS1* was reduced by *clb5Δ* or *FKH1^{hc}*, whereas *ARS1* at its native locus was unaffected. The *CLB5*-sensitivity of *HMRΔE::ARS1* did not result from formation of Sir2–4 chromatin because *sir2Δ* did not rescue origin firing in *clb5Δ* cells. These and other data supported a model in which *FKH1* and *CLB5* modulated Sir2–4 chromatin and late-origin firing through opposing regulation of a common pathway.

INTRODUCTION

Chromatin structures vary with genome position, creating structural heterogeneity along chromosomes that modulates every aspect of DNA metabolism (Fischle *et al.*, 2003). Specific DNA sequence elements form the foundation for this heterogeneity. For example, certain DNA sequences act to establish centromeric chromatin required for chromosome segregation (Cleveland *et al.*, 2003; Henikoff and Dalal, 2005). In addition, some chromatin structures, such as heterochromatin, can dominate the functional capacity of DNA sequence elements across chromosomal domains or even over an entire chromosome. Heterochromatin, for example, can delay or repress initiation by DNA replication origins and initiation of transcription from promoters (Gomez and Brockdorff, 2004; Weinreich *et al.*, 2004; Chang *et al.*, 2006). Thus, a competing balance exists between DNA sequence elements that perform different functions or establish different types of chromatin structures (Kamakaka, 1997; Donze and Kamakaka, 2002; Valenzuela and Kamakaka, 2006).

Genetic analyses of transcriptional silencing of the *HMRa* locus in *Saccharomyces cerevisiae* reveal that perturbations in the cell cycle tip the balance between the types of chromatin that can form at a particular chromosomal domain (Laman *et al.*, 1995; Fox and Rine, 1996; Ehrenhofer-Murray *et al.*, 1999).

HMRa silencing defines a form of transcription repression that requires the assembly of a specialized chromatin structure called silent chromatin (Fox and McConnell, 2005). Formation of silent chromatin requires small DNA elements called silencers that bind a collection of sequence-specific DNA binding proteins. *HMRa* contains two silencers, *HMR-E* and *HMR-I* that flank opposite ends of this ~3000-base pair locus (Loo and Rine, 1995). *HMR-E* is necessary and sufficient for *HMRa* silencing; the importance of *HMR-I* can be observed only under conditions in which silencing has been compromised (Fox *et al.*, 1995; Rivier *et al.*, 1999). *HMR-E*, the better characterized of the two silencers, contains a binding site each for the origin recognition complex (ORC), and the Rap1 and Abf1 proteins. *HMRa* silencing is not essential for yeast cell viability, but ORC is because it functions as the eukaryotic initiator, the protein complex that marks chromosomal sites as DNA replication origins (reviewed in Bell, 2002). Rap1 and Abf1 are also essential multifunctional nuclear proteins (reviewed in Shore, 1994).

The role of the *HMR-E* silencer-binding proteins is to recruit, via multiple independent protein–protein interactions, four silent information regulator (Sir) proteins, Sir1, -2, -3, and -4, nonhistone chromatin-binding proteins dispensable for cell viability but necessary for silencing (reviewed in Gasser and Cockell, 2001; Rusche *et al.*, 2003; Fox and McConnell, 2005). One important interaction occurs directly between Sir1 and ORC (Hou *et al.*, 2005; Hsu *et al.*, 2005). The Sir1-ORC complex helps recruit and/or stabilize the binding of the three other Sir proteins, Sir2, -3, and -4 to the silencer (Rusche *et al.*, 2002). Another key set of interactions occurs between Rap1 and Sir3 and Sir4 proteins (Moretti and Shore,

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2001). The multiple protein–protein interactions that recruit the four Sir proteins to HMR-E define the establishment/nucleation phase of silent chromatin assembly. In the second phase, Sir2, an enzyme, deacetylates nucleosomes neighboring HMR-E; deacetylated nucleosomes in turn promote binding of additional Sir2–4 complexes until a higher-order Sir2–4 silent chromatin structure forms at HMRa (reviewed in Rusche *et al.*, 2003; Moazed *et al.*, 2004; Fox and McConnell, 2005).

One key feature of this model is that Sir1 differs substantially from Sir2–4 because its role is confined to silencers where it enhances the assembly and/or reduces the disassembly of Sir2–4 chromatin, but is not intrinsically required either for its formation or function (Pillus and Rine, 1989, 2004; Xu *et al.*, 2006). Thus, in contrast to Sir2–4, Sir1 is not a critical structural component of HMRa silent chromatin. Another notable feature that distinguishes SIR1 from the other SIRs is that its requirement in HMRa silencing can be partially bypassed by perturbations in the cell cycle (Laman *et al.*, 1995). For example, a deletion of the major S phase cyclin CLB5 that is required for timely S phase progression (Donaldson *et al.*, 1998; Gibson *et al.*, 2004) can partially bypass the requirement for SIR1 (Laman *et al.*, 1995). In addition, mutations in the HMR-E silencer are also rescued by cell cycle perturbations caused by drugs or certain mutations (Axelrod and Rine, 1991; Laman *et al.*, 1995; Ehrenhofer-Murray *et al.*, 1999). These data provide evidence that other molecular interactions can compensate for the role of Sir1 in transcriptional silencing and reveal that specific perturbations in the cell cycle can tip the balance between silent and permissive chromatin formation at HMRa. Thus, HMRa silencing serves as an experimentally tractable model for dissecting how the cell cycle can modulate the structure and function of a defined chromatin domain.

In a previous study, we identified forkhead homologue FKH1 as a high-copy suppressor of defects in HMRa silencing caused by a *sir1Δ* mutation (Hollenhorst *et al.*, 2000). Fkh1 and its paralogue Fkh2 are evolutionarily conserved transcription factors that bind directly to promoters within a group of genes named the CLB2-cluster (reviewed in Breeden, 2000; Futcher, 2000). Transcription of these genes is repressed in late M, G1, and early S phases but is activated beginning in late S phase and through G2 and early M phases (Spellman *et al.*, 1998). Genes within this cluster encode proteins including the major G2/M phase cyclin Clb2 that drive progress through M phase. Fkh2 is the primary regulator of CLB2-cluster genes, repressing transcription of these genes in G1 phase and stimulating their transcription in late S and G2/early-M phase. (Koranda *et al.*, 2000; Kumar *et al.*, 2000; Pic *et al.*, 2000; Zhu *et al.*, 2000; Reynolds *et al.*, 2003). It is less clear how Fkh1 normally functions in CLB2-cluster transcription. FKH1 can partially compensate for loss of FKH2 because *fkh1Δfkh2Δ* cells exhibit a substantially greater reduction in CLB2-cluster transcription than *FKH1fkh2Δ* cells, indicating that Fkh1 can activate CLB2 transcription. However, paradoxically, the normal role for Fkh1 in wild-type cells seems to be as a negative regulator of CLB2-cluster transcription during G2/M, thus attenuating the level of transcription that can be achieved by Fkh2-mediated activation (Hollenhorst *et al.*, 2000, 2001; Sherriff *et al.*, 2007). Although it is unclear how the role(s) of FKH1 at the CLB2-cluster is related to its ability to affect silencing, it is known that the DNA binding domain of Fkh1 is critical for its ability to modulate SIR1-bypass HMR silencing (Hollenhorst *et al.*, 2000).

In this report, we addressed the mechanism(s) by which *FKH1^{hc}* bypasses the requirement for SIR1 in silencing. Un-

expectedly (Hollenhorst *et al.*, 2000), the genetic and cell cycle data reported here provide evidence against the role of *FKH1* in CLB2 transcription serving as the primary factor in *FKH1^{hc}*-dependent silencing. Specifically, although *FKH1^{hc}* reduced CLB2 transcription, reductions in CLB2 were insufficient to mimic *FKH1^{hc}*. Instead, the data generated through a combination of genetic analyses, Sir3-directed chromatin immunoprecipitations (ChIPs) and two-dimensional (2-D) origin mapping experiments were most consistent with a model in which *FKH1* and the S phase cyclin CLB5 act as opposing regulators that converge on a common target(s) that inhibit late replication origin firing and promote Sir2–4 chromatin assembly. The data were also consistent with the idea that this pathway favors distinct SIR1-independent molecular interactions that contribute to Sir2–4 protein association with HMR.

MATERIALS AND METHODS

Strains and Plasmids

Yeast strains used in this study (Table 1) were constructed using standard yeast molecular genetics and recombinant DNA techniques (Sambrook *et al.*, 1989; Guthrie and Fink, 1991). Five different plasmids were used in this study as indicated in the figure legends: pRS426 (Sikorski and Hieter, 1989), pCF99 (SIR1 in pRS316; Gardner *et al.*, 1999; Hollenhorst *et al.*, 2000), pCF345 (SIR1 in Yep24; Hollenhorst *et al.*, 2000), pCF480 (FKH1 in pRS426; Hollenhorst *et al.*, 2000), and pCF942 (FKH1 in pRS316). Cells were grown at 30°C in standard rich medium (YPD), in minimal medium supplemented with casamino acids (CAS) (to select for URA3-containing plasmids), or in synthetic media (SC) lacking defined supplements to select for diploids and/or plasmids as appropriate and described in the text (Guthrie and Fink, 1991). All strains were isogenic to W303-1A except the MATa cells used for mating lawns.

Semiquantitative Mating Assay

The MATa cells examined for silencing by mating efficiency were grown in log phase for 2 d, and then they were mixed with an excess of MATa cells (JRY19). The most concentrated samples of MATa cells analyzed were 5×10^6 cells/ml in a total volume of 50 μ l. Tenfold serial dilutions of this concentration were generated in 50- μ l final volumes. Six microliters of each dilution was analyzed per drop on either YPD or CAS solid agar medium, as appropriate, to determine cell counts. To the remainder of the cells, an excess of MATa cells (15 μ l of log-phase cells concentrated to 5 OD) were added and mixed. Eight microliters of this mixture was plated to synthetic solid agar medium appropriately supplemented to select for diploids (and the retention of a URA3 plasmid, if appropriate). Plating efficiencies indicated that equivalent numbers of cells were being compared for every mating comparison shown.

RNA Blots

RNA was isolated from yeast as described previously (Fox *et al.*, 1995), and 15 μ g of total RNA was analyzed per lane. RNA blot hybridization was performed with multiprimer-labeled DNA probes complementary to *a1* mRNA, CLB2 mRNA, or SCR1 RNA. The probes for *a1* mRNA and SCR1 RNA were described previously (Fox *et al.*, 1995, 1997). Template for the CLB2 probe was generated by PCR by using the following primer pair: forward, GTCCAAC-CCAATAGAAAACAC and reverse, CATGCACCGTCTGTCTCTGATG. This probe was also used in a previous study (Hollenhorst *et al.*, 2000).

Antibodies

Anti-Sir3 monoclonal antibodies were raised against full-length Sir3 purified from baculovirus-infected Sf9 cells as described previously (Georgel *et al.*, 2001), and they are available from Neoclone at <http://www.neoclone.com/> (the antibodies used to make the ChIP cocktail were 184A, 185A, and 184C). Anti-Fkh1 polyclonal antibodies were raised in rabbits (Harlan Labs, Madison, WI) to a 6xHis-Fkh1 fusion protein encoding the N-terminal 302 amino acids of Fkh1 (pCF1564) expressed in *Escherichia coli* (BL21 cells) and purified using ion exchange and nickel-affinity chromatography. These antibodies were affinity purified and used at a 1:200 dilution for protein immunoblotting (Harlow and Lane, 1999). Anti-hemagglutinin (HA) monoclonal antibodies were from Covance Research Products (Princeton, NJ).

Chromatin Immunoprecipitations

ChIP was performed as described previously (Strahl-Bolsinger *et al.*, 1997) except: Sir3 antibodies were cross-linked to protein A-Sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) by using standard methods before ChIP (Harlow and Lane, 1999). 1/50 of the immu-

Table 1. Strains used in this study

Strain	Genotype	Reference
JRY19	<i>MATa his4 leu2 trp1 ura3</i>	Gift from Jasper Rine yeast collection
JRY2234 (W303-1A)	<i>MATa ade2-1, his3-11, 15 leu 2-3,112 trp1-1 ura3-1 can1-100</i>	Gift from Jasper Rine yeast collection
JRY3009 (W303-1B)	<i>MATα ade2-1, his3-11, 15 leu 2-3,112 trp1-1 ura3-1 can1-100</i>	Gift from Jasper Rine yeast collection
CFY616	<i>MATa his4 leu2 trp1 ura3</i>	Gift from Jasper Rine yeast collection
CFY345	JRY3009 <i>HMR-SSa</i>	
CFY762	JRY3009 <i>HMR-SSa sir1Δ::LEU2</i>	
CFY1649	JRY3009 <i>HMR-SSa sir1Δ::TRP1</i>	
CFY1819	JRY3009 <i>HMR-SSa sir3Δ::LEU2</i>	Rusche <i>et al.</i> (2002)
CFY1827	JRY3009 <i>HMR-SSa sir1Δ::LEU2 sir2Δ::TRP1, LEU2::sir2N345A</i>	
CFY1265	JRY2334 <i>HMR-SSa ΔI bar1Δ::HIS3</i>	
CFY2016	JRY2334 <i>HMR-SSa ΔI bar1Δ::HIS3 fkh2Δ::HISG</i>	
CFY527	JRY3009 <i>HMR-SSa sir1Δ::LEU2 fkh1Δ::TRP1</i>	Gibson <i>et al.</i> (2004)
CFY603	JRY3009 <i>HMR-SSa sir1Δ::LEU2 fkh2Δ::HIS3</i>	
CFY1614	JRY3009 <i>HMR-SSa sir1Δ::LEU2 clb1Δ::HIS3</i>	
CFY1124	JRY3009 <i>HMR-SSa sir1Δ::LEU2 clb2Δ::TRP1</i>	
CFY2104	JRY3009 <i>HMR-SSa sir1Δ::LEU2 clb5Δ::kanMx</i>	Gibson <i>et al.</i> (2004)
CFY2279	<i>HMR-SSa sir1Δ::LEU2 clb6Δ::kanMx</i>	
CFY2268	JRY3009 <i>HMR-SSa sir1Δ::LEU2 clb5Δ::HIS3 clb6Δ::kanMx</i>	
CFY2110	JRY3009 <i>HMR-SSa sir1Δ::LEU2 clb5Δ::kanMx fkh1Δ::TRP1</i>	
CFY2446	JRY3009 <i>HMR-SSa sir1Δ::LEU2 CLB5-3xHA</i>	Gibson <i>et al.</i> (2004)
CFY110	JRY3009 <i>HMR-SSa ΔI sir1Δ::TRP1</i>	
CFY2221	JRY3009 <i>HMR-SSa rap1 sir1Δ::TRP1</i>	
CFY2133	JRY3009 <i>HMRΔE</i>	
CFY2071	JRY3009 <i>HMRΔE::ARSH4 sir1Δ::LEU2</i>	Gibson <i>et al.</i> (2004)
CFY2237	JRY3009 <i>HMRΔE::ARS1 sir1Δ::TRP1</i>	
CFY35	JRY3009 <i>HMR-SSa ΔI</i>	
CFY2230	JRY3009 <i>HMR-SSa ΔI sir1Δ::LEU2 clb5Δ::kanMx</i>	
CFY58	JRY3009 <i>HMR-SSa rap1</i>	Gibson <i>et al.</i> (2004)
CFY2218	JRY3009 <i>HMR-SSa rap1 sir1Δ::TRP1 clb5Δ::kanMx</i>	
CFY324	JRY3009 <i>HMRΔE::ARSH4</i>	
CFY2193	JRY3009 <i>HMRΔE::ARSH4 sir1Δ::LEU2 clb5Δ::kanMx</i>	
CFY321	JRY3009 <i>HMRΔE::ARS1</i>	Gibson <i>et al.</i> (2004)
CFY2236	JRY3009 <i>HMRΔE::ARS1 sir1Δ::LEU2 clb5Δ::kanMx</i>	
CFY2481	JRY2334 <i>HMRΔE::ARS1 clb5Δ::kanMx sir1Δ::LEU2 sir2Δ::TRP1</i>	
CFY2613	JRY2234 <i>hmlΔ::URA3 sir2Δ::LEU2</i>	
CFY2615	JRY2334 <i>hmlΔ::URA3 sir2Δ::LEU2 clb5Δ::kanMx</i>	Gibson <i>et al.</i> (2004)
CFY2617	JRY2334 <i>hmlΔ::URA3</i>	
CFY2619	JRY2334 <i>hmlΔ::URA3 clb5Δ::kanMx HMRΔE::ARS1</i>	

Strains were from the laboratory collection or constructed during the course of this study. The strains noted were gifts from or derived from strains from the sources in the Reference column.

nonprecipitated DNA and 1/500 of the total DNA were subjected to 26 cycles of polymerase chain reaction (PCR) by using primers specific for *HMRa* or *ADH4* or the appropriate *ARS* (Gardner and Fox, 2001). Primers to X/Ya, Ya/Z, and boundary elements were as described previously (Rusche *et al.*, 2002). PCR products were separated on a 1% agarose gel, and band intensities were quantified using video densitometry analysis and Labworks analysis software (UVP, Upland, CA).

Protein Immunoblots

To compare levels of Fkh1 expressed from a chromosomal copy of *FKH1* and from a 2 μ plasmid (*FKH1^{hc}*) quantitatively (Figure 2), crude yeast extracts were prepared as described previously (Gardner *et al.*, 1999) from wild-type cells transformed with either empty vector (pRS426; pCF225) or with a 2 μ plasmid expressing *FKH1* (*FKH1^{hc}*; pRS426 with *FKH1*; pCF480) and from a *fkh1 Δ* cells transformed with vector. To avoid distortions in the gel that could affect quantification, lysates from *fkh1 Δ* cells were used to dilute the lysates from *FKH1* or *FKH1^{hc}* cells, ensuring that the same amount of total protein was present in each lane. Four microliters of crude lysates was examined per lane by SDS-polyacrylamide gel electrophoresis (PAGE) by using a 10% acrylamide gel and standard protein blotting methods were used. To determine whether protein transfer to the blot was the same for all lanes, the blot was prestained with Ponceau S before incubation with antibody.

Cell Cycle Arrest and Release Experiments

Cells were grown and harvested in log phase (23 or 30°C) from CAS liquid medium and arrested in G1 with α -factor for 150 min (5 μ M for *bar1 Δ* cells; 25 μ M for *BAR1* cells). The cells were then released from α -factor arrest by

washing with and releasing into fresh medium. Every 15 min, aliquots were harvested for analysis of bud morphology and scored as indicated in Figure 2. Alternatively cells were harvested for analyses of DNA content by flow cytometry as described previously (Weinreich *et al.*, 1999) except that Sytox Green (Invitrogen, Carlsbad, CA) at 1 μ M was used to label DNA.

Two-Dimensional Origin Mapping

2-D origin mapping was performed as described previously (Fox *et al.*, 1995). Cells were grown at 23°C in 2 liters of complete rich medium, or, if cells contained a *URA3* plasmid, at 30°C in 2 liters of CAS medium. DNA from each sample was analyzed for replication intermediates (RIs) by digestion with HindIII for *HMR-E::ARS1* and NcoI for *ARS1*. DNA was enriched for RIs with BND cellulose after restriction digest. DNA was separated in two dimensions and examined by DNA blot hybridization by using primers specific for each region (Fox *et al.*, 1995). Probes were created using Megaprime DNA labeling system (GE Healthcare).

RESULTS

FKH1 is a high-copy suppressor of an *HMRa* silencing defect caused by a *sir1 Δ* mutation (Hollenhorst *et al.*, 2000). The role of *FKH1*, together with *FKH2*, in controlling *CLB2*-cluster transcription raised the possibility that *FKH1*-mediated silencing was related to perturbations of the cell cycle. To address this possibility and better define how Fkh1 mod-

Figure 1. *FKH1^{hc}*-silencing required recruitment of Sir3 and the catalytic activity of Sir2. (A) Steady-state levels of *a1* and *SCR1* RNAs were measured in *MAT α HMR-SSa sir1 Δ* cells (CFY762) harboring a 2 μ plasmid (lane 1, vector; pCF225), a CEN plasmid with *SIR1* (lane 2, *SIR1*-CEN; pCF99), a 2 μ plasmid with *FKH1* (lane 3, *FKH1*-2 μ ; pCF480), or a CEN plasmid with *FKH1* (lane 4, *FKH1*-CEN; pCF943). The average ratio of *a1*/*SCR1* and SD for three independent experiments is indicated below each lane. *FKH1*-CEN also reproducibly enhanced silencing slightly as measured by mating assays (Casey, unpublished data). (B) Sir3 association with *HMR*-SSa and the control locus *ADH4* was measured by ChIP with a monoclonal antibody cocktail against Sir3 (α -Sir3). Three isogenic *MAT α HMR-SSa* strains were analyzed that differed only in terms of their *SIR1* or *SIR3* genotype: *SIR1 sir3 Δ* (CFY1819), *SIR1 SIR3* (CFY345), and *sir1 Δ SIR3* (CFY762). ChIPs were performed on each strain containing either a 2 μ plasmid (vector, gray; pCF225) or a 2 μ plasmid with *FKH1* (*FKH1^{hc}*, black; pCF480). The percentage of specific PCR fragment obtained in the immunoprecipitate was determined by videodensitometry analysis using Labworks analysis software (UVP). Data and standard deviations are reported for three independent ChIP experiments. (C) ChIPs were performed as described in B except that multiple regions within the *HMR*-SSa locus were examined using primer pairs at the positions indicated in the cartoon of *HMR*a above the figure as described previously (Rusche *et al.*, 2002). Two isogenic *MAT α HMR-SSa sir1 Δ* strains were analyzed that differed only in terms of their *SIR2* genotype; *SIR2* (CFY762) or *sir2N345A* (CFY1827). Each strain harbored a plasmid containing *SIR1* (pCF99) or a 2 μ plasmid containing *FKH1* (*FKH1^{hc}*, pCF480).

ulated silencing, we compared high-copy *FKH1*-dependent (*FKH1^{hc}*-dependent) and *SIR1*-dependent silencing at the molecular and genetic levels.

FKH1^{hc}-dependent Silencing of *HMR*a Required Recruitment of Sir3 and the Catalytic Activity of Sir2

MAT α HMR-SSa sir1 Δ yeast cells are unable to mate because the sensitized synthetic silencer (*HMR*-SSa) requires *SIR1* to silence the *a*-mating type genes at *HMR*a (McNally and Rine, 1991). Thus, these cells provide a genetically useful tool for examining *SIR1* function and for identifying other mechanisms capable of transcriptional silencing. Expression of *FKH1* on a 2 μ vector (*FKH1^{hc}*) partially restores silencing to these *sir1 Δ* cells as seen by a reduction in *a1* mRNA expressed from *HMR*-SSa (Hollenhorst *et al.*, 2000; Figure 1A), thus creating *SIR1*-bypass silencing. Notably, even expression of *FKH1* from a CEN plasmid contributed to a reproducible, albeit lower level of *SIR1*-bypass silencing (Figure 1A, lane 4) that was also evident in mating assays (Casey, unpublished data).

To address the mechanism by which *FKH1^{hc}* bypasses the requirement of *SIR1* in silencing *HMR*-SSa, we first determined whether *FKH1^{hc}* reestablished *SIR*-dependent chromatin (requiring *SIR2*–4) in *sir1 Δ* cells (Figure 1B). These experiments were important because in some genetic backgrounds *HMR*a silencing can be established in the absence of Sir2–4 proteins (Rusche and Rine, 2001). To test whether Sir2–4 chromatin was reestablished by *FKH1^{hc}* in *sir1 Δ* cells,

we performed ChIPs with a cocktail of monoclonal antibodies raised against Sir3 (α -Sir3). Sir3 binding requires the other Sir proteins, Sir2 and Sir4, and it is thus a good measure of association of the Sir3-complex (Sir2–4) with a region of chromatin (Hoppe *et al.*, 2002; Luo *et al.*, 2002; Rusche *et al.*, 2002).

These experiments provided evidence that *FKH1^{hc}*-dependent silencing restored assembly of a Sir2–4 chromatin domain at *HMR*a in a substantial fraction of *sir1 Δ* cells. Sir3-directed ChIPs specifically enriched *HMR*-SSa from *SIR1* but not *sir1 Δ* cells, indicating that Sir3-association with *HMR*-SSa required Sir1 as expected based on previous characterizations of *HMR*-SSa (Gardner and Fox, 2001; Bose *et al.*, 2004) (Figure 1B). Significantly, *FKH1^{hc}*-dependent silencing in *sir1 Δ* cells, although less efficient than *SIR1*-dependent silencing (Figure 1A), reestablished measurable Sir3 binding to *HMR*-SSa (Figure 1B). The enrichment of *HMR*-SSa in these experiments required *SIR3* as none was observed in *sir3 Δ* cells, indicating that the Sir3 antibodies had the appropriate specificity (Figure 1B).

In *SIR1*-dependent silencing, Sir2–4 complexes bind regions of *HMR*a beyond the defined silencers (Rusche *et al.*, 2002). This binding to distal regions, termed spreading, requires the deacetylase activity of Sir2, an NAD-dependent histone deacetylase (Denu, 2003). To further examine the molecular nature of *FKH1^{hc}*-dependent silencing, we compared binding of Sir3 to multiple regions within and outside *HMR*-SSa by ChIPs (Figure 1C). Both *SIR1* and *FKH1^{hc}* pro-

(vector; pCF225) or *FKH1* (*FKH1^{hc}*; pCF480). The cells were mixed with an excess of *MATα* cells (CFY616) and 10-fold serial dilutions were plated on medium to select for the growth of *a/α* diploids as described in *Materials and Methods*. The most concentrated samples contained 5×10^3 cells/ μ l, and 8 μ l was analyzed in each spot. Corresponding growth controls indicated that an equal number of cells were being compared between strains (LC; data not shown). (D) Levels of Fkh1 in *MATα HMR-SSa sir1Δ* (CFY1649) cells were determined by protein immunoblot with a polyclonal antibody raised against Fkh1 (α -Fkh1). Cells, harboring either an empty 2 μ plasmid (pRS426; chromosomal; lanes 1–3) or a 2 μ plasmid containing *FKH1* (*FKH1^{hc}*; pCF480; lanes 4–6), were harvested in log-phase growth in liquid CAS medium. Two-fold serial dilutions of each protein extract were made using protein extract from *fkh1Δ* cells as diluents (lane 7) to ensure that each lane contained similar total protein amounts that facilitated accurate quantification of Fkh1 levels. The *fkh1Δ* cells showed no protein band corresponding to Fkh1 in the immunoblot (lane 7). Equal amounts of protein were loaded per lane as determined by Ponceau S staining of the blot before immunoblotting.

duced similar patterns of Sir3 binding over the *HMRa* locus. Moreover, efficient Sir3 binding required the Sir2 deacetylase activity. Specifically, Sir3 binding was examined in cells harboring a mutant version of *SIR2*, *sir2N345A*, that produces a catalytically defective version of Sir2 (Imai et al., 2000). In these *sir2N345A* mutant cells, regardless of whether the silencing was mediated through *SIR1* or *FKH1^{hc}*, Sir3 failed to associate with regions distal to the silencer, although it retained some ability to bind to the silencer (Figure 1C). Together, these data provided evidence that *FKH1^{hc}*- and *SIR1*-dependent silencing resulted in similar Sir2–4 silent chromatin domains at *HMRa* that initiated at the *HMR-E* silencer.

FKH1^{hc} Prolonged the Cell Cycle and Attenuated the CLB2 mRNA Expression Peak

Several observations provide evidence against the possibility that *FKH1^{hc}* affects *HMR* silencing directly. First, Fkh1-3xHA does not bind *HMRa* as measured by ChIPs (Simon et al., 2001; Hollenhorst, unpublished data), and tethering a Fkh1-Gal4 fusion protein directly to *HMRa* via an engineered Gal4 binding site fails to restore silencing (Fox, unpublished data), although Gal4-fusions with other proteins known to function directly at the silencer (e.g., Sirs) or to recruit *HMRa* to the nuclear periphery do (Chien et al., 1993; Lustig et al., 1996; Andrulis et al., 1998, 2004). Second, certain cell cycle perturbations enhance silencing in strains containing mutations in *SIR1* or within the *HMR-E* silencer (Axel-

rod and Rine, 1991; Laman et al., 1995; Ehrenhofer-Murray et al., 1999), and *FKH1* and its closest paralogue *FKH2* are direct regulators of transcription of the *CLB2*-cluster of genes (reviewed in Breeden, 2000) that includes *CLB2*, the major G2/M phase cyclin. Third a deletion of *FKH1* (*fkh1Δ*) causes a small but measurable effect on cell cycle progression and *CLB2* mRNA levels (Hollenhorst et al., 2000). Therefore, we hypothesized that *FKH1^{hc}* would cause changes in cell cycle progression and *CLB2* expression. To test this idea, cell bud index and *CLB2* mRNA levels were monitored during cell cycle arrest-and-release experiments (Figure 2, A and B).

MATα bar1Δ cells harboring either an empty 2 μ plasmid (vector) or a 2 μ plasmid with *FKH1* (*FKH1^{hc}*) were arrested in G1 with α -factor, released from arrest into fresh medium, and at 15-min intervals the cell population was monitored by counting the number of cells in G1(no buds), S (small buds), and G2/M (large buds) (Figure 2A). *CLB2* mRNA levels were also measured at each interval by RNA blot hybridization (Figure 2B). Compared with the vector control, *FKH1^{hc}* entered S phase with similar kinetics but it exhibited a lengthened time in S phase, consistent with the slower growth rate of *FKH1^{hc}* cells (Figure 2A). For example, although both the control cells (vector) and *FKH1^{hc}* cells entered S phase 60 min after α -factor release, at 120 min after α -factor release >60% of the *FKH1^{hc}* cells seemed to remain in S phase or early G2/M phase, whereas only ~30% of the control cells did (Figure 2A). Differences between the vector

Figure 2. *FKH1^{hc}* prolonged the cell cycle and attenuated the *CLB2* mRNA expression peak. (A) *MATα bar1Δ::HIS3* (CFY1265) cells harboring either a 2 μ plasmid (vector; pRS426), a 2 μ plasmid containing *FKH1* (*FKH1^{hc}*; pCF480), or a deletion of *FKH2* (*fkh2Δ*; CFY2016) and a 2 μ plasmid (vector) were harvested in log phase from medium lacking uracil and arrested in G1 with α -factor. The cells were then released from α -factor arrest into fresh medium, and every 15 min aliquots were harvested for analysis of bud morphology or *CLB2* mRNA levels (see B). Unbudded cells were scored in G1 phase, small-budded cells were scored in S phase, and large-budded cells were scored in M phase. A representative graph for each experiment is shown. (B) The ratio of *CLB2* mRNA to *SCR1* RNA was determined for each of the strains and time points indicated in A. Total RNA (15 μ g) as determined by absorbance at 260 λ was analyzed per lane, *CLB2* mRNA and *SCR1* RNA were probed independently, and the signals quantified by Phosphor-Imager analysis. Data from a representative experiment are shown. (C) Mating analysis of *MATα HMR-SSa sir1Δ* cells containing either wild-type *FKH2* (CFY762) or a deletion of *FKH2* (*fkh2Δ*; CFY603) and harboring 2 μ plasmids with *SIR1* (*SIR1^{hc}*; pCF345), no insert

control and the *FKH1^{hc}* cells were also evident at the level of *CLB2* mRNA expression (Figure 2B). In particular, although the temporal cycling pattern of *CLB2* mRNA was similar in both types of cells, the *FKH1^{hc}* cells produced lower levels of *CLB2* mRNA relative to *SCR1* loading control that were most evident during the peak of *CLB2* mRNA expression. The overall trend was that *FKH1^{hc}* dampened the amplitude but not the timing or duration of the *CLB2* mRNA wave.

The aforementioned data were consistent with the idea that reduced *CLB2* levels might explain the effect of *FKH1^{hc}* on *HMR* silencing, but additional analyses of *fkh2Δ* cells provided evidence against this idea. Fkh2 is the primary transcriptional activator of *CLB2*-cluster genes (Koranda *et al.*, 2000; Kumar *et al.*, 2000; Pic *et al.*, 2000; Zhu *et al.*, 2000; Reynolds *et al.*, 2003), and a deletion of *FKH2* (*fkh2Δ*) also prolongs the cell cycle and reduces *CLB2* mRNA expression (Hollenhorst *et al.*, 2000). In addition, in *fkh2Δ* cells the level of Fkh1 bound to the promoters of *CLB2*-cluster genes increases as measured by ChIPs (Hollenhorst *et al.*, 2001). Therefore, one reasonable explanation for the effects of *FKH1^{hc}* on silencing was that the increased levels of Fkh1 outcompeted Fkh2 for binding to *CLB2*-cluster promoters, thus mimicking an *fkh2Δ*-like phenotype. This explanation made two predictions. First, *FKH1^{hc}* should perturb the cell cycle and *CLB2* expression similarly to *fkh2Δ*. Second, *fkh2Δ* should enhance silencing in *MATα HMR-SSa sir1Δ* cells similarly to *FKH1^{hc}*.

Direct tests of these predictions provided evidence against this explanation. Deletion of *FKH2* (*fkh2Δ*) caused a cell cycle defect distinct from that caused by *FKH1^{hc}*; *fkh2Δ* cells showed delayed entry into S phase, but the duration of the remainder of the cell cycle was similar in *fkh2Δ* and wild-type cells (Hollenhorst *et al.*, 2000; see Figure 2A, *fkh2Δ*). In terms of *CLB2* mRNA expression, *fkh2Δ* reduced levels of *CLB2* mRNA to an even greater extent than *FKH1^{hc}*. In addition and in contrast to *FKH1^{hc}*, *fkh2Δ* delayed the peak of *CLB2* mRNA expression. Thus, compared with *FKH1^{hc}*, *fkh2Δ* had qualitatively similar but quantitatively distinct effects on the timing and amplitude of *CLB2* mRNA expression. Nevertheless, as was true for *FKH1^{hc}*, the overall effect of *fkh2Δ* was to reduce *CLB2* mRNA levels.

Therefore, we tested, in side-by-side comparisons whether *fkh2Δ* had any effect on *HMRα* silencing in *MATα HMR-SSa sir1Δ* cells or affected the efficiency of *FKH1^{hc}*-dependent silencing (Figure 2C). As determined by semiquantitative mating assays *fkh2Δ* had no substantial effect on silencing in *MATα HMR-SSa sir1Δ* cells nor did *fkh2Δ* enhance *FKH1^{hc}*-dependent silencing (Figure 2C). Therefore, *FKH1^{hc}* was not restoring silencing in *sir1Δ* cells by creating an *fkh2Δ*-like phenotype. These data also provided evidence that reductions in *CLB2* mRNA expression during the cell cycle were not sufficient to establish *FKH1^{hc}*-silencing because *fkh2Δ* cells reduced *CLB2* mRNA levels but failed to establish *SIR1*-bypass silencing. Thus, reduced levels of *Clb2* were insufficient to explain *FKH1^{hc}*-dependent silencing.

The modest levels of Fkh1 produced in *FKH1^{hc}* cells were also consistent with the conclusion that *FKH1^{hc}* cells were not achieving *SIR1*-bypass silencing by competing for Fkh2 target sites. Fkh1 levels were compared in wild-type and *FKH1^{hc}* cells by using semiquantitative immunoblotting with a polyclonal antibody against Fkh1. These experiments revealed that *FKH1^{hc}* led to an approximately fourfold increase in the steady-state levels of Fkh1 protein (Figure 2D), a relatively modest increase that was unlikely to be sufficient to compete with Fkh2 for bona fide Fkh2 target sites because Fkh2 binding, but not Fkh1 binding, is enhanced >10-fold through cooperative interactions with Mcm1 (Hollenhorst *et*

al., 2001). Thus, a modest increase in Fkh1 was sufficient to cause both the cell cycle and silencing phenotypes. These data, combined with earlier analyses of *fkh1Δ* cells that produced cell cycle and *CLB2* mRNA expression profiles that were the mirror opposite of those produced by *FKH1^{hc}* (Hollenhorst *et al.*, 2000) also provided evidence that the phenotypes detected in *FKH1^{hc}* cells reflected a normal role for Fkh1 in vivo.

Genetic Analyses Revealed that FKH1 and CLB5 Were Functioning in a Common Pathway

Although the aforementioned data indicated that reduced *CLB2* expression was insufficient to explain the *FKH1^{hc}* phenotypes, they did not address whether *CLB2* was necessary. In addition, the ability of *FKH1^{hc}* to perturb the cell cycle (Figure 2) and the documented connection between cell cycle perturbations and enhanced silencing meant that the cell cycle perturbation caused by *FKH1^{hc}* might be necessary for and/or closely associated with *FKH1^{hc}*-dependent silencing. Therefore, we asked whether deletions of specific *CLB* genes affected *FKH1^{hc}*-mediated silencing in *MATα HMR-SSa sir1Δ* cells by using RNA blot hybridization of *a1* mRNA and mating assays (Figure 3). We focused on analyzing the effects of deletions in the G2/M phase cyclins, *CLB1* and *CLB2*, because these genes are under direct *FKH* control. In addition, we analyzed the effects of deletions in the S phase cyclins *CLB5* and *CLB6* because *clb5Δ* enhances silencing in *sir1Δ* cells (Laman *et al.*, 1995) and the *FKH1^{hc}* cell cycle phenotype was consistent with the idea that *FKH1^{hc}* elongated S phase (Figure 2).

In terms of the G2/M phase cyclins, *CLB2* but not *CLB1* was required for *FKH1^{hc}*-dependent silencing (Figure 3, A and B). Specifically, *FKH1^{hc}* failed to enhance silencing in *MATα HMR-SSa sir1Δ clb2Δ* cells as determined by *a1/SCR1* RNA ratios (Figure 3A, compare vector and *FKH1^{hc}* *a1/SCR1* RNA ratios for *clb2Δ* and *CLB*). Mating assays provided independent evidence for this conclusion (Figure 3B). In contrast, *FKH1^{hc}* enhanced silencing in *MATα HMR-SSa sir1Δ clb1Δ* cells based upon both RNA blot hybridization (Figure 3A) and mating assays (Figure 3B). Thus direct measurements of the *a1/SCR1* RNA ratios and mating assays provided complementary evidence that *CLB2* was necessary for establishing *FKH1^{hc}*-dependent *SIR1*-bypass silencing at *HMR*. However, although *CLB2* was necessary it was not sufficient; *clb2Δ* failed to enhance silencing in *MATα HMR-SSa sir1Δ* as measured by *a1/SCR1* ratios (Figure 3A) or mating assays on cells grown either under selective conditions to retain plasmids (Figure 3B) or under rich conditions (Figure 3C). These data were consistent with the conclusion from the cell cycle experiments described in Figure 2 that reductions in *CLB2* were insufficient to create the *FKH1^{hc}* phenotype. Thus, even if the effect of *FKH1^{hc}* on *CLB2* expression contributes to *FKH1^{hc}*-dependent silencing, or *CLB2* functions in conjunction with *FKH1^{hc}*, other *CLB2*-independent targets or mechanism(s) also must be relevant to *FKH1^{hc}*-dependent silencing.

An earlier study demonstrated that *clb5Δ* could enhance silencing of *sir1Δ* cells (Laman *et al.*, 1995). Although *CLB2* is a direct target of Fkh2, our data provided evidence that reductions in *CLB2* were insufficient to account for *FKH1^{hc}*-dependent silencing. Therefore, we postulated that pathways or target genes affected by the S phase cyclin *CLB5* might be more immediately relevant to *FKH1^{hc}*-dependent *SIR1*-bypass silencing. In particular, if *FKH1^{hc}* and *clb5Δ* achieved *SIR1*-bypass silencing through modulation of a common pathway, then *FKH1^{hc}* and *clb5Δ* should cause quantitatively similar degrees of *SIR1*-bypass silencing, and,

Figure 3. Genetic analyses revealed that *FKH1* and *CLB5* were functioning in a common pathway. (A) *a1/SCR1* RNA ratios were determined by quantitative RNA blot hybridization for an isogenic series of *MATα HMR-SSa sir1Δ* strains that differed in terms of their *CLB* genotype (*CLB* (CFY762); *clb1Δ* (CFY1614); *clb2Δ* (CFY1124); *clb5Δ* (CFY2104); *clb6Δ* (CFY2279) and in terms of the plasmid they harbored (vector [pRS426], *SIR1^{hc}*[pCF34], and *FKH1^{hc}*[pCF48]). A normalized ratio of *a1* mRNA/*SCR1* RNA is shown on the y-axis with a value of 1.0 assigned to the *a1/SCR1* ratio calculated for *CLB* cells transformed with a plasmid harboring *SIR1* in one experiment. (B) Mating assays in an isogenic series of *MATα HMR-SSa sir1Δ* strains that differed in terms of their *CLB* genotype and in terms of the plasmid they harbored as described in A. In these experiments *clb5Δclb6Δ* (CFY2268) cells were also analyzed. (C) Because mating assays were more robust on rich media, the same cells analyzed in B were also analyzed in the absence of

exogenous plasmids after growth in YPD. (D) An isogenic series of *MATα HMR-SSa sir1Δ* cells differing in their *CLB5* and *FKH1* genotypes (*CLB5 FKH1* [CFY762]; *clb5Δ FKH1* [CFY2104]; and *clb5Δ fkh1Δ* [CFY2120]) were assessed for silencing by mating assays as described above. Mating was assessed after growth of *MATα* cells on either synthetic CAS medium to retain a *URA3* plasmid (vector) or rich medium in the absence of a plasmid (no plasmid) before selection for diploids. (E) The same cells were assessed for silencing by RNA blot hybridization of *a1* mRNA. The *a1* mRNA/*SCR1* RNA ratio was normalized as in (A). The *CLB5 FKH1^{hc}* cells were CFY762 containing a 2μ plasmid with *FKH1* (pCF480). The other cells tested in this experiment contained an empty 2μ plasmid (pCF225).

in addition, the two genes should fail to show additive interactions in terms of this phenotype. Therefore, we tested whether *clb5Δ* cells affected *FKH1^{hc}*-dependent silencing and whether *fkh1Δ* cells affected *clb5Δ*-dependent silencing.

As expected, *clb5Δ* but not *clb6Δ* enhanced silencing in *MATα HMR-SSa sir1Δ* cells (Figure 3A; compare vector *a1/SCR1* RNA ratios for *clb5Δ* and *clb6Δ* to *CLB*), consistent with the findings of a previous study (Laman et al., 1995). Based on RNA blot hybridization, *clb5Δ* and *FKH1^{hc}* enhanced *HMR-SSa* silencing to similar degrees, and *FKH1^{hc}* enhanced levels of silencing in *clb5Δ* only slightly but reproducibly (Figure 3A). The mating assays were consistent with the RNA blot hybridizations; *FKH1^{hc}* provided for some level of silencing that was independent of *CLB5* (Figure 3B, *clb5Δ*, compare vector and *FKH1^{hc}*), but it was unable to enhance silencing of *clb5Δ sir1Δ* cells as well as it enhanced silencing of *CLB5 sir1Δ* cells. These data were consistent with the idea that *FKH1^{hc}* and *clb5Δ* were impinging on a common pathway or target protein, to different extents or through different mechanisms, that was limiting for *HMRa* silencing in *sir1Δ* cells.

In contrast to *CLB5* and *CLB2*, the *CLB6* genotype had no substantial impact on *FKH1^{hc}*-dependent silencing; *clb6Δ* did not enhance silencing in *MATα HMR-SSa sir1Δ* cells nor did it perturb the ability of *FKH1^{hc}* to enhance silencing in these cells (Figure 3A, *clb6Δ*), consistent with an earlier analysis of the effect of *clb6Δ* on silencing (Laman et al., 1995). Interestingly, within the detection ranges of *a1* mRNA, *clb6Δ* consistently reduced silencing by a small amount (Figure 3A, compare vector in *clb6Δ* to *CLB* cells), perhaps by enhancing the relative activity of *CLB5* in vivo. Analyses of cells containing deletions of both *CLB5* and *CLB6* provided evidence that *CLB5* was the S phase cyclin with the most significant impact on *FKH1^{hc}*-silencing; *clb5Δ clb6Δ* produced silencing phenotypes similar to those produced by *clb5Δ* alone (Figure 3, B and C).

In these studies mating efficiencies were ~10-fold lower and somewhat more variable when measured for cells containing plasmids and grown under selective growth condi-

tions. Therefore, as an independent and additional assessment of the effect of *CLB* genotype on silencing, mating assays were performed on the same set of yeast cells described above that were instead grown on rich nonselective growth medium and in the absence of plasmids (Figure 3C). The data from these experiments were consistent with those obtained with cells harboring plasmids and grown on selective medium (compare to Figure 3B, vector).

These data provided evidence that *FKH1^{hc}* affected silencing similarly to a *clb5Δ* and might function in a common

Figure 4. *FKH1^{hc}* did not affect *Clb5* levels nor did *CLB5* genotype affect *Fkh1* levels. (A) Levels of *Clb5-3xHA* protein were determined by protein immunoblot with anti-HA antibodies in *CLB5* (CFY762), *clb5Δ* (CFY2104), and *CLB5-3xHA* (CFY2446) cells transformed with either an empty 2μ plasmid (vector) or a 2μ plasmid containing *FKH1* (*FKH1^{hc}*; pCF480). We analyzed 0.25 OD (optical density at 600 λ) cell equivalents per 4× lane, and 2× and 1× lanes contained twofold and fourfold reductions in that amount of extract, respectively. (B) Levels of *Fkh1* protein were determined by protein immunoblot with anti-Fkh1 antibodies in *CLB5* (CFY762) and *clb5Δ* (CFY2104) cells, as indicated (lanes 1–4) and in *clb5Δ* cells transformed with an empty 2μ plasmid (vector [pCF225]; lanes 5–7) or *CLB5* cells transformed with a 2μ plasmid containing *FKH1* (*FKH1^{hc}* [pCF480]; lanes 8–10). *fkh1Δ* cells (CFY527) containing vector (pCF225) were analyzed as a negative control (lane 11). We analyzed 0.25 OD cell equivalents per 4× lane and 2× and 1× were twofold and fourfold reductions, respectively, in that amount of extract.

Figure 5. *FKH1^{hc}* or *clb5 Δ* could use nonsilencer replication origins in place of the *HMR-E* silencer. (A) Structures and sequences of the E-silencer variations used in these experiments. *HMR Δ E::ARS* contains either *ARS1* or *ARS4* in the orientation shown as a complete substitute for *HMR-SS*. (B) Mating assays were performed with an isogenic series of *MAT α sir1 Δ* strains that differed in terms of their *HMR-E* (E) and/or *HMR-I* (I) silencers at *HMRa*, as indicated to the left of the figure and described in the text. The cells contained the following silencers: *HMR-SSa* (CFY1649) in place of an 800-base pair deletion that includes *HMR-E* (McNally and Rine, 1991; Palacios DeBeer and Fox, 1999); *HMR-SSa* as described above, and a 335-base pair deletion of that includes *HMR-I* (CFY110) (Fox *et al.*, 1995); *HMR-SSa* with a mutation in the Rap1 binding site (CFY2221) (McNally and Rine, 1991); a deletion of an 800-base pairs region, including *HMR-E* (CFY2133); deletion of an 800-base pairs region including *HMR-E* replaced with *ARS4* (CFY2071) or *ARS1* (CFY2237) as shown in A. Each strain was transformed with a 2 μ plasmid containing *SIR1* (*SIR1^{hc}* [pCF345]), an empty 2 μ plasmid (vector [pCF225]), or a 2 μ plasmid containing *FKH1* (*FKH1^{hc}* [pCF480])). Mating was assessed by drop tests in which 10-fold dilutions of *MAT α* cells being tested were mixed with an excess of *MATa* cells (CFY616) and grown on medium that selected for diploids retaining the plasmids. (C) Sir3-directed ChIPs were performed on *MAT α sir1 Δ HMR Δ E::ARS1 + HMR-I* cells (CFY2071) or on *MAT α sir1 Δ HMR-SSa + HMR Δ I* (CFY35) cells transformed with either *SIR1* or *FKH1^{hc}* as indicated. (D) Sir3-directed ChIPs were used to determine Sir3 association with a number of ARSs compared with *HMR-SSa* in *sir1 Δ* cells transformed with vector, *SIR1*, or *FKH1^{hc}*. (E) Mating assays were performed with an isogenic series of *MAT α* cells that differed in terms of their silencers at *HMRa* (as indicated on left of figure and described in A) and their *SIR1* or *CLB5* genotypes (as indicated at the

pathway because *FKH1^{hc}* only slightly enhanced the levels of silencing achieved in *clb5Δ* cells (Figure 3, A and B). A simple model was that *FKH1^{hc}* somehow reduced the levels or activity *CLB5*. In this model, *CLB5* function in silencing formally occurred downstream of *FKH1* and if correct, a deletion of *FKH1* (*fkh1Δ*) would have little or no effect on silencing achieved by a deletion of *CLB5* (*clb5Δ*). To test this idea, we compared silencing of *HMR-SSa* in *sir1Δ* cells that were either *clb5Δ FKH1* or *clb5Δ fkh1Δ* (Figure 3D). *clb5Δ*-dependent silencing was consistently ~10-fold better in *FKH1* cells compared with *fkh1Δ* cells as measured by mating assays (Figure 3D). As described above, mating assays were ~10-fold more efficient when cells were grown on rich medium in the absence of plasmids before selective mating. Nevertheless, regardless of how cells were grown before the mating assay (harboring an empty plasmid and grown on selective media to retain the plasmid [vector] or grown on rich media in the absence of a plasmid [no plasmid]), the mating assays revealed that *clb5Δ*-dependent silencing was reduced ~10-fold in *fkh1Δ* cells (Figure 3D). However, *clb5Δ* still clearly enhanced silencing in *fkh1Δ* cells. This effect was also evident in RNA blot hybridization experiments (Figure 3E); chromosomal *FKH1* was required for the full level of *clb5Δ*-silencing. Thus these data provided evidence that chromosomal levels of *FKH1* could affect *HMRa* silencing. Furthermore, the epistasis analyses provided evidence that *CLB5* and *FKH1* impinged on *HMR* silencing at least in part through a common pathway or target protein.

***FKH1^{hc}* Did Not Affect *Clb5* Levels nor Did *CLB5* Genotype Affect *Fkh1* Levels**

One possible explanation for some of the genetic data described above was that the *FKH1^{hc}* reduced levels of *Clb5* protein and thereby contributed to a *clb5Δ*-like phenotype. Conversely, perhaps *clb5Δ*-enhanced silencing was mediated by increases in the steady-state levels of *Fkh1* protein. To test whether *FKH1^{hc}* affected *Clb5* protein levels, *Clb5*-3xHA was monitored by protein immunoblotting in *MATα sir1Δ HMR-SSa* cells harboring either an empty 2μ plasmid (vector) or a 2μ plasmid containing *FKH1* (*FKH1^{hc}*) (Figure 4A). These data provided evidence that *FKH1^{hc}* had no effect on *Clb5*-3xHA levels. To test the converse possibility that *clb5Δ*-dependent silencing worked through increasing *Fkh1* levels, *Fkh1* was monitored in *CLB5* or *clb5Δ* cells by protein immunoblotting (Figure 4B). *Fkh1* levels were unaffected by a *clb5Δ* (Figure 4B, compare levels of *Fkh1* in *CLB5*, lanes 1 and 2) to *clb5Δ* cells (lanes 3 and 4) and compare levels of *Fkh1* in *clb5Δ* cells transformed with an empty plasmid (vector, lanes 5–7) to *CLB5* cells transformed with *FKH1^{hc}* (lanes 5–11). These data provided evidence that *clb5Δ*-dependent silencing did not result from increased levels of *Fkh1* and that *FKH1^{hc}*-dependent silencing did not result from reduced levels of *Clb5*.

***FKH1^{hc}* Could Use Nonsilencer Replication Origins in Place of the *HMR-E* Silencer**

As shown above, *SIR1*- and *FKH1^{hc}*-dependent silencing differed in terms of their relationship to the major G2 and S

phase cyclin genes, indicating that at some level these two forms of silencing relied on different sets of molecular interactions to ultimately establish the same *SIR2*–4 chromatin structures. It is well established that *Sir1* performs its silencing function primarily through its interactions with the *HMR-E* silencer, an element with a distinct arrangement of protein binding sites, most notably a binding site for ORC juxtaposed next to a binding site for *Rap1* (Fox and McConnell, 2005) (Figure 5A, *HMR-SS*). Both ORC and *Rap1* play important roles at the *HMR-E* silencer, with ORC recruiting *Sir1* and *Rap1* recruiting *Sir3* and *Sir4* through direct protein–protein interactions. To gain further insight into how *SIR1*- and *FKH1^{hc}*-dependent silencing differed in terms of their ability to establish *Sir2*–4 chromatin, their silencer requirements were compared.

Appropriate *MATα sir1Δ* yeast cells that varied only in terms of their silencers at *HMRa* were transformed with an empty 2μ vector (vector) or 2μ plasmids harboring *SIR1* (*SIR1^{hc}*) or *FKH1* (*FKH1^{hc}*). The transformed cells were compared for their ability to silence *HMRa* in mating assays (Figure 5B). As expected, *SIR1^{hc}*-dependent silencing did not require the presence of the *HMR-I* silencer (Figure 5B, line 2, *SIR1^{hc}*). In contrast, *FKH1^{hc}*-dependent silencing was abolished by deletion of *HMR-I* (Figure 5B, line 2, *FKH1^{hc}*). Thus, the two forms of silencing showed different dependencies on *HMR-I*. However, it is important to note that *SIR1^{hc}*- (and *SIR1* at chromosomal levels) is more effective than *FKH1^{hc}* at silencing *HMRa* (Figure 1A), and that deletion of *HMR-I* weakens silencing under a variety of conditions (Fox *et al.*, 1995; Rivier *et al.*, 1999). Therefore, the different requirement for *HMR-I* might simply reflect the ability of *SIR1* to silence more robustly than *FKH1^{hc}*. To test this possibility, we examined mutant silencers that we knew were defective in *SIR1*-dependent silencing for their ability to support *FKH1^{hc}*-dependent silencing (Figure 5B, lines 3–5). Importantly, these experiments revealed that several silencers existed that were better at supporting *FKH1^{hc}*- than *SIR1*-dependent silencing.

First, a mutation in the *Rap1* binding site (Figure 5B, *rap1*) in *HMR-SSa* abolishes silencing even when *SIR1* is overexpressed (McNally and Rine, 1991; Figure 5B, line 3, *SIR1^{hc}*), presumably because *Rap1* is so critical for stabilizing the binding of *Sir3* and *Sir4* proteins (Moretti and Shore, 2001). It was surprising, given that *FKH1^{hc}* silencing clearly required *Sir3* and *Sir4*, that *FKH1^{hc}* could restore some silencing to cells that contained a mutation in the *Rap1* binding site within *HMR-SSa*, whereas *SIR1* could not (Figure 5B, line 3). It was clear that both *FKH1^{hc}*- and *SIR1^{hc}*-dependent silencing required some feature of an E-silencer because an entire deletion of E (*HMRΔE*) failed to support either forms of silencing (Figure 5B, line 4). This experiment established that *FKH1^{hc}* differed from *SIR1* in terms of a requirement for a *Rap1* site in the *HMR-E* silencer.

As mentioned above, the role for ORC in *SIR1*-dependent silencing is well established (Fox and McConnell, 2005). *Sir1* shows a high specificity for silencer-bound ORCs *in vivo*; *Sir1* cannot be detected at several nonsilencer replication origins such as *ARS1*, even though such elements bind ORC (Gardner and Fox, 2001). Furthermore, substitution of the entire E-silencer with the nonsilencer replication origins *ARS1* or *ARSH4* (Figure 5A, sequences of “*HMR-E*” silencers used in Figure 5B) fails to support *SIR1*-dependent silencing (Fox *et al.*, 1995). Even when *SIR1* was overexpressed *ARS1* or *ARSH4* failed to function as effective “E-silencers” (Figure 5B, lines 5 and 6, *SIR1^{hc}* and vector). Thus, it was unexpected that efficient *FKH1^{hc}*-dependent silencing could be achieved with either *ARS1* or *ARSH4* as substitutes for the

Figure 5 (cont). top of the figure). The various strains used in these experiments were as follows: *SIR1 CLB5* with *HMR-SSa* (CFY345); *HMR-SSaΔI* (CFY35); *HMR* containing an 800-base pair deletion that includes *HMR-E* replaced with either *ARSH4* or *ARS1* (CFY325 and CFY321, respectively). *sir1Δ CLB5* versions with the silencers as listed for *SIR1 CLB5* (CFY1649, CFY110, CFY2071 and CFY2237); and *sir1Δ clb5Δ* with versions with the same silencers as listed (CFY2104, CFY2230, CFY2193, CFY2236).

HMR-E-silencer (Figure 5B, lines 5 and 6, *FKH1^{hc}*). What was particularly striking was that the level of *FKH1^{hc}*-dependent silencing achieved in cells in which either *ARS1* or *ARSH4* served as the HMR-E silencer was equivalent to that achieved in the HMR-SSa silencer background that was used in the original isolation of *FKH1* as a high-copy suppressor of a *sir1Δ* mutation (Hollenhorst *et al.*, 2000) (Figure 5B, line 1). That is, for *FKH1^{hc}*-dependent silencing and in contrast to *SIR1*-dependent silencing, either *ARS1* or *ARSH4* was as good as HMR-E in terms of functioning as an E-silencer.

As an independent assessment of the unexpected silencer requirements described above, a Sir3-directed ChIP experiment was performed (Figure 5C). These data were consistent with the silencing data; Sir3 could bind to HMRΔE::ARS1 in *FKH1^{hc}* cells but not *SIR1* cells. In contrast, Sir3 could bind to HMR-E within an HMR locus that lacked HMR-I in *SIR1* cells but not in *FKH1^{hc}* cells. These ChIP data added supporting evidence to the conclusion that *FKH1^{hc}* cells could use an ordinary ARS in place of HMR-E for Sir2–4-dependent silencing.

The data described above indicated that in *FKH1^{hc}* cells, the Sir3 protein was exhibiting some affinity for *ARS1* or *ARSH4* when present at HMR in place of HMR-E. To test whether Sir3 was exhibiting some affinity for these and other ARSs in their native locations, we used ChIP to examine Sir3 binding to a number of ARSs in *sir1Δ* (vector), *SIR1*, and *sir1Δ FKH1^{hc}* cells (Figure 5D). These data revealed that the level of Sir3 binding detected by ChIP to ARSs at their native location was only slightly above background and far below the level of Sir3 that could be detected by ChIP at HMR. Because HMR-I was critical for *FKH1^{hc}*-dependent silencing (Figure 5B, lines 7 and 8), these data were not surprising because HMR-I is an element unique to the HMR locus. Thus, although *FKH1^{hc}* may enhance the affinity for Sir3 at some nonsilencer ARSs, stable binding of Sir3 to these elements that is detectable by ChIP requires features unique to HMR, including but not necessarily only HMR-I.

In summary, *FKH1^{hc}*- and *SIR1^{hc}*-dependent silencing differed measurably in terms of silencer requirements optimal for producing Sir2–4-dependent chromatin at HMRA. Most remarkably, *FKH1^{hc}* could establish Sir2–4 chromatin by using *ARS1* or *ARSH4* in place of the HMR-E silencer, whereas *SIR1^{hc}* could not.

clb5Δ Also Could Use *ARS1* or *ARSH4* as a Substitute for HMR-E

The data concerning the relationship between *clb5Δ* and *FKH1^{hc}* in terms of *SIR1*-bypass silencing (Figure 3) raised the possibility that *clb5Δ* and *FKH1^{hc}* exerted their effects on silencing through a common pathway. If this were true, then *clb5Δ*-dependent silencing should share the same unusual silencer requirements as *FKH1^{hc}*. To test this idea, we determined whether *clb5Δ*-dependent silencing could be achieved with the same mutant silencers that effectively established *FKH1^{hc}*-dependent silencing (Figure 5E). As was true for *FKH1^{hc}*-dependent silencing, *clb5Δ*-dependent silencing required HMR-I (Figure 5E, lines 1 and 2). More strikingly, however, *clb5Δ* cells were able to use *ARSH4* or *ARS1* as effective substitutes for the HMR-E silencer (Figure 5E, compare lines 3 and 4 with line 1 for *sir1Δclb5Δ* column), whereas *SIR1* could not (Figure 5E, compare lines 3 and 4 with line 1 for *SIR1CLB5* column). In summary, *clb5Δ* and *FKH1^{hc}* could each establish *SIR1*-independent silencing at HMRA by using replication origins as substitutes for HMR-E.

clb5Δ or *FKH1^{hc}* Could Suppress HMRΔE::ARS1 Origin Activity

The relationship between origin firing and *SIR2*–4-dependent chromatin is, in general, antagonistic; wherever *SIR2*–4 silent chromatin forms, origin firing is suppressed (Stevenson and Gottschling, 1999; Zappulla *et al.*, 2002). Conversely, origin firing at the HM loci is inefficient regardless of *SIR* genotype, in part because the silencers are intrinsically ineffective replication origins (Dubey *et al.*, 1991; Palacios DeBeer and Fox, 1999; Vujcic *et al.*, 1999; Sharma *et al.*, 2001; Palacios DeBeer *et al.*, 2003).

CLB5 is the S phase cyclin most critical for activating replication origins that fire late in S phase (Donaldson *et al.*, 1998; Gibson *et al.*, 2004). Thus, in *clb5Δ* cells early S phase origins fire efficiently, whereas late S phase origins do not, and overall S phase progression is slowed because replication forks emanating from early origins must now replicate a greater fraction of the genome. In the *ARS1* substitution experiment described above (Figure 5), the entire HMR-E silencer was deleted and replaced with *ARS1*. In its native location, *ARS1* fires efficiently during early- to mid-S phase and is *CLB5* independent (Donaldson *et al.*, 1998; Gibson *et al.*, 2004). Given this collection of observations, we asked

Figure 6. *clb5Δ* or *FKH1^{hc}* suppressed HMRΔE::ARS1 origin activity. (A) 2-D origin mapping experiments were performed in *MATα HMRΔE::ARS1 sir1Δ* cells that contained either *CLB5* (CFY2237) or *clb5Δ* (CFY2236). Origin activity at HMRA was assessed with an HMR-specific probe, whereas origin activity at the native *ARS1* locus was assessed with an *ARS1*-specific probe. Two different exposures of HMRΔE::ARS1 blots are shown (dark and light) to facilitate comparison of this origin activity in *CLB5* versus *clb5Δ* cells. (B) 2-D origin mapping experiments with the cells used in A were repeated side by side with *clb5Δ sir2Δ* cells (CFY2481). (C) The DNA content in actively dividing populations of isogenic yeast cells that varied in terms of their *SIR2* or *CLB5* genotypes as indicated was determined by flow cytometry. (D) 2-D origin mapping experiments were performed in *MATα HMRΔE::ARS1 sir1Δ CLB5* cells containing either an empty 2μ plasmid (vector; pCF225) or a 2μ plasmid with *FKH1* (*FKH1^{hc}*; pCF480).

how the *ARS1* origin that replaced *HMR-E* was affected in *clb5Δ* cells by performing 2-D origin mapping experiments on *ARS1* at its native location and at *HMR* where it substituted for the *HMR-E* silencer (*HMRΔE::ARS1*) (Figure 6).

These 2-D origin-mapping experiments revealed that in wild-type (*CLB5*) cells *ARS1* functioned as a robust, efficient origin whether it was present at its native location (*ARS1*) or at *HMR*, as a substitute for the deleted *HMR-E* silencer (*HMRΔE::ARS1*) (Figure 6A, *CLB5*). However, only *ARS1* at *HMRa* (*HMRΔE::ARS1*) was sensitive to *CLB5* genotype; *clb5Δ* reduced the ability of *ARS1* to function as an origin when it substituted for *HMR-E* (*clb5Δ*, *HMRΔE::ARS1*) whereas it had no effect on *ARS1* firing at its native locus (*clb5Δ*, *ARS1*) (Figure 6A, *clb5Δ*).

Two different mechanisms could contribute to *clb5Δ*-mediated suppression of origin firing by *HMRΔE::ARS1*. First, *clb5Δ* reestablished silencing at *HMRΔE::ARS1* and thus silencing, or Sir2–4 chromatin formation, might be inhibiting firing by *HMRΔE::ARS1*. If this scenario were true, then a deletion of *SIR2* (*sir2Δ*), a gene essential for silencing, should restore efficient firing by *HMRΔE::ARS1* in *clb5Δ* cells as is observed for other cases of Sir2–4-mediated origin suppression (Zappulla *et al.*, 2002). Second, some other *SIR*-independent feature of this chromosomal region might suppress origin firing by *HMRΔE::ARS1* in *clb5Δ* cells. As discussed above, the *HM* loci fail to allow for more robust origin firing by their native silencers even in *sir* mutant cells that cannot silence. To distinguish between these mechanisms, we performed 2-D origin-mapping experiments on *HMRΔE::ARS1* in *clb5Δ sir2Δ* cells, thereby precluding the ability of *clb5Δ* to reestablish Sir2–4 chromatin at *HMRa*. The *sir2Δ* mutation failed to restore robust firing to the *HMRΔE::ARS1* origin in *clb5Δ* cells (Figure 6B), suggesting that *SIR2–4* chromatin was not the cause of *CLB5*-sensitive firing by *HMRΔE::ARS1*. However, a complicating factor in interpreting these experiments was that large replication forks were also consistently underrepresented in *sir2Δ clb5Δ* cells compared with *CLB5 SIR2* or *clb5Δ SIR2* cells raising the possibility that *sir2Δ* was having additional effects on genome replication. Nevertheless, the replication pattern generated for *HMRΔE::ARS1* in *sir2Δ clb5Δ* cells indicated that *sir2Δ* did not simply restore robust replication to *HMRΔE::ARS1*. Thus, the dependence on *CLB5* that *ARS1* acquired when it replaced *HMR-E* was independent of Sir2–4 chromatin formation.

A recent study raises the possibility that *SIR* genes, in particular *SIR2*, can negatively regulate many replication origins in the yeast genome, most probably at the level of pre-RC assembly (Pappas *et al.*, 2004). Therefore, in addition to examining the effect of a *sir2Δ* on *CLB5* sensitivity of a particular origin (Figure 6B), the effect of a *sir2Δ* on DNA replication was examined in *clb5Δ* cells by flow cytometry (Figure 6C). In the simplest case, if the majority of *CLB5*-sensitive origins were negatively regulated by *SIR2*, then a *sir2Δ* mutation would suppress the defects of a *clb5Δ*. However, examination of the DNA content in asynchronous populations of cells indicated that a *sir2Δ* did not reestablish a wild-type *CLB5* DNA-content profile. In addition, cell-cycle arrest and release experiments indicated that *sir2Δ clb5Δ* cells showed a delayed entry into M phase compared with *sir2Δ CLB5* cells, suggesting that many of the global replication defects caused by a *clb5Δ* were not rescued by a *sir2Δ* (Fox, unpublished data). Together, these data provided evidence that *SIR2* likely did not regulate many *CLB5*-sensitive origins, because *sir2Δ* did not suppress a *clb5Δ*, consistent with our data from directed analyses of *HMRΔE::ARS1* in *sir2Δ clb5Δ* cells (Figure 6B).

The genetic and cell cycle data concerning *FKH1^{hc}* were consistent with the notion that *clb5Δ* and *FKH1^{hc}* functioned in part through a shared pathway or target to modulate *HMRa* silencing. As another test of this shared relationship, we determined the effect that *FKH1^{hc}* had on origin firing by *HMRΔE::ARS1* (Figure 6D). Significantly, *FKH1^{hc}* also reduced the efficiency of origin firing by *HMRΔE::ARS1*, producing a molecular phenotype similar to that caused by a *clb5Δ* (Figure 6A, compare vector with *FKH1^{hc}*), albeit the effect of *FKH1^{hc}* on origin firing by *HMRΔE::ARS1* was less drastic than the effect of *clb5Δ* on this origin. This observation was consistent with *clb5Δ* having a more significant impact on cell growth compared with *FKH1^{hc}* (Casey, unpublished data). Nevertheless these data supported the idea that *FKH1^{hc}* and *clb5Δ* bypassed the requirement for *SIR1* in establishing Sir2–4 chromatin through effects on a similar target or pathway that can impinge on or is associated with S phase progression.

DISCUSSION

In this report, we addressed the hypothesis that Fkh1, an evolutionarily conserved transcription factor implicated in transcriptional control of *CLB2*-cluster genes (reviewed in Breeden, 2000; Futcher, 2000), modulated Sir2–4 chromatin formation at *HMRa* through an effect on cell cycle progression. Several lines of evidence presented here linked *FKH1* and the major S phase cyclin *CLB5* to a common pathway(s) or target(s). First, *FKH1^{hc}* and *clb5Δ* each bypassed the requirement for *SIR1* in silencing *HMRa* to a similar extent. Second, *FKH1^{hc}* and *clb5Δ* each had the unexpected ability to use a replication origin, *ARS1* or *ARSH4*, as an effective substitute for the *HMR-E* silencer in silencing *HMRa*. And third, *FKH1^{hc}* and *clb5Δ* each reduced origin firing by the normally robust *ARS1* origin when it substituted for the *HMR-E* silencer. Together, this set of shared phenotypes supports a model in which *FKH1* and *CLB5* impinge on a common pathway relevant to both the control of late-firing replication origins and the cell-cycle sensitive formation of Sir2–4-repressive chromatin at *HMRa*.

It was conceivable that *FKH1* and *CLB5* were part of a linear pathway in which *FKH1* affected expression of *CLB5* or vice versa as such a relationship could explain the similar phenotypes caused by *FKH1^{hc}* and *clb5Δ*. Fkh1 is, after all, a transcription factor that could directly or indirectly affect levels of Clb5. However, direct analyses of Fkh1 and Clb5 protein levels provided evidence against the simplest version of this possibility. In particular, *FKH1^{hc}* had no effect on the steady-state levels of Clb5, and, conversely, *clb5Δ* had no effect on either chromosomally produced levels of Fkh1 or the ability to overexpress Fkh1 to levels sufficient to establish *HMRa* silencing. Moreover, double mutant analyses also provided evidence against a simple linear relationship between *FKH1* and *CLB5*. If *FKH1^{hc}* enhanced *HMRa* silencing entirely through *CLB5*, we would have predicted that *FKH1^{hc}* would fail to enhance silencing at all in *clb5Δ* cells. However, *FKH1^{hc}* did enhance silencing of *clb5Δ* cells to a small but reproducible extent as determined by both mating assays and RNA blot hybridizations. Similarly, although *clb5Δ* cells required a chromosomal copy of *FKH1* for maximal *HMRa* silencing, these cells still silenced *HMRa* more effectively than corresponding *CLB5* cells. A simple model consistent with these data describes a forked pathway in which *FKH1* and *CLB5* have opposing regulatory roles on common target(s) (T) in this pathway or at least targets with shared functions (Figure 7). That is, the final activity of T depends on contributions from both *FKH1* and *CLB5*. Given

Figure 7. Two models for how *FKH1* and *CLB5* modulated origin activity and silencing at *HMRA* (A) *FKH1* positively regulates (arrow) and *CLB5* negatively regulates (flat head) a common pathway or target referred to as T. T in turn negatively regulates origin firing by normally late-firing replication origins (or origins placed within chromosomal domains that cause late firing). Robust origin firing is incompatible with the assembly of Sir2–4 chromatin. (B) *FKH1* and *CLB5* regulate target T as described in A, but origin firing is not causally linked to Sir2–4 chromatin assembly. Rather firing by late replication origins is negatively regulated, whereas Sir2–4 is positively regulated by target T.

what is known about the proteins encoded by *FKH1* and *CLB5*, a reasonable idea is that *FKH1* controls the levels of T, whereas *CLB5* negatively regulates the activity of T through phosphorylation (e.g., by the Clb5/Cdc28 kinase). Target T in turn impinges on the activity of late-firing replication origins (or at least a subset of these origins) and the assembly of Sir2–4 chromatin.

Role of *CLB2*

Our genetic and molecular studies provided evidence for how *FKH1* and *CLB5* were related, but based on our current data it is difficult to determine how the M phase cyclin *CLB2* fits into the model (Figure 7). Because *clb2Δ* can rescue some *HMR* silencing defects (Laman *et al.*, 1995) and *Fkh1* is a direct transcriptional regulator of *CLB2*, it was unexpected that a *clb2Δ* failed to mimic the silencing phenotype caused by *FKH1^{hc}*. Although *FKH1^{hc}* clearly reduced levels of *CLB2* mRNA, the inability of *fkh2Δ* cells, which also reduced the levels of *CLB2* mRNA, as well as *clb2Δ* cells to establish *SIR1*-bypass silencing provided strong evidence that reductions in *CLB2* were not sufficient to explain *FKH1^{hc}*-dependent silencing. The observation that *CLB2* was required for *FKH1^{hc}*-silencing could be because *CLB2* acts positively on target T through an *FKH1*-independent mechanism, and that the effects of *FKH1^{hc}* on *CLB2* mRNA levels were unrelated to how *FKH1* established *SIR1*-bypass silencing at *HMR*. Alternatively reduced levels of *CLB2* may be an essential component of *FKH1^{hc}* silencing, but the precise pattern and amplitude of *CLB2* mRNA expression caused by *FKH1^{hc}* is itself critical, such that a *clb2Δ* or the distinct pattern of *CLB2* reduction caused by *fkh2Δ* does not suffice to create *SIR1*-bypass silencing. A better understanding of precisely how *Fkh1* controls *CLB2* expression and/or a *FKH*-independent mode for regulating *CLB2* mRNA levels would help address this issue.

Sir2–4 Chromatin Formation and the Cell Cycle

It is well established that perturbations in the cell cycle can enhance or reduce the efficiency of silencing assessed in certain mutant backgrounds, but the mechanisms behind these effects are unclear (Axelrod and Rine, 1991; Laman *et al.*, 1995; Ehrenhofer-Murray *et al.*, 1999). In addition, studies in which expression of individual *SIR* genes is controlled with inducible promoters or temperature-sensitive mutations indicate that multiple phases of the cell cycle may modulate different distinct steps required for the ultimate

assembly of functionally silenced chromatin (Lau *et al.*, 2002; Kirchmaier and Rine, 2006; Matecic *et al.*, 2006). For example, studies using a temperature-sensitive *sir3* allele or an inducible version of *SIR1* provide evidence that Sir2–4 can rebind *HMRa* in G1 phase yet fail to establish silencing (Lau *et al.*, 2002; Kirchmaier and Rine, 2006). Rather silencing, as defined by repression of transcription of the *a1* gene, is not established until passage through the following S and M phases. Thus some of the cell cycle-regulated steps that ultimately lead to a silenced transcriptional state at *HMRa* must occur downstream of the binding of Sir proteins to *HMRa*. It was conceivable, therefore, that the effects of *FKH1^{hc}* and by inference those of several cell cycle mutants on *HMRa* silencing were mediated through similar steps and that they did not affect the efficiency of Sir binding to chromatin. Our use of the synthetic silencer (*HMR-SSa*) (McNally and Rine, 1991) allowed us to address this question. Unlike natural *HMRa* where substantial levels of Sir3 binding can be detected by ChIP even in *sir1Δ* cells (Rusche *et al.*, 2002), Sir3 binding to *HMR-SSa* was completely dependent on Sir1. This feature allowed us to demonstrate unequivocally that *FKH1^{hc}* reestablished Sir2–4 nucleation at *HMR-E* even in the absence of Sir1. Thus *FKH1^{hc}*, and by inference *clb5Δ*, bypass the requirement for *SIR1* in *HMRa* silencing at least in part by promoting efficient Sir2–4 nucleation at a silencer.

Linking Late Origin Firing and *HMRa* Silencing

The close association between silencers and DNA elements with the capacity to function as origins at the *HM* loci has spurred studies over the past two decades aimed at trying to explain the mechanistic nature of this link. Recent data focused on *HMR-E* has raised the possibility that there exists a competing relationship between the roles of ORC at origins and silencers and that the close association between these elements is a by-product of the dual functions of ORC (Palacios DeBeer and Fox, 1999; Palacios DeBeer *et al.*, 2003; McConnell *et al.*, 2006). In this model for the silencer–origin relationship, *HML* and *HMR* silencers are weak or inactive origins at least in part because robust and/or early origin firing inhibits silencing. Therefore, it was unexpected that *ARS1* and *ARSH4*, two independent and robust replication origins with no documented silencer activity, could act as effective substitutes for the *HMR-E* silencer. Indeed in a silencer-trap screen in which the *2μ* *ARS* was shown to possess weak silencer activity, direct tests of *ARS1* demonstrated it could not substitute for *HMR-E* in silencing *HMRa* (Grunweller and Ehrenhofer-Murray, 2002). This observation was confirmed in this report because neither *SIR1* cells nor *sir1Δ* cells could use *ARS1* as effectively as they used *HMR-E* for silencing *HMRa*. However, what was new and surprising was that *ARS1* could function as effectively as *HMR-E* in *sir1Δ* cells that also harbored *FKH1^{hc}* or *clb5Δ*. Thus, *ARS1* became an effective *SIR1*-independent silencer under conditions that also, notably, reduced the ability of *ARS1* to act as a replication origin. Therefore, this observation was consistent with the idea that robust origin and silencer activity were incompatible and that target T enhanced Sir2–4 chromatin formation because it inhibited origin firing by *HMRΔE::ARS1* (Figure 7A). Sir2–4 chromatin did not create a late-firing *CLB5*-sensitive origin at *HMRa* because even in the absence of silent chromatin formation (*sir2Δ*) the *HMRΔE::ARS1* origin remained suppressed in *clb5Δ* cells. However, another explanation consistent with the observations reported here was that late origin firing and silencer function were not linked causally but instead could be regulated in opposing directions by the same target T

(Figure 7B). This second version of the model is actually more consistent with the observation that *clb5Δ clb6Δ* cells could silence as effectively as *clb5Δ CLB6* cells, because *clb5Δ clb6Δ* cells restore a normal S phase (Schwob and Nasmyth, 1993), suggesting that suppression of late origin firing is not essential for Sir2–4 chromatin formation in *clb5Δ* cells.

Because silencing of *HMRa* weakened by silencer mutations can be enhanced by artificially tethering the mutant *HMRa* locus to the nuclear membrane (Andrulis *et al.*, 1998), we propose that a reasonable candidate for target T is a cell cycle-regulated protein(s) or protein complex that modulates the association of *HMRa* with the nuclear periphery, where Sir2, -3, and -4 proteins are concentrated (Palladino *et al.*, 1993; Maillet *et al.*, 1996; Taddei *et al.*, 2005). In addition, late-firing replication origins show an enhanced association with the inner nuclear membrane in both yeast and mammals (Dimitrova and Gilbert, 1999; Heun *et al.*, 2001), which also supports the hypothesis that target T represents a protein or complex that affects the association of *HMRa* with the nuclear membrane and that this association in turn can affect both origin firing and silencing.

In this interpretation, *ARS1* and *ARSH4* were equivalent to an *HMR-E* silencer weakened by mutation. That is, these origins acted as “proto-silencers” (Boscheron *et al.*, 1996), too weak to function on their own but efficient enough when combined with other Sir-stabilizing influences, such as another weak proto-silencer, *HMR-I*. This must mean that *ARS1* and *ARSH4* possess an intrinsic affinity, albeit substantially reduced compared with the native silencer, for the Sir2–4 protein complex. Thus even in the absence of Sir1, Sir2–4 proteins must have some inherent affinity for origins, or at least a component of origins, such as ORC. Consistent with this possibility, a recent study provides evidence that *SIR2*, and less effectively *SIR3* and *SIR4*, can affect the activity of many genomic origins, suggesting that these proteins may interact with many nonsilencer origins in the genome (Pappas *et al.*, 2004). This interaction may be fleeting and thus not detectable by ChIP. However, when this weak interaction is combined with additional weak Sir interaction sites provided by the *HMR-I* silencer and a higher concentration of Sir proteins that might be provided by association with the nuclear membrane, the result is sufficient stabilization energy that allows Sir2–4 proteins to establish silencing. Thus *HMRa* silencing in *clb5Δ* (or *FKH1^{hc}*) cells measured here might be effectively trapping transient interactions that occur throughout the genome between Sir2–4 complexes and origins.

In summary, the cell cycle perturbation caused by a *clb5Δ* (or *FKH1^{hc}*) associated with enhanced silencing could involve an increase in the levels and/or activity of a target T. Although it is possible that target T acts to suppress late-firing replication origins that in turn directly interfere with silencers (Figure 7A), it is also possible that both silencers and late-firing replication origins are connected to the common target T that in turn regulates the function of each of these DNA elements in opposing directions and to different degrees (Figure 7B).

The FKH1^{hc} Effect Involved Gene Targets and/or Target Sites Distinct from Fkh2 Targets

In terms of gene expression *FKH1^{hc}* had a modest but reproducible dampening effect on the wave of *CLB2*-mRNA expression during the cell cycle. These data were consistent with the idea, supported by earlier analysis of *fkh1Δ* cells (Hollenhorst *et al.*, 2000) that Fkh1 acts as a negative regulator of *CLB2* transcription at least during the stages of the cell cycle when *CLB2* mRNA expression peaks. Because

Fkh2 is a positive regulator of *CLB2* transcription, it was somewhat surprising that an *fkh2Δ* affected neither silencing on its own nor the ability of *FKH1^{hc}* to enhance silencing. Therefore, *FKH1* must act through gene targets and/or binding sites distinct from those controlled by *FKH2* to cause the phenotypes documented here. Whether *FKH1^{hc}* enhances Fkh1 association with its normal targets or also causes significant binding to new targets, and whether one or more of these targets is also critical for the *clb5Δ*-like phenotype remains to be determined.

Forkhead transcription factors (forkhead box, FOX in metazoans) have evolutionary conserved roles in cell cycle regulation, proliferation and differentiation, and modest changes in forkhead transcription factor levels have clear and pivotal effects on complex multicellular phenomena such as organogenesis and disease states (Carlsson and Mahlapuu, 2002; Gaudet and Mango, 2002). Studies of the effects of Fkh1 dosage in the single-celled yeast should contribute to unraveling how forkhead transcription factors contribute to remodeling a eukaryotic transcriptional program. Transcriptional silencing serves as sensitive barometer for examining this issue and others associated with cell cycle regulation of chromosome structure and function.

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