The role of SIR4 in the establishment of heterochromatin in the budding yeast *Saccharomyces cerevisiae*

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ABSTRACT

Heterochromatin in the budding yeast *Saccharomyces cerevisiae* is composed of polymers of the SIR (Silent Information Regulator) complex bound to nucleosomal DNA. Assembly of heterochromatin requires all three proteins of the Sir complex: the histone deacetylase Sir2, and histone binding proteins Sir3 and Sir4.

Heterochromatin establishment requires passage through at least one cell cycle, but is not dependent on replication. Inhibition of chromatin modifying enzymes may be a mechanism for how cells limit assembly. Dot1 dependent methylation of H3K79 is suggested to inhibit *de novo* assembly.

Halving the levels of Sir4 in cells causes a loss of silencing, suggesting that Sir4 protein abundance regulates the assembly of heterochromatin. We examine *de novo* assembly using a single cell assay. Half the level of Sir4 affects establishment, but not the maintenance, of silencing at *HM* loci. Additional Sir4 accelerates the rate of assembly. Epistasis analysis suggests that Dot1 dependent chromatin modification may act upstream of Sir4 abundance. We hypothesize that *dot1Δ* mutants speed assembly by disrupting telomeric heterochromatin, which liberates Sir4 to act at the *HM* loci. Deletion of *YKU70*, which specifically disrupts telomeric silencing, also speeds *de novo* assembly, without altering the methylation of histone H3.

Consistent with our model, we have shown that Sir4 abundance falls during pheromone and stationary phase arrests after which several cell cycles are required before silencing can be reestablished.

ACKNOWLEDGEMENTS

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And to Marc Larin, who has been with me every step of this journey. You ran with me when I needed speed and held me when I needed a rest. I look forward to many new adventures together.

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LIST OF ABBREVIATIONS

5-FOA	5-fluoroorotic acid
APC	anaphase promoting complex
BAH	bromo adjacent homology
CEN	centromeric plasmid
ChIP	chromatin immunoprecipitation
Dbox	R-X-X-L-X-X-X-X-N “Destruction box” APC binding
DNA	deoxyribonucleic acid
DNAPolII	DNA polymerase II
DOT	disruptors of telomeres
EUROSCARF	European *Saccharomyces cerevisiae* archive for functional analysis
FITC	fluorescein isothiocyanate
FSHD	Facioscapulohumeral muscular dystrophy
G0	stationary phase of the cell cycle
G1	gap 1 phase of the cell cycle
G2/M-phase	gap 2 / mitosis of the cell cycle
GAL	galactose
HM	hidden MAT locus
HML	hidden MAT left
HMR	hidden MAT right
HO	Homothallic switching endonuclease
HP1	heterochromatin protein 1
HU	hydroxyurea
MAP	mitogen activated protein kinase
MAT	mating type locus
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide

O-AADPR	o-acetyl ADP ribose
ORC	origin recognition complex
PBS	phosphate buffered sodium
PCR	polymerase chain reaction
PEV	position effect variegation
rDNA	ribosomal DNA locus
RENT	regulator of nucleolar silencing and telophase
RNAi	RNA interference
S-phase	synthesis phase of the cell cycle
SAS	something about silencing
SCF	Skp, Cullin, F-box containing
SIR	silent information regulator
START	start of the cell cycle, commitment to cell division
Su(Var)	suppressor of variation
TBS-T	tris buffered sodium with tween-20
TPE	telomere position effect
YEP	yeast extract peptone
YPD	yeast extract peptone dextrose
YPR	yeast extract peptone raffinose
α-factor	alpha factor, the secreted mating hormone of α cells

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INTRODUCTION

Why yeast

The budding yeast *Saccharomyces cerevisiae* is a valuable model system used by scientists for decades. *S. cerevisiae* is a single cellular eukaryotic fungus which is highly proliferative with a generation time of approximately 90 minutes and can produce colonies of genetic clones from a single cell. It contains a relatively small genome on 16 chromosomes with approximately 6600 genes and roughly 12Mb of genetic information. The entire genome of *S. cerevisiae* has been sequenced and published (Cherry et al., 1997).

Because of its status as a eukaryotic cell, *S. cerevisiae* models many facets of the general eukaryotic system, such as the processes of cell division, transcription, translation, and other biochemical processes. Budding yeast also provides unique insights into such things as polarized cell growth, cell division and cytokinesis through the mating and budding processes, and cell cycle commitment through the ability to arrest cells using chemical or physiological methods at various points in the cell cycle. Such arrests include relevant time points such as G1 prior to START where the cell makes the decision to commit to cell division or enter stationary phase (α-factor or nutrient arrest) (Duntze et al., 1970; Throm and Duntze, 1970), in S-phase by limiting protein synthesis (hydroxyurea) (Slater, 1973), or during G2/M phase (nocodazole)(Kunkel, 1979).

Why study heterochromatin formation

A powerful example of the need to regulate and change the expression of certain locations of the genome is found in *Plasmodium falciparum*, the parasite responsible for

malaria. In order to evade the immune system of the host, the parasite is able to regulate the expression of a class of genes known as *var* genes which are responsible for antigen presentation on the host cell surface (Petter et al., 2011). The *var* genes are located at the telomeres and are generally kept silent with only one *var* gene expressed at a time (Petter et al., 2011). Histone deacetylation by a Sir2 homologue, similar to that seen in the formation of budding yeast heterochromatin, is essential for the expulsion of the *Pf*H2A.Z histone variant from the telomeres that are to be silenced and for the formation of the silent chromatin structure at the location of the *var* genes (Petter et al., 2011).

The ability to maintain silencing in budding yeast is an adaptation that allows cells to switch their haploid mating type to take advantage of a bi-modal growth pattern to adapt to periods of environmental stress. Silent copies of the mating type information (held at *HML* and *HMR)* provided a genetic template for the genes at the expressed *MAT* mating type locus to be exchanged with (Astell et al., 1981; Nasmyth and Tatchell, 1980), such that the cells can mate and, through meiosis and sporulation, exchange genetic variants that may provide a survival advantage.

In humans, there are many diseases that are caused by a mis-regulation of gene expression related to heterochromatic structure. One example is Facioscapulohumeral muscular dystrophy (FSHD), which leads to loss of silencing of the telomere of chromosome 4 and aberrant expression of genes located there, resulting in asymmetrical paralysis and muscle wasting on one side extending from the face to the upper arm of patients (Hahn et al., 2010). Heterochromatin formation has also been implicated in cancer progression, as tumour suppressor genes are silenced and unable to function to prevent cancerous cell growth (Hahn 2010). Treatment with inhibitors of DNA methyltransferases

or histone acetyltransferases may be a future therapeutic option however it introduces the complex question of maintaining the useful marks of silencing in normal cells while eliminating the offending marks that are causing the cancer (reviewed in(Hahn et al., 2010; Hall et al., 2002)).

MATING AND PHEROMONE RESPONSE

How do yeast mate

Saccharomyces cerevisiae can exist as a haploid (1n) or diploid (2n) cell. The common laboratory strains are maintained as haploids for their ease of genetic manipulation as well as the stability of genetic information by not experiencing recombination as seen in diploid cells. However haploids can be easily mated and will readily form diploid cells (Figure 1). In the laboratory the ability to mate and sporulate yeast is exploited in creating new and novel strains through the combination of mutations and in an assay described further in the methods section, to allow the tracking of silencing in single cells.

a vs α cells

Saccharomyces cerevisiae diploid cells have an a/α phenotype while haploids are either **a** cells or α cells. Whether a cell is **a** or α depends on the information that is being expressed from the Mating Type (*MAT)* locus. If the cell is expressing the **a** mating type information it will be phenotypically **a,** mate as an **a** cell, and produce **a**-factor which leads to the pre-start arrest of nearby α cells (Wilkinson and Pringle, 1974). Alternatively, if a cell is expressing α information from the *MAT* locus it will be phenotypically α, mate as an

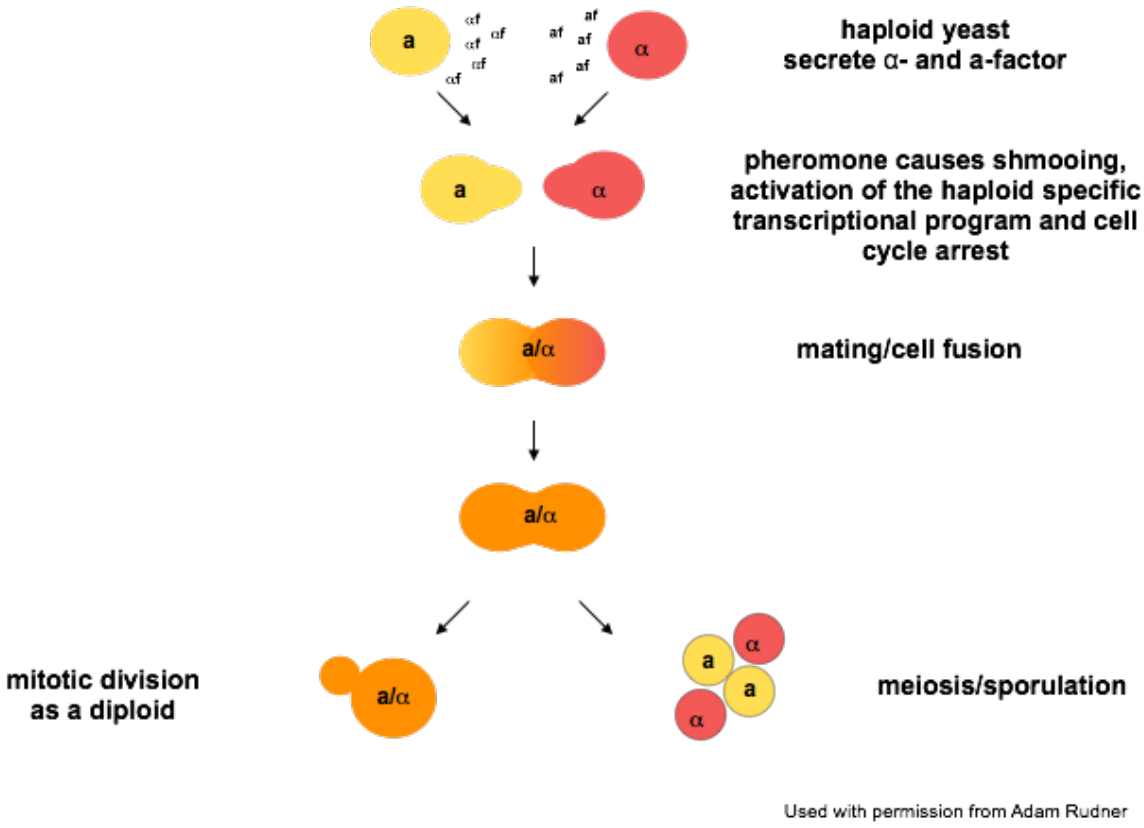

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Figure 1: Yeast can mate and undergo meiosis
Yeast cells can exist as haploid a or α cells, which secrete either **a** or α pheromone. Binding of the pheromone to its cognate receptor on cells of the opposite mating type triggers cell cycle arrest prior to start of the cell cycle, polarization, formation of a mating projection called a shmoo along the pheromone concentration gradient, and induction of a mating specific transcriptional program. Two shmoos of cells of opposite mating types can mate by fusing to form a diploid zygote. The diploid can then enter the cell cycle and divide as a diploid or, upon environmental cues that promote sporulation (low nitrogen, among others), can initiate meiosis and form 4 haploid spores: 2 a spores and 2 α spores.

α cell, and produce the pheromone α-factor that arrests **a** cells before start of the cell cycle. (Throm and Duntze, 1970). An **a** cell can mate with an α cell to produce a 2n **a**/α diploid and under favourable conditions can be sporulated through meiosis to form 4 new haploids: 2 **a** cells and 2 α cells.

In addition to the *MAT* locus there exists in the genome on the same chromosome silent copies of both of the **a** and α information to permit mating type switching in wild yeast strains (Klar et al., 1985). The silent copies are held in the Hidden Mat Right (*HMRa*) and Hidden Mat Left (*HMLα*) loci. The ability to change mating type is important for wild type yeast as it facilitates mating and gene transfer through a population of sister cells within the same colony. The HO endonuclease is used to form a double stranded break at the *MAT* locus (Kostriken et al., 1983; Strathern et al., 1982). The locus is repaired by using either *HML* or *HMR* as a template for the genetic information to be replaced at the *MAT* locus, with a preference towards the opposite mating type (Klar et al., 1982). In our laboratory strains the HO endonuclease has been deleted, ensuring that there is no mating type interconversion during our experiments. It is important that the *HM* loci remain repressed because expression of both **a** and α mating type information produces a non-mater phenotype similar to that of a diploid cell (Haber, 2012).

HMR codes for 2 genes, **a**1 and **a**2, while *HML* codes for α1 and α2. (Reviewed in (Haber, 2012)) The **a**1 gene functions only in conjunction with the *α2* transcript to repress haploid specific transcription in a diploid cell. The ***a2*** gene has no known function though it has been proposed that it may function similarly to **a**1 in repressing haploid specific transcription in conjunction with *α2* (Giesman et al., 1991). α1 is a transcription factor promoting the transcription of α specific genes (Bruhn and Sprague, 1994; Hagen et al.,

1993; Klar, 1987) needed for the α mating type. α2 has 2 roles: to repress ***a1/2*** gene expression in a haploid α cell and to form a repressor complex with **a**1 to repress haploid specific gene expression in diploids (Smith and Johnson, 1992).

When a cell expresses neither **a** nor α mating type information the default situation is for the cell to behave as an **a** cell (Osborne et al., 2009). The genes required to be expressed in response to α-factor and to mate as an **a** cell are expressed so long as *α2* is not being transcribed. This is unlike in an α cell where α2 is needed to actively repress **a** specific haploid gene expression. **a**1/2 are degraded rapidly when not required for diploid specific repression of α haploid specific genes and have no known function in the promotion of the **a** specific haploid phenotype (Haber, 2012).

Mating type switching

Laboratory strains are engineered to be a stable **a** or α haploid cell line, however wild type *S. cerevisiae* are able to take advantage of the ability to switch mating type and are termed homothallic. A tightly regulated process, mating type switching allows a cell to change its mating type in order to mate a nearby cell that is of the same mating type.

The extensive homology between the mating type loci was found by Nasmyth *et al* when they revealed via linkage analysis that *MATa* and *MATα* do indeed correspond to the silent copies located at *HML* and *HMR* and that there is enough homology that switching could occur (Nasmyth and Tatchell, 1980). The boundaries of the segments that are able to switch were later defined (Astell et al., 1981). The mechanism of switching is both simple and elegant. The *HO* endonuclease mediates a double stranded DNA break within the *MAT*

locus (Kostriken et al., 1983; Strathern et al., 1982) which is repaired by utilizing the template within the silent mating type cassettes (Strathern et al., 1982). It is the deletion of this endonuclease that renders laboratory strains heterothallic, or incapable of changing their mating type. The elegance of this system of mating type conversion lies in the frequency with which the cell chooses the *opposite* mating type cassette to perform the repair. It was shown that regardless of the genetic content of the locus, *MATα* cells consistently repaired with *HMR* and *MATa* cells consistently repaired with *HML*, implying that there is a distinct directionality to mating type switching to maximize the chances of a successful mating type conversion (Klar et al., 1982).

SILENCING AT THE MATING TYPE LOCI

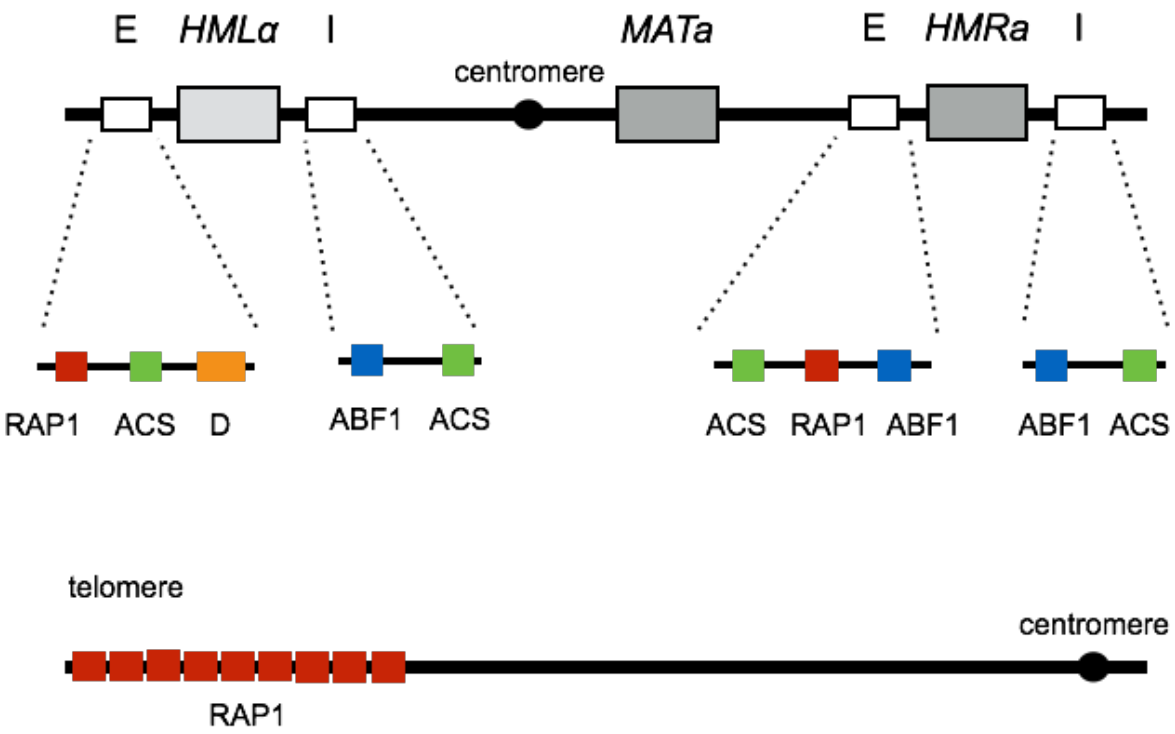
HM locus silencing

Silencing at the silent mating type loci involves the SIR complex in addition to several other factors. The silent mating type loci are called the HM loci for Hidden *MAT* Left (*HML*) and Hidden *MAT* Right (*HMR*) for their location on chromosome 3 in relation to the MAting Type (*MAT*) locus (Figure 2). *HML* contains the alpha (α) mating type genes while *HMR* contains the **a** mating type genes in silenced copies (Nasmyth and Tatchell, 1980). The role of the silenced copies is to permit the mating type switching of daughter cells to form a population of cells that are of both mating types (Nasmyth and Tatchell, 1980). Due to the importance of maintaining the cell's mating identity it is essential that the *HM* loci be kept silent. In light of this, silencing is more stable at the *HM* loci than it is at the telomeres since the *HM* loci contain specific silencer structures with binding sites for additional Sir protein recruiting factors as compared to the telomeres (Bell et al., 1993;

Mahoney and Broach, 1989; McNally and Rine, 1991; Micklem et al., 1993; Shore and Nasmyth, 1987; Sussel and Shore, 1991).

Structure of the E and I silencers

The silencers at *HML* and *HMR* consist of an *E*ssential and an *I*mportant (*E* and *I*) silencer flanking the region upstream and downstream of each site respectively (Feldman 1984). These silencers contain binding sites for Abf1, Rap1, and ORC (Bell et al., 1993; Mahoney and Broach, 1989; McNally and Rine, 1991; Micklem et al., 1993; Shore and Nasmyth, 1987; Sussel and Shore, 1991) (Figure 2). At *HML* deletion of either *E* or *I* causes no effect on silencing, though deletion of both leads to a near complete loss of silencing (Mahoney and Broach, 1989). This is in contrast to *HMR,* where the *I* site is not sufficient to maintain silencing in the absence of the *E* silencer (Mahoney and Broach, 1989). The silencing at the *HM* loci nucleates similarly to at the telomeres, however there are additional factors involved. Abf1 binding and Rap1 binding are required for proper Sir protein association and the function of the silencer (Moretti et al., 1994). ORC binds the DNA and is bound in turn by Sir1 which acts as a bridging protein to recruit Sir4 (Bose et al., 2004; Chien et al., 1993; Triolo and Sternglanz, 1996). The contributions of each set of binding sites are not equal and deletion of one site doesn't lead to complete derepression, though deletion of two of the sites leads to *HMR* derepression (Zhang et al., 2012). Rap1 binding sites make the largest contribution at *HMR-E*, followed by Orc1 and Abf1 sites respectively to the ability to establish silencing, while at *HML-E* the largest contributions are made by Orc1 and Abf1 binding (Zhang et al., 2012). A tRNA located at *HMR* may impose the cell cycle dependence of silencing, as its deletion relieves the cell cycle


E
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MATa
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HMRa
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RAP1
ACS
D
ABF1
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ACS
RAP1
ABF1
ABF1
ACS
telomere
centromere
RAP1

Figure 2: Structure of the E and I silencers
The structure of the E (essential) and I (important) silencers of *HML* and *HMR* are shown. Binding sites for ORC (on the ACS), Abf1, and Rap1 are illustrated, along with the relative positioning of the *HMLα, MAT,* and *HMRa* loci on chromosome III. Telomere ends bound by Rap1 form a telomeric silencer.

dependence and insertion at *HML* imposes the dependence for establishment of silencing (Lazarus and Holmes, 2011).

SILENCING AND SUBUNITS OF THE SIR COMPLEX

SIR complex mediated silencing

The Silent Information Regulator (SIR) complex is responsible for silencing at the mating type loci and at telomeres in *S. cerevisiae* (Aparicio et al., 1991; Laurenson and Rine, 1992; Moazed, 2001; Rine and Herskowitz, 1987; Rusche et al., 2003). The complex is composed of 3 main components: Sir2, Sir3, and Sir4 (Aparicio et al., 1991; Rine and Herskowitz, 1987; Rudner et al., 2005). These proteins are vital for silencing and the loss of any one is sufficient to prevent the formation of silenced domains in the cell (Aparicio et al., 1991; Hoppe et al., 2002; Johnson et al., 2009; Klar et al., 1979; Marshall et al., 1987; Pillus and Rine, 1989; Rine and Herskowitz, 1987).

The locations of silencing in the budding yeast cell are limited, being found primarily at the telomeres, the silent mating type loci and the rDNA loci (Aparicio et al., 1991; Rine and Herskowitz, 1987; Smith and Boeke, 1997). Silencing at the telomeres and the mating type loci are similar, requiring the involvement of specific domains, termed silencers, to recruit the SIR complex to the DNA (letter R in Figure 3). Silencer regions contain the binding site for several DNA binding proteins including Rap1, Abf1, and Orc1 (Origin Recognition Complex) (Bell et al., 1993; Mahoney and Broach, 1989; McNally and Rine, 1991; Micklem et al., 1993; Sussel and Shore, 1991). Binding of the above proteins to the silencer forms a permissive binding site for the initiation of silencing in that location.

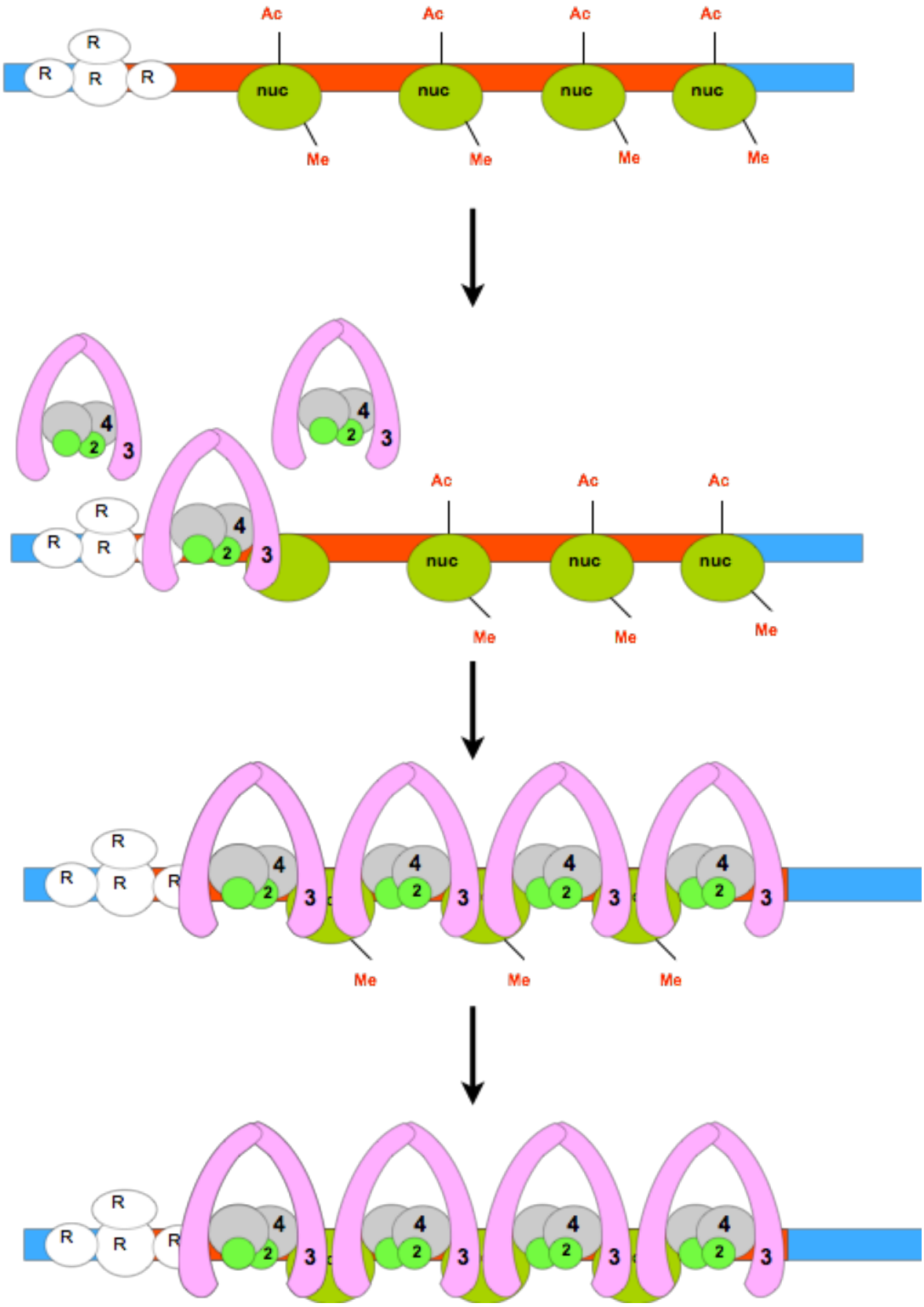
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Figure 3: Model for heterochromatin establishment
Silent chromatin initiates at silencers bound by redundant recruitment proteins (R, including Sir1, Rap1, Abf1). Prior to silencing, nucleosomes in transcriptionally active regions are acetylated on the tails of histones H3 and H4, and methylated on K79 of histone H3. The SIR complex, containing dimers of Sir2, Sir3, and Sir4, is recruited to a silencer, binds and Sir2 deacetylates H4K16 of proximal nucleosomes.Iterative rounds of deacetylation and SIR complex binding to nucleosomes and to itself are proposed to cause SIR complex spreading and extended regions of silent chromatin. Histone H3 K79 demethylation (by a yet to be isolated demethylase) is thought to occur after spreading and correlates with transcriptional repression of regions within silent chromatin (as shown in Kitada, 2012).

This is aided at the silent mating type loci by Sir1, an accessory protein for the initiation of silencing that assists in recruiting members of the SIR complex to begin silencing establishment (Bose et al., 2004; Chien et al., 1993; Triolo and Sternglanz, 1996). Once the initial recruitment of SIR proteins has occurred successive recruitment, binding, and polymerization of the SIR complex along the chromatin fibre produces the silenced domain.

The silencing described above is different than typical repression of transcription of a gene that cycles between the on and off state. Heterochromatin formation signals a more long-term form of silencing which is intended to preserve the encompassed region in an stably repressed form for several cell cycles. If a gene is not being expressed it may be because of the lack of specific initiator factors, stalled or absent RNA polymerase, or chromatin modifications such as acetylation and methylation that block transcriptional activation (Kurdistani and Grunstein, 2003). Heterochromatin differs in that it involves a heritable change to the structure of the chromatin itself to render the encompassed DNA inaccessible to transcription factors and polymerases (Gasser and Cockell, 2001).

SIR protein domains and functions

Sir1: Sir1 is involved in the establishment of silencing at the mating type loci. It is not required for silencing at telomeres or rDNA locations (Aparicio et al., 1991; Smith and Boeke, 1997). Sir1 does not have a catalytic role in silencing establishment and functions as a form of scaffold for the recruitment of the SIR complex and the initiation of silencing (Triolo and Sternglanz, 1996). It binds directly to Orc1, which is bound to the silencer binding sites and facilitates the recruitment of the initial SIR complex to the chromatin via its interaction with Sir4 (Bose et al., 2004; Chien et al., 1993; Triolo and Sternglanz, 1996).

Cells lacking Sir1 are unable to silence the silent mating type loci (Pillus and Rine, 1989) and cells lacking the Orc1 site are able to restore silencing when Sir1 is targeted to the silencer (Chien et al., 1993).

Sir2: Sir2 is an NAD+ dependent histone deacetylase and the only catalytic member of the SIR complex (Imai et al., 2000). It is highly conserved throughout the eukaryotic lineage and is the model for the Sirtuin class of enzymes found in other eukaryotes, including humans (Blander and Guarente, 2004). Sir2 acts by deacetylating the histone tails of histones H3 and H4 (Braunstein et al., 1993; Gasser and Cockell, 2001; Hoppe et al., 2002; Imai et al., 2000; Tanny et al., 2004; Yang et al., 2008). Specific residues targeted include H4K16, H3K9, H3K14 (Imai et al., 2000) though H4K16 is the acetylated residue most commonly implicated in silencing establishment (Johnson et al., 1990). The action of Sir2 is opposed by the histone acetyltransferase Sas2 (Suka et al., 2002). Acetylation of a chromatin region is associated in budding yeast with an actively transcribed region while the lack of acetylation is characteristic of a silenced region (Braunstein et al., 1993). The action of Sir2 removing the acetylation from H4K16 specifically is proposed to be essential in priming an area of chromatin for assembly of the Sir complex and the binding of the Sir3 and Sir4 proteins to the histones, since they preferentially bind deacetylated histones (Hecht et al., 1995; Johnson et al., 1990; Onishi et al., 2007) and *sir2* catalytic mutants do not exhibit the efficient Sir3/4 binding seen in *SIR2* cells (Hoppe et al., 2002). Cells which lack *SIR2* are unable to establish or maintain chromatin silencing (Rine and Herskowitz, 1987; Rudner et al., 2005; Tanny et al., 1999) but are otherwise viable. Vast over expression of SIR2 is lethal however a limited amount

of increased expression has been shown to be involved in lifespan extension in yeast (Holmes et al., 1997; Kaeberlein et al., 1999).

The Sir2 catalytic domain binds acetylated lysines and NAD+ and catalyses the removal of the acetyl group and the formation of the by-product O-AADPR (o-acetyl ADP ribose) (Tanny et al., 1999; Zhao et al., 2004). There is conflicting evidence about the involvement of O-AADPR in silencing (Chou et al., 2008; Liou et al., 2005; Martino et al., 2009). It was initially believed that the catalysis was required for silencing to be established, however the use of other histone deacetylases in place of Sir2 has shown that there is no requirement for the production of O-AADPR under normal circumstances, only for the deacetylation reaction that performs it (Chou et al., 2008). However, O-AADPR has been shown to have a positive effect in regulating the binding of Sir3 and Sir4 to chromatin (Martino et al., 2009) thus it is not an essential component of silencing establishment but does confer some advantage in silent chromatin formation when present.

Sir3: Sir 3 is an integral protein in the Sir complex and interacts with histones H3 and H4 (Buchberger et al., 2008; Carmen, 2001; Hecht et al., 1995; Johnson et al., 1990; Liou et al., 2005) as a part of its role in binding the SIR complex on chromatin. The N-terminal BAH domain of Sir3 (aa1-214) interacts with nucleosomes independently of Sir2 and silent chromatin formation, and binds preferentially to chromatin in *dot1Δ* and *sas2Δ* cells which lack H3K79 methylation and H4K16 acetylation respectively since Sir3 preferentially binds a demethylated and deacetylated template (Hoppe et al., 2002; Johnson et al., 2009; Onishi et al., 2007; Yang et al., 2008). Sir3 interacts with the C-terminal domain of Sir4 (Chang et al., 2003; Moazed et al., 1997; Moretti et al., 1994). Sir3 makes

important contacts with Rap1, a component of the silencer at both the mating type loci and at the telomeres (Shore and Nasmyth, 1987; Sussel and Shore, 1991). A Sir3 mutant lacking the Rap1 binding region has silencing defects at a weakened *HMR* (Moretti and Shore, 2001), implying that they may act somewhat redundantly to recruit Sir3 to the silencer. In contrast, at the telomeres where Rap1 forms a large component of the silencer, deletion of the Rap1 interaction domain greatly impairs the ability to silence (Moretti and Shore, 2001) since there is no significant alternative recruitment mechanism, such as *ORC*, as seen at the *HM* loci but can be rescued with Sir3 tethering in *rap1* mutant strains (Liaw and Lustig, 2006).

Sir4: Sir4 is a scaffolding protein integral to the SIR complex. It is a histone binding protein binding preferentially to demethylated and deacetylated H3 and H4 tails (Hecht et al., 1995; Onishi et al., 2007). It also acts as a bridge component in the SIR complex because it binds both Sir2 and Sir3 independently, allowing the full complex to form on chromatin (Chang et al., 2003; Moazed and Johnson, 1996; Moazed et al., 1997) since without Sir4, Sir2 and Sir3 cannot contact one another. Sir4 contains 2 domains: amino and carboxy (Marshall et al., 1987). The carboxy terminal of Sir4 mediates the interaction between Sir2-Sir4 (Moazed et al., 1997) and Sir3-Sir4 (Chang et al., 2003; Moazed et al., 1997) and forms a dimerized coiled coil with itself (Chang et al., 2003). The Sir3/4 interaction interface has been characterized and depends on residues contained within the C-terminal coiled coil of the Sir4 dimer (Chang et al., 2003; Moazed et al., 1997). Mutation of E1310R, K1324E or K1325E leads to reduced Sir3-Sir4 binding (Chang et al., 2003). The mutations of M1307N and I1311N lead to a complete loss of Sir3/4

binding and a loss of complex integrity and function (Chang et al., 2003). Sir4 has been shown to act as a dimer in solution (Moazed et al., 1997), forming a coiled coil at the C-terminal end to which the Sir3 dimer binds (Chang et al., 2003). Disruption of the coiled-coil structure leads to derepression of silenced areas, however mutations disrupting the interface abolish only telomeric and not *HM* silencing (Murphy et al., 2003). This difference could be due to the coiled coil interface acting in a different manner at *HM* loci as opposed to telomeres due to the other factors present at silencers of mating type loci versus those at the telomeres. Sir4 has been shown to bind to Rap1, a component of the silencer, when wild type versions of each are present *in vitro* (Moretti and Shore, 2001). Attraction of Sir4 to a silencer component, such as Rap1, would aid in the recruitment of Sir4 to the silencer and the initial steps in the establishment of silencing.

Comparisons with silencing in other organisms

Silencing in the budding yeast *Saccharomyces cerevisiae* is different than that of other eukaryotes, including the related yeast *Schizosaccharomyces pombe* and vertebrate species such as mice and humans. While budding yeast use a system of propagation of the SIR complex across the chromatin to block transcription and maintain a silenced state (Chang et al., 2003; Hecht et al., 1995; Johnson et al., 1990; Moazed et al., 1997; Onishi et al., 2007) the mechanism of silencing in other eukaryotes mentioned involves an RNAi dependent mechanism (Moazed, 2001). A component of the silencing in files and humans is called Swi6 or HP1, encoded by the *Su(Var)* gene family known as suppressor of variation and acts by binding to the histones in the area to be silenced (reviewed in (Moazed, 2001)). This is similar to the histone binding activities of Sir3 and Sir4 (Hecht et al., 1995; Johnson

et al., 1990; Onishi et al., 2007; Yang et al., 2008). The binding of these proteins to the histones is also dependent on the association with chromatin modifying proteins, similar to the role of Sir2 in budding yeast, such that a permissive chromatin structure can be achieved, which echoes the spreading of the SIR complex across the chromatin region (reviewed in (Moazed, 2001)). Where the two modes of silencing differ is in the introduction of the Argonaute complex and the preparation of specific small RNA molecules that, along with the Argonaute complex, bind to the chromatin to repress transcription (Reviewed in (Moazed, 2009)). The *SAS* (Something About Silencing) genes from *S. cerevisiae* are acetyltransferases implicated in silencing both the *HM* loci and the telomeres and have a sequence that is closely related to a gene involved in human Acute Myeloid leukemia (Reifsnyder et al., 1996).

Although the mechanism of silencing in budding yeast differs greatly from that of higher eukaryotes and other yeasts such as *Schizosaccharomyces pombe,* there are important similarities that can be found in both the form and function of heterochromatin across species. Basic research into heterochromatin formation will allow a better understanding of the molecular structure of silenced regions as well as what determines which areas will be expressed and which will be silenced within the genome at different times in the lifecycle or in response to environmental cues. The act of silencing also creates a reversible but heritable mark that can be used to understand the ability of a cell to faithfully pass on a specific epigenetic fate to its daughter cells. It is through the understanding of these processes of the initiation of silencing that we hope to understand not only how silencing occurs in a natural state but also how we can use the establishment of heterochromatin to prevent, treat, or cure disease in the human population.

Regulation of silencing establishment

Recent work has suggested that the chromatin modifications on histones that occur during S-phase may be influencing the S-phase requirement for silencing establishment. The need to deacetylate and demethylate histones prior to silencing establishment and spreading, as described above (Figure 3), has been implicated as a potential mechanism of limiting silencing establishment to this time period (Katan-Khaykovich and Struhl, 2005; Osborne et al., 2009).

DOT1 is a histone methyltransferase that methylates H3K79, a residue implicated in silencing establishment (Leeuwen and Gottschling, 2002; Ng et al., 2002a), as methylation of this residue is refractory to Sir3 binding to that area of the histone (Onishi et al., 2007). There is currently no known demethylase to counteract the actions of Dot1 and it is unknown if it is actively or passively removed during silencing establishment.

Since methylation of histones is a mark of actively expressed regions, it has been shown that mutation of the *DOT1* and *SET1* methyltransferases leads to formation of silent chromatin after fewer cell cycles than in a wild type cell (Katan-Khaykovich and Struhl, 2005; Lau et al., 2002; Osborne et al., 2009), however recent data has shown that *DOT1* is not required for the maintenance of silencing at *HML* (Takahashi et al., 2011). While these chromatin modifications are required to be removed to assemble silent chromatin it has not been shown whether their removal is required in the context of creating a permissive template for the SIR proteins to bind to or whether the removal stimulates a mechanism that actively promotes or regulates the assembly of new regions of silent chromatin.

Methylation of H3K79 is promoted by ubiquitination of H2BK123, which is opposed by the actions of the deubiquitinating enzyme *UBP10* (Briggs et al., 2002; Emre et al., 2005; Gardner et al., 2005; Leeuwen and Gottschling, 2002). The enzyme is also responsible for maintaining silencing at telomere proximal genes (Emre et al., 2005).

SILENCING INITIATION, SPREADING AND STABILITY

SIR complex interactions

The SIR complex is formed by the independent binding of Sir2 and Sir3 to Sir4 (Chang et al., 2003; Moazed et al., 1997) (Figure 3). Previous studies have shown that all three of these proteins appear to be present in the complex as dimers (Moazed et al., 1997; Oppikofer et al., 2013), leading to the hypothesis that the complex is formed of 6 components: 2 each of Sir2, Sir3, and Sir4. The complex is tethered to the chromatin via interactions between the SIR complex and the DNA bound histones. Sir3 and Sir4 bind the chromatin via contact with histones H3 and H4 anchoring the complex to the area (Hecht et al., 1995; Johnson et al., 1990; Onishi et al., 2007; Yang et al., 2008). The deacetylation of neighbouring nucleosomes by Sir2 is proposed to create a preferential binding site for the SIR complex, leading to successive binding of additional SIR complexes and polymerization along the chromatin (Hecht et al., 1995; Johnson et al., 1990; Onishi et al., 2007).

When Sir4 is bound to Sir2 it allosterically induces Sir2 activity (Hsu et al., 2013) and when localized where Sir4 is recruited to the silencers the deacetylation of neighbouring nucleosomes can begin, promoting the recruitment of additional SIR

complexes and the propagation of the SIR complex along the chromatin region (Hecht et al., 1995; Johnson et al., 1990; Onishi et al., 2007). The commonly accepted model for silencing establishment states that the successive rounds of deacetylation and binding of the SIR complex propagates the silencing along the chromatin template and that the region must be free of H3K79 methylation and H4K16 acetylation for the SIR complex to spread and silencing to be established (Katan-Khaykovich and Struhl, 2005; Osborne et al., 2009).

Given that the SIR proteins not only bind each other but also bind nucleosomes there are a number of factors at play for determining when and where a complex will form. The availability of the members of the SIR complex, chromatin modifications favouring SIR complex binding and the presence of silencer elements to recruit the silencing proteins are all required for the successful assembly and spreading of the SIR complex and the establishment of a silenced domain.

Telomeric silencing and position effect variegation

Silencing at the telomeres is present at all chromosome ends, though the degree of spreading along the telomere along the chromatin can vary in a process known as Telomere Position Effect variegation or (TPE). First discovered in *Drosophila melangaster* (Weiler and Wakimoto, 1995), TPE is visible as a difference in expression of telomere proximal genes based on whether the heterochromatin structure has spread to encompass and silence that gene or not. Because the extent of telomeric heterochromatin varies this can be seen as a variegated expression pattern in an organism or colony. In *Drosophila* if the telomere proximal *WHITE* gene is expressed flies have red eyes and when it is repressed the eyes are white. In the case of variegated expression the eyes are speckled red and white (Schultz,

1936; Weiler and Wakimoto, 1995). Position effect variegation is a similar effect to TPE, however it can occur at any location that is silenced. It has been shown at the yeast telomeres and *HM* loci to be regulated by similar factors such as Nat1 and Ard1 binding at telomeres and Sir1 binding at *HM* loci (Aparicio et al., 1991). Similar experiments can be performed at the yeast telomeres with either a repressible gene for growth in the absence of a certain amino acid (commonly *URA3*) or a block in the adenine biosynthetic pathway that leads to colonies of red (off), white (on), or sectored cells (off and on) (Gottschling et al., 1990).

Telomeric silencing requires the SIR complex and the presence of a silencer to nucleate silencing initiation. The silencer is made up of successive Rap1 binding sites that serve as a nucleation site for SIR complex assembly and localizes telomeric heterochromatin to the nuclear envelope (Gilson et al., 1993; Konig et al., 1996; Palladino et al., 1993) (Figure 2). Once silencing is nucleated at the silencer through the binding of the SIR complex to histones, the successive deacetylation of H4K16 by Sir2, and subsequent binding of additional SIR complex allows the spread of silencing away from the telomere ends. The degree of spreading can be influenced by the amount of Sir3 present at the telomeres (Hecht et al., 1996).

There has been some debate in the literature that the effect of PEV is not universal at the telomeres and that the strains that are most commonly used as reporters, namely *TELVII-URA3* are not reporting the natural phenomena that occur at telomeres (Takahashi et al., 2011). However these results also suggest that the changes in silencing at telomeres is dependent on the presence of H3K79 methylation while also stating that there is markedly decreased H3K79 methylation where Sir proteins are bound, implying that they are

mutually exclusive (Takahashi et al., 2011). Additionally, it was shown that not all telomeres are affected in this way by *dot1* deletion, suggesting that telomeres naturally behave slightly differently, independently of the engineering of *TELVII-URA3,* although the engineered strain does introduce a slight bias in that the *URA3* gene is placed especially close to the telomere end where silencing is more affected by methylation status of the region (Takahashi et al., 2011).

Several mutations can have a disruptive effect on telomeric silencing. Many genes, when deleted, lead to a loss of silencing phenotype, such as *yKU70* involved in DNA end binding and required for telomeric silencing, *DOT1* a H3K79 methyltransferase, *UBP10* which prevents H3K123 ubiquitination, *TLC1* the RNA component of telomerase, and many others. The DOT family of genes were found in a screen as Disruptors Of Telomeres (Singer et al., 1998) for their loss of silencing phenotype.

yKu70 and telomere specific silencing proteins

The DNA end binding protein Ku70 is implicated in both DNA repair and telomere stability (Boulton and Jackson, 1998; Lopez et al., 2011). At the telomeric Rap1 binding sites there is a competition between Ku70 and Rif1/2 for binding (Mishra and Shore, 1999). The loss of Ku70 at the telomeres leads to a loss of telomeric silencing but has little defect in the silencing of a weakened *HMR* (Mishra and Shore, 1999). Rif1/2 binds the telomeric Rap1 binding sites, in competition with Sir3 and Ku70 for binding, and binding is required for silencing establishment (Mishra and Shore, 1999; Moretti et al., 1994). Deletion of r*if1* in y*ku70Δ* cells restored the ability to silence telomeres, and when both *RIF1* and *RIF2*

were deleted in a y*ku70Δ* cell, the silencing at the telomeres and TPE returned to normal levels (Mishra and Shore, 1999).

CELL CYCLE DEPENDENCE ON THE ESTABLISHMENT OF SILENCING

History of silencing research

Miller and Nasmyth described a cell cycle dependence on the establishment of heterochromatin where it could only be established after passage through S-phase (Miller and Nasmyth, 1984). Cells were arrested with a temperature sensitive *sir3-8* allele in G0 by nutrient arrest to remove all existing silencing. Cells were then released into the cell cycle in nutrient rich media at permissive temperature. It was established that the population required between 1 and 5 cells divisions to re-establish silencing and this was dependent on passage through S-phase (Miller and Nasmyth, 1984). Later work went on to show that this dependence was not related to DNA replication (Kirchmaier and Rine, 2001; Li et al., 2001) but another event happening in S-phase the cell cycle. The window was further narrowed to between the G1 α-factor arrest and the HU arrest in S-phase (Kirchmaier and Rine, 2006). There has also been suggestions that events in M-phase may also be implicated, such as dissolution of sister chromatid cohesion (Lau et al., 2002; Martins-Taylor et al., 2004). Additionally there appear to be differences in the potential requirement for cell cycle passage at the two *HM* loci where silencing establishment at *HML* may not have the same requirements for establishment as at *HMR* (Lazarus and Holmes, 2011; Ren et al., 2010).

Strategies for measuring silencing establishment

The cell cycle dependence of silencing establishment has traditionally been measured by inducing a cell cycle arrest to synchronize a population of cells followed by a release from that arrest to follow the population through the cell cycle, while tracking a readout for silencing such as the expression of *HMRa1* mRNA (Miller and Nasmyth, 1984; Motwani et al., 2012; Ren et al., 2010). Osborne *et al* recently developed a new method of measuring the cell cycle dependence of silencing establishment by tracking pheromone response in single cells (Osborne et al., 2009) Typically the cell cycle arrest is induced prior to START, or commitment to the cell cycle, using either a nutrient starvation to enter G0, or an arrest in pheromone prior to cell cycle commitment.

SIR4 PROTEIN STABILITY AND REGULATION

SIR proteins are required for silencing establishment and maintenance

The ability of cells to regulate the content of its proteome is important to ensure that there is a balanced protein profile to match the state of the cell and its needs at that moment. Although many proteins are regulated by their gene transcription, mRNA translation, or protein degradation, no one has yet determined whether Sir4 is regulated in its abundance within the cell. It is known, however, that the amount of Sir4 in the cell is important in maintaining adequate silencing, as shown by Sussel and Shore (Sussel and Shore, 1991) where over expression of *SIR4* in a *rap1* mutant is able to restore silencing, indicating that it may be a limiting factor in the formation of heterochromatin (Sussel and Shore, 1991) while having a lower amount of Sir4 is detrimental to silencing and cells cannot

successfully form heterochromatin at a weakened *HMR* locus (Sussel et al., 1993). Given that the dosage of Sir4 appears to be important for regulating silencing at a weakened *HMR* locus we hypothesized that it would also have an effect at a native *HML* locus as well as at the telomeres of cells.

Because there is little known about the regulation of Sir4 protein levels we are unable to ascertain whether there was an active or passive regulation of the protein that may be related to the cell cycle dependence of heterochromatin establishment. Given that the most common ways to initiate a synchronous population of cells to test for cell cycle dependence of establishment of silencing is to use a chemical (Duntze et al., 1970; Wilkinson and Pringle, 1974), or genetic (Miller and Nasmyth, 1984) blockage of the cell cycle we sought to determine what regulation there is of Sir4 under these conditions. Based on the predictions seen above (Sussel and Shore, 1991; Sussel et al., 1993) regarding the ability of cells to silence with greater or lesser amounts of Sir4 we believed that the amount of the protein could be intricately tied to the cell's ability to establish heterochromatin *de novo*.

HYPOTHESIS AND OBJECTIVES

Hypothesis

general: *Saccharomyces cerevisiae* cells require that their silent mating type cassettes remain silenced during the process of mating and sporulation to retain a unique **a** or α mating type identity (Haber, 2012). The establishment of silencing at these locations has been shown to be cell cycle regulated (Katan-Khaykovich and Struhl, 2005; Kirchmaier

and Rine, 2006; Lau et al., 2002; Martins-Taylor et al., 2004; Osborne et al., 2009), in that the cell requires between one and five cell divisions before silencing is re-established after loss (Miller and Nasmyth, 1984). This regulation is not related to the replication of DNA (Kirchmaier and Rine, 2001; Li et al., 2001) or the separation of sister chromatids via cell division (Martins-Taylor et al., 2004). We hypothesize that the amount of Sir4 protein may regulate the ability to establish silencing at the mating type loci.

specific: We propose that Sir4, as an integral component of the SIR silencing complex, is a limiting factor in the rate of silencing re-establishment at the HM mating type loci and that changes in the abundance of Sir4 available at the *HM* loci change the rate of silencing establishment.

Objectives

1) To determine whether the amount of Sir4 present in the cell affects the number of cell cycles required to establish silencing at the *HML* locus.

2) To explore the importance of changes in histone modifications of a region to be silenced and the abundance of Sir4 in the ability to establish silencing.

3) To examine if cells regulate Sir4 abundance in response to prolonged arrest.

MATERIALS AND METHODS

Strain and Plasmid Construction

Appendix A lists the strains and plasmids used in this work. All strains are derivatives of the W303 strain background (W303-1a). Strains used for the single cell mating assay were a gift from Jasper Rine. All deletions and replacements were confirmed by immunoblotting, phenotype or PCR. The sequences of all primers used in this study are available upon request. The bacterial strains TG1 and DH5α were used for amplification of DNA.

All deletions were made using cassettes amplified from pAR747 (*K. lactis URA3),* P3 (*HIS3)* or the EUROSCARF collection (NATr). Double deletions were made by successive transformations and all deletions were screened against all other auxotrophies in that strain to control for mis-localization of the cassette.

GAL-SIR4 was created using integration of pAR655.

GAL-DOT1 was created using integration of pAR1097.

GAL-TLC1 was created using integration of pAR1073

β-estradiol strains were created by inserting the P-GEV cassette (gift from Mads Kaern, (Gao and Pinkham, 2000)) into pAR917 to form pAR941, which was integrated into the appropriate parents strains. *SIR4-2μ* strains were created by inserting the *SIR4* gene from pAR646 into pRS423 to form pAR696, which was then transformed into the appropriate parent strains. *SIR4-CEN* strains were created by transforming pAR646 and/or pAR722 into the appropriate parent strains.

Integration of *SIR4* was performed by transformation with pAR720 and screening for both auxotrophic growth and also increased *SIR4* expression.

Physiology

Cells cycle arrests were performed with 10μg/mL nocodazole (Sigma-Aldrich) or 25-100ng/mL α-factor (Biosynthesis) at 25 degrees Celsius. To fix cells for microscopy, ~2.0x10^6 cells were harvested and fixed with 4% paraformaldehyde in PBS pH 7.5 for 15 minutes, washed in 100mM KPO_4/1.2M sorbitol pH 7.5, sonicated, and resuspended in KPO_4/sorbitol. Samples were imaged directly using a Nikon TI microscope (Nikon) with a 60X 1.4 NA objective and FITC filter set (Chroma).

Microscopy

Cells of the indicated genotype were grown overnight in YPD to log phase, fixed in 4% paraformaldehyde for 10 minutes, washed, sonicated and resuspended in 100mM KPO_4 containing 1.2M sorbitol. Samples were imaged using a Nikon TI microscope (Nikon) with a Nikon Plan Apo 60X 1.4 NA objective and FITC filter set (Chroma) at room temperature with a Photometrics CoolsnapHQ2 camera (Photometrics). 13 fluorescent images using no ND filters and an exposure time of 2s were obtained separated by 0.5μm along the Z-axis. A single brightfield image was obtained at the central plane with an exposure time of 200ms. Example images were prepared using ImageJ software. Imaging was done in mixed populations of nocodazole (large budded) and alpha-factor (unbudded) treatment cells, treatment was determined by cell morphology. The same linear look-up-table was used for each example image. Fluorescence quantification was done using NIS-Elements software. For each cell three fluorescent foci were analyzed. A total of eight cells of each genotype were obtained in two separate experiments. Mean fluorescence in a 0.25um^2 circular ROI

was obtained for each focus. For each focus, mean fluorescence from a similar sized background ROI obtained from the same cell and focal plane was subtracted. Non-focus fluorescence measurements were obtained as described above but all images were obtained at the same Z-plane. Three measurements were obtained in each of eight cells from two separate experiments

Western Blots and Immunoprecipitation

These methods have been described previously(Rudner and Murray, 2000; Rudner et al., 2000).

The following antibodies were used for Western blots: Rabbit polyclonal α-Cdk1, α-Sir2, and α-Sir4 were used at 1:1000 in TBS-T with 4% Fat Free Milk Powder, 5% glycerol, 0.02% NaN_3. An autoclaved solution of 5% milk was used to make the 4% milk dilution buffer to increase the longevity of the antibody solution. Membranes were pre-blocked with TBS-T with 4% Fat Free Milk Powder, 5% glycerol before incubation with all primary antibodies.

Single Cell Silencing Establishment Assay

Modified from Osborne, Dudoit and Rine (Osborne et al., 2009). Cells were grown overnight on plates of either YP-Dextrose, YP-Raffinose or selective media at 30 degrees Celsius, with the exception of *ku70Δdot1*Δ (ADR5920/5921) and *ku70Δubp10*Δ (ADR5944/5945) cells which were grown at 25 degrees Celsius overnight due to a slight temperature sensitivity. A small number cells of each strain (**a** or α) were resuspended in YEP and each spotted onto a YPD plate along with a thick streak of α cells. Individual **a**

and α cells were placed next to one another and allowed to mate. Zygotes were transferred adjacent to the streak of α cells to grow at 30 degrees Celsius. Daughters were detached and placed according to pedigree position remaining adjacent to the streak of α cells. Pedigrees were scored for each zygote.

Statistical Analysis

The pairwise statistical analysis of pedigrees was performed by Corey Yanofsky as previously described (Osborne et al., 2009). Detailed statistical analysis of all combinations is provided in the appendix.

Colony Sectoring Assay

Cells of the given genotypes were grown in YPD media and plated on YPD plates and grown for 3 days at 30 degrees Celsius then placed at 4 degrees Celsius for several days for the red colour to develop in the colonies. Cells were scored using photographs of the plates to enhance the visibility of the sectors.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitations were performed as described previously (Rudner *et. al* 2005). Briefly, cultures were grown at 30 degrees Celsius to OD_{600} of 1.5, and fixed with 1% formaldehyde for 15 minutes. Cross-linking was quenched with 125mM glycine for 5 minutes, and cells were washed once with 20mM Tris-HCl (pH 7.5)-150mM NaCl. Pellets were frozen in liquid nitrogen. Cells were lysed from frozen in ChIP lysis buffer (50mM HEPES-KOH [pH 7.6], 500mM NaCl, 1mM EDTA, 1% Triton X-100). The following were added to the lysis buffer just before use: 0.1% sodium deoxycholate, 1mM PMSF, 1mM benzamidine, and leupeptin, pepstatin and bestatin to 1μg/mL each.

Chromatin in the lysates was sheared to an average size of 500bp, using the Covaris sonication system. Lysates were clarified with a 5-minute centrifugation step (15,000rpm at 4 degrees Celsius). Supernatants were collected and normalized using the Bradford assay. Input DNA was prepared from 50µL normalized lysate in 200µL 50/10-TE + 1% SDS (50/10-TE is 50mM Tris-HCl [pH 8.0], 10mM EDTA); samples were incubated at 65 degrees Celsius overnight and then processed in the same manner as the immunoprecipitates.

Sir4 and histone H3 were immunoprecipitated using 1µL affinity-purified rabbit anti-Sir4 or anti-H3 antibody, or 1µL antibody to histone H3 methylated at K79 (pan-methyl histone H3). Lysates were incubated with antibodies for 30 minutes, after which 10µL of Protein A Dynabeads beads were added to each sample and incubated at 4 degrees Celsius for 1-2h. Beads were washed three times with ChIP lysis buffer, once with Li buffer (10mM Tris-HCl [pH 8.0], 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM EDTA), and one time with 50/10-TE . Protein and cross-linked DNA were eluted in 100µL 50/10-TE + 1% SDS at 65°C for 10 minutes. This eluate was removed, and the beads were washed with 150µL 50/10-TE + 1% SDS and this wash added to the eluate. The samples were incubated at 65 degrees Celsius overnight to reverse the crosslinks, then incubated with RNase A (1µg/mL per input, and 0.1µg/mL per IP sample; 1h at 55 degrees Celsius) and 50µg/mL proteinase K (2h at 55 degrees Celsius). The samples were then extracted with an equal volume of 1:1 phenol:chloroform and extracted once with chloroform alone. DNA was precipitated with 1/10 volume 3M NaOAc (pH 5.2), 2.5 volumes ethyl alcohol and 10µg glycogen. DNA pellets were washed once with 70% ethyl alcohol, air-dried and resuspended in 50µL 10/1 TE (10mM Tris-HCl [pH 8.0], 1mM EDTA).

PCR reactions were performed with either 5µL of a 1:10 dilution of immunoprecipitated DNA, or 5µL of a 1:2500 dilution of input material. Reaction mixtures were a total volume of 12.5µL, containing PCR buffer (Invitrogen), 0.1mM deoxynucleoside triphosphates, 0.1mCi of [α-^{32}P]dCTP (Perkin Elmer), and 1µM of each primer. PCR reactions were run on 6% acrylamide (30:1 acrylamide-bis)-Tris-borate-EDTA gels for 45-60 minutes at 100V. Samples were loaded with 1.5µL loading dye (50mM Tris-HCl [pH 8.0], 50% sucrose, 4M urea, 0.05% bromophenol blue). Gels were dried and subjected to autoradiography and quantification by phosphorimaging. Relative fold

enrichment values for Sir4 and H3 were calculated as follows: [silent locus (IP)/*ACT1* (IP)]/[silent locus (input)/*ACT1* (input)]. Values are expressed as mean and standard deviation of three replicates, and scaled with average values for *sir4Δ* and *dot1Δ* set to 1.

Quantitative silencing assay

A telomeric *URA3* reporter strain (ADR7254) was transformed with a *SIR4*-CEN (pAR447) or empty CEN plasmid (pAR441). Cells were grown overnight in SC –trp media to an OD_{600} of 0.3-0.5, and arrested with 1μg/mL alpha factor or grown asynchronously. Cells were taken at hourly time points and plated at appropriate dilutions on YPD or YPD +5'-fluoroorotic acid (FOA) plates, which were left at 30 degrees Celsius for three days. Viability on FOA is expressed as the ratio of percent survival on FOA to percent survival on control plates. Values are expressed as mean and standard deviation of three replicates.

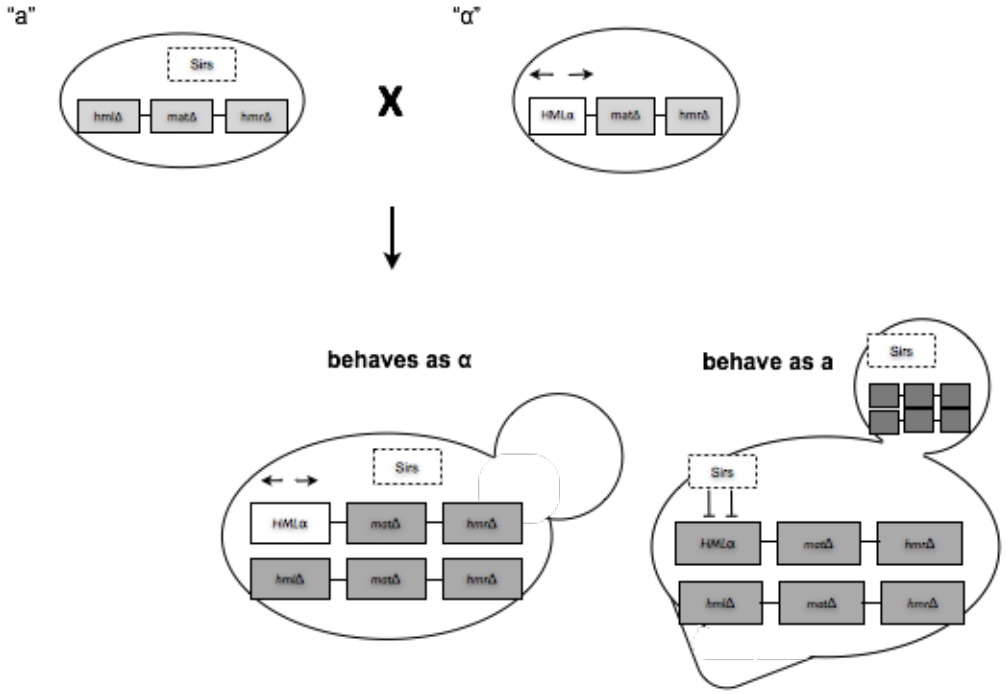
RESULTS

SIR4 is haploinsufficient for silencing at telomeres and haplodeficient at *HMLα*

Previous work has shown that *SIR4* is haploinsufficient for silencing at *HMRa* (Sussel et al., 1993). Using a telomere proximal *URA3* reporter gene (*TELVII-URA3)* to measure telomeric silencing we have shown that *SIR4* is also haploinsufficient for silencing at the telomeres (Figure 5A). The importance of Sir4 dosage in regulating silencing suggested that Sir4 abundance may regulate the establishment of heterochromatin, but this was not directly tested in earlier work (Sussel and Shore, 1991).

A novel method was recently developed to monitor the establishment of silencing at single cell resolution and we chose this method for our study because it can detect small but significant differences in the speed of establishment without relying on population averages of a read-out of transcriptional silencing. Previous work has analyzed establishment of silencing using Northern blot analysis or RT-PCR of transcripts produced from *HMRa,* and by silent chromatin-dependent changes in chromatin topology in population of cells (Ansari and Gartenberg, 1999; Katan-Khaykovich and Struhl, 2005; Lau et al., 2002; Miller and Nasmyth, 1984). The single cell assay observes single cells and measures a phenotypic response of silencing rather than the level of transcription, allowing for a simple visual screen to be performed (Osborne et al., 2009). Two engineered strains are mated (see Figure 4 for details) and the assembly of heterochromatin at *HMLα* is monitored in the resulting zygote and its progeny by their response to exogenous alpha factor pheromone that causes cell cycle arrest and polarization in cells that silence *HMLα*. The number of cell divisions required before silencing is established is analyzed by performing a pedigree analysis

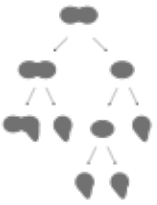
a



α cell patch

Adapted by permission from Macmillan Publishers Ltd: [Nature] Osborne et al, 2009, copyright 2009

b

Pattern 0	Pattern 1	Pattern 2	Pattern 3	Pattern 4	Pattern 5
					
0 div.	1 div.	1-2 div.	1-2 div.	2 div.	>2 div.

Adapted by permission from Macmillan Publishers Ltd: [Nature] Osborne et al, 2009, copyright 2009

*division of any of the 4 cells of the 2nd generation

Figure 4: Experimental method
A) A *hmlΔ matΔ hmrΔ* mates as a "pseudo-*MAT*a" cell (ADR4480). A *matΔ hmrΔ sir3Δ* behaves as a *MAT*α cell (ADR4481) because HML is derepressed. The resulting diploid cell behaves as a *MAT*α cell until *HMLα* has been silenced, at which point the cell switches identity and behaves as a *MAT*a cell, arresting its cell cycle and forming a shmoo in response to pheromone. B) A pedigree of daughter cells are removed from mothers and retained near the pheromone to determine how many divisions are required for the daughter cells to silence HMLalpha in a method adapted from Osborne *et al*. 2009. Different pedigrees can be placed into categories depending on the number of divisions prior to silencing of *HMLα*. Note that in patterns 5 and 6 any one of the second or third generation with a successive division falls into this category, not only the option pictured.

(Figure 4B). When *sir4*Δ/*SIR4* zygotes are analyzed in this assay I found they establish silencing at *HML* more slowly than *SIR4*/*SIR4* diploids. (Figure 5B). The wild type (*SIR4*/*SIR4)* data is similar to Osborne *et al* (2009) and verifies that we are able to efficiently replicate the assay. To test if *sir4*Δ/*SIR4* cells also have defects in silencing maintenance, the above diploid cells were subjected to quantitative mating analysis to determine whether the stability of HM silencing was affected in a *sir4*/*SIR4* background. Once *HML*α was silenced (and the diploid zygotes mate as **a** cells), there was no difference in the mating efficiency of *sir4*Δ/*SIR4* versus *SIR4*/*SIR4* diploids (Figure 5C). The pseudo-haploid diploids mate with 2-3-fold lower efficiency than control haploid *MATa* cells, perhaps due to the increase in size and ploidy in these cells compared to the control haploid cells (Figure 5C).

Increasing Sir4 levels speeds the assembly of new sites of heterochromatin

Because less Sir4 slows silencing establishment, we wondered if increasing Sir4 concentration may speed establishment. Past work has shown that increasing Sir4 improves silencing, but had not distinguished if the improvement was due to improved maintenance or changes in the rate of establishment. Using the single cell pedigree method (Osborne et al., 2009) we determined that increasing Sir4 levels changed the timing of silencing establishment at *HML*α. Low copy centromeric plasmids containing *SIR4* were transformed into the two parent strains, which were then mated to produce diploid zygotes containing additional copies of *SIR4*. Two different centromeric plasmids containing different auxotrophic markers allowed for selection of cells with further increased *SIR4* dosage.

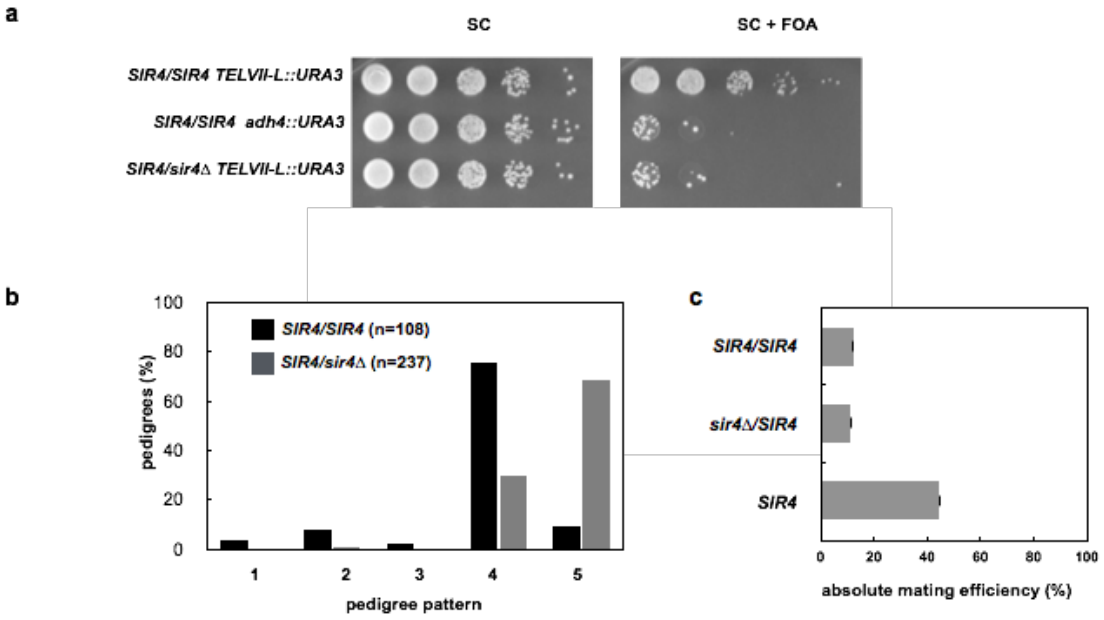

a
SC
SC + FOA
SIR4/SIR4 TELVII-L::URA3
SIR4/SIR4 adh4::URA3
SIR4/sir4Δ TELVII-L::URA3
b
SIR4/SIR4 (n=108)
SIR4/sir4Δ (n=237)
pedigrees (%)
pedigree pattern
c
SIR4/SIR4
sir4Δ/SIR4
SIR4
absolute mating efficiency (%)

Figure 5: *SIR4* is haploinsufficient for silencing at the telomeres and has a severe defect in silencing establishment at *HMLα*

A) Strains containing *URA3* at *TELVII*-L were mated to form *SIR4*/*SIR4 (*ADR2828 and 21 or 3344) and *sir4*/*SIR4 (*ADR 2830 and 21) diploids. The ability to silence *URA3* transcription was measured by the ability of cells to grow on plates containing 5-FOA. URA3 inserted at the internal *ADH4* locus was used as a control. B) Haploid cells that were either *SIR4* or *sir4*Δ were mated to form *SIR4*/*SIR4* (ADR4480x4481) and *sir4*/*SIR4* (ADR4592x4481) diploid zygotes which were monitored for establishment of silencing at *HMLα* and categorized as in Fig4B. $p<0.01$ by the likelihood ratio test. C) Quantitative mating assays were performed by crossing diploid strains formed as above that are homo- or heterozygotic for *SIR4* to an α tester strain (ADR3082) with intact *MAT* and *HM* loci, along with a control mating of wild type haploid strains. The absolute mating efficiency is the proportion of cells of each query strain that mated and formed colonies on synthetic media lacking amino acids.

Western blot analysis confirmed that the addition of SIR4-*CEN* plasmids produced additional Sir4 at levels similar to expression from the endogenous promoter (Figure 6B). Selection for a *SIR4-CEN* plasmid in one parent accelerated silencing establishment with approximately twice as many cells silencing after one cell cycle compared to the control (Figure 6A). Selection of two or four *SIR4-CEN* plasmids (one or two in each parent) showed a five and six fold increase respectively in the number of cells silencing after only one generation (see pattern 1, Figure 6A).The effect of increasing Sir4 is similar to what we and others have observed in diploids where *dot1Δ* has been deleted (Figure 6C and shown in (Osborne et al., 2009)).

The copy number of centromeric plasmids is variable in cells, so we tested if other methods of increasing Sir4 would also improve silencing establishment. Cells containing *SIR4* driven by a galactose inducible promoter (*GAL-SIR4)* were transformed with a plasmid containing *GAL4.ER.VP16 (pGEV)* allowing graded induction of Sir4 using varying estradiol concentrations, rather than induction with galactose (Gao and Pinkham, 2000) (Figure 6E). When establishment of silencing was assayed in these cells using the single cell assay we observed that cells were able to establish silencing after fewer cell generations, in a similar manner to that seen with the addition of *SIR4-CEN* plasmids (Compare Figure 6D to Figure 6A) though not at a level significantly different than the control ($p=0.084$ by likelihood ratio test). Additionally, *SIR4* was integrated at the *URA3* locus and transformants that increased Sir4 levels also improved the rate of silencing establishment (Figure 6F). These results confirmed that increasing Sir4 abundance allows for earlier silencing establishment and is not an artifact of plasmid transformation.

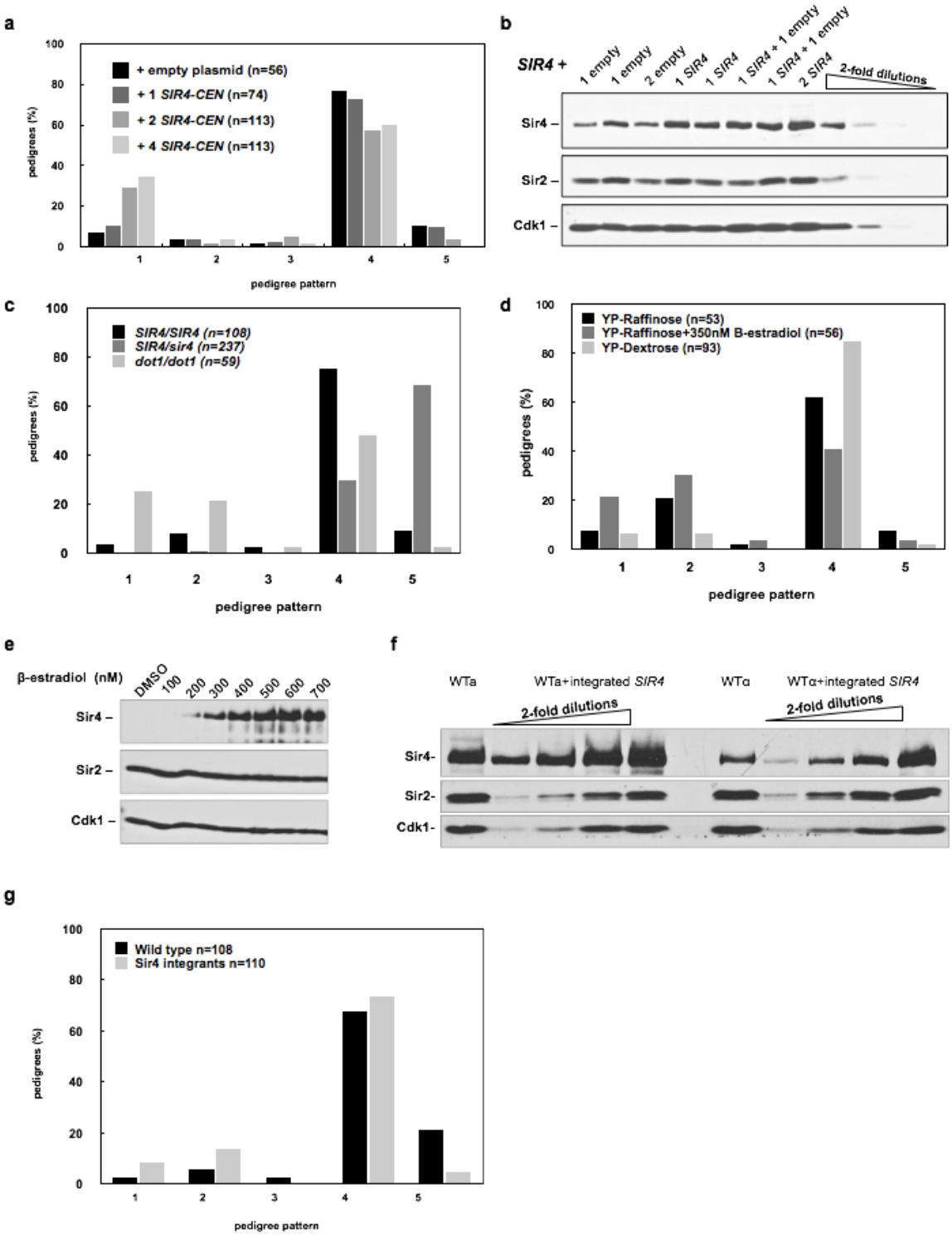

a
+ empty plasmid (n=56)
+ 1 SIR4-CEN (n=74)
+ 2 SIR4-CEN (n=113)
+ 4 SIR4-CEN (n=113)
pedigrees (%)
pedigree pattern
b
SIR4 +
1 empty
1 empty
2 empty
1 SIR4
1 SIR4
1 SIR4 + 1 empty
1 SIR4 + 1 empty
2 SIR4
2-fold dilutions
Sir4 –
Sir2 –
Cdk1 –
c
SIR4/SIR4 (n=108)
SIR4/sir4 (n=237)
dot1/dot1 (n=59)
pedigrees (%)
pedigree pattern
d
YP-Raffinose (n=53)
YP-Raffinose+350nM B-estradiol (n=56)
YP-Dextrose (n=93)
pedigrees (%)
pedigree pattern
e
β-estradiol (nM)
DMSO
100
200
300
400
500
600
700
Sir4 –
Sir2 –
Cdk1 –
f
WTa
WTa+integrated SIR4
WTα
WTα+integrated SIR4
2-fold dilutions
2-fold dilutions
Sir4-
Sir2-
Cdk1-
g
Wild type n=108
Sir4 integrants n=110
pedigrees (%)
pedigree pattern

Figure 6: Increasing intracellular Sir4 protein level leads to earlier establishment of silencing at *HMLα*.

A) One or two centromeric plasmids, each containing one additional copy of *SIR4*, were transformed into one or both mating strains prior to mating (ADR4480x4481). Individual zygote pedigrees were tracked and plotted as in Figure 5. Addition of 2 *SIR4-CEN* plasmids or 4 *SIR4-CEN* plasmids *(*pAR646 and/or pAR722) were each significantly different than the control distribution (p(2CEN)=0.002, p(4CEN)=0.0004, by the likelihood ratio test). B) Centromeric plasmids (pAR440 or pAR646) were added to *sir4Δ* (ADR4592) and *SIR4* (ADR4480) haploid cells and protein levels were measured by western blot. Sir2 level serves as a loading control. C) Haploid cells that were either *SIR4* or *sir4*Δ were mated to form *SIR4*/*SIR4* (ADR4480 and 4481) and *sir4*/*SIR4* (ADR4592 and 4481) diploid zygotes and along with *dot1Δ* haploids (ADR4631 and 4632) which were monitored for establishment of silencing at *HMLα* and categorized as in Fig4B. $p<0.01$ by the likelihood ratio test. Data is similar to that of Osborne *et al*. 2009. D) *SIR4* transcription varies with induction by β-estradiol. Cells containing *GAL-SIR4 (*ADR5389) and a hormone inducible GAL4.ER.VP16 (GEV, pAR917) *(Gao et al.)* were treated with various concentrations of β-estradiol in liquid culture at 25 degrees and Sir4 protein expression was analyzed by western blot. Cdk1 serves as a loading control. E) *GAL-SIR4* cells with the integrated β-estradiol construct were grown overnight at 30 degrees on YPRaffinose plates prior to mating on plates containing 350nM β-estradiol (ADR5389x5390). Pedigrees of individual zygotes were tracked as in A. $p=0.08$ by the likelihood ratio test. F) *SIR4* integrated into the genome was expressed from the endogenous promoter (ADR 4480, 7240, 4481, 7241, pAR720). G) *SIR4*-integrants were grown and mated on YP-Dextrose plates (ADR 7240x7241) as in Figure 5B. $p<0.01$ by the likelihood ratio test.

Further increasing Sir4 in cells is detrimental to silencing establishment. High copy *2μ-SIR4* plasmids and overexpression of *SIR4* from the galactose promoter block/slow establishment (Figure 7A-D), and also cause derepression of telomeric *URA3 (*Figure 7E*),* revealing a defect in maintenance of silencing. These findings are consistent with past data showing overabundance of Sir4 in cells disrupts silent chromatin by preventing assembly of a complete SIR complex (Cockell et al., 1995; Marshall et al., 1987; Singer et al., 1998)

Epistasis analysis of *dot1Δ* and *SIR4*/*sir4Δ*

Osborne *et al*. and others(Katan-Khaykovich and Struhl, 2005)have shown that *dot1Δ* cells speed the assembly of new sites of heterochromatin, ((Osborne et al., 2009) Figure 6C), so we wondered if changes in Sir4 abundance acted upstream, downstream or independently of Dot1 function (Figure 8). To test these three models we monitored silencing establishment in *dot1Δ SIR4*/*dot1Δ sir4Δ* diploids and found that reduction of Sir4 abundance reduces the early establishment of silencing in *dot1Δ*/*dot1Δ* diploids and slows establishment to rates similar as those of *sir4Δ*/*SIR4 (*Figure 9A*)*. This result clearly rules out a model in which Sir4 abundance functions upstream of Dot1, but cannot clearly distinguish between the two other models where Sir4 functions downstream of Dot1 or where they function in separate pathways.

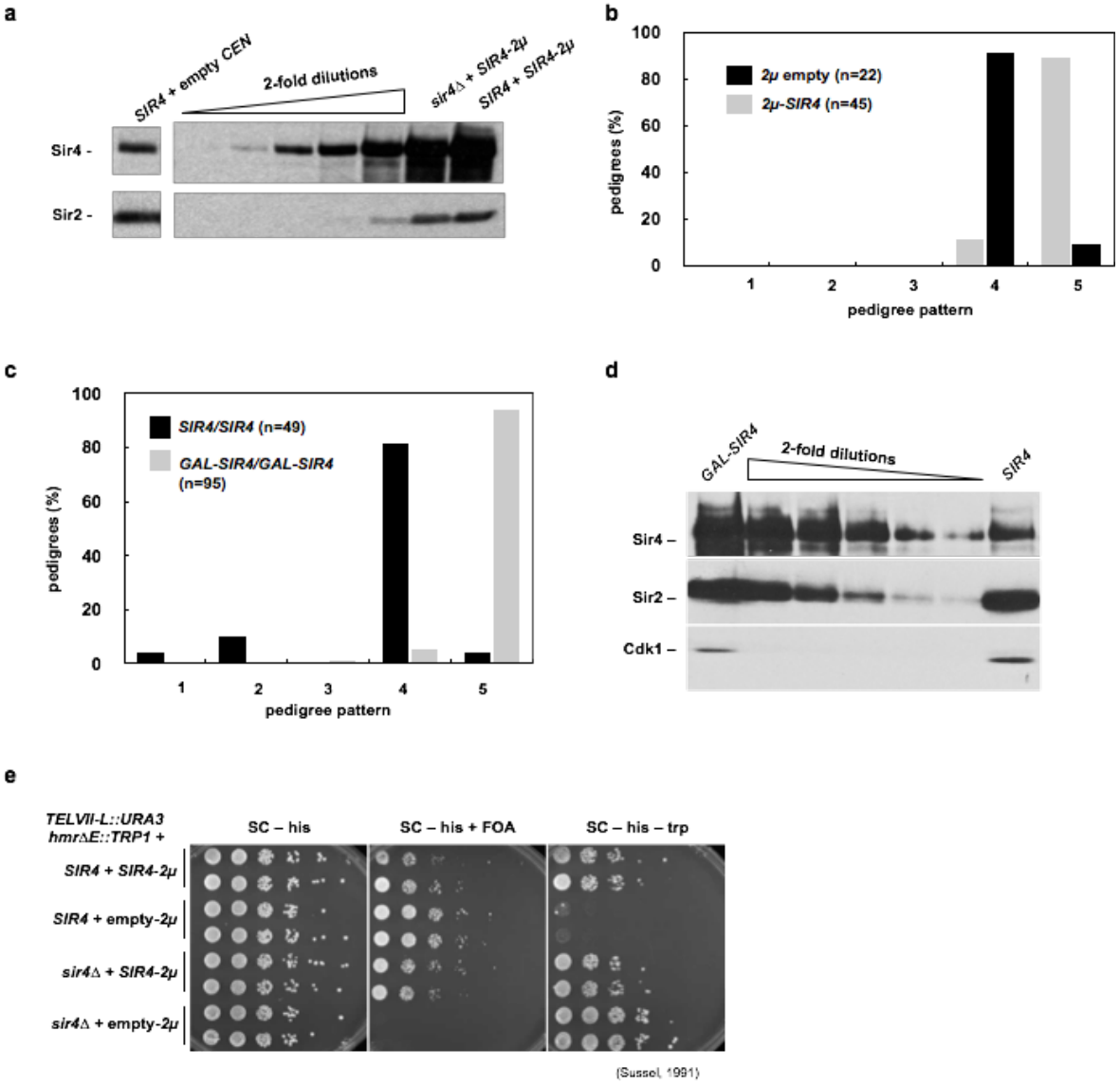
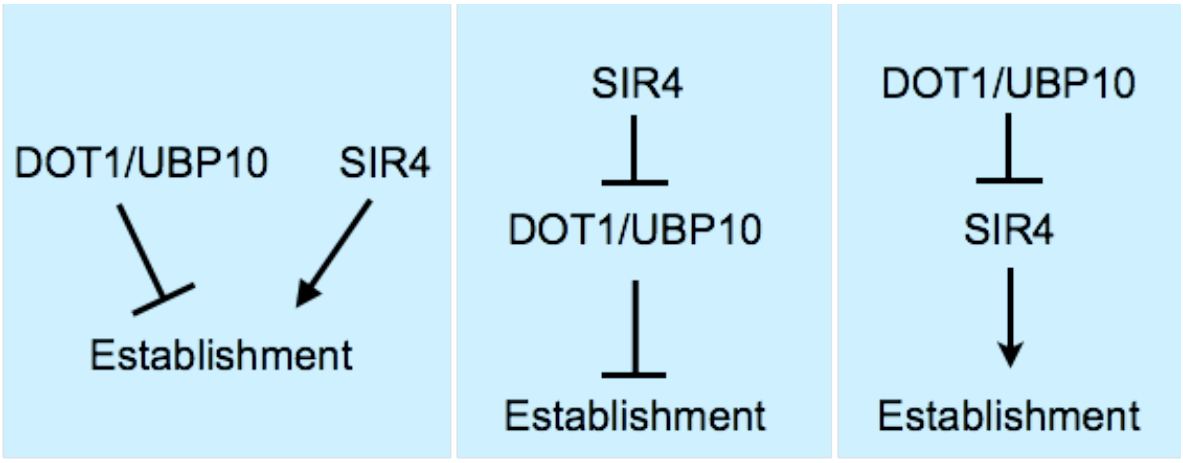

a
SIR4 + empty CEN
2-fold dilutions
sir4Δ + SIR4-2μ
SIR4 + SIR4-2μ
Sir4 -
Sir2 -
b
2μ empty (n=22)
2μ-SIR4 (n=45)
pedigrees (%)
pedigree pattern
c
SIR4/SIR4 (n=49)
GAL-SIR4/GAL-SIR4 (n=95)
pedigrees (%)
pedigree pattern
d
GAL-SIR4
2-fold dilutions
SIR4
Sir4 –
Sir2 –
Cdk1 –
e
TELVII-L::URA3
hmrΔE::TRP1 +
SC – his
SC – his + FOA
SC – his – trp
SIR4 + SIR4-2μ
SIR4 + empty-2μ
sir4Δ + SIR4-2μ
sir4Δ + empty-2μ
(Sussel, 1991)

Figure 7: *SIR4-2μ* and *GAL-SIR4* increase the Sir4 protein concentration and overexpression of *SIR4* leads to a decreased ability to establish silencing at *HMLα*. A) Cells of strain ADR4006 with or without a *SIR4-2μ* plasmid (pAR696) were grown in selective media, harvested, and blotted for Sir4 and Sir2. 2-fold serial dilutions of the SIR4-2*μ* sample was used to estimate the increase of expression of Sir4. Sir2 protein levels serve as a loading control. B) Cells with or without a *SIR4-2μ* plasmid were mated and pedigrees from the resulting zygotes categorized as described in Figure 5 (ADR4480x4481+plasmids) ($p=0.73$ likelihood ratio test) C) Haploid cells containing *GAL-SIR4* were grown on complete media containing 2% galactose, mated and pedigrees from the resulting zygotes categorized as described in Figure 5 (ADR4562x4564) ($P<0.01$ likelihood ratio test) D) Cells with *SIR4* (ADR4480) or *GAL-SIR4* (ADR4562) were grown in YP-Gal media and samples were harvested and analyzed by western blot using 2 fold serial dilutions to estimate the fold-induction of Sir4. Cdk1 levels are shown as a loading control.



Prediction:

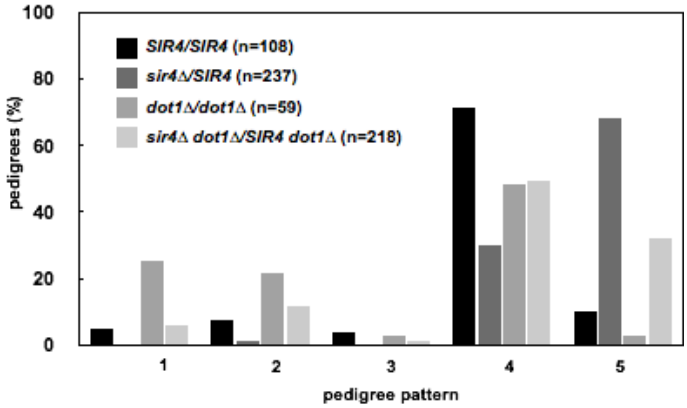
Phenotype between *dot1Δ/ubp10Δ* and *SIR4* haploid

Phenotype similar to *dot1Δ/ubp10Δ*

Phenotype similar *SIR4* haploid

Figure 8: Models of epistasis of *DOT1, UBP10,* and *SIR4*
The interactions of *DOT1, UBP10,* and *SIR4* can be categorized in one of three ways: The genes function in separate pathways to affect establishment and have an intermediate effect on silencing, *SIR4* acts upstream of *DOT1*/*UBP10* to affect establishment, or *DOT1*/*UBP10* acts upstream of *SIR4* to affect establishment. The proposed phenotypes from the single cell mating assay are listed below each model.

a


100
80
60
40
20
0
pedigrees (%)
SIR4/SIR4 (n=108)
sir4Δ/SIR4 (n=237)
dot1Δ/dot1Δ (n=59)
sir4Δ dot1Δ/SIR4 dot1Δ (n=218)
1
2
3
4
5
pedigree pattern

b

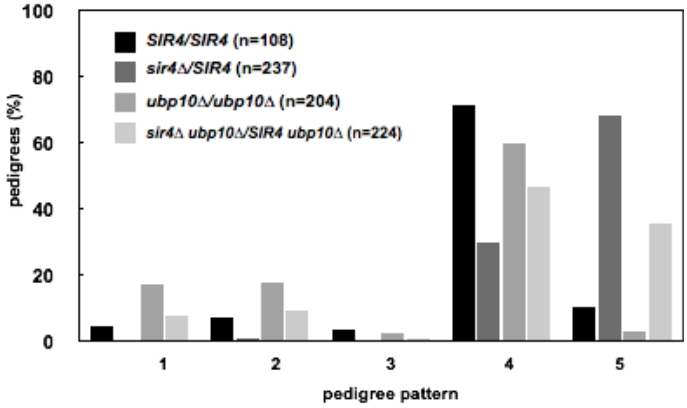

100
80
60
40
20
0
pedigrees (%)
SIR4/SIR4 (n=108)
sir4Δ/SIR4 (n=237)
ubp10Δ/ubp10Δ (n=204)
sir4Δ ubp10Δ/SIR4 ubp10Δ (n=224)
1
2
3
4
5
pedigree pattern

Figure 9: Deletion of *DOT1* or *UBP10* leads to earlier establishment of silencing at *HMLα*, but not in a *SIR4*/*sir4Δ* heterozygote diploid

A) *HML s*ilencing establishment in *sir4Δ*/*SIR4* (ADR4592 and 4481), *dot1Δ* (ADR4631 and 4632), or *sir4Δdot1Δ* (ADR5640 and 5607) haploids mated to form diploids were analyzed as in Figure 1. Pedigrees of individual zygotes were tracked and sorted as in Fig 1B. *SIR4*/*SIR4 dot1*/*dot1* distribution is significantly different than the *sir4*/*SIR4 dot1*/*dot1* and the *sir4*/*SIR4* distributions ($p<0.01$ and $p<0.01$ by the likelihood ratio test) B) *sir4Δ*/*SIR4 (*ADR4592 and 4481), *ubp10Δ* (ADR5087 and 5088), or *sir4Δubp10Δ* (ADR5550 and 5551) haploids were mated as in Figure 1. Pedigrees for individual zygotes were tracked and sorted as in Fig 5B. *SIR4*/*SIR4 ubp10*/*ubp10* distribution is significantly different than the *sir4*/*SIR4 ubp10*/*ubp10* and the *sir4*/*SIR4* distributions ($p<0.01$ and $p<0.01$ by the likelihood ratio test)

Deletion of *UBP10* lead to earlier establishment of silencing

In order to distinguish between the two remaining models we considered how Dot1, a histone modifying enzyme, might act upstream of Sir4 abundance. Although the effect of *dot1*Δ on establishment has been proposed to reflect changes in chromatin state at the *HM* loci, *dot1*Δ cells are also defective for telomeric silencing ((Takahashi et al., 2011) and Figure 10D) and this phenotype might indirectly effect the *HM* loci. Past studies have shown that telomeres and the *HM* loci compete for silencing proteins (Maillet et al., 1996; Marcand et al., 1996), and that loss of telomeric silencing can have indirect effects on other phenotypes due to relocalization of silencing proteins.

Mutation of *UBP10*, a ubiquitin protease, allowed us to examine these two possible functions of Dot1 in establishment. Unlike *dot1*Δ cells, mutation of *UBP10* is known to increase Dot1-dependent H3K79 methylation by increasing H2BK123 ubiquitination, which in turn promotes the action of Dot1 (Dover et al., 2002; Emre et al., 2005; Ng et al., 2002b; Shahbazian et al., 2005; Sun and Allis, 2002; Yang et al., 2008). Surprisingly, we found that *ubp10*Δ/*ubp10*Δ cells, like *dot1*Δ/*dot1*Δ cells speed the rate of establishment (Figure 10A), which is contrary to our original hypothesis. In looking deeper into what may link the mutant establishment phenotypes, we found that like *dot1*Δ cells, *ubp10*Δ cells have telomeric silencing defects (Singer et al., 1998), Cultures of *dot1*Δ and *ubp10*Δ cells were grown to saturation and plated on media containing 5-FOA to screen for their ability to silence telomeric *URA3*. Both mutants showed decreased viability on the drug, indicating decreased telomeric silencing (Figure 10D) and supporting a model whereby indirect effects on telomeres causes alterations in the establishment of silencing at the HM loci. Loss of telomeric silencing correlates with earlier establishment of silencing at *HML*α. We

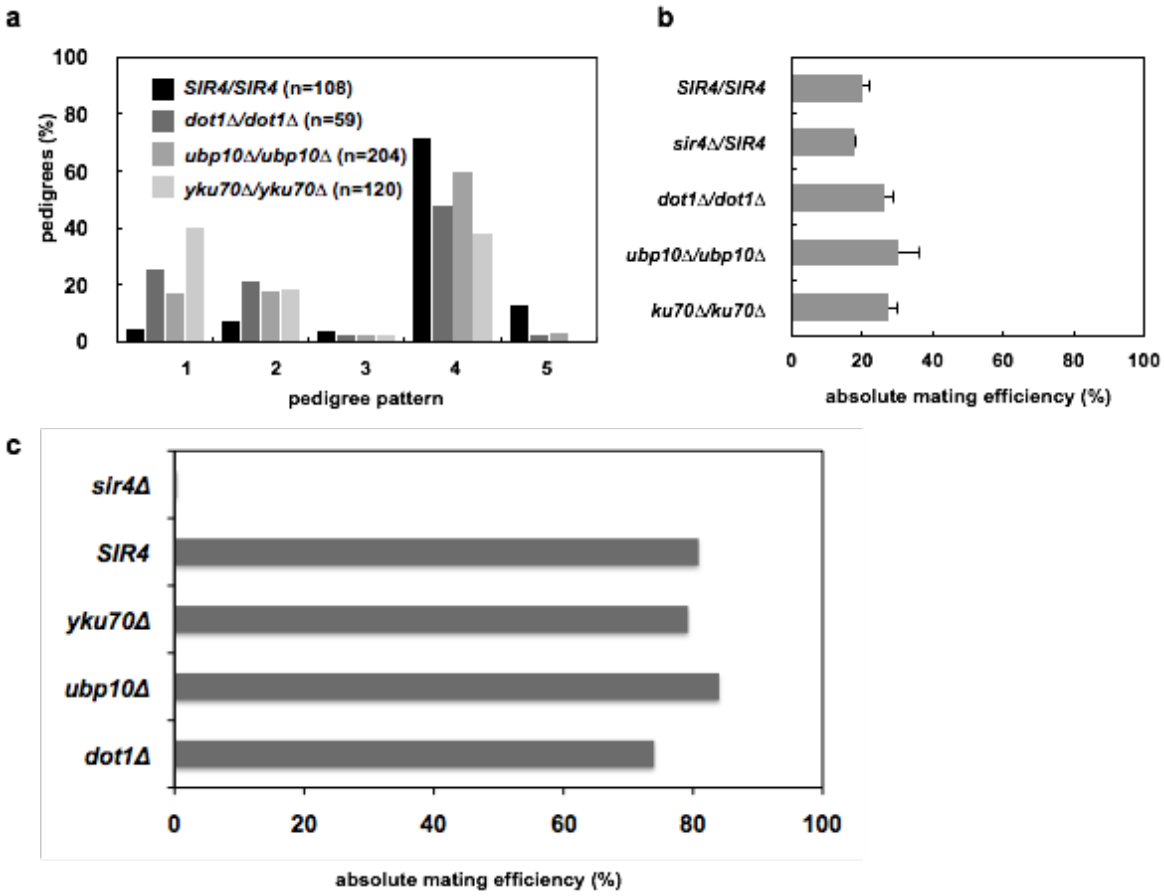

a
100
80
60
40
20
0
pedigrees (%)
SIR4/SIR4 (n=108)
dot1Δ/dot1Δ (n=59)
ubp10Δ/ubp10Δ (n=204)
yku70Δ/yku70Δ (n=120)
1
2
3
4
5
pedigree pattern
b
SIR4/SIR4
sir4Δ/SIR4
dot1Δ/dot1Δ
ubp10Δ/ubp10Δ
ku70Δ/ku70Δ
0
20
40
60
80
100
absolute mating efficiency (%)
c
sir4Δ
SIR4
yku70Δ
ubp10Δ
dot1Δ
0
20
40
60
80
100
absolute mating efficiency (%)
d
TELVII-L::URA3
hmrΔE::TRP1 +
SC
SC + FOA
wild type
sir3Δ
yku70Δ
dot1Δ
ubp10Δ

Figure 10: Deletion of *DOT1*, *UBP10,* or *yKu70* leads to earlier establishment of silencing at *HMLα*

A) *dot1Δ (*ADR4631 and 4632), *ubp10Δ* (ADR5087 and 5088) or *yku70Δ* (ADR5841 and 5842) cells were mated to produce zygotes whose pedigrees were followed as in Fig 5. *dot1Δ* data is similar to experiments published by Osborne *et al. (*2009). All deletions are significantly different than the wild type ($p<0.01$ by likelihood ratio test) B) Resultant diploids from (A) that were either *SIR4 (*ADR4480x4481), *sir4Δ/SIR4* (ADR4592x4481), *dot1Δ* (ADR4631x4632), *ubp10Δ* (ADR5087x5088), or *yku70Δ* (ADR5841x5842) were mated to a MATα tester strain (ADR3082) to determine their mating efficiency. C) Haploid a cells that were either *SIR4* (ADR4480), *sir4Δ* (ADR4592), *dot1Δ* (ADR4631), *ubp10Δ* (ADR5087), or *yku70Δ* (ADR5841) were mated to a wild type tester α strain (ADR3082). Mating efficiency was the proportion of total cells that mated and formed colonies on synthetic media lacking amino acids. D) Serial dilutions of saturated cultures of wild type (ADR4062), *sir3Δ, yku70Δ* (ADR5840), *ubp10Δ* (ADR5843), or *dot1Δ* (ADR5895) were spotted on complete media with or without 5-FOA, or -Trp minimal media. Cells which grow on media containing 5-FOA are able to silence the *TELVII-L::URA3* gene.

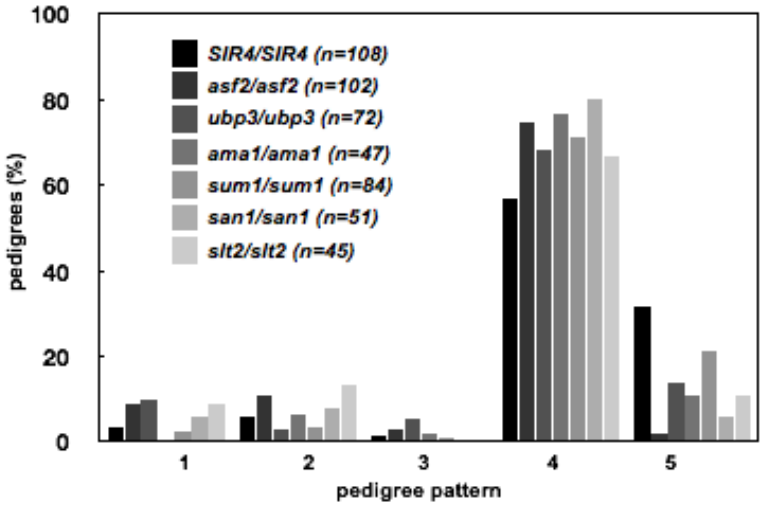
therefore propose that the earlier establishment at *HML* in *dot1*Δ or *ubp10*Δ cells may be promoted by a loss of telomeric silencing as opposed to histone modification status.

Like *SIR4*/*sir4Δ* cells, *ubp10Δ*/*ubp10Δ* and *dot1Δ*/*dot1Δ* cells (and *dot1Δ* and *ubp10Δ* haploids) have no effect on maintenance of *HM* silencing as assayed by quantitative mating assays (Figure 10B and C). Also like *dot1Δ*/*dot1Δ* cells, *ubp10Δ SIR4*/ *ubp10Δ sir4Δ* cells slow establishment to rates similar to those of *SIR4*/*sir4Δ* cells (Figure 9B). These data support a model whereby Dot1 and Ubp10 function upstream of Sir4 abundance by regulating telomeric heterochromatin, and by extension, the amount of free Sir complex available to establish silencing at the *HM* loci.

Deletion of y*KU70* leads to earlier establishment of silencing at *HMLα*

To test if loss of telomeric silencing could cause an earlier establishment phenotype we deleted *yKU70,* a component of the Ku complex involved in telomeric silencing, telomere structure, and double strand break repair (Boulton and Jackson, 1998; Lopez et al., 2011). yKu70 is not known to regulate H3K79 methylation and its deletion has no effect on maintenance of silencing at *HMLα* (Figure 10B and C and data not shown). Like *ubp10*Δ cells, K79 methylation of histone H3 is similar in *yku70*Δ cells to wild type cells at the *HM* loci in the presence or absence of silent chromatin (Figure 11B). It has however, been shown to have an overlapping function with Sir1 at the *HM* loci (Patterson and Fox, 2008). *yku70*Δ homozygotes, like *dot1*Δ cells, show a dramatic increase in the number of cells

a


100
80
60
40
20
0
pedigrees (%)
SIR4/SIR4 (n=108)
asf2/asf2 (n=102)
ubp3/ubp3 (n=72)
ama1/ama1 (n=47)
sum1/sum1 (n=84)
san1/san1 (n=51)
slt2/slt2 (n=45)
1
2
3
4
5
pedigree pattern

b

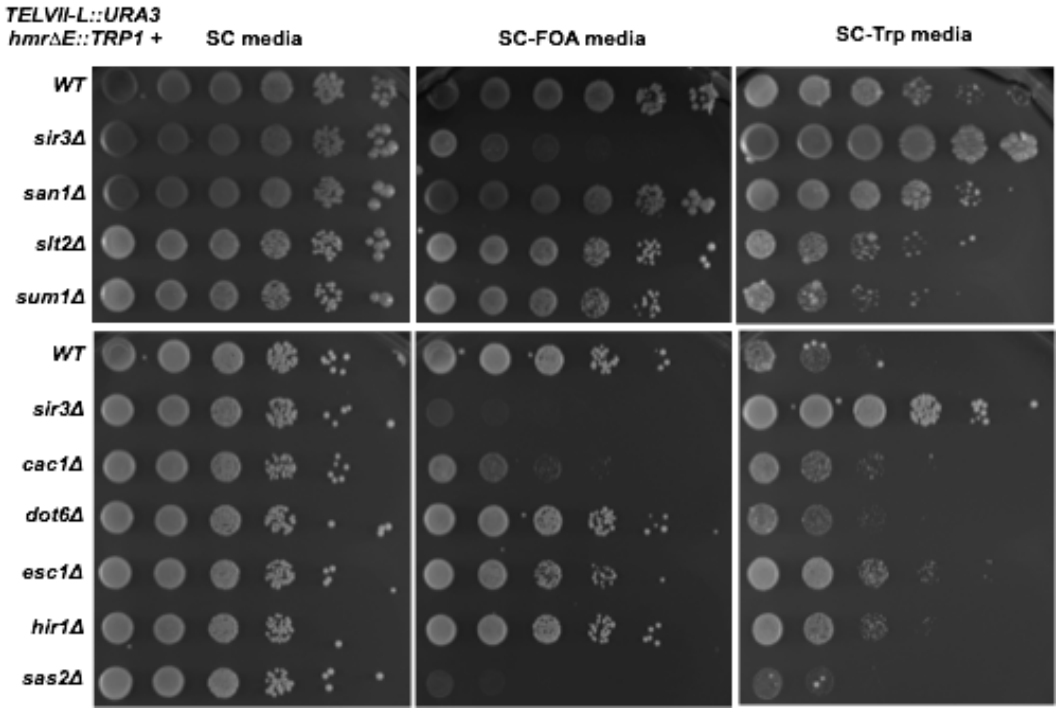

TELVII-L::URA3
hmrΔE::TRP1 +
SC media
SC-FOA media
SC-Trp media
WT
sir3Δ
san1Δ
slt2Δ
sum1Δ
WT
sir3Δ
cac1Δ
dot6Δ
esc1Δ
hir1Δ
sas2Δ

Figure 11: Other candidate genes do not lead to a decreased ability to establish silencing at *HMLα*

A) Haploid cells with *asf2Δ* (ADR4520 and 4522), *ubp3Δ* (ADR4992 and 4993), *ama1Δ* (ADR5001 and 5002), *sum1Δ* (ADR5090 and 5091), *san1Δ* (ADR5110 and 5111), or *slt2Δ* (ADR5838 and 5839) were mated to form the listed diploids and pedigrees were analyzed as in Figure 5B. None are significantly different than the control by likelihood ratio test ($p>0.5$ for all) B) Serial dilutions of saturated cultures of wild type (ADR4062), *sir3Δ*, *san1Δ* (ADR6119), *slt2Δ* (ADR5838), *sum1Δ* (ADR6121), *cac1Δ* (ADR6128), *dot6Δ* (ADR6124), *esc1Δ* (ADR6122), *hir1Δ* (ADR6126), or *sas2Δ* were plated on complete media, complete media containing 5-FOA, and complete media lacking tryptophan. Cells which grow on media containing 5-FOA are able to silence the *TELVII-L::URA3* gene. Cells which grow on -trp media are able unable to silence the *hmrΔE::TRP1* gene.

which were able to establish silencing after only one generation (Figure 10A, pattern 1). Like *dot1*Δ and *ubp10*Δ, the *yku70*Δ early establishment phenotype is blocked by the *SIR4*/*sir4*Δ heterozygotes (data not shown). Double mutant diploids for the above deletions were also tested for epistatic effects using the single cell mating assay and none of the pairwise deletions showed a significant difference in the ability to silence compared to either single mutant (Figure 12 A -C). Given the data above regarding the loss of telomeric silencing and the *HM* silencing phenotype, it indicates that these mutants all function in a very similar manner to promote earlier establishment of silencing.

We also examined the mutants for silencing maintenance and found that all of the three mutants were able to maintain established silencing at HM loci similarly to the wild type cells (Figure 10B and C).

To further confirm the telomeric silencing phenotype and validate *yKU70* as a control we deleted the two Rap1 Interacting Factor genes (*RIF1*/*RIF2*), which we hypothesized would restore telomeric silencing in a *yku70Δ* mutant and, by extension, alleviate the earlier establishment of silencing seen at *HMLα*. The *ku70Δ rif1Δ rif2Δ*/*ku70Δ rif1Δ rif2Δ* cells had a silencing phenotype similar to that of wild type cells, indicating that the return of telomeric silencing affected the ability to establish silencing at *HMLα* (Figure 13).

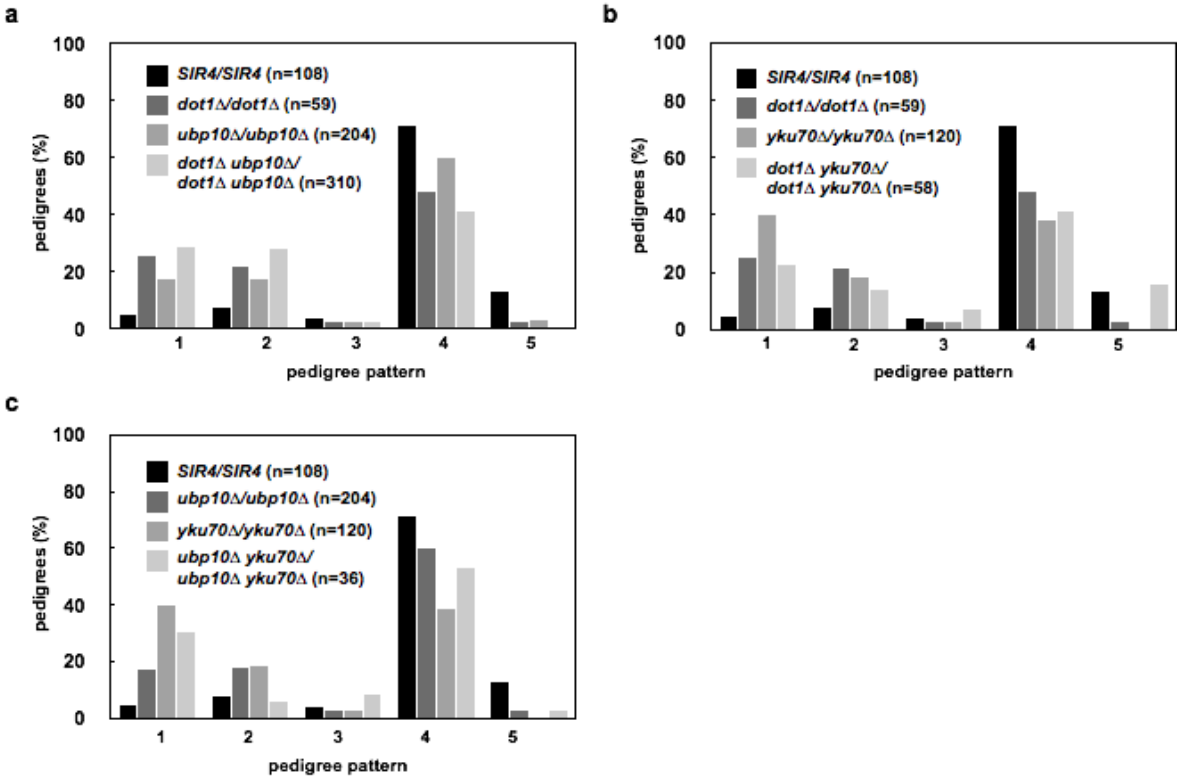

a
100
80
60
40
20
0
pedigrees (%)
SIR4/SIR4 (n=108)
dot1Δ/dot1Δ (n=59)
ubp10Δ/ubp10Δ (n=204)
dot1Δ ubp10Δ/
dot1Δ ubp10Δ (n=310)
1
2
3
4
5
pedigree pattern
b
100
80
60
40
20
0
pedigrees (%)
SIR4/SIR4 (n=108)
dot1Δ/dot1Δ (n=59)
yku70Δ/yku70Δ (n=120)
dot1Δ yku70Δ/
dot1Δ yku70Δ (n=58)
1
2
3
4
5
pedigree pattern
c
100
80
60
40
20
0
pedigrees (%)
SIR4/SIR4 (n=108)
ubp10Δ/ubp10Δ (n=204)
yku70Δ/yku70Δ (n=120)
ubp10Δ yku70Δ/
ubp10Δ yku70Δ (n=36)
1
2
3
4
5
pedigree pattern

Figure 12: Double deletions have no effect on the ability of cells to establish silencing at *HMLα* and there is no epistatic effect between *dot1Δ, ubp10Δ* and *yku70Δ*.
A, B, C) Haploid cells that are *dot1Δubp10Δ* (ADR5171 and 5172), *dot1Δyku70Δ* (ADR5944 and 5945) or *ubp10Δyku70Δ* (ADR5920 and 5921) were mated to produce the listed zygotes and pedigrees were analyzed as in Figure 5B. None of the distributions were significantly different than the control. $p<0.5$ for each by likelihood ratio test.

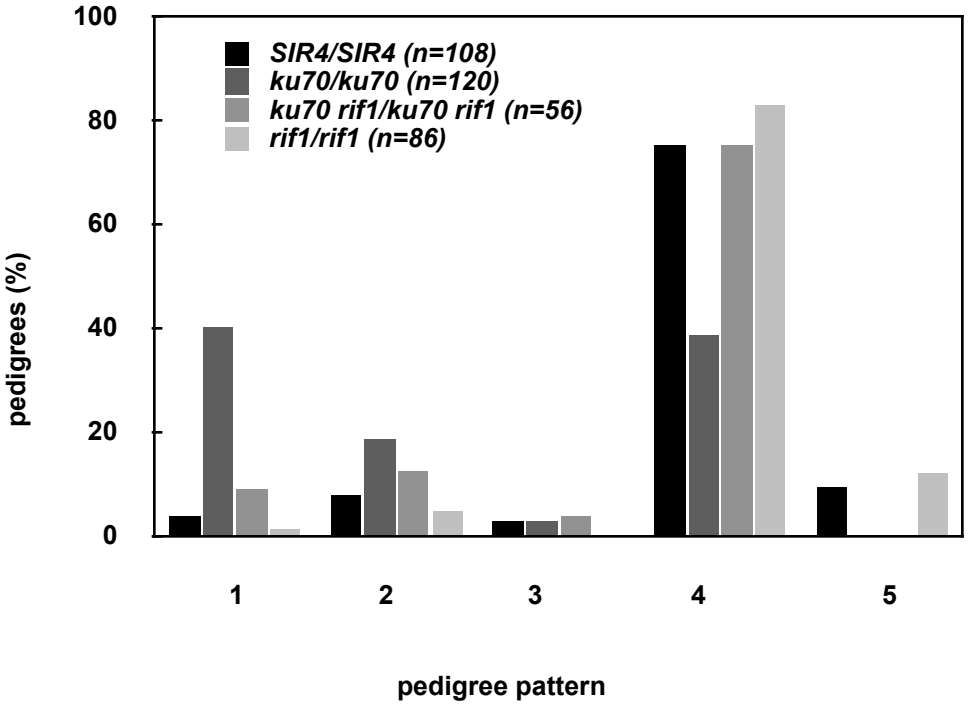

100
80
60
40
20
0
pedigrees (%)
SIR4/SIR4 (n=108)
ku70/ku70 (n=120)
ku70 rif1/ku70 rif1 (n=56)
rif1/rif1 (n=86)
1
2
3
4
5
pedigree pattern

Figure 13: Deletion of *RIF1* reverses the effect of *yku70Δ* on the establishment of silencing at *HMLα*

Haploid cells that are wild type (ADR 4480 and 4481), *ku70Δ* (ADR5841 and 5842), *ku70Δrif1Δ* (ADR7972 and 7975) or *rif1Δ* (ADR7962 and 7966) were mated to produce the listed zygotes and pedigrees were analyzed as in Figure 5B. The *rif1Δ* distribution was significantly different than the control. $p<0.5$ by likelihood ratio test. The *ku70Δrif1Δ* was significantly different than both the control and *ku70Δ* distributions, $p>0.05$ by the likelihood ratio test.

Deletion of other proteins previously implicated in silencing does not promote earlier heterochromatin establishment at *HMLα*

In the course of the experiments described above I tested additional mutants for their role in silencing establishment. Deletion of *SAN1* (a ubiquitin ligase thought to regulate Sir4 (Dasgupta, 2004)), *ASF2* (a putative antisilencing protein (Le et al., 1997)*),* *UBP3* (a ubiquitin protease, inhibitor of silencing (Baker et al., 1992; Moazed and Johnson, 1996))*,* *AMA1* (activator of the anaphase promoting complex, (APC) (Cooper et al., 2000))*,* *SUM1* (previously implicated in silencing (Irlbacher, 2005; Klar et al., 1985))*,* or *SLT2* (cell wall integrity (Cid et al., 1995)) have no statistically significant effect on the the rate of *de novo* silencing establishment, nor do mutations in *SIR4* that remove potential destruction boxes that target substrates for Anaphase Promoting Complex-dependent proteolysis (Figure 11A, and L. Sunstrum, E. Williams and A. Rudner, unpublished data, Table 3). Additional strains potentially implicated in silencing were tested for telomeric and *HM* silencing defects (Figure 11B), but not used in the single cell mating assays.

Sir4 protein occupancy is lower at telomeres in *dot1Δ* and *yku70Δ* cells and unchanged at *HMLα* and *HMRa*

Given our hypothesis that mutants which cause a loss of telomeric silencing will liberate Sir4 and increase the concentration of Sir4 available at the *HM* loci, Katherine Harding used chromatin immunoprecipitation (ChIP) to assay the amount of Sir4 bound to telomeres in the mutant strains. Wild type, *dot1Δ, ubp10Δ,* and *yku70Δ* cells were harvested and immunoprecipitated using a Sir4 antibody. Occupancy of Sir4 was

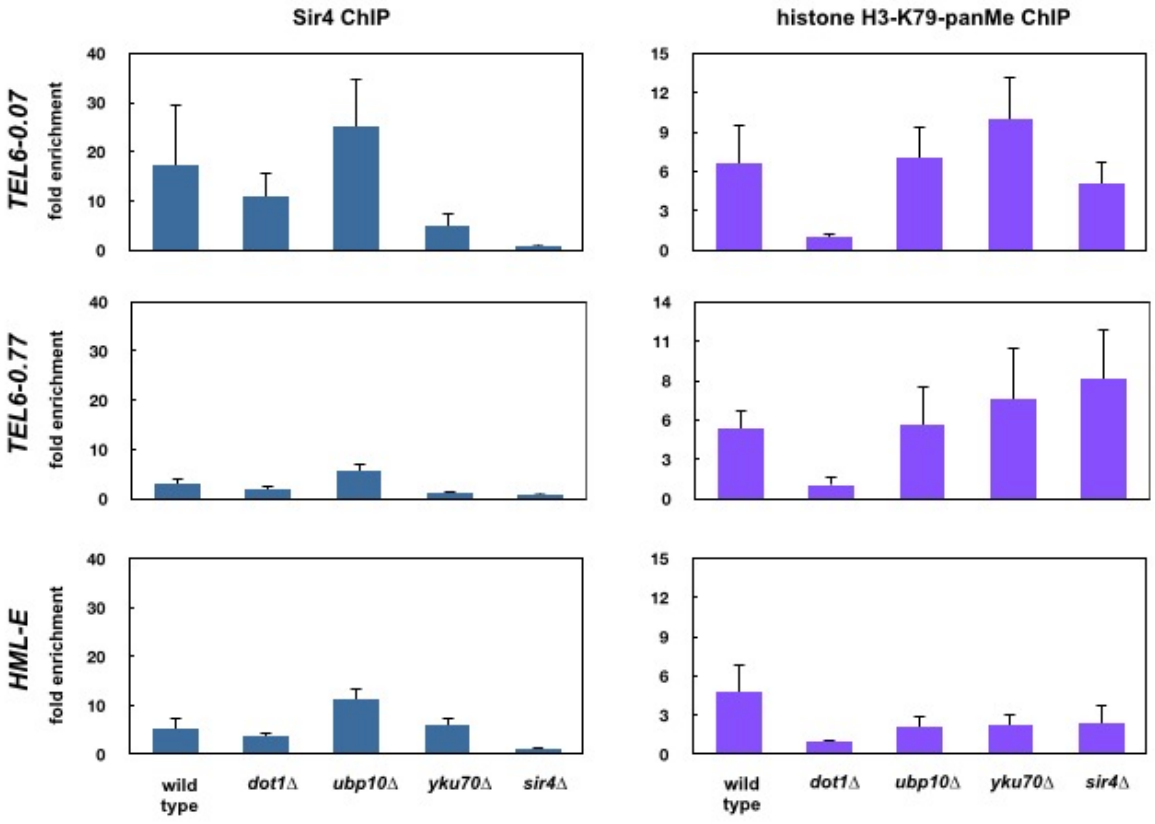
determined by PCR of target locations at the telomere, *HML-E,* and a control locus (*ACT1)*. The amount of Sir4 present at the *HM* loci remained similar in the mutant strains tested, while the amount of Sir4 localized to telomeres correlated with the extent of telomeric silencing (Figure 14, K. Harding, preliminary data).

Deletion of *yku70* has no effect on H3K79 methylation

We tested whether the deletion of *YKU70* or *UBP10* would impact the H3K79 methylation status of histones in the silenced regions. At *TEL-VI*, where Sir4 localization is reduced in *yku70Δ,* there is slightly higher H3K79 methylation, while at *HML* H3K79 methylation was similar or higher that that of wild type cells when there is no change in steady state silencing (Figure 14B), confirming that the establishment phenotype shared between the deletions of *DOT1* and *yKU70* does not correlate to the status of histone H3K79 methylation.

***DOT1* plays two roles in silencing establishment and maintenance**

As previously reported, *dot1Δ* cells have a defect in silencing at weakened silencers (Osborne et al., 2009; Singer et al., 1998), while others have shown that *dot1Δ* cells have improved establishment of silencing at the native *HML* locus (Figure 6C and Osborne 2009). Previous work examined silencing at *HMR* using strains that insert *ADE2* at the *HMRa* locus and weakens a portion of the silencer element (*hmrΔA-ADE2*) (Mano et al., 2013; Valenzuela et al., 2008). Repression of *ADE2* causes yeast to change color from



Katherine Harding and Adam Rudner

Figure 14: Sir4 protein distribution is altered in deletion mutants, as is the methylation status of histones in *dot1Δ* but not *ubp10Δ* or *yku70Δ* cells.

Chromatin immunoprecipitation (ChIP) was performed using antibodies against Sir4 and pan-methylated histone H3K79, on cells fixed with 1% formaldehyde as previously described. The resulting DNA was used as a template for PCR at the listed locations with radiolabelled nucleotides. (ADR22, 6168, 6162, and 6183)

white to red, and using this simple readout silencing (and switching between ON and OFF states) can be assessed by the percentage of red, white and sectored colonies (Figure 15). Because individual sectored colonies can switch between ON and OFF states several times, it was proposed that this assay may measure establishment, and the first experiments that showed Sir4 dosage regulates silencing were performed using this strain (Sussel and Shore, 1991).

This assay is much less labour intensive than the single cell assay we have used, so we therefore tested if *dot1Δ, ubp10Δ,* or *yku70Δ* improved or weakened silencing at *hmrΔA-ADE2,* along with the control *esc8Δ*. If this assay measured establishment of silencing we predicted that these mutants would improve silencing, and increase the percentage of red colonies in the population, similar to what was originally found for increasing Sir4 dosage (Sussel and Shore, 1991). However, all four strains had defects in silencing *hmrΔA*, and the percentage of white colonies increased. We conclude that the presence of a weakened silencer (*ΔA*) behaves similarly to deleting *SIR1* and reveals silencing defects in *dot1Δ* and *ku70Δ* (Osborne et al., 2009; Patterson and Fox, 2008). This assay implies that *dot1Δ* and *yku70Δ* can have both positive and negative effects at the *HM* loci: the negative effects are direct and are only seen when the silencer is weakened, while the positive effects are indirect and are caused by a loss of telomeric silencing. We also conclude that *hmrΔA-ADE2* is likely a sensitive assay for maintenance of silencing and not establishment.

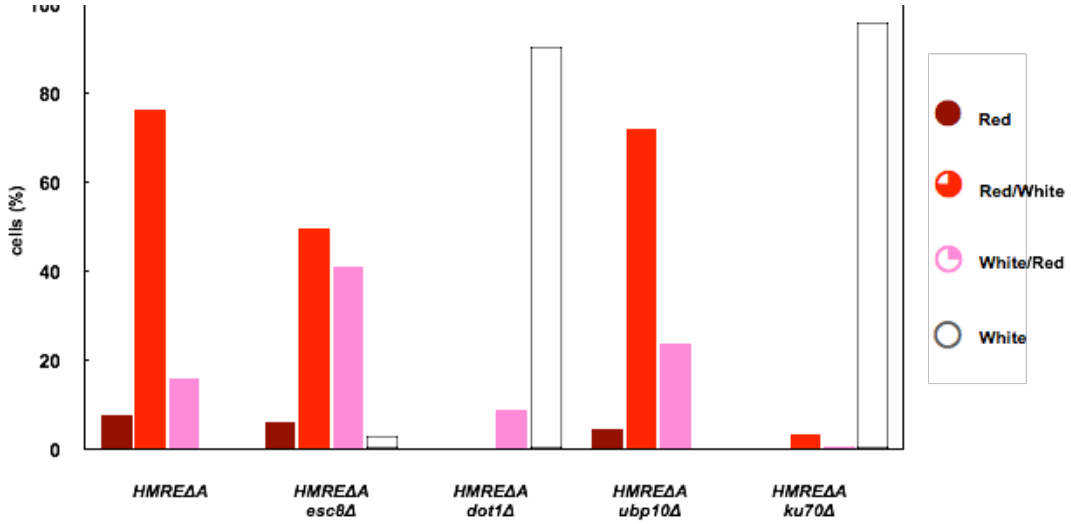

cells (%)
80
60
40
20
0
HMREΔA
HMREΔA esc8Δ
HMREΔA dot1Δ
HMREΔA ubp10Δ
HMREΔA ku70Δ
Red
Red/White
White/Red
White

Figure 15: Weakened *HMR* shows defects in silencing maintenance in mutant strains
A) Cells were grown in YPD and plated on YPD plates and grown at 30 degrees for 3 days the placed at 4 degrees to allow the red colour to develop. Cells were analyzed for the ability to silence the *ADE2* locus (red or white) and switch between silenced and unsilenced states (sectored colonies), and scored for colony colour and sectoring. Representative of 1-5 independent experiments per strain (ADR6144 n=854, 6145 n=1111, 6184 n=452, 6185 n=203, 6186 n=396)

Sir4 protein decreases in α-factor

Our data is consistent with a model that the abundance of Sir4 is the primary mechanism used to regulate the establishment of silent chromatin. However, it is unknown whether Sir4 abundance is regulated during periods when cells are not able to establish silent chromatin. Past work has shown that establishment cannot occur during pheromone arrest, so we examined Sir4 protein levels during these arrests. When cells are arrested in α factor the level of Sir4 protein falls, while arrest in nocodazole has no effect on Sir4 levels (Figure 16A). After arrest in α factor and a release back into the cell cycle, the level of Sir4 rises again to the pre-arrest level over 2 cell cycles (Figure 16B), which is similar to the amount of time that has been reported for *de novo* silencing to occur and similar to what is seen in the single cell establishment assays ((Osborne et al., 2009) and Figure 4B). This dramatic decrease in Sir4 abundance in pheromone arrest has not previously been reported and, since silencing appears to remain stable during the arrest, we wanted to assess if the decrease in Sir4 was affecting preferentially the soluble protein fraction or the chromatin bound Sir4. Despite low levels of total Sir4 protein in cells, ChIP confirms that chromatin-associated Sir4 is maintained at high levels at *TEL-VI* and *HMLα* after five hours of exposure to mating pheromone (Figure 17). The measurement of Sir4 level at *TEL-VI* was performed near to the telomere end. Preliminary data shows that the loss of Sir4 is more substantial further from the telomere ends upon pheromone exposure (K. Harding, unpublished data).

Sir4 localizes to foci within the nucleus (Palladino et al., 1993) and as an independent measure of the persistence of chromatin bound Sir4 we measured the fluorescence of the foci. There are no changes in the intensity of the intra-nuclear *SIR4-*

GFP foci in pheromone or nocodazole arrest (Figure 18, N. Lianga, unpublished data). These data suggest that although total cellular Sir4 protein levels are decreasing, Sir4 is retained at silenced loci during pheromone exposure.

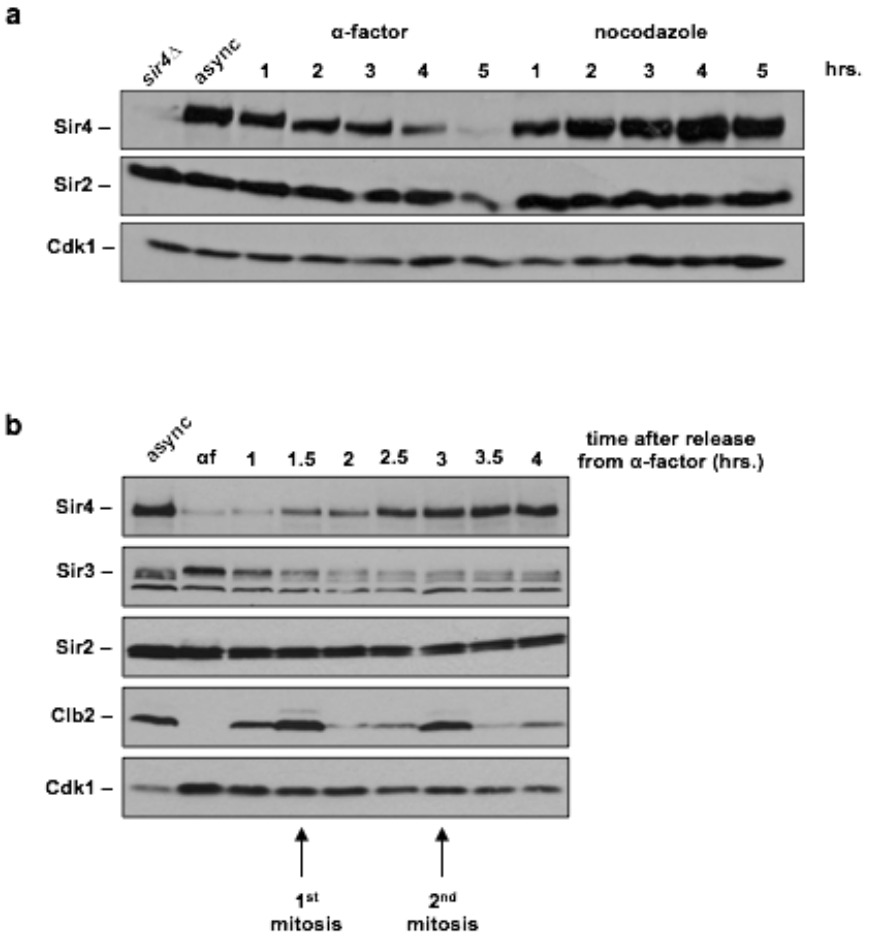

a
α-factor
nocodazole
sir4Δ
async
1
2
3
4
5
1
2
3
4
5
hrs.
Sir4 –
Sir2 –
Cdk1 –
b
async
αf
1
1.5
2
2.5
3
3.5
4
time after release from α-factor (hrs.)
Sir4 –
Sir3 –
Sir2 –
Clb2 –
Cdk1 –
1st mitosis
2nd mitosis

Figure 16: Sir4 protein levels decrease upon exposure to α-factor.
A) Cells were grown in the presence of 1μg/ml α-factor or 10μg/ml nocodazole at 25 degrees (ADR4006). Samples were harvested every hour and protein levels were analyzed by western blot. Cdk1 is shown as a loading control. B) Asynchronous cells were arrested in α factor at 25 degrees. The pheromone was washed out and cells were returned to YPD media at 25 degrees. Cells were harvested at the indicated times and analyzed by western blot. CDK1 is shown as a loading control.

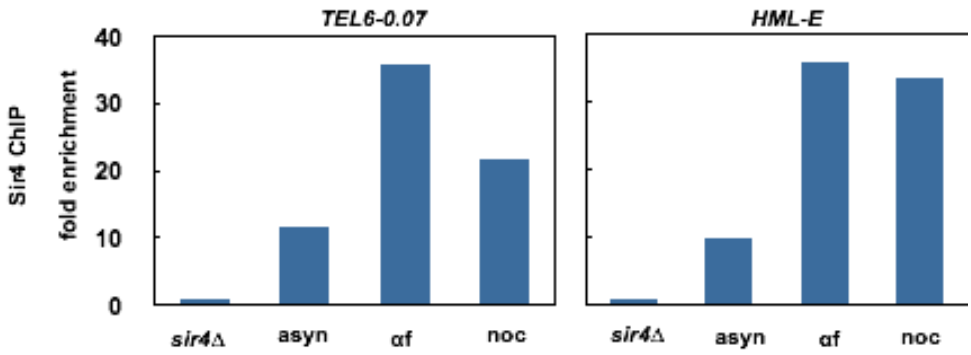

TEL6-0.07
HML-E
Sir4 ChIP
fold enrichment
40
30
20
10
0
sir4Δ
asyn
αf
noc
sir4Δ
asyn
αf
noc

Figure 17: Sir4 remains localized to silent regions in periods of pheromone arrest
Cells were cultured in 1μg/ml α-factor or 10μg/ml nocodazole at 25 degrees. Cells were fixed and ChIP was performed using the α-SIR4 antibody and probed at the listed loci, as well as the control locus *ACT1*. (ADR4006 and 3387)

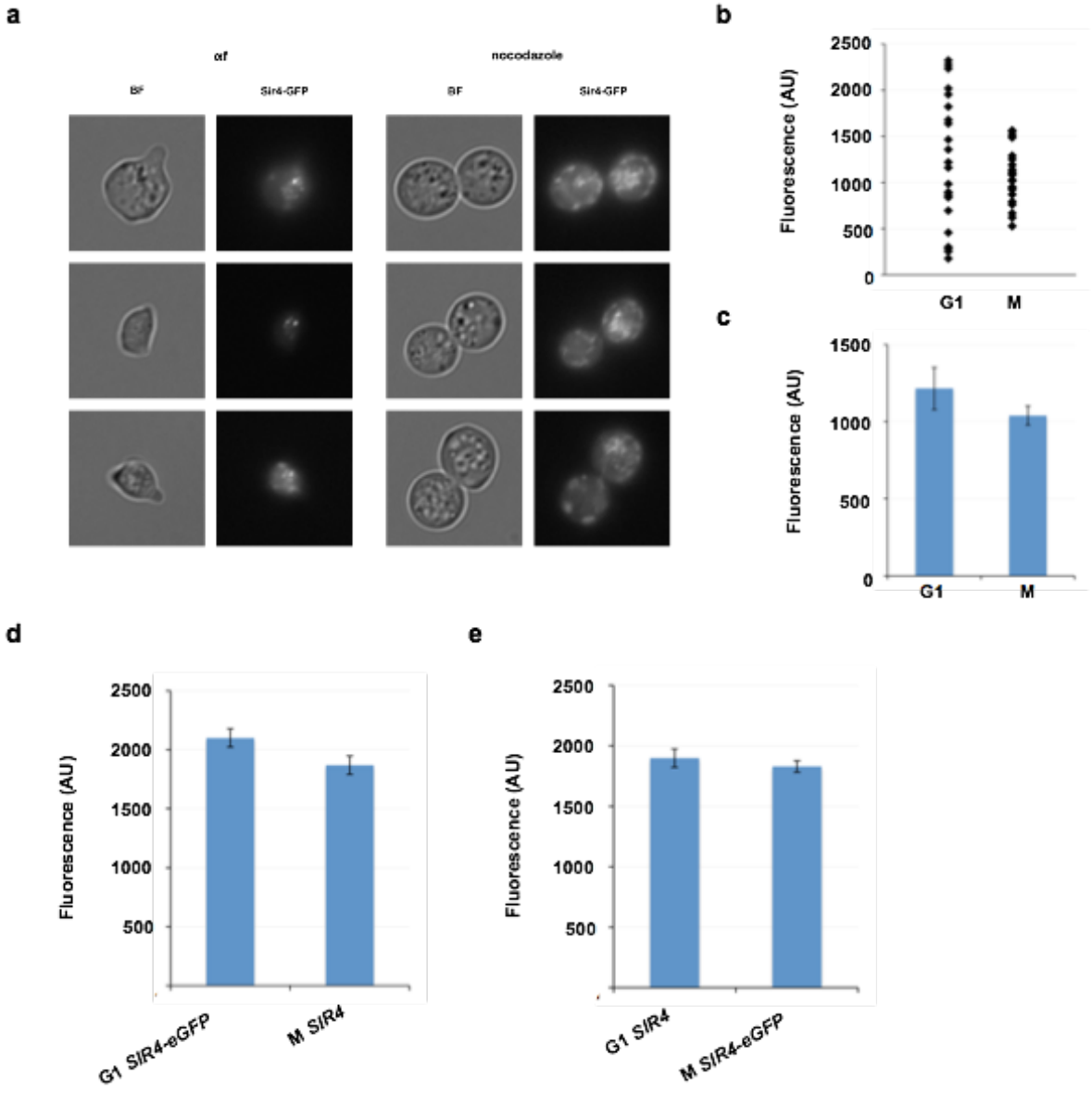

a
αf
nocodazole
BF
Sir4-GFP
BF
Sir4-GFP
b
Fluorescence (AU)
2500
2000
1500
1000
500
0
G1
M
c
Fluorescence (AU)
1500
1000
500
0
G1
M
d
Fluorescence (AU)
2500
2000
1500
1000
500
G1 SIR4-eGFP
M SIR4
e
Fluorescence (AU)
2500
2000
1500
1000
500
G1 SIR4
M SIR4-eGFP

Figure 18: Sir4 persist in α-factor arrested cells
A, B, C) *SIR4-eGFP* (ADR3810) cells were grown overnight to log phase and arrested in either α-factor or nocodazole for 5 hours, fixed and imaged using a fluorescence microscope. α-factor or nocodazole arrested example cells are shown in A). The relative fluorescence of Sir4-eGFP foci was determined in the images from A) (n=24 for each treatment). The relative fluorescence of each foci is displayed in B) and the average fluorescence for each treatment is displayed in C) (mean +/- SEM) (difference in average fluorescence is not statistically significant, p=0.254, student's two-tailed t-test). D) Background fluorescence images of wildtype (ADR4006) cells treated for 5 hours with nocodazole and *SIR4-eGFP* (ADR3810) cells treated with α-factor were obtained as in A). The average fluorescence over background is displayed (mean +/- SEM) (n=12 for each treatment). E) Background fluorescence images of wildtype (ADR4006) cells treated for 5 hours with α-factor and *SIR4-eGFP* (ADR3810) cells treated with nocodazole were obtained as in A). The average fluorescence over background is displayed (mean +/- SEM) (n=12 for each treatment).

DISCUSSION

Silencing is dependent on SIR protein concentration as well as histone modifications

Prior studies have shown that the abundance of the SIR proteins, specifically Sir4, has an impact on silencing (Benbow and DuBois, 2008; Sussel et al., 1993). Using weakened *HMR* silencers, integrating additional copies of *SIR4* improved silencing at these sites ((Sussel et al., 1993) and Figure 6) and in diploids with only one copy of *SIR4* both a weakened *HMR* and telomeric *URA3* are derepressed (Sussel et al., 1993)(Figure 5). Although Sussel and Shore proposed that Sir4 dosage effects reflected a role in establishment, their experiments could not distinguish between establishment and maintenance of silencing. We have directly monitored establishment of silencing in our report using a native silencer, *HML,* rather than the modified version of *HMR* favoured in the past. The use of a single cell assay, such as the one developed by Osborne *et al* and utilized in this study, allows us to carefully examine the timing of heterochromatin establishment (Osborne et al., 2009). Plate based assays use colony colour (*ADE2* expression) or expression of auxotrophic markers (like *URA3*) to assay silencing within populations of cells (Leeuwen and Gottschling, 2002) after approximately 20 divisions on a plate. While informative for the behaviour of a population, these studies mask heterogeneity in the population and do not provide the resolution to look at events that occur over 1-3 divisions. Single cell analysis removes the need to differentiate between intermediate states of repression within cells versus a mixed population of derepressed and fully repressed cells and allows for statistical analysis of data that shows finite differences in the timing of silencing establishment. As we have seen in our single cell silencing experiments, there is a measurable level of variation between cells of any given genotype

that would be presented as an average in most population based assays, while the techniques we used were able to discretely categorize the behaviour of the population on a cell by cell level. Our results suggest that one copy of *SIR4* is not sufficient to efficiently establish silencing at a native *HMLα* locus, while additional *SIR4* on a centromeric plasmid, via integration of *SIR4,* or by β-estradiol induction of *SIR4* leads to more rapid establishment (Figure 6).

Past work has shown that deletion of *DOT1*, the histone H3K79 methyltransferase also significantly speeds establishment of silencing. We therefore explored the relationship between Dot1 and changes in Sir4 abundance using traditional genetic epistasis (Figure 8). Using a double mutant (*SIR4 dot1Δ*/*sir4Δ dot1Δ*) we concluded that *dot1Δ* was able to only slightly rescue the defect of a *SIR4*/*sir4Δ* mutant, thus the changes in Sir4 dosage are able to largely block the earlier establishment phenotype from deletion of *DOT1* and they must work via independent mechanisms or through Dot1 regulation of Sir4 (Figure 8).

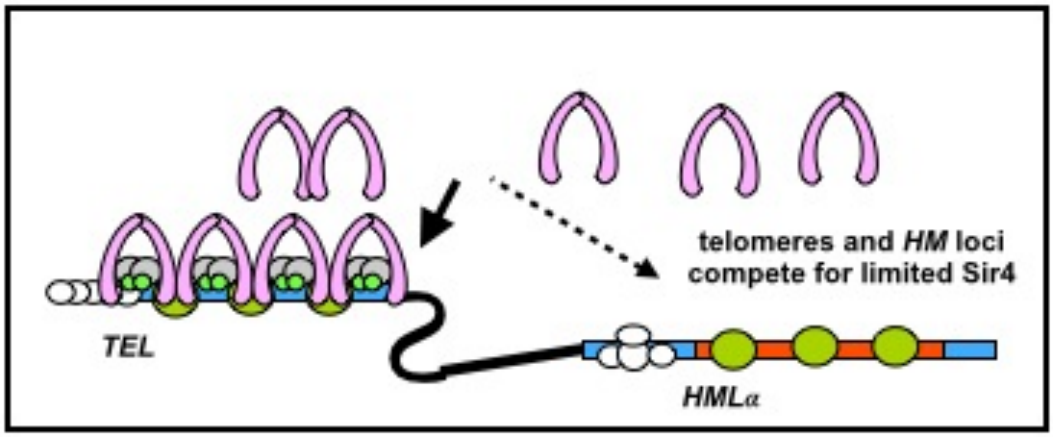
Dot1 and Ubp10 are both chromatin modifying enzymes which have opposite effects on H3K79 methylation status when they are deleted: *dot1Δ* cells lack the ability to methylate H3 while *ubp10Δ* cells are unable to deubiquitinate the H2BK123ub which promotes methylation of H3K79, instead allowing the feed forward mechanism to proceed unchecked (Emre et al., 2005; Leeuwen et al., 2002; Ng et al., 2002a). These two mutants might be predicted to have opposing phenotypes when silencing establishment is tested at *HM* loci owing to the decrease and increase of methylation respectively. Interestingly, we found that *dot1Δ* and *ubp10Δ* had the same earlier establishment phenotype at *HMLα*, as well as loss of silencing seen at the telomeres in both mutants (Singer et al., 1998). Given the somewhat incongruous data, we hypothesized that the loss of silencing at the telomeres

observed in both strains may indirectly promote establishment by liberating Sir4 from telomeres to *HML* (Figure 19).

Competition for limiting amounts of Sir4 between the telomeres and *HM* loci has been noted in the past (Marcand et al., 1996) where *rap1* mutants eliminated telomeric silencing but had better silencing at internal loci. Past work (Maillet et al., 2001) has shown that Sir proteins can be mobilized from telomeres to other sites, and we have shown that the loss of telomeric silencing in *dot1*Δ, *ubp10*Δ, and *yku70*Δ, each of which have known telomeric silencing defects, may liberate Sir4 from telomeres and increase the pool of free Sir4 available to establish silent chromatin at *HMLα* (Figure 10D). This new evidence suggests that past models implying that the removal of histone modifications is the rate-limiting step in the establishment of heterochromatin (Hecht et al., 1995; Katan-Khaykovich and Struhl, 2005; Lau et al., 2002; Moazed, 2001; Onishi et al., 2007) may be incomplete, since *yku70*Δ cells do not affect histone methylation yet are still able to efficiently establish heterochromatin (Figure 14). Our data supports the model that deletion of *DOT1, UBP10,* or *YKU70* eliminates telomeric silencing and liberates Sir4 and the SIR complex within the nucleus. The increased concentration of unbound SIR complex can then target the HM loci, allowing the cell to overcome boundaries to silencing caused by histone modifications. Previous data has implicated certain modifications in the silencing process, specifically the need for mature heterochromatin to have deacetylated H4K16 and demethylated H3K79 (Hecht et al., 1995; Johnson et al., 1990; Katan-Khaykovich and Struhl, 2005; Kitada et al., 2012; Onishi et al., 2007; Osborne et al., 2009) and our model preserves the need for these modifications in mature heterochromatin however questions their involvement in the establishment of silencing.

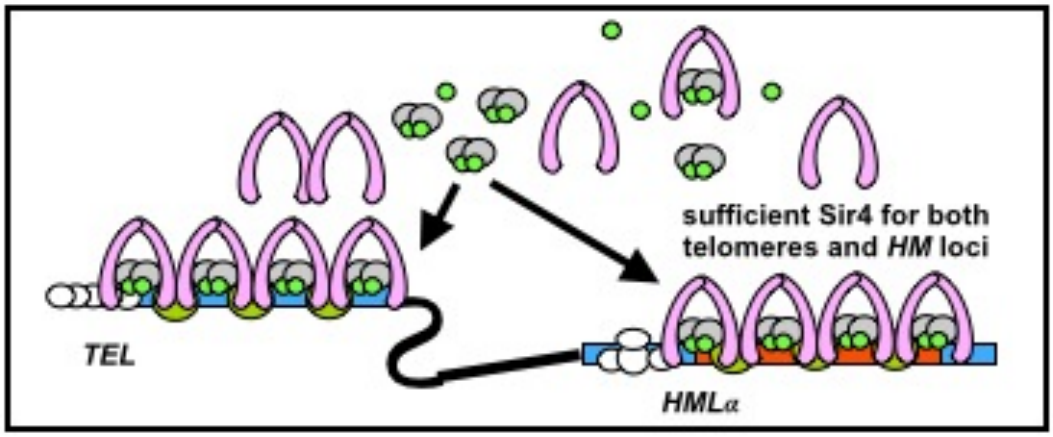
A

de novo assembly of heterochromatin at *HMLα*



B

de novo assembly of heterochromatin in cells with increased Sir4



C

de novo assembly of heterochromatin in *dot1*Δ or *yku70*Δ cells

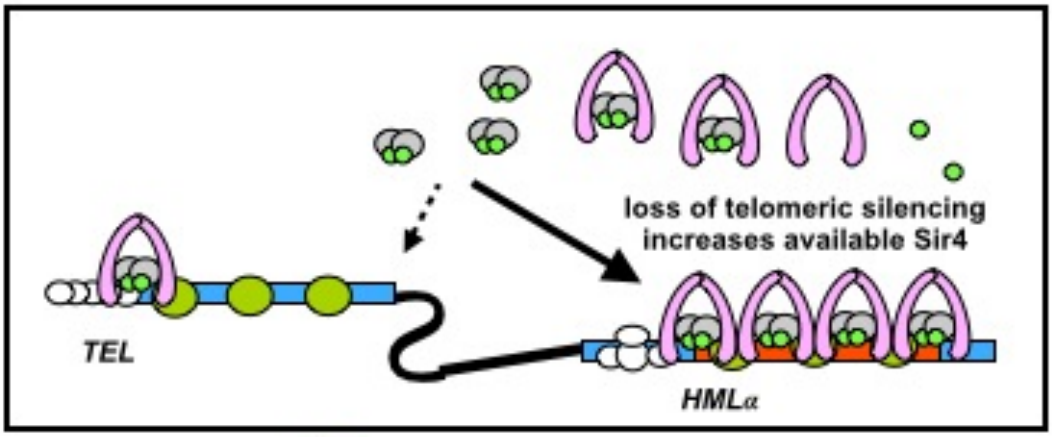


Figure 19: Model for the dissociation and re-location of Sir4 in silencing mutants. The loss of any one of *DOT1, UBP10,* or *yKU70* lowers the affinity of the SIR complex for the telomeres, resulting in a loss of silencing and reduced affinity of the SIR complex for the telomeres. Loss of the SIR complex from the telomeres allows it to be recruited to the HM loci and strengthen existing silencing or speed re-establishment.

yKu70 functions in telomeric silencing by inhibiting the Rif1 and Rif2 proteins, which compete with the SIR complex for binding to Rap1, the primary recruitment protein that binds to the SIR complex at telomeres. In the absence of *YKU70*, Rif1 and Rif2 effectively block SIR complex binding to Rap1, and the loss of telomeric silencing in *yku70*Δ can be suppressed by the deletion of *RIF1* (Mishra and Shore, 1999). As further support of our model for establishment of silencing we have shown that *yku70*Δ *rif1*Δ diploids completely reverse the earlier establishment phenotype we observe in *yku70*Δ diploids. (Figure 13). These results support our hypothesis that loss of telomeric silencing can promote earlier establishment of silencing at the *HM* loci.

Use of silencing assays to study silencing establishment vs maintenance

We were also able to make an important distinction about the establishment versus maintenance of silencing in our mutant strains. Many assays claim to study the establishment of silencing, however they are unable to isolate the single division that defines the establishment step and instead look at a marker later in the silencing timeline, such as repression of mRNA transcription or colony colour switching, that requires several generations to visualize (Gottschling et al., 1990; Miller and Nasmyth, 1984; Motwani et al., 2012; Ren et al., 2010). In our study we have shown that once silencing has been established it is stably maintained through several generations (Figure 5C, 10B and C). Although mating of each of the engineered diploid strains was less efficient than mating to a control haploid due to their unique ploidy, there was no difference in diploid mating

efficiency in situations where establishment was slower (*SIR4*/*sir4*) or faster (*dot1*Δ, etc). These data imply that once the *SIR4*/*sir4* mutants silence *HML* this silencing remains stable (Figure 5C, 10B). The maintenance of a silenced region is critical to the ability of a cell to maintain its identity. In yeast the loss of silencing and expression of both forms of mating type information leads to a sterile diploid phenotype and an inability to mate (Derr et al., 1991; Osborne et al., 2009).

The repercussions of a loss of silencing in higher mammalian organisms such as humans are much more profound than an inability to mate. The loss of silencing can cause disease and genetic mis-regulation such as the ability to maintain a chromosomal histone modification, known as an imprint, is essential in the pathology of Prader-Willi/Angelman syndrome, where a specific ICR (imprint control region) is modified so as to control the expression of neighbouring genes, whose aberrant expression causes the disease phenotype. (reviewed in (Hahn et al., 2010)). Heterochromatin formation is also linked to cancer where the tumorigenetic genes are misrelated due to defects in methylation status and are inappropriately active instead of being heterochromatically repressed (Hahn et al., 2010).

hmrΔA measures silencing stability not establishment

The assays for looking at establishment of silencing have long been proxies for looking at the actual establishment step. For example, looking at a population average of mRNA expression to determine when silencing occurs could be showing one of two things: 50% of cells are fully silenced and 50% are fully expressed, or 100% are half-silenced. Another assay which has been proposed to monitor the establishment of silencing is

monitoring the expression of an *ADE2* gene inserted adjacent to the weakened *hmrΔA* silencer. Cells that are maintained in a white colony colour are fully derepressed while those that are red are repressed and unable to finish adenine biosynthesis, hence the red colour. In an otherwise wild type strain many colonies will have sectors of red and white, and the switching between these two states may reflect changes in establishment or stability (maintenance).

Our data has shown that this assay does not measure the establishment of silencing *dot1Δ* and *yku70Δ* mutants, which speed establishment, are defective in silencing *hmrΔA-ADE2*, suggesting that in the context of a weakened silencer the loss of these two proteins blocks the assembly of silent chromatin. Similar results have been seen when these mutants are combined with *sir1*Δ, which also creates cells that have weakened silencers. Past work has proposed that the loss of histone H3K79 methylation in a *dot1Δ* mutant leads to promiscuous binding of Sir3 across the genome (Leeuwen et al., 2002), and in cells with weakened silencers, they may be unable to compete for recruitment of Sir3 to levels sufficient to establish silencing. In cells with a native silencer the loss of histone H3K79 methylation may cause few problems in the recruitment of Sir3 and silencing

This assay could be used in future as a screening tool to select mutants that may require follow up, however it does not give a complete look at the behaviour of the silenced chromatin at the weakened *hmrΔA* locus and thus should be used with caution.

Regulation of silencing at telomeres and differences at *HML vs HMR*

Our work and the work of others has made use of strains that contain the *URA3* reporter inserted proximal to *TELVII-L*. Two recent studies (Rossmann et al., 2011; Takahashi et al., 2011) have questioned the ability of telomeric URA3 reporter assays to measure telomeric heterochromatin, and showed that the *TELVII-L* reporter (Figure 5A and others) may exaggerate the strength of telomeric silencing. These studies questioned the value of the *TELVII-L* reporter because in their studies they found that although transcription at this locus was increased in *dot1*Δ cells, transcription at other telomeric locations was unchanged. Through the use of microarrays these studies assessed transcription in populations of cells and the epigenetic differences in silencing between a small number of the cells may have been undetectable in their assay. The silencing at *TELVII-L* has repression in approximately 50% of cells, thus silencing at that telomere would be detectable in their assay.

Additionally, recent experiments have shown conflicting results whether silencing establishment at *HML* has the same cell cycle requirements as at *HMR* (Lazarus and Holmes, 2011; Ren et al., 2010). These studies suggest that silencing establishment may be regulated slightly differently at *HMR* and *HML*, thus a future experimental direction for this project would be to create a *HMR*α mutant similar to those used in the single cell mating assay to directly compare whether there are differences in the establishment of silencing depending on the location and surroundings of the α-mating type information. This would assist in quantifying what, if any, components of the *HML* or *HMR* locus confer a benefit or detriment to the ability to establish silencing at those loci.

Sir4 protein levels may impart a delay that resembles cell cycle dependence

In simplest terms, a key component for a cell to be able to establish silencing is to have all of the required proteins available at the point when silencing is to be established. In the case of Sir4, cells exposed to mating pheromone or arrested in stationary phase have lower Sir4 levels than asynchronous cells (Figure 16). However, ChIP data suggests that if silencing has been previously established at these sites, the SIR proteins will remain in place despite total Sir4 levels decreasing (Figure 17), implying that silencing is stable throughout the arrest. This is similar to the finding that Sir3 remains localized at the HM loci when Sir3 protein levels are decreasing (Motwani et al., 2012). Previous studies which have shown a cell cycle dependence for the establishment of heterochromatin (Kirchmaier and Rine, 2001; 2006; Lau et al., 2002; Lazarus and Holmes, 2011; Martins-Taylor et al., 2004; Miller and Nasmyth, 1984; Ren et al., 2010) have arrested cells in either a pheromone-induced G1 arrest or in stationary phase, and thus Sir4 protein abundance may have been decreased at the start of these experiments. These researchers didn't consider the simple model that one of the Sir proteins was limiting during their experiments, and instead explored whether replication is required for establishment, and if turnover of histone modifications was an important rate limiting step. While *de novo* establishment requires sufficient Sir4, silencing establishment may not be regulated during a normal cell cycle when Sir4 levels are stable. Instead, establishment may be limited only during periods when Sir4 levels decrease, including pheromone arrest and stationary phase. Further research into understanding how cells regulate Sir4 abundance will help to determine how cells choose to limit *de novo* silencing establishment.

Stability of silent chromatin

Although the level of Sir4 in prolonged pheromone arrest may be insufficient to establish new sites of silent chromatin, past work has shown that existing sites of silent chromatin are more stable during a pheromone arrest (Aparicio and Gottschling, 1994), and maintaining proper mating identity is critical during pheromone exposure. We investigated whether the drop of Sir4 during pheromone exposure explains why increasing Sir4 promotes earlier establishment of silencing at *HMLα*. Newly mated zygotes are placed next to a patch of cells producing α factor. Cells are exposed to pheromone from their mating partner in the process of mating and zygote formation which should lead to reduced levels of Sir4 as suggested by exogenous exposure data (Figure 16). Our data supports this hypothesis by showing that silencing establishment can be rescued, and promoted to occur earlier, by increasing the amount of Sir4 in the cell. Once silencing has been established however, we have shown that zygotes maintain the ability to silence *HMLα* equally well and maintain their mating type, regardless of whether they are haploid or diploid for *SIR4*. The stability of existing sites of heterochromatin would imply that two factors are at play: low levels of Sir4 prevent new sites of heterochromatin from forming, and changes in levels of Sir4 (or another factor) prevent disassembly of existing sites. Further study will probe the regulation of Sir4 protein levels via transcription, translation, or stability during the process of heterochromatin formation and determine its implications on the establishment and maintenance of silenced regions.

How do cells regulate Sir4 abundance

The drop in Sir4 during pheromone arrest may be caused by cell cycle arrest or by a pheromone induced pathway. In order to differentiate these two possibilities we have attempted to monitor Sir4 stability during a G1 arrest that is independent of pheromone using *cdc28-4,* a temperature sensitive allele of *CDC28* (*CDK1*) (data not shown) however in this mutant, as well as in wild type strains, Sir4 is stabilized at elevated temperatures, making it difficult for us to assay the level of Sir4 in the cell and determine if the cell cycle arrest is responsible for the lowered protein level. Future work would be able to overcome this obstacle by using a triple-Cln (*cln1Δ cln2Δ cln3Δ*) mutant (Cross, 1990; Richardson et al., 1989) in which all three of the Cln G1 cyclin proteins are deleted. In this situation the cells cannot pass START of the cell cycle and they arrest in G1 (Cross, 1990; Richardson et al., 1989). However if a single Cln is expressed inducibly the cells can still cycle, as the Clns are redundant (Cross, 1990; Richardson et al., 1989). If Sir4 levels do not decrease during G1 arrest we can then test if addition of pheromone after G1 arrest triggers a drop in Sir4 that would be heritable to the next generation.

In order to understand the role that Sir4 levels play in *de novo* establishment of silencing and the implications for the model of cell cycle dependence we first need to examine what is regulating Sir4 protein levels. Using alternative methods to arrest cells prior to START that do not involve use of pheromone or nutrient starvation is key to elucidating whether the drop in Sir4 levels is pheromone or arrest dependent. If it is arrest dependent that would indicate that a Cdk substrate may be important in regulation of Sir4 protein levels. However, if it is a pheromone dependent phenomenon that will indicate that a systematic screening of members of the pheromone response pathway will be required in

order to determine the factors responsible for Sir4 regulation. In either case degradation of Sir4 may be a key component to its regulation. Further research into possible degradation schemes, such as SCF dependent degradation or APC mediated degradation will be required, in addition to investigating the possibility of control at the translational level. It has been shown that there is inhibition of translation during pheromone treatment that was linked to cell polarization inherent in the pheromone mediated arrest (Goranov et al., 2009) and that telomere localization changes in pheromone arrest (Stone et al., 2000), potentially destabilizing the telomeres and liberating a pool of Sir4 that was once telomere bound.

A revised model for SIR dependent *de novo* silencing establishment

Our findings shed new light on the previous model of the cell cycle dependence of heterochromatin establishment. We have found that the ability of a cell to establish heterochromatin may be related to the abundance of Sir4 in the cell and not a specific cell cycle requirement. Half the level of Sir4 in a cell leads to a decreased ability to establish silencing at *HMLα,* while increasing the Sir4 concentration leads to earlier establishment of silencing. Our analysis also suggests that mutations that speed establishment of silencing cause this change indirectly by disrupting telomeric silencing and increasing the quantity of Sir4 available to establish a new site of heterochromatin (Figure 19). Past work that defined a cell cycle requirement used pheromone or nutrient arrests during which Sir4 levels decrease dramatically. Therefore, although these arrests do create a block to new establishment and are likely physiologically relevant for determining cell identity, it is still unknown if specific cell cycle events regulate de novo establishment or if only the slow re-

accumulation of Sir4 over two cell cycles drives a delay in establishment. Given that the standard protocol for synchronizing cells involves a pheromone or nutrient starvation arrest, and both of these lead to a decrease in Sir4 levels, we believe that more research needs to be completed to determine if the current model stands, or needs to be amended to include Sir4 protein level fluctuations as a result of arrest.

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Lisa Sunstrum and Elizabeth Williams: APC mutant analysis and strain creation

APPENDIX A

Table 1: Strains used in this study

Strain #	Relevant Genotype	Mating type	Reference
W303	ura3-1, leu2-3,112 trp1-1, his3-11, ade2-1, can1-100		All strains are W303 background unless owtherwise noted
ADR21		a	Rudner lab collection
ADR22		alpha	Rudner lab collection
ADR2828	TELVII-L::URA3 x ADR21	diploid	Gottschling 1990 & This study
ADR2830	ADH4::URA3 x ADR21	diploid	Gottschling 1990 & This study
ADR2828	TELVII-L::URA3 x ADR3344	diploid	Gottschling 1990 & This study
ADR3082	his1 (not W303)	a	Fred Winston
ADR3344	sir4Δ::NAT SIR4-CEN-HIS3 (pAR450)	alpha	This study
ADR3387	sir4Δ::NatR	a	This study
ADR3810	SIR4-eGFP-KanR	a	This study
ADR4006	bar1Δ	a	Rudner lab collection
ADR4062	hrmΔE::TRP1, TEL-VII-URA3, BAR1+	a	Rudner lab collection
ADR4480	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG	a	Jasper Rine JRY8828
ADR4481	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2	alpha	Jasper Rine JRY8829
ADR4520	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG asf2Δ::HIS	a	This study
ADR4522	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, asf2Δ::HIS	alpha	This study
ADR4562	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, SIR4-URA	a	This study
ADR4564	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, SIR4-URA	alpha	This study
ADR4592	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, sir4Δ::HIS3	a	This study

ADR4593	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, sir4Δ::HIS3	alpha	This study
ADR4631	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, dot1Δ::spHIS5	a	This study
ADR4632	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, dot1Δ::spHIS5	alpha	This study
ADR4992	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, ubp3Δ::cURA3	a	This study
ADR4993	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, ubp3Δ::cURA3	alpha	This study
ADR5001	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, ama1Δ::cURA3	a	This study
ADR5002	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, ama1Δ::cURA3	alpha	This study
ADR5087	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, dot4Δ::cURA3	a	This study
ADR5088	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, dot4Δ::cURA3	alpha	This study
ADR5090	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, sum1Δ::cURA3	a	This study
ADR5091	matD::KAN, sir3D::TRP1, hmrD::HYG, lys2, sum1Δ::cURA3	alpha	This study
ADR5110	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, san1Δ::cURA3	a	This study
ADR5111	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, san1Δ::cURA3	alpha	This study
ADR5171	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, dot1Δ::spHIS5, ubp10Δ::cURA3	a	This study
ADR5172	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, dot1Δ::spHIS5, ubp10Δ::cURA3	alpha	This study
ADR5389	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, pGEV-LEU, SIR4-URA	a	This study
ADR5390	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, pGEV-LEU, SIR4-URA	alpha	This study
ADR5550	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, dot4Δ::cURA3, sir4Δ::HIS3	a	This study
ADR5551	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, dot4Δ::cURA3, sir4Δ::HIS3	alpha	This study
ADR5607	matD::KAN, sir3D::TRP1, hmrD::HYG, lys2, sir4Δ::HIS3, dot1Δ::cURA3	alpha	This study

ADR5640	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, sir4Δ::HIS3, dot1Δ::cURA3	a	This study
ADR5838	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, slt2Δ::HIS3	a	This study
ADR5839	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, slt2Δ::HIS3	alpha	This study
ADR5840	hrmΔE::TRP1, TEL-VII-URA3, ku70Δ::HIS3	a	This study
ADR5841	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, ku70Δ::HIS3	a	This study
ADR5842	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, ku70Δ::HIS3	alpha	This study
ADR5843	hrmΔE::TRP1, TEL-VII-URA3, ubp10Δ::HIS3	a	This study
ADR5895	hrmΔE::TRP1, TEL-VII-URA3, dot1Δ::HIS3	a	This study
ADR5920	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, ku70Δ::HIS3, ubp10Δ::cURA3	a	This study
ADR5921	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, ku70Δ::HIS3, ubp10Δ:cURA3	alpha	This study
ADR5944	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, ku70Δ::HIS3, dot1Δ::cURA3	a	This study
ADR5945	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, ku70Δ::HIS3, dot1Δ::cURA3	alpha	This study
ADR6119	hrmΔE::TRP1, TEL-VII-URA3, san1Δ::KanR	a	This study
ADR6121	hrmΔE::TRP1, TEL-VII-URA3, sum1Δ::KanR	a	This study
ADR6122	hrmΔE::TRP1, TEL-VII-URA3, esc1Δ::KanR	a	This study
ADR6124	hrmΔE::TRP1, TEL-VII-URA3, dot6Δ::KanR	a	This study
ADR6126	hrmΔE::TRP1, TEL-VII-URA3, hir1Δ::KanR	a	This study
ADR6128	hrmΔE::TRP1, TEL-VII-URA3, cac1Δ::KanR	a	This study
ADR6144	Dahmr::ADE2	a	This study
ADR6145	Dahmr::ADE2, esc8Δ::KanMx	a	This study
ADR6162	dot1Δ::KanMX	alpha	This study

ADR6168	ubp10Δ::KanMX	alpha	This study
ADR6183	ku70Δ::KanMX	alpha	This study
ADR6184	Dahmr::ADE2, dot1Δ::KanMX	a	This study
ADR6185	Dahmr::ADE2, ubp10Δ::KanMX	a	This study
ADR6186	Dahmr::ADE2, ku70Δ::KanMX	a	This study
ADR7240	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, SIR-URA3	a	This study
ADR7241	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, lys2, SIR4-URA3	alpha	This study
ADR7962	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, rif1Δ::spHIS3	a	This study
ADR7966	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, rif1Δ::spHIS3	alpha	This study
ADR7972	matΔ::KAN, hmlΔ::NAT, hmrΔ::HYG, rif1Δ::spHIS3, ku70Δ::3HA-klTRP1	a	This study
ADR7975	matΔ::KAN, sir3Δ::TRP1, hmrΔ::HYG, rif1Δ::spHIS3 ku70Δ::natNT2	alpha	This study

Table 2: Plasmids used in this study

Plasmid number	Genotype	Background
pAR440	CEN-HIS	DH5a
pAR646	SIR4(Mlu1)-HIS3-CEN	DH5a
pAR655	SIR4-HIS	DH5a
pAR696	SIR4-2μ-HIS3	DH5a
pAR720	SIR4(Mlu1)-URA int	DH5a
pAR722	SIR4(Mlu1)-CEN-URA	DH5a
pAR869	SIR4(Mlu1) -NAT-CEN	
pAR917	β-estradiol construct	Mach1 e.coli
pAR941	β-estradiol construct in pRS405 (Bodos 105) integrating vector	DH5a
pRS416/Bodos112	CEN-URA	
pRS423	HIS3-2μ	

APPENDIX B

Table 3: Statistical analysis data

Run ID	Ctrl	Treatment	Significant p<0.1	Deviance	p value
1	1 cen	2 cen	yes	13.4	0.0094
2	1 cen	4 cen	yes	17.7	0.0014
3	2 cen	4 cen	no	5.5	0.24
4	wt	2 mi	no	2.1	0.73
5	wt	dot1	yes	30.3	0
6	wt	GAL sir4	yes	165	0
7	wt	asf2	no	8.6	0.073
8	wt	ubp3	no	4.3	0.36
9	wt	ama1	no	4.1	0.4
10	dot1	ubp10	no	3.8	0.44
11	wt	sum1	no	7.1	0.13
12	wt	san1	no	4.3	0.36
13	wt	dot1 ubp10	yes	80.7	0
14	dot1	dot1 ubp10	no	3.9	0.42
15	ubp10	dot1 ubp10	yes	25.6	0.00004
16	wt	dot1 sir4	yes	26.3	0.00003
17	dot1	dot1 sir4	yes	51.9	0
18	wt	ubp10 sir4	yes	32.7	0
19	ubp10	ubp10 sir4	yes	47.5	0
20	sir4	dot1 sir4	yes	81.3	0
21	sir4	ubp10 sir4	yes	71.1	0
22	wt	ku70	yes	69.6	0

23	dot1	ku70	no	8.1	0.87
24	ubp10	ku70	yes	27.3	0.00002
25	wt	slt2	no	4.9	0.29
26	wt	B-est	yes	16.1	0.0029
27	dmso	B-est	no		
28	wt	ku70 ubp10	yes	18.4	0.0011
29	ku70	ku70 ubp10	yes to .5 only	10.6	0.03
30	ubp10	ku70 ubp10	no	8.8	0.066
31	wt	ku70 dot1	yes	19.4	0.0007
32	ku70	ku70 dot1	yes	26.3	0.00003
33	dot1	ku70 dot1	yes to .5 only	10.3	0.036
34	wt	ku70 sir4	yes	25.5	0.00004
35	ku70	ku70 sir4	yes	98.9	0
36	sir4	ku70 sir4	yes	17.8	0.0013
37	wt	sir4 haplo	yes	116	0
39	tlc1-ctrl	tlc1-gal	no	1.64621	0.648957
40	wt	tlc1ctrl	no	4.47636	0.34536
41	wt dex	gal-dot1 gal/ gal	yes	17.44293	0.001585
42	wt dex	gal-dot1 raff/gal	yes	23.17442	0.000117
43	gal-dot1 gal/gal	gal-dot1 raff/gal	no	4.11074	0.249752
44	wt	rad6	no	3.23084	0.519964
45	wt	wt b-est plate	no	4.7665	0.312106
46	wt dex	b-est on ypd	no	4.94274	0.293223
47	b-est on ypd	b-est on ypR	no	3.57075	0.467202

48	b-est on ypR	b-est on 100R	no	5.68457	0.223976
49	b-est on ypR	b-est on 350R	no	8.21387	0.084051
50	b-est on ypR	b-est on 750R	no	2.72949	0.604064
51	b-est on ypR	b-est on 1000R	yes to .5 only	10.60818	0.031339
52	b-est on 350R	b-est dot1 on 350	yes	62.59836	0
53	dot1 on ypR	b-est dot1 on 350	no	9.02138	0.060567
54	dot1 on ypR	dot1 on 350	yes to .5 only	12.6767	0.012968
55	b-est on 350R	b-est ku70 on 350R	yes	19.30832	0.000684
56	ku70 on ypR	b-est ku70 on 350R	no	4.32959	0.363235
57	ku70 on ypR	ku70 on 350	no	8.65206	0.07041
58	wt from ints	sir4 int 4xwt	yes to .5 only	10.8264	0.028586
59	wt from ints	sir4 int wtx14	yes	32.18991	0.000002
60	wt from ints	sir4 int 1x1	yes	14.70633	0.005351
61	wt from ints	sir4 int 1x14	yes to .5 only	12.50641	0.013957
62	wt from ints	sir4 int 4x14	yes	22.13686	0.000188
63	wt from ints	sir4 int 1xwt	yes	15.42096	0.003903
64	wt from ints	sir4 int wtx1	no	3.39175	0.494528

65	wt	ku70 new	yes	19.20865	0.000715
66	dot1	ku70 new	no	5.75497	0.21821
67	ubp10	ku70 new	no	3.24257	0.518082
68	WT	rif1	no	6.449826176	0.167979321
69	WT	ku70	yes	69.60000	0.000000
70	WT	ku70 rif1	yes	11.93913704	0.017809643
71	WT	ku70 rif1 rif2	no	7.994881512	0.091765871
72	ku70	ku70 rif1	yes	25.68635578	1.11E-05
73	ku70	ku70 rif1 rif2	yes	37.21562483	1.63E-07

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Michelle Parsons, Katherine Harding, Noel Lianga, Carole Doré , Adam D Rudner. The abundance of SIR4 regulates *de novo* assembly of silent chromatin

Parsons, M Lianga, N Pilon, S and Rudner, A. The role of Sir4 in the *DE NOVO* ASSEMBLY of heterochromatin in budding yeast. ASCB Annual Meeting. Denver, CO 2011

Parsons, M and Rudner, A. The role of Sir4 in the establishment of heterochromatin in budding yeast. FASEB Summer Conferences: Yeast Chromosome Structure, Replication and Segregation. Carefree, AZ 2010

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Parsons, M Lianga, N and Rudner, A. SIR CONTRIBUTES TO *DE NOVO* HETEROCHROMATIN FORMATION. University of Ottawa Cell Dynamics Retreat. Keane, ON 2013

Parsons, M Lianga, N Pilon, S and Rudner, A. The role of Sir4 in the *DE NOVO* ASSEMBLY of heterochromatin in budding yeast. University of Ottawa Student Poster Day. Ottawa, ON 2012 *Winner of BMIGSA "Most Visually Appealing Poster" award*

Parsons, M Lianga, N Pilon, S and Rudner, A. The role of Sir4 in the *DE NOVO* ASSEMBLY of heterochromatin in budding yeast. ASCB Annual Meeting. Denver, CO 2011

Parsons, M and Rudner, A. The role of Sir4 in the *DE NOVO* ASSEMBLY of heterochromatin in budding yeast. FASEB Summer Conferences: Yeast Chromosome Structure, Replication and Segregation. Carefree, AZ 2010

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