Rep and Sir Protein Influence on $2\mu$m Gene Regulation

by

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Abstract

The naturally occurring 2µm circle in *Saccharomyces cerevisiae* maintains a high copy number of 60-100 molecules per cell regulated by plasmid-encoded proteins Rep1, Rep2, and Flp1. Rep1 and Rep2 are both essential for maintaining this copy number and promoting equal partitioning of the circle to mother and daughter cells. Stable segregation of the circle is dependent on Rep1 and Rep2 and their interaction with the cis-acting locus, *REP3*. Rep1 and Rep2 proteins also work together to autoregulate their own transcription and control the expression of the *FLP1* gene, important in plasmid amplification. We are examining the mechanism of transcriptional repression on the 2µm circle, specifically testing for the influence of Sir proteins.

In previous studies, we observed transcriptional repression when we replaced *HML* silencers with *REP3*. We also found that overexpression of *REP1* and *REP2* leads to the disruption of silencing at the *HMR* locus. In this study, two hybrid assays confirm an interaction between Rep1p-Rep2p and Rep2p-Rep2p, and reveal a new interaction between Rep2p-Sir2p. We observe significant repression of basal level *HIS3* transcription when Rep proteins are tethered to the promoter. Although background *HIS3* transcription by Rep1 appears to be influenced by Sir4, Rep2 mediated repression does not depend on Sir proteins.

We have also studied the effect of Sir proteins on the gene expression of both a 2µm derived single copy plasmid and on endogenous 2µm plasmids. Analysis of *FLP1* message fails to show any significant influence of Sir proteins in controlling
2\textmu m gene repression. Through the study of 2\textmu m gene regulation we hope to better understand the mechanism of transcriptional repression on the 2\textmu m circle.
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**Introduction**

The term “plasmid” was coined by J. Lederberg in the 1950s to describe autonomously replicating extrachromosomal structures. These DNA structures have been widely studied especially in bacteria and are mostly found as double-stranded circular molecules. Plasmids vary in size, structure, copy number, mode of replication and propagation, as well as function. The ability to carry important genes such as those encoding for antibiotic resistance or restriction enzymes allow plasmids to successfully proliferate within host cells (Feinbaum, 1998). By the 1970s, naturally occurring plasmids were used to construct vectors important for the development of recombinant DNA technology to improve medicinal, agricultural and commercial practices (Helinski, 2004). In molecular biology, vectors have been widely used to study segregation and replication mechanisms in simple eukaryotic model systems. So when naturally occurring 2µm circles were first discovered in yeast in the late 1960s, scientists quickly exploited its biology to use the plasmid for vector design and site-specific recombination (Broach, 1997; Sinclair, 1967).

**2µm Circle Biology**

The 2µm circle, a double stranded circular DNA consisting of 6318 base pairs, is found in most laboratory *Saccharomyces cerevisiae* strains. Although it does not confer any particular selective phenotype to the host, 2µm containing cir<sup>+</sup> strains tend to have a slight growth disadvantage of 1% compared to its isogenic cir<sup>−</sup> counterpart (Futcher and Cox, 1983). The extrachromosomal element remains stable at an unusually high copy number of 60 to 100 molecules per cell, contributing
approximately 1.5-5% of the total nuclear DNA. As a selfish DNA element extant only for its autonomous propagation, the plasmid genome is devoted to two main functions: its ability to equipartition and to maintain a steady copy number (Futcher et al., 1988).

The structural organization of the 2µm genome is pertinent to its mode of propagation. Like chromosomes, 2µm plasmids undergo compaction through histone associations forming nucleosome structures (Nelson and Fangman, 1979). The nucleosome consists of 146 base pairs of DNA wrapped around two copies of H2A, H2B, H3, and H4 core histone proteins (Luger et al., 1997). Although the exact location of association is unknown, studies suggest that the nucleosomes are loosely bound and exhibit shifting influenced by 2µm plasmid encoded- products (Veit and Fangman, 1985).
**Figure 1**: A diagram of the genes and important elements on the 2µm circle. The arrows indicate the four different gene sizes. *REP3* does not generate protein products but acts as a binding site and anchorer. The two dark FRT bars represent the sites of inversion, while the ARS element is the initiation site for DNA replication.

Within the 6 Kb plasmid resides four protein encoding genes, required only for the maintenance of the 2µm circle (Figure 1). Among these, the largest open reading frame, *FLP1*, encodes for the Flp1 recombinase, which acts on two FRT sequences on the plasmid as part of a mechanism to increase plasmid copy number. *RAF1*, the smallest coding region, encodes the D protein for 2µm plasmid gene regulation. The *REP1* and *REP2* genes encode products involved in both gene regulation and plasmid segregation. *REP3* and ARS sequences act as protein binding sites important for plasmid segregation and replication (Volkert et al., 1989).

Most plasmids express gene products that present a selective advantage to the host system; however very little evidence suggests that yeast systems benefit from hosting 2 µm plasmids. The fact that the 2µm genes encode proteins for plasmid...
maintenance instead of yeast growth factors or resistance factors also makes it unlikely that the 2µm plasmid improves strain fitness in any major way (Volkert et al., 1989). The one phenotype exhibited in yeast cells that can be attributed to 2µm circles is the observation of nibbled colony morphology. Nibbled colonies were first observed in strains containing a mutation in the NIB1 gene. These strains had elevated 2µm plasmids, which eventually caused cell death. The unusual morphology and nib1’s lethality was dependent on the 2µm circles because isogenic cir⁰ strains reverted back to wild type phenotypes (Holm, 1982). Rather than conferring a growth advantage to the host, so far host systems appear to have a greater affect on 2µm circle functionality.

Initiation of DNA replication originates at the autonomous replication sequence (ARS) in both chromosomes and plasmids. Most ARS containing plasmids segregate asymmetrically due to a maternal inheritance bias. In this segregation bias, ARS plasmids such as extrachromosomal rDNA circles (ERCs) accumulate in the mother cell and reduce life span, while producing plasmid free daughter cells (Sinclair and Guarente, 1997). Under normal conditions, the 2µm ARS initiates a single replication event during S phase (Zakian et al., 1979). The cis-acting element, REP3 (STB), helps confer equal partitioning and contributes to the propagation of stable high-copy number plasmids between mother and daughter thereby bypassing the maternal inheritance bias (Broach and Volkert, 1991; Murray and Szostak, 1983; Sinclair and Guarente, 1997). Since naturally occurring 2µm plasmids do not accumulate in old yeast cells, yeast life span is not adversely affected (Falcon et al., 2005).
Plasmid Segregation

2µm Segregation Factors

The persistence of the 2µm plasmid occurs through the REP3 locus, which is central to the plasmid partitioning system. Located adjacent to the ARS sequence, REP3 consists of five and a half tandem repeats of 62 base pairs and does not produce functional transcripts. As a cis-acting element, the locus only stabilizes plasmids if located within the plasmid. REP3 acts independently from ARS but is dependent on both Rep1 and Rep2 proteins (Jayaram et al., 1985; Kikuchi, 1983). Yeast plasmids lacking the REP3 locus or either of the Rep proteins exhibit decreased stability. In the presence of both Rep1 and Rep2 proteins, the REP3 locus functions as an anchoring element which secures the 2µm circles to large cellular structures (Gartenberg and Wang, 1993).

REP3 binding activities as well as monohybrid assays demonstrate that both Rep1 and Rep2 are capable of interacting with REP3 (Hadfield et al., 1995; Velmurugan et al., 1998). Fluorescence microscopy studies have verified the nuclear localization of Rep proteins and find colocalization of Rep1 and Rep2 (Ahn et al., 1997; Scott-Drew and J.A., 1998). In addition to direct interaction with REP3, Rep1 and Rep2 proteins interact with one another and form a protein complex. Specifically, the amino-terminal end of Rep1 protein is required for its interaction with Rep2. Furthermore, evidence suggests self-associations between Rep1-Rep1 and Rep2-Rep2 proteins (Ahn et al., 1997; Velmurugan et al., 1998).
Localization to Distinct Foci

Localization studies in cir² and cir⁺ strains show that Rep1 and Rep2 proteins colocalize in distinct foci in the presence of the REP3 locus. Therefore, both protein-protein and protein-DNA interactions may assist in the compartmentalization of the Rep proteins (Ahn et al., 1997). The signal for nuclear compartmentalization resides within the last 20-30 amino acids of the carboxy-terminal tails for both Rep1 and Rep2 protein (Scott-Drew and Murray, 1998; Velmurugan et al., 1998). So far studies analyzing the exact location of the foci have been contradictory, with some believing that foci associate with nuclear lamina and others finding evidence for localization near the mitotic spindle (Scott-Drew and J.A., 1998; Velmurugan et al., 2000; Wu et al., 1987). The foci itself does not significantly change between haploid and diploid strains as well as between strains differing in copy plasmid number. With the advent of mitosis, foci number tend to double from 4 to 8 clusters and appear to segregate with chromosomes concurrently (Scott-Drew and J.A., 1998; Velmurugan et al., 2000).

Exploiting Yeast Segregation Machinery

The 2µm circle’s ability to utilize nucleosomes for compaction and cellular replication machinery suggest its potential to borrow other chromosomal systems for its propagation. Therefore it is conceivable that such a plasmid could depend on the chromosomal segregation apparatus for plasmid inheritance and stability. Chromosomal segregation requires a number of steps and proteins such as Ipl1 which help in normal spindle pole function (Biggins et al., 1999). Strains expressing mutant
Ipl1 proteins were found to exhibit similar missegregation of chromosomes and 2µm plasmids (Velmurugan et al., 2000).

The most compelling correlation between the Rep-REP3 system and the chromosomal segregation apparatus is the discovery of Scc1 protein’s association with REP3 in a Rep dependent fashion. Scc1 is a protein found within the yeast cohesin complex which convenes at the centromeres and other binding sites to help hold sister chromatids together (Mehta et al., 2002; Mehta et al., 2005). The H3 histone variant Cse4p/CENP-A, required for kinetochore assembly, was originally believed to exist only at host chromosome centromeres. However, Rep proteins have been recently discovered to help incorporate Cse4p onto REP3 sites to aid partitioning (Hajra et al., 2006). During S phase, Rep1’s ability to recruit cohesin to REP3 leads to the cohesion between 2µm plasmids. The segregation itself occurs in a sister to sister fashion, where plasmids are believed to hitch a ride on chromosomes and segregate to the daughter cells (Ghosh et al., 2007).

**2µm Amplification System**

The 2µm plasmid’s stability is ensured by a second mechanism, which regulates copy number amplification through the Flp-FRT recombination system. In an event where missegregation produces cells with lower than usual copy number, the Flp recombinase system becomes activated. The recombinase system is coupled to the 2µm plasmid replication and remains inactive in conditions of stable copy number, allowing for a single round of replication during S phase for each individual plasmid. In conditions of decreased copy number the plasmid has devised a unique
method in which plasmid encoded proteins act upon the DNA sequence during replication to control copy number (Broach, 1997; Volkert et al., 1989).

**Flp recombinase and FRT**

The one essential protein required for 2µm plasmid amplification is Flp1, a tyrosine based site-specific recombinase. Flp1 is involved in catalyzing a recombination reaction between two 599 base pair regions known as FRT or Flp1 recognition target sites (Gates and Cox, 1988; Hartley and Donelson, 1980). These FRT regions divide the 2µm circle approximately in half and possess different orientations. Two Flp proteins cooperatively bind to each FRT site. Once bound, Flp’s nucleophilic tyrosine cuts the DNA backbone and introduces a single stranded cleavage to produce protruding ends that can anneal (Andrews et al., 1985; Senecoff et al., 1985). The overhanging strand can then exchange with its recombination partner creating a DNA Holliday junction. Finally, a second round of cleavage and ligation steps via activation of two more Flp proteins completes the recombination process (Chen et al., 2000; Chen and Rice, 2003; Jayaram et al., 1988; Parsons et al., 1988). Flp’s strand cleavage does not require any high energy source such as ATP and is a rather poor catalyst with low turnover number. Its weak enzyme activity causes recombination to occur at a low frequency (Broach, 1997; McLeod et al., 1986). Fluctuations in Flp1 proteins tightly regulate recombination events. Since Flp recombination is coupled with plasmid replication, changes in recombination events affect the number of 2µm plasmids replicated.
**Replication**

Under steady state conditions, replication begins at the 2µm circle’s origin of DNA replication, or ARS sequence. The 2µm ARS lies closer to the proximal FRT and farther away from the distal FRT. As the fork moves bidirectionally, they converge opposite from the ARS site producing two plasmids. This plasmid replication process utilizes the same proteins and gene products required for replication on the chromosome (Brewer and Fangman, 1987; Huberman et al., 1987). The Flp-FRT interaction along with the location of the FRT sites in relation to the ARS allows for a unique recombination event to take place for amplification.

**Plasmid Amplification**

The best supported amplification model was proposed by Futcher in the 1980s. The model circumvents the possibility of multiple replication initiation events and instead proposes that changes in replication fork direction lead to amplification. In this model, activation of Flp-FRT recombination is triggered when 2µm circle levels decrease and Flp1 protein is produced. Once a diverging replication fork passes the proximal FRT during replication, recombination between a replicated and an unreplicated FRT site causes the replication forks to move in the same direction, as seen in Figure 2. Unidirectional replication forks continue to produce multiple copies of the plasmid until a second recombination event terminates the amplification process (Futcher, 1986). Although the importance of Flp-FRT recombination is understood, there is little experimental evidence that directly confirms Futcher’s model.
Figure 2: A diagram representing a favored model of amplification. As replication begins at the ORI or ARS sequence and forks move bidirectionally, inversion of the original two FRT sites changes the direction of one of the forks. The inversion of FRT sites result in two forks moving in the same direction so that multiple copies of 2µm circles are created. The FRT inversion is mediated by the action of Flp proteins which form a holiday junction intermediary (Chen and Rice, 2003; Ghosh et al., 2007).

2 µm Gene Regulation

Since studies observe only a single replication event during S phase for 2µm circles, regulation of amplification may primarily be affected by fluctuations in Flp1 concentrations. Although protein modifications and degradation can regulate
concentration, regulation of \textit{FLP1} gene transcription appears to be the major point of control in the activation and inactivation of amplification.

\textbf{Gene Repression Regulates 2\textmu m Amplification}

Several studies suggest that \textit{FLP1} transcription is regulated by the plasmid encoded proteins, Rep1, Rep2, and D. If increasing \textit{FLP1} transcription leads to increases in plasmid copy number, the plasmid must repress the \textit{FLP1} gene in steady state conditions. Studies show that Rep1 and Rep2 proteins act as negative regulators or repressors of \textit{FLP1} gene transcription (Murray et al., 1987; Som et al., 1988; Veit and Fangman, 1988). Strains containing frameshift mutations within \textit{REP1} and \textit{REP2} genes or temperature sensitive \textit{REP1} genes exhibit at least a 20 fold increase in \textit{FLP1} and \textit{REP3} transcripts and a smaller increase in \textit{REP1} and \textit{REP2} genes (Veit and Fangman, 1988). An independent study observed Rep1 and Rep2-dependent regulation of \textit{REP1} and \textit{FLP1} transcription. In this study, overexpressing \textit{REP1} and \textit{REP2} caused a 100 fold decrease in \textit{FLP1} and a 20 fold decrease in \textit{REP1} on a 2\textmu m derived plasmid (Som et al., 1988). Both studies suggest that Rep1 and Rep2 proteins act together to regulate 2\textmu m genes at the level of transcription. The Som et al study also observed that Rep1 and Rep2 negatively regulated \textit{RAF} transcription. The repression of \textit{RAF} product appears to prevent \textit{FLP1} expression because the D protein antagonizes Rep1 and Rep2 action (Murray et al., 1987).

\textbf{2 \textmu m Gene Regulation and Amplification}

Copy number control depends primarily on \textit{FLP1} gene regulation, which contributes to the activation of the amplification system. The regulation of \textit{REP1} is
also important because it implements a negative feedback loop to tightly maintain 2µm circle copy number at 60-100 molecules per cell. In a model suggested by the Broach lab, high concentrations of Rep proteins caused by high plasmid copy numbers, repress *FLP1* and *REP1* message. Repression of *FLP1* inactivates amplification preventing cells from producing further plasmids, while repression of *REP1* inhibits production of more Rep proteins. In cells containing low copy number, low Rep protein concentrations lift repression off of *REP1* and *FLP1*, thereby activating the Flp amplification system (Som et al., 1988). Figure 3 represents a schematic of this model for 2µm gene regulation. Gene regulation provides a quick method of maintaining steady state copy number, important for both the plasmid and its host. As a plasmid that does not confer any selective advantage, a sufficiently high copy number is required for propagation; however, excessive copy number can be deleterious to the host if plasmids titrate away essential segregation and replication factors from the chromosome.
Figure 3: An illustration of proposed sites of silencing by Rep protein complexes. This Broach model claims that Rep protein complexes negatively regulate the expression of \textit{REP1}, \textit{FLP1}, and \textit{RAF1} genes (Som et al., 1988).

\textbf{Silencing}

Regulation of 2\textmu m genes requires the Rep1 and Rep2 proteins, but the mode of repression is still unknown. Other transcriptional factors may be involved in modulating plasmid gene expression by collaborating with Rep1 and Rep2. Like segregation, Rep proteins may interact with important host factors and employ mechanisms of repression similar to silencing at the chromosomes.

\textbf{Silencing in yeast}

Silencing refers to structurally repressed chromosomal regions which help block transcription and recombination. The condensed chromosome structure, known as heterochromatin, consists of tight nucleosome units containing short DNA fragments wrapped around several histone proteins. Post-translational modifications
on N-terminal histone tails such as those for H3 and H4 are necessary for chromosome condensation (Bi and Broach, 1997; Luger et al., 1997).

In yeast, silencing occurs at three distinct loci: the mating type loci, the telomeres, and the rDNA repeats. In haploid cells, the mating type locus consists of a MAT locus expressing either a or α genes. On the same chromosome as the MAT locus, the HMR and HML loci encode a and α genes respectively; however, HMR and HML are both transcriptionally silenced (Abraham et al., 1984). Silencing is also observed at the telomeres, but unlike the HM loci, silencing is rather weak and can spread farther into the chromosome. The ability to silence reporter genes adjacent to telomeres is termed the telomeric position effect and is frequently used to study silencing (Gottschling et al., 1990; Renauld et al., 1993). The rDNA silencing region consists of ~150-200 tandem rDNA repeats, which can easily undergo recombination to form extrachromosomal rDNA circles. Silencing of rDNA is necessary to suppress recombination and prevent deleterious accumulation of rDNA circles (Gottlieb and Esposito, 1989; Petes, 1977).

**Components of Silenced Chromatin**

Silencing depends on several cis and trans-acting elements. The cis-acting DNA elements, E and I silencers, are only found surrounding HML and HMR. These silencers contain binding sites for trans-acting proteins such as Rap1, Abf1, and ORC (Rusche et al., 2003). Rap is a DNA-binding protein that has a role in both gene repression and activation. Rap proteins are recruited to HM and telomere repeats (Shore, 1994). Abf1 is also a transcriptional activator responsible for the expression of many genes, while the origin recognition complex (ORC) helps initiate DNA
replication (Bell, 1993; Planta et al., 1995). Although these elements are necessary for some loci, the major structural components of all heterochromatin include Sir proteins and histones H3 and H4.

Among the three silent information regulator proteins, Sir2 is the only repressor that is an NAD-dependent histone deacetylase. As a histone deacetylase, Sir2 is responsible for nucleosome modifications through the hypoacetylation of H3 and H4 (Braunstein et al., 1993). In vivo, the Sir proteins are found in a complex but studies observe mainly a Sir2-Sir4 interaction with Sir3 later recruited to the complex (Moazed et al., 1997). Unlike the telomeres and HM loci, only Sir2 is required for silencing of the rDNA repeats (Smith et al., 2002). Sir3 and Sir4 proteins lack enzymatic activities and instead contribute to heterochromatin structure. Sir4 associates with silencer binding proteins and also has a role in tethering the telomeres to the nuclear periphery, thereby restricting the movement of heterochromatin (Ansari and Gartenberg, 1997). Sir3, Sir4, and Rap1 have all been shown to localize to distinct foci along the nuclear periphery (Cockell et al., 1995; Palladino et al., 1993).

**Basis of This Study**

2m circle copy number regulation depends on tight regulation of *FLP1* gene transcription, mediated by the Rep1 and Rep2 proteins. *FLP1* transcription is significantly repressed under normal circumstances. A number of prior observations suggested to us that Sir proteins might be involved in this repression of transcription.
Common Properties of Rep and Sir Proteins

Rep and Sir proteins appear to share several similar properties. The Sir proteins that bind to the *HM* cis-acting silencers and the interacting Rep proteins on the *REP3* locus both localize to similar discrete foci within the nucleus. Like Sir protein foci, the Rep protein clusters were initially thought to localize to the nuclear periphery (Wu et al., 1987); however more recent fluorescence studies claim that Rep clusters localize to mitotic spindles instead (Palladino et al., 1993; Velmurugan et al., 2000). The Sir4 and Rap1 proteins are known anchors, which help inhibit DNA rotation by tethering associated DNA sequences to nuclear components (Ansari and Gartenberg, 1997). Similarly, *REP3* in conjunction with Rep1 and Rep2 proteins exhibits an anchoring capability, where plasmids remain tethered to foci locations within the nucleus (Gartenberg and Wang, 1993). Besides the ability to anchor, the Rep-*REP3* system is involved in stable plasmid segregation. Plasmids containing either *HMR* E silencers or telomere repeat sequences demonstrated similar stable segregation in the presence of Sir2, Sir3, and Sir4 proteins (Longtine et al., 1992). The ability of silencers and silencing proteins to have additional anchoring and segregation properties led our lab, in a prior study, to examine *REP3* as a silencer.

Preliminary 2µ-silencing Studies:

In a *REP3* study conducted by the Holmes lab, the *REP3* anchor was positioned at the chromosome to examine how localizing the DNA sequence influences silencing. The results suggest a *REP3* mediated silencing phenomena dependent on Sir and Rep proteins. Surprisingly, silencing at *HML* appeared to improve in the absence of 2µm plasmids and decrease at *HMR* with the
overexpression of Rep1 and Rep2 proteins (Figure 4). According to a model supporting these observations, it was suggested that Rep proteins antagonize Sir protein silencing by titrating Sir proteins away from respective silencing regions (Papacs et al., 2003). In this study we examined Rep and Sir interactions to further investigate this theory.
Figure 4: A pictorial depiction of the results obtained from the REP3 mediated silencing study. Replacing natural silencers with a REP3 sequence increased silencing at HML in the presence of Sir and Rep proteins. At HMR, overexpression of Rep1 and Rep2 disrupted silencing, while the absence of the 2µm plasmid increased repression of HML.

In a search for novel cis-acting elements that could mediate silencing, the Ehrenhofer-Murray lab found that a fragment containing the 2µm ARS sequence could aid silencing at the HMR locus. This silencing remained dependent on SIR2,
SIR3, and SIR4. When the Sir2 histone deacetylase homologs HST1 to HST4 were tested, only Hst3 proteins appeared to affect the new silencer. This study also found Hst3 to be physically bound to 2µm circle promoters and important in the regulation of FLP1 (Grunweller and Ehrenhofer-Murray, 2002).

Questions Addressed in this Thesis:

The many similarities and genetic interactions between the Rep and Sir systems led us to hypothesize that repression of FLP1 gene transcription on the 2µm plasmid is a Sir protein mediated phenomena. We speculate that Rep proteins recruit Sir proteins to 2µm genes to aid in heterochromatin formation and prevent transcription of both FLP1 and REP1. Two types of experiments were conducted to directly test our hypothesis. To uncover potential Rep-Sir protein interactions, we used a qualitative two hybrid assay. We also used strains bearing conditional alleles of the SIR genes to directly examine the role of Sir2 and Sir3 in repressing FLP1 and REP1 transcription.
Materials and Methods

Media

Most strains were grown in YPD or YPraffinose media consisting of 1% yeast extract, 2% Bactopeptone and 2% dextrose or raffinose respectively. For galactose induction experiments, galactose was added to strains that were pre-grown in YPraffinose to increase activity of the GAL10 promoter, creating a final 2% galactose solution. Two hybrid strains were grown in synthetic dextrose media instead. For the selection of strains containing pOBD2 or pOAD plasmids, cultures were grown in SDC media lacking tryptophan or media lacking leucine respectively. Unlike yeast cultures, bacterial strains were grown in Luria Broth (LB). Bacterial strains containing plasmids were selected by growth in LB media with 100 µg/ml ampicillin.

Strains

Table 1 shows a list of strains used in this study. YSH513, YSH514, and YSH625 strains were used for tethering and two hybrid assays. These strains contain a GAL4 promoter upstream of the HIS3 reporter gene. The YSH513 and YSH514 strains are nearly identical except that YSH513 expresses the MATa gene while YSH514 expresses the MATa gene. YSH625 is a derivative of YSH514 but has SIR2, SIR3, and SIR4 deletions.

The remaining strains were used for transcriptional assays. The YSH425 strain contains genomic REP1 and REP2 genes under the GAL10 promoter. This strain lacks the endogenous 2µm circle and only produces high levels of REP1 and REP2 transcripts in YPgalactose media. For this particular study SIR2 was deleted.
from this background strain and a single copy 2µm derived plasmid was introduced. YSH491 and YSH494 strains are derived from YSH189 but contain $SIR2$ and $SIR3$ temperature sensitive mutations respectively. We grew YSH491 and YSH494 strains at 37 °C to inactivate Sir2p and Sir3p and studied the influence on transcription.
## Table 1: List of Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>YSH513</td>
<td>MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4D gal80D LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</td>
<td>Yeast Resource Center/Seattle Project (YRC)</td>
</tr>
<tr>
<td>YSH514</td>
<td>MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4D gal80D LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</td>
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<td>YSH625</td>
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<td>M. Hickman</td>
</tr>
<tr>
<td>YSH189</td>
<td>MATα ura3 ade2 lys1 his5 leu2 can1 + 2µ+</td>
<td>J. Broach</td>
</tr>
<tr>
<td>YSH491</td>
<td>MATα ura3 ade2 lys1 his5 leu2::sir2-614ts-LEU2 can1 Dsir2::URA3 2µ+</td>
<td>Matecic</td>
</tr>
<tr>
<td>YSH494</td>
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<td>S. Holmes</td>
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<td>YSH425</td>
<td>MATα hmrDA::ADE2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 GAL+ cir0 leu2-3,112::LEU2-GAL10-REP1 trp1::TRP1-GAL10-REP2</td>
<td>L. Papacs</td>
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<td>YSH513 Δsir2::URA3</td>
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<td>YSH513 Δsir3::URA3</td>
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</tr>
<tr>
<td>14α</td>
<td>MATα his1</td>
<td>M. Smith</td>
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<tr>
<td>17α</td>
<td>MATα his1</td>
<td>M. Smith</td>
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## Table 2: List of Plasmids

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<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>pOAD</td>
<td>ARS1, GAL4-AD, LEU, CEN4</td>
<td>YRC</td>
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<tr>
<td>pOBD2</td>
<td>ADH1 promotor, ADH1 terminator, GAL4-AD, TRP1, CEN4</td>
<td>YRC</td>
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<tr>
<td>CV21f</td>
<td>CEN4-URA3, LacZ-flp, rep1, rep2, and rep3 2-micron derived plasmid</td>
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<tr>
<td>pAG25</td>
<td>NAT-MX4</td>
<td>Goldstein</td>
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<td>pSH105</td>
<td>Δsir3::URA3</td>
<td>S. Holmes</td>
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<td>pAW3</td>
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<tr>
<td>Primers</td>
<td>Sequence (5' to 3')</td>
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<td>Sp178</td>
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<td>pOBD2 &amp; pOAD</td>
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<td>RT-PCR for ACT1</td>
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A. Genetic Assays

Plasmid Construction

The pOAD and pOBD2 plasmids containing the Gal4 activation domain and Gal4 binding domain respectively were used for the two-hybrid or tethering assays. The objective of these genetic assays was to assess protein-protein interactions by constructing Gal4-BD and Gal4-AD protein fusions through gap repair. Primers were designed to amplify entire gene fragments encoding for desired proteins such as Sir and Rep proteins. These primers, listed in Table 3, consist of sequences that have end homology to plasmid regions flanking the GAL4BD or GAL4AD sequences. Once amplified using the polymerase chain reaction (PCR), the fragments were ready to be transformed into competent yeast cells with the appropriate vectors, pOAD or pOBD2. Before transforming these vectors, the plasmids were cut using the NcoI and PvuII restriction enzymes to prevent the ends from rejoining during transformation, thereby increasing the efficiency of gap repair with PCR fragments.

Transformed cells with repaired plasmids were identified through growth on selective plates. To select for potential pOAD and pOBD2 fusions, transformed cells were grown in SDC-LEU and SDC-TRP plates respectively. Uncut pOAD and pOBD2 vectors were transformed as positive controls while cells lacking these vectors were used as negative controls. Growth of transformed yeast containing these cut vectors indicate baseline repair of plasmids and were used to compare transformed cells that have achieved gap repair with PCR fragments. To ensure successful gap repair in the transformed strains, PCR was used to check for the presence of the gene and its correct location on the plasmid.
Several methods were used to isolate constructed pOAD and pOBD2 plasmid from checked strains. In doing so, these fused plasmids could readily be available for transforming into various strains. In past studies, purifying plasmid DNA using the regular EPICENTRE DNA extraction kit has proven to be difficult. Therefore strains containing the desired fused plasmids were subject to a smash and grab procedure, which yields both chromosomal and plasmid DNA. Then the extracts were transformed into bacterial strains for amplification and purification of plasmid DNA. Plasmid DNA was transformed into bacteria either chemically or through electroporation. Since the pOAD and pOBD2 vectors contain a beta lactamase gene encoding for ampicillin resistance, bacterial cells containing these fused plasmids were selected for in L-AMP plates. After plasmid purification, the plasmids were subject to a final PCR check and ready for transformation.
Figure 5: The genomic maps of pOBD2 and pOAD vectors. The bait and prey sequences were inserted within the \textit{GAL4-DBD} or the \textit{GAL4AD} domains. Each plasmid can be selected for in bacteria because of the expression of beta lactamase. For selection in yeast, \textit{pOBD2} vectors expressed \textit{TRP1} while \textit{pOAD} vectors expressed \textit{LEU2}.
Two Hybrid Strains

The plasmids containing the AD fused with prey genes were transformed into YSH514, while plasmids containing BD fusions with bait genes were transformed into YSH513 strains. These wild type haploid strains were mated to produce a final diploid strain containing both pOAD and pOBD2 fusion plasmids. For the mating assay, we spotted individual colonies and spotted overlapping colonies for mating on YPD plates. Positive controls consisting of a known MATα and MATa haploid strain was spotted on the same YPD plate. The YPD plates were grown overnight at 30°C and then replica plated on SDC-LEU-TRP plates to select for diploid cells which have incorporated both pOAD and pOBD2 fused plasmids. These diploid strains were freshly streaked out on SDC-LEU-TRP plates and used for two-hybrid assays.

Mating requires haploid strains to exhibit differentiation primarily through their expression of either a MATα or MATa gene. In any given condition, yeast cells possess both α and a genes at the HML and HMR locus respectively. However, endogenous Sir proteins repress HML and HMR genes and express genes only at the MAT locus (Rusche et al., 2003). In strains lacking Sir proteins, the cells do not exhibit haploid characteristics and cannot undergo mating. Therefore in our YSH625 strain which lacks the SIR2, SIR3, and SIR4 genes, the plasmids were transformed sequentially instead of using a mating assay. Cut pOBD2 plasmids and bait gene PCR fragments were transformed first. After a PCR check for proper gene-plasmid fusion, a second transformation was conducted on SDC-TRP-LEU plates with cut pOAD and prey gene PCR fragments. A final PCR was done to check for presence of both prey-pOAD and bait-pOBD2 plasmids.
Sir Deletion Strains for Tethering Assay

The tethering assay was utilized to observe the effects of localizing Rep1 and Rep2 to the GAL4 promoter. Therefore, two-hybrid strains containing only pOBD2 plasmids fused with REP1 and REP2 bait genes were necessary for this assay. In addition to transformation in wild type YSH513 and SIR2, SIR3, and SIR4 deleted YSH625 colonies, strains containing only SIR2 deletions and strains containing only SIR3 deletions were produced for this study. The plasmids pAW3 and pSH105 were used to knockout SIR2 and SIR3 genes respectively in YSH513 strains bearing pOBD2 plasmids. By digesting pAW3 with the restriction enzymes, SallI and XbaI, a Δsir2::URA3 fragment could be produced and isolated using a gel purification kit. The resulting fragment contains end homology with the endogenous SIR2, so that the cell can undergo homologous recombination to knockout the gene. SIR3 was knocked out in a similar fashion by first isolating the Δsir3::URA3 fragment from the pSH105 plasmid by digesting with HindIII and BamHI restriction enzymes. Once isolated, these fragments were transformed into the YSH513 background strain containing the different pOBD2-bait genes and selected for in SDC-URA-TRP plates.

A PCR gene disruption method was also used to knockout SIR2 or SIR3 genes from the wild type YSH513 strain. The NAT gene on the pAG25 plasmid was amplified using primers with tails identical to SIR2 or SIR3 sequences at the beginning and end of the reading frame. PCR with specific primers results in either a Δsir2::NAT or a Δsir3::NAT fragment. Once transformed, these fragments undergo homologous recombination to knock out and replace either SIR2 or SIR3 with the NAT reporter gene. Cells containing SIR knockouts were selected for in YPD-NAT plates.
Once the SIR knockout fragments were transformed, the strains were checked by mating with 17α strains in the mating assay described above. The wild type YSH513 strain was also mated with 17α as a positive control, which grew on SD plates. The success of SIR knockout was assessed by the absence of diploid cell growth on the same SD plate.

**Serial Dilution Assays**

The two-hybrid assay and the tethering assay provide information on protein-protein interactions and their affect on basal level *HIS3* transcription by comparing growth phenotypes on plates lacking specific amino acids. Two hybrid strains containing different bait-BD and prey-AD fused plasmids were selected in 2 ml of SDC-TRP-LEU media and grown overnight at 30°C. Unlike the two-hybrid strains, strains used for the tethering assay were selected for in SDC-TRP media. In order to normalize the density of the overnight cultures, the optical density (OD$_{600}$) was measured; cultures were then diluted with water and equalized to the least dense culture. A total of 200 μl of equalized cultures were pipetted into the first column in 96 well plates. The adjacent columns were 10 fold dilutions of previous lanes. In order to create the serial dilutions, 20 μl of equalized cultures were added to 180 μl of water in the adjacent wells. This method of dilution was continued to obtain up to 1,000 or 10,000 fold dilutions of original equalized cultures. Once the serial dilutions were ready, the multi-channel pipette was used to spot 4μl of cultures from each column onto control and experimental plates.
Two or three sets of plates were spotted for each experiment to create replicates. The plates were then incubated at 30°C for 1-5 days and the results scanned and stored as a picture file. The particular strains used for two-hybrid and tethering assays contain a UAS, or upstream activation sequence, region adjacent to the HIS3 reporter gene. To observe changes in HIS3 expression, strains were grown in SDC-TRP-LEU-HIS plates. A control SDC-TRP-LEU plate was utilized in order to show the presence of both pOAD and pOBD2 fusion plasmids. To select for the pOBD2 variants for the tethering assay, strains were grown on control SDC-TRP plates and experimental SDC-TRP-HIS plates. For the two-hybrid assay, positive interactions between bait and prey proteins were assessed by growth above background on SDC-TRP-LEU-HIS plates. The background control consists of strains containing just the pOAD plasmid which encodes for a non-interacting activation domain.
Figure 6: A diagram depicting the two hybrid assay. The prey protein fused to the activation domain interacts with transcriptional machinery. The bait fused binding domain protein binds to the UAS GAL4 promoter. Only an interaction between prey and bait proteins should result in HIS3 transcription.

B. Transcriptional Assays

cDNA Preparation

The objective of my project is to assess how 2µm circle gene expression is affected in strains lacking Sir proteins. Gene expression can be analyzed through the amount of mRNA transcribed from a given cell. Although RNA studies were originally analyzed by northern blot hybridization, in this study we utilize a more sensitive reverse transcriptase PCR technique to analyze 2µm gene expression.

Different strains were grown in appropriate media overnight and induced within different environments. Cell densities were measured using the spectrophotometer to ensure an OD$_{600}$ of 0.1 to 0.4. To begin RNA extraction, cells were spun down and incubated in the presence of TES solution and acid phenol. Each step was carefully conducted on ice or at cold temperatures to prevent RNA degradation. After several acid phenol and chloroform extractions, solutions
containing RNA were precipitated for at least one hour and suspended in deionized water. A spectrophotometer was used to determine RNA concentration so that 1 µg of RNA sample could be isolated for DNase treatment. By incubating the RNA samples with DNAse and buffer from Ambion, the objective of the protocol was to ensure degradation of DNA so that only RNA transcripts remain present. The success of the DNAse treatment was checked using a diagnostic PCR, which was expected to result in an absence of products. The final DNAse treated RNA samples were subject to the Ambion’s RETROscript Kit to produce cDNA. The samples were incubated with dNTP mix, buffer, RNase Inhibitor, and reverse transcriptase and finally analyzed by PCR to amplify the FLP1 and/or REP1 gene.

**Gal-inducible Strain Construction**

In order to study the effects of overexpression of REP1 and REP2 on 2µm circle gene expression, the YSH425 strain was struck out and used as the background control strain. The YSH421 strain, which originally hosted Broach Lab’s 2µm derived plasmid CV21f, was tested and found to be faulty. The plasmid DNA was first extracted through a smash-and-grab procedure and then subject to either chemical transformation or electroporation for amplification in bacterial strains. After cells grew on selective L-AMP plates, plasmid DNA was isolated from the bacterial cells. A diagnostic PCR was conducted to confirm the presence of the CV21f plasmid. The CV21f plasmid was then transformed into YSH425 in order to confirm Broach findings and selected for in SDC-URA plates (Som et al., 1988). To test how the absence of Sir2 protein affects this system, the SIR2 gene was deleted from the YSH425 strain. As mentioned before, PCR was used on pAG25 plasmid to
isolate $Asir2::\text{NAT}$ fragments. YSH425 cells that took up the fragment were selected for in YPD-NAT plates and checked using a mating type assay for SIR2 knockout. The 2µm-derived CV21f plasmid was finally transformed into the SIR2 knockout strain for our final experimental strain.

**Overexpression of REP1 and REP2**

For this inducible system, YSH425 strains were grown overnight in 10 ml of YPraffinose media while strains containing CV21f plasmids were selected for in YPraffinose-URA media. From each culture, 4.5ml of culture was removed into another tube containing 0.5ml of 20% galactose media. The addition of 2% total galactose induces overexpression of REP1 and REP2. The control YPraffinose cultures and experimental cultures were grown for an additional 3-5 hours for galactose induction. Once induced, samples grown in both galactose and raffinose media were subject to RNA extraction, DNAse treatment, and finally reverse transcriptase reaction. For this particular system, due to transcription of the genomic REP1 transcript, only FLP1 message from CV21f was analyzed using RT-PCR. The ACT1 transcript was amplified and used as an internal control in order to normalize FLP1 message between different strains and conditions.

In this particular transcriptional assay, the YSH425 strain was used as a negative control because the strain lacks endogenous 2µm circles. The YSH425 strain containing CV21f was used as a positive control and was analyzed to confirm a Broach Lab study that claims that overexpressing Rep proteins leads to a decrease in FLP1 expression (Som et al., 1988). This strain was then used to compare FLP1 expression in the same strain lacking endogenous SIR2 gene.
2µm Gene Expression in Sir\textsuperscript{ts} Strains

YSH189 (wild type), YSH491 (Sir2\textsuperscript{ts}), and YSH494 (Sir3\textsuperscript{ts}) strains were grown in 10 ml of YPD media overnight at a permissive temperature of 23ºC. Five ml of each overnight cultures were separated and incubated at 37ºC for 1-2 hours in order to inactivate Sir2 or Sir3 proteins, while the rest were continued at 23ºC. The OD\textsubscript{600} was measured for each 23ºC and 37ºC culture. \textit{FLP1} and \textit{REPI} transcripts were analyzed from the expression of the endogenous 2µm plasmids by RT-PCR. Like the Rep overexpression system, the \textit{ACT1} gene was used as an internal control for transcript normalization.

PCR Parameters

For \textit{FLP1}, \textit{REPI}, \textit{α1}, and \textit{ACT1} gene amplification, 0.6µl of cDNA template was used in PCR. Detection of different genes required various PCR cycling parameters and number of cycles. \textit{FLP1} and \textit{REPI} detection required 4 minutes at 94ºC, 30 seconds at 94ºC, 30 seconds at 53ºC, 1.5 minutes at 72ºC, and a final 3 minutes at 72ºC at a total of 30 cycles. \textit{ACT1} message was detected at the same PCR temperature settings but was run for 25 cycles instead. At 30 cycles, \textit{α1} message was detected with the following cycling parameters: 4 minutes at 94ºC, 30 seconds at 94ºC, 30 seconds at 50ºC, 1.5 minutes at 72ºC, and 3 minutes at 72ºC.

Quantitative Analysis of 2µm Genes

The \textit{FLP1} and \textit{REPI} PCR fragments for both the Rep overexpression and Sir\textsuperscript{ts} system were added to native loading buffer and run through native acrylamide gels.
The bands were stained with SYBR gold and analyzed on a phosphoimager. The 
*REP1* and *FLP1* band intensities normalized to the internal control represent the
amount of gene expression. *FLP1* and *REP1* bands from galactose or nonpermissive 
temperatures conditions were then normalized to expression from wild type 
conditions.
Results

A. Rep-Sir Interaction Assays

Two Hybrid Assays

The possibility of an in vivo Rep and Sir interaction was suggested as an explanation for observations noted in a study establishing REP3 as a silencer (Papacs et al., 2003). To test potential interactions, we used a two hybrid technique. The yeast two hybrid assay has long been utilized as a reliable genetic technique to test for protein-protein interactions indirectly through expression of a reporter gene. The basic design of this technique relies on the ability to separate modular domains of transcriptional factors into an activation and binding domain. As two separate entities, these protein domains are unlikely to join together to have a functional role in recruiting polymerase to the GAL4 promoter upstream of the HIS3 reporter gene. By engineering a chimeric protein, one containing a prey protein fused to the activation domain and one containing a bait protein fused to the DNA binding domain, an interaction can be determined through HIS3 expression. If proteins interact, the activation domain is brought into proximity to the binding domain, reconstituting a transcriptional factor so that the transcriptional machinery can express the reporter gene (Young, 1998).

The two hybrid assays were designed such that Rep proteins were fused to the binding domain for tethering to the GAL4 promoter. To rule out any HIS3 transcription occurring from the tethering of just the binding domain, a pOBD2 vector control experimental plate was included in the wild type strain as seen in Figure 7 and in SIR deleted strains in Figure 10. Each set of plates contain different pOAD variants.
expressing proteins such as Rep1, Rep2, Sir2, Sir3, and Sir4 fused to the Gal4 activation domain. Strains expressing only the activation domain, AD, act as negative controls due to the inability to localize to the \textit{GAL4} promoter. The set up for the experiment includes a SDC-TRP-LEU plate demonstrating the presence of strains bearing both the pOBD and pOAD vectors. To observe a decrease or increase in \textit{HIS3} expression, strains were grown in SDC-TRP-LEU-HIS plates so that change in growth compared to the control could be observed.

**Interactions in Wild Type Strains**

In the presence of just a DNA binding domain, proteins fused to the activation domain should not be recruited to the UAS promoter. However, the system still expresses \textit{HIS3}, as seen in Figure 7, due to background transcription. Addition of the AD-fusion proteins does not affect the level of background transcription. Like Figure 7, the experimental plate in Figure 8 shows equal growth along each row representing strains that express different AD-fused proteins. The strains in Figure 8 also express Rep1-BD and do not exhibit increased growth compared to the AD vector control strain on the first row. The little growth that is observed among the different strains represents background growth, similar to the background growth occurring with the control strains. Therefore, we fail to observe an interaction with the Rep1-BD protein in this assay.

Next we tested the ability of the same set of AD-fusion proteins to interact with a Rep2-BD fusion. Compared to the AD control strain, an obvious increase in growth is seen in strains expressing Rep1-AD and Rep2-BD, seen on the second row. Aside from this one growth phenotype, the background \textit{HIS3} transcription is
noticeably repressed throughout the plate in Figure 9. After repeating this two hybrid assay with independent cultures a minimum of three more times, similar observations were made; prior studies also suggest an interaction between Rep2 and Rep1 (Ahn et al., 1997; Velmurugan et al., 1998).
Two Hybrid Assay Control

**Figure 7:** The experimental plate contains strains expressing only a binding domain with each row representing expression of various activation domain protein fusions. Strains containing just BD are controls for the two hybrid experiment. The left plate confirms the presence of both pOBD and pOAD fusion plasmids through growth on SDC-TRP-LEU plates. Strains containing just the single binding domain results in background *HIS3* transcription level.
Two Hybrid Assay with Rep1 Tethered

Figure 8: Unlike the previous control plate, the strains contain Rep1 fused to the binding domain. The rows represent the same activation domain fused proteins expressed within each strain. On the experimental SDC-TRP-LEU-HIS plate, decreased background HIS3 expression is observed. Compared to the AD control, no significant increase in growth is observed.
Two Hybrid Assay with Rep2 Tethered

Figure 9: In this set of strains, proteins with Rep2 fused to the binding domain are expressed. Unlike the control plate, the background *HIS3* growth is absent when Rep2 is tethered. However, an increase in growth appears in strains containing both Rep2 and Rep1 proteins. This result is consistent with previous studies indicating a Rep2 and Rep1 interaction (Ahn et al., 1997).
Interactions in Strains Lacking Endogenous Sir Proteins

The wild type YSH513 and YSH514 strains used contain endogenous levels of Sir proteins. In the HIS3 system AD-fused Sir2, Sir3, or Sir4 could potentially be competing with these endogenous Sir proteins. Any Sir proteins recruited to chromatin structures could also lead to repressed states thereby decreasing the polymerase complex’s ability to transcribe genes. To uncover interactions that may have been masked by Sir silencing of the reporter gene, the same two hybrid assays were conducted in YSH625, a strain lacking the SIR2, SIR3, and SIR4 genes. Similar to wild type, background HIS3 transcription also appears with YSH625 strains expressing only the DNA binding domain, as observed in Figure 10. This background HIS3 transcription appears once again in YSH625 strains expressing Rep1-BD in Figure 11. Since there does not seem to be an apparent increase in growth compared to the AD vector control strain on this SDC-TRP-LEU-HIS plate, we still fail to find evidence for a positive interaction between Rep1 and Rep or Sir proteins using this particular set of fusion proteins. However, we do find evidence for protein interactions with the Rep2-BD fusion in this strain (Figure 12). Compared to the AD vector control row, increased growth is observed with Rep1-AD, Rep2-AD, and Sir2-AD expressing strains. Several two hybrid assays with independent cultures produced similar observations. Therefore, our results suggest a positive interaction between Rep2 and Rep1, Rep2 and Rep1, as well as Rep2 and Sir2. Assays conducted by independent labs also found evidence to suggest a Rep1-Rep2 interaction as well as Rep1 and Rep2 self associations (Ahn et al., 1997; Velmurugan et al., 1998).
Two Hybrid Assay Control in $\Delta sir2\Delta sir3\Delta sir4$ Strains

Figure 10: The experimental plate contains strains expressing only a binding domain with each row representing various activation domain protein fusions. As before, the pOBD2 containing strains are controls for the experiment. The strains used here are identical to the wild type strains used for Figures 7-9 but have $SIR2$, $SIR3$, and $SIR4$ deletions. Growth on the right panel represents background $HIS3$ expression from a leaky $HIS3$ promoter.
Two Hybrid Assay with Tethered Rep1 in $\Delta sir2\Delta sir3\Delta sir4$ Strains

**Figure 11**: Two hybrid assay in strains lacking Sir2, Sir3, and Sir4 proteins. These strains produce Rep1 fused to the binding domain with various proteins fused to activation domain described along each row. Compared to the AD control, increase in growth is not observed. The absence of increased growth does not rule out potential interactions, which may be masked by disruptions in protein structure.
Two Hybrid Assay with Tethered Rep2 in $\Delta sir2\Delta sir3\Delta sir4$ Strains

Figure 12: This final experimental plate contains strains lacking the SIR2, SIR3, and SIR4 genes. These strains produce Rep2 fused binding domains with various proteins fused to activation domain. When rows are compared the AD control, in addition to Rep1 and Rep2 interactions, increase in growth appears in strains containing Rep2BD and Rep2AD proteins. Increase in growth is also observed between Rep2 and Sir2 protein.
Testing for Sir Protein Influence on Rep-mediated HIS3 Repression

Two hybrid assays query protein interactions by comparing growth phenotypes between experimental and control strains. In the course of conducting these assays, we noticed changes in HIS3 background expression when comparing the pOBD, Rep1-BD, and Rep2-BD controls. To examine the possibility that tethering Rep1 or Rep2 could mediate repression of basal HIS3 transcription we conducted the experiment shown in Figure 13. We expressed the BD plasmids in a strain lacking pOAD plasmids. HIS3 expression was assessed via growth on SDC-TRP-HIS plates. As seen in the first (wild type) panel, the SDC-TRP-HIS plate exhibits a decrease in basal level HIS3 growth when Rep1 and Rep2 is tethered. To test whether this Rep mediated repression is dependent on SIR genes, the same background HIS3 transcription was tested in a SIR2 deleted strain, a SIR3 deleted strain, and a strain lacking all endogenous SIR genes. Deletion of SIR2 or SIR3 does not affect Rep mediate repression of HIS3. However, simultaneous disruption of SIR2, SIR3, and SIR4 abolishes Rep1’s ability to repress the reporter gene but does not appear to affect Rep2 mediated HIS3 repression.
Tethering Assay: Sir Influence on Rep Mediated \textit{HIS3} Expression

**Figure 13:** The following experiment was conducted to look at \textit{HIS3} expression in YSH513 derived strains. Growth on SDC-TRP-HIS plates show the extent of leaky \textit{HIS3} expression in YSH513 strains and how tethering Rep to the promoter affects growth. Whereas normal background \textit{HIS3} is expressed when only a binding domain is tethered, background growth is abolished when either Rep1 or Rep2 is tethered. The same result is observed in background strains lacking Sir2p or Sir3p, indicating that this repression is Sir2 and Sir3 independent. When \textit{SIR2}, \textit{SIR3}, and \textit{SIR4} genes are deleted, Rep1 tethered repression is eliminated.
B. Transcriptional Assays

To stably maintain and propagate 2µm plasmids in host strains, regulation of 2µm genes is essential. The 2µm FLPI gene is regulated by an efficient gene repression mechanism; when copy number of the plasmid declines, repression is relieved to initiate amplification of the 2µm circle. Since transcription of the FLPI gene determines the activation of the amplification system, the mode of repression at FLPI is important in controlling Flp1 concentrations. We hypothesize that the 2µm circle might use Sir proteins and similar silencing mechanisms to regulate 2µm gene expression. To assess the influence of SIR on 2µm gene transcription, cDNA from FLPI and REP1 mRNA transcripts have been quantified in the presence and the absence of functional Sir proteins.

Rep1 and Rep2 Overexpression and 2µm Gene Expression

The first system we utilized to assess 2µm gene expression was designed based on a prior study conducted by the Broach lab. In this study, FLPI, REP1, REP2, and RAF1 transcripts were quantified from a single copy 2µm-derived plasmid, CV21f, in the presence of overexpressed Rep1 and Rep2 proteins. According to the study, strains overexpressing genomic REP1 and REP2 resulted in a significant decrease in FLPI transcript and a smaller yet noticeable decrease in REP1 transcript (Som et al., 1988). We introduced the Broach lab’s CV21f plasmid into our background YSH425 strain, containing chromosomal GAL10-REP1 and GAL10-REP2, and into a Δsir2 version of this strain. The original YSH425 strain lacks endogenous 2µm plasmids and serves as a negative control for the specificity of our FLPI mRNA detection as seen on the first two lanes of Figure 14A. The strains were
each grown in raffinose and galactose media in order to compare the system at wild
type or overexpressed levels of Rep1 and Rep2. We measured *FLP1* message from
different strains and conditions using reverse transcriptase PCR. Results were
expressed as the ratio between *FLP1* and *ACT1* internal control for each culture. Each
strain was then normalized to its respective raffinose control condition. From Figure
14A alone, no major difference in *FLP1* message was observed between conditions or
between strains. Figure 14B represents the average normalized *FLP1* message from
four different sets of cultures. Our results indicate only a slight decrease in *FLP1*
transcription in wild type and no change in *FLP1* in ΔSir2 deleted strains when *REP1*
and *REP2* were overexpressed. Taking into account experimental error, our results
failed to show the 100-fold *FLP1* repression observed by the Broach lab.
Rep Overexpression on *FLP1* message in CV21f

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control YSH425</th>
<th>YSH425 CV21f</th>
<th>Δsir2-YSH425 CV21f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*FLP1* expression from CV21f

![FLP1 and ACT1 bands](image)

**Figure 14**: This transcriptional assay was conducted on strains containing genomic *REP1* and *REP2* under the *GAL10* promoter. **A.** The *FLP1* bands for each strain in the presence and absence of galactose was assessed relative to an internal *ACT1* control. The first two lanes represent the background strain which lacks *FLP1* gene, while the next four lanes represent the experimental measurements of *FLP1*. **B.** This
graphic representation is the average normalized $FLP1$ expression from CV21f in wild type or $\Delta$sir2 strains. This graph includes cumulative data from four independent experiments and standard deviations.

Since we were unable to replicate Broach results, we could not address how $SIR2$ deletions affect regulation. The results may differ from expected because of faulty strains or an overestimation of the extent to which Rep proteins repress $FLP1$ in previous experiments. To troubleshoot the background strain YSH425, we took cDNA cultures and tested for the overexpression of $REP1$ and $REP2$ in raffinose and galactose conditions by RT-PCR. Figure 15 indicates that $REP1$ mRNA was overexpressed but $REP2$ mRNA was not induced under galactose conditions. The YSH425 strain fails to overexpress both Rep1 and Rep2 simultaneously and therefore is a poor candidate for testing Sir influence on 2µm gene expression through Rep1 and Rep2.
Troubleshooting the Background Strain

YSH425 Background Strain

Figure 15: To check whether Rep1 and Rep2 were being overproduced, we amplified REP1 and REP2 message under the GAL10 promoter. When the background YSH425 strain was induced with galactose, an obvious increase in REP1 transcript is observed. However, we failed to observe REP2 message upon galactose induction.

2μm Gene Expression in Sir<sup>ts</sup> Strains

In the second system used, we sought to directly address how Sir proteins influence endogenous 2μm gene expression. We utilized strains that contain temperature sensitive SIR alleles so that when cultures are switched from room temperature (23°C) to nonpermissive temperature at 37°C, the Sir proteins become nonfunctional. We used RT-PCR to measure both FLP1 and REP1 mRNA levels in these conditions.
Two techniques were used to check for the presence of temperature sensitive alleles in the YSH491 and YSH494 strains. Figure 16 displays a mating assay to test for Sir2 and Sir3 protein inactivation. Since active Sir proteins are required for silencing and haploid characteristics, inactivation of Sir prevents mating ability and inhibits growth on SD plates. Figure 16 shows diploid growth for wild type, Sir2\textsuperscript{ts}, and Sir3\textsuperscript{ts} strains at room temperature but does not show growth for Sir2\textsuperscript{ts} and Sir3\textsuperscript{ts} alleles when grown at 37°C. Although this mating test was important for verifying temperature sensitive strains, we did not know if 2 hours of incubation at 37°C was sufficient to completely inactivate Sir2 or Sir3 proteins. Therefore the inactivation of Sir2 and Sir3 proteins was assessed by checking the \( \alpha_1 \) message of cDNA cultures. Figure 17 confirms this expectation because \( \alpha_1 \) message only appears when Sir2 and Sir3 proteins are inactivated at 37°C for both YSH491 and YSH494 respectively.
Confirming Temperature Sensitivity

Figure 16: A mating assay to test the mating characteristics of the YSH189, YSH491, and YSH494 strains on SD plates. After spotting the sample haploid strains with a 17α strain overnight, the colonies were replica plated on SD plates. Strains lacking functional Sir proteins behave as diploid cells and fail to mate. As expected, when plates were shifted to the non-permissive temperature at 37°C, Sir proteins became inactivated and cultures failed to mate.

Sir<sup>ts</sup> System Check through α1 Message

Figure 17: The Sir<sup>ts</sup> cultures were incubated at NPT for 1-2 hours. To test whether this short incubation period was sufficient to inactivate Sir2 and Sir3, we amplified α1 message. The strains normally express a mating type but in the absence of functional Sir proteins begin to express α1. As expected, when YSH491 and YSH494 strains are shifted to 37°C α1 message is expressed as the Sir proteins become inactivated.
As shown in Figure 18A, three strains were tested for \textit{FLP1} expression, each grown at 23°C or 37°C. For quantification, each product was expressed as a ratio to an \textit{ACT1} internal control and normalized to the permissive temperature control. Figure 18B is a graphic representation of three separate experiments and the normalized \textit{FLP1} expression of each strain. In general there is about a 50% increase in \textit{FLP1} expression for the wild type YSH189 strain when shifted to 37°C. With both the Sir2\textsuperscript{ts} and Sir3\textsuperscript{ts} strains, shifting to 37°C did not significantly increase or decrease \textit{FLP1} transcription. Taking into account the error attributed to fluctuations from different experiments, the cultures express almost the same level of \textit{FLP1} message with or without the inactivation of Sir2 and Sir3. Therefore, we fail to find evidence to suggest that Sir proteins affect \textit{FLP1} transcription.

We also measured \textit{REP1} message in these strains. It is apparent in Figure 19A that shifting both Sir2\textsuperscript{ts} and Sir3\textsuperscript{ts} alleles to 37°C results in a decrease in \textit{REP1} message. After repeating the experiments with two different cultures, the graphic representation of normalized \textit{REP1} message in Figure 19B shows an overall decrease in \textit{REP1} transcript when Sir2 and Sir3 proteins are inactivated.
Influence of Sir2p and Sir3p on endogenous 2µm circle *FLP1* expression

### Table 1

<table>
<thead>
<tr>
<th>Strains</th>
<th>YSH189</th>
<th>YSH491</th>
<th>YSH494</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temp (°C)</strong></td>
<td>23</td>
<td>37</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>23</td>
<td>37</td>
</tr>
</tbody>
</table>

*FLP1*

*ACT1*

**A.**

**Figure 18:** A *FLP1* transcriptional assay in strains containing active or inactive Sir2 and Sir3 proteins. B. Shifting YSH189 strains to nonpermissive temperature leads to an overall increase in *FLP1* message. When Sir2 or Sir3 protein becomes inactive, there is no significant increase or decrease in *FLP1* message.
Influence of Sir2p and Sir3p on endogenous 2µm circle *REP1* expression

**A.** The above gel represents the RT-PCR of *REP1* and *ACT1* message. There is a significant decrease in *REP1* expression when Sir2 or Sir3 proteins become inactivated at nonpermissive temperatures. **B.** The graph depicts average normalized *REP1* expression from two independent experiments. When the background strain is shifted to 37ºC, there is a slight increase in *REP1* expression. Although there appears to be a large variability, when *SIR2*<sup>ts</sup> and *SIR3*<sup>ts</sup> strains were shifted to 37ºC a significant decrease in *REP1* expression is observed.

<table>
<thead>
<tr>
<th>Strains</th>
<th>YSH189</th>
<th>YSH491</th>
<th>YSH494</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
<td>Sir2&lt;sup&gt;ts&lt;/sup&gt;</td>
<td>Sir3&lt;sup&gt;ts&lt;/sup&gt;</td>
</tr>
<tr>
<td>Temp (ºC)</td>
<td>23</td>
<td>37</td>
<td>23</td>
</tr>
<tr>
<td>Normalized <em>REP1</em></td>
<td>1</td>
<td>1.23±0.35</td>
<td>1</td>
</tr>
</tbody>
</table>
Discussion

Several earlier studies demonstrated Rep protein’s ability to negatively regulate important 2\(\mu\)m genes involved in copy number maintenance (Som et al., 1988; Veit and Fangman, 1988). However, the exact mechanism of Rep repression on 2\(\mu\)m genes has yet to be established.

Recruitment of the Sir protein complex is required for the formation of heterochromatin in yeast at the mating type loci, rDNA repeats, and telomeres. Heterochromatin is less accessible to transcriptional machinery, thereby preventing gene expression and establishing a mechanism of gene regulation (Rusche et al., 2003). The regulation of 2\(\mu\)m encoded genes involves repression and derepression of FLPI to adjust copy number. For its segregation mechanism, the 2\(\mu\)m circle has been shown to exploit the host’s segregation machinery via cohesin for its own stable propagation (Ghosh SK, 2007). We hypothesized that the repression of the 2\(\mu\)m gene expression may depend on host silencing machinery including recruitment of Sir proteins. Two independent studies noted REP3 as a novel silencer and its ability to silence in the presence of Sir proteins. Several similarities in segregation, localization, and anchoring between the Sir proteins and 2\(\mu\)m encoded products also led us to test our hypothesis.

When REP3 mediated silencing was found to be dependent on Sir and Rep proteins in a study previously conducted in the Holmes lab, it was suggested that Sir and Rep proteins may be competing for a common anchoring site or interacting (Papacs et al., 2004). To examine the possibility that Sir and Rep proteins may
interact, we used the two hybrid assay. With this qualitative study, we found positive interactions between Rep1-Rep2, Rep2-Rep2, and Rep2-Sir2 proteins. Rep1-Rep2 interactions were easily detected in wild type strains; however, Rep2-Rep2 and Rep2-Sir2 interactions were only observed when endogenous SIR genes were deleted.

Several independent labs have verified Rep1-Rep2 interactions and self associations (Ahn et al., 1997; Velmurugan et al., 1998). Although these labs have noted a Rep1-Rep1 interaction, our results do not indicate any such interactions.

Rep2-Sir2 interactions have not been previously observed by other labs, but we consistently observed this positive interaction. Sir2 proteins are known to form complexes with Sir3 and Sir4 in order to create silenced chromatin. In the presence of endogenous Sir proteins, Sir2-AD protein may have recruited Sir complexes to the reporter gene promoter and prevented expression of HIS3. In the absence of endogenous Sir proteins, the reporter gene can be open to the transcription machinery and result in a positive growth phenotype if one exists. This potentially explains why a Rep2-Rep2 interaction was observed only in SIR deleted strains. The Rep2-AD protein is most likely interacting with Sir2 and recruiting the Sir complex to the Rep2-BD and GAL4 promoter for HIS3 silencing in the wild type strain. So only when endogenous Sir proteins are deleted, do we easily detect increased HIS3 expression and interpret it as a positive interaction between Rep2-Rep2 and Rep2-Sir2.
Two Hybrid Assay Limitations

Our results suggest a positive interaction in strains with Rep2-BD and Rep1-AD, but we do not observe similar interactions with strains containing Rep2-AD and Rep1-BD. As seen in Figure 8, we did not observe increased growth in strains expressing Rep1-BD and Rep1-AD as well as Rep1-BD and Rep2-AD even though interactions have been previously reported to exist (Ahn et al., 1997; Velmurugan et al., 1998). One of the limitations to the two hybrid assay is its ability to result in false negatives. In this particular case, fusing the Rep1 protein to a binding domain may have disrupted its structure or function and thereby prevented an increase in growth. However, fusing the Rep1 protein to the activation domain does not appear to alter the protein because a positive interaction is observed with Rep2. Further studies using functional assays can be conducted to check whether or not Rep1-BD’s inability to interact is dependent on a loss of function or change in structure. If the binding domain does prove to change Rep1 structure or function, the two hybrid assay can be conducted using strains containing a LexA operator and LexA fusions instead of a GAL4 promoter and BD fusions.

Rep1 and Rep2 Confers Repression of HIS3 Transcription

In the two hybrid assays, different protein fusions were tethered to the GAL4 promoter. We noticed differences in the background HIS3 transcription depending on what BD fusion was expressed. Therefore to visualize these differences between strains containing the three different pOBD vectors, a tethering assay was utilized. As seen on the left-most panel in Figure 13, we observed a decrease in leaky HIS3 transcription only when Rep1 or Rep2 was fused to the binding domain. The
repression of the *HIS3* reporter can be attributed to either the tethered Rep proteins themselves or the recruitment of a repressor via natural interactions with Rep proteins. If this Rep mediated *HIS3* repression occurs through a Sir dependent mechanism in this constructed *GAL4* system, it could provide strong evidence for Sir dependence on natural 2μm gene promoters. When we analyzed background *HIS3* in Δ*sir2* or Δ*sir3* strains, Rep tethered repression remained the same compared to wild type strains. However, when all endogenous *SIR* genes were deleted, Rep1 mediated repression was alleviated. The results suggest that Rep2 tethered repression of *HIS3* is *SIR* independent, but that Rep1’s ability to repress depends on either Sir4 or the entire Sir protein complex. For future studies, it is necessary to replicate our results and observe recovery of Rep1 mediated silencing in *SIR4* deleted strains to rule out the influence of the entire Sir complex on this type of repression.

**Do Sir Proteins Affect Rep-mediated *HIS3* Repression or Not?**

Several anomalies arose from the tethering assays. The two hybrid screen suggests a Rep2-Sir2 interaction; however deletion of *SIR2* does not affect Rep2’s ability to repress *HIS3*. The results imply that Sir2 exhibits a non-silencing role when bound to Rep2. The binding of Rep2 with Sir2 itself may inactivate Sir2 function by impeding Sir2’s catalytic site. Both Sir3 and Sir4 proteins do not appear to interact with Rep2p or influence Rep2’s ability to silence. Therefore the *HIS3* repression that is observed when Rep2 is tethered seems to work independently of endogenous Sir proteins.

Rep1 appears to repress the reporter gene in a *SIR* dependent fashion. The tethering assay in general suggests that Rep1 and Rep2 have two different
requirements for their ability to silence. This is quite unexpected because both Rep1 and Rep2 proteins were previously believed to repress as a Rep complex rather than work individually. Since this seems unlikely, further testing must be done to confirm this SIR dependence on Rep1 mediated repression by checking the strain and designing new experiments.

Sir Influence on Gene Expression of the Single Copy CV21f Plasmid

The possibility of a Sir dependent Rep1 mediated repression of HIS3 on a GAL4 system led us to continue to address whether Sir proteins influence 2µm gene expression. We began by analyzing the expression of the FLP1 gene in the presence of elevated Rep1 and Rep2 protein levels. The objective of this particular experiment was to understand how the deletion of endogenous Sir proteins affect Rep protein’s ability to negatively regulate the FLP1 gene from a single copy CV21f plasmid. Whereas the Broach study observed a 100-fold decrease in FLP1 expression in the presence of high Rep1 and Rep2 concentrations (Som et al., 1988), we failed to confirm these results, possibly due to problems with Rep2 induction. Without a reliable strain, we were unable to assess how Sir2 proteins influence FLP1 regulation through Rep1 and Rep2.

Sir Influence on Endogenous 2µm Circle

As an independent test of Sir influence, we quantified FLP1 and REP1 message from endogenous 2µm circles in Sir2-ts and Sir3-ts strains grown at 23°C and 37°C. Within the same strain, adjusting temperatures can modify Sir2 or Sir3 activity. In our studies, FLP1 mRNA message does not appear to change significantly with the
inactivation of Sir2 or Sir3 protein at 37ºC. Prior studies indicate an importance in regulating \textit{REPI} transcription, which indirectly controls amplification by regulating \textit{FLPI} transcription (Som et al., 1988; Veit and Fangman, 1988). In our \textit{REPI} transcriptional assays, we noticed that inactivation of Sir2 or Sir3 protein decreased wild type \textit{REPI} mRNA levels by 50%. Therefore, our results suggest that the presence of Sir proteins typically increase \textit{REPI} expression. If Sir proteins were involved in 2µm gene repression, as we hypothesized, the inactivation of Sir2 or Sir3 should have caused a significant increase in both \textit{FLPI} and \textit{REPI} message. Aside from a Sir-independent mechanism of silencing 2µm genes, the transcriptional assays suggest differences between \textit{FLPI} and \textit{REPI} regulation. Therefore the mechanism and strength of repression may be tightly modulated between different genes on the 2µm circle.

\textbf{Model for 2µm Circle Gene Regulation}

The objective of this study was to address whether or not a Sir dependent silencing mechanism is utilized to repress transcription of the \textit{FLPI} and \textit{REPI} genes on 2µm circles. Transcriptional assays and tethering assays primarily suggest that the repression of 2µm genes is Sir independent. Although two hybrid assays do show a Rep2-Sir2 interaction, deletion of \textit{SIR2} does not eliminate Rep2’s ability to repress the reporter gene. The deletion of \textit{SIR2, SIR3}, and \textit{SIR4} and its influence on Rep1’s ability to repress remains the one observation supporting our hypothesis. Therefore we must verify Sir influence on Rep1 tethered repression before concluding that our hypothesis is null.
Results from *REP1* transcriptional analysis were initially baffling in terms of our hypothesis and the conventional role of Sir as silencing proteins. Assuming that Sir proteins were actively silencing throughout the 2µm circle, inactivation of Sir2 or Sir3 protein should lead to an increase in 2µm gene expression. Alternatively, if Sir proteins do not actively mediate gene repression, inactivation of the silencing proteins should exhibit little or no change in 2µm gene expression as seen with *FLP1* levels in Figure 18. Sir proteins normally create condensed chromatin structures to prevent transcription of genes. If Rep protein complexes are recruited to *REP1* and *FLP1* promoters, two hybrid results suggest that Sir proteins may also be localized there. According to transcriptional assays, Sir proteins do not contribute to the repression of 2µm genes. Thus, any Sir proteins recruited to 2µm gene promoters should have a non-silencing role and may be competing with other Rep proteins for similar binding sites. Figure 20 is a pictorial representation of how inactivating Sir proteins may affect *FLP1* and *REP1* expression. Since we have identified a Rep2-Sir2 interaction, the inactivation of Sir2 or Sir3 may disrupt this interaction and allow the binding of other Rep proteins to the same binding site, increasing the overall number of Rep complexes at the promoter. At the *REP1* promoter, an increase in Rep protein recruitment when Sir2 or Sir3 is inactivated may describe the decrease in *REP1* expression observed in Figure 19.

At the *FLP1* promoter, Sir complex and Rep complex may also be competing for the same Rep binding site. If the *FLP1* gene is maximally repressed, an increase in Rep recruitment through Sir inactivation should not change *FLP1* expression. As an indirect regulator of amplification, the expression of Rep proteins can exhibit more variation and flexibility. Therefore *REP1* gene expression may be more subject to
fluctuations and more sensitive to the environment. Although the transcriptional assay for REP1 must be repeated, this model provides a plausible mechanism to explain the observations so far.

A. **REP1 Expression**

![Diagram of REP1 Expression](image)

B. **FLP1 Expression**

![Diagram of FLP1 Expression](image)

**Figure 20**: A model for Sir and Rep protein influence on **REP1** and **FLP1** expression. 
**A.** The inactivation of Sir2 or Sir3 may allow for recruitment of Rep complex increasing the overall repression on **REP1**. **B.** However, if **FLP1** is already regulated in wild type conditions, an increase in Rep recruitment at nonpermissive temperatures may result in no change in **FLP1** transcript levels.

**What is the Role of Sir Proteins on the 2μm Plasmid?**

Based on our results, we propose that gene regulation on the 2 μm circle occurs in a mechanism distinct from Sir-dependent silencing. Although conventional Sir-dependent silencing is not observed, Sir proteins may still be recruited to 2μm genes through Sir2-Rep2 interactions. This would suggest that recruited Sir proteins have non-silencing roles on the endogenous plasmids. Sir proteins are involved in
several roles other than silencing such as aging. At the rDNA repeats, complexes containing only Sir2 proteins are recruited to create silent chromatin. Sir2 inhibits recombination machinery access to the rDNA repeats and suppresses recombination (Fritze, 1997; Gottlieb and Esposito, 1989; Smith and Boeke, 1997). Sir2 also appears to contribute to cohesin deposition, specifically Scc1 proteins, to various sites. At the rDNA repeats, Sir2 is involved in forming cohesin structures to prevent unequal recombination between sister chromatids (Kobayashi et al., 2004). On the 2µm circle, Sir2 proteins may interact with Rep2 through Scc1 and contribute to the 2µm circle segregation pathway and less so to 2µm gene regulation. If Sir proteins are likely to have a role in recombination, they may recruit cohesin complexes to FRT sites and prevent sister-plasmid recombination. For future investigations, we may wish to address changes in 2µm plasmid segregation in SIR deleted strains. Before studies are tested to address the potential role of Sir proteins, it may be important to examine the localization of Sir and Rep proteins within the 2µm circle so that Sir and Rep protein function can be specified to certain 2µm plasmid sites. Overall, my thesis research demonstrates that 2µm genes are regulated in a Sir-independent manner. To better understand the mechanism of 2µm gene regulation, further tests can be conducted targeting other proteins such as Sir2 homologues or known gene repressors.

**How Does FLPI Get Repressed?**

So how might Rep proteins mediate repression if Sir proteins are not involved? Rep proteins may be novel repressors or recruit protein complexes that contain other repressors that work in a SIR independent mechanism. Although Sir proteins are a major contributor to repression of genes in yeast, many other modes of
repression exist. The Sir2 homologues Hst3 and Hst4 deacetylate H3 K56 in an NAD+ dependent manner. Several studies have discovered that strains lacking \textit{HST3} or \textit{HST4} result in silencing defects (Celic et al., 2006; Freeman-Cook et al., 1999; Yang et al., 2008). Since Hst3 and Hst4 proteins are so similar to Sir2 function, these homologues may potentially be the major player in Rep mediated silencing. Along with Sir2, Rep2 might interact with Sir2 homologues, Hst3 or Hst4, to stimulate repression of \textit{HIS3} at the \textit{GAL4} promoter. So in \textit{SIR2} deleted strains, the presence of active Hst3 or Hst4 might prevent the loss of silencing. In one study, silencing exhibited by the 2µm ARS sequence was found to be dependent on the \textit{HST3} gene. The study also noted the physical presence of Hst3 histone deacetylase at the 2µm plasmid genes and found that deleting \textit{HST3} upregulated \textit{FLP1} transcripts (Grunweller and Ehrenhofer-Murray, 2002). Like deacetylation, posttranslational modifications such as sumoylation in histones have also been shown to cause transcriptional repression. SUMO, which are small ubiquitin-like proteins, can attach to lysine residues and modulate a variety of functions such as facilitate protein-protein interactions or protect substrates from proteolysis (Nathan et al., 2006; Nathan et al., 2003; Verger et al., 2003). SUMO appears to attach to Flp1, Rep2, and Rep1 proteins and accounts for a 40 fold increase in regular 2µm plasmid levels in a SUMO pathway mutant strain (Chen et al., 2005). Thus, it is possible that sumoylated Rep proteins recruit SUMO to histones on the \textit{GAL4} promoter and thereby cause transcriptional repression of \textit{HIS3} gene. In addition to contributions to silent chromatin, sumoylation can directly modify Flp proteins and affect Flp recombination. SUMO proteins may be influencing 2µm gene products directly or by attracting other proteins that can modify their activities. Nevertheless, understanding
the exact mechanism of 2µm gene regulation and amplification requires further investigation.
References


