The 2-μm plasmid encoded protein Raf1 regulates both stability and copy number of the plasmid by blocking the formation of the Rep1–Rep2 repressor complex

Syed M. A. Rizvi¹, Hemant K. Prajapati¹, Purba Nag² and Santanu K. Ghosh¹,*

¹Department of Biosciences and Bioengineering, Indian Institute of Technology, Bombay, Powai, Mumbai 400076, Maharashtra, India and ²Signal Transduction Laboratory, QIMR Berghofer Medical Research Institute, 300 Herston Road, Herston, Brisbane Queensland 4006, Australia

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ABSTRACT

The 2-μm plasmid of the budding yeast Saccharomyces cerevisiae achieves a high chromosome-like stability with the help of four plasmid-encoded proteins (Rep1, Rep2, Raf1 and Flp) and several host-encoded proteins. Rep1 and Rep2 and the DNA locus STB form the partitioning system ensuring equal segregation of the plasmid. The Flp recombinase and its target sites FRTs form the amplification system which is responsible for the steady state plasmid copy number. In this work we show that the absence of Raf1 can affect both the plasmid stability and the steady state copy number. We also show that the Rep proteins do bind to the promoter regions of the 2-μm encoded genes, as predicted by earlier models and Raf1 indeed blocks the formation of the Rep1–Rep2 repressor complex not by blocking the transcription of the REP1 and REP2 genes but by physically associating with the Rep proteins and negating their interactions. This explains the role of Raf1 in both the partitioning and the amplification systems as the Rep1–Rep2 complex is believed to modulate both these systems. Based on this study, we have provided, from a systems biology perspective, a model for the mechanism of the 2-μm plasmid maintenance.

INTRODUCTION

The high copy number 2-μm plasmid of the Saccharomyces strains (1,2) is a classic example of selfish DNA elements present in the nucleus without jeopardizing the fitness of its host (reviewed in (3,4)). Even though it confers no obvious advantage to its host, it is among the most stable plasmids with a loss rate of about 10⁻⁵ per cell per generation (5–8). Owing to its high mitotic stability and copy number, components of the 2-μm plasmid are being used successfully for heterologous high copy gene expression in yeast. This near chromosome-like stability is due to two plasmid borne systems, namely the partitioning and the amplification systems comprising of the four proteins (Rep1, Rep2, Raf1 and Flp), a partitioning locus STB and two recombination sites FRTs (9–13). These proteins and DNA loci are involved in the faithful segregation and the maintenance of steady state copy number of the plasmids. Rep1 and Rep2 proteins along with the cis-acting locus STB form the partitioning system that helps in the equal segregation of the plasmids between the mother and the daughter. If the copy number drops due to missegregation, the amplification system is activated till the steady state copy number is restored. Once the steady state is reached, the amplification system is presumably switched off. The amplification system comprises of the plasmid encoded recombinase Flp and its target sites FRTs. The amplification of the copy number is achieved as the Flp-mediated recombination switches between the theta mode and a rolling circle mode of plasmid replication, as proposed by Futcher (14). A relatively less studied but an important member among the plasmid encoded genes is RAF1.

The partitioning and the amplification systems together ensure the stable maintenance of the 2-μm plasmid at a high copy number in the cell (6,15,16). However, as the proteins involved in the partitioning and amplification systems are not mutually exclusive, perturbations in one are likely to affect the other. The communication between the partitioning and amplification systems is likely mediated by the Rep1 and Rep2 proteins. The roles of the Rep proteins in the utilization of several host factors required for equal plasmid partitioning are well documented (3,17–25). In addition, Rep1 and Rep2 form a bipartite repressor that regulates plasmid gene expression, in particular, to prevent inappropriate or unregulated plasmid amplification (15,26,27).
Whereas FLP, REP1 and RAF1 genes are subject to down-regulation by the Rep1–Rep2 repressor, REP2 appears to be constitutively expressed (27). However, there is no direct evidence for the interaction of the Rep proteins with the plasmid genes presumed to be under their control.

Genetic evidence implicates Raf1 in triggering a prompt amplification in response to low plasmid copy number (PCN) by antagonizing the repressor and thus enhancing FLP expression (15). It is not clear whether Raf1 acts by disrupting the formation of the heteromeric repressor, or by blocking the action of the mature repressor at the FLP promoter. The role of Raf1 is not limited to PCN maintenance, but also extends to partitioning (28). Raf1 has been shown to associate with STB (29), and its absence results in increased plasmid missegregation (28). However, the mechanisms by which Raf1 promotes plasmid stability are not understood.

In this report, we have analyzed the interrelationships between Raf1 and the Rep1–Rep2 repressor to better understand the interplay between the 2-μm plasmid partitioning and amplification systems. We provide direct evidence for the interactions of Rep1, Rep2 and Raf1 with promoters of the 2-μm plasmid genes. Results from yeast two hybrid, competitive yeast two hybrid and bimolecular fluorescence complementation (BiFC) assays demonstrate that Raf1 physically interacts with Rep1 and Rep2. Raf1–Rep1 and Raf1–Rep2 interactions are independent of Rep2 and Rep1, respectively. These interactions disrupt Rep1–Rep2 repressor formation, and provide a molecular explanation for Raf1 being a Rep1–Rep2 antagonist. We integrate these protein interactions into a model in which a repressor amplified negative feedback loop sets up cell cycle-dependent oscillations of the Rep1–Rep2 repressor, and possibly of the Flp, Rep1 and Raf1 proteins as well. The rhythmic change in the repressor levels proposed by the model is typical of several biochemical oscillators (30). The model is consistent with the experimental observation that the Rep protein levels increase from a minimum at the G1 phase, peaks at the S/G2 stage and then decreases till the end of the mitosis (11).

To explain the role of Raf1 in copy number amplification, it has been hypothesized that Raf1 promotes transcriptional activation of Flp expression by antagonizing the Rep1–Rep2 repressor complex (16,29). Raf1 may either disrupt formation of the heteromeric repressor or may block its role as a negative regulator of Flp gene expression. Raf1 has also been implicated to play a role in the partitioning system to promote plasmid segregation, although the mechanistic details are not well understood (28). In this report we sought to test the above hypothesis to unveil the role of Raf1 in further detail.

The communication between the partitioning and amplification systems is likely mediated by the Rep1–Rep2 repressor complex. It generates sufficient scientific curiosity to address how faithful plasmid partitioning and maintenance of a steady state PCN are simultaneously achieved through the Rep1–Rep2-mediated cross-talk between the partitioning and the amplification systems. Functions of the Rep proteins through utilization of the host factors in equal segregation of the plasmids are well documented (3,17–25). In a recent study, Prajapati et al, demonstrated that along with the microtubules and Kip1 motor (23,31), the microtubule-associated proteins Bik1 and Bim1 play a significant role in the faithful segregation of the 2-μm plasmid (32), adding yet another class of host factors to the working model of plasmid segregation. To link Rep1–Rep2 complex with Flp-mediated amplification functions, several studies have proposed that the complex forms a bipartite repressor and attenuates Flp activity by repressing its transcription. A feedback repression by the Rep1–Rep2 complex also attenuates the expression of REPI and RAF1 (15,16,27). Raf1, on the other hand, has been proposed to antagonize Rep1–Rep2 repressor due to its ability to act as a positive regulator of Flp which has been inferred by observing an increased level of FLP transcripts when Raf1 is over-expressed although no effect on the transcript level of REP2 was reported (27).

Although it has been demonstrated that the disruption of RAF1 leads to the missegregation of the plasmid (28) and Raf1 has the ability to associate with STB (29), the mechanism by which Raf1 promotes the stability is not yet understood. In this report cells bearing the 2-μm plasmids devoid of RAF1 gene were used to demonstrate that Raf1 can influence both the equal partitioning and the copy number of the plasmids. All the interactions pertaining to the transcriptional control of the plasmid encoded genes leading to faithful maintenance have been predicted primarily through indirect evidences based on which a model of plasmid maintenance has been proposed where Rep1–Rep2 complex acts as a repressor of transcription and Raf1 as an anti-repressor (16,27). However, there has been no direct evidence demonstrating the interaction of either the Rep proteins or Raf1 to the promoters to modulate transcription. Moreover, there was no evidence to demonstrate the interaction of Raf1 directly with Rep1–Rep2 complex to attenuate the repressor activity (16,27). In this study we have provided direct evidence to validate the model by demonstrating the interactions of Rep1, Rep2 and Raf1 to the promoters of the 2-μm encoded proteins. With the help of yeast two hybrid assay, competitive yeast two hybrid assay and BiFC assay we have also demonstrated that Raf1 physically interacts with both the Rep proteins independently and while doing so it indeed blocks the formation of the Rep1–Rep2 repressor complex. The 2-μm plasmid has the capability to revert to a steady state optimum for its stable maintenance in its host. It has been demonstrated that the expression of the Rep proteins is not constitutive and goes through a cyclic change during the cell growth. The cellular concentration of the Rep proteins increases from a minimum at the G1 phase, reaches a maxima at the S/G2 stage and then decreases till the end of the mitosis (11). This rhythmic change in the concentration is suggestive of a system-level characteristic typical of many biochemical oscillators (33). Toward the end, we have proposed that the interactions among the 2-μm plasmid encoded proteins form a repressor amplified negative feedback system that maintains a stably oscillating level of the Rep1–Rep2 complex and possibly Flp, Rep1 and Raf1 proteins which is crucial for faithful propagation of the plasmids at a steady state copy number within the host.
MATERIALS AND METHODS

Reagents, plasmids and yeast strains

Reagents are listed in the Supplementary Data. Plasmids are listed and described in Supplementary Table S1. Yeast strains are listed and described in Supplementary Table S2.

Bi-molecular fluorescence complementation (BiFC) assay

Earlier studies have provided extensive cell biological evidence for the localization and dynamics of Rep1 and Rep2 (11), but the complex itself has never been visualized. A system based on BiFC (34) (see the Supplementary Data for BiFC tagging and Supplementary Figure S5) was developed to visualize the complex and its dynamics. BiFC was confirmed by detecting the fluorescence in the presence of 2% galactose. Cells were grown overnight in SC-Raffinose and were subcultured in fresh SC-Raffinose till the OD_{600} reached 0.5–0.8. Dextrose or galactose was then added to a final concentration of 2%, and samples were harvested at different time intervals. The cells were washed with and resuspended in 0.1M phosphate buffer before imaging. Imaging was done with the YFP filter (Zeiss filter set 46 (excitation: BP 500/20; beamsplitter: FT 515; emission: BP 535/30)).

Other methods

The other methods are described in detail in the Supplementary Data.

RESULTS

Raf1 is required for plasmid stability

Earlier studies with Raf1 demonstrated its role in the plasmid stability (28) and its ability to bind to the plasmid partitioning locus, STB (29), however, the mechanism through which Raf1 affects plasmid segregation is not well understood. The role that Raf1 plays in the maintenance of plasmid could be through a combined effect of this protein both on the partitioning and the amplification systems. This is because Raf1 also affects the expression of FLP (and hence the PCN (16)) and therefore deletion of RAFl is not only expected to affect the partitioning but also the amplification system (16,27). It is instructive to note that the stability of the 2-μm plasmid is affected by the gene dosages of REP1 and REP2 (10). Therefore, a change in the PCN can alter the relative stoichiometry of Rep1 and Rep2 which, in turn, affects the localization and function of the Rep proteins at STB as well as the function of the Rep–Rep2 complex as a transcriptional repressor (26). Consequently, the plasmid missegregation is a concerted effect of both the loss of partitioning and the variation in the PCN. Unlike earlier experiment (28), in this study the effect of Raf1 on the 2-μm plasmid stability has been visualized using a fluorescently labeled 2-μm derived plasmid, pSV1 as described earlier (25,35). pSV1, harboring lac operator array appeared as green foci within the yeast cells expressing lac repressor fused to GFP (Figure 1A). Budded cells with separated DAPI (post anaphase) were analyzed for the distribution of the plasmid foci. Equal number of foci found in the mother and daughter nuclei of a cell is counted as ‘equal segregation’ whereas unequal distribution of foci is counted as ‘mis segregation’ or ‘no segregation’. Since pSV1 harbors only the partitioning locus STB, it segregates equally in the [Cir–] cells where Rep proteins are provided in trans from the endogenous 2-μm plasmid whereas it misseggregates in the [Cir+] cells (36), due to absence of any native plasmid and hence the Rep proteins. To find if Raf1 acts as an independent component of the partitioning system or if it acts through the FLP recombinase, the segregation of pSV1 was visualized and the extent of missegregation or ‘no segregation’ (unequal distribution of plasmid foci) was measured in wild-type [Cir–], [Cir+] and raf1Δ strains. The extent of missegregation was found to be much higher in the raf1Δ strain as compared to wild-type [Cir+] (Figure 1B). As expected [Cir+] cells showed highest ‘no-segregation’ population type (all the plasmid foci segregated to the mother, Figure 1B green bar). Further validation of the plasmid segregation analysis was performed using plate-based plasmid stability assay as described in Supplementary Data using another 2-μm derived plasmid, YEpLac181 harboring an auxotrophic marker (LEU2) (Figure 1A). Plasmid stability was assayed by calculating the plasmid bearing cells following a growth in the non-selective media. Consistent with the cell biological assay the instability (%) of YEpLac181 was found to be minimum for the [Cir+] wild-type strain but the instability increased gradually and consistently for raf1Δ, and [Cir+] strains (Figure 1B). To analyse if Raf1 functions independent of its role in FLP expression, the plasmid instability was also measured for flpΔ and raf1ΔflpΔ double deletion strains. Comparison of the average instability of the plasmid in raf1Δ with raf1ΔflpΔ strain revealed a slight difference (paired t-test \( P = 0.14, 95\% \) confidence interval) while comparison of flpΔ and raf1ΔflpΔ strains revealed a significant difference (paired t-test \( P = 0.04, 95\% \) confidence interval) in the average instability (Figure 1C). These results are consistent with the earlier study (28) and suggest that Raf1 might have a role in promoting equal segregation of the plasmids independent of its effect on FLP and hence PCN. Surprisingly, Raf1 when over-expressed from a GAL10 promoter also showed an increased plasmid loss rate much higher than the [Cir+] wild-type strain (Figure 1C). This increased instability was not due to the growth medium (SC-galactose) since there was no difference in the instability (paired t-test \( P = 0.299, 95\% \) confidence interval) when raf1Δ was grown in either YPD or SC-galactose.

Raf1 influences plasmid amplification and hence the copy number

A direct consequence of FLP activation function of Raf1, as proposed in the previous study (15), suggests that there should be an increase in the PCN when RAFl is deleted. To measure this perturbation, we performed qPCR based copy number measurements, as described in the Supplementary Data, for raf1Δ, flpΔ and raf1ΔflpΔ mutants. As expected, there was a substantial decrease in the PCN in the flpΔ mutant. However, to our surprise PCN almost doubles in a raf1Δ strain (Figure 1D). This result apparently contradicts the hitherto believed model of the 2-μm plasmid maintenance (15,16,27), where Raf1 is described as an acti-
Figure 1. Plasmid loss, PCN and FLP expression increases in a raf1Δ strain: (A) Schematic showing the segregation of the native 2-μm plasmid and the 2-μm derived plasmid during mitosis. The native 2-μm plasmid is depicted by circles and the 2-μm derived plasmids whose stability is measured is depicted by dots. (B) Lowermost values show the percentage of cells with extreme mother bias where the mother receives all the plasmids while the daughter receives none (No segregation). Values in the middle show the percentage of cells showing the disproportionate distribution of the plasmids between mother and daughter (misseggregation). Values at the top show normal segregation where the mother and the daughter cells receive an equal number of plasmid foci. (C) Plasmid stability was measured as the percentage of cells growing on the selective media (i.e. the cells retaining the plasmid) following prolonged growth under non-selective condition and instability (i%) was calculated based on the initial and final stabilities as mentioned earlier (39). Cells were either grown in YPD or SC-galactose. Paired t-test P-values are shown between the pairs of instability values for statistical significance. (D) Copy number assay: qPCR based copy number measurements to study the effect of Raf1 and Flp on the amplification system. SGY2023, SGY2033 and SGY2029 were used to measure the PCN in raf1Δ, flpΔ and raf1ΔflpΔ strains, respectively. Raf1 was either overexpressed or deleted in W303 [Cir+] to measure the PCN. Both the deletion and over-expression of Raf1 showed similar effect on the PCN. Average PCN was increased to around 100 copies per cell when Raf1 was either deleted or over-expressed. Apart from increased average copy number, the variance was also observed to increase. (E) mRNA quantification by RT-qPCR: Quantification of the mRNA level of the three plasmid encoded genes (REP1, REP2 and FLP) showed more than 2-fold increase in the FLP mRNA level in raf1Δ as compared to the wild-type strain while no change in the mRNA level of REP1 or REP2 was observed.
vector of \( FLP \) and therefore it is expected that the removal of \( RAF1 \) should result in attenuated \( FLP \) activity and reduced PCN. To investigate whether the increase in PCN in \( raf1 \Delta \) strain is due to increased Flp activity, the expression levels of \( FLP \), \( REP1 \) and \( REP2 \) genes were measured by assaying the mRNA levels using RT-qPCR in the \( raf1 \Delta \) strain and were compared to the expression level in the wild-type strain (Figure 1E). \( FLP \) was found to be expressed at more than 2-fold higher level in the \( raf1 \Delta \) strain as compared to the wild-type strain, while no difference was observed for either \( REP1 \) or \( REP2 \). In an earlier study (16,27), Rep1or Rep2 over-expression was shown to reduce \( FLP \) mRNA level, however, a prolonged over-expression of Rep1 but not Rep2 caused only a marginal increase in the \( FLP \) mRNA level. This observation suggests that the effect of concentration of Rep1 on the \( FLP \) transcription is not linear, possibly due to the presence of a feedback transcriptional repression of \( REP1 \) itself by the Rep1–Rep2 complex (15,16,27). Moreover, the mRNA level was assayed by northern analysis while in this study we have used RT-qPCR which can detect much lesser variations in the mRNA level. This observation suggests that the effect of concentration of Rep1 on the \( FLP \) transcription is not linear, possibly due to the presence of a feedback transcriptional repression of \( REP1 \) itself by the Rep1–Rep2 complex (15,16,27). Moreover, the mRNA level was assayed by northern analysis while in this study we have used RT-qPCR which can detect much lesser variations in the mRNA level. The reason for a high expression of Flp and consequently a higher PCN in \( raf1 \Delta \) strain could be due to a change in stoichiometry of Rep1–Rep2 repressor complex. Intriguingly, as Rep1 is attenuated by a negative feedback loop by the Rep1–Rep2 complex which can be antagonized by Raf1, the regulatory circuit controlling the expression of the plasmid encoded genes is such that both the deletion and the over-expression of Raf1 has similar effect on the stoichiometry of the Rep1–Rep2 complex (see Figures 1C, D and 4A). Moreover, the increase in the PCN, with a concomitant decrease in the stability (Figure 1) in \( raf1 \Delta \) cells further supports the proposition that the reduced plasmid stability of the 2-\( \mu \)m derived plasmid is not an effect of reduced PCN in these cells, suggesting that Raf1 might have a direct effect on the Rep1–Rep2-mediated partitioning pathway. Since it was observed that the loss rate of the 2-\( \mu \)m derived plasmid increases due to both Raf1 over-expression and deletion (albeit to different extent) (Figure 1) and the PCN increases due to \( RAF1 \) deletion, we investigated if the copy number also shows the same trend as the plasmid instability. The PCN was therefore measured in a wild-type [Cir\(^+\)] cell with over-expressed Raf1 (see Supplementary Data for the description of PCN measurement) and was found that the average PCN also increases as it happens in the \( RAF1 \) deleted cells (Figure 1D). Thus the PCN follows the same trend as the plasmid loss rate and hence this result supports the hypothesis that both deletion and over-expression of Raf1 has identical effect on the plasmid maintenance. Interestingly, Figure 1D shows another intriguing effect of the perturbation of Raf1 on the copy number control mechanism. In both the cases (deletion and over-expression) while the PCN increases, an increase in the overall spread of the data points can also be clearly seen. This observation suggests that the copy number control mechanism loses its stringency and the PCN varies over a larger range of values. This increase in the noise could be due to the loss of a second layer of control that Raf1 provides in addition to the negative feedback control of \( REP1 \) expression by the Rep1–Rep2 repressor complex.

**RAF1 binds to the partitioning locus STB**

We found that the plasmids show an increased mother bias due to lack of Raf1 (Figure 1B). The most obvious reason for this increased mother bias can be attributed to a direct influence of Raf1 on the partitioning system similar to what has been reported earlier (28). However, whether this effect of Raf1 on the partitioning system is indirect due to a fluctuation in the PCN in \( raf1 \Delta \) was not addressed. We observed an increased plasmid loss rate when \( FLP \) was deleted along with \( RAF1 \) (Figure 1C) confirming a Flp (hence PCN) independent effect of Raf1 on the plasmid stability. To validate our hypothesis that Raf1 may be directly involved in partitioning, we investigated the association of Raf1 with the partitioning locus \( STB \) in \( vitr o \) using ChIP (Chromatin Immunoprecipitation) and monohybrid assays. Both the assays showed that Raf1 binds to the partitioning locus \( STB \) in a [Cir\(^+\)] strain but not in a [Cir\(^0\)] strain (Figure 2A–C) which is in contrast to the earlier finding that demonstrated the interaction of Raf1 with \( STB \) in \( vitr o \) in the absence of the Rep proteins (29). The result was further verified by rescuing the Raf1–STB interaction in [Cir\(^+\)] strain by providing 3HA fused Rep1 or Rep2 driven by an inducible \( GALI \) promoter. Flag tagged Raf1 was expressed in the same strain driven by the \( GALI0 \) promoter from a \( CEN \) plasmid, and \( STB \) was provided with an episomal plasmid (YPEp). ChIP was performed, and \( STB \) was pulled down by both anti-\( HA \) and anti-FLAG antibodies. In all the ChIP experiments Raf1 consistently showed pull down at \( STB \) when either Rep1 or Rep2 was present (Figure 2D). Since all the tagged proteins were driven by the \( GALI \) promoter, dextrose was taken as a negative control. To validate the ChIP assay data, monohybrid assay was performed (Figure 2B), \( STB \) was cloned upstream of the \( HIS3 \) reporter in the plasmid pHISi-1 (Clonetech). Raf1 fused to Gal4 activation domain (AD) was expressed through pGAD424 vector. Rep1 fused to AD was used as a positive control for the monohybrid experiment. All the monohybrid assays were done in [Cir\(^+\)] strains.

**RAF1 might not act as a transcription factor**

This study and other studies demonstrate that Raf1 affects both PCN and plasmid stability at the same time (28,29) see Figure 1). What could be the underlying mechanism through which Raf1 affects both the partitioning and the amplification systems together? From the studies it can be proposed that Raf1 does so by altering the activity of Flp and the Rep1–Rep2 complex. There are two hypotheses—Raf1 may alter the activity of the proteins by controlling the expression of the corresponding genes by occupying their promoters as a transcription factor or it may directly interact with these proteins to bring about the changes. To address the former hypothesis, we looked for the localization of Raf1 at the promoters (\( P_{REP1} \), \( P_{REP2} \), \( P_{FLP} \), \( P_{RAF1} \)) of the 2-\( \mu \)m encoded genes. The promoters were validated and their strength was measured by cloning a 200-bp upstream region of each of the four ORFs upstream of a LacZ reporter. The expression of the reporter was assayed qualitatively by a colony lift filter assay and quantitatively by liquid \( \beta \)-galactosidase assay. Under all the four
Raf1 binds to the partitioning locus STB but not to the promoters: (A) ChIP (chromatin immuno precipitation) assay demonstrating the localization of Raf1 at the partitioning locus STB. Both Rep1 and Rep2 were used as a positive control for the assay. (B) Monohybrid assay showing the binding of Raf1 to STB (C) ChIP assay showing the binding of Raf1 to STB in the presence (Cir +) and absence (Cir −) of the 2-µm plasmid. (D) Galactose inducible 3HA tagged Rep1 or 3HA tagged Rep2 were integrated in the chromosome by pCM26 or pCM2, respectively. Similarly, 3XFLAG tagged Raf1 driven by a galactose inducible promoter was expressed by the plasmid pSG2028. WCE: whole cell extract, +Ab: antibodies added, −Ab: antibodies not added, Gal: galactose, Dex: dextrose, αHA: anti-HA antibodies, αFLAG: anti-FLAG antibodies, downward arrow depicts pull down. (E) All the four promoters (PREP1, PREP2, PFLP and PRaF1) were immuno-precipitated using anti-FLAG antibodies against the FLAG tagged Raf1. Raf1 showed no pull down at any of the promoters. STB was used as the positive control. The occupancy of the HA fused Rep proteins at the promoters was also verified by ChIP using anti-HA antibodies. All the promoters along with STB showed IP with the Rep proteins. While Rep1 showed equal pull down at all the promoters and at STB, Rep2 showed less pull down at STB as compared to the promoters. A linear form of the 2-µm circle is represented at the bottom with the sequence of the amplicon targeted for the qPCR. The values for the enrichment per input is one order higher for Rep2 as compared to that of Rep1; the scale is, therefore, different for the 3HA-Rep1 and 3HA-Rep2 ChIP results. The comparison among different targets (PREP1, PREP2, PFLP, PRaF1 and STB) is performed from the same ChIP experiment.
promoters, LacZ expression was observed, albeit with different levels of expression (Supplementary Figure S2) indicating that the regions of the plasmid cloned upstream of the LacZ reporter can indeed fire LacZ expression and hence contain promoter activity. However, we found that Raf1 does not bind to any of the promoters (Figure 2E, top panel). This suggests that Raf1 might not be involved in the regulation of the activities of the 2-μm proteins by occupying their gene promoters. The Rep proteins however, showed pull down at all the four promoters. This observation is in agreement with the model proposed in the earlier studies (15,16,27) that suggest the binding of the Rep1–Rep2 complex at the promoters of the 2-μm plasmid encoded genes. Surprisingly, Rep2 showed at least 10-fold more enrichment per input at all the promoters and STB as compared to Rep1. It is difficult to infer if this difference in the pull down efficiency is physiologically relevant, since all ChIP assays were performed with overexpressed Rep1 and Rep2. Nevertheless, assuming an equal level of expression of both Rep1 and Rep2, it can be said that under identical conditions, Rep2 has a higher affinity for the promoters and STB. This is consistent with the fact that Rep2 is predominantly a nuclear protein and is sufficient in single copy to maintain the plasmid stability (26). Furthermore it has previously been demonstrated by in vitro assays that Rep2 has a higher affinity towards STB (29).

**Raf1 physically interacts with the Rep proteins**

Earlier studies (15,16) and the data presented above support the notion that Raf1 can function as an antagonist to Rep1–Rep2 repressor complex and can alter the Flp activity (Supplementary Figure S6 and Figure 1E, respectively). Since in the above section we failed to observe the interaction of Raf1 with any of the promoters of the plasmid borne ORFs, we hypothesize that Raf1 does its function through physically interacting with Rep1, Rep2 or Flp. We tested this hypothesis by performing yeast two-hybrid assay (37) and found that Rep1 and Rep2 interact with Raf1 independently in both the [Cir°] (Supplementary Figure S1) and [Cir°] strains, however, no such interaction was observed between Raf1 and Flp (Supplementary Figure S6) or between Raf1 itself (Supplementary Figure S1). These results suggest that the observed effect of Raf1 on Rep1–Rep2 complex may be due to a physical blockage of the formation of this complex and the effect of Raf1 on Flp may be mediated indirectly via Rep1–Rep2 complex. Although this result supports the hypothesis that Raf1 acts as an anti-repressor by blocking the Rep1–Rep2 interaction, more direct evidence was required to establish this hypothesis. A competitive two-hybrid assay and BiFC assay (Figure 3) was performed to validate this hypothesis.

**Raf1 blocks the formation of the Rep1–Rep2 repressor complex**

From above and the previous studies it can be gleaned that Raf1 blocks the formation of the Rep1–Rep2 repressor complex through physical interactions. We wished to demonstrate the blockage of Rep1–Rep2 by Raf1 with a more direct experiment. We hypothesized that if Raf1 indeed blocks the Rep1–Rep2 interaction, its overexpression should result in a reduced Rep1–Rep2 interaction leading to the reduced expression of the HIS3 reporter in a yeast two-hybrid assay using AD-Rep1 and BD-Rep2. To test this hypothesis three strains were constructed to perform competitive two-hybrid assay—(i) AD-fused Rep1 and BD-fused Rep2 with Cu(II) inducible 3XFLAG-Raf1, (ii) AD-fused Rep1 and BD-fused Rep2 with galactose-inducible 3XFLAG-Raf1 and (iii) AD-fused Rep1 and pGBD vector (negative control) with Cu(II) inducible 3XFLAG-Raf1. The over-expression of 3XFLAG-Raf1 upon induction with CuSO4 has been verified in the first and third strains (Figure 3B). It was observed that in the presence of Cu(II) (100 and 200 μM CuSO4) the interaction of Rep1 and Rep2 was slightly but reproducibly reduced due to the over-expression of Raf1 (see the first row in each panel of Figure 3A) but no effect on the interaction of Rep1 and Rep2 was observed in the absence of Raf1 (see the second row in each panel of Figure 3A). No growth was observed in the negative control irrespective of Raf1 over-expression (see the last row in each panel of Figure 3A). However, a similar assay using monohybrid strain harboring either AD-Rep1 or AD-Rep2 shows that Raf1 does not block the interaction of the Rep proteins with the STB (Supplementary Figure S3). These results indicate that Raf1 attenuates Rep1–Rep2 complex through protein-protein interactions. However, Raf1 cannot negate Rep1–STB or Rep2–STB interactions suggestive of different binding surfaces of these proteins are involved in protein–protein or DNA–protein interactions. To further confirm our hypothesis that Raf1 physically blocks Rep1–Rep2 interaction, we performed a BiFC (Bimolecular Fluorescent Complementation) assay used to study the physical interaction between two proteins (38). For this, the interaction between VN173-Rep1 and VC155-Rep2 was investigated in the presence of either Raf1 or one of the un-tagged Rep1 or Rep2 (driven by GAL promoter) as competing partner. It was expected that 3XFLAG-Raf1, 3XHA-Rep1 or 3XHA-Rep2 would compete for interaction with VN173-Rep1 and VC155-Rep2 and will lead to depleted fluorescence. The strains for the BiFC assay was constructed as explained in the Supplementary Table S2. While VN173-Rep1 was driven by the leaky CUP1 promoter, VC155-Rep2 or VC155-Flp was driven by the inducible GAL1 promoter. BiFC was observed between Rep1 and Rep2 but not between Rep1 and Flp (Figure 3C, panels I and II and Supplementary Figure S4) thus confirming that the signal observed due to Rep1–Rep2 interaction is a true signal and not an artifact. Importantly, the BiFC signal was found to be both nuclear and as a localized dot within the nucleus. The dot-like signal segregated between the mother and the bud during cell division in a fashion similar to the chromosome segregation suggesting that the localized BiFC designates the STB bound Rep1–Rep2 complex (Supplementary Figure S4). The strains showing BiFC were then transformed with integrative plasmids harboring galactose-inducible Raf1 or Rep1 or Rep2 to study the effect of the over-expression of these proteins on the Rep1–Rep2 interaction (see Supplementary Data for strain construction). The effect of over-expression on the BiFC signal was quantified using FACS analysis as the change in the signal intensity after induction with galactose. Strain (SGY2058) not containing the YFP (or BiFC) fragments (see Supple-
Figure 3. Raf1 blocks the formation of the Rep1–Rep2 repressor complex: (A) Competitive two-hybrid assay demonstrates that Raf1 blocks the formation of the Rep1–Rep2 repressor complex. Two-hybrid strains bearing AD-fused Rep1 and BD-fused Rep2, or AD-fused Rep1 and empty pGBD vector were transformed with 3XFLAG tagged Raf1 driven by CUP1 promoter (first and third row in each block). A two-hybrid strain bearing AD-fused Rep1 and BD-fused Rep2 with galactose inducible Raf1 (second row in each block) was used as a positive control since no over-expression of Raf1 was observed due to CuSO4 induction. A slight decrease in the HIS3 expression was observed in the 200 μM CuSO4 concentration (first row in the third block). (B) The overexpression of Raf1 was confirmed by western blot in all the three strains used for the competitive two-hybrid experiment. (C) FACS analysis of the BiFC after 4 h of galactose induction shows a reduction in the shift of the fluorescence when Raf1 is over-expressed (panel III) compared to panels II, IV and V where Rep1–Rep2 interaction was not challenged by Raf1 over-expression. (D) For each time point the shift in the peak was measured and was plotted against the duration of induction by galactose. Raf1 over-expression clearly shows a reduced fluorescence. The promoters upstream of the individual cassettes are shown by double arrows in C and D.
DISCUSSION

The maintenance of the endogenous 2-µm plasmid in high copies in the nucleus of various *Saccharomyces* strains is believed to be a function of the interplay between the partitioning and the amplification systems borne by the plasmid. A genetic circuit that regulates the expression of the plasmid-encoded genes is at the centre of this interplay. From the earlier experimental evidence, it appears that Rep1–Rep2 complex plays a pivotal role in controlling this partitioning and copy number maintenance. In this study we provide biochemical evidence that Rep1–Rep2 complex indeed binds to the 2-µm gene promoters to regulate the expression. However, we cannot conclude whether this binding is direct or indirect through other mediators. Biochemical assay using purified Rep proteins and the DNA targets might reveal this. Importantly, we have characterized the Raf1 function and provided evidence that this protein can physically associate and negate the function of Rep1–Rep2 complex. This way Raf1 can serve as a fine tuner between the partitioning and the amplification systems. This work reveals another layer of molecular mechanism to explain the remarkable stability of the 2-µm plasmids at a high copy number.

**Raf1 plays a role in both the partitioning and the amplification of the 2-µm plasmid and promotes faithful plasmid segregation**

Raf1 has been considered a minor player in the entire scheme of the 2-µm plasmid maintenance mechanism and its role in the stable maintenance of the 2-µm plasmid has been previously demonstrated (28) but with little mechanistic details. In this study, we verified the effects of both RAF1 deletion and over-expression on both plasmid segregation and copy number. Deletion of Raf1 caused increased plasmid loss rate of the 2-µm derived plasmid but so did the Raf1 over-expression (Figure 1). The increased plasmid loss rate due to deletion was less severe than the over-expression. Since the plasmid stability is directly influenced by the Rep1–Rep2 complex and Raf1 is known to affect the plasmid maintenance only when both Rep1 and Rep2 are present (15), we speculated that the increased loss rate in both the deletion and over-expression strains is due to a decrease in the concentration of the Rep1–Rep2 complex. Following the same rationale, we speculated that the PCN should show a similar trend as the plasmid loss rate since the former is directly influenced by the FLP activity which is attenuated by the Rep1–Rep2 complex (16). As expected, the PCN followed the same trend as the plasmid loss rate (Figure 1D) along with an increase in the *FLP* mRNA (Figure 1E). Moreover, the PCN showed more variation among different samples when Raf1 was either over-expressed or deleted, suggesting that not only there is a decrease in the *FLP* attenuation, the control on the expression of *FLP* is also weakened. This increase in the noise might have resulted from the loss of the fine control that Raf1 provides on PCN under its native expression regime. As expected, the deletion of *FLP* resulted in a slight increase in the plasmid loss rate (Figure 1C), possibly due to the reduced PCN (Figure 1D). However, the plasmid loss rate in *RAF1* deleted strain was found significantly higher than the strain where *FLP* was deleted (Figure 1C, *P* = 0.04) and remained more or less same in the strain where both *RAF1* and *FLP* were deleted (Figure 1C, *P* = 0.14). These results suggest that the plasmid loss rate is primarily affected by the *RAF1* deletion, and *FLP* deletion has little effect on the plasmid stability.

**Raf1 binds to STB**

Using ChIP assay, we have demonstrated in *vivo* Raf1–STB interaction in the presence of the Rep proteins but not in their absence (Figure 2A–C) and that Raf1–STB interaction can be re-established in a [Cir*] cell if either Rep1 or Rep2 is provided in *trans* (Figure 2D). The interaction of Raf1 with STB, independent of any other plasmid-encoded protein, has previously been demonstrated in *vivo* by Surface Plasmon Resonance (SPR) study, but a similar result was not achieved with the gel retardation assay (29). More contradicting results were obtained such as Raf1 was not identified in a mono-hybrid library screening using STB as the bait but was identified through candidate approach where, Raf1–STB interaction was detected in a monohybrid strain harboring transcriptional AD-Raf1 fusion protein (22). It was also observed that at a low Raf1 level Raf1–STB interaction was lost (29). These observations suggest a transient interaction between Raf1 and STB. In this study, we could not detect Raf1–STB interaction independent of the Rep proteins (Figure 2), neither did our results produce higher enrichment per input at *STB* with Raf1 compared to the Rep proteins (Figure 2E), which is contradictory to the earlier findings using SPR where Raf1–STB interaction was shown to be independent of the Rep proteins, and a stronger
interaction was observed between Raf1 and STB as compared to both Rep1–STB and Rep2–STB interactions (29). These contradictions between earlier findings and our observation may arise due to the transient but rapid Raf1–STB interaction in the absence of the Rep proteins which could be detected by SPR but not by other assays (ChiP and gel retardation) where a more stable interaction is captured. We suspect, that under native conditions the Raf1–STB interaction is stabilized by the Rep proteins through a stable interaction of Raf1 with the Rep proteins (Supplementary Figure S6).

**Rep1 and Rep2 but not Raf1 bind to the 2-μm gene promoters**

The model for the transcriptional control circuit of the 2-μm plasmid maintenance proposes the binding of the Rep1–Rep2 complex to the promoters of the plasmid-encoded ORFs (summarized in (22)). However, it was only speculated that there are promoters immediately upstream of the four 2-μm ORFs that can be occupied by the Rep1–Rep2 complex. In this study, we cloned a 200 bp upstream region of the four ORFs upstream of the LacZ reporter to demonstrate that these fragments can indeed possess promoter activity as they drive the expression of LacZ (Supplementary Figure S2). The quantitative estimation of β-galactosidase specific activity revealed the relative strengths of these promoters, with RAF1 promoter being the weakest. ChiP assay was performed confirming that both Rep1 and Rep2 bind at the Rep1, FLP and RAF1 promoters as per the hypothesis (Figure 2E, (22)). To be noted, a positive and consistent binding was also observed at the Rep2 promoter. Since Rep2 is believed to not undergo any transcriptional control by the Rep1–Rep2 complex (15) and therefore binding by either Rep1 or Rep2 at the Rep2 promoter is unlikely, although a binding at the promoter may not necessarily warrant for a transcriptional repression. Moreover, STB and 5′ regions of FLP and REP2 share homology with a consensus sequence (TC(T rich)13,15 ATCTTG) suggesting that REP2 promoter may contain a potential target sequence for the Rep1 and Rep2 interaction. It should be noted that the presence of this consensus sequence varies in number among the 5′REP2 (one repeat between −10 to +20 of REP2 ORF), the 5′FLP (three repeats within −90 to +10 of FLP ORF) and the STB (three repeats within the PsI and SnaBI fragment) regions (39). We speculate that the binding of the Rep proteins at the Rep2 promoter is possible but with lesser stringency due to the presence of only one repeat. On the other hand, ChiP with Raf1, as expected, did not show enrichment at any region of the 2-μm plasmid chromatin except for the STB (Figure 2E). This is in agreement with the earlier studies (29) and the observation that deletion of RAF1 alone can lead to plasmid instability (Figure 1). However, it is paradoxical that while Raf1 binds to the STB through the Rep proteins, it does not do so at the promoters. While this may imply that there are two distinct kinds of the Rep1–Rep2 complexes (one at the STB and other at the promoters), no other observation from earlier studies or from this study obviates such an assumption. On the contrary it is well known that the chromatin architecture of STB is distinct from the rest of the plasmid (19,20,40,41). Therefore, it is possible that the interaction and dynamics of the Rep1–Rep2–STB and Rep1–Rep2–PFp may be affected not only by the sequence of the DNA target loci but also by the chromatin architecture of these loci. In that case presence of Rep proteins may not be the only determinant for Raf1 binding. Importantly, in a previous study a substantial changes in the chromatin structure was demonstrated at the 5′ regions of FLP and REP1 ORFs and at STB in cells devoid of Rep1 or Rep2 or Raf1 (42). Therefore, we believe that our observation of Rep proteins binding to these regions and at REP2 and RAF1 promoters might bring about the biological functions through

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**Figure 4.** The 2-μm regulatory network: the hypothesized model to explain how Raf1 might affect both the segregation and the copy number of the 2-μm plasmid. (A) The proposed model for the 2-μm control circuit. Rep1–Rep2 repressor complex connects a negative feedback loop (number 1) and a positive feedback loop (number 2) to maintain the stability and PCN through changes in the cellular concentration of Rep1–Rep2 complex. (B) The negative feedback loop (number 1) in the absence of Raf1 (Z) controls the intracellular concentration of the Rep1–Rep2 repressor complex (Y) with a delay due to intermediate processes like transcription, translation and post translation modification of Rep1 (X). (C) The repressor-amplified negative feedback motif formed due to the presence of Raf1 (Z) and hence the addition of a positive feedback loop (number 2) in tandem with the delayed negative feedback loop causes an upward shift in the intracellular concentration of the Rep1–Rep2 repressor complex (Y). (D) The decrease in the intra-cellular concentration of the Rep1–Rep2 repressor complex due to absence of Raf1 results in a decreased plasmid stability and an increased Flp concentration due to de-repression of FLP and hence increased PCN as shown by arrows.
making crucial changes in the chromatin structure of these regions.

**Raf1 blocks the formation of Rep1–Rep2 repressor complex**

The central component of the network that ensures stable high copy maintenance of the 2-µm plasmid is the Rep1–Rep2 repressor complex (Figure 2E). It has been proposed that Raf1 controls Flp activity either by blocking the interaction of the Rep1–Rep2 repressor complex with the promoter of *FLP* (by occupying the promoter and hence blocking the accessibility of the Rep1–Rep2 complex to the promoter) or by blocking the physical interactions among the Rep proteins themselves to form the Rep1–Rep2 complex so that the complex cannot bind to the *FLP* promoter as a repressor (16); however, no direct evidence was available to support either of this hypothesis. Our data suggests that the former hypothesis might not be true since Raf1 does not occupy the promoter (Figure 2E). We provide evidence in support of the latter hypothesis by showing that Raf1 directly hinders Rep1–Rep2 interactions (Figure 3) perhaps by physically associating with these proteins (Supplementary Figure S6). However, we found that Raf1 cannot restrict association of Rep1 or Rep2 with *STB* (Figure 2D and Supplementary Figure S3) when the Rep proteins were overexpressed along with Raf1. In an alternate experiment where the Rep proteins were driven by their native promoters, quantitative estimation demonstrated that the occupancy of the Rep proteins at *STB* was reduced (in case of Rep2) or completely abrogated (in case of Rep1) due to the overexpression of Raf1 (Supplementary Figure S7). It should be noted that in this experiment the Rep proteins were expressed from a single copy integrated form of the 2-µm plasmid and therefore resulted in very low dosage. The result should be interpreted cautiously, especially for Rep1 which is known to be insufficient for plasmid maintenance at low dosage (27).

Both the Rep proteins have an N-terminal oligomerization domain and a C-terminal DNA binding domain. The oligomerization domain is required for association of Rep1 or Rep2 with themselves (Rep1–Rep1/Rep2–Rep2) and the formation of the Rep1–Rep2 complex (43). From our results we propose that Raf1 interacts with the N-terminal oligomerization domain of both Rep1 and Rep2 leading to a blockage of oligomerization site and the formation of the Rep1–Rep2 complex. Whereas, the Rep proteins can still bind to the *STB* through their C-terminal DNA binding domain. It has been demonstrated that the overexpression of Raf1 leads to an increased *FLP* expression when both Rep1 and Rep2 are present, but there is no effect on the expression of *FLP* when either of them is absent (16). Our data that Raf1 blocks the formation of the Rep1–Rep2 repressor complex also explain the dependence of Flp activation function of Raf1 on the presence of the Rep proteins. These results support the proposition that Raf1 blocks the formation of Rep1–Rep2 complex and hence the repression of *FLP*. An obvious prediction of this model is that the PCN should decrease in a *raf1Δ* strain as Flp is de-repressed. However, a higher PCN was observed which apparently contradicts this paradigm (Figure 1D). This increase in PCN is unlikely due to plasmid missegregation as shown in *raf1Δ* strain (Figure 1). This is because all the copy number assay experiments were performed in non-selective (YPD) growth conditions and therefore, any increase in the copy number is not due to the selection of plasmid bearing cells in the population or due to the effect of plasmid missegregation. Removal of *FLP* caused highest drop in PCN (Figure 1D) as expected; however, removal of *RAF1* in *flpΔ* strain caused an increase in PCN albeit below the steady state level observed in the wild-type strain. Since Raf1 itself has no amplification function and in the absence of Flp the amplification system is completely defunct, it is difficult to explain this higher steady state copy number as any indirect activation of the amplification system.

Based on the above observations we propose a working model (Figure 4) for the transcriptional regulation and the resulting observable effect on the plasmid stability and PCN. According to the model, the Rep1–Rep2 complex is central to the two pathways that control both the plasmid stability and the PCN. The observed effect of the removal of *RAF1* on the stability and PCN might result from a decreased concentration of active Rep1–Rep2 complex. Interestingly, a similar phenotype is expected if Raf1 is overexpressed (Figure 1C and D) indicating a balanced Raf1 dosage is essential for maintaining a certain level of Rep1–Rep2 concentration optimized to provide steady state PCN. We argue that the feedback loops operating among Rep1, Rep2 and Raf1 as discussed below are crucial to understand how plasmids are stably maintained at a steady state level of copy number.

**A Rep1–Rep2 repressor amplified negative-feedback motif may create a stable control system**

Oscillatory networks are common in the living systems. They lie at the core of the biological rhythms and are involved in synchronization of the myriad cellular processes. Sustained oscillations of the networks can be achieved through a variety of regulatory motifs ranging from simple two-component time-delayed negative feedback loops to complex multi-component hysteresis oscillators constructed by interlinked negative and positive feedback loops (30). We analyzed our results from this perspective to envisage a systems level explanation for rhythmic events such as the steady state PCN maintenance through the synchronization of Flp-mediated copy number amplification with the cyclic cell cycle dependent efficient plasmid segregation process (44). We propose that these cyclic and synchronous events are a result of a multi-component oscillatory network formed by two feedback loops. The regulatory network that maintains a stable steady state PCN results from the two feedback loops (1 and 2) shown in Figure 4A and C and is reminiscent to a repressor amplified negative-feedback motif discussed elsewhere (33). In this network, the two feedback loops are connected through the Rep1–Rep2 repressor complex. The first feed-back loop (numbered 1) can be viewed as a negative-feedback loop where the product (repressor complex Rep1–Rep2) represses its own expression. Since *REP2* is shown to be unaffected by the repressor complex (27) and hence its dosage does not affect the control network, it can be reasonably assumed to be expressed constitutively and a non-limiting factor for the con-
trol network. Under this assumption, the cellular concentration of the Rep1–Rep2 complex will be affected only by the fluctuations in the cellular concentration of Rep1. Consequently, the first negative-feedback loop can be perceived as a Rep1 negative-feedback where Rep1 represses its own expression. The first loop can also be seen as a negative-feedback loop with a time delay by a series of intermediate processes such as time delay due to the intermediate processes of transcription-translation-SUMOylation of Rep and Flp proteins-transport into the nucleus etc. (45–47). This time delay in feedback gives rise to an oscillatory response and causes the repressor (Rep1–Rep2) to oscillate around a steady state level (30,33). In the absence of the positive feedback loop when Raf1 is absent (Figure 4B and D), the oscillations maintain a certain average steady state concentration of the repressor as operated by loop number 1 alone and the concentration is believed to be, as discussed below, less than what is observed in the wild-type. However, introduction of a positive feedback loop by Raf1 (number 2), as in the wild-type shifts the average steady state concentration of the repressor. The new oscillatory response formed due to the introduction of Raf1 is similar to a three component repressor amplified negative-feedback loop (33) and Figure 4C where the repressor (Rep1–Rep2 complex) represses the transcription of both Rep1 and Raf1. The Rep1–Rep2/Raf1 positive feedback loop pushes the Rep1–Rep2 complex away from the steady state, causing an upward shift in the average steady state concentration of the repressor due to a positive feedback from Raf1. Thus, this two-looped system approach explains the decreased stability and an increased PCN (and increased FLP mRNA) in the raf1Δ cells perhaps due to an overall decrease in the average steady state concentration of the Rep1–Rep2 repressor. It is important to investigate whether under native condition in the raf1Δ cells the steady state level of Rep1–Rep2 is indeed lower than the wild-type. Efforts to test this by expressing the VN173-Rep1 and VC155-Rep2 under their native promoters failed repeatedly due to several technical problems. VN173-Rep1 fusion in the native plasmid could not be achieved due to gross destabilization of the plasmid. Transformation of a wild-type [Cir+] strain or a VC155-Rep2 circle plasmid could not be achieved due to gross destabilization. However, introduction of a positive feedback loop by Raf1 (number 2), as in the wild-type shifts the average steady state concentration of the repressor. The new oscillatory response formed due to the introduction of Raf1 is similar to a three component repressor amplified negative-feedback loop (33) and Figure 4C where the repressor (Rep1–Rep2 complex) represses the transcription of both Rep1 and Raf1. The Rep1–Rep2 (Rep1–Rep2 complex) represses the transcription of both Rep1 and Raf1. The Rep1–Rep2/Raf1 positive feedback loop pushes the Rep1–Rep2 complex away from the steady state, causing an upward shift in the average steady state concentration of the repressor due to a positive feedback from Raf1. Thus, this two-looped system approach explains the decreased stability and an increased PCN (and increased FLP mRNA) in the raf1Δ cells perhaps due to an overall decrease in the average steady state concentration of the Rep1–Rep2 repressor. It is important to investigate whether under native condition in the raf1Δ cells the steady state level of Rep1–Rep2 is indeed lower than the wild-type. Efforts to test this by expressing the VN173-Rep1 and VC155-Rep2 under their native promoters failed repeatedly due to several technical problems. VN173-Rep1 fusion in the native plasmid could not be achieved due to gross destabilization of the plasmid. Transformation of a wild-type [Cir+] strain or a VC155-Rep2 [Cir+] strain (where all the native plasmids harbored VC155-Rep2) with VN173-Rep1 tagging cassette yielded sick colonies on the selective plates after transformation. Alternatively, Rep1 and Rep2 expressed from a chromosomally integrated version of the 2-µm plasmid (pFV14) were fused with VN173 and VC155, respectively at their N-termini. However, in such cells BiFC between VN173-Rep1 and VC155-Rep2 driven by their native promoters was not observed. We suspect that this lack of BiFC was due to a very low concentration of the intracellular VN173-Rep1–VC155-Rep2 complex as they were expressed from single copy genes. Nevertheless, this approach provides a theoretical platform to explain the rhythmic changes in the components of the 2-µm system synchronized with the cell cycle events. The oscillations in Rep1–Rep2 complex can be fine-tuned to match the cell cycle events by changing the kinetic parameters of the control system. Further investigation measuring the variations in the stoichiometry of Rep1–Rep2 complex in the presence and absence of RAF1 and coupling that information with a detailed modeling and analysis of the control network can help in better understanding the overall dynamics of the 2-µm plasmid maintenance mechanism.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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