Published online 26 September 2022 *Nucleic Acids Research, 2022, Vol. 50, No. 18* ***10571–10585***
https://doi.org/10.1093/nar/gkac810

The yeast 2-micron plasmid Rep2 protein has Rep1-independent partitioning function

Anastasiia Mereshchuk, Peter S. Johnstone, Joyce S.K. Chew and Melanie J. Dobson*

Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, Nova Scotia B3H 4R2, Canada

Received April 25, 2022; Revised August 18, 2022; Editorial Decision September 07, 2022; Accepted September 08, 2022

ABSTRACT

Equal partitioning of the multi-copy 2-micron plasmid of the budding yeast *Saccharomyces cerevisiae* requires association of the plasmid Rep1 and Rep2 proteins with the plasmid *STB* partitioning locus. Determining how the Rep proteins contribute has been complicated by interactions between the components. Here, each Rep protein was expressed fused to the DNA-binding domain of the bacterial repressor protein LexA in yeast harboring a replication-competent plasmid that had LexA-binding sites but lacked *STB*. Plasmid transmission to daughter cells was increased only by Rep2 fusion expression. Neither Rep1 nor a functional RSC2 complex (a chromatin remodeler required for 2-micron plasmid partitioning) were needed for the improvement. Deletion analysis showed the carboxy-terminal 65 residues of Rep2 were required and sufficient for this Rep1-independent inheritance. Mutation of a conserved basic motif in this domain impaired Rep1-independent and Rep protein/*STB*-dependent plasmid partitioning. Our findings suggest Rep2, which requires Rep1 and the RSC2 complex for functional association with *STB*, directly participates in 2-micron plasmid partitioning by linking the plasmid to a host component that is efficiently partitioned during cell division. Further investigation is needed to reveal the host factor targeted by Rep2 that contributes to the survival of these plasmids in their budding yeast hosts.

INTRODUCTION

In the budding yeast *Saccharomyces cerevisiae*, autonomously replicating (*ARS*) plasmids lacking a partitioning element are preferentially retained in the mother nucleus during cell division (1). This maternal bias arises from the constricted nature of the bud neck which limits passive diffusion of the plasmids from the mother to daughter during the relatively brief duration of yeast mitosis (2). This bias benefits daughter cells by limiting inheritance of extrachromosomal ribosomal DNA circles and other factors that accumulate in aging mother cells, contributing to loss of reproductive capacity (2,3).

Previous studies have shown that daughter cell inheritance of *ARS* plasmids can be increased by multiple mechanisms. Conditions that lengthen G2/M allow more time for the plasmids to diffuse through the bud neck constriction (2). The presence of telomeric sequences and silencer elements confers partitioning (4,5). For these, improved inheritance depends on recruitment of the Sir4 protein (Silent Information Regulator 4) to those sequences (6,7), and the ability of the Sir4 partitioning and anchoring domain (Sir4PAD) to act as a natural tether, linking the plasmids to the nuclear envelope, or to telomeres by interacting with proteins at those sites (2,7–10). Transmission to daughter cells was also increased by artificially tethering *ARS* plasmids directly to nuclear components, such as the nuclear pore (11), or nuclear membrane (2), that are segregated, albeit unequally, at cell division (9,12). More effective is the inclusion of a centromeric (*CEN*) sequence, which enables *ARS* plasmid capture and equal partitioning by the spindle apparatus, just as it does for chromosomes (13). Spindle-mediated segregation was also achieved by tethering Ask1, a component of the kinetochore microtubule-binding Dam1 complex (11,14,15), to an acentric *ARS*-plasmid, or to a chromosome with an inactivated *CEN,* although in both cases, partitioning was quantitatively not as efficient as that provided by a natural centromere (15).

The 2μm plasmid, one member of a family of multi-copy, circular double-stranded DNA plasmids found only in budding yeast (16–18), also overcomes the maternal bias in inheritance (1). The plasmid achieves this by having a mechanism that partitions plasmid copies equally (or nearly equally) between the mother and daughter nuclei at cell division, and a system for correcting plasmid copy number in rare cases of plasmid mis-segregation (19–21). Together, these give the 2μm plasmid an almost chromosome-level fidelity of inheritance (22).

Equal partitioning of the plasmid requires association of the Rep1 and Rep2 proteins with a repeated sequence at the plasmid *STB* stability locus (23–25). The Rep proteins have been shown to form homo- and hetero-complexes *in*

*To whom correspondence should be addressed. Tel: +1 902 494 7182; Fax: +1 902 494 1355; Email: dobson@dal.ca

vitro and *in vivo* (26–28), and to co-localize with the plasmid copies which are observed to cluster in a small number of foci in the nucleus (27,29). The nature of the complex the Rep proteins form at *STB* is unclear. The addition of *STB* to an *ARS* plasmid is sufficient to promote stable high-copy inheritance provided the host cells contain native 2μm plasmid (termed [cir^+]) to supply the Rep proteins in *trans* (23,24). This feature has been exploited for the development of many widely-used yeast expression and cloning vectors (30). In a strain lacking the 2μm plasmid (termed [cir^0]), or one where either Rep1 or Rep2 are not expressed, an *ARS* plasmid containing *STB* behaves like one lacking *STB* or *CEN* and displays a strong maternal bias in inheritance with few daughter cells receiving plasmid copies (1).

Considerable circumstantial evidence suggests the complex of Rep proteins at *STB* overcomes maternal bias by physically linking plasmid copies to host chromosomes in a way that ensures the plasmids will be equally partitioned when the chromosomes segregate during host cell division (20,29,31–35). Consistent with this model, conditions that destabilize the spindle apparatus or impair chromosome segregation also disrupt 2μm plasmid inheritance (29,33). Further support comes from the observed co-localization of plasmid foci with nuclear chromatin (31,32), and with meiotic (36) and mitotic (37) chromosomes. Whether these associations represent direct linkage of plasmids to the chromosomes has yet to be determined.

In addition to their role in plasmid partitioning, the Rep proteins control plasmid copy number by negatively regulating expression of the plasmid genes (38–40). Increases in 2μm plasmid copy number are triggered when Flp, a site-specific recombinase encoded by the plasmid, catalyzes recombination between its target sites in two inverted repeats on the plasmid during replication. This inverts one of the two bi-directionally-oriented replication forks, converting copying to a rolling-circle mode which produces a multimeric copy of the plasmid from a single replication initiation event (41,42). The multimer is subsequently resolved into multiple monomeric copies by Flp-mediated or homologous recombination. At normal plasmid copy number, cellular Rep protein levels are high enough to ensure repression of the *FLP* gene, preventing any further Flp-mediated increase in plasmid copy number (38,39,42). Raf, the fourth protein encoded by the 2μm plasmid, can alleviate Rep protein-mediated repression of the plasmid genes by competing with Rep2 for Rep1 association (38,43,44).

Although the Rep proteins, likely in the form of a Rep1/Rep2 heterodimer (43), target the two divergent promoter regions on the 2μm plasmid to repress transcription (38,39,43), it is only their association with *STB* that confers plasmid partitioning function (23,24,45). This difference may reflect the number and arrangement of Rep protein target sites offered by the *STB* repeats and the recruitment of host factors to *STB*, many of which are known to function in chromosome segregation. Among these, the RSC2 (Remodels Structure of Chromatin) complex seems to play an early and critical step in establishing a functional Rep protein partitioning complex at *STB* (46,47). The presence of a functional RSC2 complex and both Rep proteins at *STB* are prerequisites for recruitment of other host factors needed for the 2μm plasmid to be efficiently partitioned. These include the centromere-specific histone H3, Cse4 (48), a kinesin family nuclear motor protein Kip1 (49), and two ring-shaped SMC (Structural Maintenance of Chromosomes) complexes, cohesin (32,47) and condensin (37). Cohesin and condensin both play critical roles in chromosome segregation and genome organization mediating inter- and intra-chromosomal linkages by topologically entrapping DNA strands (50–53). Studies of a single-copy 2μm reporter plasmid indicate the RSC2 complex and Rep protein-dependent recruitment of cohesin to *STB* maintains cohesion between newly-replicated sister plasmids until anaphase, when their separation coincides with that of the segregating chromosomes, contributing to equal partitioning of the plasmid copies (32,47,54,55). Mis-segregation of a 2μm plasmid reporter plasmid when condensin was inactivated suggests condensin recruited to *STB* also contributes to 2μm plasmid inheritance, possibly by compacting the clusters of replicated sister plasmids into formations that favour symmetric attachment to sister chromatids or by directly bridging interaction with the chromosomes (37).

Other host proteins found at *STB* include two microtubule-associated proteins, Bik1 and Bim1 (56), which share an overlapping essential role in chromosome segregation (57,58). Bim1 recruits Bik1 to the Dam1 microtubule-binding complex of the outer kinetochore complex formed at centromeres, inducing Dam1 complex oligomerization into rings that enable kinetochore capture by the microtubule plus-end (57). In cells lacking Bik1, Kip1 was no longer recruited to *STB*, and in the absence of either Bik1 or Bim1, a single-copy 2μm reporter plasmid showed increased mis-segregation, and decreased recruitment of cohesin to *STB,* supporting both proteins contributing to the function of the plasmid partitioning complex (56). However, there is no evidence to suggest a kinetochore complex is assembled at *STB* (33).

Despite progress in identifying *STB*-interacting host proteins that contribute to 2μm plasmid partitioning, the role of the Rep proteins in this process has yet to be established. Both Rep proteins seem to be required for recruitment of critical host proteins to *STB,* but the dependence of Rep1 on interaction with Rep2 for post-translational stability (43,59) raises the possibility that the major role of Rep2 might be to protect Rep1 from turnover, and possibly to promote Rep1 nuclear localization or retention (27,35). Consistent with Rep1 being the key determinant of *STB* recognition, Rep1 is efficiently targeted to *STB* in the absence of Rep2, while Rep2 association with *STB* is reduced in the absence of Rep1 (59). Whether Rep1 directly recognizes the *STB* repeat DNA is unknown. Rep1 has not been shown to display DNA-binding activity *in vitro* in the absence of host proteins (60). In contrast, Rep2 was found to bind DNA using a southwestern assay, showing a preference for the *STB* repeat sequence, which could indicate sequence specific binding but could also represent a preference for AT-rich DNA (61). It is currently unclear how this activity contributes to function of the Rep protein/*STB* partitioning complex.

The interactions of the Rep1 and Rep2 proteins and their dependence on *STB* have made it difficult to disentangle the contribution each makes to 2μm plasmid partitioning. In this study, we investigated the ability of the Rep proteins to contribute to plasmid inheritance in the absence of one an-

other by artificially tethering them to an otherwise unstable *ARS* plasmid lacking *STB*. Tethered Rep2 displayed partitioning function that did not require the presence of Rep1 or a functional RSC2 chromatin remodeler. Analysis of truncated and mutated versions of Rep2 showed the carboxy-terminal 65 residues were required and sufficient for this activity. Mutation of a basic motif in this domain conserved in the non-Rep1-related partitioning proteins encoded by other 2μm family plasmids impaired this Rep1-independent inheritance and Rep1/*STB*-dependent 2μm plasmid partitioning. Mutation of the basic motif did not affect Rep2 DNA-binding activity or Rep2 interaction with itself, Rep1 or Raf suggesting this motif may mediate Rep2 association with a host protein. Our findings suggest the RSC2 complex and Rep1 enable recruitment and positioning of Rep2 proteins on the *STB* repeats where the Rep2 carboxy termini may link to a host component that is partitioned during cell division. Further study is needed to identify the host factor/s targeted by Rep2 and to determine how this interaction contributes to the Rep protein/*STB*-mediated inheritance of 2μm plasmids in their yeast host.

MATERIALS AND METHODS

Strains and media

Yeast strains used in this study were derived in a W303/1A background (*MAT***a***ade2-1 ura3-1 leu2-3,-112 his3-11,-15 trp1-1*) and are listed in Supplementary Table S1. Strains containing native 2μm plasmid are designated cir^+ and those lacking the plasmid are termed cir^0. Yeast were cultured at 28˚C in YPAD (1% yeast extract, 2% Bacto Peptone, 0.003% adenine, 2% glucose), in synthetic complete (SC) (0.67% Difco yeast nitrogen base without amino acids, 2% glucose, 1% Difco casamino acids, 0.003% adenine, 0.002% uracil, 0.002% tryptophan), or in synthetic defined (SD) medium (0.67% Difco yeast nitrogen base without amino acids, 2% glucose, 0.003% adenine, 0.002% uracil and all required amino acids) (62). Specific amino acids or bases were omitted from SD or SC medium to select for the presence of plasmids in transformed yeast. For induction of galactose-inducible promoters, 2% glucose was replaced with 2% raffinose for pre-cultures and with 2% galactose for induction. Media were solidified with 2% Bacto agar. *Escherichia coli* strain DH5α and yeast were cultured and manipulated according to standard protocols.

Plasmids

Plasmids and oligonucleotides used as primers for polymerase chain reactions (PCR), for site-directed mutagenesis, or as linkers for addition of restriction sites, are shown in Supplementary Tables S2 and S3, respectively. Phusion Polymerase was used for all PCR-based cloning, as recommended by supplier (Thermo Scientific). All plasmid sequences generated by PCR were confirmed by sequencing.

Plasmids for expression of 2μm plasmid proteins in yeast. *HIS3*-tagged *ARS*/*CEN* single-copy yeast plasmids (pMM7 series) to provide constitutive expression in yeast of the DNA-binding domain of the bacterial repressor protein LexA (amino acids 1–87; $LexA_{BD}$) or $LexA_{BD}$ fused to wild type, truncated or mutant versions of 2μm Rep1, Rep2 and Raf proteins were derived from previously described 2μm-based plasmids (pSH2-1 backbone) (43,59,61) by isolating the respective EcoRV fragment encoding $LexA_{BD}$ or the $LexA_{BD}$ fusion, with flanking *ADH1* promoter and terminator sequences from each, and ligating these with a 4.5 kb PvuII fragment from the *CEN*/*HIS3* cloning vector pRS313 (63).

Plasmids for galactose-inducible expression of Rep1, Rep2 and Raf in yeast. A *LEU2*-tagged *ARS*/*CEN* plasmid (pRSGAL-LEU) and derivatives of pRSGAL-LEU that express untagged Rep1, Rep2 and Raf in yeast under the control of the *GAL1* promoter (pRSGAL-LEU-REP1, pRSGAL-LEU-REP2 and pRSGAL-LEU-RAF) have been previously described (43). A plasmid to express an untagged dimerization mutant of Rep2 (pRSGAL-LEU-rep2(AA)) was similarly constructed (43).

TRP1-tagged ARS plasmids. The *TRP1*-tagged *ARS* plasmids pTRP1/ARS and pTRP1/ARS/lexAop8 were created by replacing a 600-bp BamHI/NruI fragment in the plasmid pYR7 (64) with a BamHI/ScaI fragment encoding the *UASΔGAL1* promoter region, obtained from the one-hybrid vector pJL638 (65) or amplified from the genome of the one-hybrid reporter yeast strain CT/MD/3a (66), respectively. In the latter, two copies of a 78-bp oligonucleotide inserted at the cloning site in pJL638, each encoding two colE1 (overlapping) operator sequences, provide eight tandem LexA binding sites ($lexA_{op8}$) in the pTRP1/ARS/lexAop8 plasmid (67).

2μm-based plasmids. Plasmid pAS4, an *ADE2*-tagged flp^- version of the 2μm circle that can be propagated in *E.coli* and yeast, and pAS4Δrep2, pAS4 with deletion of the *REP2* gene, have been previously described (59,61). To facilitate replacement of the *REP2* coding region in pAS4 with mutant versions, a unique in-frame NotI site was introduced immediately upstream of the *REP2* stop codon, creating plasmid pAS27. An open reading frame (ORF) encoding amino acids 1–231 of Rep2 with the SV40 Large T antigen nuclear localization signal (NLS) (N- PKKKRKV-C) fused in-frame at the C-terminus was generated by PCR and used to replace the *REP2* ORF in pAS27, generating plasmid pAS27-$rep2_{1\text{-}231}$-NLS. The SV40 NLS has previously been shown to restore Rep2 nuclear targeting and partitioning function when substituted for the C-terminal 20 amino acids of the Rep2 protein (28).

Plasmids encoding Rep2 with four arginine-to-alanine substitutions (4RA) in a basic motif. A mutant version of the *REP2* gene ($rep2_{4RA}$) in which arginine codons at positions 248, 249, 251 and 253 were changed to ones encoding alanine was generated by PCR-mediated site-directed mutagenesis. The $rep2_{4RA}$ ORF was cloned in the vector pMM7 to enable expression in yeast fused to $LexA_{BD}$ (pMM7-$rep2_{4RA}$) or used to replace the wild type *REP2* ORF in the *ADE2*-tagged 2μm plasmid pAS27 (pAS27-$rep2_{4RA}$) to assess the effect on Rep1-mediated plasmid partitioning.

Plasmid inheritance assays

To measure the ability of $LexA_{BD}$ fusion proteins to promote plasmid inheritance, yeast cells lacking native 2μm plasmid [cir^0] (strain JP49/6b) were co-transformed with two plasmids, one *TRP1*-tagged (pTRP1/ARS or pTRP1/ARS/lexAop8), and the other a *HIS*3-tagged *CEN*/*ARS* plasmid that would constitutively express $LexA_{BD}$ (pMM7) or a $LexA_{BD}$ fusion protein (pMM7 series). Co-transformants were cultured to early stationary phase (∼8–10 generations) in medium selective for the presence of both plasmids (SD lacking histidine and tryptophan, SD-his-trp). The mitotic stability of the *TRP1*-tagged plasmid (defined as the percentage of cells containing the plasmid in a population of cells grown in medium selective for the plasmid) was determined from the ratio of viable colonies obtained by plating on medium selective for both plasmids (SD-his-trp) *versus* selective only for the *CEN*/*ARS* $LexA_{BD}$-protein-expressing plasmid (SD lacking histidine, SD-his). The plasmid loss rate during culture in medium non-selective for the *TRP1*-tagged plasmid was determined as previously described (68)(Supplementary Data).

To assess the effect of expressing an untagged 2μm plasmid protein in cells expressing $LexA_{BD}$ fusion proteins, yeast cells were co-transformed with three plasmids, p*TRP1*/*ARS*/*lexAop8*, a *HIS*3-tagged *CEN*/*ARS* plasmid that would constitutively express $LexA_{BD}$ or a $LexA_{BD}$ fusion protein, and a *LEU2*-tagged *ARS*/*CEN* plasmid encoding a 2μm plasmid protein under the control of a galactose-inducible promoter (*GAL1p*) or that did not encode a protein (pRSGAL-LEU-based plasmids). The transformants were cultured in medium selective for the presence of the three plasmids and containing galactose as the carbon source, SD(gal)-his-leu-trp. The percentage of cells containing the *TRP1*-marked plasmid was determined by measuring plating efficiency on SD(gal)-his-leu-trp versus on SD(gal)-his-leu medium.

To measure the mitotic stability of *ADE2*-tagged 2μm-based plasmids, [cir^0] yeast (strain AG8/5) transformants were and cultured in SC medium lacking adenine for 10–12 generations and the percentage of cells containing the plasmid determined by plating on selective (lacking adenine) versus non-selective (adenine-containing) SC medium.

Statistical analysis

For all plating assays, results presented are the average with standard deviation from analyzing a minimum of five independent yeast transformants for each unless otherwise indicated. Statistical significance was assessed using a two-tailed Student's *t*-test.

Western blotting analysis

Protein was extracted from yeast cultures and analyzed by western blotting as previously described (43). Rabbit polyclonal anti-LexA (Invitrogen), rabbit polyclonal anti-$Gal4_{AD}$ (Sigma) and mouse monoclonal anti-Pgk1 (Abcam) were used as primary antibodies with Horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Novex) and anti-mouse IgG (Sigma) as secondary antibodies. Signals were detected by chemiluminescence using a Clarity Western ECL Substrate Kit (BioRad) and images captured using a VersaDoc 4000MP imaging system and Quantity One software (BioRad) or by autoradiography. Images were processed using Adobe Photoshop CS6 and figures prepared using Adobe Illustrator CS6.

RESULTS

Rep1-independent partitioning function of the 2μm Rep2 protein

Determining the contributions made by Rep1 and Rep2 to 2-micron plasmid partitioning has been complicated by their interaction with each other and with Raf, their joint role in regulating transcription of their encoding genes, and by uncertainty in how they associate with the repeated sequence at the *STB* locus. To disentangle the roles of the individual components, we took advantage of an approach previously used to demonstrate partitioning activity in which a protein of interest is artificially tethered to an unstable *ARS* plasmid and the effect on inheritance of the plasmid monitored (2,7,11). Here, we assessed whether either of the Rep proteins or the Raf protein, when fused to the DNA-binding domain of the bacterial repressor protein LexA ($LexA_{BD}$) and expressed in yeast cells in the absence of any other 2μm plasmid components, would affect inheritance of an *ARS* plasmid containing LexA-binding sites, For the assay, yeast cells lacking native 2μm plasmid (cir^0) were transformed with two plasmids, the first, a *TRP1* gene-tagged *ARS* plasmid that either contained (pTRP1/ARS/lexAop8) or lacked (pTRP1/ARS) a tandem array of LexA-binding sites, and the second, a *HIS3*-tagged *CEN*/*ARS* plasmid, that would constitutively express $LexA_{BD}$ or $LexA_{BD}$ fused to Rep1, Rep2 or Raf. The transformed yeast cells were cultured in medium selective for the presence of the two plasmids, and the percentage of cells that contained the *TRP1*-tagged plasmid was determined using a plating assay (Figure 1A).

As expected for the maternal bias in inheritance of an *ARS* plasmid (1,69), only ∼15% of the cells transformed with the *TRP1*-tagged *ARS* plasmid lacking LexA target sites were able to form colonies on medium lacking tryptophan, irrespective of the $LexA_{BD}$ fusion protein expressed. Mitotic stability of the plasmid containing LexA target sites was similarly low in cells expressing $LexA_{BD}$ or $LexA_{BD}$ fused to Rep1 or Raf. In contrast, 30–50% of cells expressing the $LexA_{BD}$-Rep2 fusion protein and transformed with the pTRP1/ARS/lexAop8 plasmid were able to form colonies on medium lacking tryptophan. Plating assays showed that inheritance of the *HIS3*-tagged *CEN*/*ARS* expression plasmid did not differ between these cell cultures which, as expected for centromere-based plasmids, were stably maintained, irrespective of whether $LexA_{BD}$ or $LexA_{BD}$-Rep2 was encoded (Supplementary Data Figure S1).

To determine whether the failure of the Rep1 and Raf proteins to affect plasmid inheritance when tethered to an *ARS* plasmid might be due to low levels of the fusion proteins, total protein was extracted from the pTRP1/ARS/lexAop8 yeast transformants analyzed in Figure 1A and analyzed by western blotting (Figure 1B).

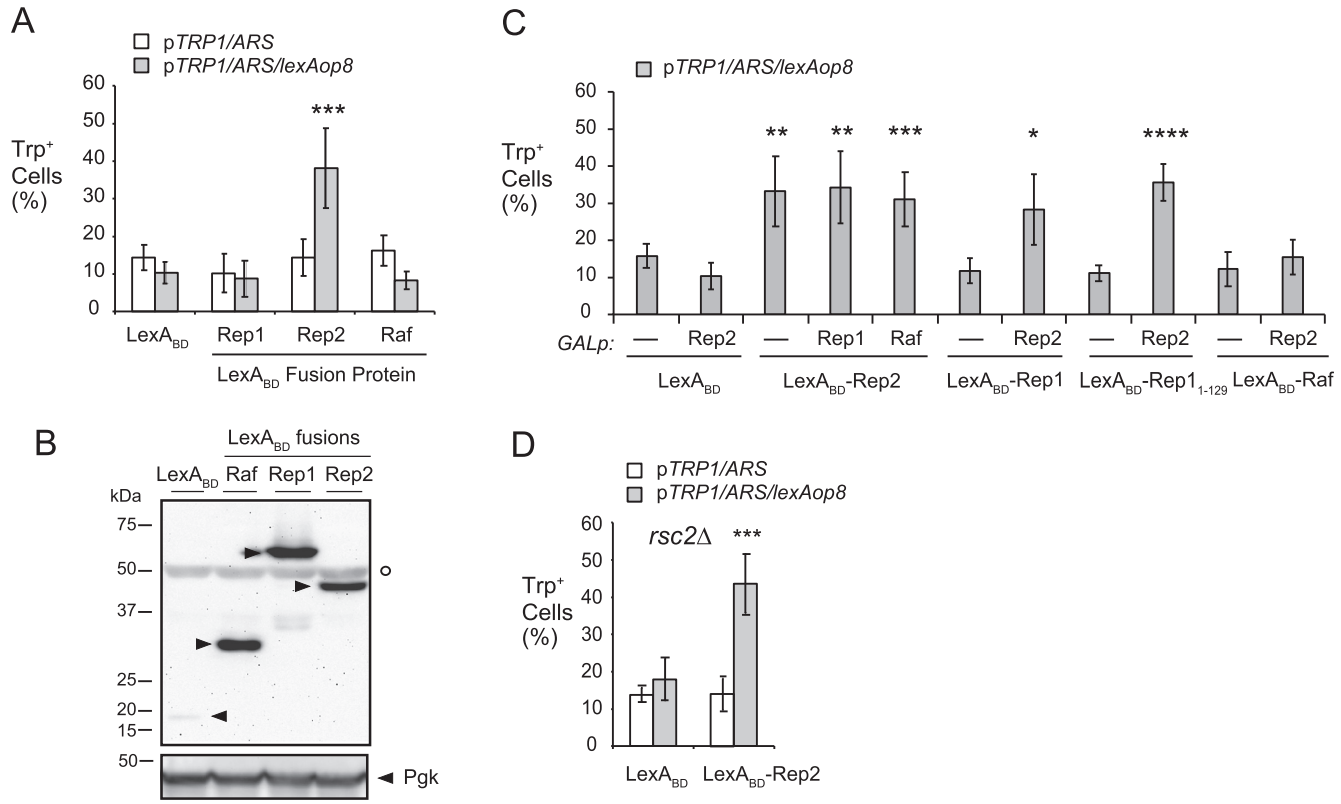

Figure 1. Tethering Rep2 to an *ARS* plasmid lacking *STB* promotes plasmid inheritance that is not dependent on Rep1 or affected by absence of Rsc2. (**A, B**, **D**) Yeast cells lacking native 2μm plasmid were co-transformed with two plasmids: an *ARS*/*CEN HIS3*-tagged plasmid that would express either the DNA binding domain of LexA ($LexA_{BD}$) or the indicated protein fused to $LexA_{BD}$, and a *TRP1*-tagged *ARS* plasmid either containing (pTRP1/ARS/lexAop8) or lacking (pTRP1/ARS) an array of 8 LexA-binding sites. The co-transformed yeast cells were cultured overnight in medium selective for the presence of both plasmids. (**C**), as in (A), except yeast also contained a *LEU2*-tagged *ARS*/*CEN* plasmid that expressed an untagged version of the indicated 2μm plasmid protein under the control of a galactose-inducible promoter (*GALp*) or that did not encode a protein (–). The transformants were cultured overnight in medium selective for the presence of the plasmids and containing galactose as the carbon source. (A, C, D) The percentage of Trp^+ cells (an indication of inheritance of the *TRP1*-marked plasmid) was determined by a plating assay. (D) As in (A) except the yeast lacked the *RSC2* gene. Results represent the average (±s.d.) from assaying five independent co-transformants for each combination of plasmids for (A and D**)** and six for (C). Asterisks indicate significance of increased inheritance of the pTRP1/ARS/lexAop8 plasmid relative to the cells expressing only $LexA_{BD}$ as determined by a two-tailed Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). (B) Total protein was extracted from the yeast transformants containing the pTRP1/ARS/lexAop8 plasmid used for the plating assay in (A) and analyzed by western blotting with antibodies specific for LexA (top) and a yeast host protein Pgk1 (bottom). Species with the mobility expected for the LexA fusion protein being expressed are indicated with an arrowhead. An open circle denotes a non-LexA host protein detected by the antibody.

The steady-state levels of the Rep1 and Raf $LexA_{BD}$ fusion proteins were similar to those of the $LexA_{BD}$-Rep2 fusion protein and therefore high enough to promote plasmid inheritance if they had been capable of independently doing so when tethered to the *ARS* plasmid. These results also demonstrate that the higher mitotic stability associated with tethered Rep2 was not merely due to a greater abundance of the $LexA_{BD}$-Rep2 fusion protein.

We also assessed the ability of the $LexA_{BD}$-Rep2 fusion protein to promote inheritance of the pTRP1/ARS/lexAop8 plasmid in the absence of selection for the *TRP1* marker gene on the tethered plasmid. Plating assays showed that after ten generations in medium containing tryptophan, a significantly higher percentage of the $LexA_{BD}$-Rep2-expressing cells still contained the *TRP1*-tagged plasmid and had a lower rate of loss of the pTRP1/ARS/lexAop8 plasmid than cells expressing $LexA_{BD}$ (1.7% versus 6.0% loss per generation) (Supplementary Data Figure S2). Taken together these results are consistent with Rep2 tethering increasing transmission of the *ARS* plasmid to daughter cells.

To determine whether the results obtained from plating cell cultures reflected a difference in plasmid transmission by individual mother cells, a pedigree analysis was performed in which buds were separated from mother cells by micromanipulation and scored for inheritance of the pTRP1/ARS/lexAop8 plasmid. Consistent with the cell culture plating assays, even when the $LexA_{BD}$-expressing mother cells contained sufficient levels of the *TRP1*-tagged plasmid to produce a viable tryptophan-prototrophic colony, only 25% of daughters were able to form a colony on medium lacking tryptophan, as compared to mother cells expressing $LexA_{BD}$-Rep2, where 67% of daughters were able to do so, indicating they had received the plasmid (Supplementary Data FigureS3).

The increased transmission of the pTRP1/ARS/lexAop8 plasmid by mother cells expressing $LexA_{BD}$-Rep2 is consistent with the plasmid being actively partitioned at cell divi-

sion by being tethered to Rep2, but in principle, could also occur if plasmid copy number was significantly increased allowing more plasmids to reach daughter cells by passive diffusion. To assess this possibility, the copy number of the *TRP*1-tagged plasmid in transformants cultured under selective conditions was quantified. The average number of pTRP1/ARS/lexAop8 plasmids in the cell population did not significantly differ between yeast expressing $LexA_{BD}$ (12 ± 5 copies per cell) versus those expressing $LexA_{BD}$-Rep2 (13 ± 6 copies per cell) (Supplementary Data Figure S4). Moreover, when the fraction of the population containing these copies is considered, the copy number of the *TRP1*-tagged plasmid per plasmid-containing cell was significantly higher in the $LexA_{BD}$-expressing cultures (56 ± 13 copies per Trp^+ cell) than in those expressing $LexA_{BD}$-Rep2 (24 ± 12 copies per Trp^+ cell) (Supplementary Data Figure S4). These results indicate that the increased transmission of the pTRP1/ARS/lexAop8 plasmid associated with $LexA_{BD}$-Rep2 expression cannot be attributed to higher copy number, and instead supports tethered Rep2 enabling partitioning of the *ARS* plasmid.

Tethered Rep1 allows Rep2-dependent partitioning of an *ARS* plasmid

The ability of Rep2 to mediate partitioning of an *ARS* plasmid when bound to the plasmid through a heterologous DNA-binding domain, raises the possibility that partitioning of the native 2 μm plasmid may depend on Rep2 linking the plasmid to a segregating host component, with Rep1 being required to target Rep2 to the plasmid *STB* locus. Our previous studies of Rep protein interactions with synthetic *STB* repeat sequences *in vivo* support a model in which Rep1 plays the key role in recognizing sequence elements in the *STB* repeats, either directly or through interaction with a host factor that is targeted to those sites, while Rep2 association with *STB* is more dependent on interaction with Rep1 (66,70). Based on this model, we might expect that if Rep1 were tethered to an *ARS* plasmid and could recruit Rep2, the plasmid could be partitioned without requiring the presence of the *STB* sequence. To test this, we assessed the mitotic stability of the *TRP1*-tagged *ARS* plasmid containing LexA binding sites in yeast expressing $LexA_{BD}$ or $LexA_{BD}$ fusion proteins, as before (Figure 1A). However, this time, native Rep1, Rep2 or Raf, were also expressed in the cells under the control of a galactose-inducible promoter to see if any of these three 2μm proteins, which have been shown to interact with each other and to associate with the *STB* locus *in vivo*, would affect inheritance of the tethered plasmid. As observed in Figure 1A, if no other 2μm plasmid protein was expressed, mitotic stability of the pTRP1/ARS/lexAop8 plasmid was only increased by expression of the $LexA_{BD}$-Rep2 fusion (Figure 1C). The percentage of cells containing the plasmid was not further increased by expression of Rep1 or Raf, both of which associate with Rep2 and help maintain Rep2 steady-state levels *in vivo* (43,59). This shows that the mitotic stability conferred by $LexA_{BD}$-Rep2 was not limited by lack of these stabilizing partner proteins, nor did their presence interfere with this Rep2-mediated inheritance.

In contrast to the lack of impact 2μm partner protein expression had on tethered Rep2, the mitotic stability of the pTRP1/ARS/lexAop8 plasmid was increased if Rep2 was expressed in cells where either Rep1, or the Rep1 domain required and sufficient for Rep2 interaction (residues 1–129) (61), was expressed fused to $LexA_{BD}$. The improvement in inheritance conferred by Rep2 expression did not significantly differ between the two Rep1 fusions (Supplementary Data Figure S5A) or from that provided by expression of $LexA_{BD}$-Rep2 alone (Figure 1C) ($P = 0.39$ and $P = 0.59$ for $LexA_{BD}$-Rep2 expression as compared to the Rep1 and $Rep1_{1–129}$ $LexA_{BD}$ fusions, respectively, with Rep2). Western blotting analysis showed the improvement was not due to higher steady-state levels of the Rep1 fusion proteins in the cells expressing Rep2 (Supplementary Data Figure S5B). While the difference was not significant, improvement conferred by the full-length Rep1 fusion might have been affected by the presence of the Rep1/Rep2 heterodimer in the cells (27,40), an effect not imposed when Rep2 was associated with the Rep1 truncation. Co-expression of the Rep proteins at levels higher than those provided here has previously been shown to inhibit cell growth and cell cycle progression (27,40). Taken together, the data suggests that recruitment of Rep2 to the plasmid, either directly by tethering through the $LexA_{BD}$ moiety, or indirectly through interaction with Rep1 or the Rep1 amino-terminal domain, was sufficient to mediate this improved partitioning. Interestingly, although Raf also interacts with Rep2 (43), the mitotic stability of the pTRP1/ARS/lexAop8 plasmid in cells expressing the $LexA_{BD}$-Raf fusion protein was not increased by expression of Rep2. This may reflect differences in the way Rep1 and Raf associate with Rep2. Raf interacts with the Rep2 domain required for self-association (Rep2 residues 58–231), and unlike Rep1, may compete with Rep2 for this interaction (43). Alternatively, Rep2 association with the tethered Raf fusion protein might not be sufficiently robust or might not position Rep2 in a way that allows linkage to a nuclear component that could enable the tethered plasmid to be delivered more efficiently to daughter cells.

Rep1-independent Rep2-mediated plasmid partitioning is not affected by absence of Rsc2

RSC complexes are members of the SWI/SNF family of ATP-dependent chromatin remodelers (71), able to slide nucleosomes along DNA and destabilize or eject them (72). In yeast, RSC complexes are required for remodeling centromere and centromere-flanking chromatin to the form needed for kinetochore function (73), and regulate the differential association of cohesin with centromeres and chromosome arms during mitosis (47). They also function in DNA replication and repair processes (71) and are major contributors to establishing accessible chromatin at gene promoter regions (74). Of the two RSC complexes found in the budding yeast *S. cerevisiae* (75), RSC2 differs from the RSC1 complex in having the Rsc2 protein rather than the Rsc1 protein as a component and is the more abundant of the two (76). Cells lacking either the Rsc1 or Rsc2 protein are viable, while absence of both is lethal, indicating some functional redundancy between the two, but only deletion

of the gene encoding Rsc2 (*rsc2Δ*), and not of the one encoding Rsc1, leads to a high rate of loss of the 2μm plasmid (77). Chromatin immunoprecipitation (ChIP) has revealed the presence of the Rsc2 protein and other RSC complex subunits at the 2μm plasmid *STB* locus (46,47). This along with a dramatic alteration in micrococcal nuclease sensitivity of *STB* chromatin in yeast lacking the Rsc2 protein, but not when the Rsc1 protein was absent (77), are consistent with the RSC2 complex being directly involved in establishing the chromatin configuration needed for partitioning competence at *STB*.

Here, we assessed whether partitioning of an *ARS* plasmid mediated by tethered Rep2 would resemble Rep protein-dependent inheritance of a 2μm plasmid in requiring a functional RSC2 complex. The mitotic stability of the pTRP1/ARS/lexAop8 plasmid was increased when the $LexA_{BD}$-Rep2 fusion was expressed in *rsc2Δ* cells (Figure 1D) to the same extent as observed in cells with a wild type *RSC2* gene (Figure 1A), with the increase being dependent on the *TRP1*-tagged *ARS* plasmid having LexA-binding sites. The ability of tethered Rep2 to mediate plasmid partitioning in the absence of the Rsc2 protein suggests the requirement for the RSC2 complex is a feature only of the Rep protein/*STB*-mediated plasmid partitioning either for reconfiguring *STB* chromatin or to enable productive Rep1 association, steps that may be bypassed when Rep2 is not dependent on Rep1 for linkage to the plasmid.

The C-terminal 65 amino acids of tethered Rep2 are required and sufficient to promote *ARS* plasmid inheritance

Previous studies have identified domains in Rep2 sufficient for self-association and for interactions with Rep1, Raf and DNA (43,61). To determine which portion of Rep2 might be responsible for promoting inheritance of the tethered plasmid, truncated and mutant versions of Rep2 were expressed in yeast fused to $LexA_{BD}$ and assessed for their ability to mediate partitioning of the *TRP1*-tagged *ARS* plasmid containing LexA binding sites (Figure 2A). Consistent with this partitioning activity not requiring Rep1, fusions lacking the domain required for Rep1 association, (residues 1–57, $LexA_{BD}$-$Rep2_{58\text{-}296}$) (61), or with an amino acid substitution that abolishes Rep1 interaction (D22N, $LexA_{BD}$-$Rep2_{D22N}$) (43), were still able to promote inheritance of the tethered plasmid. In contrast, a $LexA_{BD}$-Rep2 fusion with leucine-to-alanine substitutions at positions 185 and 186 which abolish Rep2 dimerization ($LexA_{BD}$-$Rep2_{AA}$) and one lacking the C-terminal 65 amino acids of Rep2 ($LexA_{BD}$-$Rep2_{1–231}$) (43), were unable to promote inheritance of the tethered plasmid. Western blotting analysis showed similar steady state levels for all these $LexA_{BD}$ fusions indicating that the failure of the latter two to promote plasmid inheritance was not due to reduced expression (Figure 2B). Having demonstrated that the C-terminal 65 residues of Rep2 were required for this partitioning activity, we then tested a $LexA_{BD}$ fusion containing only this C-terminal portion of Rep2 ($LexA_{BD}$-$Rep2_{232–296}$) to see if this domain was sufficient. Rather surprisingly, given this truncation lacks the domain required for dimerization, this fusion protein was as proficient as the full-length wild type Rep2 $LexA_{BD}$ fusion at promoting inheritance of the *TRP1*-tagged *ARS* plasmid containing LexA binding sites. This apparent discrepancy suggested there might be a difference in the way the full-length 296 amino acid Rep2 protein was configured when bound to the tandem array of LexA binding sites when it was no longer able to dimerize, as compared to the fusion containing only the C-terminal 65 residues of Rep2.

Although non-functional in the tethering assay, the $Rep2_{L185A,\ L186A}$ dimerization mutant was previously shown to be competent for Rep1-dependent 2μm plasmid partitioning (43). This raised the possibility that in the native context, the $Rep2_{L185A,\ L186A}$ dimerization mutant was able to function due to Rep1 interaction and might also do so in the tethering assay. We tested this by expressing the full-length Rep2 dimerization mutant in yeast where Rep1 or the Rep1 amino-terminal domain (1–129) were tethered to an *ARS* plasmid as $LexA_{BD}$ fusions as in Figure 1C. The Rep2 dimerization mutant proved to be as efficient as wild-type Rep2 at improving mitotic stability of the *ARS* plasmid tethered to either of the Rep1 fusions (Supplementary Data Figure S5A). As was seen for wild type Rep2, the average mitotic stability was slightly better when the Rep2 mutant was expressed with the truncated version of Rep1. If the difference reflects an inhibition imposed by co-expression of full-length Rep1 with Rep2 (27,40), it seems Rep2 dimerization is not required for this effect. Overall, the results of the tethering assay are consistent with Rep1 interaction compensating for loss of Rep2 dimerization. One possible mechanism would be if Rep1 interaction disrupted an intramolecular association between the Rep2 amino-terminal domain and a more C-terminal portion of Rep2. This association might normally be outcompeted by Rep2 dimerization and if not blocked, render the C-terminal domain inaccessible.

Potential evidence for such an intra-molecular association comes from i*n vitro* baiting assays in which a Rep2 truncation containing only the amino-terminal domain (residues 1–58) was pulled down by full-length Rep2, but with an efficiency much lower than the pull-down of a Rep2 truncation lacking this domain that retained the dimerization domain (residues 58–231) (43,61). If Rep1 interaction rescued partitioning function for the tethered Rep2 dimerization mutant by disrupting an inhibitory intramolecular association, deletion of the Rep2 amino-terminal domain might be expected to do the same. Consistent with this hypothesis, inheritance of the *ARS* plasmid was increased when the amino-terminal 57 residues of the tethered Rep2 dimerization mutant were deleted ($LexA_{BD}$-$Rep2_{58\text{-}296(AA)}$) (Figure 2A). The mitotic stability was lower than that conferred when wild-type Rep2 lacking this domain was tethered to the plasmid, but this was likely due to the lower steady-state level of the $LexA_{BD}$-$Rep2_{58\text{-}296(AA)}$ fusion protein (Figure 2B). Taken together, the results of the tethering assays demonstrate that the C-terminal 65 amino acids of Rep2 are required and sufficient to mediate this Rep1-independent plasmid partitioning. They also suggest that in the absence of dimerization, the full-length Rep2 protein may be prevented from providing this function due to an intramolecular association between the amino-terminus and

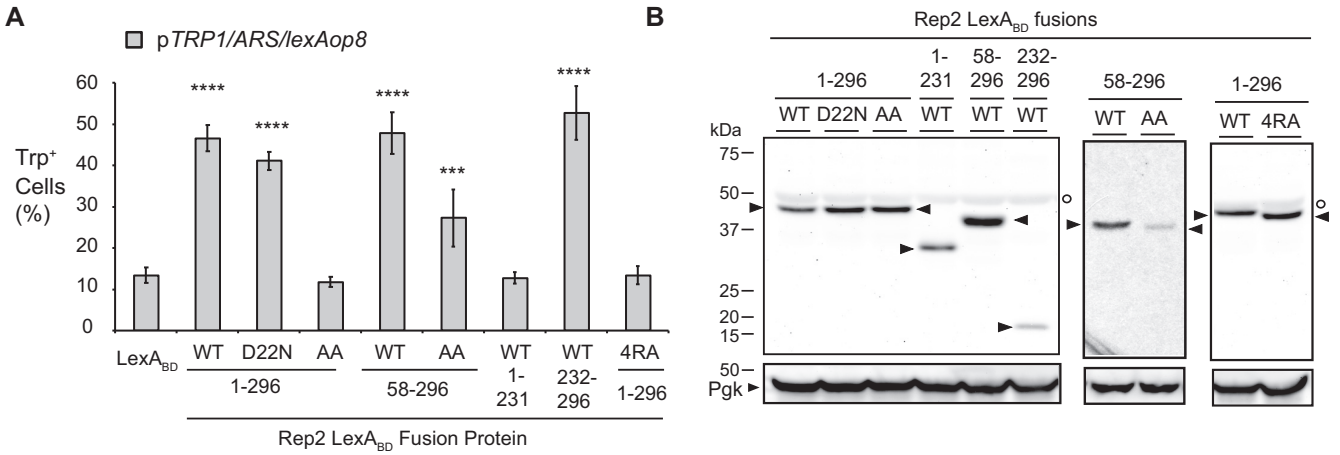

Figure 2. The C-terminal 65 residues of tethered Rep2 are required and sufficient for promoting plasmid inheritance in the absence of Rep1 and this activity is lost when a basic motif in this domain is mutated. Yeast lacking native 2μm plasmid were co-transformed with two plasmids: an *ARS/CEN HIS3*-tagged plasmid that would express either $LexA_{BD}$ or $LexA_{BD}$ fused to the indicated version of Rep2, either full-length (1–296), or truncated (1–231, or 58–296, or 232–296), and either wild type (WT), or mutant (D22N or L185A, L186A (AA)), or with four arginine-to-alanine substitutions in the C-terminal basic motif (4RA). The co-transformed yeast were cultured overnight in medium selective for the presence of both plasmids. (**A**) The percentage of Trp^+ cells (an indication of inheritance of the *TRP1*-marked plasmid) was determined by a plating assay. Results represent the average (±s.d.) from assaying six independent co-transformants for each combination of plasmids. Asterisks indicate significance of increased inheritance of the pTRP1/ARS/lexAop8 plasmid relative to the cells expressing only $LexA_{BD}$ as determined by a Student's two-tailed *t*-test ($^{****}P < 0.0001$). **(B)** Total protein was extracted from the co-transformed yeast cultures in (A) and analyzed by western blotting with antibodies specific for LexA (top) and a yeast host protein Pgk1 (bottom). Species with the mobility expected for the $LexA_{BD}$ fusion protein being expressed are indicated with an arrowhead. An open circle denotes a non-LexA host protein detected by the antibody.

a more carboxy-terminal region of the protein, with Rep1 interaction or deletion of the Rep2 amino-terminal domain being able to restore function.

Arginine-to-alanine substitutions in a basic motif in the Rep2 C-terminal domain abolishes Rep1-independent plasmid partitioning function

Although members of the 2μm family of plasmids in other species of budding yeast do not share DNA sequence identity with the 2μm plasmid of *S. cerevisiae*, all have a similar organization with two unique regions separated by a pair of large, inverted repeats (16,18,78). Each member encodes a highly conserved Flp site-specific recombinase with target sites in the repeats, a recognizable Rep1 homolog, and a second protein that, where studied, has been shown to be required along with the Rep1 protein for plasmid partitioning but which lacks sequence similarity to the 2μm Rep2 protein (79–82). A GLAM2 search (83) identified a potential bipartite basic motif common to the C-terminal domain of Rep2 and several of these Rep2-functionally equivalent proteins, raising the possibility that the element might contribute to the partitioning function of these proteins (43). To assess this, codons in the *REP2* ORF specifying four arginines (residues 248, 249, 251 and 253) were mutated to encode alanines. Rep2 with this mutant basic motif (Rep2-4RA), fused to $LexA_{BD}$ ($LexA_{BD}$-$Rep2_{4RA}$), was unable to promote inheritance of the pTRP1/ARS/lexAop8 plasmid (Figure 2A). Western blotting showed that this loss of partitioning function was not due to a reduced steady state level of the mutant protein (Figure 2B). Two-hybrid interaction of the Rep2 protein with Rep1, Rep2 and Raf was also unaffected by the four arginine-to-alanine amino acid substitutions, suggesting that the overall structure of the Rep2 protein was not grossly perturbed by these changes (Supplementary Data Figure S6).

Mutation of the basic motif in the Rep2 C-terminal domain or deletion of this domain impairs 2μm plasmid inheritance

Since the arginine-to-alanine substitutions in Rep2 abolished its ability to mediate partitioning of an *ARS* plasmid to which it was directly tethered via a $LexA_{BD}$ moiety, we wanted to know whether loss of this basic patch in Rep2 would also compromise Rep1-dependent partitioning of a 2μm plasmid and to what extent the entire C-terminal domain contributes to this function. To test this, we made use of an *ADE2*-tagged 2μm plasmid, pAS27. pAS27 contains a complete copy of the 2μm plasmid and is partitioning competent, even in yeast cells lacking native 2μm, but is defective for Flp-mediated amplification due to disruption of the *FLP* gene. These features make the plasmid ideal for assaying plasmid partitioning as Flp might otherwise compensate for copy number decreases resulting from plasmid missegregation events (59,61). Derivatives of pAS27 were created in which the wild-type *REP2* coding region was replaced with one encoding full-length Rep2 with the basic motif mutated (pAS27-$rep2_{4RA}$) or one that would express a truncated version of Rep2 with the C-terminal 65 amino acids deleted and replaced with the SV40 large T antigen nuclear localization signal (NLS) (pAS27-$rep2_{1-231}$-NLS). Nuclear localization of Rep2 has previously been shown to be lost when the C-terminal twenty amino acids were deleted but that replacing these residues with the SV40 NLS was able to restore Rep2 nuclear localization and plasmid partitioning function (28). The *ADE2*-tagged plasmids were assayed for mitotic stability in yeast lacking native 2μm plasmid and with a deletion of the chromosomal *ADE2* gene

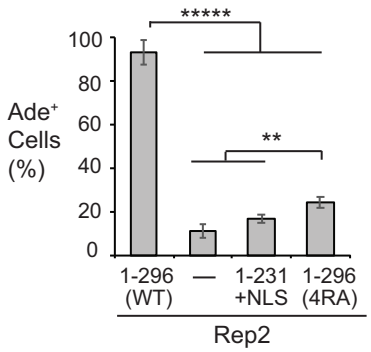

Figure 3. Deletion of the C-terminal 65 residues of Rep2 or alteration of a basic motif in this domain impairs 2-micron plasmid inheritance. Yeast cells lacking native 2μm plasmid and with a deletion of the genomic *ADE2* gene were transformed with *ADE2*-tagged amplification-defective (*flp*$^-$) versions of a 2μm-based plasmid in which the *REP2* gene was wild type (WT), or deleted (–), or had been replaced with one that encoded Rep2 residues 1–231 fused to the SV40 viral nuclear localization signal (+NLS) or full-length Rep2 with four arginine-to-alanine substitutions in the C-terminal domain basic motif (4RA). Transformed yeast cells were cultured in medium lacking adenine. The percentage of Ade$^+$ cells (an indication of inheritance of the *ADE2*-marked 2μm plasmid) was determined by a plating assay. Results represent the average (±s.d.) from assaying four independent transformants for each. Data for the plasmid lacking *REP2* are from a previous study (84). Asterisks indicate significance of differences as determined by a two-tailed Student's *t*-test ($^{**}P < 0.01$, $^{*****}P < 0.000001$).

(Figure 3). The pAS27 plasmid with the wild type copy of the *REP2* gene was inherited as efficiently as previously reported for the *ADE2*-tagged *flp*$^-$ 2μm plasmid pAS4 (61) with >90% of cells containing the plasmid when the yeast were cultured under selective conditions in medium lacking adenine. In contrast, only 17% and 24% of cells in cultures transformed with the pAS27 derivatives encoding Rep2 lacking the C-terminal 65 amino acids but fused to the SV40 NLS, or full length Rep2 with the mutant basic motif (4RA), respectively, contained plasmid when grown under the same conditions. The slightly higher mitotic stability of pAS27-rep2(4RA) relative to pAS27-rep2(1-231-NLS) or the *ADE2*-tagged 2μm plasmid lacking a *REP2* gene (84), could indicate the Rep2-4RA mutant retains some limited function, but taken together the results demonstrate that the 65 carboxy-terminal residues of Rep2 are required for 2μm plasmid partitioning and this function is lost when the GLAM2 basic motif in this domain is mutated.

One possible explanation for the failure of the Rep2-4RA mutant to support partitioning of the 2μm-based plasmid would be if mutation of the basic motif affected Rep2 nuclear localization. Although deletion of the C-terminal 20 residues of Rep2 has been shown to lead to loss of nuclear localization (28), the more amino-terminally positioned conserved basic motif might still be part of the nuclear localization signal. To assess this, cells expressing GFP-tagged versions of Rep2 were examined by fluorescence microscopy. Like wild-type Rep2, the Rep2-4RA mutant protein was observed to localize to the nucleus, even in cells lacking native 2μm plasmid, demonstrating the conserved basic motif was not part of the Rep2 nuclear localization signal (Supplementary Data Figure S7).

Another possibility for the effect of the 4RA mutation on Rep2 plasmid partitioning function would be if substitution of these basic residues led to loss of the DNA-binding activity of Rep2. We assessed this by expressing wild type and 4RA mutant versions of the C-terminal 65 residues of Rep2 in bacteria as hexa-histidine-tagged thioredoxin (Trx) fusion proteins. We have previously shown that this domain of Rep2 is sufficient for binding *STB* DNA in a southwestern assay (61). Here, we found the Rep2-4RA mutant protein to be as efficient as wild type Rep2 at binding *STB* DNA (Supplementary Data Figure S8). While DNA binding observed in a southwestern assay may not reflect protein-DNA associations that would occur *in vivo*, the results suggest that these four arginine-to-alanine substitutions may impair some function of the Rep2 C-terminal domain other than *STB* DNA binding.

DISCUSSION

For budding yeast, the distribution of plasmids lacking an active partitioning system (*ARS*-plasmids) during cell division is skewed strongly towards the mother nucleus (1). This maternal bias must be overcome for equal (or nearly equal) mother-to-daughter plasmid segregation. In this study, the ability of the 2μm plasmid Rep2 protein to overcome this bias when artificially tethered to an *ARS*-plasmid was observed by monitoring plasmid inheritance in cell populations and in a pedigree analysis. Unlike Rep protein-mediated partitioning of the native 2μm plasmid, the mitotic stability conferred by tethered Rep2 did not require Rep1, a functional RSC2 chromatin remodeling complex, or the presence on the plasmid of the 2μm *STB* partitioning locus.

We further showed that a small carboxy-terminal domain of Rep2 was required and sufficient for this Rep1-independent partitioning function and that four arginine-to-lysine substitutions in a basic patch in this domain led to loss of this function. Rep2 with these substitutions was also compromised for Rep1-dependent partitioning of a 2μm-based plasmid which suggests this carboxy-terminal domain of Rep2 may perform the same essential role in partitioning for both situations.

Efficiency of plasmid partitioning mediated by tethered Rep2 relative to that conferred by the Rep protein/*STB* partitioning complex

Current evidence supports a model for 2μm plasmid partitioning in which plasmids become attached to mitotic chromosomes in a way that enables roughly equal numbers to be segregated into the two products of cell division (20,34,35,37,55). Studies of a fluorescently-tagged single-copy 2μm reporter plasmid suggest this is achieved by two separate mechanisms, both dependent on the Rep protein complex formed at the plasmid *STB* locus. The first involves random attachment of plasmid copies to chromosomes, overcoming the maternal bias in plasmid inheritance, and the second promotes symmetric tethering of newly-replicated sister plasmids to sister chromatids to ensure the copies are equally partitioned between the mother and daughter cell during host cell division (34,35,54).

Replication-assisted and Rep protein-dependent recruitment of the host cohesin complex to the *STB* locus and timely assembly of the spindle apparatus were found to be required for the equal partitioning (35). However, in the absence of plasmid replication or under conditions of cohesin depletion or delayed spindle assembly, the Rep protein/*STB* system was still sufficient to almost completely eliminate the maternal bias in plasmid inheritance, although plasmid copies then segregated independently of one another (35).

Mitotic stabilities reported for 2μm-based plasmids tend to be high (70-90%), even in the absence of Flp-mediated plasmid amplification (61,85), but can vary depending on the plasmid marker gene and the host genetic background (85–87). In our study, although tethering Rep2 to an *ARS* plasmid increased the percentage of cells containing the plasmid under selective growth conditions (∼15% when untethered *versus* up to 50% when tethered) (Figure 1A), the increase was less than might be expected if the partitioning was as efficient as that conferred by the 2μm plasmid Rep protein/*STB* system.

The mitotic stability conferred here by tethered Rep2 might have been limited by the cells being required to maintain a *CEN*-based plasmid for expression of the $LexA_{BD}$-Rep2 fusion protein in addition to the *TRP1*-tagged *ARS* plasmid with the LexA binding sites, or by the way the Rep2 proteins, fused to the DNA-binding domain of LexA ($LexA_{BD}$, residues 1–87), were tethered to the plasmid. In the variant of the 2μm plasmid found in most lab strains of *S. cerevisiae*, the *STB* locus contains a tandem array of five 62–63-bp repeats where Rep2 associates with Rep1 to mediate plasmid partitioning (25) (70). One-hybrid assays show a single copy of one of these repeats is sufficient for Rep1 and Rep2 association *in vivo*, while a minimum of two, tandemly arrayed, is able to confer Rep protein-mediated partitioning, with four tandem repeats increasing the efficiency to the level provided by the native locus (66). In comparison, although the tandem array of eight LexA operators on the *ARS* plasmid makes it unlikely that the inheritance of the tethered *ARS* plasmid was limited merely by insufficient Rep2 molecules being bound, the spacing of the LexA target sites or the avidity of binding of the $LexA_{BD}$-Rep2 fusions might have affected the efficiency of plasmid inheritance tethered Rep2 could confer. Alternatively, the mitotic stability conferred by tethered Rep2 might be the maximum that could be delivered in the absence of other components of the partitioning complex formed at *STB*.

The role of Rep1 in 2μm plasmid partitioning

The extensive sequence conservation between Rep1 homologs encoded by different 2μm family plasmids (16,18) (our unpublished data), support Rep1 being a key determinant in the partitioning process. Despite this, tethered Rep1 failed to display partitioning activity independently of Rep2 in this study (Figure 1A). It is possible that $LexA_{BD}$-Rep1 fusion protein levels were not high enough for function to be detected in the absence of Rep2, or that fusion of the LexA DNA-binding domain to the Rep1 amino-terminus interfered with Rep1 partitioning function. The latter seems less likely given this fusion was able to substitute for native Rep1 in Rep/*STB*-dependent 2μm plasmid partitioning (data not shown). Perhaps in the native context, Rep1 does not act as a direct tether. Rep1 function might be restricted to positioning Rep2 on the *STB* repeats, enabling Rep2 attachment to a host factor, and with Rep2 allowing cohesin to be recruited (32,47). Alternatively, Rep1 might need association with RSC2-remodeled *STB* chromatin to adopt a configuration that could confer partitioning function independently of Rep2, one that tethered Rep1 could not adopt.

Is the partitioning activity displayed by tethered Rep2 responsible for the residual mitotic stability of 2μm plasmids in *rsc2Δ* yeast?

In yeast lacking the Rsc2 subunit of the RSC2 complex, Rep1 no longer localizes to the subnuclear foci where it would normally be found with the 2μm plasmid (77), ChIP and one-hybrid analyses show Rep1 (but not Rep2) association with *STB* is lost, and cohesin fails to be recruited to *STB*, resulting in 2μm plasmid mis-segregation (25,77). We do not know how Rep1 is targeted to *STB* or why this association is lost when Rsc2 is absent. Cell cycle arrest and release studies show the Rep proteins are present on the *STB* repeats for most of the cell cycle except for a brief period as cells exit G1 (25,46). Reassembly of the partitioning complex occurs in stages during S phase with some RSC2 complex subunits (Rsc8 and Rsc58) arriving before the Rep proteins (and the Kip1 motor protein) appear, and others, notably the Rsc2 and Sth1 catalytic subunits, appearing after the Rep proteins, and coincident with acquisition of the Cse4 centromeric histone. The cohesin subunit Mcd1 (indicative of cohesin presence) arrives last (25,32,46). This timeline suggests neither Rep protein depends on presence of the full RSC2 complex at *STB* for their initial recruitment to the *STB* partitioning locus (25,46). Further support comes from a one-hybrid assay where Rep protein interaction with a tandem array of only two *STB* repeats, rather than the native array of five, was assessed (88). In contrast to the longer array, Rep1 association was not lost in the *rsc2Δ* yeast, if anything, it was increased, while that of Rep2 was slightly decreased. This suggests neither Rep protein required Rsc2 for initial recruitment to *STB*, although the associations of both might be altered by its absence (88).

The difference in Rep1 *STB* association in these studies may reflect ineffective acquisition of Cse4 by the two-repeat array. Although an array of two repeats would be marginally long enough to accommodate a single Cse4-containing nucleosome (89), a previous study showed a minimum of three *STB* repeats was required and sufficient for optimal Cse4-*STB* association (90). Based on the previously proposed stages in the assembly of the 2μm partitioning complex (25,46), Rep1 may become dependent on Rsc2 for retention at *STB* only after Cse4 is incorporated, a step that would be significantly enhanced for the longer *STB* repeat array. Subsequent RSC2 complex-mediated repositioning or remodeling of the *STB* nucleosomes, or contacts mediated by the AT-hook DNA-binding motif and bromodomains in the Rsc2 protein (76), may be needed to stabilize Rep1 on the Cse4-containing nucleosomes in a configuration with Rep2 that enables replication-dependent capture of cohesin (35,46).

The discovery here of Rep2 partitioning function that does not depend on Rep1 (Figure 1A) may explain why the loss of Rep1 association with the native *STB* repeat array in *rsc2Δ* yeast does not impact 2μm plasmid maintenance as severely as absence of either Rep1 or Rep2 (46,59,66). Unlike cells lacking Rep1, Rep1 association with *STB* in the *rsc2Δ* cells may be lost only after Rep1 has helped recruit and position Rep2 on the *STB* repeat array. The retained Rep2 might be able to improve transmission to the daughter by linking the plasmid to a segregating host factor, as it did when tethered to the *ARS* plasmid. However, in the absence of sustained Rep1 association and cohesin recruitment, this linkage might not be sufficient to fully overcome the mother bias in inheritance and enable equipartitioning of the plasmid (55). Mitotic stability of 2μm-derived plasmids in *rsc2Δ* yeast varies with genetic background (46,59,77), but is comparable to that demonstrated here for an *ARS* plasmid tethered to Rep2 (Figure 1A) (59). It is therefore plausible that the Rep1-independent partitioning activity Rep2 exhibited in the tethering assay is responsible for this residual mitotic stability.

Potential Rep2 basic motif interaction targets

Although the mechanism by which Rep2 tethering improves *ARS* plasmid mitotic stability remains to be established, the extent of improvement was similar to that provided by tethering to Sir4 or Sir4PAD (7), to the nucleopore protein Mlp1 (11) or the Yif1 integral nuclear envelope protein (2) or when the plasmid was linked to the Ask1 component of the microtubule-binding Dam1 complex (11,14). While it is possible that the partitioning activity displayed by tethered Rep2 is unrelated to the way the 2μm plasmid links to host chromatin (32,35), this degree of stabilization suggests Rep2 could function as the tether that links the 2μm plasmid to chromosomes. Rep2 linkage might maintain plasmid association with segregating chromosomes during anaphase after the RSC2 complex subunits and cohesin have dissociated from *STB* (46,73). Alternatively, Rep2 association with a different segregating host factor or a component of the mitotic spindle apparatus might represent an independent contribution to plasmid maintenance.

If artificially tethered Rep2 confers plasmid partitioning by linking the *ARS* plasmid to a host factor, our analysis of truncated versions of Rep2 suggests the interaction is mediated by the highly basic C-terminal 65 amino acids of Rep2 and disrupted by four arginine-to-alanine substitutions (4RA) in a conserved basic motif in this domain (Figure 2A). The inability of this mutant version of Rep2 to mediate partitioning of a 2μm-based plasmid (Figure 3) suggests this Rep2 domain likely serves the same function for the Rep protein/*STB* partitioning complex. The motif could mediate Rep2 interaction with a chromosome-associated protein or be required for Rep2 to bind target chromosomal DNA sequences, tethering the plasmid to those sites. The latter seems less likely given that the 4RA mutation did not affect DNA-binding by this domain *in vitro* (Supplementary Figure S8). The domain might have multiple functions, with the DNA-binding activity enabling Rep2 association with the *STB* repeat sequence and the basic motif specifying attachment to a chromosome-associated protein, thereby linking the plasmid to chromosomes.

Potential candidates for a factor targeted by the Rep2 C-terminal basic motif would be host proteins already known to be required for 2μm plasmid partitioning and those found to associate with the Rep2 protein. Among these, the Kip1 motor protein co-immunoprecipitated with Rep2, but not with Rep1 (49), and although spindle-, rather than chromosome-associated, could help move Rep2-tethered plasmids to nuclear positions where chromosome attachment is favoured or the chance for plasmid transmission through the bud neck to the daughter improved. However, the dependence of Kip1 on Rep1 for association with *STB*, and the less profound impact of Kip1 absence on 2μm plasmid partitioning (35,49) relative to that caused by mutation of the C-terminal basic motif in Rep2 (Figure 3), make Kip1 a less likely target for attachment to Rep2 through this motif.

Similarly, while absence of either of the microtubule-associated proteins Bim1 and Bik impairs segregation of a 2μm-based plasmid, the impact is not as severe as that caused by absence of the Rep proteins (56), or as observed here when the Rep2 basic motif was mutated (Figure 3). This, and the lack of dependence of Bik1 and Bim1 on the Rep proteins for association with *STB*, suggests neither are the Rep2 attachment target. Bik1 and Bim1 did require each other for *STB* association and it has been suggested they might act as adapters to link the 2μm plasmid to the microtubules, facilitating plasmid movement, possibly by association with the Kip1 motor protein, to nuclear sites where acquisition of cohesin and attachment to chromosomes might be favoured (56).

Possible clues as to the nature of the host factor targeted by Rep2 come from the study in which fluorescently tagged versions of the Rep proteins were expressed in mammalian Cos-7 cells (35). Rep2 localized on the mitotic chromosomes in the absence of Rep1 and when expressed with Rep1, the Rep proteins co-localized in foci that were symmetrically positioned (and non-randomly distributed) on sister chromatids. In the absence of 2μm plasmid in these cells, the pattern could reflect Rep1-directed interaction of the Rep proteins with chromosomal sequences resembling Rep1 protein target sites on the 2μm plasmid (66,70) or Rep2-directed binding of Rep protein complexes to DNA sequences on the mammalian chromosomes that resemble the AT-rich *STB* repeat sequence or association with a conserved chromatin component that was enriched at those loci.

Chromatin candidates for Rep2 recognition could be components of the RSC, cohesin and condensin complexes, all of which are highly conserved from yeast to mammals (52,53,91). In yeast, RSC complexes localize to centromeres and centromere-proximal regions (47), to numerous tRNA gene and other RNA polymerase III promoters, and to some specific RNA polymerase II promoters where they regulate gene expression (71,92). Multiple subunits of the RSC2 chromatin remodeling complex co-purified with TAP-tagged Rep1 and Rep2 suggesting association with either one or both Rep proteins may be direct (46). The Rsc2 subunit is unlikely to be the Rep2 target given that partitioning of the Rep2-tethered *ARS* plasmid was not perturbed by

deletion of the gene encoding Rsc2 (Figure 1D). However, one of the other subunits that reassemble at *STB* early in S phase before the Rep proteins reassociate (46) could directly recruit Rep2 to *STB* or bridge attachment of Rep2 to chromosome-bound RSC complexes.

The Rep protein-dependent association of the cohesin and condensin complexes with the *STB* locus suggests a direct role for each in 2μm plasmid partitioning, although it is possible that the 2μm plasmid mis-segregation observed when they are inactivated is an indirect consequence of chromosome mis-segregation (32,37). In yeast, cohesin and condensin are enriched at centromere-proximal regions and at the repeated ribosomal DNA array locus but are also found at distinct discrete sites along the chromosome arms that differ between the two (93). Evidence suggests both complexes are loaded on the chromosomes by the cohesin loading factor Scc2/Scc4 (93), with cohesin relocating to intergenic regions where transcription converges (94) while condensin remains enriched at tRNA genes and RNA polymerase II-transcriptionally silenced regions and sites where DNA replication forks converge (93,95). The pattern of association of the Rep proteins with the mammalian mitotic chromosomes could reflect the Rep proteins being targeted to regions enriched for condensed chromatin, or a subset of sites where cohesin is concentrated (52,93). The localization of fluorescently-tagged 2μm reporter plasmids near centromeres and telomeres of yeast mitotic chromosomes, with a preference for the latter (37), regions enriched in heterochromatin, and a two-hybrid interaction between the Rep proteins and the Brn1 subunit of condensin, could indicate the complex is directly recruited to *STB* by the Rep proteins. This could allow the plasmid to be linked to the chromosomes (29,37). It is currently unclear if the association with condensin is direct, as it could be bridged by a host protein that interacts with Brn1, or if only one of the Rep proteins is involved, as the assay was undertaken in a strain that contained native 2μm plasmid where the endogenous Rep proteins could bridge an interaction with the other Rep protein. Genome-wide chromatin capture studies are now needed to identify the chromosomal sites where the Rep proteins localize which may reveal how the 2μm plasmids hitchhike on segregating chromosomes.

Evolutionary considerations

Although the identity of the host factor the Rep2 C-terminal domain may bind to promote plasmid inheritance remains to be determined our results support Rep2 being a key participant in mediating partitioning of the 2μm plasmid in addition to providing Rep1-supportive functions. This is somewhat surprising given the lack of sequence similarity between Rep2 and the Rep2 functional-equivalent partitioning proteins encoded by other members of the 2μm circle family of plasmids (16,18,43,78), Members of the family may have acquired a different gene that encodes a protein capable of providing the same function as Rep2, namely, interaction with Rep1 and attachment to a chromosomal component. However, the similar position of the open reading frame encoding these in each plasmid relative to the position of the genes encoding Flp and Rep1 and the inverted repeats, and the presence in seven of the eight proteins of a C-terminal region enriched in basic residues with a match to the bipartite basic motif mutated here, suggests a common origin (43).

The 2μm plasmid family may resemble the papillomaviruses and Epstein–Barr and Kaposi's sarcoma-associated herpesviruses which also depend on tethering to host chromosomes for their inheritance and for which the DNA-binding domains of their respective partitioning proteins, despite lacking sequence identity, have similar structures (96). Taken together with the restricted distribution of the 2μm family of plasmids in the Saccharomycetes budding yeast (16,97), the differences between Rep2 and the other Rep1-interacting partitioning proteins may reflect rapid divergent evolution from a common gene in concert with changes to the respective partitioning loci which also differ between members of this family of plasmids (16,18,78). Such differences are not without precedent. Kinetochore proteins and the centromeric sequences on which kinetochores assemble have been able to significantly evolve without loss of chromosome segregation function (98). Functional studies are now needed to determine whether the 2μm plasmid Rep2 protein and the dissimilar counterparts encoded by other members of the 2μm family of plasmids are directly involved in attaching their genomes to the chromosomes of their respective budding yeast hosts.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank members of the Dalhousie University Yeast Molecular Biology Group for helpful discussions, Jordan Pinder for creation of plasmid pAS27, and Lois Murray for advice regarding fluorescent DNA southwestern assays.

FUNDING

Natural Sciences and Engineering Research Council of Canada [Discovery Grant 155268 to M.J.D.]; Natural Sciences and Engineering Research Council of Canada [Undergraduate Student Research Award to P.S.J.]. Funding for open access charge: Natural Sciences and Engineering Research Council of Canada.
Conflict of interest statement. None declared.

REFERENCES

1. Murray,A.W. and Szostak,J.W. (1983) Pedigree analysis of plasmid segregation in yeast. *Cell*, **34**, 961–970.
2. Gehlen,L.R., Nagai,S., Shimada,K., Meister,P., Taddei,A. and Gasser,S.M. (2011) Nuclear geometry and rapid mitosis ensure asymmetric episome segregation in yeast. *Curr. Biol.*, **21**, 25–33.
3. Sinclair,D.A. and Guarente,L. (1997) Extrachromosomal rDNA circles - a cause of aging in yeast. *Cell*, **91**, 1033–1042.
4. Kimmerly,W.J. and Rine,J. (1987) Replication and segregation of plasmids containing *cis*-acting regulatory sites of silent mating-type genes in *Saccharomyces cerevisiae* are controlled by the *SIR* genes. *Mol. Cell. Biol.*, **7**, 4225–4237.
5. Longtine,M.S., Enomoto,S., Finstad,S.L. and Berman,J. (1992) Yeast telomere repeat sequence (TRS) improves circular plasmid segregation, and TRS plasmid segregation involves the RAP1 gene product. *Mol. Cell. Biol.*, **12**, 1997–2009.

6. Moretti,P., Freeman,K., Coodly,L. and Shore,D. (1994) Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. *Genes Dev.*, **8**, 2257–2269.
7. Ansari,A. and Gartenberg,M.R. (1997) The yeast silent information regulator Sir4p anchors and partitions plasmids. *Mol. Cell. Biol.*, **17**, 7061–7068.
8. Taddei,A., Hediger,F., Neumann,F.R., Bauer,C. and Gasser,S.M. (2004) Separation of silencing from perinuclear anchoring functions in yeast Ku80, Sir4 and Esc1 proteins. *EMBO J.*, **23**, 1301–1312.
9. Andrulis,E.D., Zappulla,D.C., Ansari,A., Perrod,S., Laiosa,C.V., Gartenberg,M.R. and Sternglanz,R. (2002) Esc1, a nuclear periphery protein required for Sir4-based plasmid anchoring and partitioning. *Mol. Cell. Biol.*, **22**, 8292–8301.
10. Schober,H., Ferreira,H., Kalck,V., Gehlen,L.R. and Gasser,S.M. (2009) Yeast telomerase and the SUN domain protein Mps3 anchor telomeres and repress subtelomeric recombination. *Genes Dev.*, **23**, 928–938.
11. Khmelinskii,A., Meurer,M., Knop,M. and Schiebel,E. (2011) Artificial tethering to nuclear pores promotes partitioning of extrachromosomal DNA during yeast asymmetric cell division. *Curr. Biol.*, **21**, R17–R18.
12. Khmelinskii,A., Keller,P.J., Lorenz,H., Schiebel,E. and Knop,M. (2010) Segregation of yeast nuclear pores. *Nature*, **466**, E1.
13. Clarke,L. and Carbon,J. (1980) Isolation of a yeast centromere and construction of functional small circular minichromosomes. *Nature*, **287**, 504–509.
14. Kiermaier,E., Woehrer,S., Peng,Y., Mechtler,K. and Westermann,S. (2009) A Dam1-based artificial kinetochore is sufficient to promote chromosome segregation in budding yeast. *Nat. Cell Biol.*, **11**, 1109–1115.
15. Lacefield,S., Lau,D.T. and Murray,A.W. (2009) Recruiting a microtubule-binding complex to DNA directs chromosome segregation in budding yeast. *Nat. Cell Biol.*, **11**, 1116–1120.
16. Volkert,F.C., Wilson,D.W. and Broach,J.R. (1989) Deoxyribonucleic acid plasmids in yeasts. *Microbiol. Rev.*, **53**, 299–317.
17. Toh-e,A., Tada,S. and Oshima,Y. (1982) 2μm DNA like plasmids in the osmophilic haploid yeast *Zygosaccharomyces rouxii*. *J. Bacteriol.*, **151**, 1380–1390.
18. Murray,J.A., Cesareni,G. and Argos,P. (1988) Unexpected divergence and molecular coevolution in yeast plasmids. *J. Mol. Biol.*, **200**, 601–607.
19. Rizvi,S.M.A., Prajapati,H.K. and Ghosh,S.K. (2018) The 2 micron plasmid: a selfish genetic element with an optimized survival strategy within *Saccharomyces cerevisiae*. *Curr. Genet.*, **64**, 25–42.
20. Sau,S., Ghosh,S.K., Liu,Y.T., Ma,C.H. and Jayaram,M. (2019) Hitchhiking on chromosomes: a persistence strategy shared by diverse selfish DNA elements. *Plasmid*, **102**, 19–28.
21. Chan,K.M., Liu,Y.T., Ma,C.H., Jayaram,M. and Sau,S. (2013) The 2 micron plasmid of Saccharomyces cerevisiae: a miniaturized selfish genome with optimized functional competence. *Plasmid*, **70**, 2–17.
22. Futcher,A.B. and Cox,B.S. (1983) Maintenance of the 2 μm circle plasmid in populations of *Saccharomyces cerevisiae*. *J. Bacteriol.*, **154**, 612–622.
23. Jayaram,M., Li,Y.-Y. and Broach,J.R. (1983) The yeast plasmid 2 μm circle encodes components required for its high copy propagation. *Cell*, **34**, 95–104.
24. Kikuchi,Y. (1983) Yeast plasmid requires a *cis*-acting locus and two plasmid proteins for its stable maintenance. *Cell*, **35**, 487–493.
25. Yang,X.M., Mehta,S., Uzri,D., Jayaram,M. and Velmurugan,S. (2004) Mutations in a partitioning protein and altered chromatin structure at the partitioning locus prevent cohesin recruitment by the *Saccharomyces cerevisiae* plasmid and cause plasmid missegregation. *Mol. Cell. Biol.*, **24**, 5290–5303.
26. Ahn,Y.-T., Wu,X.-L., Biswal,S., Velmurugan,S., Volkert,F.C. and Jayaram,M. (1997) The 2μm-encoded Rep1 and Rep2 proteins interact with each other and colocalize to the *Saccharomyces cerevisiae* nucleus. *J. Bacteriol.*, **179**, 7497–7506.
27. Scott-Drew,S. and Murray,J.A. (1998) Localisation and interaction of the protein components of the yeast 2 mu circle plasmid partitioning system suggest a mechanism for plasmid inheritance. *J. Cell Sci.*, **111**, 1779–1789.
28. Velmurugan,S., Ahn,Y., Yang,X., Wu,X. and Jayaram,M. (1998) The 2μm plasmid stability system: analyses of the interactions among plasmid and host encoded components.*Mol. Cell. Biol.*, **18**, 7466–7477.
29. Velmurugan,S., Yang,X., Chan,C., Dobson,M.J. and Jayaram,M. (2000) Partitioning of the 2μm circle plasmid of *Saccharomyces cerevisiae*: functional coordination with chromosome segregation and plasmid-encoded Rep protein distribution. *J. Cell Biol.*, **149**, 553–566.
30. Mereshchuk,A., Chew,J.S.K. and Dobson,M.J. (2021) Use of Yeast Plasmids: Transformation and Inheritance Assays. *Methods Mol. Biol.*, **2196**, 1–13.
31. Scott-Drew,S., Wong,C.M. and Murray,J.A. (2002) DNA plasmid transmission in yeast is associated with specific sub-nuclear localisation during cell division. *Cell Biol. Int.*, **26**, 393–405.
32. Mehta,S., Yang,X.M., Chan,C.S., Dobson,M.J., Jayaram,M. and Velmurugan,S. (2002) The 2 micron plasmid purloins the yeast cohesin complex: a mechanism for coupling plasmid partitioning and chromosome segregation. *J. Cell Biol.*, **158**, 625–637.
33. Mehta,S., Yang,X., Jayaram,M. and Velmurugan,S. (2005) A novel role for the mitotic spindle during DNA segregation in yeast: Promoting 2 micron plasmid-cohesin association. *Mol. Cell. Biol.*, **25**, 4283–4298.
34. Liu,Y.T., Ma,C.H. and Jayaram,M. (2013) Co-segregation of yeast plasmid sisters under monopolin-directed mitosis suggests association of plasmid sisters with sister chromatids. *Nucleic Acids Res.*, **41**, 4144–4158.
35. Liu,Y.T., Chang,K.M., Ma,C.H. and Jayaram,M. (2016) Replication-dependent and independent mechanisms for the chromosome-coupled persistence of a selfish genome. *Nucleic Acids Res.*, **44**, 8302–8323.
36. Sau,S., Conrad,M.N., Lee,C.Y., Kaback,D.B., Dresser,M.E. and Jayaram,M. (2014) A selfish DNA element engages a meiosis-specific motor and telomeres for germ-line propagation. *J. Cell Biol.*, **205**, 643–661.
37. Kumar,D., Prajapati,H.K., Mahilkar,A., Ma,C.H., Mittal,P., Jayaram,M. and Ghosh,S.K. (2021) The selfish yeast plasmid utilizes the condensin complex and condensed chromatin for faithful partitioning. *PLoS Genet.*, **17**, e1009660.
38. Murray,J.A., Scarpa,M., Rossi,N. and Cesareni,G. (1987) Antagonistic controls regulate copy number of the yeast 2 mu plasmid. *EMBO J.*, **6**, 4205–4212.
39. Som,T., Armstrong,K.A., Volkert,F.C. and Broach,J.R. (1988) Autoregulation of 2μm circle gene expression provides a model for maintenance of stable plasmid copy levels. *Cell*, **52**, 27–37.
40. Reynolds,A.E., Murray,A.W. and Szostak,J.W. (1987) Roles of the 2 micron gene products in stable maintenance of the 2-micron plasmid of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **7**, 3566–3573.
41. Futcher,A.B. (1986) Copy number amplification of the 2μm circle plasmid of *Saccharomyces cerevisiae*. *J. Theor. Biol.*, **119**, 197–204.
42. Volkert,F.C. and Broach,J.R. (1986) Site-specific recombination promotes plasmid amplification in yeast. *Cell*, **46**, 541–550.
43. McQuaid,M.E., Pinder,J.B., Arumuggam,N., Lacoste,J.S.C., Chew,J.S.K. and Dobson,M.J. (2017) The yeast 2-micron plasmid Raf protein contributes to plasmid inheritance by stabilizing the Rep1 and Rep2 partitioning proteins. *Nucleic Acids Res.*, **45**, 10518–10533.
44. Rizvi,S.M.A., Prajapati,H.K., Nag,P. and Ghosh,S.K. (2017) 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. *Nucleic Acids Res.*, **45**, 7167–7179.
45. Jayaram,M., Sutton,A. and Broach,J.R. (1985) Properties of *REP3*: a *cis*-acting locus required for stable propagation of the *Saccharomyces cerevisiae* plasmid 2μm circle. *Mol. Cell. Biol.*, **5**, 2466–2475.
46. Ma,C.H., Cui,H., Hajra,S., Rowley,P.A., Fekete,C., Sarkeshik,A., Ghosh,S.K., Yates,J.R. 3rd and Jayaram,M. (2013) Temporal sequence and cell cycle cues in the assembly of host factors at the yeast 2 micron plasmid partitioning locus. *Nucleic Acids Res.*, **41**, 2340–2353.
47. Huang,J., Hsu,J.M. and Laurent,B.C. (2004) The RSC nucleosome-remodeling complex is required for cohesin's association with chromosome arms. *Mol. Cell*, **13**, 739–750.
48. Hajra,S., Ghosh,S.K. and Jayaram,M. (2006) The centromere-specific histone variant Cse4p (CENP-A) is essential for functional chromatin architecture at the yeast 2-micron circle partitioning locus and promotes equal plasmid segregation. *J. Cell Biol.*, **174**, 779–790.

49. Cui,H., Ghosh,S.K. and Jayaram,M. (2009) The selfish yeast plasmid uses the nuclear motor Kip1p but not Cin8p for its localization and equal segregation. *J. Cell Biol.*, **185**, 251–264.
50. Nasmyth,K. and Haering,C.H. (2005) The structure and function of SMC and kleisin complexes. *Annu. Rev. Biochem.*, **74**, 595–648.
51. Hassler,M., Shaltiel,I.A. and Haering,C.H. (2018) Towards a unified model of SMC complex function. *Curr. Biol.*, **28**, R1266–R1281.
52. Nasmyth,K. and Haering,C.H. (2009) Cohesin: its roles and mechanisms. *Annu. Rev. Genet.*, **43**, 525–558.
53. Paul,M.R., Hochwagen,A. and Ercan,S. (2019) Condensin action and compaction. *Curr. Genet.*, **65**, 407–415.
54. Ghosh,S.K., Huang,C.C., Hajra,S. and Jayaram,M. (2010) Yeast cohesin complex embraces 2 micron plasmid sisters in a tri-linked catenane complex. *Nucleic Acids Res.*, **38**, 570–584.
55. Ghosh,S.K., Hajra,S. and Jayaram,M. (2007) Faithful segregation of the multicopy yeast plasmid through cohesin-mediated recognition of sisters. *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 13034–13039.
56. Prajapati,H.K., Rizvi,S.M., Rathore,I. and Ghosh,S.K. (2017) Microtubule-associated proteins, Bik1 and Bim1, are required for faithful partitioning of the endogenous 2 micron plasmids in budding yeast. *Mol. Microbiol.*, **103**, 1046–1064.
57. Dudziak,A., Engelhard,L., Bourque,C., Klink,B.U., Rombaut,P., Kornakov,N., Janen,K., Herzog,F., Gatsogiannis,C. and Westermann,S. (2021) Phospho-regulated Bim1/EB1 interactions trigger Dam1c ring assembly at the budding yeast outer kinetochore. *EMBO J.*, **40**, e108004.
58. Westermann,S., Drubin,D.G. and Barnes,G. (2007) Structures and functions of yeast kinetochore complexes. *Annu. Rev. Biochem.*, **76**, 563–591.
59. Pinder,J.B., McQuaid,M.E. and Dobson,M.J. (2013) Deficient sumoylation of yeast 2-micron plasmid proteins Rep1 and Rep2 associated with their loss from the plasmid-partitioning locus and impaired plasmid inheritance. *PLoS One*, **8**, e60384.
60. Hadfield,C., Mount,R.C. and Cashmore,A.M. (1995) Protein binding interactions at the *STB* locus of the yeast 2μm plasmid. *Nucleic Acids Res.*, **23**, 995–1002.
61. Sengupta,A., Blomqvist,K., Pickett,A., Zhang,Y., Chew,J.S.K. and Dobson,M.J. (2001) Functional domains of yeast plasmid-encoded Rep proteins. *J. Bacteriol.*, **183**, 2306–2315.
62. Burke,D., Dawson,D. and Stearns,T. (2000) In: *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
63. Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.
64. Struhl,K., Stinchcomb,D.T., Scherer,S. and Davis,R.W. (1979) High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 1035–1039.
65. Li,J.J. and Herskowitz,I. (1993) Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. *Science*, **262**, 1870–1874.
66. McQuaid,M.E., Polvi,E.J. and Dobson,M.J. (2019) DNA sequence elements required for partitioning competence of the *Saccharomyces cerevisiae* 2-micron plasmid *STB* locus *Nucl. Acids Res.*, **47**, 716–728.
67. Estojak,J., Brent,R. and Golemis,E.A. (1995) Correlation of two-hybrid affinity data with in vitro measurements. *Mol. Cell. Biol.*, **15**, 5820–5829.
68. Murray,J.A. and Cesareni,G. (1986) Functional analysis of the yeast plasmid partition locus STB. *EMBO J.*, **5**, 3391–3399.
69. Hinnen,A., Hicks,J.B. and Fink,G.R. (1978) Transformation of yeast. *Proc. Nat. Acad. Sci. U.S.A.*, **75**, 1929–1933.
70. McQuaid,M.E., Mereshchuk,A. and Dobson,M.J. (2019) Insights into the DNA sequence elements required for partitioning and copy number control of the yeast 2-micron plasmid. *Curr. Genet.*, **65**, 887–892.
71. Lorch,Y. and Kornberg,R.D. (2017) Chromatin-remodeling for transcription. *Q. Rev. Biophys.*, **50**, e5.
72. Clapier,C.R., Kasten,M.M., Parnell,T.J., Viswanathan,R., Szerlong,H., Sirinakis,G., Zhang,Y. and Cairns,B.R. (2016) Regulation of DNA translocation efficiency within the chromatin remodeler RSC/Sth1 potentiates nucleosome sliding and ejection. *Mol. Cell*, **62**, 453–461.
73. Hsu,J.M., Huang,J., Meluh,P.B. and Laurent,B.C. (2003) The yeast RSC chromatin-remodeling complex is required for kinetochore function in chromosome segregation. *Mol. Cell. Biol.*, **23**, 3202–3215.
74. Kubik,S., O'Duibhir,E., de Jonge,W.J., Mattarocci,S., Albert,B., Falcone,J.L., Bruzzone,M.J., Holstege,F.C.P. and Shore,D. (2018) Sequence-Directed Action of RSC Remodeler and General Regulatory Factors Modulates +1 Nucleosome Position to Facilitate Transcription. *Mol. Cell*, **71**, 89–102.
75. Cairns,B.R., Lorch,Y., Li,Y., Zhang,M., Lacomis,L., Erdjument-Bromage,H., Tempst,P., Du,J., Laurent,B. and Kornberg,R.D. (1996) RSC, an essential, abundant chromatin-remodeling complex. *Cell*, **87**, 1249–1260.
76. Cairns,B.R., Schlichter,A., Erdjument-Bromage,H., Tempst,P., Kornberg,R.D. and Winston,F. (1999) Two functionally distinct forms of the RSC nucleosome-remodeling complex, containing essential AT hook, BAH, and bromodomains. *Mol. Cell*, **4**, 715–723.
77. Wong,M.C., Scott-Drew,S.R., Hayes,M.J., Howard,P.J. and Murray,J.A. (2002) RSC2, encoding a component of the RSC nucleosome remodeling complex, is essential for 2 micron plasmid maintenance in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **22**, 4218–4229.
78. Utatsu,I., Sakamoto,S., Imura,T. and Toh-e,A. (1987) Yeast plasmids resembling 2 micron DNA: regional similarities and diversities at the molecular level. *J. Bacteriol.*, **169**, 5537–5545.
79. Utatsu,I., Utsunomiya,A. and Toh-e,A. (1986) Functions encoded by the yeast plasmid pSB3 isolated from *Zygosaccharomyces rouxii* IFO 1730 (formerly *Saccharomyces bisporus var. mellis*). *J. Gen. Microbiol.*, **132**, 1359–1365.
80. Toh-e,A. and Utatsu,I. (1985) Physical and functional structure of a yeast plasmid, pSB3, isolated from *Zygosaccharomyces bisporus*. *Nucleic Acids Res.*, **13**, 4267–4283.
81. Chen,X.J., Cong,Y.S., Wesolowski-Louvel,M., Li,Y.Y. and Fukuhara,H. (1992) Characterization of a circular plasmid from the yeast *Kluyveromyces waltii*. *J. Gen. Microbiol.*, **138**, 337–345.
82. Araki,H., Jearnpipatkul,A., Tatsumi,H., Sakurai,T., Ushio,K., Muta,T. and Oshima,Y. (1985) Molecular and functional organization of yeast plasmid pSR1. *J. Mol. Biol.*, **182**, 191–203.
83. Frith,M.C., Saunders,N.F., Kobe,B. and Bailey,T.L. (2008) Discovering sequence motifs with arbitrary insertions and deletions. *PLoS Comput. Biol.*, **4**, e1000071.
84. Sengupta,A. (2000) In: *Molecular analysis of yeast plasmid segregation. Ph.D. Thesis*. Dalhousie University.
85. Dobson,M.J., Yull,F.E., Molina,M., Kingsman,S.M. and Kingsman,A.J. (1988) Reconstruction of the yeast 2μm plasmid partitioning mechanism. *Nucleic Acids Res.*, **16**, 7103–7117.
86. Cashmore,A.M., Albury,M.S., Hadfield,C. and Meacock,P.A. (1986) Genetic analysis of partitioning functions encoded by the 2um circle of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **203**, 154–162.
87. Christianson,T.W., Sikorski,R.S., Dante,M., Shero,J.H. and Hieter,P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene*, **110**, 119–122.
88. McQuaid,M.E. (2015) In: *Contributions of DNA sequence elements, plasmid-encoded proteins and host proteins to maintenance of the yeast 2-micron plasmid. Ph.D. Thesis*. Dalhousie University.
89. Furuyama,S. and Biggins,S. (2007) Centromere identity is specified by a single centromeric nucleosome in budding yeast. *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 14706–14711.
90. Huang,C.C., Hajra,S., Ghosh,S.K. and Jayaram,M. (2011) Cse4 (CenH3) association with the *Saccharomyces cerevisiae* plasmid partitioning locus in its native and chromosomally integrated states: implications in centromere evolution. *Mol. Cell. Biol.*, **31**, 1030–1040.
91. Tang,L., Nogales,E. and Ciferri,C. (2010) Structure and function of SWI/SNF chromatin remodeling complexes and mechanistic implications for transcription. *Prog. Biophys. Mol. Biol.*, **102**, 122–128.
92. Ng,H.H., Robert,F., Young,R.A. and Struhl,K. (2002) Genome-wide location and regulated recruitment of the RSC nucleosome-remodeling complex. *Genes Dev.*, **16**, 806–819.
93. D'Ambrosio,C., Schmidt,C.K., Katou,Y., Kelly,G., Itoh,T., Shirahige,K. and Uhlmann,F. (2008) Identification of cis-acting sites for condensin loading onto budding yeast chromosomes. *Genes Dev.*, **22**, 2215–2227.
94. Lengronne,A., Katou,Y., Mori,S., Yokobayashi,S., Kelly,G.P., Itoh,T., Watanabe,Y., Shirahige,K. and Uhlmann,F. (2004) Cohesin

relocation from sites of chromosomal loading to places of convergent transcription. *Nature*, **430**, 573–578.
95. Wang,B.D., Eyre,D., Basrai,M., Lichten,M. and Strunnikov,A. (2005) Condensin binding at distinct and specific chromosomal sites in the *Saccharomyces cerevisiae* genome. *Mol. Cell. Biol.*, **25**, 7216–7225.
96. Coursey,T.L. and McBride,A.A. (2019) Hitchhiking of viral genomes on cellular chromosomes. *Annu. Rev. Virol.*, **6**, 275–296.
97. Malik,H.S. and Henikoff,S. (2009) Major evolutionary transitions in centromere complexity. *Cell*, **138**, 1067–1082.
98. Drinnenberg,I.A., Henikoff,S. and Malik,H.S. (2016) Evolutionary turnover of kinetochore proteins: a ship of Theseus?*Trends Cell Biol.*, **26**, 498–510.