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# Stable propagation of the yeast 2 micron plasmid: Equal segregation by hitchhiking on chromosomes.

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# Stable propagation of the yeast 2 micron plasmid: Equal segregation by hitchhiking on chromosomes.

**by**

**Keng-Ming Chang, B.Sc.**

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# Dedication

To my family and my friends

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**Stable propagation of the yeast 2 micron plasmid: Equal segregation by hitchhiking on chromosomes.**

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The 2 micron plasmid of *Saccharomyces cerevisiae* resides in the nucleus as an extra-chromosomal element with a steady state copy number of 40-60 per cell. As a benign but selfish DNA element, the plasmid utilizes a self-encoded partitioning system and an amplification system to ensure its stable, high-copy propagation. The partitioning system consists of the plasmid encoded proteins, Rep1 and Rep2 and a *cis*-acting partitioning locus *STB*. The Rep proteins, together with several host factors, assembled at *STB* couple plasmid segregation to chromosome segregation. A plasmid lacking an active partitioning system is subject to a 'diffusion barrier', which causes it to be retained in the mother cell with a strong bias (mother bias). Currently available evidence favors the hitchhiking model for plasmid segregation, in which the tethering of plasmids to chromosome provides the basis for faithful plasmid partitioning. However, direct

evidence to support this hypothesis has been difficult to obtain because of the small size of the budding yeast nucleus and the poor resolution of chromosomes in live cells or in chromosome spreads.

In this study, we have attempted to verify the hitchhiking model using single copy derivatives of the 2 micron plasmid as reporters. We demonstrate, using two single copy reporters present in the same nucleus, that plasmid association with chromosome spreads is authentic, and is dependent on the partitioning system. By using a strategy that forces all chromosomes to stay in either the mother or the daughter compartment, we show that plasmid segregation can be uncoupled from nuclear envelope segregation. However, plasmid segregation cannot be uncoupled from chromosome segregation under this condition. This tight coupling between plasmid and chromosome segregation is consistent with the hitchhiking model for plasmid segregation.

The plasmid partitioning complex is assembled *de novo* at *STB* during each cell cycle during the G1-S window. Plasmid replication or cell cycle cues that signal cellular DNA replication appear to trigger this assembly. Furthermore, there is an apparent temporal hierarchy in the association and dissociation of protein factors at *STB*. When DNA replication is delayed or blocked, the dissociation of factors from *STB* from the previous portioning cycle and the association of factors for the new partitioning cycle are delayed or blocked, respectively. The precise role of replication in plasmid segregation has not been elucidated. We have addressed this question by blocking either plasmid replication or all cellular DNA replication. We find that replication is not required for plasmid to overcome mother bias. However, replication is critical for the equal

segregation of sister plasmid copies. These results provide a refinement of the hitchhiking model by suggesting that sister plasmids tether to sister chromatids in a replication-dependent manner and hitchhike on them during chromosome segregation.

Finally, we have attempted to reconstitute the 2 micron plasmid partitioning system in mammalian cells with the goal of exploiting their larger nuclear size and the considerably higher chromosome resolution they provide. In experiments completed so far, we show that Rep2 expressed in COS7 cells localizes to chromosomes, and Rep1 does so in the presence of Rep2. Furthermore, they show co-localization on sister chromatids in a symmetric fashion, implying that plasmids associated with them are likely to follow suit. These observations suggest, by extrapolation, the Rep1-Rep2 assisted association of sister plasmids with sister chromatids in yeast as well, and are consistent with the refined hitchhiking model for plasmid segregation.

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# CHAPTER 1

# Introduction

## 1.1 Persistence of selfish extra-chromosomal genetic elements

There are two alternative perceptions of selfish genetic elements. In one view posited by Dawkins (Dawkins, 1976), a selfish element enhances its own fitness by conferring some benefit on its host, whose well-being is essential for its survival. In a distinct view proposed by Crick, Doolittle and Sapienza (Doolittle and Sapienza, 1980; Orgel and Crick, 1980), the selfishness of a genetic element is manifested by its ability to replicate and spread efficiently, regardless of whether it is beneficial or not to its host. The studies presented in this thesis concern mechanisms of selfishness of the latter type.

When a genetic element does not provide an advantage to its host organism, there is no obvious selective pressure for its maintenance over evolutionary time. The long-term survival of such a selfish genome poses a challenge, and must require specialized molecular strategies. Fundamental to such strategies is the ability of the element to replicate efficiently, often using the host replication machinery. Subsequent to replication, there must be a mechanism that ensures the distribution of the replicated copies to mother and daughter cells at the time of cell division. Finally, the steady state population of the element in a cell must be retained at a near constant level to guard against dilution and eventual elimination.

A fail-safe mode of stable propagation for a selfish element is to be integrated into the chromosome or chromosomes of its host, as exemplified by bacterial insertion

sequences, transposons and repeated DNA elements as well as integrated states of prokaryotic and eukaryotic viruses (Burt and Trivers, 2006). Extra-chromosomal selfish DNA molecules, which by definition cannot become an integral part of the host genome, face a more arduous challenge for their continued propagation in host populations over multiple generations. Circular and linear plasmids found in prokaryotes, circular plasmids present in select species of budding yeasts and episomes of certain mammalian viruses accomplish this task through a variety of replication-partitioning-copy number control mechanisms. The investigations summarized in this thesis focus on the functional attributes of the partitioning system utilized by the 2 micron plasmid of the budding yeast *Saccharomyces cerevisiae.*

### 1.1.1 Segregation of extra-chromosomal circles in prokaryotes

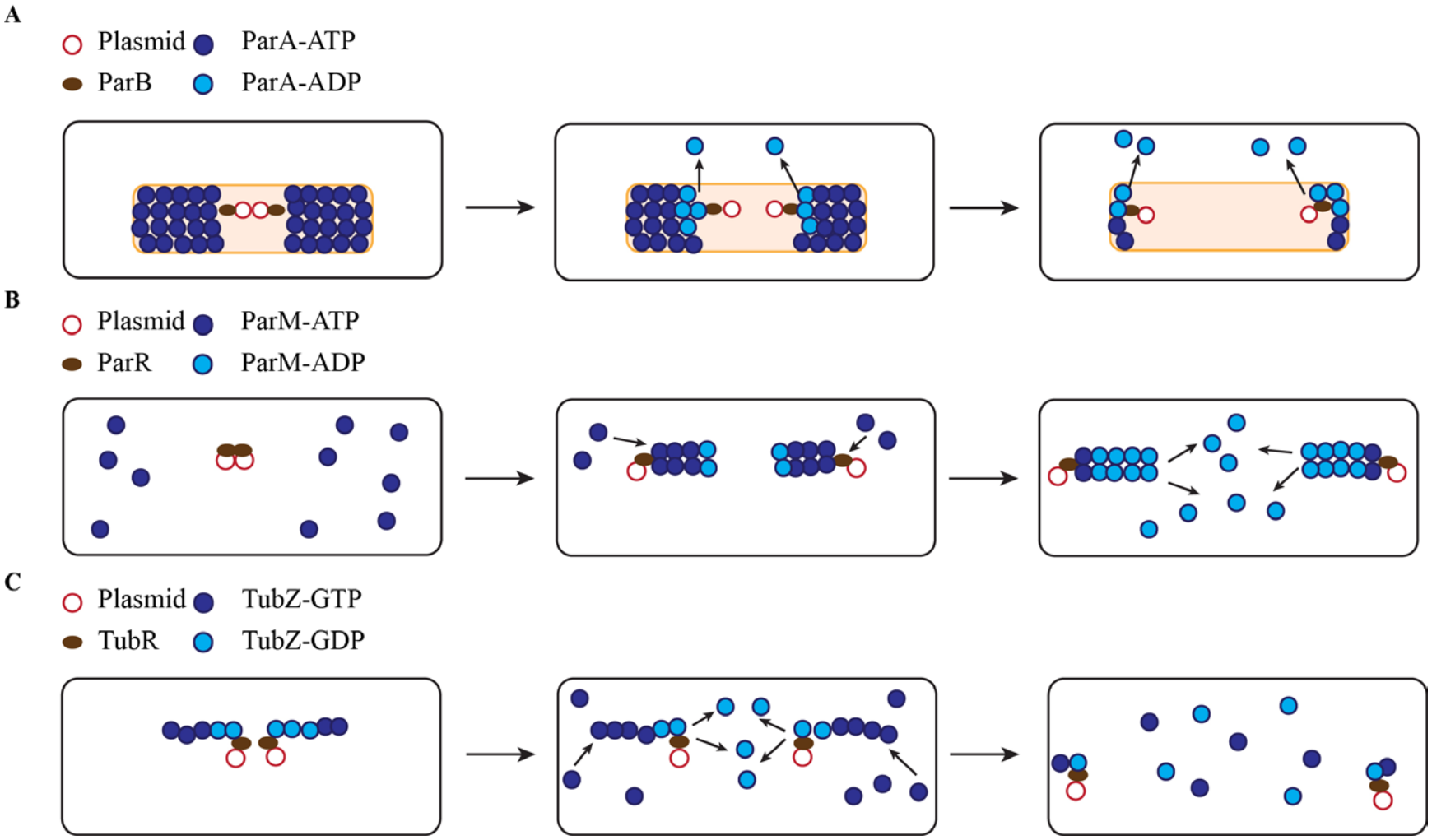
The extra-chromosomal elements in prokaryotes consist of conjugative and non-conjugative plasmids as well as viruses such as P1 that do not integrate into the host chromosome. The F factor of *Escherichia coli*, responsible for sex determination, can have either an autonomous existence as an episome or can be integrated into the chromosome. The majority of prokaryotic plasmids have a circular organization, and can vary in copy number from one or a few to tens of plasmid molecules per cell. The mechanisms for stability differ among plasmids, depending on their copy numbers. Most high copy plasmids segregate by a random mechanism. The chance of a cell losing plasmid during cell division decreases as the copy number increases. Differences in copy number due to unequal segregation can be corrected by regulation at the level of replication. Higher than normal copy number suppresses replication and lower than

normal copy number induces multiple rounds of replication. Therefore the mean plasmid copy number in the population can be maintained at a steady state value.

Random segregation is not useful for the stable propagation of low copy number plasmids. For a copy number of one, the predicted loss rate under random segregation would be roughly 30% (based on Poisson distribution). Low copy plasmids harbor partitioning mechanisms for actively segregating replicated molecules to daughter cells during division. The general organization of bacterial plasmid partitioning systems consists of two partitioning proteins (Par proteins) and a partitioning locus (Par locus), often referred to as the plasmid centromere (Gerdes et al., 2000; Jayaram et al., 2004a; Schumacher, 2012). The Par locus consists of multiple copies of a consensus repeat element. One of the Par proteins is a DNA binding protein that interacts with the Par locus. The second Par protein, which interacts with its DNA binding Par partner, is an active ATPase belonging to the Walker family or the actin family of ATPases or a tubulin related GTPase. The energy of hydrolysis of the NTP is utilized in different ways to move plasmid molecules to locations on opposite sides of the division septum. Variations of this shared organizational and functional theme underlie the partitioning schemes of plasmids in prokaryotes.

In the type I partitioning system of the low copy P1 plasmid, the ParA protein (an ATPase) polymerizes along the nucleoid in an ATP-dependent manner and interacts with its partner ParB protein bound to the *par*S plasmid centromere (Ringgaard et al., 2009; Vecchiarelli et al., 2010). This interaction stimulates ATP hydrolysis causing the retraction of the filament along with plasmid. The asymmetric localization of the filament

along the nucleoid provides the motive force for plasmid partitioning (Fig 1.1A). By contrast, in the type II system, the actin-like ParM ATPase forms dynamic filaments that grab the plasmid sisters by binding to ParR (ParM partner) associated with *par*C (par locus) (Salje et al., 2010). The orientation of the replicated sister plasmids with respect to the growing filaments is such that they are moved away from each other to be localized in daughter cells (Fig 1.1B). The type III system is characterized by a tubulin based mechanism for plasmid segregation (Chen and Erickson, 2008; Larsen et al., 2007; Tang et al., 2007). In this system, the active nucleotide is GTP instead of ATP. The nucleotide binding Par protein forms double stranded filaments that associate with the second Par protein bound to the par locus. This interaction stimulates GTP hydrolysis and causes treadmilling, with the elongation of the filament at the plus end and its regression at the minus end (Aylett et al., 2010; Salje et al., 2010). As a result, the attached plasmid will be relocated towards opposite poles of the cell. In all three systems, a critical step in plasmid segregation is the polymerization of a nucleotide binding protein into filaments that searches for and associates with the second partitioning protein bound to the plasmid. The generation of the motive force by NTP hydrolysis assists in pushing sister plasmids apart by filament extension, or pulling them away from each other by filament contraction or localizing them in a biased fashion by treadmilling and filament translocation. The type III tubulin based mechanism has features reminiscent of the spindle based mechanism for the segregation of eukaryotic chromosomes. (Fig 1.1C)




**(Figure 1.1 , full caption next page.)**

**Figure 1.1 The three Types of systems of bacterial plasmid segregation.**

(A) In the type I system, the ATP-bound form of the partitioning protein, [ParA-ATP], binds DNA along the nucleoid. When ParA-ATP binds to ParB, its ATPase activity is stimulated. ParA-ADP will be released from the nucleoid. The dynamic interaction between ParA and the ParB-centromere complex causes an uneven distribution of the ParA molecules along the nucleoid. This will provide the motive force for plasmid segregation towards opposite poles. The ATP bound form of ParA is regenerated from the ADP bound form by nucleotide exchange. The figure is modified from Vecchiarelli et al., 2010. (B) In the type II system, the plasmid binding protein ParR interacts with the ParM ATPase. This DNA-protein complex nucleates the oligomerization of ParM into a filament, which pushes the plasmid sisters away from mid-cell position towards the poles. Filament elongation occurs by the addition of ParM monomers at the end proximal to the plasmid centromere (*par*C). ATP hydrolysis and conversion of ParM-ATP to ParM-ADP result in the disassembly of the filament at the distal end. Nucleotide exchange converts the ParM-ADP into the active ParM-ATP form. The figure is modified from Gerdes et al., 2014. (C) In the type III system, the filament formed by the Par GTPase (TubZ) binds to its partner Par protein (TubR) associated with the centromere loci of a pair or sister plasmids. The stimulation of GTPase following this interaction results in the shortening of the filament at the centromere proximal end (referred to as the minus end) and growth of the filament at the distal end (plus). This treadmill-like action drags the plasmid towards the cell pole. The figure is adapted from Ni et al., 2010.

### 1.1.2 Extra-chromosomal circles and their maintenance in eukaryotes

In contrast to prokaryotes, eukaryotes generally do not harbor plasmids. A rare exception to this rule is a subset of fungal species belonging to the Saccharomycetaceae lineage (Malik and Henikoff, 2009; Volkert et al., 1989). The circular DNA plasmids present in these yeasts are similar in size as well as genetic organization, suggesting their evolutionary relatedness (Blaisonneau et al., 1997; Utatsu et al., 1987). The 2 micron plasmid in *S. cerevisiae* is the most well characterized representative within the group of budding yeast plasmids.

The 2 micron plasmid resides in the nucleus at a steady state copy number of 40-60 molecules per cell (Jayaram et al., 2004c). As a benign but selfish extra-chromosomal element, the plasmid utilizes a self-encoded partitioning system and a copy number maintenance system for its high-copy propagation with nearly the same stability as chromosomes. The plasmid partitioning system appears to direct several host factors towards fulfilling its segregation needs. Following replication of each plasmid molecule once per cell cycle, the replicated copies are distributed evenly into daughter cells. A decrease in plasmid copy number, caused by occasional plasmid missegregation, is corrected by a DNA amplification mechanism. However, the plasmid engenders regulatory functions that prevent over-amplification, which would impose an undue metabolic burden on the host. Thus, the plasmid appears to have optimized its selfishness to maximize the benefits it derives from the host while minimizing the fitness cost to the host (Jayaram et al., 2004b; 2004c). Benign as well as malignant parasite genomes, widely distributed from yeast to mammals, appear to share certain common or related

molecular strategies for their long-term propagation. The predominant majority of such genomes are present as chromosomal integrants. The average number of copies of an element per host genome is apparently limited within a narrow range for the integrated elements as well. The 2 micron plasmid system provides a simple model to study how selfish genetic elements establish stable maintenance in a eukaryotic host.

The episomes of viruses of the gamma herpes and papilloma families represent plasmid-like circular DNA elements that are present in the nuclei of infected mammalian cells. They persist stably for extended periods of time during the latent phase of infection. The well characterized members among these viruses include Epstein-Barr virus (EBV), Kaposi's sarcoma associated herpes virus (KSHV) and human and bovine papillomaviruses (HPV and BPV, respectively). These viral episomes can undergo two types of replication: a tightly regulated mode of replication once per cell cycle during latency, and an amplification mode of multiple rounds of replication during acute infection (Doorbar, 2007; Hammerschmidt and Sugden, 1988; Ilves et al., 2003; Kadaja et al., 2009; Mesri et al., 2010; Murata and Tsurumi, 2013). These replication modes are analogous to the steady state and copy number restoration modes of replication of the 2 micron plasmid, respectively.

The common strategy employed by the viral episomes to ensure their persistence in the host is tethering to the host chromosomes and segregating by hitchhiking. A virally encoded partitioning protein, which interacts with a partitioning locus on the viral genome, mediates chromosome tethering by interacting with a chromatin binding host protein (Botchan, 2004; Frappier, 2004; Ilves et al., 1999; Lehman and Botchan, 1998;

You et al., 2004). In some instances, the viral partitioning protein may directly associate with the host chromosomes. The advantage to the virus of tethering to chromosomes is two-fold. The virus not only gains the sophistication of chromosome segregation but also avoids its loss in the cytoplasm due to disassembly and reassembly of the nuclear envelope during the open mitosis of mammalian cells.

#### 1.1.2.1 Viral episomes and chromosome tethering: maintenance of EBV episomes

Epstein-Barr virus (EBV), a large double-stranded circular DNA approximately 172 kbp long, is a well-studied example for an episomal virus that persists in cells via chromosome tethering (Kanda et al., 2001; Lindner and Sugden, 2007; Nanbo et al., 2007). The main *cis* element responsible for its replication and maintenance is called oriP. It is composed of a sequence segment with a **d**yad **s**ymmetry (DS) and a **f**amily of **r**epeat (FR) elements (Baer et al., 1984; Reisman et al., 1985). DS is required for replication initiation during the latent stage, while FR is required for transcription activation and episome retention. Both the DS and FR segments contain the binding site for the viral protein EBNA-1. The DS segment contains four binding sites for the protein whereas FR contains 21 copies of an imperfect 20 bp repeat element with 20 high affinity binding sites (Reisman et al., 1985; Summers et al., 1996; Wysokenski and Yates, 1989). EBNA-1 associates with the host EBNA-1 binding protein 2 (hEBP2) to assist partitioning of the episome copies formed by viral replication. EBP2 is a conserved nucleolus-associated protein among eukaryotes, which functions in ribosome biogenesis (Kapoor and Frappier, 2003a; Tsujii et al., 2000; Wu, 2000). As hEBP2 also binds to

chromosomes, the EBNA1-hEBP2 interaction promotes tethering of viral episomes to chromosomes (Kanda et al., 2007). The segregation of EBV episomes during the latent stage appears to be non-random, suggesting that the viral episomes do not associate with chromosomes in a random manner (Nanbo et al., 2007). In mitotic chromosome spreads EBNA-1 foci are seen as symmetrically localized on sister chromatids (Kanda et al., 2007). Furthermore, EBV genomes are localized in between paired EBNA1 foci. Additional evidence suggests that a pair of EBV sisters formed by replication tend to associate with identical or closely spaced sites on sister chromatids (Kanda et al., 2007). It is suggested that as sister chromatids separate from each other and move into daughter cells in a one-to-one fashion during anaphase, the associated episomes achieve non-random (symmetric) segregation. (Fig. 1.2)

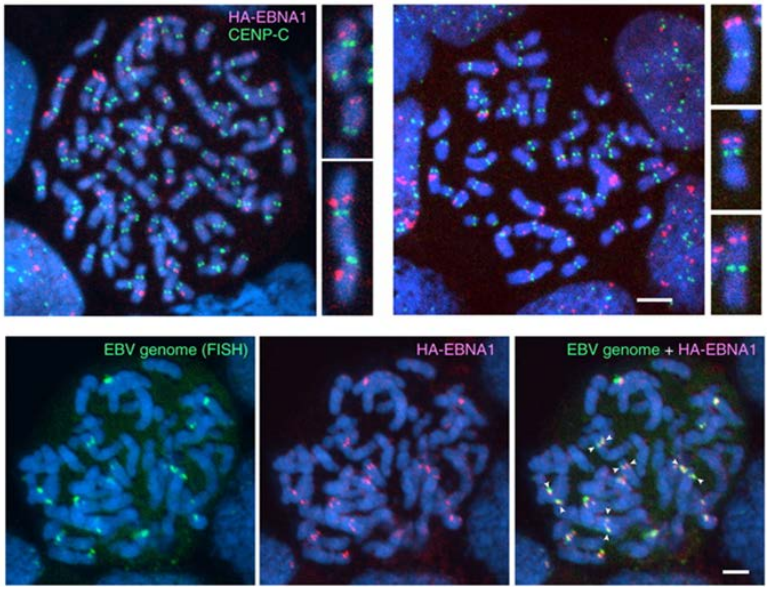



**Figure 1.2 Symmetrical distribution of EBNA-1 and EBV genomes on sister chromatids.**

(A) The localization of EBNA-1 (red) and a centromere binding protein CENP-C (green) on mitotic chromosome spreads is performed by immunofluorescence assay (Kanda et al., 2007). (B) The localization of EBV genome (green) and EBNA-1 (red) in mitotic chromosome spreads is scored by FISH (fluorescence in situ hybridization) and immunofluorescence, respectively. The figure is adapted from Kanda et al. 2007.

The symmetric mode of chromosome tethering and segregation in association with sister chromatids are unlikely to be general features of viral episomes. For example, BPV-1 episomes are not symmetrically distributed on chromosomes (Oliveira et al., 2006). However, the interaction between a virus encoded protein, which binds directly to the viral partitioning locus, and a chromosome binding host protein as the key underlying strategy for chromosome tethering appears to operate across viral families.

#### 1.1.2.2 Potential relatedness between the partitioning mechanisms of viral episomes and yeast plasmids

As briefly noted earlier, viral episomes of mammals and the yeast 2 micron plasmid share certain similarities in their life styles. They normally undergo regulated replication, and are partitioned efficiently during cell division. However, they are also capable of raising their copy number by amplification under special circumstances. As will become evident from the concluding sections of this chapter, several lines of circumstantial evidence suggest that the 2 micron plasmid might also utilize chromosome tethering for its stable maintenance (Cui et al., 2009; Ghosh et al., 2007; Hajra et al., 2006; Huang et al., 2011; Jayaram et al., 2004c; Liu et al., 2013; Ma et al., 2013; Mehta et al., 2002; Velmurugan et al., 2000). However, unlike viral episomes, the plasmid does not face the danger of being excluded from the nucleus into the cytoplasm during mitotic cell divisions. Budding yeasts, like fungi in general, carry out closed mitosis, without breakdown of the nuclear envelope. It is possible that chromosome tethering by viral episomes and the yeast plasmid represent the evolutionary divergence of a common survival strategy utilized by eukaryotic extra-chromosomal elements. Alternatively the

viral and plasmid systems may have independently arrived at chromosome associated partitioning via convergent evolution. In either case, it is not surprising that selfish genomes might acquire, by common or distinct evolutionary routes, mechanistically related strategies that elevate their segregation fidelity to nearly that of the chromosomes of their hosts.

## 1.2 The 2 micron plasmid: highly stable extra-chromosomal circles in S. *cerevisiae*

The stability of the 2 micron plasmid is comparable, within α factor of 10, to that of the *S. cerevisiae* chromosomes (a plasmid loss rate of $10^{-5}$ to $10^{-4}$ per cell division). As mentioned previously, the functional organization of the plasmid, its replication origin aside, is comprised of the plasmid partitioning and amplification systems. Under its normal life style, during steady state growth of the host cells, the doubling of plasmid copy number during S phase is followed by equal segregation during anaphase. The amplification system is brought into play only in cells experiencing a drop in copy plasmid copy number from the normal steady state value.

### 1.2.1 The organization of the 2 micron plasmid genome

The 6,318 bp 2 micron plasmid genome (Hartley and Donelson, 1980) contains four *cis*-acting DNA elements and four protein coding regions. All of the *cis*-acting loci and *trans*-acting proteins are essential for the stable, high copy maintenance of the plasmid (Broach et al., 1979; Chang et al., 2013; Hartley and Donelson, 1980). The plasmid genome is divided into two unique regions by a pair of 599 bp inverted repeats,

each of which includes the target (*FRT* = Flp recombination target) site for the plasmid coded site-specific recombinase Flp. The recombination between the two *FRT* sites mediated by Flp leads to two isomeric forms of the 2 micron plasmid (Broach, 1982) (Fig 1.3). The recombination reaction is critical in controlling the copy number of the plasmid (Futcher, 1986; Volkert and Broach, 1986). The Raf1 protein is also involved in copy number control, and is believed to act by positively regulating *FLP* gene expression (Murray et al., 1987; Reynolds et al., 1987; Som et al., 1988). The plasmid replication origin (*ORI*), which partially overlaps with one of the inverted repeats, is functionally analogous to origins present on chromosomes. The replication of each plasmid occurs once per cell cycle by a single firing of *ORI* early during the S phase (Zakian et al., 1979). The two plasmid encoded proteins Rep1 and Rep2, together with the *STB* (**st**a**b**ility conferring) locus, are responsible for the efficient partitioning of the 2 micron circle during cell division (Jayaram et al., 1983; Kikuchi, 1983). The *STB* locus is located a few hundred bp away from the *ORI*. *STB* can be divided into two sub-regions, *STB*-proximal and *STB*-distal, based on their relative locations with respect to *ORI* (Murray and Cesareni, 1986). *STB*-proximal contains five tandem direct repeats of a 60 bp AT-rich consensus element, and is primarily responsible for the partitioning function. *STB*-distal contains a transcription terminator that prevents transcription from going through the *STB*-proximal and *ORI* regions (Fig 1.4). Maintaining *STB*-proximal as a transcription-free zone may be important for its partitioning function (Jayaram et al., 1983; Murray and Cesareni, 1986). Its location with respect to *ORI* may also be important

in this regard. It has been generally noted that 2 micron derived artificial plasmids can vary considerably in their stability, depending on the location of *STB* within them.

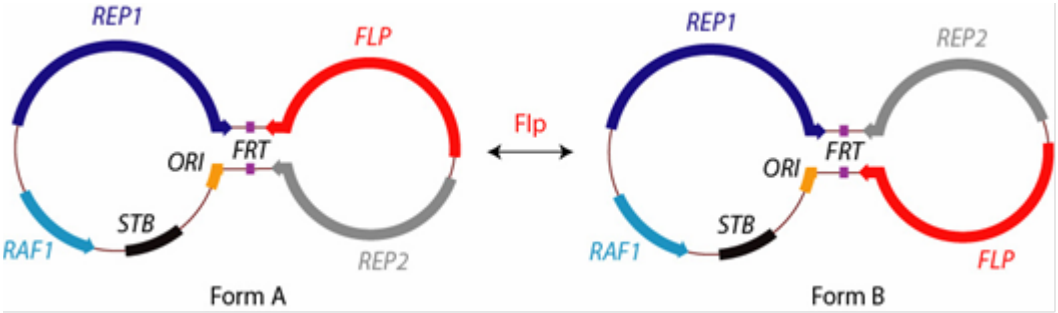



**Figure 1.3 Genetic and functional organization of the 2 micron plasmid.**

The arrangement of the *cis*-acting (*ORI*; *STB*; *FRT* sites) and *trans*-acting (*REP1*; *REP2*; *FLP*; *RAF1*) loci are indicated in the schematic representation of the 2 micron plasmid genome. The parallel lines denote a 599 bp inverted repeat, within which the *FRT* sites are located. Association of the Rep1 and Rep2 proteins with the *STB* locus is an essential step for equal plasmid segregation. The A and B forms of the plasmid arise as a result of Flp mediated recombination between the *FRT* sites. A Flp recombination event coupled to bi-directional plasmid replication provides the trigger for the amplification reaction (see Fig. 1.5). Raf1 serves as the positive regulator of amplification by helping to turn on Flp expression. Rep1 and Rep2 form a bipartite negative regulator that represses Flp. Raf1 is thought to antagonize the Rep1-Rep2 repressor. This figure is adapted from Velumurugan et al., 2003.

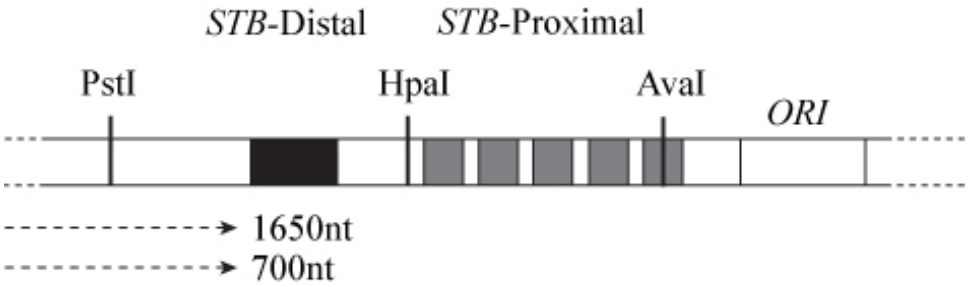



**Figure 1.4 Organization of the 2 micron circle *STB* element.**

The *STB* locus is located between the PstI and AvaI sites on the large unique region of the 2 micron plasmid genome, and can be further divided into two sub-regions: *STB*-proximal (HpaI-AvaI) and *STB*-distal (HpaI-PstI), relative to the position of the plasmid replication origin (*ORI*). *STB*-proximal contains 5 tandem direct repeats of a 60 bp consensus AT-rich element, and appears to encompass the functional part in plasmid partitioning. *STB*-distal contains a transcription terminator, which prevents transcription from going through *STB*-proximal region. Two plasmid transcripts directed toward the *STB*-proximal are terminated within *STB*-distal. *STB*-proximal also contains a silencer element, which can down-regulate the activity of a promoter placed proximal to it. The figure is adapted from Chang et al., 2013.

### 1.2.2 The 2 micron plasmid partitioning system

The 2 micron plasmid partitioning system, composed of two plasmid coded proteins (Rep1, Rep2) and a partitioning locus (*STB*) assembled from an iterated set of a consensus sequence element, is similar in organization to the bacterial partitioning systems described earlier. However, there appears to be little or no functional similarity between the yeast and bacterial systems. Neither Rep1 nor Rep2 protein contains peptide motifs typical of NTPase activity. The association of Rep1 and Rep2 with *STB* appears to be dependent upon a host factor (or factors) (Hadfield et al., 1995; Yang et al., 2004), although weak binding of Rep2 to *STB* DNA in vitro has been demonstrated (Sengupta et al., 2001). It is nevertheless possible that the yeast plasmid Rep-*STB* system and the bacterial *par* system might have diverged from a common ancestral partitioning system, and then adapted to their widely differing biological contexts. The tyrosine family site-specific recombinases (Grindley et al., 2006), to which Flp belongs, are widely prevalent among prokaryotes but absent in eukaryotes, except for the budding yeast lineage. It is not unlikely, therefore, that the yeast plasmid might have had a bacterial origin, and might have been acquired by an ancestor of budding yeasts by horizontal transmission.

Since the main focus of this thesis is the functional characterization of the 2 micron plasmid partitioning system, several of its known attributes will be emphasized, under appropriate sections throughout this thesis. For now, it may be noted that the *STB* locus provides a platform for the assembly of the plasmid partitioning complex in a Rep1 and Rep2 assisted manner. Among the host factors identified at *STB* are components of the RSC2 chromatin remodeling complex, the nuclear motor Kip1, the cohesin complex

and the histone H3 variant Cse4, which replaces histone H3 in the specialized nucleosome at the point centromere of budding yeast chromosomes (Cui et al., 2009; Ghosh et al., 2007; Hajra et al., 2006; Huang et al., 2011; Ma et al., 2013; Mehta et al., 2002; Wong et al., 2002). In addition to Cse4, the other *STB*-associated host factors are also present at centromeres and play important roles in chromosome segregation. In contrast to the situation at centromeres, the amounts of cohesin and Cse4 detected at *STB* are highly sub-stoichiometric. The functional significance, if any, of this substoichiometric association is not understood. Since the 2 micron plasmid forms foci containing groups of plasmid molecules (see below **1.2.5**), cohesin and Cse4 may act at the level of the entire group rather than individual plasmid molecules (Ghosh et al., 2007; Huang et al., 2011). This group behavior might be advantageous to the plasmid in limiting its utilization of host factors whose primary functions are in chromosome transactions, including segregation. An alternative possibility, though quite unlikely, is that a subset of the host factors present at *STB* may not be relevant to plasmid segregation. It has been argued, based upon evolutionary considerations, that the unusual point centromere of *S. cerevisiae* might have originated from the partitioning locus of an ancestral 2 micron plasmid (Malik and Henikoff, 2009). If this is true, the association of common host factors at *STB* and *CEN* may reflect their shared evolutionary history and not necessarily their requirement in present day plasmid partitioning.

An *STB* reporter plasmid colocalizes in the yeast nucleus with Rep1 and Rep2 proteins; by contrast an *ARS* reporter plasmid, containing a replication origin but lacking *STB*, does not. Furthermore, Rep1 and Rep2 colocalize in chromosome spreads prepared

from mitotic cells in a mutually dependent, but *STB*-independent, manner. An *STB* plasmid is also found associated with chromosome spreads, but only when both Rep1 and Rep2 are expressed in the host strain (Velmurugan et al., 2000). Viewed in the context of the possibility of chromosome-associated plasmid segregation (the 'hitchhiking model'), these findings suggest that Rep1 and Rep2 direct the 2 micron plasmid foci to their tethering sites on the chromosomes. Experiments testing the deeper implications of the hitchhiking model form the central core of this thesis.

### 1.2.3 2 micron plasmid amplification system: controlling the plasmid copy number

The plasmid amplification system is a safeguard against a decrease in copy number due to rare missegregation events (Futcher, 1986; Volkert and Broach, 1986). However, under normal growth conditions, the amplification system is rarely triggered, indicating the high efficiency of the partitioning system. Density shift-equilibrium gradient centrifugation experiments have shown that, during steady state growth, over 95% of the plasmid molecules undergo only one round of replication during one cell generation (Zakian et al., 1979).

According to the currently accepted model, the amplification reaction is initiated by a recombination event, during bi-directional replication, between a copy of the duplicated origin-proximal *FRT* site and the unduplicated origin-distal *FRT* site (Futcher, 1986; Volkert and Broach, 1986) (Fig 1.5). The result of such a carefully timed recombination event is the inversion of one replication fork with respect to the other. The two forks will then chase each other around the circular template to produce a concatemer

consisting of multiple tandem copies of the plasmid. Individual plasmid molecules can be resolved from this amplified DNA by Flp mediated site-specific recombination or by homologous recombination mediated by the host machinery. The Raf1 protein serves as a positive regulator of *FLP* gene expression, thereby accelerating the amplification response. The Rep1 and Rep2 proteins appear to form a bipartite repressor for the negative regulation of *FLP* and *RAF1* expression. Raf1 is thought to antagonize the Rep1-Rep2 repressor. The balance between the negative and positive regulation of *FLP* expression is the key to plasmid maintenance without significant fluctuations from its steady state copy number (Murray et al., 1987; Reynolds et al., 1987; Som et al., 1988). The Rep1-Rep2 repressor also negatively regulates *REP1* expression, but appears not to affect *REP2* expression. Thus, the level of the Rep1 protein may provide an indirect readout of the plasmid copy number as well as determine the effective concentration of the repressor. This modulation of the repressor as a function of copy number is at the heart of 2 micron plasmid gene regulation for ensuring a quick amplification response as needed without the risk of runaway amplification.

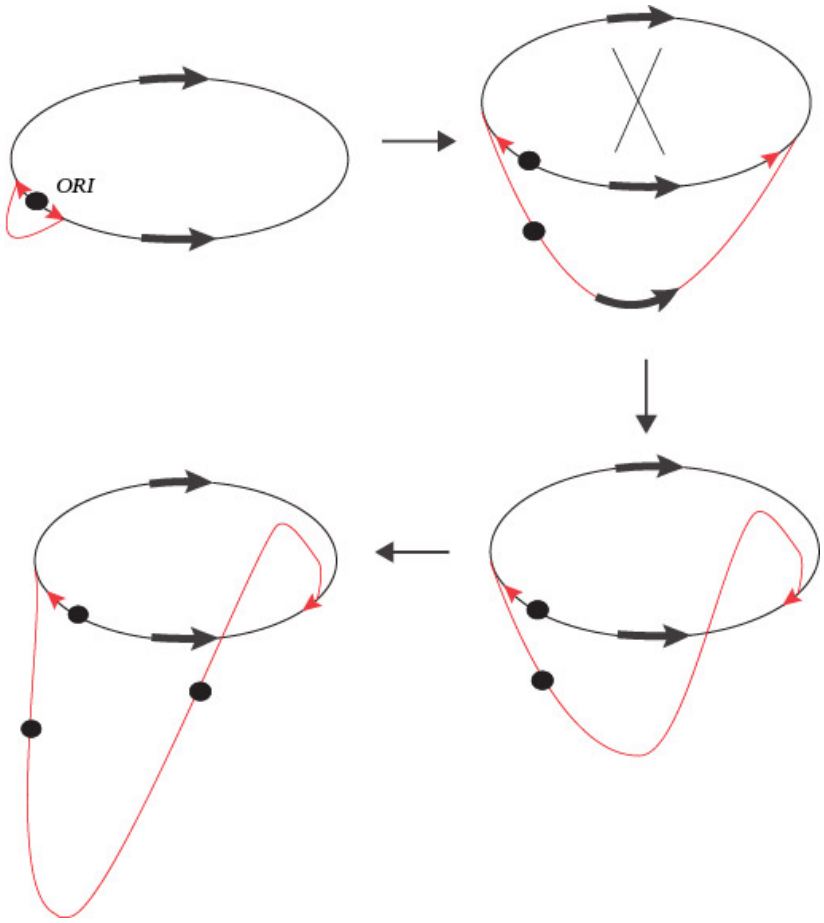



**Figure 1.5 The 2 micron plasmid amplification system for copy number maintenance.**

The amplification model (Futcher, 1986) postulates a Flp mediated recombination event during bidirectional plasmid replication when only the origin-proximal *FRT* site has been duplicated. The crossover between the distal *FRT* site and one of the two copies of the proximal *FRT* site inverts one replication fork with respect to the other. As a result, replication switches to a uni-directional rolling circle mode. The amplified DNA containing tandem copies of the plasmid can be subsequently resolved into single plasmid units. A second recombination event can restore bi-directional replication to stop the amplification. The *FRT* sites and their relative orientation are shown by the thick black arrows. The red arrowheads denote the leading edges of replication.

### 1.2.4 Chromosome segregation in budding yeast

Since the central hypothesis tested in the present study concerns chromosome associated segregation of the yeast plasmid, the essential features of chromosome segregation that directly pertain to the rationale and design of several experiments are briefly outlined here.

As the survival of a cell is critically dependent on a normal complement of chromosomes, chromosome segregation is a highly coordinated and tightly regulated event with multiple checkpoints. Each chromosome is replicated only once during the S phase of a cell cycle. Sister chromatids thus formed are held together by a multi-subunit protein complex called the cohesin complex, so as to promote the bi-oriented attachment of their kinetochores to the mitotic spindle during G2/M (Guacci et al., 1997; Michaelis et al., 1997). The proper alignment of sister chromatids generates tension as a result of the pulling force exerted by the spindle in opposite directions (Santaguida and Musacchio, 2009). The spindle checkpoint ensures correct tension before permitting the progression of the cell cycle beyond metaphase. At the onset of anaphase, this checkpoint is inactivated, and securin is degraded to release separase, which disassembles the cohesin complex by cleavage of its Mcd1 subunit (Uhlmann, 2001; Uhlmann et al., 1999). The unpaired sister chromatids are then pulled apart towards opposite cell poles as a result of spindle dynamics (Buvelot et al., 2003; DeLuca, 2007; Onn et al., 2008; Pan and Chen, 2004; Tanaka et al., 2000; Uhlmann, 2001) (Fig. 1.6). One of the key experimental strategies employed in the present studies is to design single copy versions of *STB* reporter plasmids, so that the behavior of plasmid sisters formed by replication

can be followed under conditions of normal chromosome segregation or under conditions that force missegregation of sister chromatids.

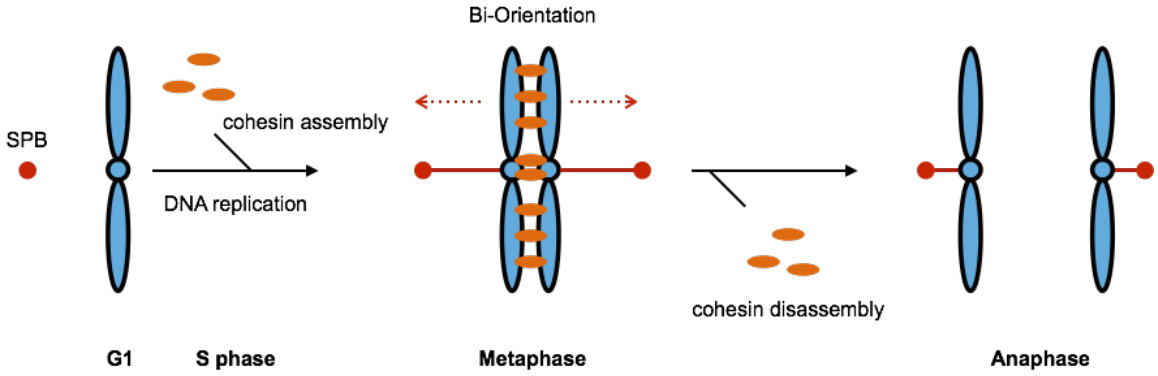



**Figure 1.6 Faithful segregation of sister chromosomes formed by replication.**
The cohesin complex associates with chromosomes during late G1 phase, and establishes cohesion of replicated sister chromatids during S phase. The cohesed sisters are bi-oriented on the mitotic spindle (red line) by the attachment of sister kinetochores to microtubules emanating from opposite spindle pole bodies (SPB). When cohesin is disassembled at anaphase, the sister chromatids separate and move to opposite cell poles.

### 1.2.5 2 micron plasmid segregation in the context of chromosome segregation: physical and/or functional connections?

Fluorescence-tagged reporter plasmids containing the *STB* locus, and complemented by Rep1 and Rep2 from the endogenous 2 micron circle or by ectopic expression systems, have been fundamental to our understanding of the possible mechanisms for 2 micron plasmid segregation (Ghosh et al., 2007; Velmurugan et al., 2000). In haploid cells, a multi-copy reporter plasmid is seen as 3-5 foci, suggesting that plasmid molecules form themselves into groups or clusters. Each focus appears to act as a

self-contained unit in segregation, as the foci tend to segregate in an n: n fashion in anaphase cells. Segregation assays based on multi-copy reporter plasmids are not quite precise, as the number of plasmid copies within each focus is unknown. Furthermore, occasionally plasmid foci tend to overlap or coalesce with each other, introducing some uncertainty in their numbers. These shortcomings have been overcome by designing single copy (or close to single copy) reporter plasmids. Following replication, the segregation of the two sister plasmid copies is either equal (1:1) or unequal (2:0).

Previous cell biological analyses suggest that the dynamics and kinetics of 2 micron plasmid segregation are nearly identical to those of chromosome segregation (Velmurugan et al., 2000). By contrast, a plasmid lacking *STB* behaves quite differently. These observations suggest that plasmid segregation is somehow coupled to chromosome segregation through the Rep-*STB* system. This notion is further supported by a number of genetic experiments as well. In the chromosome missegregation mutant *ipl1-2*, at the non-permissive temperature, the 2 micron plasmid foci tend to remain in the same cell compartment as that occupied by the bulk of the missegregating chromosomes (Velmurugan et al., 2000). The Rep-*STB* dependent coupling of the plasmid and chromosomes is also observed during chromosome missegregation under the influence of several kinetochore mutants: *ndc10, ctf7, ctf13* and *ndc80* (Mehta et al., 2002). In principle, the apparent functional similarities between plasmid and chromosome segregation may be due to physical association between the two, or may be independent of such association. The segregation behavior of single copy *STB* plasmids suggest that the 2 micron plasmid may follow a pairing-unpairing mechanism analogous to that of

sister chromatids (Ghosh et al., 2007). In principle, this one-to-one segregation could occur in a chromosome-independent manner. However, based on cumulative evidence from several experiments (Cui et al., 2009; Liu et al., 2013; Mehta et al., 2005; Velmurugan et al., 2000), the hitchhiking model, based on plasmid-chromosome tethering, is currently favored.

Although the integrity of the mitotic spindle is required for equal plasmid segregation, the role of the spindle in this process appears to be indirect. Delaying spindle assembly until G2/M in an otherwise normal cell cycle does not affect chromosome segregation, but causes the 2 micron plasmid to missegregate (Mehta et al., 2005). The presence of an intact mitotic spindle during pre-G2/M phase of the cell cycle is important for the coupling between plasmid and chromosome segregation (Cui et al., 2009; Mehta et al., 2005). It is quite unlikely that the plasmids attach directly to the spindle in a bi-oriented fashion (analogous to kinetochores), and are pulled apart by spindle forces. When a reporter plasmid contains two copies of *STB*, it does not exhibit instabilities that are typical of dicentric plasmids. Furthermore, efforts to detect proteins of the kinetochore complex at *STB* have not yielded positive results. Thus, the effects of spindle manipulations and mutations that disrupt normal chromosome segregation on 2 micron plasmid segregation are most easily explained by assuming that the plasmid segregates as a chromosome tethered entity. If validated, the hitchhiking model would establish a common logic for the stable maintenance for yeast plasmids and viral episomes in eukaryotic cells.

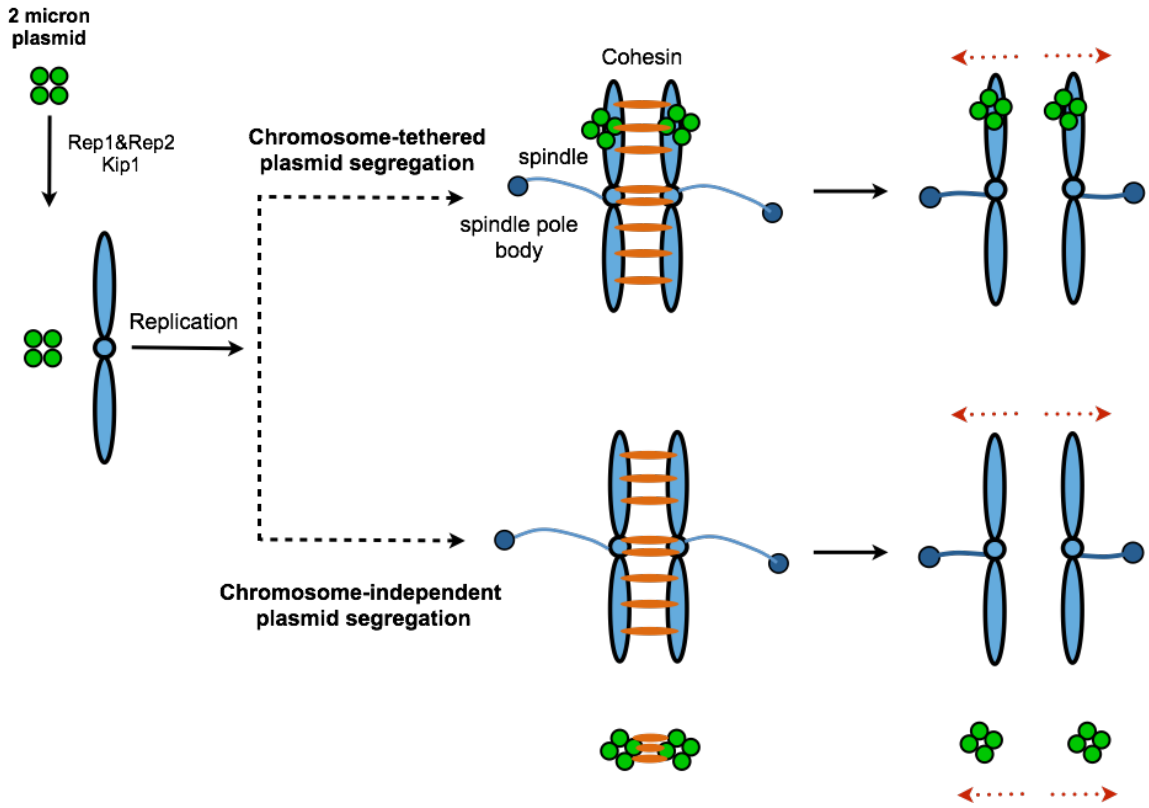



**Figure 1.7 Possible models for the segregation of the 2 micron plasmid.**

Based on current evidence, the Rep proteins together with the Kip1 motor protein (and perhaps other host factors) are thought to help localize the 2 micron plasmid to its nuclear address in a spindle-dependent process. At this "partitioning center", the plasmid may readily access chromosome segregation factors or may physically associate with chromosomes (tethering). In the chromosome-associated (hitchhiking) plasmid segregation model, replicated plasmid clusters paired by the cohesin complex attach to sister chromatids, which are also bridged by cohesin. When cohesin is cleaved during anaphase, plasmids would segregate by hitchhiking on sister chromatids. In the chromosome independent plasmid segregation model, equal plasmid segregation occurs by utilizing a subset of chromosome segregation factors, but without direct attachment to chromosomes. In either model, the individual plasmid molecules within the replicated clusters must have a precise organization to achieve equal segregation.

### 1.2.6 Plasmid replication and the association of the partitioning complex with *STB*

As noted earlier, several host factors are associated with the *STB* locus, and assist the plasmid partitioning process. The loading of these host factors (RSC2 chromatin remodeling complex, the nuclear motor Kip1, the cohesin complex and the histone H3 variant Cse4) on the *STB* locus are all Rep1-Rep2 dependent (Cui et al., 2009; Huang et al., 2011; Ma et al., 2013; Mehta et al., 2005). Both the assembly and the disassembly of the partitioning complex are temporally regulated. While the recruitment of factors occurs within a narrow window of the cell cycle, their disassembly takes place over a wider span of the cell cycle. When DNA replication is blocked or delayed, the assembly/disassembly events are also blocked or delayed (Ma et al., 2013). This observation suggests that the partitioning clock is reset during each cell cycle and that replication serves as a cue for the resetting event. However, the mechanism by which replication contributes to the equal segregation of the 2 micron plasmid is not understood.

### 1.2.7 Overcoming the challenges for segregation during budding yeast mitosis by the 2 micron plasmid partitioning system

Extra-chromosomal circles in yeast, despite the capacity for proficient replication, are quite unstable, as illustrated by the high loss rate of *ARS* plasmids. The obstacles to stable propagation faced by the *ARS* plasmids are also relevant to the 2 micron plasmid. However, the Rep-*STB* system is dedicated to overcoming them, thus ensuring the long-term stability of the 2 micron plasmid.

As noted previously, despite the high absolute copy number of the 2 micron plasmid, its effective copy number for partitioning is much lower due to the apparent organization of groups of plasmids into a small number of foci (Velmurugan et al., 2000; 1998). Furthermore, random segregation of these foci and correction of copy number by the amplification system can be ruled out during normal steady state growth conditions. Few plasmid molecules, if any, undergo more than one round of replication (Zakian et al., 1979), implying that the Rep-*STB* system is adept at promoting equal plasmid distribution into mother and daughter cells. Plasmids that lack this partitioning system fail to segregate equally, being trapped disproportionately in the mother compartment (mother bias) (Gehlen et al., 2011; Murray and Szostak, 1983; Velmurugan et al., 2000). The barrier to free diffusion of plasmid molecules may be attributed to the shape of the nucleus with a marked constriction at the bud neck coupled with the short duration of mitosis and/or the association of plasmids with nuclear membrane components that are impeded from entering the daughter cell (Gehlen et al., 2011; Shcheprova et al., 2008). Clearly, the role of the partitioning system is in overcoming this diffusion barrier responsible for the mother bias.

According to the hitchhiking model, the mechanism for negating the mother bias is Rep-*STB* mediated plasmid tethering to chromosomes. However, random tethering of plasmid foci to chromosomes will not be sufficient to achieve the high efficiency of equal segregation suggested by the near absence of plasmid amplification in normally growing cells. For the simplest case of two plasmid foci formed by replication, random tethering cannot do better than 50% equal segregation as chromosomes assort independently. This

value will decrease further as the foci number increases. One will therefore have to invoke a non-random mode of association of the 2 micron plasmid foci with chromosomes. Since sister chromatids segregate in a one-to-one fashion, a simple model that accommodates equal plasmid segregation would posit that pairs of plasmid foci associate with sister chromatids (symmetric tethering) (Fig 1.8). A previous study shows that, when the meiosis I-specific monopolin complex is inappropriately expressed during a mitotic cell cycle, there is an increase in the co-segregation of sister chromatids (Monje-Casas et al., 2007; Petronczki et al., 2006). The normal function of the monopolin complex is to clamp down sister kinetochores so that they are mono-oriented on the spindle during meiosis I. As a result, homologues segregate from each other without the separation of sisters. The monopolin-induced co-segregation of sister-chromatids during mitosis is unbiased toward the mother or daughter cell. *STB* plasmid sisters formed by the replication of a single copy plasmid also show increased co-segregation in the presence of monopolin. Furthermore, there is nearly perfect correlation between the increase in co-segregation frequencies between the reporter chromosome and the reporter plasmid. This suggests that replicated copies of an *STB* plasmid might be tethered symmetrically on sister chromatids to achieve high stability (Liu et al., 2013). A large number of experiments described in this thesis were designed to verify predictions of the hitchhiking model for plasmid segregation.

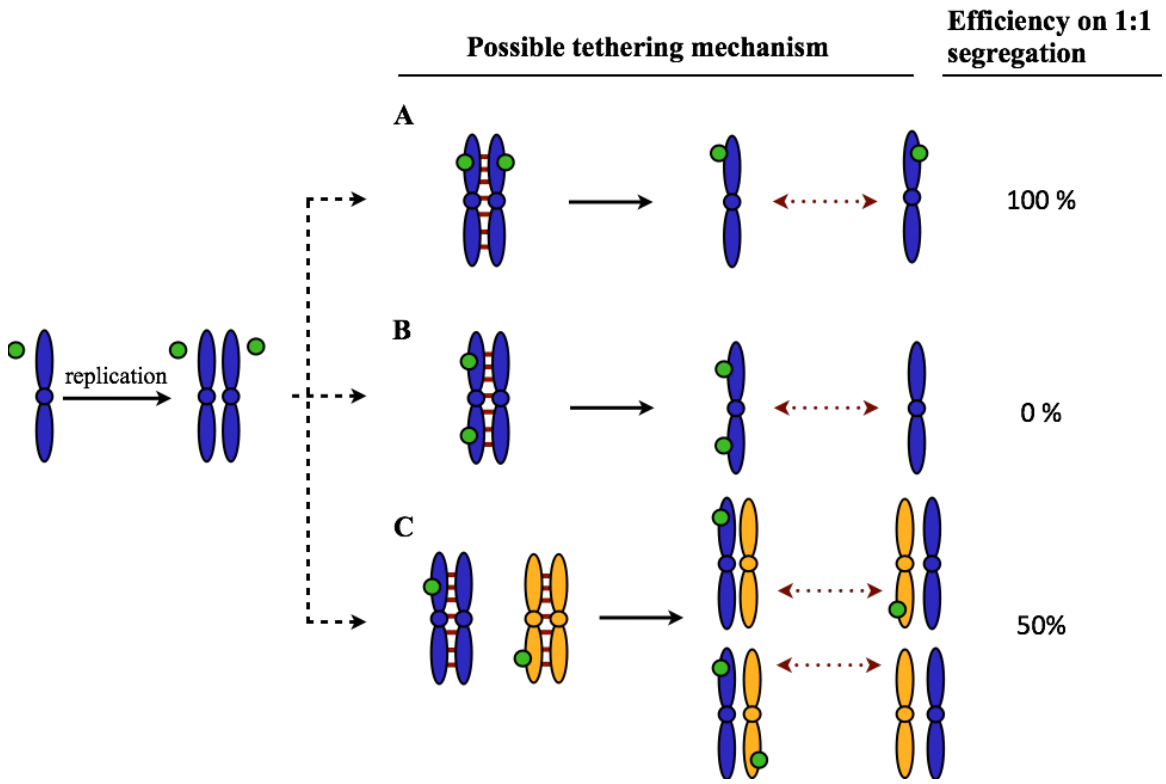



**Figure 1.8 Different types of potential tethering of replicated plasmid copies to chromosomes**

When a plasmid (green circle) is replicated, the replicated plasmid copies may associate with a chromosome (or chromosomes) in more than one fashion. (A) In a symmetric mode of tethering, the replicated plasmids are tethered to a pair of sister chromatids. In this mode of hitchhiking, the efficiency of equal segregation is the same for the plasmid and the chromosome (~100%). (B) In an asymmetric mode of tethering, the two plasmid copies are associated with only one of the two sister chromatids. The result is plasmid missegregation. (C) If the two plasmids tether to different chromosomes (random tethering), they have a 50% chance of equal segregation (as the chromosomes assort independently). This schematic diagram applies strictly to only a single copy derivative of the 2 micron plasmid. Experiments utilizing single copy *STB* reporter plasmids are generally consistent with the mechanism depicted in A. Furthermore, the foci formed by a multi-copy reporter plasmid appear to function as independent units in segregation. As each focus likely contains more than one plasmid copy, the plasmid molecules formed by replication will have to be organized precisely for them to achieve equal segregation by hitchhiking.

## 1.3 Organization of the thesis

This introductory chapter (**Chapter 1**) is followed by six chapters. In **Chapter 2**, the experimental procedures employed for this study are summarized. **Chapter 3** embodies the results of experiments aimed at addressing the potential association of 2 micron derived reporter plasmids with yeast chromosomes. In **Chapter 4**, plasmid-chromosome tethering is more critically scrutinized against the possibility of plasmid tethering to the nuclear membrane. The potential role of DNA replication in overcoming mother bias and/or imparting equality of plasmid segregation is addressed in **Chapter 5**. Attempts to reconstitute, at least partially, the 2 micron plasmid partitioning system in mammalian cells are described in **Chapter 6**. The rationale was to take advantage of the higher resolution of mitotic chromosomes in such cells to more stringently verify plasmid-chromosome association. In the final chapter (**Chapter 7**), the current picture of 2 micron plasmid partitioning emerging from the cumulative results from **Chapters 3-6** is presented.

# CHAPTER 2

# Materials and Methods

The materials and methods employed in this study are summarized in this chapter.

## 2.1 Yeast strains and plasmids

The yeast strains and the plasmids utilized in various assays are listed in Table 2.1 and Table 2.2, respectively. The relevant genotypes of the strains and the properties of the plasmids are indicated, along with the pertinent references, where appropriate. The strains and plasmids are ordered according to the sequence of the chapters in which they are first referred to. Furthermore, the figure numbers corresponding to the experiments in which they were utilized are also given in the two Tables.

## 2.2 Mammalian cell lines

COS7 (**C**V-1 (simian) in **O**rigin, and carrying the **S**V40 genetic materials) cells, a fibroblast-like cell line derived from monkey kidney tissue, were used to study the localization of the Rep proteins. HEK 293 cells (**H**uman **E**mbryonic **K**idney) and HEK 293T cells (HEK 293 cells with SV40 Large T-antigen) were also used for studying the localization of Rep proteins as well as the stability of an *STB* containing plasmid. All cell lines were grown in DMEM (**D**ulbecco's **M**odified **E**agle **M**edium) (from Cellgro) supplied with 10% FBS (**F**etal **B**ovine **S**erum) (from Atlantic Biology).

## 2.3 Mammalian expression vectors

The vectors employed for protein expression in mammalian cells are schematically diagrammed in Fig. 2.1. Their attributes are outlined in the legend to the figure.

## 2.4 [cir+] and [cir0] yeast strains; *STB* and *ARS* reporter plasmids

The yeast strains containing the native 2 micron plasmid are denoted as [$cir^+$], and those lacking the plasmid are designed as [$cir^0$]. Reporter plasmids derived from the 2 micron circle (containing *ORI* and the *STB* locus), and complemented by the Rep1 and Rep2 proteins, are considered to be representative of native 2 micron circles. The *STB* reporter plasmids present in [$cir^+$] strains receive the Rep proteins from the endogenous plasmid. In a [$cir^0$] strain, these proteins can be complemented by expressing them from a constitutive or an inducible promoter, for example, the *ADH* promoter or the *GAL* promoter, respectively. Reporter plasmids that are capable of replication (utilizing the 2 micron circle origin or a chromosomal origin) but lack the *STB* locus (*STB*-) are considered as *ARS* (**a**utonomously **r**eplicating **s**equence) plasmids. *STB* plasmids present in the [$cir^0$] strains without being complemented by Rep1 and Rep2 also behave as *ARS* plasmids.

**Experimental strains**: In the list provided below, the genotypes are detailed against the strain numbers. The presence of a plasmid in a strain is indicated at the end of its genotype.

**Table 2.1 Strains**

| Chapter 3 | | | |
|---|---|---|---|
| **Strain** | **Genotype** | **Source/ ref.** | **Figure numbers** |
| MJY3016 | *MAT***a** *ade2::GFP-LacI::ADE2 his3-11 leu2-3, 112 trp1 ura3-1* [cir$^{+}$] / pSG1:: *TRP1* | (Ghosh et al., 2007) | Fig. 3.2-3.3, 3.5-3.6, 3.8 |
| MJY3017 | *MAT***a** *ade2::GFP-LacI::ADE2 his3-11 leu2-3, 112 trp1 ura3-1* [cir$^{0}$] / pSG1:: *TRP1* | (Ghosh et al., 2007) | Fig. 3.2 |
| MJY3020 | *MAT***a** *his3-11 leu2::GFP-LacI::LEU2 trp1 ura3-1 RFP- TetR* [cir$^{+}$] / pSG1:: *TRP1* pSG2:: *URA3* | (Ghosh et al., 2007) | Fig. 3.4 |
| MJY3021 | *MAT***a** *his3-11 leu2::GFP-LacI::LEU2 trp1 ura3-1 RFP- TetR* [cir$^{0}$] / pSG1:: *TRP1* pSG2:: *URA3* | (Ghosh et al., 2007) | Fig. 3.4 |
| MJY3022 | *MAT***a** *his3-11::GAL1p- REP1-GAL10p-REP2::HIS3 leu2::GFP-LacI::LEU2 trp1 ura3-1 RFP- TetR* [cir$^{0}$] / pSG1:: *TRP1* pSG2:: *URA3* | (Ghosh et al., 2007) | Fig. 3.4 |
| MJY9017 | *MAT***a** *ade2::GFP-LacI::ADE2 his3-11 leu2::GAL10p-Rep2::LEU2 trp1 ura3-1* [cir$^{0}$] / pSG1:: *TRP1* | This study | Fig. 3.3 |
| MJY9027 | *MAT***a** *ade2::GFP-LacI::ADE2 his3-11 leu2-3, 112 trp1 ura3-1::GAL1p-Rep1::URA3* [cir$^{0}$] / pSG1:: *TRP1* | This study | Fig. 3.3 |
| MJY9028 | *MAT***a** *ade2::GFP-LacI::ADE2 his3-11 leu2::GAL10p-Rep2::LEU2 trp1 ura3-1::GAL1p-Rep1::URA3* [cir$^{0}$] / pSG1:: *TRP1* | This study | Fig. 3.3 |
| MJY9031 | *MAT***a** *ade2::GFP-LacI::ADE2 his3-11 11 leu2::GA1p-Rep2-3HA::LEU2 trp1 ura3-1::GAL1p-Rep1::URA3* [cir$^{0}$] | This study | Fig. 3.3 |
| MJY9032 | *MAT***a** *ade2::GFP-LacI::ADE2 his3-11 11 leu2-2,112 trp1 ura3-1::GAL1p-Rep1::URA3* [cir$^{0}$] | This study | Fig. 3.3 |

Table 2.1 continued

| | | | |
|---|---|---|---|
| MJY9033 | *MAT***a** *ade2::GFP-LacI::ADE2 his3-11 11 leu2::GA1p-Rep2-3HA::LEU2 trp1 ura3-1* [cir$^0$] | This study | Fig. 3.3 |
| MJY9025 | *MAT***a** *ade2 his3-11::kip1Δ::HisMX leu2-3, 112 trp1 ura3-1::GFP-LacI::URA3* [cir$^+$] / pSG1:: *TRP1* | This study | Fig. 3.7 |
| MJY9029 | *MAT***a** *ade2::GFP-LacI::ADE2 his3-11::rsc2Δ::HisMX leu2-3, 112 trp1 ura3-1* [cir$^+$] / pSG1:: *TRP1* | This study | Fig. 3.8 |
| MJY9030 | *MAT***a** *ade2 his3-11 leu2-3, 112 trp1 ura3-1::GFP-LacI::URA3* [cir$^+$] / pSG1:: *TRP1* | This study | Fig. 3.7 |
| **Chapter 4** | | | |
| **Strain** | **Genotype** | **Source/ ref.** | **Figure numbers** |
| MJY7188 | *MAT* **a** *can1-100 leu2 ura3-1 trp1-1::$P_{GAL}$-SCC1RRDD-3HA::TRP1 his3-11,15::pSTB-::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] / pAJ2979::*URA3* | This study | Fig. 4.3 |
| MJY7189 | *MAT* **a** *can1-100 ura3-1 trp1-1::$P_{GAL}$-SCC1RRDD-3HA::TRP1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3-11,15::pSTB-::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] / pAJ2979::*URA3* | This study | Fig. 4.3 |
| MJY7190 | *MAT* **a** *can1-100 leu2 ura3-1 trp1-1::$P_{GAL}$-SCC1RRDD-3HA::TRP1 his3-11,15::pSTB::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] / pAJ2979::*URA3* | This study | Fig. 4.3 |
| MJY7191 | *MAT* **a** *can1-100 ura3-1 trp1-1::$P_{GAL}$-SCC1RRDD-3HA::TRP1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3 11,15::pSTB::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] / pAJ2979::*URA3* | This study | Fig. 4.3 |
| MJY9139 | *MAT***a** *ade2 his3-11 leu2::TetR-GFP::LEU2 trp1 ura3-1* [cir$^+$] / pSG2::*URA3* | This study | Fig. 4.1 |
| MJY9140 | *MAT***a** *ade2 his3-11 leu2::TetR-GFP::LEU2 trp1 ura3-1* [cir$^+$] / pTL26::*TRP1* | This study | Fig. 4.1 |
| MJY9141 | *MAT***a** *ade2 his3-11 leu2::TetR-GFP::LEU2 trp1 ura3-1 Mlp1-TetR::KanMX* [cir$^+$] / pSG2::*URA3* | This study | Fig. 4.1 |
| MJY9142 | *MAT***a** *ade2 his3-11 leu2::TetR-GFP::LEU2 trp1 ura3-1 Mlp1-TetR::KanMX* [cir$^+$] / pTL26::*TRP1* | This study | Fig. 4.1 |
| MJY9143 | *MAT***a** *ade2 his3-11 leu2::TetR-GFP::LEU2 trp1 ura3-1 Nup2-TetR::KanMX* [cir$^+$] / pSG2::*URA3* | This study | Fig. 4.1 |

Table 2.1 continued

| | | | |
|---|---|---|---|
| MJY9144 | *MAT***a** *ade2 his3-11 leu2::TetR-GFP::LEU2 trp1 ura3-1 Nup2-TetR::KanMX* [cir$^+$] / pTL26::*TRP1* | This study | Fig. 4.1 |
| **Chapter 5** | | | |
| **Strain** | **Genotype** | **Source/ ref.** | Figure numbers |
| MJY5060 | *MAT* **a** *can1-100 trp1-1 ura3-1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3-11,15::pSTB::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^0$] | (Liu et al., 2013) | Fig. 5.3 |
| MJY5062 | *MAT* **a** *can1-100 trp1-1 leu2::($P_{GAL}$-RecR::LEU2)X2 ura3-1::$P_{GAL}$-REP1 REP2::URA3 his3-11,15:: pSTB::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^0$] | (Liu et al., 2013) | Fig. 5.3 |
| MJY7120 | *MAT* **a** *can1-100 trp1-1 ura3-1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3-11,15::pSTB-::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] | (Liu et al., 2013) | Fig. 5.3-5.4 |
| MJY7122 | *MAT* **a** *can1-100 trp1-1 ura3-1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3-11,15::pSTB::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] | (Liu et al., 2013) | Fig. 5.3-5.4 |
| MJY7124 | *MAT* **a** *can1-100 trp1-1 leu2::($P_{GAL}$-RecR::LEU2)X2 CENIV::TetOx448::URA3 ura3-1:: $P_{GAL}$-3MYC-CDC5::URA3 $P_{GAL}$-3HA-MAM1::KanMX6 his3-11,15::pSTB::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] | (Liu et al., 2013) | Fig. 5.6 |
| MJY7127 | *MAT* **a** *can1-100 trp1-1 leu2::($P_{GAL}$-RecR::LEU2)X2 CENIV::TetOx448::URA3 ura3-1:: $P_{GAL}$-3MYC-CDC5::URA3 $P_{GAL}$-3HA-MAM1::KanMX6 his3-11,15::pSTB-::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] | (Liu et al., 2013) | Fig. 5.6 |
| MJY7128 | *MAT* **a** *can1-100 trp1-1 leu2::($P_{GAL}$-RecR::LEU2)X2 CENIV::TetOx448::URA3 ura3-1:: $P_{GAL}$-3MYC-CDC5::URA3 $P_{GAL}$-3HA-MAM1::KanMX6 his3-11,15::pSTB::HIS3 ade2-1::$P_{URA3}$-TetR-GFP::ADE2* [cir$^+$] | (Liu et al., 2013) | Fig. 5.6 |
| MJY7187 | *MAT* **a** *can1-100 trp1-1 ura3-1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3-11,15::pSTB-/ORIΔ::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] | This study | Fig. 5.3-5.4 |
| MJY7188 | *MAT* **a** *can1-100 leu2 ura3-1 trp1-1::$P_{GAL}$-SCC1RRDD-3HA::TRP1 his3-11,15::pSTB-::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] / pAJ2979::*URA3* | This study | Fig. 5.5 |

Table 2.1 continued

| | | | |
|---|---|---|---|
| MJY7189 | *MAT* **a** *can1-100 ura3-1 trp1-1::$P_{GAL}$-SCC1RRDD-3HA::TRP1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3-11,15::pSTB-::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] / pAJ2979::*URA3* | This study | Fig. 5.5 |
| MJY7190 | *MAT* **a** *can1-100 leu2 ura3-1 trp1-1::$P_{GAL}$-SCC1RRDD-3HA::TRP1 his3-11,15::pSTB::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] / pAJ2979::*URA3* | This study | Fig. 5.5 |
| MJY7191 | *MAT* **a** *can1-100 ura3-1 trp1-1::$P_{GAL}$-SCC1RRDD-3HA::TRP1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3-11,15:: pSTB::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] / pAJ2979::*URA3* | This study | Fig. 5.5 |
| MJY7195 | *MAT* **a** *can1-100 trp1-1 ura3-1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3-11,15::pSTB/ORIΔ::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] | This study | Fig. 5.3-5.4 |
| MJY7196 | *MAT* **a** *can1-100 ura3-1 trp1-1::$P_{GAL}$-SCC1RRDD-3HA::TRP1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3-11,15:: pSTB /ORIΔ::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] / pAJ2979::*URA3* | This study | Fig. 5.5 |
| MJY7197 | *MAT* **a** *can1-100 ura3-1 trp1-1::$P_{GAL}$-SCC1RRDD-3HA::TRP1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3-11,15::pSTB-/ORIΔ::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] / pAJ2979::*URA3* | This study | Fig. 5.5 |
| MJY7200 | *MAT* **a** *can1-100 trp1-1 ura3-1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3-11,15::pSTB-/ORIΔ::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^0$] | This study | Fig. 5.3-5.4 |
| MJY7201 | *MAT* **a** *can1-100 ura3-1 leu2::($P_{GAL}$-RecR::LEU2)X2 trp1-1::pSTB/ORIΔ::TRP1 his3-11,15::pSTB/ORIΔ::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] | This study | Fig. 5.3, 5.6 |
| MJY7202 | *MAT* **a** *can1-100 trp1-1 leu2::($P_{GAL}$-RecR::LEU2)X2 ura3-1::$P_{GAL}$-REP1 REP2::URA3 his3-11,15 pSTB/ORIΔ::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^0$] | This study | Fig. 5.3, 5.6 |
| MJY7203 | *MAT* **a** *can1-100 ura3-1 leu2::($P_{GAL}$-RecR::LEU2)X2 trp1-1::pSTB/ORIΔ::TRP1 his3-11,15::pSTB/ORIΔ::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^0$] | This study | Fig. 5.3, 5.6 |

Table 2.1 continued

| | | | |
|---|---|---|---|
| MJY7204 | *MAT* **a** *can1-100 ura3-1 trp1-1::$P_{GAL}$-SCC1RRDD-3HA::TRP1 leu2::($P_{GAL}$-RecR::LEU2)X2 his3-11,15:: pSTB/ORIΔ::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^0$] / pAJ2979::*URA3* | This study | Fig. 5.3, 5.6 |
| MJY7212 | *MAT* **a** *can1-100 leu2::($P_{GAL}$-RecR::LEU2)X2 ura3-1::$P_{GAL}$-3MYC-CDC5::URA3 $P_{GAL}$-3HA-MAM1:: trp1-1::pSTB/ORIΔ::TRP1 his3-11,15::pSTB/ORIΔ::HIS3 ade2-1::$P_{HIS3}$-GFP-LacI::ADE2* [cir$^+$] | This study | Fig. 5.6 |
| MJY 9146 | MATa ade2-1 can1-100 leu2-3, 112 his3-11, 15::GAL cdc6::hisG trp1-1 ura3::URA3 GAL-ubiR-CDC6 [cir$^+$] /pSV1 :: *LEU2* | This study | Fig. 5.8 |

**Table 2.2 Plasmids**

**Chapter 3**

| Plasmid | Salient features | Source/Ref. | Figure numbers |
|---|---|---|---|
| pSG1 | *$P_{Gal1}$-CEN3-STB-ORI* and 256 copies of Lac operator cloned in YEpLac112 (*TRP1*) | (Ghosh et al., 2007) | Fig. 3.2-3.8 |
| pSG2 | *$P_{Gal1}$-CEN3-STB-ORI* and 112 copies of TetO operator cloned in pRS306 (*URA3*) | (Ghosh et al., 2007) | Fig. 3.4 |

**Chapter 4**

| Plasmid | Salient features | Source/Ref. | Figure numbers |
|---|---|---|---|
| pAJ2979 | pRS316-Nup49-mcherry (*URA3*) | Dr. Arlen Johnson's lab | Fig. 4.3 |
| pSG2 | *$P_{Gal1}$-CEN3-STB-ORI* and 112 copies of TetO operator cloned in pRS306 (*URA3*) | (Ghosh et al., 2007) | Fig. 4.1 |
| pTL26 | *$P_{Gal1}$-CEN3-STB-ORI* and 112 copies of TetO operator cloned in pRS304 (*TRP1*) | MJ laboratory | Fig. 4.1, 4.3 |
| pCM218 | p*STB* plasmid excision cassette (RS-*ORI-STB*-[LacO]$_{256}$-RS)* cloned in pRS403 (*HIS3*) | (Liu et al., 2013) | Fig. 4.3 |

Table 2.2 continued

| | | | |
|---|---|---|---|
| pTL29 | p*STB*- plasmid excision cassette (RS-*ORI*-[LacO]$_{256}$-RS)* cloned in pRS403 (*HIS3*) | (Liu et al., 2013) | Fig. 4.3 |

**Chapter 5**

| Plasmid | Salient features | Source/Ref. | Figure numbers |
|---|---|---|---|
| pSV1 | 256 copies of Lac operator sequence cloned in YEpLac181 (*LEU2*) | (Velmurugan et al., 2000) | Fig. 5.7 |
| pAJ2979 | pRS316-Nup49-mcherry (*URA3*) | Dr. Arlen Johnson's lab | Fig. 5.5 |
| pCM218 | p*STB* plasmid excision cassette (RS-*ORI*-*STB*-[LacO]$_{256}$-RS)* cloned in pRS403 (*HIS3*) | (Liu et al., 2013) | Fig. 5.3- 5.6 |
| pTL29 | p*STB*- plasmid excision cassette (RS-*ORI*-[LacO]$_{256}$-RS)* cloned in pRS403 (*HIS3*) | (Liu et al., 2013) | Fig. 5.3- 5.6 |
| pTL44 | p*STB*-/*ORI*Δ plasmid excision cassette (RS-[LacO]$_{256}$-RS)* cloned in pRS403 (*HIS3*) | This study | Fig. 5.3- 5.6 |
| pTL47 | p*STB*/*ORI*Δ plasmid excision cassette (RS-*STB*-[LacO]$_{256}$-RS)* cloned in pRS403 (*HIS3*) | This study | Fig. 5.3- 5.6 |
| pTL56 | *STB*/*ORI*Δ plasmid excision cassette (RS-*STB*-[LacO]$_{256}$-RS)* cloned in pRS404 (*TRP1*) | This study | Fig. 5.3- 5.6 |

**Chapter 6**

| Plasmid | Salient features | Source/Ref. | Figure numbers |
|---|---|---|---|
| pcDNA3.1 Hyg+ | Expression vector designed for transient expression in mammalian cells (Invitrogen) with Hygromycin as selective marker | Dr. Chris Sullivan's lab | |
| pEGFP-C1 | Vector for expressing and visualizing a protein of interest fused to EGFP on N-terminus. (Clontech) | Dr. Chris Sullivan's lab | |

Table 2.2 continued

| | | | |
|---|---|---|---|
| pDsRed Express-C1 | Vector for expressing and visualizing a protein of interest fused to EGFP on N-terminus. (Clontech) | Dr. Chris Sullivan's lab | |
| pKC6 | pEGFP-Rep1 | This study | Fig. 6.1-6.4 |
| pKC7 | pEGFP-Rep2 | This study | Fig. 6.1 |
| pKC13 | pDsRed-Express-Rep1 | This study | Fig. 6.1, 6.6 |
| pKC14 | pDsRed-Express-Rep2 | This study | Table. 6.1 Fig. 6.1-6.4 |
| pKC18 | pEGFP-Nodamura Virus B2 | This study | Fig. 6.2 |
| pKC28 | pKC-Rep2 (pDsRed-Rep2-DsRedΔ) | This study | Fig. 6.6 |
| pKC35 | pEGFP-Rep1+SV40NLS | This study | Fig. 6.2 |
| pKC83 | pDsRed-Rep2ΔN120 | This study | Table. 6.1 Fig. 6.3 |
| pKC84 | pDsRed-Rep2ΔC120 | This study | Table. 6.1 Fig. 6.3 |
| pKC85 | pDsRed-Rep2ΔN30 | This study | Table. 6.1 Fig. 6.3 |
| pKC86 | pDsRed-Rep2ΔN60 | This study | Table. 6.1 Fig. 6.3 |
| pKC87 | pDsRed-Rep2ΔN90 | This study | Table. 6.1 Fig. 6.3 |
| pKC92 | pDsRed-Rep2ΔN13 | This study | Table. 6.1 Fig. 6.3 |
| pKC94 | pDsRed-Rep2ΔN180 | This study | Table. 6.1 Fig. 6.3 |
| pKC95 | pDsRed-Rep2ΔN210 | This study | Table. 6.1 Fig. 6.3 |
| pKC96 | pDsRed-Rep2ΔN240 | This study | Table. 6.1 Fig. 6.3 |
| pKC117 | pcDNA3.1 Hyg+ d2EGFP (*SV40 ORI* -) | This study | Fig. 6.6 |
| pKC118 | pcDNA3.1 Hyg+ d2EGFP *STB* (*SV40 ORI* -) | This study | Fig. 6.6 |

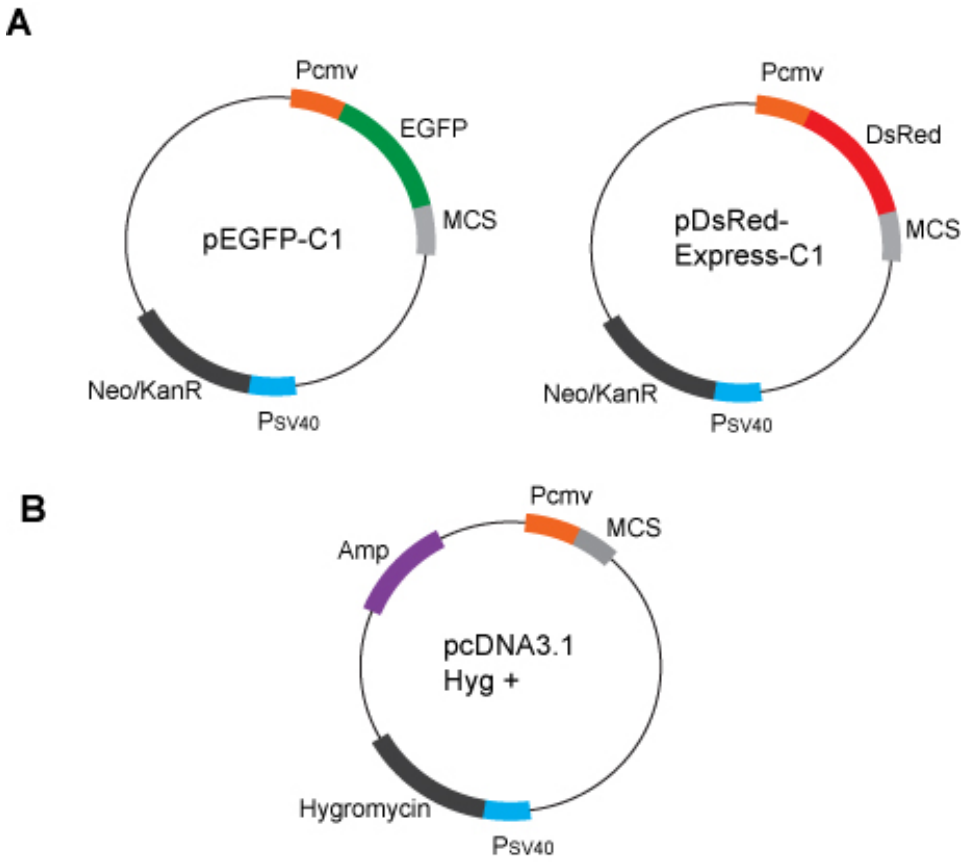



**Figure 2.1 Expression vectors for mammalian cells.**

(A) pEGFP-C1 and pDsRed-Express-C1 vectors were used for expressing GFP- or DsRed-fusion proteins. Fusions to the C-terminus of EGFP or DsRed retained the fluorescence properties of the native protein, permitting the localization of the fusion proteins *in situ*. High level protein expression was achieved from the strong CMV promoter. A Kozak sequence was positioned appropriately upstream of the EGFP/DsRed coding sequence to optimize translation efficiency. Gene fusions in the correct reading frame were constructed by introducing DNA fragments into the MCS (**M**ultiple **C**loning **S**ite). The SV40 origin present in the vectors supported their replication in mammalian cells expressing the SV40 T-antigen. (B) pcDNA3.1 Hyg+ vector was used to express a protein in its native form. This vector was also engineered to harbor the beneficial features of the other two: the Kozak sequence, the CMV promoter and the SV40 origin.

## 2.5 Culture conditions

Yeast strains were grown in complex medium or synthetic dropout medium with appropriate supplements at 30°C.

## 2.6 Synchronization of yeast cells

For G1 synchronization, *MATa* cells in early log phase were treated with α-factor at a final concentration of 10 μg/ml for *BAR1* strains. The incubation varied from 2 to 3 hours, depending on individual strains. Samples were examined under a light microscope to ensure >90% of cells were arrested with typical "shmoo" form.

For G2/M arrest, cells in mid-log phase were treated with a final concentration of 15 μg/ml nocodazole (Sigma) dissolved in DMSO. After 2 to 3 hours incubation, >85% cells were arrested at G2/M with a large-budded phenotype. The arrested cells contained a single DAPI-stained DNA mass close to the bud neck.

For some experiments, G1 arrested cells were released in the presence of nocodazole. They were arrested at the G2/M stage as large budded cells with a single DAPI mass.

## 2.7 Single-copy derivatives of reporter plasmids

Two strategies were employed to generate single-copy reporter plasmids in this study. The first one was to clone into the plasmid a *CEN* sequence, which can be conditionally inactivated by driving transcription through it from the *GAL* promoter. An active *CEN* is dominant over *STB* in copy number control. The second strategy was to integrate the plasmid into a chromosome locus, and then excise it in circular form by a

site-specific recombination event. In the $P_{GAL}$-*CEN* system, the plasmid was maintained close to one (but not precisely one) copy per cell when the carbon source was glucose or raffinose (no transcription through the *CEN* sequence). *CEN* was inactivated at the appropriate time during an assay by adding 2% galactose to the medium (inducing high level transcription through the *CEN* sequence). In the integrant plasmid system, the plasmid sequence was flanked by two direct copies of the target site for the R site-specific recombinase from *Zygosaccharomyces rouxii* (Araki et al., 1992). The recombinase was expressed at the desired time to excise the plasmid from the chromosome. The plasmid would be exactly one copy per cell in G1 arrested cells (before DNA replication).

The $P_{GAL}$-*CEN* system described in published work (Ghosh et al., 2007) was made use of for experiments described in **Chapter 3** and **Chapter 4**. The specific plasmid constructs and their carbon source-dependent behavior are presented in Fig. 3.1. The plasmid excision strategy was employed for assays included in **Chapter 4** and **Chapter 5**. The different types of single copy reporter plasmids constructed by the excision strategy are diagrammed in Fig. 4.2 and Fig. 5.1. For experiments requiring two single copy reporter plasmids, they were integrated into different chromosomes, and excised simultaneously by R mediated recombination.

### 2.8 Plasmid or chromosome visualization in live cells

The method for visualizing the segregation of a reporter plasmid or a chromosome was based on the interaction between an operator array ($[LacO]_{256}$ or $[TetO]_{224}$) harbored by each and the cognate repressor fused to a fluorescent protein (Straight et al., 1996).

Strains were engineered to express GFP-repressor or repressor-RFP or (in a few cases) both hybrid repressors ($P_{HIS3}$-*GFP-LacI* for the Lac system, $P_{URA3}$-*TetR-GFP* or $P_{URA3}$-*TetR-RFP* for the Tet system). A tagged chromosome or a single copy reporter plasmid was visualized as a single fluorescent focus (Ghosh et al., 2007); a multi-copy *STB* reporter plasmid as 3-5 foci (Velmurugan et al., 2000).

### 2.9 Antibodies used in this study

The mouse monoclonal anti-HA (HA.11) antibody was supplied by Covance, CA. The mouse monoclonal anti-GFP (LGB-1) antibody and the mouse monoclonal anti-Fibrillin (11C1.3) antibody were obtained from Abcam. For Rep1 detection, a polyclonal peptide-directed antibody was used. The Rep1 antibody was diluted 1:250 and the others 1:500 for detection by immunofluorescence.

### 2.10 Immunofluorescence assays

**Yeast:** Immunofluorescence assays were performed for localizing proteins or plasmids in chromosome spreads (see below) (Mehta et al., 2005; Velmurugan et al., 2000) immobilized on glass slides. The spreads were first blocked with 1 mg/ml BSA for 15 min at room temperature. The primary antibody was added and the slides were incubated in a humid chamber at room temperature for 3 h. The slides were washed with 1x PBS, and incubated with the secondary antibody (conjugated to a fluorescent dye) for 1 h at room temperature. DNA was stained using 1 μg/ml DAPI in 1x PBS. Slides were mounted with mounting medium and cover glass, and examined by fluorescence microscopy.

**Mammalian cells:** Cells were seeded on cover slides in 6 wells plates. After 48 hr from the time of transfection, cells were fixed with 4% paraformaldehyde in 1x PBS for 30 min. They were permeabilized with 0.1% TritonX-100 in 1x PBS for 5 min. After washing with 1x PBS twice, cells were blocked with 1 mg/ml BSA for 1hr. The slides were incubated with the primary antibody for 3hr. They were then washed with 1x PBS thrice (10 min each time), and were incubated with the secondary antibody for 2hr. After three washes with 1x PBS, slides were stained with 1 μg/ml Hoechst 33342 (Sigma) in 1x PBS for 5 min. After mounting with the mounting medium and cover glass, the slides were examined by fluorescence microscopy.

### 2.11 Fluorescence microscopy

The observations were performed using an Olympus BX-60 microscope. Images were taken at room temperature at 100× (oil NA 1.30 objective) using a Photometrics Quantix camera (Roper Scientific), and then processed by MetaMorph (Universal Imaging Corporation) and PhotoShop CS4 (Adobe Systems, Inc.). Z-series sectioning of the yeast nucleus, deconvolution of the stacks, and their 2D projections were performed as detailed previously (Mehta et al., 2005; Velmurugan et al., 2000).

### 2.12 Plasmid association with chromosome spreads; plasmid segregation

#### 2.12.1 The $P_{GAL}$-*CEN* system

In Chapter 3 and Chapter 4, the reporter plasmids for the chromosome spread assays and a subset of the segregation assays were based on the $P_{GAL}$-*CEN* system, which

keeps the copy number close to one per nucleus. Over 80% of the cells contained a single plasmid dot when assayed by fluorescence microscopy.

Yeast strains containing a (nearly) single copy plasmid were grown in 2% raffinose medium to early log phase before treatment with α-factor. After 1 hr, cells were transferred to 2% glucose or 2 % galactose medium for an additional 2 hr in the presence of α-factor. Galactose-induced transcription would inactivate the *CEN* sequence present on the plasmid. Cells were washed, and were released from α-factor into glucose or galactose medium. Samples were collected at desired time points. Cells were examined prior to release to ensure that 80% or more of the cells contained a single focus of the reporter plasmid. The remaining cells contained mainly two plasmid foci. Very few cells contained more than two plasmid foci or no foci. Plasmid association with chromosomes was probed in G1 cells or in cells at different times along the cell cycle. Plasmid segregation was assayed in anaphase cells, after excluding those cells that contained more than two foci (indicating the presence of more than one plasmid focus prior to replication). The general procedure is outlined in Fig. 2.2.

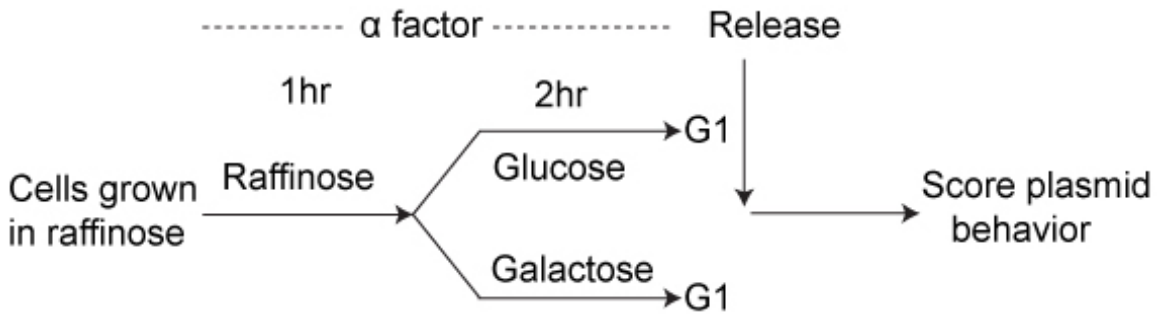



**Figure 2.2 The general experimental scheme for assays utilizing reporter plasmids based on the $P_{GAL}$-*CEN* system.**

The centromere present on the reporter plasmid maintains its copy number close to one per cell, and confers high plasmid stability. The centromere is active in raffinose or glucose medium, but is inactivated in galactose medium (by high level transcription through it) during a single experimental cell cycle.

### 2.12.2 Plasmid excision system

In **Chapter 4** and **Chapter 5**, the reporter plasmids were obtained by the plasmid excision strategy. The yeast strains containing the integrated form of a plasmid were grown in 2% raffinose medium. At an $OD_{600}$ of ~0.2, cells were arrested in G1 with α-factor. After 2.5 hr, galactose was added to a final concentration of 2% to induce the expression of the R recombinase from the *GAL* promoter. After 3 hr in galactose, the reporter plasmid could be nearly completely excised from its integrated form. Cells were then released into the cell cycle in galactose medium, and plasmid segregation was assayed in anaphase cells. The general experimental scheme is outlined below (Fig. 2.3)

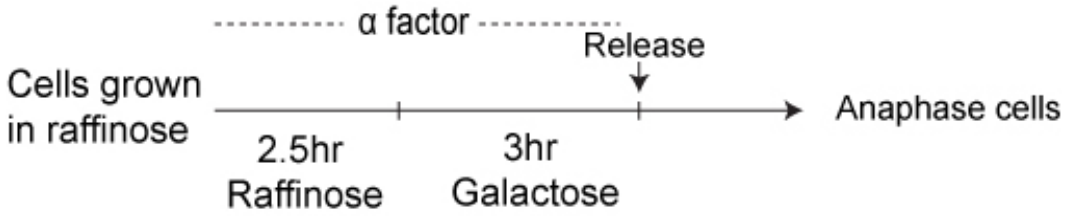



**Figure 2.3 The experimental procedure for analysis of a single copy reporter plasmid generated by excision from the chromosome.**

Cells arrested in G1 were shifted to galactose medium for inducing the R recombinase. Plasmid excision was nearly complete in 3 hr. Cells were released into the cell cycle, and plasmid segregation was assayed in anaphase cells.

### 2.12.3 Segregation of a multi-copy reporter plasmid

In **Chapter 5**, the segregation of a multi-copy *STB* reporter plasmid (Velmurugan et al., 2000) was assayed in one experiment, with DNA replication blocked by Cdc6 depletion. Yeast strains harboring the reporter plasmid were grown to an $OD_{600}$ of ~0.4 in galactose medium to permit Cdc6 expression (Ma et al., 2013). The cells were then manipulated to arrest them in G1 with Cdc6 almost completely depleted. The cells were released into a replication blocked cell cycle (in presence of glucose), and plasmid segregation was scored in anaphase cells. Further experimental details are described in **Chapter 5** (**5.2.6).**

## 2.13 Preparation of yeast chromosome spreads

Chromosome spreads from mitotic cells were prepared by following previously employed procedures (Mehta et al., 2005; Velmurugan et al., 2000) with minor modifications. Spheroplasts were obtained by treating cells with zymolyase. 20 µl aliquots of the spheroplasts (~ 5 $x10^7$ cells/ml) were transferred on to pre-cleaned glass

slides, and were mixed gently with 40 μl of 4% paraformaldehyde/3.4% sucrose. 80 μl of 1% lipsol was added, followed by 80 μl of 4% paraformaldehyde/3.4% sucrose. The mixtures were spread evenly on the slides. After overnight incubation at room temperature, the slides were washed three times with 1ml of 0.4% photoflo-200 (Kodak) and once with 1× PBS for 10min before proceeding to immunofluorescence assays.

### 2.14 DNA analysis by Southern blotting

The excision efficiency of the single copy reporter plasmid was assayed by Southern blot analysis. Total yeast genomic DNA was digested with desired restriction enzymes, and was fractionated by electrophoresis in 1% agarose gels. DNA was transferred to Hybond-XL membrane according to the manufacturer's protocol (GE Healthcare). Hybridization was performed using a $^{32}$P-labeled plasmid-specific probe. Bands were detected and quantitated by phosphorimaging using a Typhoon Trio phosphorimager and ImageQuant software (GE Healthcare)

### 2.15 Transfection of mammalian cells

For protein localization in fixed cells, COS7 and 293/293T cells were seeded on cover glass in 6-well plates. When cells reached 70% confluence, cells were transfected using Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's instructions. Approximately 4 μg of total plasmid DNA were used for each transfection. After 6-8 hr of transfection, the medium was replaced to reduce the toxicity of Lipofectamine. Cells were assayed for protein localization (or plasmid localization) after 48 hr of transfection.

COS7 cells, used for chromosome spread assays, were initially seeded on Superfrost Plus microscope slides (Fisherbrand) in 10 cm dishes. When cells reached 50% confluence, they were transfected with ~24 μg of plasmid DNA. After 24 hr, cells were taken through the steps for synchronization and enrichment of mitotic cells.

For plasmid stability assays, 293/293T cells were transfected with Lipofectamine 2000, using a modified time scheme described in **Chapter 6 (6.2.7).**

### 2.16 Enrichment of mitotic cells

Transfected cells that reached ~70% confluence (24 hr after transfection) were synchronized in S phase by incubation in the presence of 2 mM thymidine for 12-16 hours. Cells were then washed with 1x PBS (room temperature) twice and DMEM (37° C) once. Fresh DMEM with 10% FBS was added, and cells were incubated at 37° C. After 5hr, they were released from thymidine, and were incubated for 5 hours in the presence of 30ng/ml colcemid. This procedure enriched mitotic cells, which were rounded in appearance when observed under a light microscope.

### 2.17 Mitotic spreads of mammalian chromosomes

The mitotic chromosome spread assays followed previously described protocols (Zheng et al., 2005). Slides, on to which synchronized cells were transferred, were washed with 1x PBS, and were incubated with H1 buffer (10mM Tris pH 7.4/10mM NaCl/5mM MgCl2) for 15 min at room temp. The slides were then incubated with H2 buffer (25% PBS) for an additional 15 min. They were immediately spun for 3 min at 1500 rpm in a cytocentrifuge, and were fixed in 3.2% paraformaldehyde in 1x PBS for 20

min. Cells were permeabilized by incubating the slides in 0.1% Triton X-100 in 1x PBS for 15min at 4° C. The slides were then taken through the standard steps for immunofluorescence analysis.

**2.18 Plasmid stability assay in mammalian cells**

The plasmid retention assay was modified from a published procedure (Silla et al., 2010). Briefly, HEK 293 /293T cells were transfected with protein expression plasmids and reporter plasmids by lipofectamine 2000 transfection procedure. After 24hr of transfection, 12 well plates were seeded at a cell density of $3x10^5$ cells/well. At set time points after transfection, cells from each well were collected in 500ul. Collected samples were analyzed by flowcytometry (Accuri C5 Cytometer, BD Bioscience). The total number of GFP (or DsRed) positive cells in each 500 [illegible] was estimated. These numbers were normalized to that for the first time point (48 hr after transfection), which is taken as 1. Cell doubling rate for each sample analyzed was estimated from the increase in cell counts during time intervals measured from the start point (48 hr after transfection).

# CHAPTER 3

## Preliminary validation of 2 micron plasmid-yeast chromosome association for plasmid segregation

Chromosome spread assays were performed in order to clarify if the 2 micron plasmid is tethered to chromosomes. Instead of using a multi-copy *STB* reporter plasmid, as was done in prior studies, a single copy (or very nearly single copy) reporter plasmid was employed to increase the sensitivity of the assay. The conclusions from these studies are as follows. **1.** The *STB* reporter plasmid associates with the chromosome spreads, but only in the presence of both Rep1 and Rep2. The efficiency of this association is only slightly lower than that of a *CEN* reporter plasmid (by ~10%), which served as a positive control in these assays. **2.** Spindle integrity is required for *STB* plasmid-chromosome association. When the mitotic spindle is disassembled, plasmid presence in chromosome spreads is greatly diminished. However, plasmid-chromosome association can be re-established when the spindle is restored. **3.** In the absence of the nuclear motor protein Kip1, there is a modest decrease in the fraction of plasmid containing chromosome spreads. Previous work demonstrated that *kip1Δ* increases the frequency of unequal plasmid segregation. **4.** When Rsc2, a component of the RSC chromatin remodeling complex, is depleted, there is >30% drop in the association of the *STB* plasmid with chromosomes. Lack of Rsc2 has been shown to increase the loss rate of the 2 micron plasmid. **5**. In order to affirm the validity of the spread assay, the simultaneous presence

of two single copy *STB* plasmids in chromosome spreads was probed. The frequency of binary plasmid association with chromosomes was significantly greater than that expected for independent association of each plasmid with a certain probability. **6**. The net results suggest that Rep1-Rep2 dependent presence of the *STB* plasmid in chromosome spreads signify authentic plasmid-chromosome association relevant to plasmid segregation.

### 3.1 Introduction

The equal segregation of the 2 micron plasmid, mediated by the Rep1-Rep2-*STB* partitioning system, is intimately coupled to that of chromosome segregation (Ghosh et al., 2007; Velmurugan et al., 2000; Yang et al., 2004). Analyses of *STB* reporter plasmids visualized by FLORS (fluorescence labeling by the operator-repressor system) suggest that the plasmid foci closely resemble chromosomes in their dynamics and segregation during the mitotic cell cycle (Velmurugan et al., 2000). Time lapse-FLORS analyses of a single (or a very low) copy *STB* reporter plasmid against a *CEN* reporter plasmid in cells containing both the plasmids also lead to similar conclusions (Ghosh et al., 2007). In these assays, the two plasmids are distinguished by differential fluorescence tagging, green (GFP-LacI-LacO) in one case and red (TetR-RFP-TetO) in the other. Similar experiments also reveal that sister *STB* plasmids formed by replication, one pair labeled green and the other red, segregate 1:1 nearly 80% of the time, that is, each daughter cell receives one green and one red plasmid. In an analogous assay with two differentially fluorescence tagged *CEN* plasmids, the sister-to-sister segregation is nearly 100%. These

early observations form the basis for the notion that the Rep-*STB* system confers chromosome-like segregation behavior on the 2 micron plasmid.

An important question raised by the cell biological studies of multi-copy and single copy *STB* reporter plasmids is whether equal plasmid segregation is directly dependent on chromosome segregation, or can occur even when chromosome segregation is interfered with. So far, nearly all efforts to uncouple plasmid segregation from chromosome segregation, other than by inactivating the Rep-*STB* system, have been unsuccessful (Mehta et al., 2002; 2005). An exception is an altered cell cycle program in which the assembly of the spindle is delayed until G2/M, that is, until after chromosome replication and plasmid replication have been completed. Attempts to establish conditions that force chromosome missegregation while maintaining normal plasmid segregation have also been unproductive (Mehta et al., 2005).

Two plausible models for plasmid segregation consistent with the results summarized above may be considered. First, the plasmid directly utilizes the chromosome segregation mechanism for its own partitioning with the assistance of the Rep-*STB* system. Alternatively, the Rep-*STB* system facilitates the association of the plasmid with chromosomes, thus indirectly taking advantage of the chromosome segregation machinery. The first model seems unlikely for the following reasons. First, it is difficult to imagine that a simple partitioning system consisting of just two plasmid coded proteins and a partitioning locus would be sufficient to recapitulate the chromosome segregation pathway, which is dependent on a large number of protein factors and whose fidelity is ensured by multiple surveillance mechanisms (Bouck et al.,

2008; Clarke and Bachant, 2008; Ghosh et al., 2006; McIntosh et al., 2003). Second, the kinetochore complex, whose crucial role is to attach chromosomes to the mitotic spindle via their centromeres, does not appear to be assembled at *STB*. Third, delaying spindle assembly until G2/M does not affect normal chromosome segregation whereas it disrupts *STB* plasmid segregation. The second model for plasmid segregation, by tethering to chromosomes and hitchhiking on them, is therefore favored. The integrity of the mitotic spindle prior to or at the time of plasmid replication may be important for plasmid-chromosome association. The nuclear motor protein Kip1 may also contribute to this spindle effect on plasmid segregation (Cui et al., 2009).

As a first test of the hitchhiking model for plasmid segregation, we wished to follow the association of *STB* reporter plasmids with chromosome spreads prepared from mitotic cells. The mitotic chromosomes in the budding yeast are not as strongly condensed as in higher eukaryotic cells, and it is impossible to obtain resolution of individual chromosomes Furthermore, the chromosome spreads are not entirely free of components of the nuclear envelope or of sub-nuclear scaffold structures. In spite of these technical limitations, we have now extended and refined previous chromosome spread analyses by employing single copy reporter plasmids under conditions where the Rep-*STB* system is functional or non-functional as well as by utilizing mutations that are known to affect normal plasmid segregation.

In previous yeast chromosome spread analyses, a multi-copy reporter plasmid was found to be absent in spreads prepared from cells treated with nocodazole to depolymerize microtubules (Mehta et al., 2005). The nuclear motor protein Kip1 has also

been found to be required for normal nuclear localization of a nearly single copy *STB* plasmid as well as its segregation (Cui et al., 2009; Ghosh et al., 2007). These two pieces of data suggest that the spindle and Kip1 motor may act cooperatively to transport the 2 micron plasmid to its normal nuclear location, where it might have a high probability of tethering to a chromosome.

Results from previous analyses show that an *STB* containing reporter plasmid can be localized in yeast chromosome spreads from a [$cir^+$] strain but not from a [$cir^0$] strain (Velmurugan et al., 2000). The difference between [cir+] and [cir0] strains is that the former contains Rep1 and Rep2 proteins supplied by the native plasmid molecules whereas the latter lacks these proteins. It was noticed that Rep1 and Rep2 proteins can localize to chromosome spreads even in the absence of the 2 micron plasmid or of an *STB* containing reporter plasmid derived from it. These results suggest that 2 micron plasmid tethering to chromosomes, if it does occur, is likely mediated by Rep proteins. However, as noted before, the limited resolution of the yeast system makes it hard to draw definitive conclusions regarding the direct association between plasmid and chromosomes. The presence of a reporter plasmid in the chromosome spreads preparations may simply result from association of the plasmid and certain chromosomal anchoring regions with common locations on the nuclear matrix. By using a single copy reporter plasmid, I wished to address whether such a plasmid is tethered to chromosomes and whether such tethering is relevant to its segregation.

### 3.2 Results

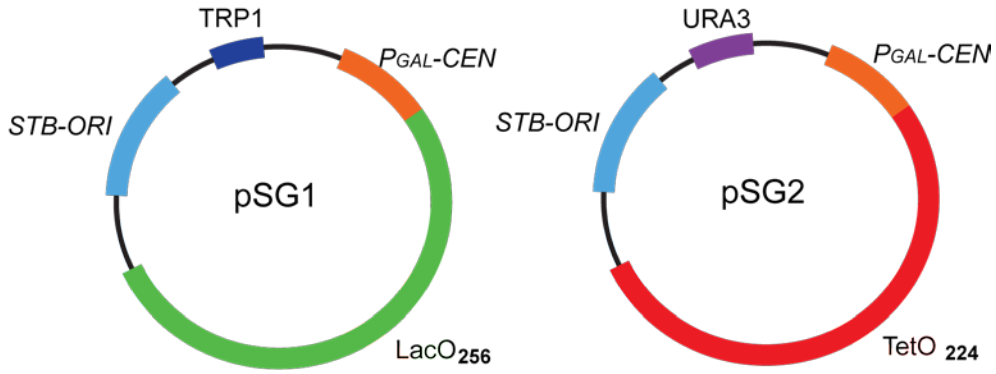
### 3.2.1 Rationale of the single copy *STB* plasmid design: advantages over a multi-copy reporter plasmid in addressing chromosome tethering

As pointed out, previous work using a multi-copy reporter plasmid suggested that the Rep-*STB* system promotes plasmid association with chromosome spreads. This plasmid is present, on average, as 3-5 foci per nucleus. We were concerned that the authenticity and specificity of plasmid-chromosome association might be compromised by the multiple foci number, increasing the chances of artifacts. As yeast chromosome spreads are not comprised exclusively of chromosomes, and the chromosomes themselves form a congressed mass, the random presence of a plasmid focus in a spread was potentially high. We therefore performed chromosome spread assays using a single copy reporter plasmid, so as to minimize the chance association of the plasmid with the spreads.

The single copy *STB* plasmid pSG1 described in previously published work (Ghosh et al., 2007) (Fig. 3.1A) includes a copy of *CEN* in its backbone, which is responsible for bringing down the copy number to nearly one. The *CEN* is placed immediately downstream of the *GAL* promoter, and can be conditionally inactivated by inducing high-level transcription in the presence of galactose. As a result, the plasmid can be made to behave as an *STB* plasmid in galactose medium when provided with Rep1 and Rep2. Conversely, it can be made to behave as a true *CEN* plasmid in glucose medium in the absence of the Rep proteins. Furthermore, in galactose medium in the absence of the Rep proteins, the plasmid behaves as an *ARS* plasmid (lacking a partitioning system), as neither *CEN* nor *STB* will be functional (Fig. 3.1B). The plasmid is present as a single

focus in >90% of the glucose grown cells. Thus, the effective volume occupied by it in the nucleus is one third to one fifth of that occupied by a multi-copy reporter plasmid (Fig. 3.2A). The chances of accidental interaction of the plasmid with chromosomes are reduced by α factor of 3-5. Furthermore, the methodology and time scale of the chromosome spread assays is such that no cell division occurs during this period, and consequently there is no change in plasmid copy number per cell due to potential missegregation. A second single copy *STB* plasmid used in a subset of the assays was conceptually similar in its organization to pSG1.

A




B

| | Glucose | Galactose |
|---|---|---|
| [cir $^{+}$] (Rep1+; Rep2+) | CEN+; STB+ | CEN-; STB+ |
| [cir $^{0}$] (Rep1 -; Rep2-) | CEN+; STB - | CEN-; STB - |

**Figure 3.1 The design of *CEN-STB* single copy reporter plasmids.**

(A) Single copy 2 micron reporter plasmids (pSG1; pSG2) contain both *CEN* and *STB* sequences. The *CEN* sequence is placed downstream of the *GAL* promoter, and can be conditionally inactivated. The LacO or TetO arrays present in these plasmids provide binding sites for GFP-LacI or TetR-RFP, respectively, expressed in the host strains. The plasmids can thus be followed by fluorescence microscopy. (B) The *CEN* or *STB* or both in a single copy plasmid can be inactivated depending on the [cir$^{0}$] or [cir$^{+}$] status of the host as well as the carbon source (glucose or galactose) present in the medium.

### 3.2.2 Association of a single copy *STB* plasmid with chromosome spreads in the presence of Rep1 and Rep2

First we assayed chromosome spreads prepared from [cir$^+$] cells grown in glucose (*CEN* active) and arrested in G1 using α-factor. The [cir$^+$] strain contains the native 2 micron plasmid, which serves as the source of Rep1 and Rep2. The plasmid was present in ~93% of chromosome spreads under this condition (Fig. 3.2B). When the assay was repeated in a [cir$^0$] (but otherwise isogenic) host strain, the fraction of plasmid containing spreads remained more or less the same (data not shown). Thus, when *CEN* alone is active or both *CEN* and *STB* are potentially active in the plasmid, it is almost always present in the spreads.

Next, we prepared chromosome spreads from glucose-grown [cir+] and [cir$^0$] cells arrested in G1 with α-factor after shifting them to galactose medium (*CEN* inactive). The single copy *STB* plasmid was present in ~80% of the chromosome spreads from the [cir$^+$] strain (*STB* active) (Fig. 3.2B). By contrast, in the [cir$^0$] strain (lacking the Rep proteins; *STB* inactive), the plasmid was associated with <20% of the spreads (Fig. 3.2B).

The above results suggest that a single copy *STB* reporter plasmid associates with chromosomes with high efficiency when provided with the Rep proteins. As pointed out earlier, this interpretation is subject to the caveat that the spreads are not comprised of chromosomes exclusively, but also contain other nuclear associated entities. A single copy *ARS* plasmid is rarely present in chromosome spreads, regardless of the presence or absence of Rep proteins. A *CEN* plasmid is almost always localized in the spreads, indicating that such a plasmid serves as a good proxy for chromosomes.

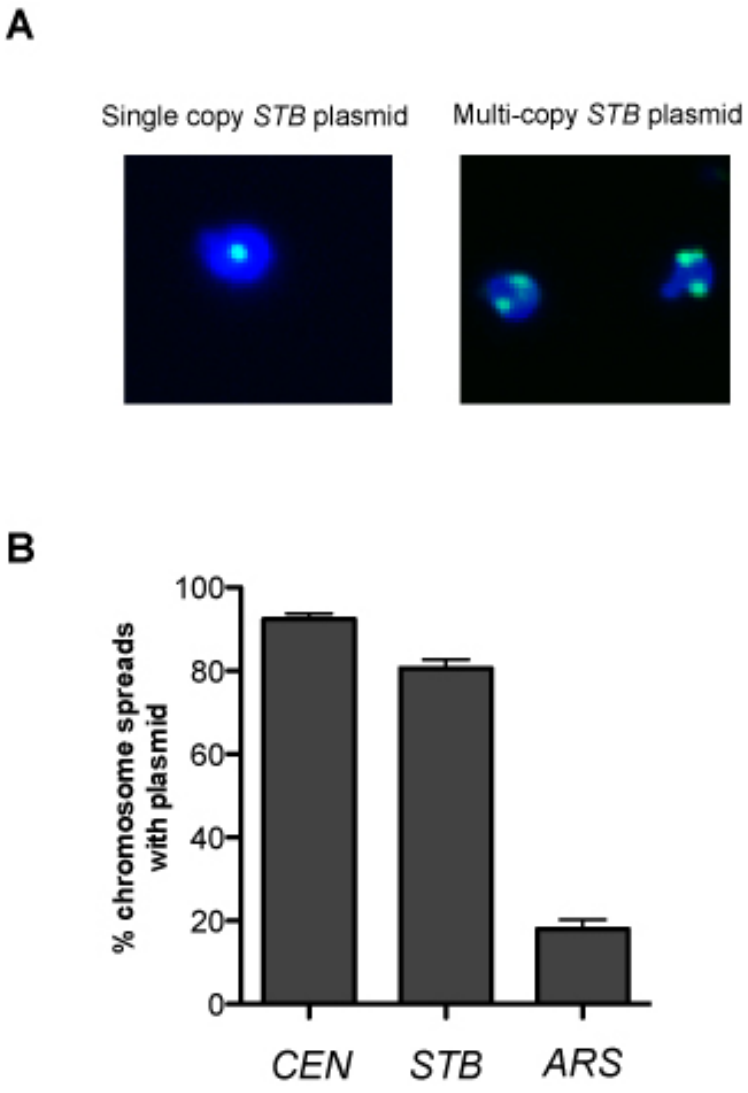



**Figure 3.2 A single copy reporter *STB* plasmid associates with chromosome spreads.**

(A) In chromosome spread preparations, a single copy *STB* reporter plasmid pSG1 ([cir$^+$]; galactose) was seen as a single focus (left). A multi-copy *STB* reporter plasmid was present as more than one focus, usually 3-5 foci per spread (right). The chromosomes were revealed as a blue mass by staining them with DAPI. The plasmid was visualized by the green fluorescence of GFP-LacI associated with it. (B) Chromosome spreads were prepared under specific conditions that induce the single copy reporter plasmid to behave as a *CEN*, *STB* or *ARS* plasmid. The histograms represent the fraction of spreads containing the plasmid signal. The bars indicate standard error of the mean (SEM).

### 3.2.3 Absolute requirement of Rep1 and Rep2, but not other *trans*-acting 2 micron plasmid factors, for plasmid-chromosome association

In the assays summarized in Fig 3.2B, the endogenous 2 micron plasmid present in a [$cir^+$] strain was utilized as the source for Rep1 and Rep2 proteins. In order to verify that the association of the *STB* plasmid with chromosomes is promoted by Rep1 and Rep2 proteins, but not by other plasmid components, these proteins were expressed one at a time or together in a [$cir^0$] experimental strain. In this set of assays, the inducible *GAL* promoter controlled the expression of *REP1* and/or *REP2*, integrated at a chromosomal locus.

Neither Rep1 nor Rep2, by itself, was able to associate with chromosome spreads; however, when co-expressed, both proteins were detected in the vast majority of chromosome spreads (Fig. 3.3A). The presence of an *STB* plasmid was not required for Rep1-Rep2 association with chromosome spreads. Next, the association of the single copy *STB* reporter plasmid with chromosome spreads was probed when it was supplied with either Rep1 or Rep2 alone or with both the proteins. In the presence of only one of the Rep proteins, the *STB* plasmid was no better than an *ARS* plasmid in its chromosome association (<20%). By contrast, expression of both Rep1 and Rep2 raised the percentage of plasmid-chromosome association to ~70%. (Fig. 3.3B)

The combined results from Fig. 3.2B and Fig. 3.3B suggest that the fraction of plasmid containing chromosomes spreads from the [$cir^0$] strain in the presence of Rep1 plus Rep2 is similar to that observed in the [$cir^+$] strain. In addition, the fraction of spreads containing the Rep proteins (in the absence of a reporter plasmid) and that

containing the reporter plasmid complemented by Rep1 plus Rep2 are nearly the same. Taken together these findings indicate that, as far as the contributions from the 2 micron plasmid are concerned, Rep1 and Rep2 are necessary and sufficient for the association of an *STB* plasmid with chromosomes.

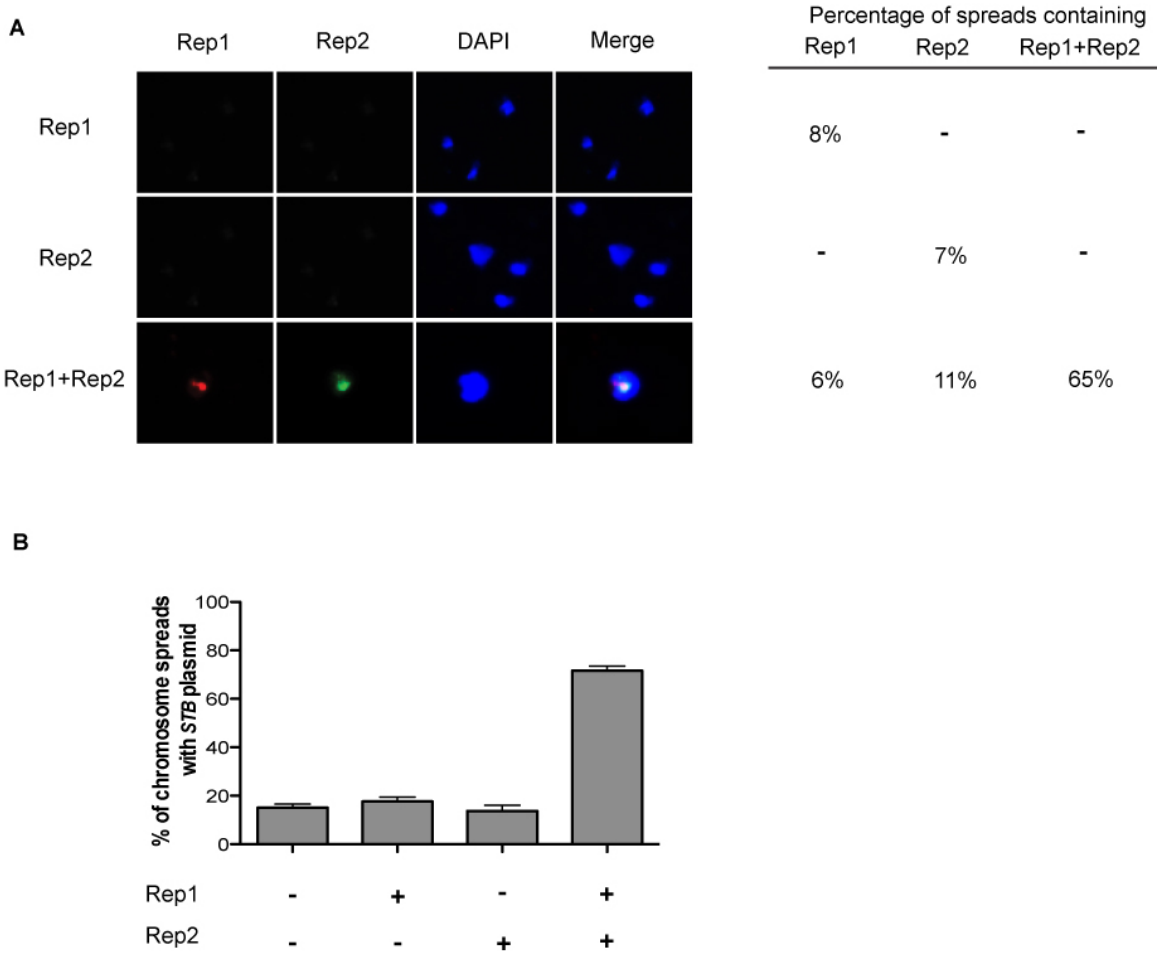



**Figure 3.3 A single copy reporter plasmid present in a [cir$^0$] strain associates with chromosome spreads when Rep1 and Rep2 are expressed.**

(A) The presence of Rep1 or Rep2 in chromosome spreads was assayed in a [cir$^0$] strain expressing Rep1 or Rep2 or both. The proteins were detected by immunofluorescence. The red and green signals represent Rep1 and Rep2 respectively. (B) Chromosome spreads were prepared after shifting glucose grown cells to galactose medium in the presence of α-factor to inactivate the plasmid-borne *CEN* and to impose G1 arrest. The fractions of plasmid containing spreads are presented as bar graphs. The bars denote standard error of the mean (SEM).

### 3.2.4 Simultaneous association of two single copy reporter plasmids with chromosomes

As already noted, there is some uncertainty in equating the presence of a plasmmid in chromosome spreads with its actual tethering to chromosomes. To minimize this uncertainty, we increased the sensitivity of the chromosome spread analysis by using two single copy plasmids harbored by the experimental strain as reporters. If Rep-*STB* mediated plasmid-chromosome association is real, the expectation is that the simultaneous presence of the two reporters in the spreads would be high, and would be nearly the same as that of a single reprter observed in previous assays (Fig. 3.2B).

The two reporters were quite similar in their organization, and each contained an identical *ORI-STB* fragment from the 2 micron circle (Fig. 3.1A). They were distinguished by their fluorescence, green (LacO-[GFP-LacI]) or red (TetO-[TetR-RFP]). The chromosome spreads were divided into four groups based on whether they harbored only one of the two plasmids (I and II) or both plasmids (III) or neither plasmid (IV). The two reporters were detected together in ~90% of the spreads (Class III) when their *CEN* sequences were kept active (Fig. 3.4). When each of the plasmids contained an active *STB* (with Rep1 and Rep2 supplied) but an inactive *CEN*, the fraction of the Class III spreads was ~80%. When each plasmid was maintained in its *ARS* form (inactive *CEN* and *STB*), the spreads lacking either plasmid (Class IV; ~70%) far outnumbered the other three classes. Furthermore, the Class III spreads (containing both plasmids) constituted the smallest fraction.

The *CEN* and *STB* plasmids behave in a similar fashion in that their association with chromosome spreads remains high, and virtually unaltered, when the number of reporter plasmids is doubled. The *ARS* plasmid shows a diametrically opposite pattern, the simultaneous association of the two reporter plasmids with chromosome spreads being significantly lower than that of the poor association of each individual plasmid. A reasonable interpretation of these data is that the Rep proteins actively promote the tethering of an *STB* plasmid to yeast chromosomes. In the absence of the Rep-*STB* system, a plasmid has a very low probability 'p' of being trapped in a chromosome spread. The observed value for the simultaneous presence of two *ARS* plasmids in a spread is consistent with the expectation that they behave independently ($p \times p = p^2$).

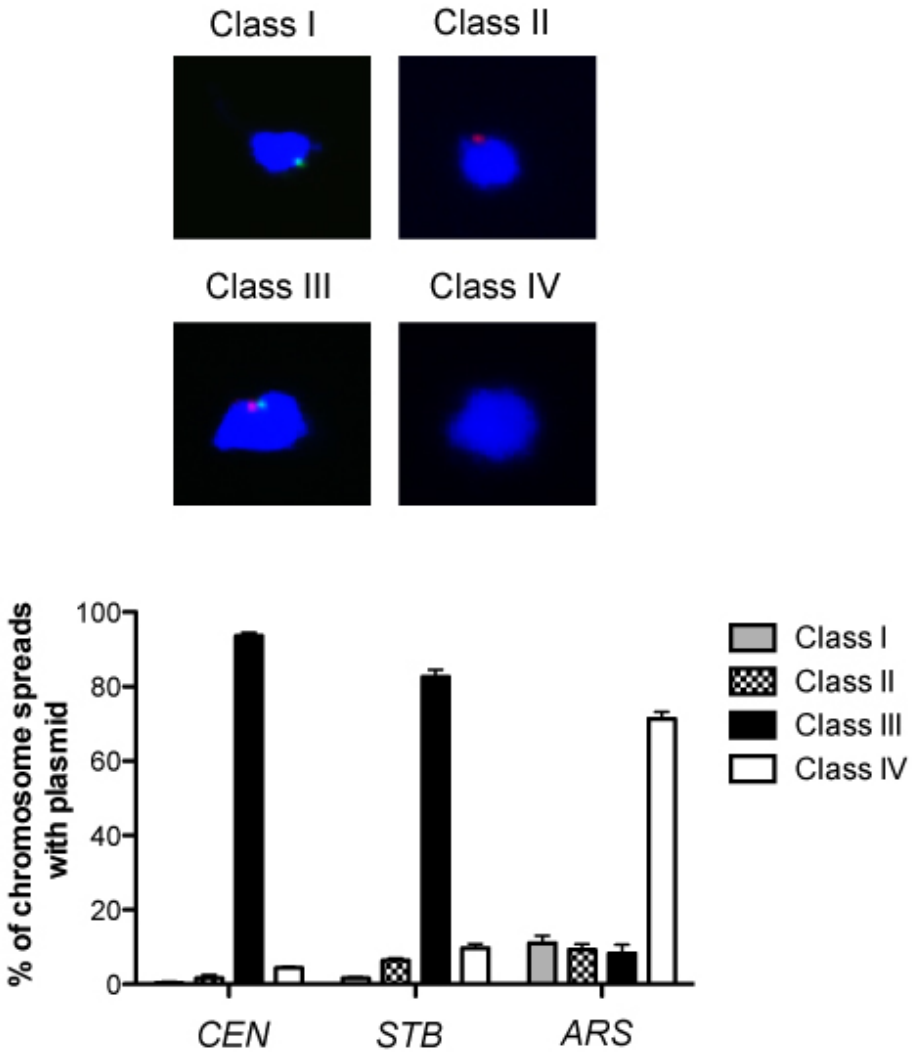



**Figure 3.4 A dual-reporter chromosome spread assay supports Rep1-Rep2 dependent association of the 2 micron plasmid with chromosomes.**

Two single copy reporter plasmids were introduced into the same host strain expressing GFP-LacI and TetR-RFP. The two plasmids were visualized as green and red foci by virtue of the LacO and TetO arrays, respectively, harbored by them. The chromosome spreads prepared under each experimental condition were separated into four classes, depending on whether they contained only one plasmid or both plasmids or lacked plasmids. Each class of spreads obtained for the *CEN*, *STB* and *ARS* states of the reporter plasmids is indicated by the bar graphs. The bars denote standard error of the mean (SEM).

### 3.2.5 Spindle integrity is required for the *STB* plasmid chromosome association

The integrity of the mitotic spindle is required for equal segregation of the 2 micron plasmid; however the role of the spindle appears to be indirect (Mehta et al., 2005). A possible role for the spindle is in mediating the association of the plasmid with chromosomes. We utilized the chromosome spread assay employing a single copy reporter plasmid to test this notion.

First we performed a kinetic analysis of the association of the *CEN* and *STB* forms of the reporter plasmid with chromosome spreads as a function of the cell cycle (Fig. 3.5). Cells were first arrested in G1 with α-factor, and after releasing them, chromosome spreads were prepared at various time points corresponding to the progression of a cell cycle. The times for cell cycle completion in glucose and galactose medium were 90 min and 120 min, respectively. The fractions of plasmid-containing spreads remained more or less unchanged during the cell cycle for both the *CEN* and *STB* plasmids.

Next, the assay was repeated by releasing the cells from G1 in the presence of nocodazole to arrest them in G2/M. The spreads prepared from this cell cycle stage showed similar values for the presence of the *CEN* plasmid for normal as well as nocodazole treated cells (~90%). However, there was a sharp reduction in the presence of the *STB* plasmid in spreads prepared from the nocodazole treated cells (from ~80% to ~25%). We then followed the plasmid in chromosome spreads after removing nocodazole, and allowing the cells to recover in drug-free medium for 45 min (glucose) or 60 min (galactose). Previous experiments showed that the mitotic spindle was fully restored by this time, and cells were predominantly in anaphase/early telophase stage.

The association of the *STB* plasmid with chromosome spreads was restored to slightly less than normal (~65%) following spindle reassembly (Fig. 3.6).

The association of the 2 micron plasmid with chromosomes appears to be maintained throughout a normal cell cycle. The time resolution of the assays is not sufficient to rule out transient periods of dissociation of the plasmid from chromosomes. Plasmid-chromosome association is disrupted by spindle disassembly, and can be re-instated to a considerable extent by restoring spindle integrity.

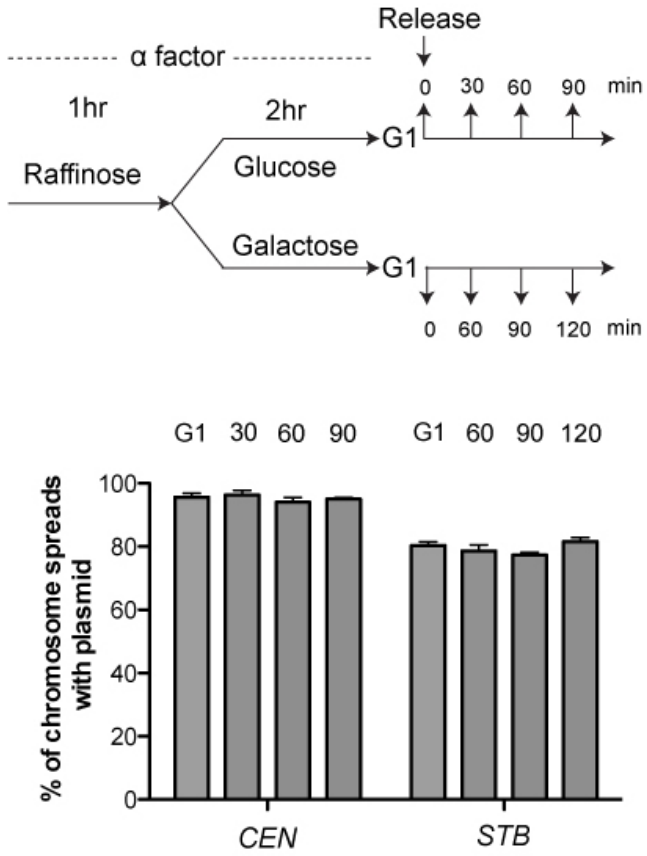



**Figure 3.5 The association of an *STB* plasmid with chromosome spreads is maintained throughout cell cycle.**

The experimental scheme is outlined at the top. Following G1 arrest, cells were released into the cell cycle, and spreads were prepared at different time points (Glucose: 0 min, 30 min, 60 min, 90 min / Galactose: 0 min, 60 min, 90 min, 120 min). The bar graphs represent the fractions of spreads containing the plasmid in its *CEN*-active (left) and *STB*-active (right) forms. The bars denote standard error of the mean (SEM).

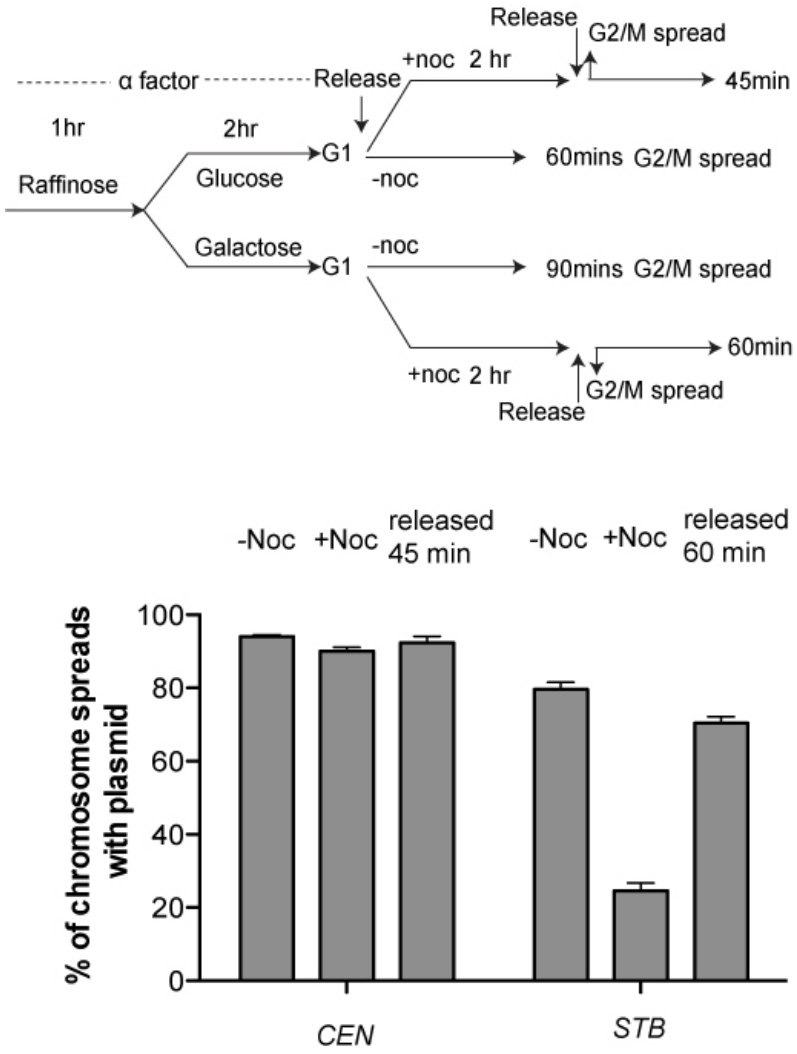



**Figure 3.6 Spindle disruption blocks the association between an *STB* plasmid and chromosome spreads.**

The experimental scheme is described at the top. G1 arrested cells were released into the cell cycle in medium with or without nocodazole. The predominant fraction of the cells was arrested at G2/M after 2 hr in the nocodazole containing medium. The G2/M stage during the normal cell cycle was identified by large budded cells containing a single DAPI mass near the bud neck. The spindle length in such cells, visualized by immune-staining for tubulin, was ~1μm. Chromosome spreads prepared from G2/M stage cells and from nocodazole treated cells after their recovery in drug-free medium for 45 min (glucose) or for 60 min (galactose) were scored for the presence of the plasmid. The graphs represent the fractions of plasmid positive spreads, with the bars denoting standard error of the mean (SEM).

### 3.2.6 Plasmid-chromosome association in a *kip1Δ* strain

Kip1 is a kinesin family nuclear motor in *S. cerevisiae* that shares redundant functions with Cin8 (Hildebrandt and Hoyt, 2000; Tytell and Sorger, 2006). These spindle associated proteins play important roles in microtubule dynamics during mitosis. They are also associated with centromeres, and may contribute to chromosome segregation in ways that are not directly related to their spindle functions. Kip1 is also recruited to *STB* in a Rep1-Rep2 dependent manner, and is required for normal 2 micron plasmid segregation (Cui et al., 2009). The requirement for an intact mitotic spindle and for Kip1 in 2 micron plasmid segregation suggests their mechanistically overlapping or related roles in this process, although the molecular nature of this connection remains unknown. We therefore tested whether the association of a single copy *STB* reporter plasmid with chromosomes is compromised in the absence of Kip1.

There was no difference in the extent of *CEN* plasmid association with chromosome spreads between the wild type and *kip1Δ* strains (Fig. 3.7A) in G1 arrested cells or cells at the G2/M stage (60 min after release from G1 arrest). However, there was a 10 to 15 % decrease in *STB* plasmid association with chromosome spreads at both G1 and G2/M stages (~90 min after release from G1 arrest) (Fig. 3.7A).

Chromosome spreads were also analyzed from anaphase/telophase cells (~120 min after G1 release) recognized by well separated nuclei in the mother and daughter compartments (Fig. 3.8B). At this time point in the cell cycle, >90% of the cell population showed this phenotype. The fractions of *STB* plasmid containing spreads in the wild type and *kip1Δ* strains were unaltered or only slightly reduced in anaphase cells

compared to G1 or G2/M cells. However, there were two classes of plasmid containing spreads at anaphase, those with a single plasmid focus and those with two plasmid foci. The latter class of spreads was increased by *kip1Δ*) compared to the wild type (~14%).

The lack of Kip1 causes a modest but significant reduction in the association between the *STB* plasmid and chromosomes, and this reduction appears to be independent of the cell cycle stage. However the effect of *kip1Δ* is considerably smaller than that of microtubule depolymerization. Since plasmid replication is nearly 100% during a cell cycle and is not affected by *kip1Δ* (as inferred from previous studies), the chromosome spreads with two plasmids indicate missegregation of plasmid sisters. The increase in this class of spreads in the absence of Kip1 compared to its presence (Fig. 3.7B) is in agreement with a role for Kip1 in 2 micron plasmid segregation suggested by previous studies (Cui et al., 2009).

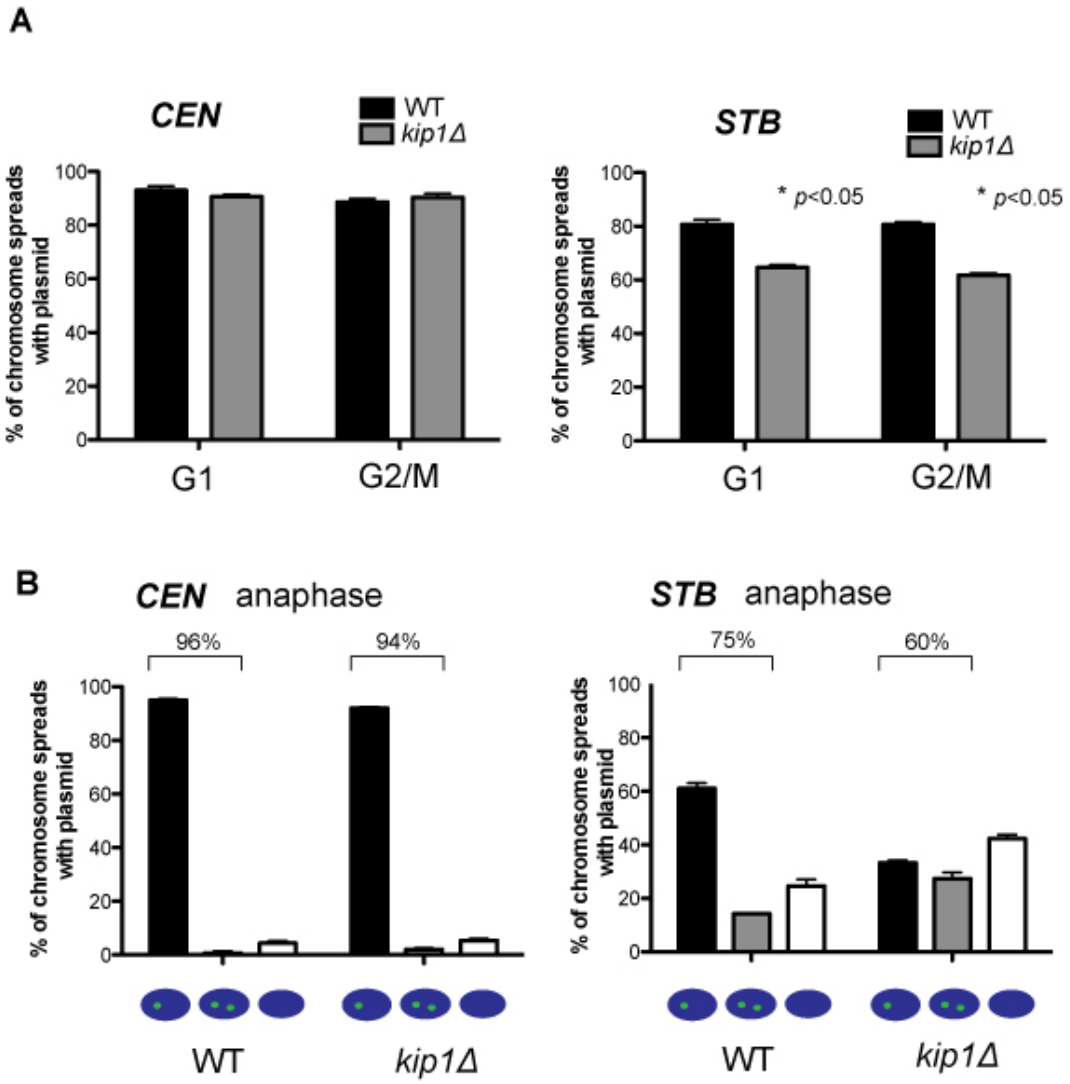



**Figure 3.7 Association of the *STB* plasmid with chromosome spreads from anaphase cells in the wild type and *kip1*Δ strains.**

(A) The percentage of plasmid containing spreads in the wild type and *kip1*Δ strains is plotted with the vertical bars indicating the SEM. The decrease due to lack of Kip1 is significant, ($p<0.05$) (B) Chromosome spreads were analyzed at 90 min (glucose), 120 min (galactose) after the cells were released from G1 arrest. ~90% of the cell population at this time was in the anaphase/telophase stage, as suggested by two DAPI staining chromosome masses that were well separated from each other in the mother and daughter compartments. The smaller fraction of chromosome spreads containing two plasmid foci were grouped separately from those containing a single plasmid focus.

### 3.2.7 STB plasmid association with chromosome spreads is reduced by *rsc2Δ*

The Rsc2 protein is a component of the RSC chromatin remodeling complex, which belongs to the SWI/SNF family of remodeling complexes of *S. cerevisiae* (Cairns et al., 1996; Wong et al., 2002; Yukawa et al., 2002). There are two versions of the RSC complex, RSC1 and RSC2 with multiple shared components. Rsc1 and Rsc2 are unique to the RSC1 and RSC2 complexes, respectively. Whereas *rsc1*Δ or *rsc2*Δ is tolerated by cells, the double deletion causes lethality. The dispensability of either Rsc1 or Rsc2 is consistent with the redundant functions of the RSC1 and RSC2 complexes. Lack of Rsc2, but not that of Rsc1, causes an increase in the loss rate of the 2 micron plasmid (Wong et al., 2002), suggesting a role for the RSC2 complex in the functional organization of the *STB* chromatin. Consistent with this notion, the interaction between Rep1 and *STB* is disrupted in an *rsc2*Δ strain without affecting Rep2-*STB* interaction (Yang et al., 2004). Furthermore, recent experiments have demonstrated the interaction of other RSC components, Rsc8 and Rsc58, with the Rep-*STB* system (Ma et al., 2013). Conditional inactivation of Rsc8 or Rsc58 also causes an increase in the rate of missegregation of an *STB* reporter plasmid. Based on the Rsc2-dependence for normal Rep1-*STB* interaction and the requirement of the RSC2 complex for normal plasmid segregation, we have now examined the effect of *rsc2*Δ on the association of an *STB* plasmid with chromosome spreads.

There was a ~30% decrease in the number of *STB* plasmid containing spreads in the *rsc2*Δ strain compared to the wild type at the G1 stage (Fig. 3.8). There was a further drop (by 10%) in plasmid-chromosome spread association due to *rsc2*Δ when cells were

analyzed at 60 min after release from G1. These cells correspond to the G2/M stage of the cell cycle.

The absence of Rsc2 adversely affects *STB* plasmid association with chromosomes, the magnitude of the effect being intermediate between those of spindle disassembly and *kip1*Δ. Furthermore, the requirement of Rsc2 in plasmid-chromosome association appears to be even more important after plasmid replication has been completed. Such a functional modulation, depending on the cell cycle stage, has not been observed for Kip1.

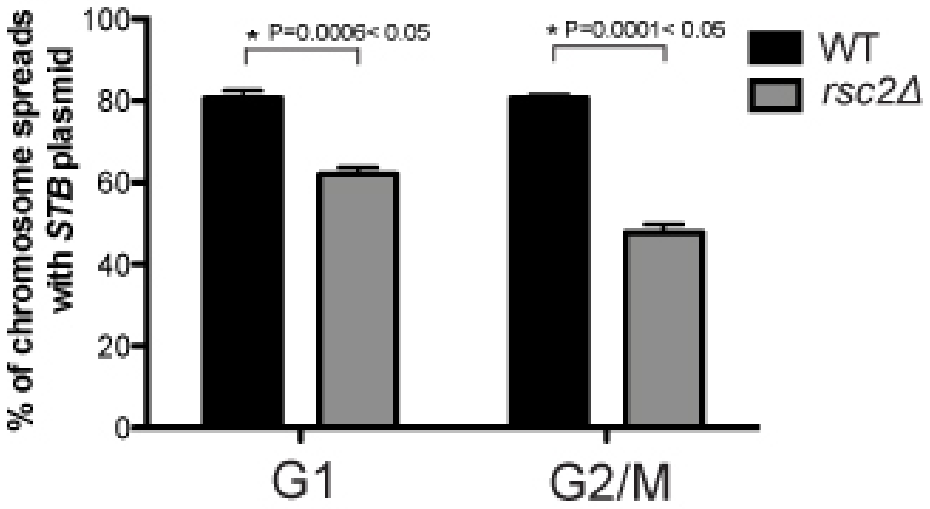



**Figure 3.8 Association of an *STB* plasmid with chromosomes in an *rsc2*Δ strain.**
The chromosome spread assays were performed in the wild type and *rsc2*Δ strains using G1 arrested cells or G2/M cells.

## 3.3 Discussion

The experiments described in this chapter were aimed at testing the hitchhiking model for the segregation of the 2 micorn plasmid from two different aspects. One important criterion for the validity of this model is the physical association between the plasmid and chromosomes. The results from chromosome spread assays performed under a variety of conditions satisfy this criterion.

### 3.3.1 Evidence supporting the tethering of the 2 micron plasmid to chromosomes

A single copy *STB* reporter plasmid associates with chromosome spreads with an efficiency that is only slightly lower than that of a *CEN* plasmid. This association is absolutely dependent upon both Rep1 and Rep2 proteins. In the absence of a functional Rep-*STB* system, there is a conspicuous drop in the frequency of plasmid presence in chromosome spreads. The association between the *STB* plasmid and chromosomes is maintained throughout the cell cycle. The functional relevance of plasmid-chromosome association is further supported by the fact that the efficiency for the simultaneous association of two single copy *STB* plasmids with chromosome spreads is almost the same as that for the association of one single copy *STB* plasmid. By contrast, the simultaneous presence of two single copy *ARS* plasmids in chromosome spreads is quite rare, markedly less frequent than the presence of one single copy *ARS* plasmid. The magnitude of the difference is consistent with a certain small probability for the accidental association of a plasmid with chromosome spreads even when the Rep-*STB* system is non-functional. This is not surprising, as chromosome spreads are not pure

chromosome preparations, but also include additional nuclear materials to different extents. Based on the cumulative observations on the single and dual reporter plasmid assays in the absence and presence of a functional partitioning system, we conclude that the Rep proteins promote the tethering of the 2 micron plasmid to *S. cerevisiae* chromosomes.

The 2 micron plasmid-chromosome association is functionally analogous to the association of viral episomes to mammalian chromosomes (Botchan, 2004; Frappier, 2004; Ilves et al., 1999; Lehman and Botchan, 1998; You et al., 2004). For example, the virally encoded partitioning proteins EBNA1 and E2 are critical in tethering EBV (Epstein-Barr virus) and HPV (human papilloma virus), respectively, to chromosomes. The viral partitioning proteins partner with distinct cellular proteins in this process, hEBP2 in the case of EBNA1 and Brd4 in the case of HPV (Kapoor et al., 2005; McBride et al., 2012; Nayyar et al., 2009; Van Tine et al., 2004). The identity of host protein partner(s) of Rep1-Rep2 that assist 2-micron plasmid-chromosome association is yet to be revealed.

### 3.3.2 Functional relevance of *STB* plasmid-chromosome association to 2 micron plasmid segregation

The fact that the integrity of the Rep-*STB* system is absolutely required for plasmid-chromosome association strongly suggests that this association is important for equal segregation of the 2 micron plasmid. This notion is further supported by the impairment of plasmid-chromosome association under three conditions that have been

previously demonstrated to decrease the fidelity of 2 micron plasmid segregation, namely disruption of spindle integrity, *kip1*Δ and *rsc2*Δ.

The largest decrease in plasmid-chromosome association is caused by spindle disassembly, which can be fully reversed by restoring the spindle. The absence of Kip1 causes a more modest decrease in the extent of plasmid-chromosome association. If Kip1 acts in concert with the spindle in promoting plasmid tethering to chromosomes, the simplest expectation is that *kip1*Δ and spindle disassembly should have had the same or similar effects. It is possible that Kip1 function is at least partially redundant. Alternatively, Kip1 may be required only under certain conditions. For example, in association with the spindle, it may help retrieve plasmids that become temporarily dissociated from chromosomes and re-establish plasmid-chromosome association. Previous single generation segregation assays using a multi-copy reporter plasmid showed that *kip1*Δ increases the rate of unequal plasmid segregation, but rarely causes plasmid loss (or total missegregation) (Cui et al., 2009). The increase in the fraction of anaphase chromosome spreads containing two plasmids, rather than one, observed in the present study using a single copy *STB* reporter plasmid is consistent with the higher rate of unequal 2 micron plasmid segregation caused by *kip1*Δ. Finally, the association between an *STB* plasmid and chromosomes is also diminished by *rsc2*Δ, which has been shown to elevate the loss rate of the 2 micron plasmid. In our analyses, the effect of *rsc2*Δ is intermediate between that of spindle disassembly and *kip1*Δ. The stronger effect of *rsc2*Δ at G2/M compared to G1 may signify its role in mediating the tethering of

replicated plasmids to chromosomes. Such a role would be relevant if there is temporary detachment of plasmids from chromosomes at the time of their replication.

### 3.4 Summary and perspectives

The chromosome spread assays with single copy reporter plasmids enhance the confidence in the hitchhiking model for 2 micron plasmid segregation, initially proposed on the basis of purely circumstantial lines of evidence. To be unequivocally certain that the plasmid foci seen in the chromosome spreads are not associated with nuclear membrane fragments contaminating these preparations, we have conducted a set of experiments described briefly in the next chapter (**Chapter 4**). Subsequent chapters (**a**) address the role of plasmid replication in coupling plasmid segregation to chromosome segregation and (**b**) probe potential plasmid-chromosome association at single chromosome resolution offered by mammalian cells.

# CHAPTER 4

## 2 micron plasmid segregation is not coupled to nuclear membrane

The experiments described in this chapter were aimed at distinguishing between the two possibilities suggested by the chromosome spread assays (**Chapter 3**): (**a**) plasmid-chromosome tethering and (**b**) plasmid-nuclear membrane tethering (perhaps less likely). All assays were performed using single copy reporter plasmids. The salient findings are as follows. **1**. When an *ARS* plasmid (lacking *STB*) is tethered to the nuclear membrane via Mlp1 or Nup2, its equal segregation frequency increases but does not reach the level of an *STB* plasmid. **2**. When an *STB* plasmid is similarly tethered to the nuclear membrane, its equal segregation frequency decreases from the normal value. **3**. In a mitotic cell cycle in which the entire set of sister chromatids is forced to missegregate, the *STB* plasmid sisters follow the chromosomes and not the nuclear membrane. These results argue against nuclear envelope tethering, and in favor of chromosome tethering, as the strategy for the equal segregation of the 2 micron plasmid.

### 4.1 Introduction

The reason why yeast plasmids require an active partitioning system for efficient segregation is the operation of a diffusion barrier that enhances the retention of plasmid molecules in the mother cell (Gehlen et al., 2011). The nature of this diffusion barrier is not fully resolved. One set of experiments suggests that the strong mother bias (9:1) of *ARS* plasmids is because of their association with nuclear pores, which do not diffuse

freely along the membrane into the daughter cell (Shcheprova et al., 2008). Retention of extra-chromosomal rDNA circles by a similar nuclear pore association has been suggested for their preferential accumulation in mother cells (Shcheprova et al., 2008). The burden posed by these DNA circles is one of the causes for aging in yeast and for limiting the replicative life span (RLS) of a cell. However, the idea that nuclear pores are not freely diffusible has been challenged (Khmelinskii et al., 2010). It is likely that some components of the pore complex are transferred to the daughter whereas others are restricted from such transfer. More recent experiments suggest that the diffusion barrier has both geometric and temporal features (Gehlen et al., 2011). The geometric constraint arises from the constricted nature of the nuclear membrane at the bud-neck. The temporal constraint results from the relatively short duration of the mitotic cell cycle, leaving insufficient time for plasmid molecules to equilibrate between mother and daughter compartments. Chromosomes overcome this barrier with the help of the force generated by the spindle based mitotic apparatus. DNA and protein molecules attached to the chromosomes will also be free of the barrier. Similarly, in principle, membrane proteins that can freely access the daughter compartment will be able to transport cargo molecules associated with them across the mother-daughter boundary without impediment.

The rationale behind the strategies employed in the present set of experiments is two-fold. First, we compare the effects of tethering an *STB* plasmid or an *ARS* plasmid to a membrane protein that is not subject to the diffusion barrier on the segregation efficiency of each. If the function of the Rep-*STB* system is to promote plasmid-membrane association, the two tethered plasmids are expected to show similar

segregation frequencies, comparable to that of a standard *STB* plasmid (non-tethered). Second, the segregation of an *STB* plasmid or an *ARS* plasmid is monitored under conditions when chromosomes missegregate *en masse* to one cell compartment while the nuclear membrane spans both cell compartments. A chromosome-tethered plasmid would be seen exclusively in the compartment in which the chromosome mass is entrapped. A membrane-tethered plasmid would have roughly equal chance of being in the chromosome-containing or chromosome-free compartment.

## 4.2 Results

### 4.2.1 Altering plasmid stability by artificially tethering to components of the nuclear pore complex

Previous studies have shown that the stability of *ARS* plasmids (multi-copy) can be increased when they are artificially tethered to Mlp1 (**M**yosin-**L**ike **P**rotein 1) or to Nup2 (**N**uclear **P**ore), which are components of nuclear pore complex (Khmelinskii et al., 2011). Direct plasmid visualization by fluorescence-tagging them have further rvealed their increased partitioning into daughter cells (that is, overcoming the normal strong mother bias) (Khmelinskii et al., 2011). We applied an analogous strategy to test whether the tethering of a plasmid (harboring *STB* or lacking it) to the nuclear pore complex (NPC) will mimic the Rep-*STB* system with respect to its segregation.

#### 4.2.1.1 Reporter plasmids for the segregation assays

The reporter plasmids were analogous to the ones described in **Chapter 3**, whose copy number was kept close to one per nucleus by virtue of the galactose-regualtable

*CEN* sequence present in them (Fig. 3.1). The plasmids differed from each other in either containing or lacking *STB*. They contained $TetO_{224}$, and were introduced into [$cir^+$] host strains expressing GFP-TetR to visualize them as single green foci in individual cells. The experimental strains were also engineered to express Mlp1-TetR or Nup2-TetR, while the control strains lacked the expression cassettes. These fusion proteins promoted the association of the plasmids with the nuclear membrane by their interaction with the TetO array.

#### 4.2.1.2 Segregation of plasmids tethered to the nuclear membrane

Raffinose-grown cells were arrested in G1, transferred to glucose or to galactose (to inactivate *CEN*), released in glucose (*CEN* active) or in galactose (*CEN* inactive), and plasmid segregation patterns were analyzed in anaphase cells (large budded with two well separated nuclear masses revealed by DAPI staining) (Fig 4.1A). The three classes of segregation scored were 1:1 (equal), 2:0 (mother-biased missegregation) and 0:2 (daughter-biased missegregation).

When *CEN* was active (in glucose), the equal segregation frequency was >95% regardless of the presence or absence of Mlp1-TetR or Nup2-TetR (Fig. 4.1B, D). We had expected to see a drop in *CEN* mediated segregation due to the presence of these fusion proteins, but this was not the case. Presumably, the kinetochore-spindle attachment was dominant over Mlp1 and Nup2 mediated envelope tethering, thereby excluding the less efficient membrane-associated mode of segregation. When *CEN* was inactived, the *ARS* plasmid showed only 15% equal segregation in the absence of Mlp1-TetR or Nup2-TetR (Fig 4.1C). Furthermore, a strong mother bias (2:0) was seen in the missegregation

class, as expected. When complemented by the fusion proteins, the equal segregation frequency rose to 46.2% (Mlp1-TetR) and 39.5% (Nup2-TetR) (Fig. 4.1C). Missegregation still favored the mother (2:0). By contrast to the *ARS* plasmid, the *STB* plasmid showed 73% equal segregation in the absence of the tethering proteins (Fig 4.1E), and this value was reduced significantly by their presence (39.4%, Mlp1-TetR and 33.5%, Nup2-TetR) (Fig. 4.1E). These observations suggest that nuclear envelope tethering is not only not neutral towards *REP-STB* mediated partitioning but is deleterious to it. In other words, normal 2 micron plasmid segregation is highly unlikely to be mediated by membrane tethering.

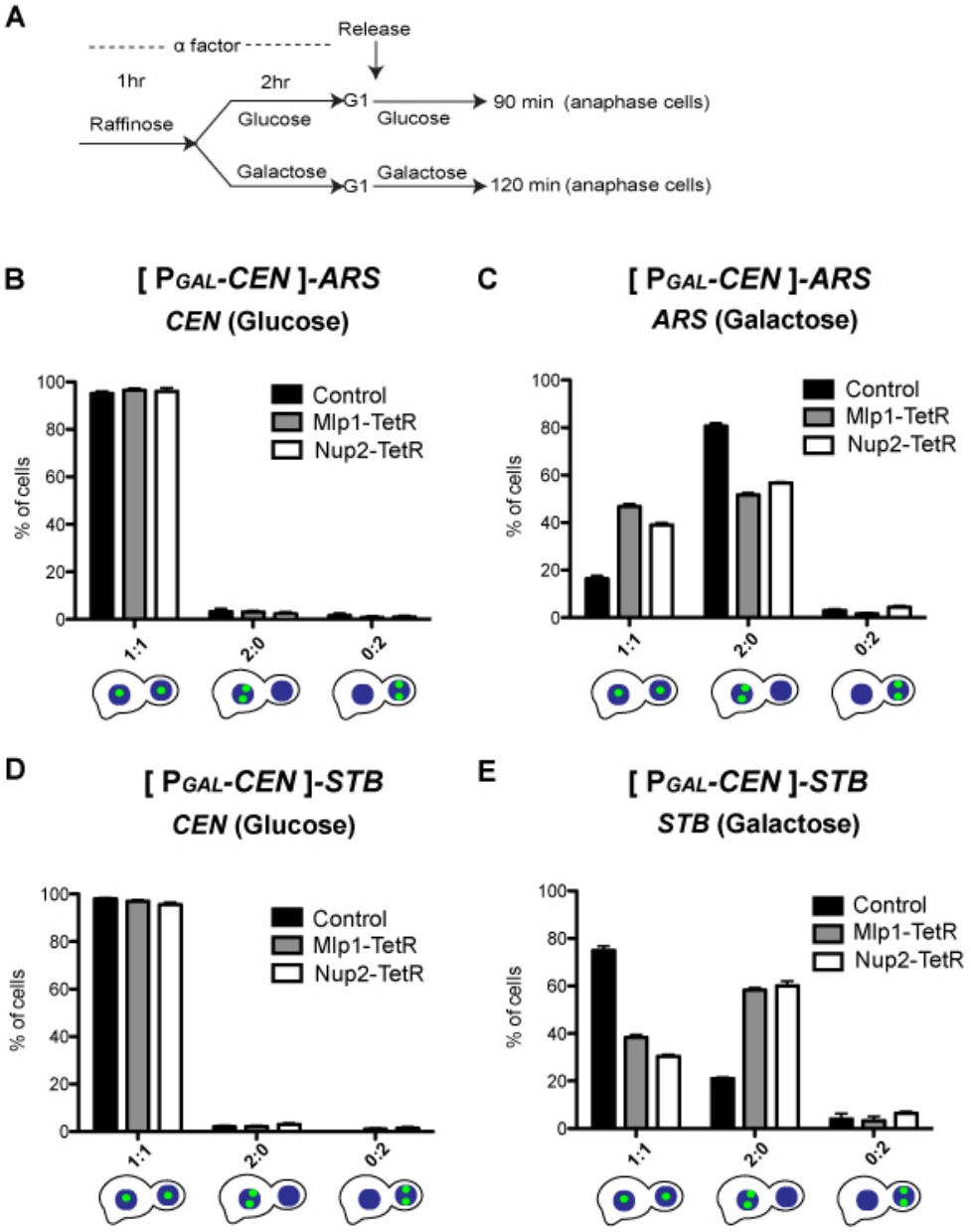



**Figure 4.1 Segregation of *CEN*, *ARS* and *STB* plasmids in the presence and absence of proteins that can potentially tether them to the nuclear envelope.**

The reporter plasmids contained *ARS1* or the 2 micron circle derived *STB-ORI* segment in addition to *CEN*. They were made to behave as single copy *CEN*, *ARS* or *STB* reporter plasmids by manipulating the carbon source (A). Panels B, D refer to glucose as the carbon source (*CEN* active) and panels C, E denote galactose as the carbon source (*CEN* inactive; *ARS* plasmid, top; *STB* plasmid, bottom). 'Control' indicates the absence of either Mlp1-TetR or Nup2-TetR.

### 4.2.2 Over-expression of Mcd1(nc) leads to confinement of all paired sister chromatids to the mother or daughter cell compartment

Previous studies suggested, but could not prove, chromosome-associated segregation of the 2 micron plasmid. Earlier genetic experiments revealed the tendency of a multi-copy *STB* reporter plasmid to follow the bulk of the chromosomes when their normal segregation was disrupted by conditional mutations (Velmurugan et al., 2000). However, in these assays complete missegregation of chromosomes was not accomplished. An occasional plasmid focus or a few such foci remained in the cell compartment lacking most of the chromosomes (visualized by DAPI). It was not possible to conclude with confidence whether these minority foci segregated independently of chromosomes, or whether they were still associated with the small number of chromosomes present in the same cell compartment (but were not readily visible by DAPI staining). A more recent study followed sister plasmids formed from a single copy *STB* reporter plasmid and a pair of sister chromatids during a contrived mitotic cell cycle in which the monopolin complex was overexpressed to misdirect sister chromatids to the same cell compartment (Liu et al., 2013). There was a strong correlation between chromosomes and the plasmid in sister co-segregation. However, the magnitude of such co-segregation, for the chromosome and the plasmid, was no higher than 30-35%.

In the present analysis, we prevented the disassembly of the cohesin complex (de Gramont et al., 2007; Uhlmann et al., 1999), which holds sister chromaitds together from the time of DNA replication until the onset of anaphase, during a cell cycle. The purpose was to maintain every pair of sister chromatids bridged by cohesin together throughout

anapahse. To accomplish this inappropriate sister-to-sister pairing, we utilized the expression of a non-cleavable form of the Mcd1 subunit (R180D/R268D) of cohesin Mcd1(nc) (Uhlmann et al., 1999) during the experiemntal cell cycle. Cells harbored a normal copy of the *MCD1* gene, and were maintained in glucose. After G1 arrest, they were shifted to galactose to induce the over-expression of the *GAL*-promoter controlled *mcd1-nc* gene, causing the native cohesin to be a minor fraction of the total cohesin pool. When the cells were released from arrest, they progressed through the cell cycle, and in a considerable fraction of cells the nuclear membrane (outlined by fluorescence-tagged Nup49; a component of the nuclear pore complex) extended into the bud compartment. However, the entire chromosome mass remained in the mother or in the daughter compartment. This experimental set up, shown in Fig. 4.2, permitted us to address plasmid-chromosome association versus plasmid-membrane association in the same assay.

#### 4.2.2.1 Single copy reporter plasmids for following how Mcd1(nc) affects their distribution in mother and daughter cells

The single copy reporter plasmids for this set of assays were generated by a different strategy than the one employed in prior experiments. Here the reporter plasmid was first integrated into a chromosome (Chr XV) of the haploid host strain as a unit copy (Fig. 4.2). The plasmid was flanked by two direct copies of the target site for the *R* site-specific recombinase (from *Zygosaccharomyces rouxii* (Araki et al., 1992)) placed under the control of the *GAL*-promoter. By shiting the G1 arrested cells to galactose, the plasmid was excised as a precisely single copy reporter. At the same time, expression of

Mcd1(nc) was also turned on. The plasmid was visualized as a green focus (LacO-[GFP-LacI] interaction), and the nuclear memebrane was outlined by mcherry-Nup49 expressed in the host strain. After releasing the cells, plasmid distribution patterns were scored only in those cells that showed clearly separated nuclear envelopes in mother and daughter. In the control plasmid, also released from the chromosome by R recombinase mediated excision, *STB* was deleted but the replication origin was intact (Fig. 4.2B). This *STB*-plasmid is hereafter referred to as an *ARS* plasmid.

#### 4.2.2.2 Segregation of a fluorescence-tagged pair of sister chromatids with respect to the entire chromosome mass under the influence of Mcd1(nc)

In order to ensure that sister chromatids never (or almost never) separate from each other and all sisters stay together, which is the critical premise of this analysis, the assay was also performed in a strain lacking the R recombinase. Here, the integrated plasmid would function as a reporter for the segregation of Chr XV sisters cohesed by Mcd1(nc). As shown in Fig. 4.3A, Chr XV sisters (green) were always associated with the DAPI mass in either the mother or the daughter compartment. Furthermore, the sisters were coalesced as a single fluorescent dot, indicating that the cohesin complex bridging them was not disassembled. In this analysis, we did not come across a cell in which one fluorescent dot each was present in mother and daughter.

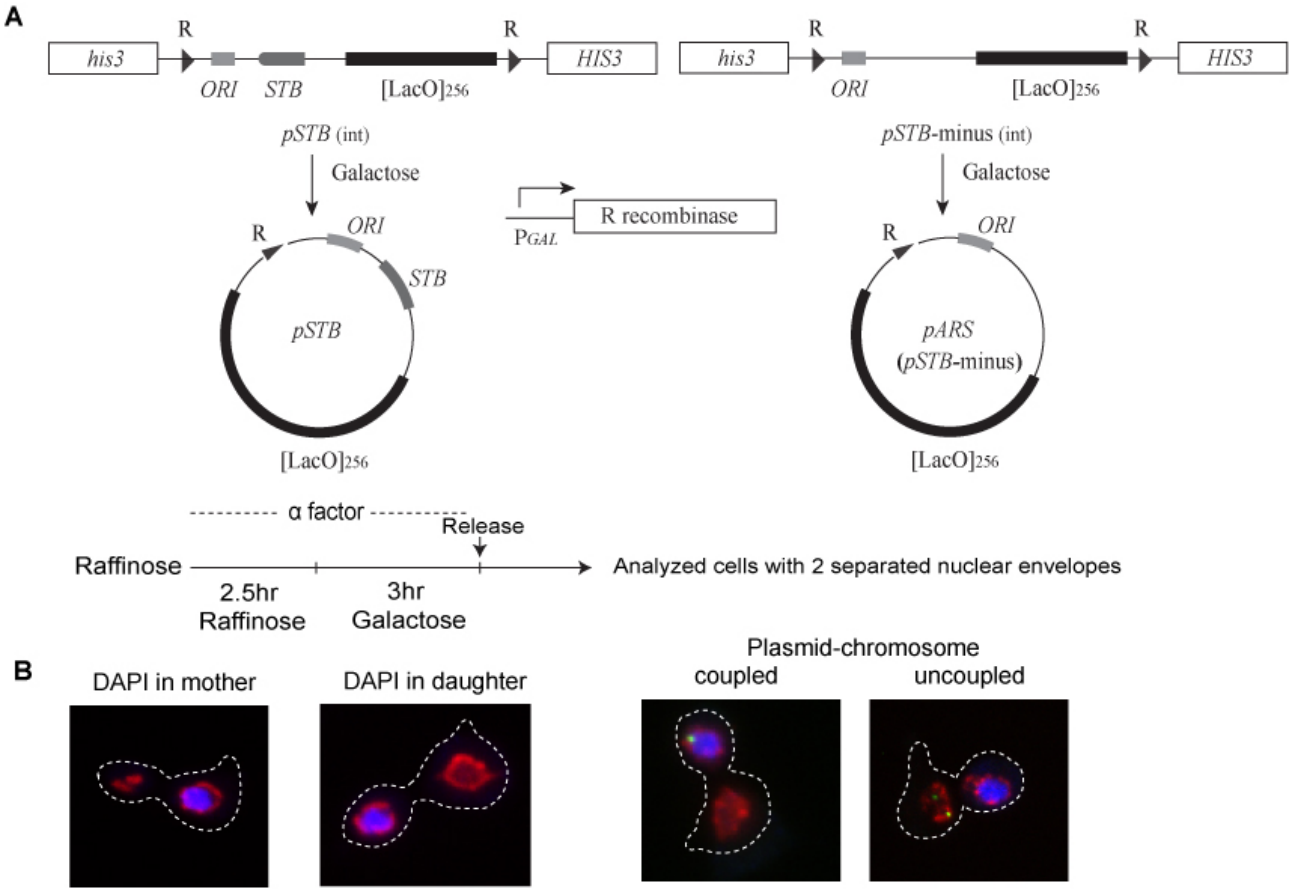



**Figure 4.2 Nuclear division and chromosome or plasmid localization when cohesin disassembly is blocked.**

(A) The strategy for generating the single copy *STB* or *ARS* plasmid by excision from the chromosomally integrated state is schematically diagrammed. The protocol for following the distribution of plasmid sisters in the mother and daughter compartments, with cohesin disassembly blocked, is outlined. (B) When non-cleavable Mcd1(nc) was overexpressed relative to native Mcd1, the entire chromosome mass (visualized by staining with DAPI) was localized either in the mother or daughter cell compartment. Nuclear segregation was followed by labeling the nuclear envelope by mcherry-Nup49. Although normal nuclear elongation and division were perturbed under this conditon, a sufficiently large fraction of cells revealed the presence of the nuclear membrane in the daughter.

#### 4.2.2.3 Plasmid distributions with respect to the chromosomes when cohesin disassembly is blocked

We then followed the plasmid patterns in cells containing the chromosomes in the mother compartment and those containing the chromosomes in the daughter compartment. The *STB* plasmid showed a very strong tendency to stay with the chromosomes (Fig 4.3B) in both types of cells. In a very small number of cases, one plasmid dot was located in the chromosome-free compartment (1:1; Fig. 4.3A). There was no instance when both plasmid sisters were uncoupled from the chromosomes (presence of plasmid only in the cell compartment lacking chromosomes). The plasmid sisters associated with the chromosomes were seen as one dot or two separate dots in roughly equal proportions. The mean fluorescence intensity of the single dots (in closely related experiments) was approximately equal to the sum of the intensities of the two separated dots, suggesting that plasmid replication was not affected under the experiemntal regimen. The implications of this observation, in light of the assembly of cohesin at *STB*, are considered under 'Discussion'.

The *ARS* plasmid behavior was strikingly different from that of the *STB* plasmid. In a high proportion of cells containing chromosomes in the mother, the plasmid sisters were also present in the mother (Fig 4.3C). Unlike the *STB* plasmid sisters, the *ARS* plasmid sisters appeared most often as two dots, a single coalesced dot being quite rare. The apparent coupling of the *ARS* plasmid to chromosomes in this group of cells was misleading, and reflected the normal mother bias of the plasmid, as revealed by cells with

chromsomes confined to the daughter. In the latter class of cells, either both plasmid sisters (or at least one of them) stayed in the mother >70% of the time. Interestingly, there was a clear increase in the 1:1 plasmid distribution (in mother and daughter) when chromosomes were trapped in the daughter. Since chromosomes occupy much of the nuclear volume and contribute significantly to the nuclear mass, they may accidentally sequester a small plasmid molecule without specifically interacting with it.

The distribution pattterns of the *STB* plasmid in the two groups of cells, containing chromososmes in the mother or in the daughter, reveal the strong coupling of the plasmid to chromosomes and the lack of such coupling to the nuclear membrane. Conversely, these groups of cells provide no indication that the *ARS* plasmid is coupled to the nuclear membrane. The cells containing chromosomes only in the daughter aslo provide strong evidence against the coupling of the *ARS* plasmid to chromosomes.

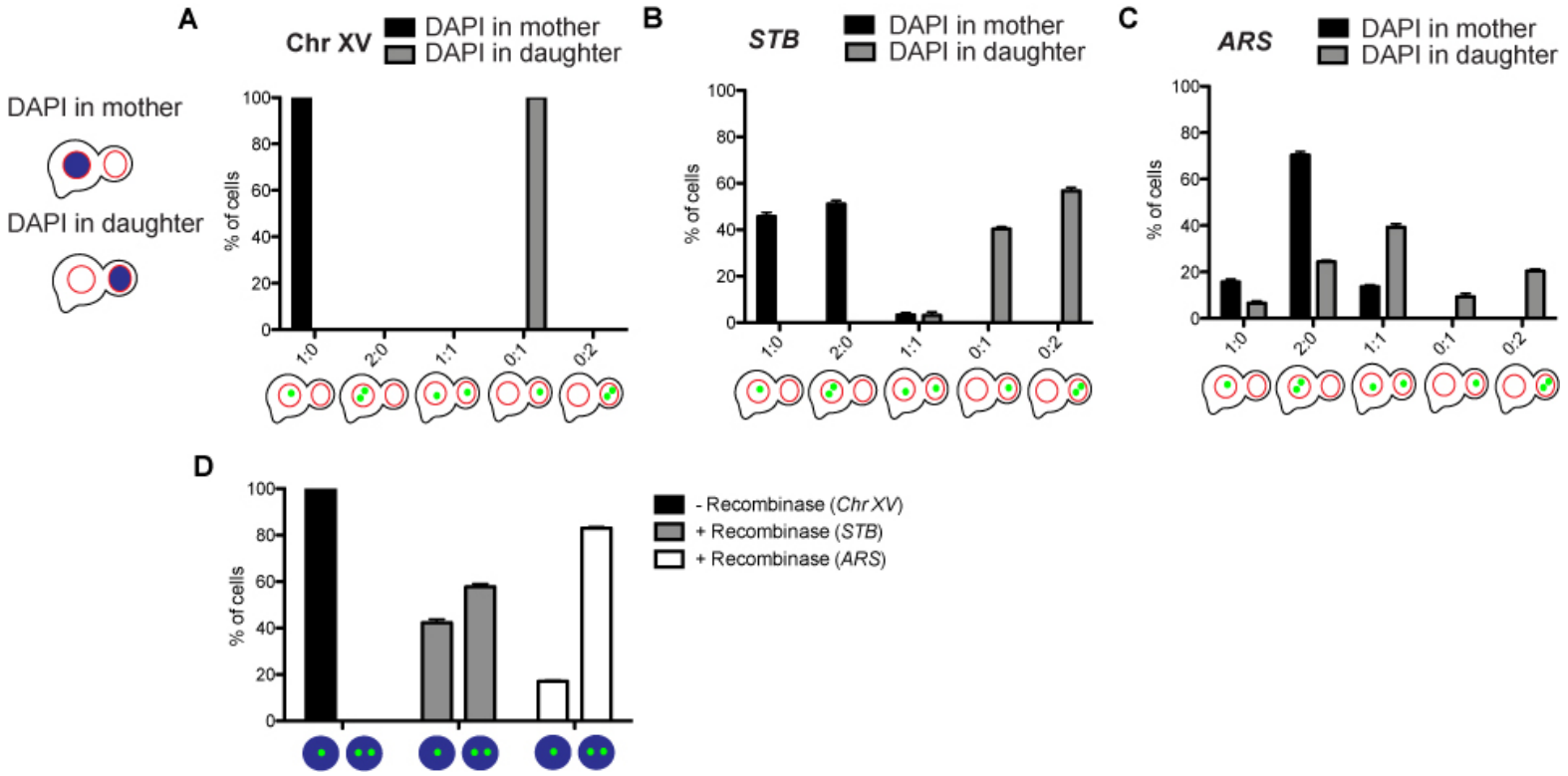



**Figure 4.3 Chromosome and reporter plasmid patterns under overexpression of Mcd1(nc).**

Cells with separated nuclear envelopes (mcherry-Nup49, red circle) were assayed to score chromosome or plasmid distributions. (A) In the experiemntal strain lacking the R recombinase, the integrated plasmid sequence was not excised, but served as a fluorescence tag on chromosome XV. The cohesed Chr XV sisters (seen as one green dot) always migrated with whole chromosome mass (stained with DAPI). (B) The *STB* plasmid sisters (seen as one dot or two dots in roughly equal proportions) were predominantly associated with chromosomes, regardless of their presence in the mother or the daughter. (C) The *ARS* (*STB*-) plasmid sisters (seen predominantly as two dots) were frquently unassociated with chromosomes present in the daughter cells. (D) Within the subset of cells containg the plasmid sisters only in one cell compartment, the fractions containing one dot varsus two dots were plotted. The *STB* plasmid far outnumbered the *ARS* plasmid in the one-dot population. Chr XV sisters appeared as one dot in every case.

## 4.3 Discussion

The results from the experiments in which plasmids were tethered to the nuclear membrane or cohesin disassembly was prevented during a cell cycle strongly support the notion that the 2 micron plasmid associates with chromosomes and not with the nuclear membrane.

### 4.3.1 Tethering to the nuclear membrane improves the segregation efficiency of an *ARS* plasmid but not an *STB* plasmid

When an *ARS* plasmid is tethered to the nuclear membrane with the assistance of Mlp1 or Nup2, its equal segregation efficiency is elevated. Clearly, the mother bias can be overcome to a large degree when the plasmid is associated with a membrane protien that can diffuse into the daughter cell. However, Mlp1 or Nup2 mediated plasmid segregation is still considerably inferior to that mediated by the Rep-*STB* system. The effect of membrane tethering on an *STB* plasmid is the reverse of that on the *ARS* plasmid in that its equal segregation efficiency is significantly lowered. We conclude that the 2 micorn plasmid does not segregate by membrane-associated diffusion.

### 4.3.2 *STB* plasmid sisters show nearly perfect coupling to chromosomes when sister chromatid separation is blocked

The results from the assays depicted in Fig. 4.3 can be represented in terms of the coupling strength between a plasmid and chromsomes (Fig. 4.4). When chromosomes occupy the mother compartment, both the *STB* and *ARS* plasmids show nearly perfect coupling to chromosomes (Fig 4.4A). When chromosomes occupy the daughter, the *STB*

plasmid still retains the same coupling strength. By contrast, the coupling breaks down for the *ARS* plasmid. When the presence of one of the two plasmid sisters with the chromosomes is considered as one-half coupling, the extent of *ARS* plasmid coupling to chromosomes is ~49% (Fig. 4.4A). This is quite likely an overestimate, as one of the plasmid copies could be randomly entrapped by the chromosome mass. When the more stringent criterion for coupling is applied (that is, both plasmid sisters need to be chromosome-associated), the extent of coupling between the *ARS* plasmid and chromosomes drops to ~29% (Fig. 4.4B).

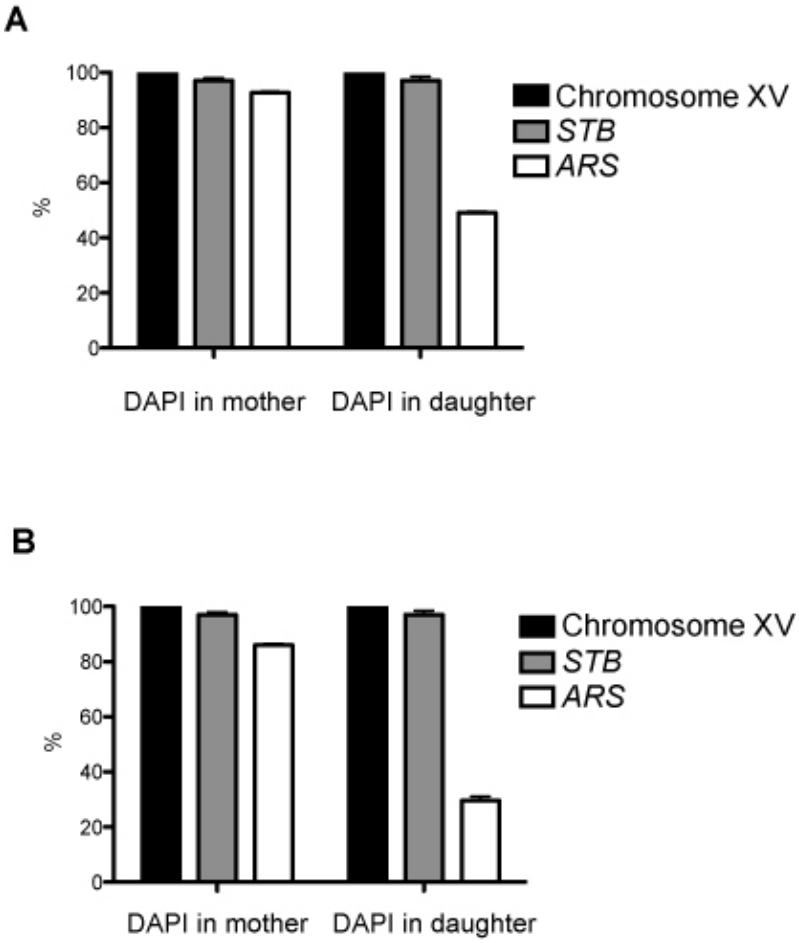



**Figure 4.4 Plasmid-chromosome coupling strength.**

(A) In this analysis, the association of both plasmid sisters with chromosomes was taken as 100% coupling; that of only one plasmid sister with chromosomes was assigned 50% coupling. (B) The analysis differed from that in A in that the dissociation of even one plasmid sister from the chromosomes was designated as 0% coupling. Because of the strong mother bias of the *ARS* plasmid, the apparent robust coupling seen in cells with chromosomes confined to the mother is deceptive.

### 4.3.3 The coalescence of *STB* plasmid sisters is likely promoted by the cohesin complex

Previous work showed that the cohesin complex is assembled at *STB* at the same time during the cell cycle when cohesin is assembled at chromosomal loci (Mehta et al., 2002). The disassembly of cohesin from chromosomes and from the plasmid, mediated by the cleavage of Mcd1 by the separase enzyme, occurs at early anaphase (Mehta et al., 2002; Uhlmann et al., 1999). Experiments with single copy *STB* plasmids suggest that sister plasmids are held together topologically by a single cohesin ring (on average) encircling them (Ghosh et al., 2010). It is possible that pairing by cohesin helps the association of plasmid sisters with sister chromatids. However, the assembly of cohesin at *STB* is highly substoichiometric (Ghosh et al., 2010). Perhaps cohesin acts in a catalytic fashion at *STB*. Once the initial pairing of sisters is established, it may be stablized by the Rep proteins (likely in conjunctin with host factors), and cohesin may then dissociate from *STB* in a subset of the paired plasmids. According to earlier work, under a normal cell cycle, 70-80% of *STB* plasmid sisters (fluorescence-tagged) are paired, as judged by their presence as a single fluorescent dot in metaphase nuclei (unpublished data).

In the present analyses, only about half of the *STB* plasmid sisters are paired by the single dot criterion (Fig. 4.3D). Since the cohesin was non-cleavable, one might have expected a significantly higher fraction to remain paired. The lower value would be consistent with the turn-over of cohesin from *STB* after establishing plasmid-pairing. It should be noted, though, that the cells also contained native (and hence cleavable) cohesin, although the overexpressed non-cleavable cohesin was more abundant. Since

there is only one cohesin complex on average for a pair of *STB*s (Ghosh et al., 2010), a certain fraction of the plasmid sisters would be paired by native cohesin, which would be disassembled by the action of separase. Such plasmids may contribute to the increase in the two plasmid dot population noted in the present assays.

### 4.4 Summary and Conclusions

The results presented in this chapter extend and strengthen those detailed in **Chapter 3**. The possibility that the Rep-*STB* system may counteract mother bias by tethering the 2 micron plasmid to the nuclear membrane can be ruled out. The alternative mechanism of plasmid tethering to chromosomes and plasmid segregation by hitchhiking appears to be more likely.

# CHAPTER 5

## Role of DNA replication in 2 micron plasmid segregation

The segregation of the 2 micron plasmid is linked to DNA replication. The plasmid partitioning complex is assembled *de novo* at *STB* as cells transition from G1 to S phase. We used two strategies to dissect the role of DNA replication in plasmid segregation. In one, plasmid replication was blocked specifically without affecting cellular DNA replication. In the other, all DNA replication, plasmid as well as chromosomal, was arrested. The key findings are listed here. **1.** A single copy *STB* plasmid lacking the replication origin is able to overcome 'mother bias' when the Rep-*STB* system is active. **2**. However, analysis of two *ORI*-minus single copy *STB* plasmids suggests that replication is required for equal *STB* plasmid segregation. **3.** The strength of chromosome-plasmid coupling under conditions of cohesin disassembly (Mcd1(nc); see **Chapter 4**) is also lower. **5**. The tightly correlated co-segregation of sister copies of a single copy *STB* plasmid and a pair of sister chromatids during a monopolin-directed deviant mitotic cell cycle breaks down for a pair of *ORI*-minus single copy *STB* plasmids. **6.** When all DNA replication in the cell is blocked by Cdc6 depletion, a multi-copy *STB* reporter plasmid can still overcome mother bias but the equal segregation frequency is reduced.

### 5.1 Introduction

Each round of 2 micron plasmid replication is mediated by a newly assembled partitioning complex at the *STB* locus. The time at which components of the old

partitioning complex are fully ejected from *STB* and Rep1, Rep2 and host factors are recruited for another round of partitioning coincides with (or immediately precedes) the start of DNA replication (Ma et al., 2013). Plasmid replication occurs very early during S phase (Zakian et al., 1979). A possible connection between replication and partitioning has been suspected, but has not been carefully studied.

### 5.1.1 Temporal hierarchy in the assembly and disassembly of the plasmid partitioning complex with respect to DNA replication

Host factors that associate with *STB* include components of the SWI/SNF related RSC2 chromatin remodeling complex, the spindle-associated motor protein Kip1, the histone H3 variant Cse4 and the yeast cohesin complex (Cui et al., 2009; Ghosh et al., 2007; 2010; Huang et al., 2011; Mehta et al., 2002; Wong et al., 2002; Yang et al., 2004). Using short-interval ChIP (**ch**romatin **i**mmuno**p**recipitation) analysis, an approximate temporal sequence in the assembly of protein factors at *STB* and their subsequent dissociation from *STB* has been derived (Fig. 5.1) (Ma et al., 2013). Based on the current data, the assembly pathway starts in late G1, and is completed with the recruitment of the cohesin complex during S phase. Presumably, plasmid replication and pairing of replicated copies by cohesin mark an important execution point in the partitioning pathway. Components of the RSC2 complex exit from *STB* after cohesin assembly, and cohesin itself is disassembled during early anaphase (Ma et al., 2013; Mehta et al., 2002). Cse4, persists until late telophase, perhaps until the time of spindle disassembly. Rep1, Rep2 and Kip1 remain at *STB* even after cytokinesis through the subsequent G1 stage. They dissociate

from *STB* at the G1-S window, clearing the way for the *de novo* assembly of the partitioning complex.

When DNA replication is delayed by deleting the genes for two B-type cyclins (*clb5 clb6*), the dissociation of Rep1 from *STB* is also correspondingly delayed (Ma et al., 2013). Similarly, when DNA replication is blocked by depleting the initiator protein Cdc6, Rep1 does not dissociate from *STB* during the time course of the analysis. Collectively, these observations suggest that DNA replication directly or indirectly provides the signal for the removal of the spent partitioning complex from *STB* and the assembly of a fresh partitioning complex. It is not known whether the passage of the replication fork through *STB* is required for the renewal of the partitioning complex, or whether cellular cues that trigger DNA replication also provide the signal for initiating the plasmid partitioning pathway.

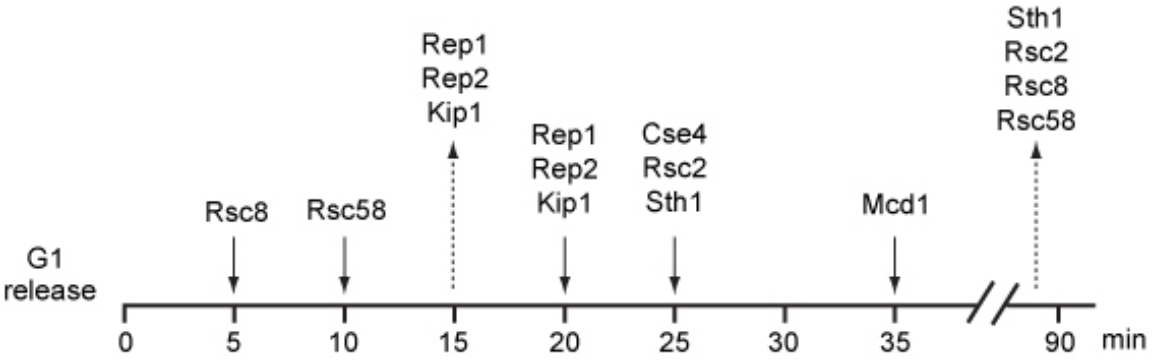



**Figure 5.1 Sequence of association and dissociation of plasmid partitioning factors at *STB*.**

The time-lines depicting the assembly and disassembly of the plasmid partitioning complex are based on high time-resolution ChIP analyses (Ma et al., 2013). Rsc2, Rsc8, Rsc58 and Sth1 are components of RSC2 complex, and Mcd1 is representative of the cohesin complex. The G1-S window of the cell cycle marks the complete dissociation of the old partitioning complex and the assembly of a new partitioning complex.

### 5.1.2 Monopolin-induced co-segregation of sister chromatids and sister *STB* plasmids

The first cell division during meiosis (meiosis I) contrasts with mitotic cell division and the second meiotic cell division (meiosis II) in that sister chromatids segregate to the same cell pole, while homologues segregate to opposite cell poles. The reductional division of meiosis I is followed by the equational division of meiosis II (in which sister chromatids segregate from each other) to complete the process of haploidization and the generation of four gametes. The co-orientation of sister chromatids on the spindle during meiosis I is promoted by the monopolin complex, which clamps sister kinetochores, with the assistance of the cohesin complex associated with centromeres and the Ipl1 kinase (Corbett et al., 2010; Petronczki et al., 2006; Rabitsch et al., 2003; Yu and Koshland, 2007). The monopolin complex is composed of Mam1, a meiosis-specific protein, together with Csm1 and Lrs4, which are nucleolus-associate proteins present during both mitosis and meiosis (Huang et al., 2006; Smith et al., 1999; Tóth et al., 2000; Waples et al., 2009). The functional monopolin complex is formed when the Csm1-Lrs4 complex, released from the nucleolus under the regulation of the polo-like kinase Cdc5, associates with Mam1. When Cdc5 is depleted, the co-orientation of sister chromatids becomes defective due to the impaired formation of the monopolin complex. (Clyne et al., 2003; Lee and Amon, 2003; Rabitsch et al., 2003). When Mam1 and Cdc5 are overexpressed during mitosis, the reconstituted monopolin complex causes sister chromatids to co-segregate at ~30% frequency (Brito et al., 2010; Monje-Casas et al., 2007).

In a recent study, the segregation behavior of a single copy *STB* reporter plasmid was followed with respect to that of a tagged chromosome during a mitotic cell cycle in which monopolin was expressed. The extent of sister plasmid co-segregation under this condition almost perfectly matches that of the tagged sister chromatids (Liu et al., 2013). This strong correlation, in the context of the hitchhiking model, is suggestive of the association of sister plasmids with sister chromatids.

### 5.1.3 Does replication promote *STB* plasmid-chromosome coupling?

In principle, DNA replication could be a critical event in coupling sister plasmids with sister chromatids. Replication promotes the cohesin-mediated pairing of sister chromatids, keeping them in close proximity. Cohesin also seems to promote the establishment of plasmid pairing, although the mechanisms for the maintenance of plasmid and chromosome cohesion may not be identical. Thus, replication-assisted formation of paired sister chromatids and sister plasmids would be conducive to their mutual association. The experiments assembled under 'Results' investigate how plasmid-chromosome association and plasmid segregation are affected by interfering with DNA replication.

## 5.2 Results

### 5.2.1 Single copy fluorescence-tagged *ORI*-plus and *ORI*-minus *STB* reporter plasmid systems

The reporter plasmids for these studies were generated by the R recombinase mediated excision from their chromosomal locations (**Chapter 2**) (Liu et al., 2013). The

excision was carried out in G1 arrested cells by inducing the R recombinase (Araki et al., 1992) from the *GAL* promoter (Fig. 5.2A) These plasmids harboring the $[LacO]_{256}$ array were visualized by their association with GFP-LacI expressed in the host strains from the *HIS3* promoter. A control plasmid lacking the *STB* locus is referred to as an *STB*-minus or an *ARS* reporter. In a subset of the reporter plasmids, the 2 micron circle replication origin (*ORI*) was removed by a ~300 bp deletion (spanning ~100 bp upstream and downstream of the ~100 bp *ORI*) (Broach, 1982; Broach and Hicks, 1980). Plasmids lacking the origin are referred to as *ORI*-minus or *ORIΔ* (Fig. 5.2A,B). In certain experiments requiring a pair of *ORI*-minus plasmids, two almost identical *STB ORI*-minus plasmids were integrated into two different chromosomal loci. The segregation of the excised copies of these DNA circles was analyzed (**5.2.3**) (Fig. 5.2B).

A previous study showed that 3 hr galactose induction in G1 arrested cells achieved >90% plasmid excision, while no excision could be detected in the absence of induction (Liu et al., 2013). Similar kinetics of induction were followed in the present experiments as well. In those cases where two copies of a plasmid were excised simultaneously from two distinct chromosomal locations, each of the two plasmids was excised nearly quantitatively after 3 hr induction of the recombinase (Fig. 5.2C).

To follow plasmid behavior in the presence and absence of the Rep proteins, analyses were performed in $[cir^+]$ and $[cir^0]$ strains, respectively. In certain experiments, Rep1 and Rep2 were complemented in the $[cir^0]$ hosts by expressing them inducibly from the bidirectional *GAL1-GAL10* promoter.

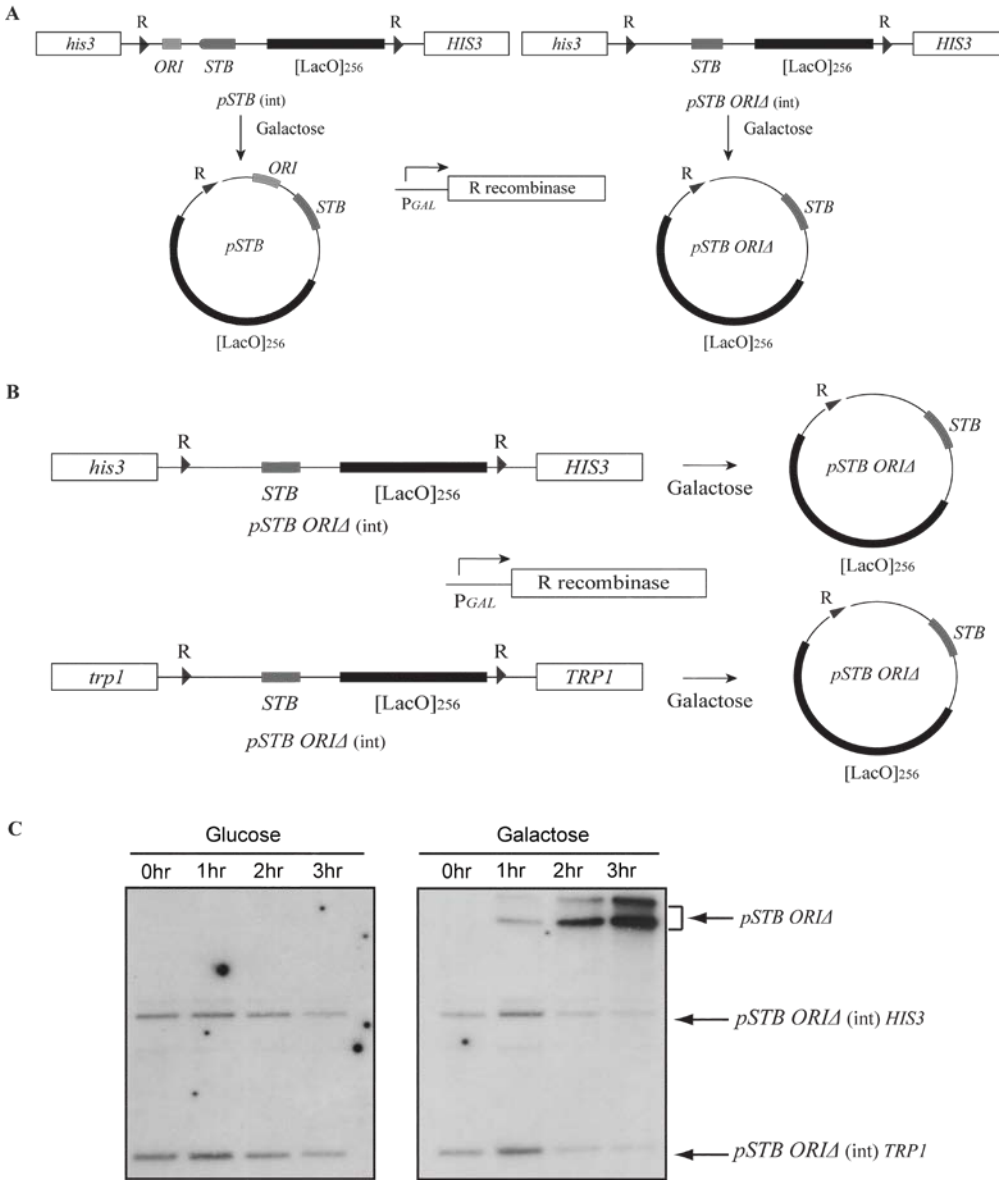



**Figure 5.2 Single-copy reporter plasmids excised from their integrated states**

(A)Single copy reporter plasmids with or without a replication origin were generated in G1 arrested cells by recombination mediated excision from their chromosomal locales (at *HIS3* and *TRP1*). The control plasmids lacking *STB* (p*STB*-minus or p*ARS*); not shown here) were also generated by the excision reaction (B) Two nearly identical copies of a single copy reporter plasmid lacking *ORI* (p*STB ORIΔ*) were obtained by recombination within integrants located at two distinct chromosomal loci. (C) Time course of dual plasmid excision over a 3 hr period of galactose induction was followed by Southern blot analysis. Excision of both plasmid copies was nearly complete in 3 hr. The separation of the bands corresponding to the linear forms of the *ORIΔ* plasmids is larger than that expected from their respective sizes. This could be due to the spontaneous deletion of some of the LacO repeats from one of them.

### 5.2.2 Lack of Replication does not abolish the ability of an *STB* plasmid to overcome mother bias

In budding yeast, plasmids lacking an active partitioning system show a strong mother bias imposed by a diffusion barrier between the nuclear chambers of the mother and daughter (Gehlen et al., 2011). Overcoming this bias is central to equal plasmid segregation. We assayed the segregation patterns of the *ORI*-plus and *ORI*-minus single copy reporter plasmids in anaphase cells. The three expected patterns for the replication-competent plasmids are: 1:1 (equal), 2:0 (plasmids in mother) and 0:2 (plasmids in daughter). As noted earlier, two plasmid sisters may occasionally coalesce to give the appearance of a single plasmid focus, however with twice the fluorescence intensity of one plasmid copy. Such foci were grouped into the 2:0 and 0:2 classes. An *ORI*-minus plasmid would be present in the mother (1:0) or the daughter (0:1). The ability of a reporter plasmid to overcome mother bias was expressed as $O_{mb}$, the ratio of plasmid foci present in the daughter ($F_d$) to the total plasmid foci present in both mother and daughter ($F_d + F_m$), $O_{mb} = F_d/(F_d + F_m)$.

For the *ORI*-plus *STB* reporter plasmid, $O_{mb}$ was estimated as 47.3% in the presence of the Rep proteins ([$cir^+$] strain) and ~18% in their absence ([$cir^0$] strain) (Fig. 5.3A) The corresponding *ARS* plasmid (lacking *STB*) gave an $O_{mb}$ nearly identical to the smaller of the two values. This result was consistent with previous observations of mother bias (Gehlen et al., 2011; Murray and Szostak, 1983). The $O_{mb}$ values for the *ORI*-minus *STB* plasmid were virtually unchanged in the [$cir^+$] (41.7%) and the [$cir^0$] (~15%) hosts from those of its *ORI*-plus counterpart. When Rep1 and Rep2 were

overexpressed in the [$cir^0$] strain, the $O_{mb}$ of the *ORI*-minus *STB* plasmid rose higher (66.4%) than that in the [$cir^+$] strain (Fig. 5.3B). This was unexpected, as a perfect $O_{mb}$ is 50%, signifying equal plasmid segregation in every cell. Under an excess of Rep1 and Rep2, the *STB* plasmid not only overcomes the mother bias completely, but tends to show a slight daughter bias. The *ORI*-minus *STB*-minus plasmid had a low $O_{mb}$ of ~15% in the [$cir^+$] host.

The outcomes from this analysis show that the strong mother bias of an *ARS* plasmid is unchanged by preventing its replication. Thus, the act of replication does not contribute to mother bias. Conversely, the competence of the Rep proteins in freeing an *STB* plasmid from mother bias is independent of the ability of the plasmid to replicate. Thus, replication appears to have no role in the mechanism by which the Rep-*STB* system counteracts the plasmid-diffusion barrier.

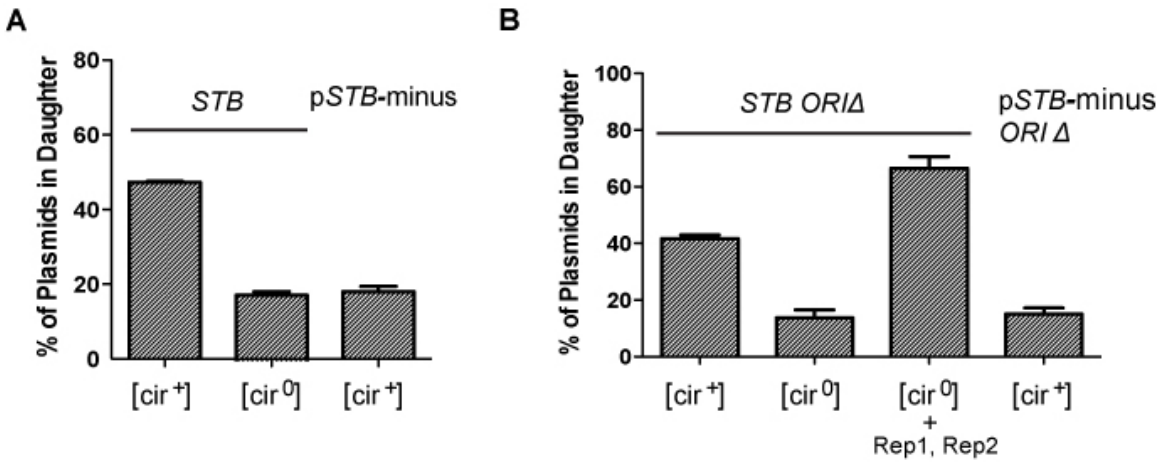



**Figure 5.3 The extent of mother bias in reporter plasmids with or without the ability for replication.**

The distributions of the single copy plasmids (excised from chromosomal integrants) were analyzed in mother and daughter nuclei in anaphase cells. The ability to overcome mother bias was estimated as $O_{mb} = F_d/(F_d + F_m)$, $F_d$ and $F_m$ being the number of plasmid foci in mother and daughter nuclei, respectively. (A) *ORI*-plus reporter plasmids harboring the *STB* locus or lacking it (*ARS*) were analyzed in the indicated host strains. (B) Similar assays were performed after deleting *ORI* from the plasmids represented by the analysis in A. The Rep1 and Rep2 proteins were supplied in the [$cir^0$] strain by expressing them from the bi-directional *GAL1-GAL10* promoter.

### 5.2.3 Analysis of the segregation of two copies of an *STB* reporter plasmid lacking *ORI*

The segregation patterns (1:1), (2:0) and (0:2) of two copies of a single copy *ORI*-minus *STB* plasmids, obtained by excision in G1 cells (Fig. 5.2B), were followed after their progression to anaphase. These plasmids are referred to as a pseudo-sister pair to distinguish them from an authentic sister pair resulting from the replication of an *ORI*-plus single copy plasmid. The equal segregation of the pseudo-sister pair was reduced to

46.1% compared to that of the sister pair (63.7%). The 2:0 (plasmids in mother) and 0:2 (plasmids in daughter) segregation types for the pseudo-sisters were ~37% and ~17%, respectively (Fig. 5.4). These numbers are entirely consistent with the $O_{mb}$ value of ~40% estimated for this plasmid. The predictions for the 2:0, 1:1 and 0:2 classes based on an $O_{mb}$ of 0.4 are **36**% ($0.6^2$), **48**% (2 x 0.6 x 0.4) and **16**% ($0.4^2$), respectively.

The significant reduction in 1:1 segregation of the pseudo-sister plasmid pair compared to the sister pair, and the almost perfect agreement of this segregation type with $O_{mb}$, suggests that equal plasmid segregation mediated by the Rep-*STB* system is dependent on plasmid replication. In the absence of replication, the segregation of the pseudo-sisters is accounted for by the ability of Rep-*STB* to overcome mother bias. Thus, active plasmid partitioning involves more than just counteracting the diffusion barrier.

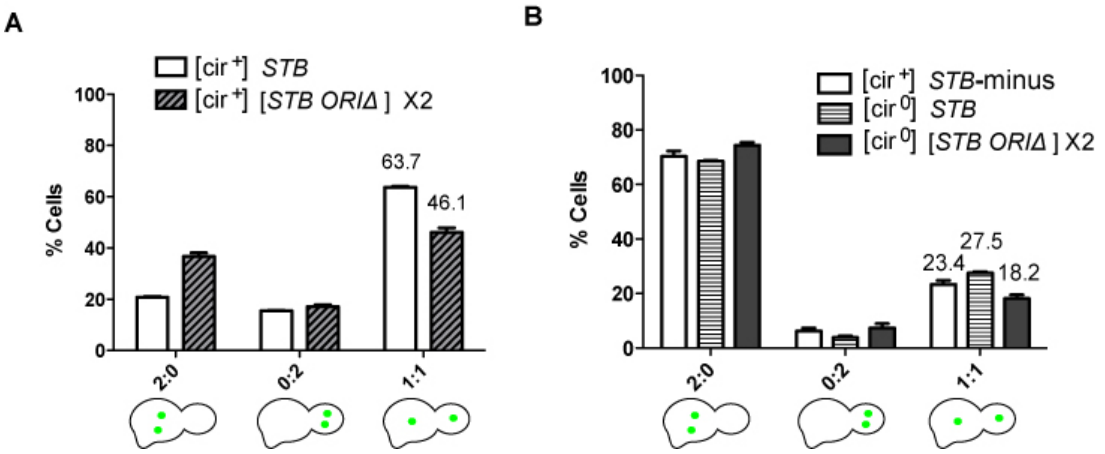



**Figure 5.4 Segregation patterns of authentic *STB* plasmid sisters and pseudo-sisters.**

The two plasmid copies formed by the replication of a single copy *STB* plasmid are authentic sisters (*STB* in A and B). The two identical plasmid copies generated by recombination in G1 cells, and do not contain an *ORI* sequence, are pseudo-sisters (*STB ORI*□in A and B). The plasmid sisters lacking *STB* (*ARS* sisters) are denotes as *STB*-minus in B. (A) The segregation patterns of a pair of *ORI*-plus *STB* plasmid sisters and a pair of *ORI*-minus *STB* plasmid pseudo-sisters were followed in a [cir$^+$] strain. (B) The segregation patterns of the same pair of *STB* plasmid sisters and pseudo-sisters as in A were followed in a [cir$^0$] strain. The sisters resulting from the replication of an *ORI*-plus *STB*-minus (*ARS*) plasmid were also analyzed in a [cir$^+$] strain. The common factor in this experiment is the absence of a functional Rep-*STB* system. The segregation behaviors of the three plasmids were very similar, the prominent common feature being the strong mother bias (2:0) in every case.

### 5.2.4 The effect of plasmid replication on plasmid-chromosome coupling under overexpression of Mcd1(nc)

We have already demonstrated in **Chapter 4** the high degree of coupling between an *STB* plasmid and chromosomes under the overexpression of Mcd1(nc) to block the disassembly of cohesin assembled on sister chromatids (**Chapter 4.2.2**). Under this condition, all chromosomes stay together in the mother or the daughter, while the nuclear membrane is distributed in a majority of cells into both mother and daughter. To

address a possible role for plasmid replication in plasmid-chromosome coupling, the Mcd1(nc) strategy was combined with blocking plasmid DNA replication.

Here, an *ORI*-plus plasmid would give rise to two sister copies during S phase, where as an *ORI*-minus plasmid would stay as one copy. The sister pair could segregate as 1:1, 2:0 or 0:2, the first and second numbers in these ratios indicating plasmid presence in the mother and daughter, respectively. The replication-blocked single copy circles would segregate as 1:0 or 0:1. All chromosomes would be present either in the mother or in the daughter.

Plasmid distributions were scored only in those anaphase cells with two well separated nuclear envelopes in mother and daughter, as described earlier (**Chapter 4.2.2**). Furthermore, only the subset of such cells that contained the DAPI mass in the daughter was included in this analysis. As already explained, the strong mother bias in the absence of a partitioning system would give the mistaken impression of tight plasmid-chromosome coupling in cells containing chromosomes in the mother.

The strong coupling of the *ORI*-plus *STB* plasmid (~95%) with chromosomes was lowered to (~70%) when *ORI* was deleted (*ORI*-minus) from this plasmid (Fig. 5.5A). The *ORI*-plus *STB*-minus (*ARS*) plasmid showed a low coupling to chromosomes (~50%), which was reduced even more (~16%) when the *ORI* sequence was deleted from it. In these plots, the 1:1 class (one plasmid focus each in the chromosome-containing and chromosome-free nuclei) was assigned 50% coupling (compared to 100% coupling for two plasmid foci present with the chromosomes). When the 1:1 class was instead taken to mean no coupling (0%), as plotted in Fig. 5.5B, the estimated coupling values for the

*ORI*-plus *STB* plasmid and *ORI*-plus *ARS* (*STB*-minus) plasmid were ~93% and ~30%, respectively. The values for the *ORI*-minus circles would remain the same in Fig. 5.5 A and B, as they were present as one copy (and would not include the 1:1 class).

The nearly identical coupling strengths of the *STB* plasmid sisters in the plots of Fig. 5.5A (~95%) and Fig. 5.5B (~93%), reveal that both plasmid copies are coupled to chromosomes in the predominant majority of cells. The sharp decline from the low coupling of ~50% for the *ARS* plasmid (*STB*-minus) sisters (Fig. 5.5A) to ~30% (Fig. 5.5B) by the stringent criterion indicates that, in most cases, only one of the two plasmid copies was present with chromosomes in the daughter .

These findings suggest, from the perspective of the hitchhiking model, that the normal association of the 2 micron circle with chromosomes, relevant to equal segregation, is dependent on plasmid replication. However, the higher coupling of an *STB* plasmid lacking *ORI* compared to an *ARS* plasmid (*ORI*-plus; *STB*-minus) indicates that the Rep-*STB* system establishes plasmid-chromosome association, even though such association may be less efficient and/or functionally less competent. The low level coupling of an *ARS* plasmid with the chromosomes in the daughter cell may be accounted for by the propensity of the chromosome mass to entrap a plasmid molecule during its migration to the daughter compartment. The additional drop in this coupling when *ORI* is deleted suggests that the physical state of the plasmid during its replication, perhaps the release from an interaction that restricts its diffusion, may enhance the probability of the plasmid being dragged into the daughter by chromosome movement.

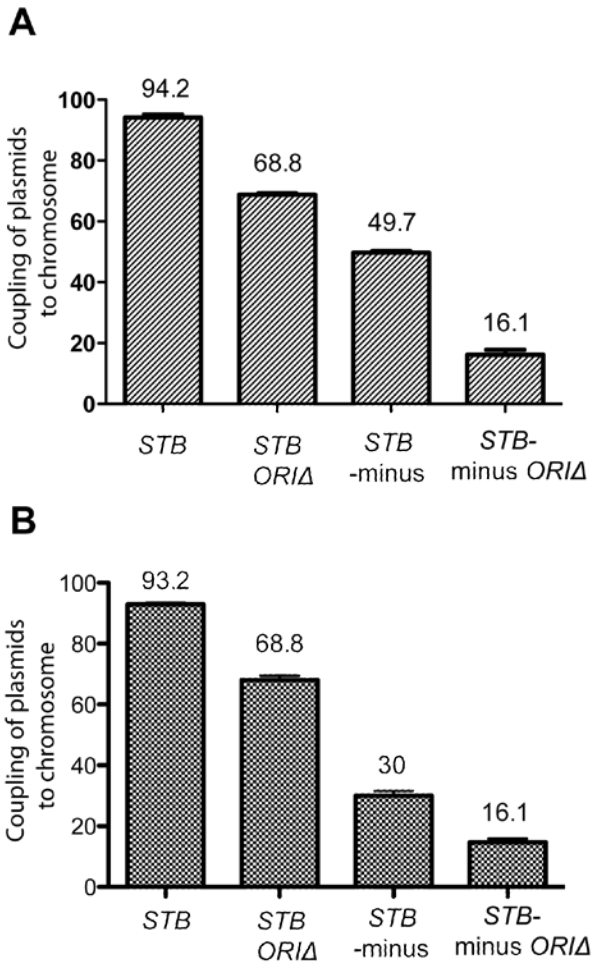



**Figure 5.5 The coupling of reporter plasmid with the chromosomes present in the daughter cell under expression of Mcd1(nc).**

In estimating the coupling, only those anaphase cells containing chromosomes in the daughter, but nuclear membrane in mother and daughter, were considered. (A) For the *ORI*-plus plasmids, the presence of two plasmid copies and one plasmid copy with the chromosomes were assigned coupling values of 100% and 50%, respectively. The absence of the plasmid in the daughter was assigned 0% percent coupling. (B) The difference from (A) is that these graphs represent coupling values when the presence of only one of the two plasmid copies with the chromosomes was also assigned 0%. In (A) and (B), the coupling for the *ORIΔ* circles are unchanged, as they were present as single copies either with the chromosomes in the daughter (100% coupling) or away from the chromosomes in the mother (0% coupling).

### 5.2.5 Segregation of *STB* plasmid pseudo-sisters when the monopolin complex is expressed during mitosis

As noted before, the distortion in the segregation of a pair of sister chromatids and that of a pair of *STB* sister plasmids by monopolin during a mitotic cell cycle (towards co-segregation) are nearly identical (Liu et al., 2013; Monje-Casas et al., 2007; Petronczki et al., 2006). We wished to know whether the observed strong correlation also holds for a pair of pseudo-sisters. In these assays, chromosome IV with the $[lacO]_{256}$ sequence integrated near the centromere served as the chromosome reporter. Galactose induction in G1 arrested cells not only caused the excision of the reporter plasmids but also turned on the *MAM1* and *CDC5* genes in the experimental strains, promoting the assembly of the monopolin complex.

In anaphase cells, in the presence of monopolin, there was drop in equal segregation (1:1) of chromosome IV from 100% to 66% (34% co-segregation; 2:0 or 0:2) (Fig 5.6A). This co-segregation was essentially unbiased towards the mother (2:0, ~18.5%) or the daughter (0:2, ~15.5%). A similar quantitative increase in co-segregation (~24.4%) due to monopolin was noted for the *ORI*-plus *STB* plasmid as well (Fig. 5.6B). The co-segregation was only slightly biased towards the mother (2:0, ~13.3%; 0:2,~11.1%). Monopolin had little or no effect on the segregation of an *ORI*-plus plasmid lacking *STB* (*ARS* plasmid), on its low equal segregation frequency (1:1) or its distinct mother bias (2:0) (Fig. 5.6C). However, a reduction in the extent of mother bias was noted in the presence of monopolin (2:0, 50.9%; 0:2, 22.6%) compared to its absence (2:0, 70.2%; 0:2 6.3 %). These results are consistent with those from a previous study

(Liu et al., 2013). The segregation patterns of the *STB* plasmid pseudo-sisters (two identical copies of the *ORI*-minus *STB* plasmid) were nearly identical in the wild type and monopolin expressing strains (Fig. 5.6D).

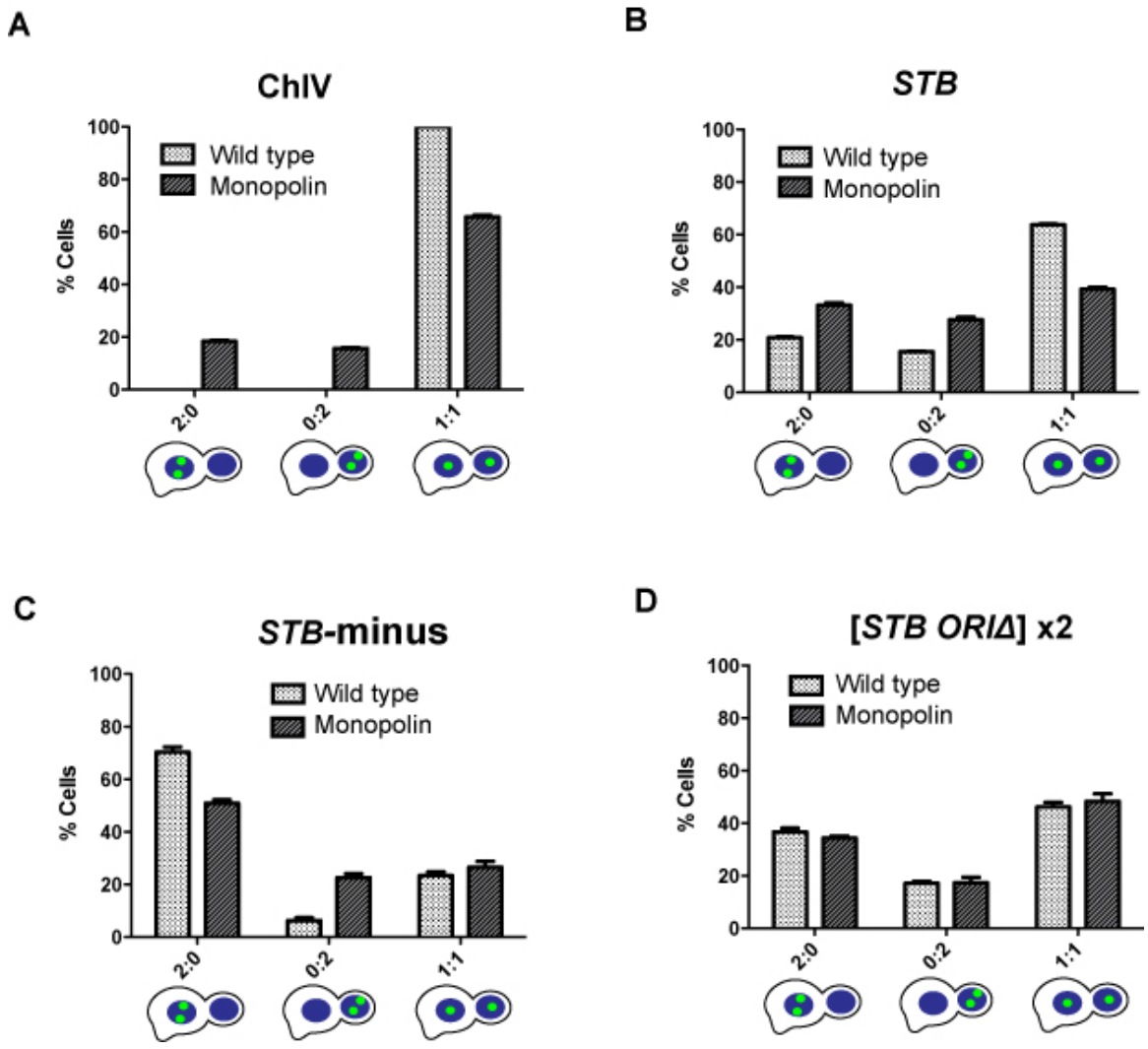



**Figure 5.6 Segregation of reporter plasmids and chromosome IV during a mitotic cycle in which the monopolin complex was expressed.**

Assembly of the monopolin complex was induced during the mitotic cycle by turning on *MAM1* and *CDC5* expression in G1 cells and then releasing them from G1 arrest. All segregation assays were performed in [cir$^+$] strains. The 1:0 and 0:1 types (coalesced plasmid foci) were binned into the 2:0 and 0:2 groups, respectively. 'Wild type' refers to a normal mitotic cell cycle in the absence of monopolin.

The quantitative effects of monopolin on the segregation of a chromosome or a plasmid are most easily visualized by representing the data in the form of radar plots. The details of this analysis have been described previously (Liu et al., 2013). The three relevant variables are denoted as $V_e$, $V_m$ and $V_d$, representing equal segregation, co-segregation biased towards mother and co-segregation biased towards the daughter. The sum of these variables is 100%. Their changes due to an altered condition (in our case, monopolin directed mitosis) are $\Delta V_e$, $\Delta V_m$ and $\Delta V_d$, with the appropriate algebraic signs, such that $\Delta V_e + \Delta V_m + \Delta V_d = 0$. This inequality follows from the fact that a decrease in Ve will be equal to the sum of the increases in $V_m$ and $V_d$. As the equal segregation frequencies are not the same for the chromosome and the individual plasmids, and these frequencies differ between the wild type and monopolin expressing strains, the $\Delta V$ values are normalized to $\Delta V'$ values for plotting the graphs. The normalization is done with respect to the relevant equal segregation frequencies during normal mitosis. As an example, consider the case where the equal segregation frequencies for a plasmid are 63.7% and 39.2% in the absence (wild type) and the presence of monopolin, respectively. Then, $\Delta V_e$ = -24.5% ; $\Delta V_e'$ -24.5% / 63.7% = -38.5%.

The strong correlation between the *ORI*-plus *STB* reporter plasmid and chromosome IV (the reporter chromosome) is evident from the near congruence of the two triangles (blue and red) representing them (Fig. 5.7A). The plasmid lacking *STB* (the *ARS* plasmid) does not show this correlation (red triangle). The *ORI*-minus *STB* plasmid, characterized by the purple triangle, is not correlated with the chromosome and the *ORI*-plus *STB* plasmid (Fig. 5.7B), or with the *ORI*-plus *STB*-minus (*ARS*) plasmid (Fig. 5.7A,

B). The shapes of the triangles in Fig. 5.7B illustrate another important point as well. The distinction between the monopolin effects on the sister plasmids versus pseudo-sister plasmids is manifested prominently with respect to ΔV'e (change in co-segregation frequency) but barely with respect to $\Delta V'_m$ or $\Delta V'_d$ (mother or daughter bias). This result reinforces the earlier inference that equal plasmid segregation is dependent upon replication whereas overcoming mother bias by the Rep-*STB* system is not.

Taken together, the results of this analysis suggest that eliminating mother bias, which is possible in the absence of plasmid replication, does not fully recapitulate the attributes of 2 micron plasmid partitioning. The strong coupling to chromosomes in equal segregation or in missegregation is lost when the plasmid cannot replicate. This important role for plasmid replication is perhaps reflected in the resetting of the partitioning clock during each cell cycle at or immediately prior to DNA replication (G1-S transition stage).

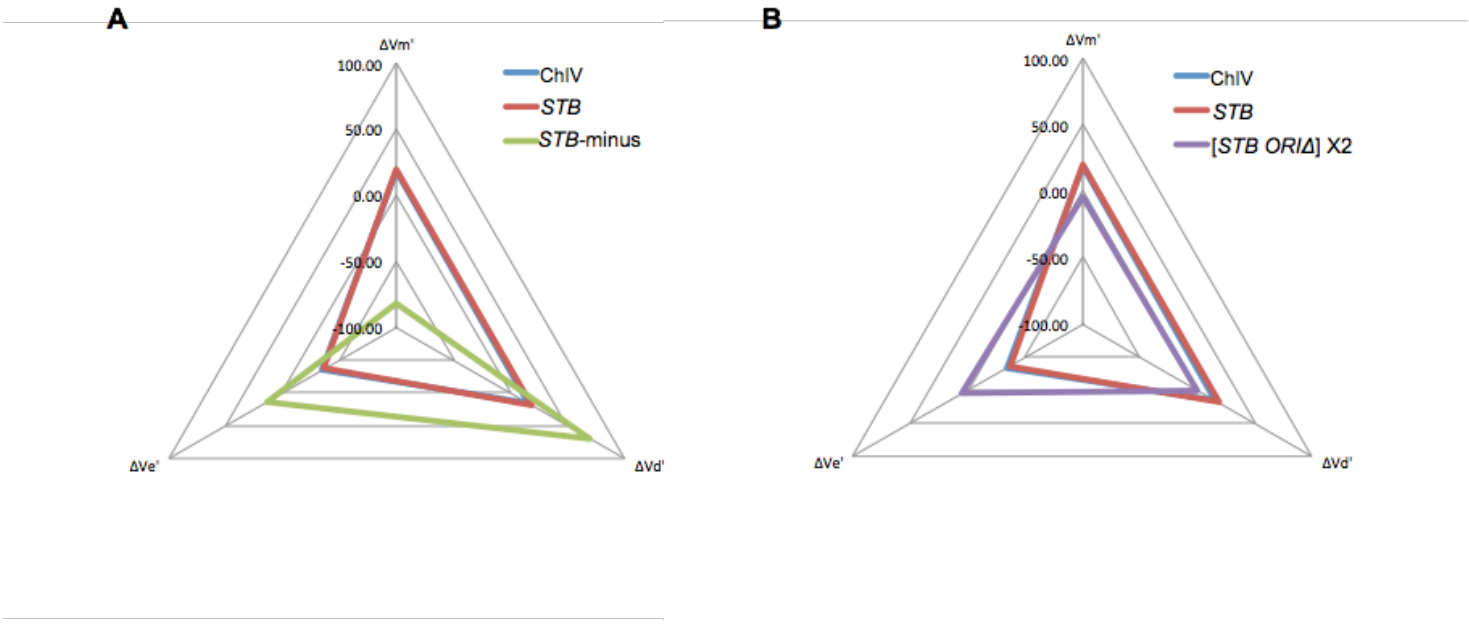



**Figure 5.7 Correlations between the segregation patterns of a reporter chromosome and reporter plasmids under monopolin expression during a mitotic cell cycle.**

The normalized values ΔV' are represented as radar plots to show the extent of correlation between a chromosome and a plasmid in how their segregations are distorted by monopolin. The strong correlation between the *ORI*-plus *STB* plasmid and the chromosome IV is contrasted by the lack of such chromosome-correlation for the *ORI*-plus *STB*-minus (*ARS*) plasmid or the *ORI*-minus *STB*-plus plasmid.

### 5.2.6 Segregation of a multi-copy reporter *STB* plasmid during a Cdc6-depleted cell cycle

In all the experiments described above, plasmid replication was specifically blocked by deleting the *ORI* sequence. As such, we could not completely dissociate the lack of plasmid replication from the absence of *ORI* per se as being responsible for the observed effects. The *ORI* sequence is the binding site for the Orc complex, which plays a key role in initiating eukaryotic DNA replication (Tsakraklides and Bell, 2010). The Orc complex has also been shown to function in gene silencing and in regulating the

expression of genes linked to replication origins (Shor et al., 2009). To rule out potential indirect effects of removing *ORI*, we followed the effects of blocking total DNA replication in the cell on the segregation of a multi-copy *STB* reporter plasmid harboring an intact *ORI*. Because of the technical difficulties, we have not been able to perform this analysis with the single copy *STB* plasmid used in the previous experiments.

In order to block replication, we depleted the initiation factor Cdc6, which associates with the replication origin bound by Orc to form the pre-replicative complex (Tanaka et al., 1997) The host strain harbored *cdc6* deletion, but could be maintained in galactose medium by the presence of a chromosomally integrated cassette, p*GAL-ubi-CDC6*. The N-terminal ubiquitin conjugated Cdc6 has a shorter half-life than native Cdc6, so that it does not accumulate to significant levels during a cell cycle, and can be completely depleted under non-inducing conditions (Piatti et al., 1996). The depletion protocols requires the incubation of mid-log phase cells in hydroxyurea/galactose for one doubling followed by their transfer to glucose medium (without hydroxyurea) in the presence of α factor (Severin et al., 2001). After releasing the G1-arrested cells, the segregation of the *STB* reporter plasmid was monitored in anaphase (Fig 5.8A). During a Cdc6-depleted cell cycle, the unreplicated chromosomes segregate roughly equally to mother and daughter by a poorly understood process that has been termed 'reductional mitosis'.

The multi-copy fluorescence-tagged *STB* reporter plasmid, which was also used in a number of previous experiments (Velmurugan et al., 2000), formed 3 to 6 foci in a haploid nucleus. The majority of anaphase cells (~70%) contain equal (or nearly equal)

number of foci in the mother and daughter nuclei, suggesting that each focus (containing more than one plasmid copy) acts as a unit in segregation. However, since an occasional focus may overlap or be coalesced with another focus (and the plasmid copy number in individual foci might vary), the segregation assay based on plasmid foci counting has a certain element of subjectivity. It lacks the precision of the single copy plasmid segregation assay.

In order to confirm that Cdc6 depletion was efficient and replication was blocked under our assay conditions, we counted the average plasmid foci number in G1 cells as well as in anaphase cells under replication-permissive (galactose) and replication-arrested (glucose; Cdc6 depleted) conditions. When cdc6 was depleted, the DAPI distributions in the mother and daughter were roughly equal, suggesting that the haploid chromosome number was reduced to approximately half in each compartment. The average plasmid foci count in G1 was ~4 for both groups of cells (Fig. 5.8B). However, in anaphase, this number was ~8 for cells maintained in galactose but ~5 for cells transferred to glucose (Fig 5.8 B). The lack of increase in plasmid foci number in the latter group of cells verifies that Cdc6 depletion was effective and that plasmid replication was blocked.

We then followed the segregation of the *STB* reporter plasmid in the presence and absence of Cdc6. First, we checked the ability of *STB* plasmids to overcome mother bias by estimating the plasmid foci distributed in daughter cells as a fraction of all plasmid foci (in mother plus daughter). There was only a slight decrease in this fraction (from 46.8% to 43.5%) when the Cdc6 was depleted (Fig 5.9A). This result, which is similar to

that obtained with the single copy *ORI*-minus *STB* plasmid (Fig 5.2B), indicates that the *STB* plasmid can overcome mother bias in the absence of plasmid replication.

We further monitored the segregation of the plasmids in individual anaphase cells, and expressed equal (or almost equal) segregation as the sum of N:N and N:(N-1) classes of cells (N being the number of plasmid foci in one of the two nuclei). The rest of the segregation types (N:N') was grouped as unequal segregation. The equal segregation frequency fell from 72% to 60.5% in the absence of DNA replication (Fig. 5.9B). The corresponding drop for the *ORI*-minus *STB* plasmid relative to the *ORI*-plus *STB* plasmid was from ~64% to ~47% (Fig. 5.4A). In the unequal segregation class, the higher plasmid foci number was skewed towards the mother (mother bias) for the replication-competent and replication-blocked plasmids.

Thus, blocking DNA replication by deleting the plasmid origin or by inhibiting initiation of replication does not prevent the 2 micron circle from overcoming mother bias. Association with chromosomes is likely the critical step in bias elimination. However, in the absence of plasmid replication, equal segregation of the plasmid is adversely affected. We suspect that the nature of plasmid-chromosome association in the absence of plasmid replication (or of all DNA replication) is distinct from that in the presence of normal plasmid and chromosome replication. In other words both plasmid and chromosome replication would be critical for equal plasmid segregation, as would be consistent with the hitchhiking of sister plasmids on sister chromatids. The role of chromosome replication in plasmid segregation cannot be stringently tested, as we are

unable to conceive an experimental design that would permit plasmid replication while preventing chromosome replication.

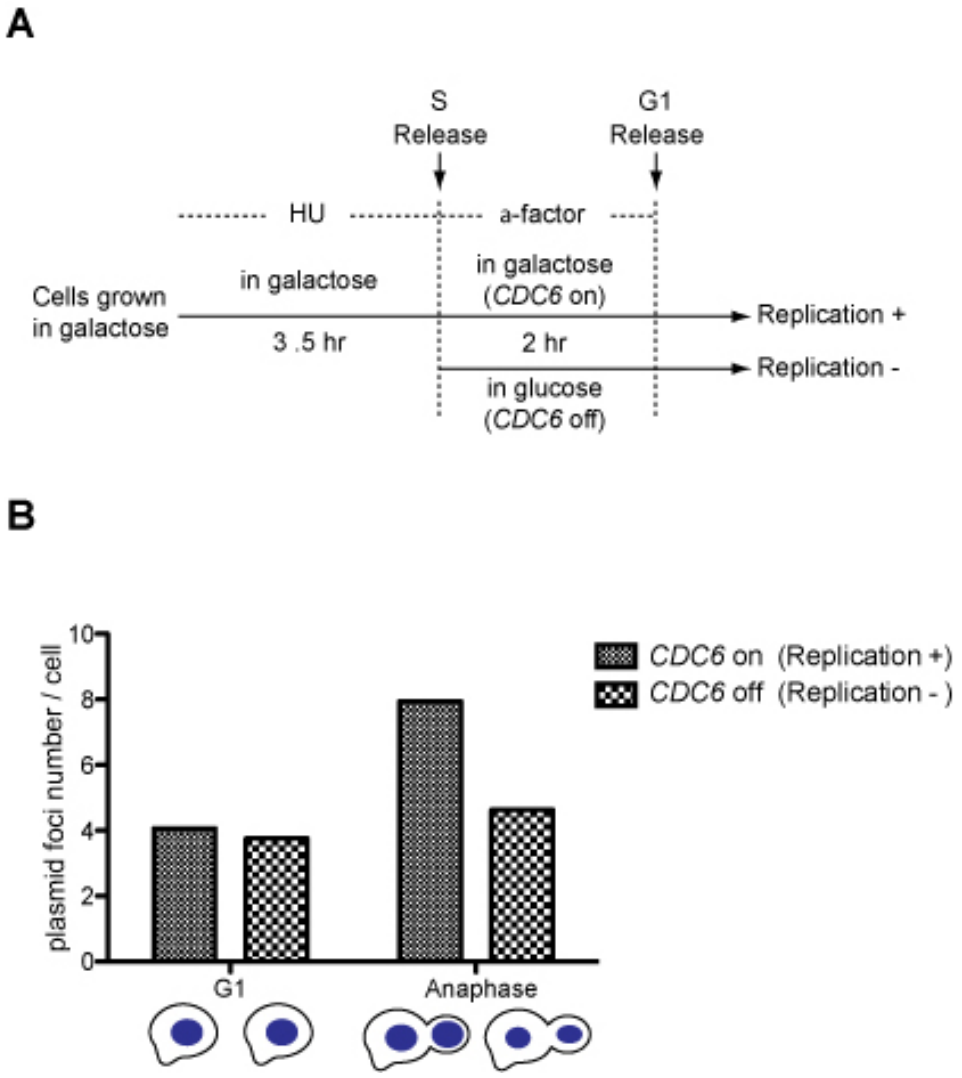



**Figure 5.8 Depletion of Cdc6 in G1 arrested cells blocks replication in the subsequent cell cycle.**

(A) Cells were grown in galactose to keep the expression of Ubi-Cdc6 on. They were arrested (or delayed) in S phase with hydroxyurea (HU), and then released in glucose medium in the presence of α factor. The control cells were released in galactose medium containing α factor. The presence of α factor caused cell cycle arrest in G1. (B) After release from G1 (in galactose or in glucose) the cells were examined by microscopy to identify large budded cells with two separated DAPI masses in the mother and daughter. The plasmid foci were counted in G1 cells and anaphase cells, and are expressed as the number of foci per cell (one nucleus in G1 cells and two nuclei in anaphase cells). The schematic representation of the DAPI staining regions in cells are meant to convey that, even when *CDC6* is off, chromosomes distribute roughly equally between mother and daughter; however each receives only half the number of haploid chromosomes.

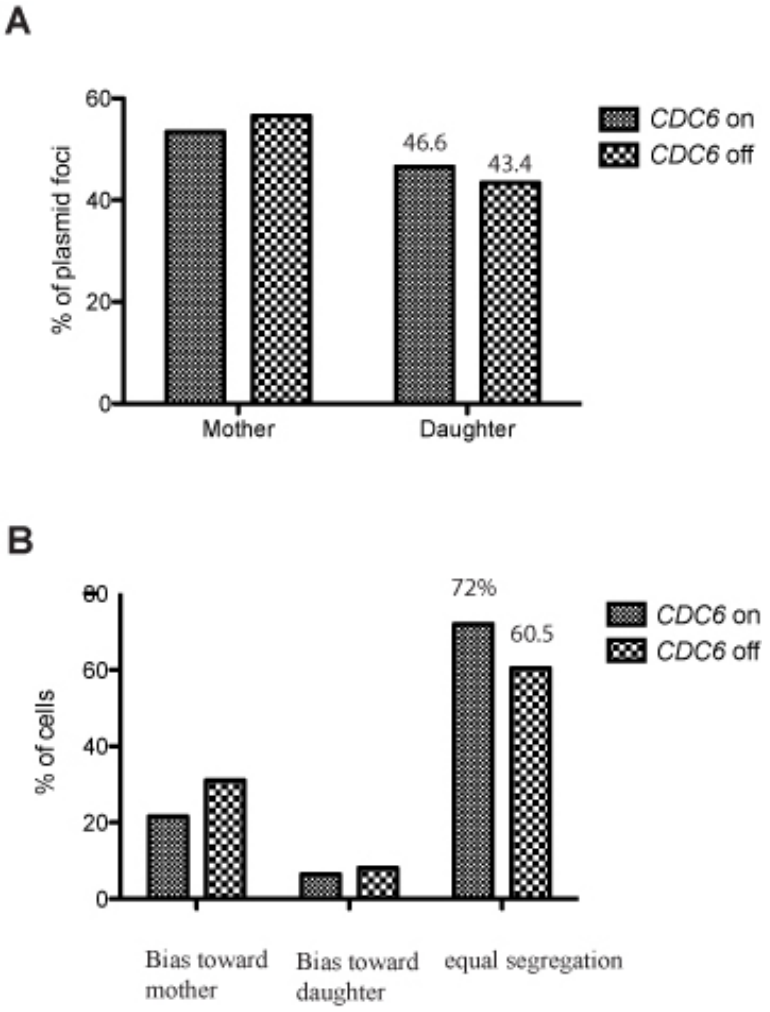



**Figure 5.9 Segregation of a multi-copy *STB* reporter plasmid under depletion of Cdc6.**

(A) After initiating the cell cycle in the presence or absence of Cdc6, plasmid foci were counted in anaphase cells. The fractions of plasmid foci in mother and daughter nuclei are plotted. (B) The equal (or almost equal) segregation class is plotted as the sum of two classes (N: N and N: (N-1) foci ratios between the two nuclei of an anaphase cell). The unequal segregation class included all other types of segregation. This class is plotted by splitting cells containing higher plasmid foci number in the mother (biased towards mother) from those containing higher number of plasmid foci in the daughter (biased towards the daughter).

## 5.3 Discussion

The primary objective of the experiments described in this chapter was to understand the possible role of plasmid replication in the equal segregation of the 2 micron plasmid. The majority of experiments carried out with single copy reporter plasmids, together with one analysis using a multi-copy reporter plasmid, are consistent with the following conclusions. First, in the absence of replication, an *STB* plasmid is able to overcome the mother bias with the help of the Rep proteins. Second, overcoming mother bias cannot, by itself, guarantee equal segregation, which is strictly dependent on plasmid replication. Both these results can be accommodated by a common explanation based on the ability of the Rep-*STB* system to mediate plasmid-chromosome tethering.

### 5.3.1 How does the Rep-*STB* system overcome mother bias?

Regardless of the specific mechanism involved, for overcoming the normal mother bias, a plasmid will have to associate with a nuclear entity that can diffuse between the mother and daughter compartments or is actively partitioned between the two. In the former case, the diffusion rates will have to be sufficiently high to establish near equilibrium state within the duration of a mitotic cell cycle. As pointed out earlier, the geometric bottle neck posed by the shape of the budding yeast nucleus and the relatively short time of mitosis are strong impediments to equilibration of freely diffusing plasmid molecules between mother and daughter. Plasmid tethering to chromosomes, which is the basic premise of the hitchhiking model, would be conducive to overcoming the bias, as chromosomes are partitioned equally between mother and daughter.

A single copy *STB* plasmid lacking *ORI* overcomes the mother bias to a large extent when supplied with the Rep proteins, but this is not the case for a plasmid lacking *STB* or for a DNA circle lacking both the origin and *STB*. If the Rep proteins can promote the tetehring of the *ORI*-minus *STB* plasmid to a chromosome, it will have a 50-50 chance of being in the mother or the daughter. The observed value of 40-45% plasmid presence in the daughter cell may suggest that the Rep-*STB* system is not 100% efficient in tethering the reporter plasmid to a chromosome. Based on a number of published results, it is clear that various 2 micron circle derived plasmids cannot achieve the same stability as the native plasmid. Their stabilities are often two orders of magnitude lower (loss rate of $10^{-3}$ to $10^{-2}$ per division), but still much higher than those of *ARS* plasmids (lacking *STB*). The equal segregation frequency of single copy *STB* reporter plasmids in the presence of the Rep proteins in our hands is only between 65-75% in the single cell cycle assay, indicating the limitation of the reconstituted segregation assay. Under the same conditios, the equal segregation frequency of singe copy *ARS* plasmids is only between 20-30%.

The results with the multi-copy *STB* reporter plasmids in the absence of both plasmid and chromosome replication (Cdc6 depletion; reductional mitosis) also fit into the interpretations based on the single copy plasmids. Although counting multiple plasmid foci is less accurate than counting one focus formed by a single copy plasmid or two foci formed by its sister or pseudo-sister copies, there are roughly equal number of foci per mother or daughter nucleus in a cell population under normal DNA replication and in the absence of DNA replication. Again, the slightly higher number observed for

the mother nucleus might indicate the upper limit to the efficiency of the reconstituted partitioning system in tetehring plasmid to chromosomes, and in turn, ovecoming the mother bias.

### 5.3.2 Equal segregation of the 2 micron circle is promted by plasmid replication

If the replicated copies of a single copy *STB* plasmid are tethered to two separate chromosomes, they have a 50% chance of equal segregation (1:1) and a 25% chance of being in the mother (2:0) or in the daughter (0:2). Random plasmid-chromosome tethering will tend to decrease or eliminate bias, but will not useful for efficient equal segregation. As the plasmid copy number increases, the equal segregation frequency will decrease. For a copy number of 2 (with four plasmid copies formed after replication), the expected frequency of 2:2 segregation will be 6/16 = 37.5%. If sister plasmid copies are tethered to sister chromatids, the predicted frequency of equal segregation will be 100%, regardless of the copy number.

The observed equal segregation frequency for a single copy *ORI*-plus *STB* plasmid is ~64%, higher than the 50% predicted for random tethering and the ~46% observed for the pseudo-sisters of the corresponding *ORI*-minus plasmid. These results suggest that, within the limits of efficiency of the system, the Rep proteins, in conjunction with *STB*, mediates the tethering of sister plasmids to sister chromatids. For this mode of ‘symmetric’ tethering, plasmid replication is essential, as indicated by the behavior of the pseudo-sisters. This conclusion is further supported by comparing the sister plasmids to pseudo-sisters under a mitotic cell cycle in which the monopolin complex promotes sister chromatid co-segregation to a limited extent. The contrasting

behaviors of *ORI*-plus and *ORI*-minus *STB* containing circles when cohesin disassembly is blocked (forcing maximal sister chromatid co-segregation) are also consistent with this interpretation. The strong correlation (or coupling) observed between sister chromatids and sister plasmids under these aberrant mitotic cell cycles breaks down for plasmid pseudo-sisters or for lone single copy circles blocked in replication.

A number of previous studies suggest the assembly of the partitioning complex at *STB* culminates in the recruitment of the cohesin complex, which establishes the cohesion of sister plasmids (Ghosh et al., 2010; Ma et al., 2013; Mehta et al., 2002). A cohesed pair of plasmids will have a high probability of attaching to symmetrically located tethering sites on a pair of sister chromatids that are also held together by cohesin (Nasmyth and Haering, 2009; Onn et al., 2008). In this model, symmetric tethering is favored by proximity. In the absence of replication, a pair of pseudo-sisters is unlikely to be bridged by cohesin, even though cohesin may associate separately with each of the *STB* locus present on the individual plasmids. Prior evidence demonstrates that the assembly of cohesin after the replication of an *STB* plasmid has been completed is not functional for plasmid cohesion (Mehta et al., 2005). Thus, according to our current thinking, the 2 micron plasmid can associate with chromosomes by random tethering in the absence of replication, but must go through replication to establish symmetric tethering on sister chromatids. The former mode of plasmid-chromosome interaction is sufficient to alleviate or overcome mother bias without promoting efficient equal plasmid segregation, while the latter accomplishes both the elimination of bias and equal segregation.

While the above model is attractive and easily applied to the single copy plasmid system, it is not clear how it will operate in the context of a group of plasmid molecules clustered into a focus (as inferred from current cell biological assays). Such a focus, which appears to be the unit of segregation, must have a high-order organization, which must be duplicated during plasmid replication, in order for sister clusters to segregate equally from each other.

### 5.3.3 A replication-independent effect of *ORI* on plasmid-chromosome coupling

Although the results based on *ORI*-minus plasmid constructs are accounted for primarily on the basis of replication arrest, some replication-independent effect of the presence of an *ORI* sequence in *cis* cannot be ruled out. For example, in results not presented here, we have seen a ~20-30% decrease in the association of an *ORI*-minus *STB* plasmid with chromosome spreads compared to its *ORI*-plus counterpart. Furthermore, within the missegregating classes for the *ORI*-plus plasmid sisters and the *ORI*-minus pseudo-sisters, the mother bias (2:0 class > 0: 2 class) is considerably larger for the latter. If chromosome association were not affected by the absence of *ORI*, there should have been little or no difference between the two types of plasmids in their bias values. Finally, in a mitotic cell cycle in which cohesin disassembly is blocked, the coupling of the *ORI*-minus circle to chromosomes is lower than that of the *ORI*-plus sisters. Since all chromosomes are partitioned into either the mother or the daughter under this regimen, a pair of plasmids associated with chromosomes should have co-partitioned with them, regardless of whether their association is with sister or non-sister chromatids. Thus while *STB* alone is sufficient for the Rep1-Rep2 mediated tethering of

plasmids to chromosomes, the presence of an *ORI* close to it (within ~300 bp in the native 2 micron circle) may augment the efficiency of tethering. The Orc complex which binds to the origin and/or other protein factors that interact with Orc may play a role in this indirect effect of *ORI* in alleviating mother bias.

### 5.4 Summary and perspectives

The Rep-*STB* system promotes the tethering of the 2 micron plasmid to chromosomes. The tethering can be of two types. In the first, two plasmid copies are tethered to chromosomes in a random fashion. In the second, sister plasmids are tethered to sister chromatids. Random tethering is mediated even in the absence of plasmid replication, while symmetric tethering is replication-dependent. Thus, during a normal cell cycle, the 2 micron circle is able to overcome mother bias and achieve equal segregation through a common mechanism.

In the final set of experiments summarized in **Chapter 6**, we have directed our efforts to partially reconstituting the 2 micron circle partitioning system in mammalian cells in the hope of better understanding the nature of plasmid-chromosome association by exploiting the ability to resolve individual chromosomes cytologically.

# CHAPTER 6

## Attempts to reconstitute the yeast plasmid partitioning system in mammalian cells

The experiments that embody this chapter are based on the rationale that the higher chromosome resolution afforded by mammalian cells will provide better verification of the hitchhiking model for 2 micron plasmid partitioning, provided the Rep-*STB* system can be reconstituted (at least partially) in these cells. The progress made towards this goal is summarized here. **1.** When expressed in mammalian cells, Rep2 by itself (but not Rep1) is localized on the chromosomes throughout the cell cycle. The distribution of Rep2 foci is not uniform along the lengths of chromosomes, but reveals localized regions of preferential deposition. **2.** Rep1 can localize on the chromosomes only in the presence of Rep2, and co-localizes with the latter. **3**. In mitotic chromosome spreads, the Rep1 and Rep2 proteins show a symmetric pattern of localization on sister chromatids. **4**. In a plasmid retention assay, a fluorescence-tagged *STB* reporter plasmid shows slightly higher stability in the presence of both Rep1 and Rep2. However, the significance of this increase, compared to plasmid stability in the presence of Rep1 or Rep2 alone, is not certain at this time.

### 6.1 Introduction

In **Chapter 3**, we employed yeast chromosome spread assays to test plasmid-chromosome association as a preliminary criterion for the hitchhiking model for 2 micron

circle segregation. Although the results from this analysis are consistent with the model, the small size of the yeast nucleus and the poor resolution of the mitotic chromosomes introduce some uncertainty in their interpretations. Mammalian cell systems are vastly superior to budding yeast in cell biological assays because of their large nuclei, increased chromosome size and the resolution of the highly condensed individual mitotic chromosomes. These features provide the requisite level of stringency for testing the basic tenets of the hitchhiking model by fluorescence microscopy-based assays. However, for the analysis to be practical, we should be able to reconstitute at least key aspects of the 2 micron plasmid partitioning system in mammalian cells. The template for these studies is provided by the experimental approaches behind the well-established chromosome-association of viral episomes, in particular, Epstein-Barr virus (EBV) (Kanda et al., 2001; 2007; Nanbo et al., 2007; Silla et al., 2010).

Analogous to the 2 micron plasmid, EBV replicates once per cell cycle during latency, and segregates with high efficiency during cell division. The extra-chromosomal replication of the EBV episome requires a *cis*-acting latent replication origin (oriP) and the *trans*-acting viral protein EBNA-1 (Yates et al., 1985; 1984). EBNA-1 also mediates tethering of viral episomes to chromosomes, by interacting with the chromatin binding host protein hEBP2 on the one hand and with oriP on the other (Kapoor and Frappier, 2003b; Nayyar et al., 2009; Wu, 2000). EBNA-1 is localized on chromosomes, whether or not an oriP-containing plasmid is present in the cell (Nanbo et al., 2007). Furthermore, a significant fraction of the EBNA-1 spots in mitotic chromosomes shows a symmetric disposition on sister chromatids (Kanda et al., 2007). An oriP/LacO reporter plasmid,

visualized by its association with the GFP-LacI, is localized on condensed mitotic chromosomes under the expression of EBNA-1(Kanda et al., 2001).

The strategies we have developed, patterned after the EBV analyses, are as follows. **1**. First, express Rep1 and Rep2 proteins, individually and together, in COS7 cells to test if either or both of these proteins can be localized on chromosomes. **2**. If successful, test whether an *STB*/LacO reporter plasmid is directed to chromosomes with the assistance of the Rep proteins. **3**. Furthermore, test whether the stability of this non-replicating plasmid is increased in cells expressing the Rep proteins.

We have successfully completed the first of the above three objectives. The results are in agreement with the hitchhiking model. The last two strategies have not yielded conclusive results mainly due to technical limitations. Attempts to overcome these impediments are in progress.

## 6.2 Results

### 6.2.1 Expression and localization of Rep1 and Rep2 in mammalian cells

Preliminary experiments by expressing Rep1 and Rep2 individually in COS7 (**C**V-1 (simian) in **o**rigin, and carrying the **S**V40 genetic material) cells suggested that the latter is likely associated with chromosomes. The localization assays were therefore carried out first with Rep2, followed by Rep1.

Rep1 and Rep2 carry their own nuclear localization signals that cause their accumulation within the yeast nucleus (Ahn et al., 1997). We assumed that these signals are likely sufficient for their nuclear import in mammalian cells as well. Rep1 and Rep2

coding sequences were fused in frame with EGFP and DsRed coding sequences, respectively, in the SV40 based pEGFP-C1 and pDsRed-Express-C1 vectors (Clontech). The expression of the fusion proteins was under the control of the $P_{CMV}$ promoter (see Material and Methods, Fig. 2.1). COS7 cells, transfected with these plasmids, were fixed with 4% paraformaldehye after 48 hr and stained with Hoechst 33342 to visualize chromosomes. Rep1 and Rep2 fusion proteins were examined by fluorescence microscopy.

### 6.2.2 Rep2 is localized on chromosomes throughout the cell cycle in COS7 cells

DsRed-Rep2 was found in the nucleus in COS7 cells when it was expressed alone (in the absence of Rep1). In the interphase cells, DsRed-Rep2 formed foci within the Hoechst stained chromosome regions (Fig. 6.1A). In mitotic cells, DsRed–Rep2 foci were confined to the condensed chromosomes (Fig. 6.1B). Rep2 was not uniformly present over the chromosomes; rather, there were several hot spots for its localization. Overall, Rep2 was found to bind to chromosomes throughout the cell cycle. Furthermore, the halo regions in the Hoechst stained chromosome regions (for example, see Fig. 6.1A, C white arrow), were free of Rep2 foci. Using antibodies to the nuclear marker, Fibrillarin, we verified that these halo regions represent the nucleolus (Fig 6.1C).

In budding yeast, the localization of Rep1 and Rep2 on chromosome spreads is a mutually dependent event. This might potentially be due to a technical artifact in the preparation of yeast chromosome spreads. The current notion, based on the absence of Rep1-DNA interaction and extremely weak Rep2-DNA interaction *in vitro* (Sengupta et al., 2001), is that chromosome association of Rep1/Rep2 is mediated with the assistance

of a host factor (Hadfield et al., 1995). The association of Rep1 and Rep2 to a yeast chromatin binding protein may depend on a bifurcated recognition motif split between Rep1 and Rep2. Or, the interaction between Rep1 and Rep2 may be required to expose the recognition motif localized within one of them.

Based on the notion of a mediator protein for the chromosome-association of the Rep proteins, it seems likely that Rep2 can interact with a mammalian protein that binds to chromatin directly or does so indirectly by interacting with a chromatin binding protein. The enrichment of DsRed-Rep2 in certain chromosome regions in mitotic COS7 cells might indicate preferred interaction regions, or might result from the specialized organization of specific chromosome locales in their condensed state. The exclusion of Rep2 from the nucleolus suggests that ribosomal DNA (rDNA) is unlikely to be a target site for Rep2 localization.

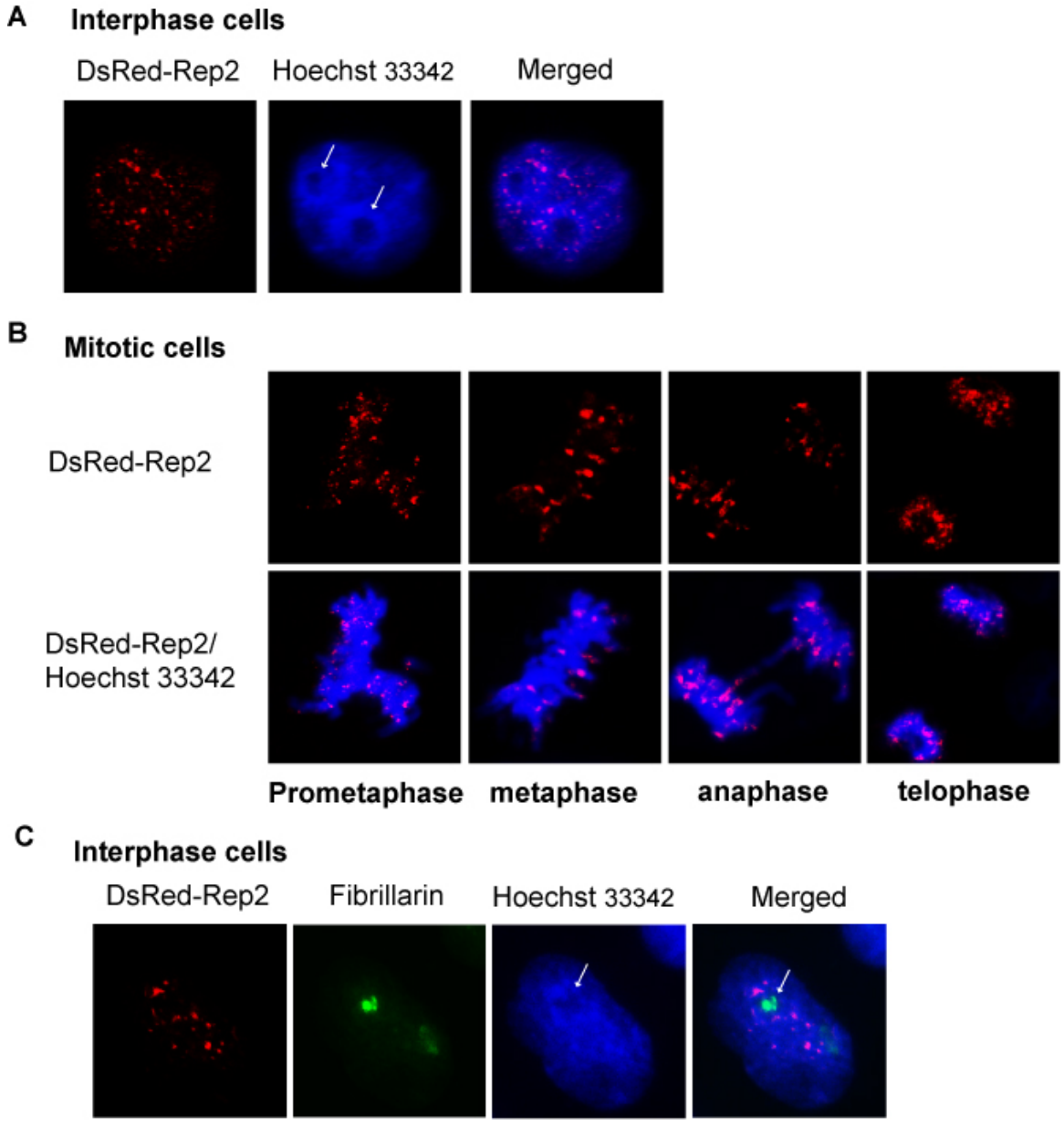



**Figure 6.1 DsRed-Rep2 localizes on chromosomes in COS7 cells.**

DsRed-Rep2 was monitored in COS7 cells at 48 hours following transfection. Localization on chromosomes was observed throughout the cell cycle, in interphase cells (A) and in cells at different mitotic stages (B). Chromosomes were stained using Hoechst 33342. (C) Fibrillarin, stained green by immune-fluorescence, was used as a nucleolar marker. The arrows indicate the halo region within which Fibrillarin was entirely contained.

### 6.2.3 Rep1 localizes on chromosomes in the presence of Rep2

When EGFP-Rep1 was expressed in the COS7 cell line, Rep1 protein stayed in the cytosol in nearly all of the cells expressing the protein (Fig. 6.2A). However, when DsRed-Rep2 and EGFP-Rep1 were co-expressed, Rep1 co-localized with Rep2 on chromosomes throughout the cell cycle. Similar results were obtained when the fluorescent tags were switched between the two proteins, EGFP-Rep2 and DsRed-Rep1 (data not shown). In order to confirm that the co-localization was not due to potential interaction between the EGFP and DsRed proteins, we expressed a cytosolic protein (Nodamura Virus B2 protein (NMV B2) as EGFP-NMV B2 together with DsRed-Rep2. Under this co-expression condition, Rep2 stayed in the nucleus while the EGFP-NMV B2 protein remained in the cytosol (Fig. 6.2B). Even in mitotic cells, with their disassembled nuclear membranes, there was no interaction between EGFP-NMV B2 and DsRed-Rep2 (Fig. 6.2B).

Overall, the above results suggested that the native NLS of Rep1 is perhaps not functional in COS7 cells. Consistent with this inference, when the NLS from SV-40 T-antigen was fused to EGFP-Rep1, the protein was almost entirely intra-nuclear in its localization. The pattern of localization was distinct from that of Rep2. The fluorescence from EGFP-Rep1 was confined to the nucleolus, as was ascertained by its presence in the halo regions marked by Fibrillarin (Fig. 6.2C).

Although Rep1 does not enter the nucleus on its own, it can do so when provided with a strong NLS, or when Rep2 is also expressed in the same cell. The localization of Rep1 under these two conditions shows completely distinct patterns. Consistent with the

known interaction between Rep1 and Rep2, under co-expression, the two proteins form coincident foci over chromosomes that recapitulate the foci formed by Rep2 alone. The nuclear localized Rep1, in the absence of Rep2, is restricted to the nucleolus. Perhaps Rep1 and Rep2 interact with distinct host proteins, accounting for the distinct nuclear localization of each protein in the absence of the other. Results from co-expression assays (data not shown) reveal that EGFP-Rep1(SV40-NLS) is displaced from the nucleolus in the presence of DsRed-Rep2, and co-localizes with the latter. Thus, Rep1-Rep2 interaction must be dominant over the interaction responsible for the nucleolar localization of Rep1.

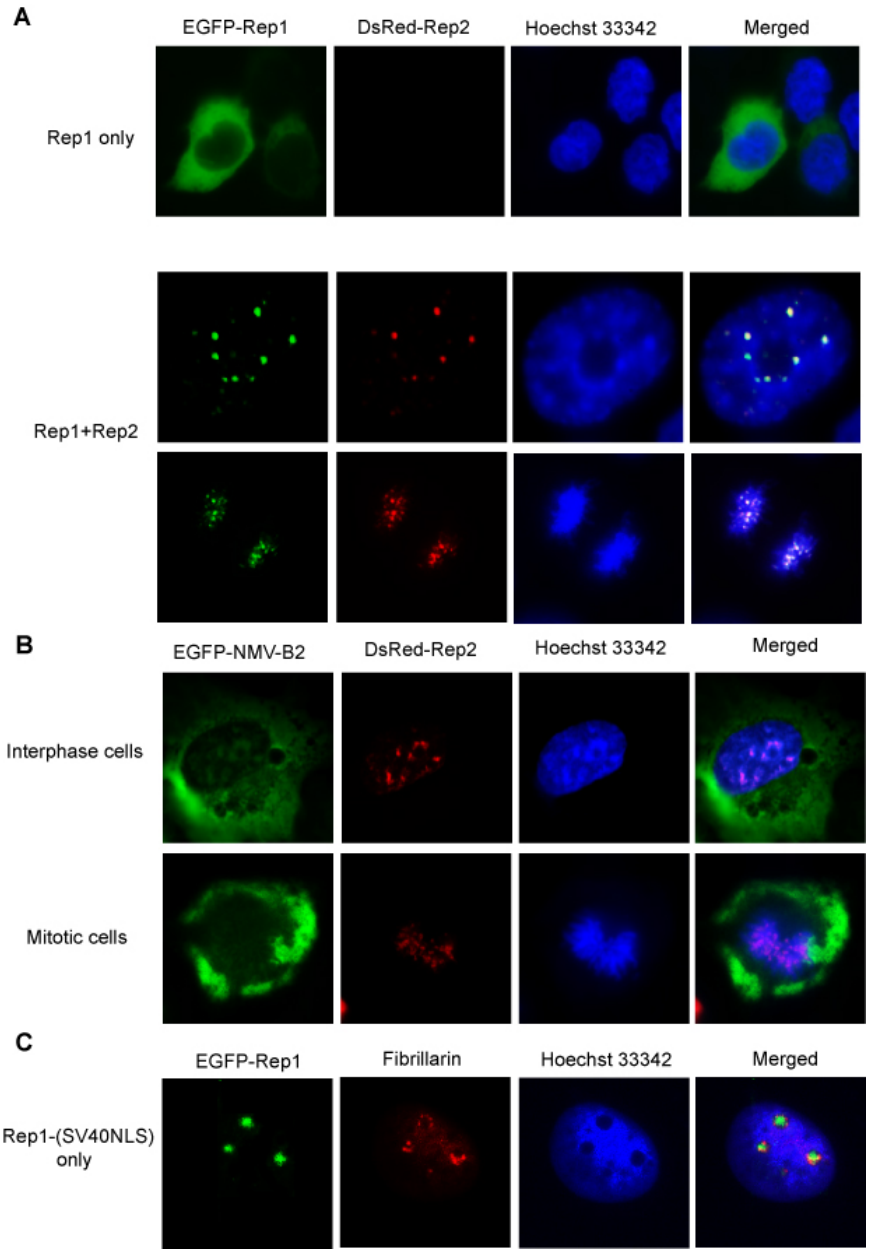



**Figure 6.2 EGFP-Rep1 can localize on chromosomes in the presence of DsRed-Rep2.**

(A) In COS7 cells, EGFP-Rep1 was localized almost exclusively in the cytosol. When DsRed-Rep2 was co-expressed with EGFP-Rep1, both proteins were located in the nucleus in interphase cells and on chromosomes in mitotic cells. (B) DsRed-Rep2 and EGFP-NMV-B2, visualized by immunofluorescence, were not co-localized in interphase or mitotic cells. (C) EGFP-Rep1(SV40NLS) could enter the nucleus efficiently, but was localized in nucleolus marked by Fibrillarin.

### 6.2.4 Functional characterization of Rep2 and Rep1 association with mammalian chromosomes

Since Rep2 was seen binding to chromosomes throughout the cell cycle, we wished to delineate the potential protein domain(s) required for Rep2-chromosome association. A series of truncated Rep2 proteins were expressed in the pDsRed-Express-C1 vector, as described for the full-length protein.

The native NLS of Rep2 is located within the C-terminal 20 amino acids of Rep2 (between residues 277-296). A C-terminal deletion of 120 residues, replaced by the SV40 NLS referred to earlier in the context of Rep 1 expression and localization, permitted the entry of Rep2(1-176) into the nucleus. The protein was distributed throughout the nucleus, was excluded from the nucleolus, and was not associated with chromosomes in mitotic cells (Fig. 6.3A). A series of N-terminal deletions were examined more closely. Removal of up to 90 amino acids from the N-terminus did not affect the nuclear entry of Rep2 (91-296) or the spotted pattern in interphase nuclei and in mitotic chromosomes, typical of full-length Rep2. Two larger deletions, Rep2(121-296) and Rep2(181-296) showed the spotted pattern in interphase nuclei but a more diffused association with mitotic chromosomes (Fig. 6.3B). Rep2(211-236) and Rep2(241-296) showed granular aggregation in interphase nuclei (Fig. 6.3C). It is unclear whether such aggregates are associated with the nucleolus or with other granular bodies. While the shorter of the two showed diffused association with mitotic chromosomes, the longer version of the truncated Rep2 was surprisingly absent from the chromosomes (Fig 6.3B). Perhaps the 211-240 amino acid region masks the ability of the downstream peptide to interact with

the host factor responsible for chromosome-association. The different phenotypes of the truncated Rep2 proteins are displayed in Fig. 6.3 and summarized in Table 5.1.

The deletion analysis demonstrates that the N-terminal 90 amino acids of Rep2 are not required for its normal (or near normal) chromosome-association. However, Rep2(91-296) was unable to promote Rep1 association with chromosomes (Fig. 6.3 D). A much shorter N-terminal deletion Rep2(31-296) also showed a similar phenotype. These findings are consistent with previous results from in vivo (in yeast) and in vitro assays indicating that interaction with Rep1 is mediated, at least in part, by the N-terminal 58 amino acid region of Rep2 (Sengupta et al., 2001).

**Table 6.1 Summary of the localization and Rep1-intreraction phenotypes of truncated DsRed-Rep2 derivatives**

| Truncated Rep2 | Rep1 interaction | Nuclear entry | Interphase cell nuclear localization pattern | Chromosome-association |
|---|---|---|---|---|
| Full length Rep2 (a.a. 1-296) | + | + | Spotted; not in nucleolus | Yes; spotted |
| Rep2 ΔN13 (a.a. 14-296) | + | + | Spotted; not in nucleolus | Yes; spotted |
| Rep2 ΔN30 (a.a. 31-296) | | + | Spotted; not in nucleolus | Yes; spotted |
| Rep2 ΔN60 (a.a. 61-296) | | + | Spotted; not in nucleolus | Yes; spotted |
| Rep2 ΔN90 (a.a. 91-296) | | + | Spotted; not in nucleolus | Yes; spotted |
| Rep2 ΔN120 (a.a. 121-296) | | + | Spotted; not in nucleolus | Yes; diffused pattern |
| Rep2 ΔN180 (a.a. 181-296) | | + | Spotted; not in nucleolus | Yes; diffused pattern |
| Rep2 ΔN210 (a.a. 211-296) | | + | Accumulation in granules | No |
| Rep2 ΔN240 (a.a. 241-296) | | + | Accumulation in granules | Yes; diffused pattern |
| Rep2 ΔC120 (a.a. 1-176) | | | Cytosol | No |
| Rep2 ΔC120 (a.a. 1-176)-SV40NLS | + | + | Evenly distributed in nucleus; not in nucleolus | No |

The truncated DsRed-Rep2 constructs were transfected into COS7 cells, and were assayed for their localization in interphase cells and mitotic cells. Their ability to promote the nuclear localization of EGFP-Rep1 was also tested. The competence of a Rep2 derivative in nuclear localization and its positive interaction with Rep1 (based on their nuclear co-localization) is indicated by a '+' sign under the respective columns. The different types of interphase nuclear localization and chromosome association described here are illustrated by the representative micrographs shown in Fig. 6.3.

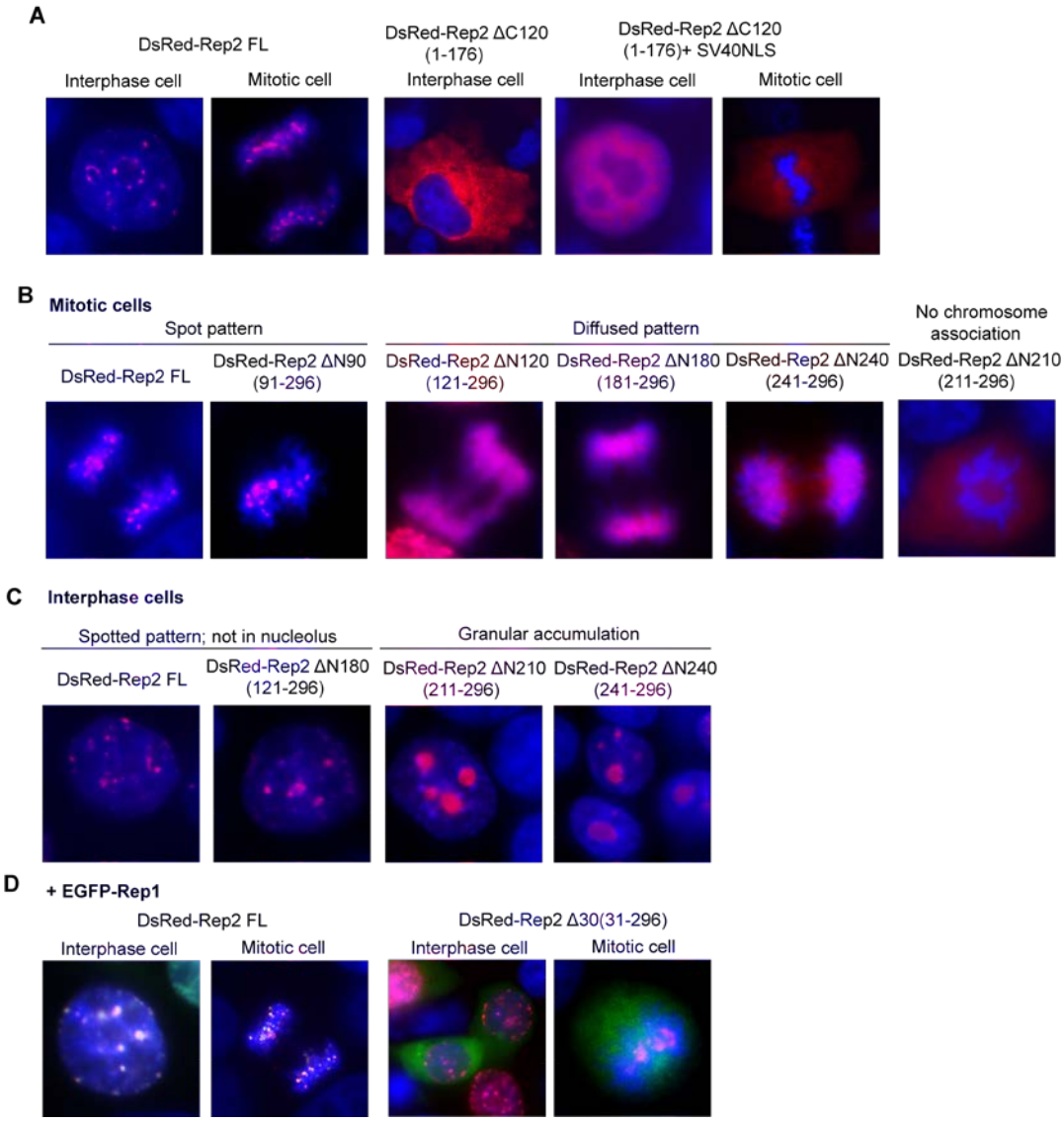



**Figure 6.3 Different phenotypes in the nuclear and chromosomal localizations of truncated DsRed-Rep2 derivatives carrying N-terminal deletions and one C-terminal deletion.**

The DsRed-Rep2 derivatives harboring N-terminal deletions showed either a spotted pattern (A, B) or a diffusely spread pattern (B) on mitotic chromosomes. Two types of patterns were observed in interphase cells. One consisted of sharp spots while the other included larger granular aggregates (C). Even a short N-terminal deletion abolished Rep2 mediated nuclear localization of EGFP-Rep1 (D).Deletion of the C-terminal 120 residues caused this Rep2 derivative to be cytoplasmic by removing its native NLS (A; middle panel). Addition of the SV40-NLS to this truncated Rep2 restored its nuclear localization (A; second panel from the right) but not chromosome association (A; right most panel). FL = Full-length Rep2.

### 6.2.5 Rep1 and Rep2 are localized symmetrically on mitotic chromosomes

In order to more critically characterize the chromosome-association of Rep1 and Rep2, a mitotic chromosome spread assay was performed. COS7 cells are particularly suited for this analysis because of their large nuclear size and the better quality of spreads obtained from them compared to other cell lines. Cells co-transfected with the EGFP-Rep1 and DsRed-Rep2 expression plasmids were first blocked in G1/S by thymidine treatment. After release, they were arrested in metaphase by colcemid treatment, and processed for preparing chromosome spreads (**Chapter 2**; Materials and Methods).

Rep1 and Rep2 were clearly localized on chromosomes, and formed foci along the chromosome arms (Fig.6.4). Furthermore, Rep1 and Rep2 foci were predominantly coincident. Strikingly, >70% of Rep foci showed symmetric localization on sister chromatids. In individual spreads, most chromosomes contained at least one or two foci per chromatid, and very few were free of foci. The foci were, in general, distributed along chromosome arms (~66%), with a significant number present at or proximal to telomeres (~26%; at chromosome tips). Localization at or near centromeres was relatively rare (~8%).

The presence of the Rep proteins on individual chromosomes unequivocally establishes their ability for chromosome tethering, at least in mammalian cells. This observation reinforces the validity of the yeast chromosome spread assays, suggesting that the 2 micron plasmid tethers to chromosomes in a Rep1and Rep2 assisted fashion. It is likely that the host chromatin binding protein that promotes the chromosomal localization of Rep2 is evolutionarily conserved between budding yeast and mammals.

Alternatively, a conserved peptide domain within otherwise unrelated proteins may be responsible for interacting with Rep2 in the two evolutionarily distant organisms. From the present analysis, we cannot say whether there is preferential association of Rep1/Rep2 with a specific subset of chromosomes, or whether there are preferred sites for Rep1/Rep2 localization within a given chromosome. We will be examining a large number of mitotic spreads to see if this information can be reliably extracted.

A logical deduction from the specific localization of Rep1 and Rep2 at *STB* (Hadfield et al., 1995; Sengupta et al., 2001; Velmurugan et al., 2000) is that the association of the Rep proteins with chromosomes would likely mirror the association of an *STB* plasmid with chromosomes. The mammalian chromosome spread results provide strong, even if indirect, support to the hitchhiking model for 2 micron circle segregation. Furthermore, they are consistent with the conclusions from the analyses of single copy reporter plasmids in yeast (**Chapter 5**) (Ghosh et al., 2007; Liu et al., 2013) that sister plasmid copies formed by replication tether to, and hitchhike on, sister chromatids.

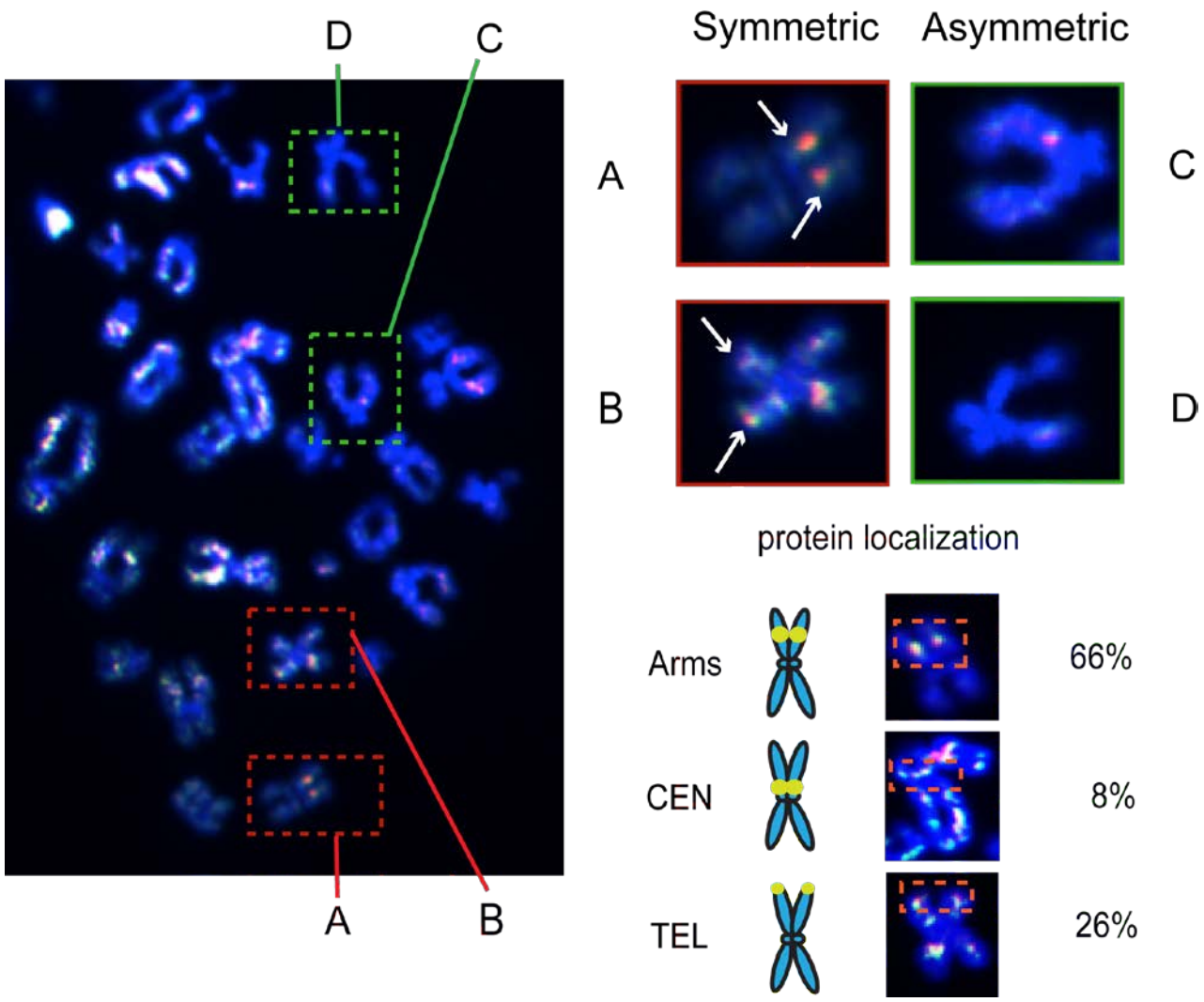



**Figure 6.4 DsRed-Rep2 and EGFP-Rep1 show symmetric co-localization on mitotic chromosomes.**

DsRed-Rep2 and EGFP-Rep1 were co-expressed in COS7 cells. Cells were synchronized in G1/S by thymidine block, released and arrested at metaphase by colcemid treatment. Chromosome spreads were prepared from metaphase cells. The red (Rep2) and green (Rep1) signals were co-localized in nearly all cases. The symmetric localization of the protein foci on sister chromatids was seen for >70% of the foci scored. The frequencies of Rep1-Rep2 localization followed the order: chromosome arms > telomeres (TEL; chromosome tips) > centromeres (CEN).

### 6.2.6 Rep2 alone is not sufficient for *STB* plasmid–chromosome association

The experiments in this section and the following one (**6.2.5** and **6.2.6**) were prompted by the results from the chromosome spread assays. The two critical questions addressed are: (1) can an *STB* containing plasmid be localized on mitotic chromosomes with the assistance of the Rep proteins, and (2) can the nuclear retention of such a plasmid be prolonged in the presence of the Rep proteins? Unfortunately, the results from these experiments are inconclusive at this point, primarily due to technical difficulties. We will attempt to overcome these impediments, and establish cleaner systems for investigating *STB*-plasmid association with mammalian chromosomes and increased plasmid stability through chromosome association.

A previous in vitro study suggests that Rep2 contains the DNA binding domain for *STB* locus (Sengupta et al., 2001). However, this association is weak, and may be augmented in vivo in yeast by Rep1 and as yet uncharacterized host factor or factors (Hadfield et al., 1995). Since Rep2 by itself is localized on mammalian chromosomes, it seemed reasonable to us that Rep2 alone may be able to mediate the chromosome-tethering of an *STB* reporter plasmid. To address this possibility, an *STB*-LacO yeast plasmid used in previous experiments (**Chapter 5.2.6,** an yeast *STB*/LacO plasmid (pSV1)) (harboring a yeast replication origin but lacking replication potential in mammalian cells) was employed in this assay. The plasmid could be visualized in cells expressing EGFP-LacI. COS7 cells were first co-transfected with EGFP-LacI and DsRed-Rep2 expression plasmids. After 24 hr, the *STB*/LacO reporter plasmid was transfected into these cells, and they were examined by fluorescence microscopy 12 hr

later. The number of plasmid foci within the nucleus in interphase cells and the number of plasmid foci present on chromosomes in mitotic cells were counted.

We did not see an increase in the nuclear localization of the plasmid in interphase cells in the presence of DsRed-Rep2; nor did DsRed-Rep2 increase plasmid-chromosome association in mitotic cells (Fig. 6.5). Few, if any, plasmid foci were co-localized with DsRed-Rep2.

It is possible that, in addition to Rep2, Rep1 is also required for mediating plasmid-chromosome tethering. However, providing the two proteins together may not help if a yeast protein that promotes Rep1-Rep2 association with *STB* does not have a functional homologue in mammalian cells. Our attempts to supply both Rep1 and Rep2 in COS7 cells by transfection were unsuccessful. We found that over the duration of the assay, the fraction of cells within the population expressing both proteins was quite low. The experiments will have to be re-designed to provide conditions for the optimal supply of both Rep1 and Rep2 in a sufficiently large cell population.

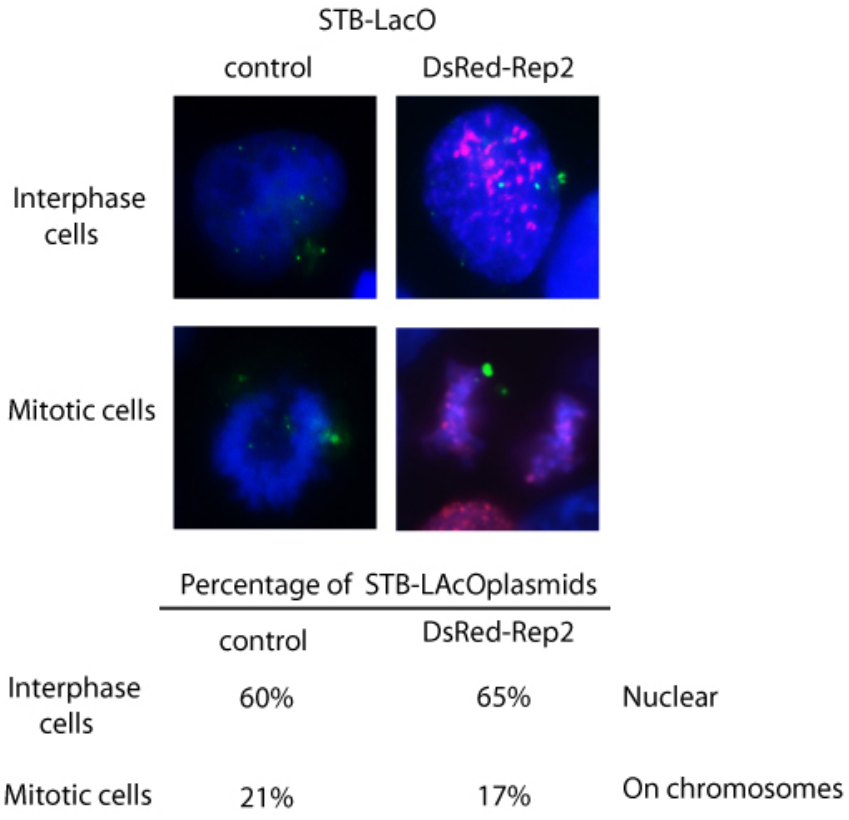



**Figure 6.5 Localization of an *STB*/LacO reporter plasmid in the mammalian cells.**

The *STB*/$[LacO]_{256}$ reporter plasmid was visualized as dots, or occasionally as a cluster of dots, by the green fluorescence from EGFP-LacI bound to the LacO array. In interphase cells, the percentage of plasmid foci within the nucleus relative to the total foci population (inside and outside the nucleus) was scored. In mitotic cells, the number plasmid foci located on condensed chromosomes was expressed as a fraction of all nuclear plasmid foci. The control cells were transfected with the reporter plasmid but not with the expression vector for DsRed-Rep2

### 6.2.7 Retention of the *STB* reporter plasmid in HEK293T cells by the expression of Rep1 and Rep2 proteins

A non-replicating plasmid present within the nucleus of a mammalian cell will be diluted out in the population as a function of the number of cell divisions. For an initial copy number 'n' of the plasmid that is partitioned equally at each division, the first

division will reduce the copy number to (n/2) per cell, the second to (n/4) per cell and so on until a downstream division event will generate a cell containing one plasmid copy and a cell without the plasmid. Each subsequent division of the plasmid-containing cell will generate a plasmid-free cell, and each division of the plasmid-free cell will generate two plasmid-free cells. For n = 2, at the end of three generations, 75% of the cell population (six cells out of eight) will not contain the plasmid. Assuming that chromosome association promotes the distribution of plasmids into both daughter cells during a division event (either by promoting equal or nearly equal plasmid segregation and/or by preventing plasmid exclusion into the cytoplasm as a result of nuclear membrane disassembly), the loss rate per generation will be higher for a plasmid that cannot tether to chromosomes compared to one that can do so. The plasmid retention assays described below is based on this rationale.

Our experimental strategy was similar to that employed in a recent study to follow segregation of BPV episomes in a growing cell population (Silla et al., 2010). A reporter plasmid harboring *STB* and designed to express d2EGFP (a fast degradable form of EGFP; life-time of ~2 hr) from the pCMV promoter was constructed in the pcDNA3.1 Hyg+ vector for these assays (Material and Methods, Fig. 2.1). A cell containing the plasmid can be differentiated from one lacking it by the presence or absence of the green fluorescence signal. The experiments were done using HEK293T cells, as they gave optimal transfection efficiencies. The SV40 origin sequence was deleted from the reporter plasmid to ensure that it would not replicate in the host cells. Rep1 was expressed as DsRed-Rep1 from the pDsRed-express-C1 vector (Material and Methods,

Fig. 2.1). Rep2 was expressed as the native protein from the same vector by deleting the sequences coding for DsRed. The fluorescence tag on Rep1 allowed us to optimize the transfection conditions for the Rep2-dependent nuclear localization of Rep1. By transfecting the DsRed-Rep1 and Rep2 expression vectors at a ratio of 1:9, >80% of the cells showed nuclear localized signal for Rep1 at 48hr after transfection (implying that they contained both Rep1 and Rep2 in the nucleus).

The procedure for the plasmid retention assay is schematically outlined in Fig. 6.6A. At 24 hr following transfection of HEK293T cells, they were seeded into 12 well-plates at a density of $3x10^5$ cells per well. Cell samples were withdrawn at 48 hr, 72 hr, 96 hr, 120 hr and 144 hr post-transfection, and were analyzed by flow-cytometry. The growth medium was also refreshed at these times to allow the cells to continue to divide normally. The generation time under these conditions was ~20 hr. Cells were sorted into 4 different types: GFP+, DsRed+, GFP+/DsRed+, GFP-/DsRed-. The plasmid retention rates were estimated in terms of the changes in the total number of GFP+ cells in the population with time in the presence of DsRed-Rep1 plus Rep2 as well as under various control conditions (Fig. 6B-E).

The data plotted in Fig. 6B-E were normalized by assigning a value of 1.0 for the total number of GFP+ cells (harboring the reporter plasmid) present in the initial population (at 48 hr). With passage of time, cells would be dividing, thus increasing the total number of cells in the population. The increase in the GFP+ cells with time would depend on the efficiency of segregation of the reporter plasmid. For one doubling time, if all the cells received the plasmid the number of GFP+ cells would also double from 1 to

2. For <100% segregation efficiency, this number 'n' would be $1 \leq n \leq 2$. Since the cell division time was not significantly altered for the different samples compared in an assay, the changes in 'n' from 1 at 48 hr to different values at later times reflected true differences in plasmid stability in these samples.

In the absence of the Rep proteins, the changes in GFP+ cells with time showed nearly identical trends for a reporter plasmid containing *STB* and a control plasmid lacking *STB* (Fig. 6.6B). When DsRed-Rep1, DsRed-Rep2 or DsRed protein was expressed alone, the changes in the number of GFP+ cells (signifying the presence of the *STB* reporter plasmid) were similar (Fig. 6.6C). At 72 hr, there was an increase in these cells, followed by a return to a value of ~1 by 120 hr. When both Rep1 and Rep2 were supplied, the GFP+ cells continued to increase till 96 hr, and more or less plateaued thereafter for the duration of the assay (Fig. 6.6D). By contrast, with expression of DsRed-Rep2 alone, the small increase in GFP+ cells till 96 hr was followed by a drop to ~1 by 120-144 hr. Comparable results were obtained when the assays were repeated in HEK293 cells. The slopes for the rise in GFP+ cells were smaller for the expression of DsRed-Rep1 or DsRed-Rep2 alone compared to the co-expression of both proteins (Fig. 6.6E). Furthermore, the slopes were reversed for the descending portion of the graphs between 96 and 120 hr.

Although the overall results could be interpreted as indicating plasmid stabilization in dividing cells by the combined action of Rep1 and Rep2, we are uncomfortable with drawing strong conclusions from these experiments. The variables in the system (multiple plasmid transfections, unknown stabilities of the expression

plasmids and uncertainties in the expression levels of Rep1 and Rep2 in individual cells) were too difficult to control, and could have affected the data. A better experimental scheme will have to be designed in the future for investigating Rep-Rep2 mediated stability of an *STB* plasmid in mammalian cells.

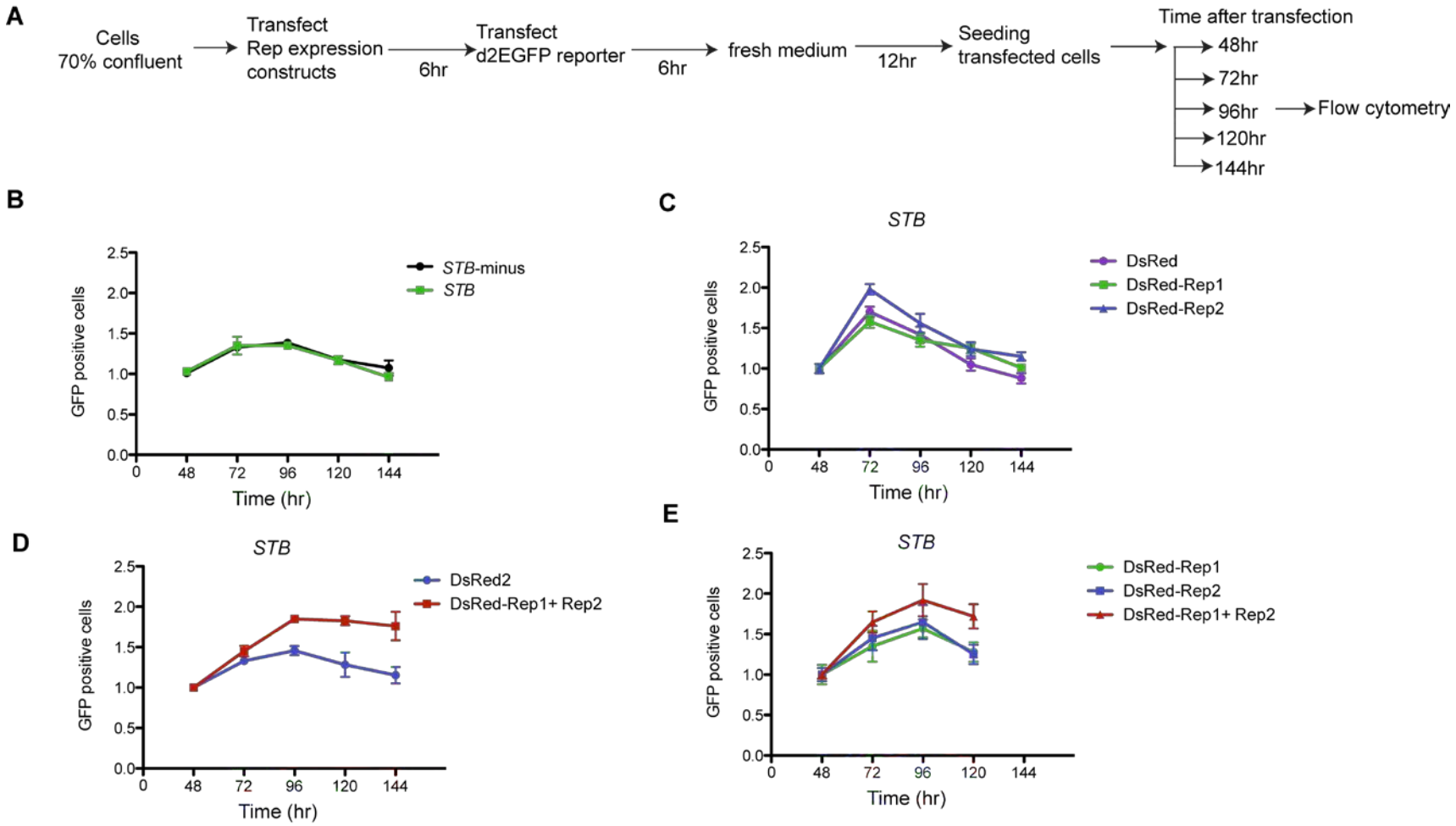



**Figure 6.6 Retention of an *STB* reporter plasmid in HEK293 and 293 cells.**

(A) The experimental scheme for the plasmid retention assay is schematically outlined. (B)-(D) Experiments were performed using the HEK 293T cell line. In (B), a reporter plasmid carrying *STB* or lacking *STB* was assayed without expressing any of the proteins indicated in (C) and (D). (C), (D). The *STB* reporter plasmid was assayed under the expression of the indicated proteins. (E) The analysis was similar to that in (C) or (D), except that transfections were carried out in HEK293 cells. The X-axis indicates the time elapsed after transfection.

## 6.3 Discussion

The attempts to reconstitute components of the 2 micron plasmid partitioning system in mammalian cells have been partially successful. We were able to express the Rep1 and Rep2 proteins in COS7 cells, and functionally characterize their localization on chromosomes. However, attempts to localize an *STB* reporter plasmid to chromosomes in a Rep1 and Rep2 dependent manner were not successful. Although preliminary assays suggest that the stability of a non-replicating *STB* plasmid in HEK293T or HEK293 cells may be enhanced by the Rep1 and Rep2 proteins, these results need to be more rigorously verified using better experimental designs.

### 6.3.1 Cellular localization of Rep1 and Rep2 expressed in mammalian cells

Previous immunofluorescence studies in yeast showed that Rep2 was localized in the nucleus whereas Rep1 was dependent on Rep2 for its nuclear localization (Scott-Drew and Murray, 1998). We have now observed a similar pattern for the nuclear localization of these proteins in COS7 cells: autonomous nuclear localization by Rep2 and Rep2-dependent nuclear localization by Rep1. Our early analysis using GFP-fusions of the Rep proteins showed that both Rep1 and Rep2 can localize to the nucleus in yeast, independently of each other, and their NLS sequences are located close to the C-terminus of each protein (Velmurugan et al., 1998). It is possible that Rep1 harbors a weaker NLS compared to Rep2. One of the three candidate NLS sequences present in Rep2, PTKKRRV (a. a. 275-281), is quite similar to the strong NLS harbored by the SV40 T antigen (Makkerh et al., 1996; Scott-Drew and Murray, 1998). The efficient nuclear

localization of Rep2 in mammalian cells, as opposed to the inability of Rep1 to do so, is therefore not surprising.

When a strong exogenous NLS is fused to Rep1, its localization becomes almost entirely nuclear in COS7 cells. Furthermore, it is preferentially localized in the nucleolus, which is distinct from the more widely distributed (and punctated) localization of Rep2 in interphase nuclei and on mitotic chromosomes. When Rep1 (with or without an exogenous NLS) is co-expressed with Rep2, the two proteins are co-localized with the typical Rep2 pattern. These observations are consistent with the ability of Rep1 and Rep2 to interact with each other and with the dominance of Rep2 in determining the specific pattern of localization.

### 6.3.2 Symmetric co-localization of Rep1 and Rep2 on mitotic chromosomes: implications for the hitchhiking model for 2 micron plasmid segregation

The association of Rep1 and Rep2 with yeast chromosome spreads occurs in a mutually dependent fashion (Mehta et al., 2002) (**Chapter3**; **3.2.2**). It was rather surprising to find that Rep2 by itself associates with mitotic chromosomes in COS7 cells. Perhaps Rep2 is able to interact with a chromatin-associated host factor in these cells. The Rep2 association is non-uniform along chromosome arms, suggesting preferred target sites. Rep2 localization at or near telomeres as well as centromeres has also been seen. The chromosome localization of Rep1, when co-expressed with Rep2, is identical to that of the latter.

The most striking feature of the pattern of Rep1-Rep2 association with chromosomes is the remarkable symmetry between sister chromatids. This symmetric

distribution, seen in >70% of the Rep1-Rep2 foci analyzed, further supports the existence of specific chromosome locales where the proteins are preferentially deposited. The pattern is reminiscent of the EBV system, where the EBNA1 protein tends to form symmetric foci along sister chromatids (Kanda et al., 2007). The extent of the symmetry observed for the Rep proteins is higher than that reported for EBNA1 (Kanda et al., 2007). Based on the reasonable assumption that *STB* plasmids associated with Rep1 and Rep2 would be symmetrically tethered to sister chromatids, the present findings have strong implications for the proposed hitchhiking model for 2 micron plasmid segregation. The segregation behavior of single copy plasmids in yeast during normal mitosis and experimentally induced aberrant forms of mitosis is most consistent with plasmid sisters hitchhiking on sister chromatids (Ghosh et al., 2007; Liu et al., 2013). This specialized hitchhiking model, supported by indirect evidence from yeast experiments, is strengthened by directly visualizing the symmetric disposition of Rep1 and Rep2 on sister chromatids in mammalian cells.

### 6.4 Summary and perspective

The most significant outcome from the partial reconstitution of the 2 micron plasmid partitioning system in mammalian cells is the support it lends to the hitchhiking model for plasmid segregation in yeast. A more complete reconstitution of the system in its non-native context is challenging, and will have to overcome significant technical hurdles. Nevertheless, directly visualizing *STB*-reporter plasmids co-localized with the Rep proteins on chromosomes and following their segregation during a cell cycle are important future goals. Construction of stable cell lines expressing Rep1 and Rep2 from

constitutive as well as inducible promoters would be a critical advancement in realizing these goals.

# CHAPTER 7

## Yeast 2 micron plasmid segregation: an overview

Here we present an overview of the mechanism for the segregation of the 2 micron plasmid by combining the outcomes from the present study with what is already known from previous studies.

### 7.1 Replication dependent equal segregation of sister plasmid copies: hitchhiking on sister chromatids

A single copy *STB* plasmid is associated with a chromosome prior to replication as indicated by chromosome spreads from yeast cells at the G1 stage (**Chapter 3**) (Fig. 3.2). During S phase, chromosomes replicate, and sister chromatids become bridged by the cohesin complex. Cohesin also promotes the pairing of sister plasmids. If the plasmid replicates in association with the chromosome or in close proximity to it, the plasmid-chromosome attachment will be re-established, now between the sisters (Fig. 7.1A). If the plasmid dissociates from the chromosome for its replication, the sister copies of the plasmid formed by replication may tether to a different pair of sister chromatids (Fig. 7.1B). At the metaphase stage, sister chromatids are bi-oriented on the mitotic spindle, and so are the sister plasmids by association. During anaphase, the cohesin is degraded, leading to the equal segregation of the sister plasmids as hitchhikers on the sister chromatids. If the sister kinetochores are mono-oriented on the spindle at some frequency (monopolin-directed mitosis), the sister plasmids also co-segregate to the same cell pole at a nearly identical frequency (**Chapter 5**). When all sister chromatids are, in effect,

cosegregated to the same pole (by the assembly of non-cleavable cohesin on chromosomes), the sister plasmids show nearly absolute coupling to the entire chromosome set in occupying the mother or the daughter compartment (**Chapter 5**). These results support the specialized hitchhiking model for plasmid segregation, in which sister plasmids hitchhike on sister chromatids.

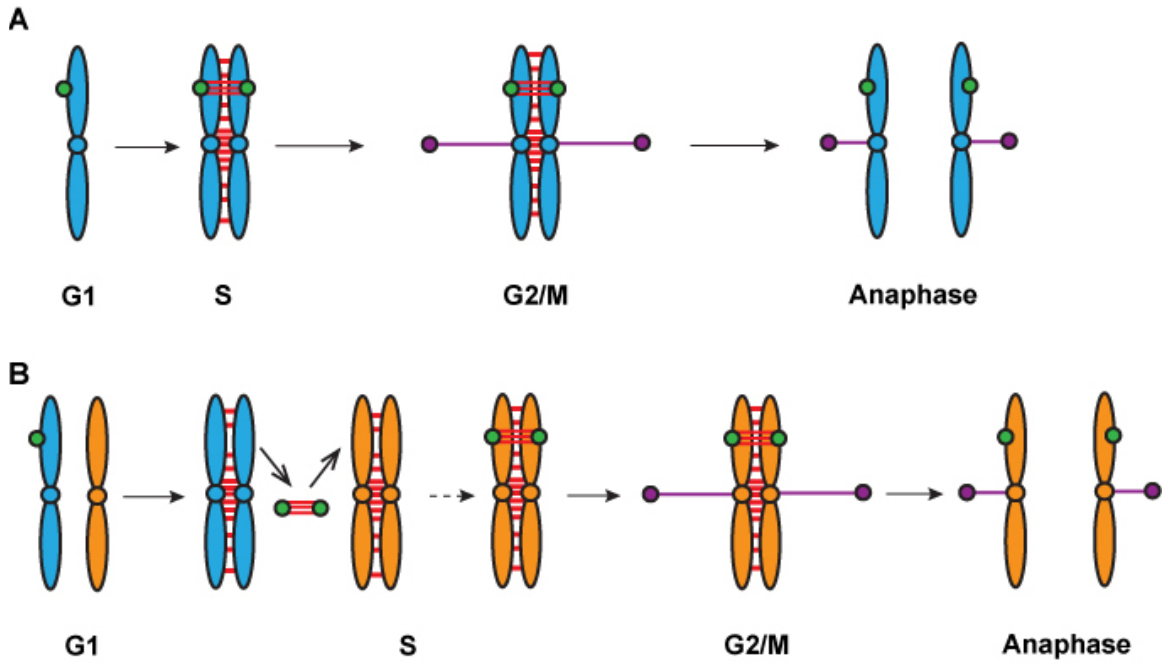



**Figure 7.1 Hitchhiking of sister plasmid copies on sister chromatids.**

(A) A single copy plasmid associated with a chromosome replicates in this associated state. The sister plasmids have a high probability of associating with sister chromatids formed by replication of the same chromosome. Pairing of sister chromatids by cohesin and cohesin-assisted pairing of sister plasmids would provide the drive for this mode of association through proximity effects. (B) The plasmid replicates after detaching from the chromosome that it was tethered to initially. The paired plasmid sisters may now associate with any of the sixteen pairs of sister chromatids with roughly equal probability. Regardless of (A) or (B), in the course of normal mitosis, the bi-orientation of sister chromatids during G2/M followed by cohesin disassembly during anaphase ensures equal plasmid segregation. In case a pair of sister chromatids with the attached sister plasmids should be mono-oriented on the spindle, both will co-segregate to the same cell pole.

### 7.2 Plasmid replication is not required for alleviating mother bias

When the replication origin is deleted from a single copy *STB* plasmid, it can still largely overcome mother bias (**Chapter 5**). In conformity with the hitchhiking model, the plasmid would be associated with one chromosome of a pair of sister chromatids, and has a 50-50 chance of ending up in the mother compartment or daughter compartment (Fig. 7.2A). The property of the Rep-*STB* system to tether plasmids to chromosome is necessary and sufficient to overcome or nearly eliminate mother bias. The origin sequence itself may have a small positive effect on tethering, and may contribute indirectly in a minor way towards reducing mother bias.

When there are two copies of an *ORI*-minus plasmid (pseudo-sister plasmids), each has a high probability of tethering to two different chromosomes (Fig. 7.2B). They will have a 50% chance of equal segregation, and a 25% chance of being in the mother or the daughter (without bias) (Fig. 7.2B). In the low probability event of the two plasmid copies being tethered to the same chromosome, they will missegregate to the mother or daughter, again without bias (Fig. 7.2C). Thus, while random chromosome association helps reduce the mother bias, it is not conducive to equal plasmid segregation. It can at best provide 50% equal segregation (for a single copy replication-competent plasmid), with the efficiency of equal segregation decreasing as the copy number goes up.

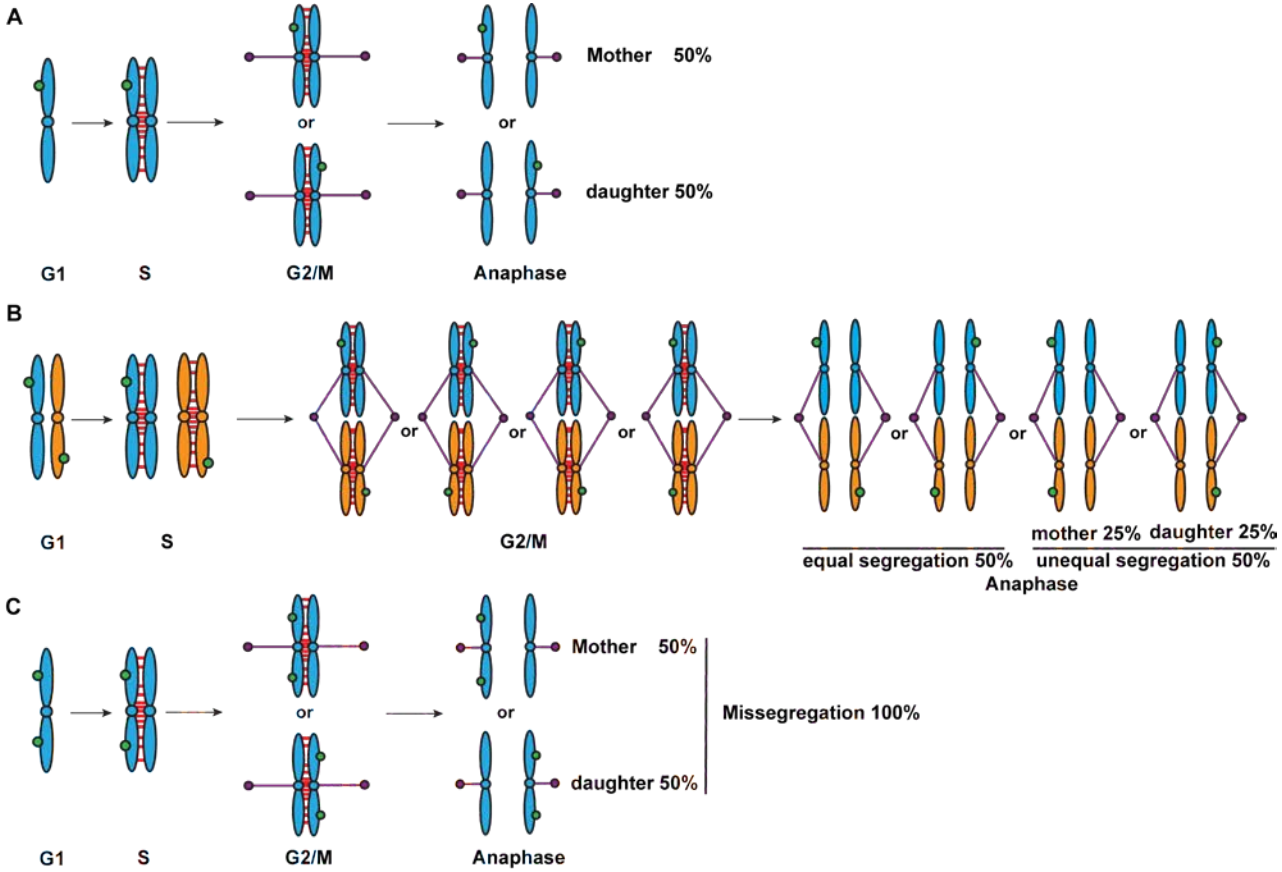



**Figure 7.2 Effect of random chromosome association of one or two replication-blocked plasmid copies on bias versus equal segregation.**

(A) A chromosome-associated plasmid has an equal probability of being in the mother or daughter after chromosomes have segregated. (B) When two plasmid copies are associated with two different chromosomes, they have equal chances for equal segregation (50%) or unequal segregation(25%, mother; 25%, daughter), as the chromosomes segregate by independent assortment. (C) When the plasmids are tethered to the same chromosome, they will missegregate 100% of the time (50% mother, 50% daughter).

### 7.3 Association of Rep1 and Rep2 with mammalian chromosomes

While the experiments in *S. cerevisiae* supports the predictions of the hitchhiking model with sister plasmids piggy-backing on sister chromatids, it is almost impossible to directly prove the validity of this model using the cell biological tools currently available for the yeast system. We have sought mammalian cells as a potential alternative, where direct proof for the hitchhiking model might be obtained. The efforts have so far been only partially successful. We find that the Rep2 protein associates with mitotic mammalian chromosomes, and promotes the chromosome-association of Rep1 when the two are co-expressed (**Chapter** 6) (Fig. 6.4). They co-localize on mitotic chromatin spreads, being predominantly distributed in a symmetric fashion on sister chromatids along their arms (Fig. 7.3A). They are seen to be co-localized at or near centromeres (Fig. 7.3B) at a low frequency, also at or near telomeres (Fig. 7.3B) with higher frequency. Again, the telomere and centromere proximal localizations of the Rep proteins are symmetric. If one imagines copies of the *STB* reporter plasmids associated symmetrically with sister chromatids with the assistance of the symmetrically positioned Rep1-Rep2, plasmid segregation would be equal and efficient, in accordance with the hitchhiking model.

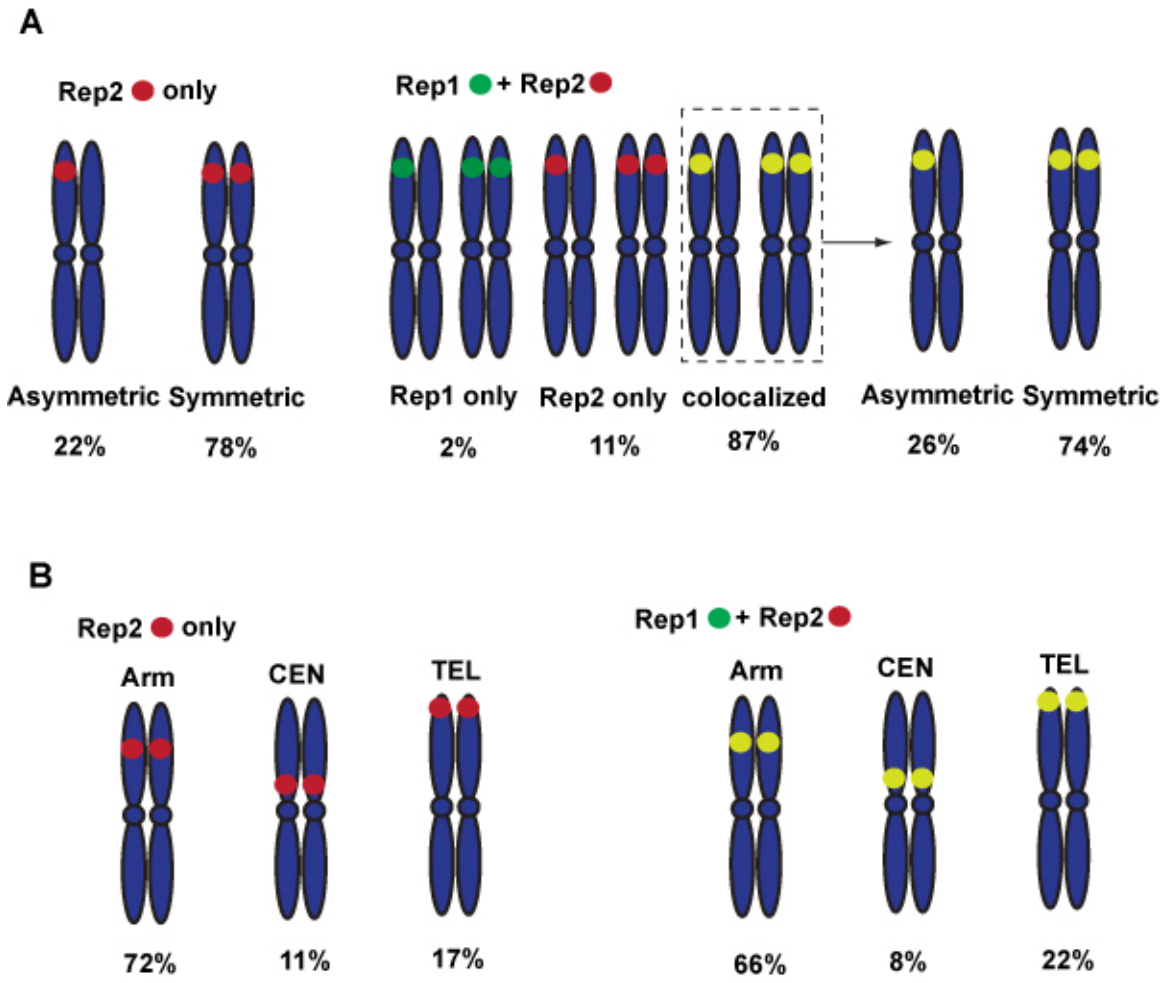



**Figure 7.3 Localization of Rep1 and Rep2 on mammalian mitotic chromosomes.**

(A) When Rep2 was expressed alone, nearly 80% of the protein foci located on mitotic chromosomes were symmetrically disposed on sister chromatids (left). When both Rep1 and Rep2 were co-expressed, they showed >85% co-localization on mitotic chromosomes (middle). In approximately 75% of the cases, the co-localization was symmetric (right). (B) The localization of Rep2 (left) or the co-localization of Rep1 and Rep2 (right) was primarily along chromosome arms and less frequently at or near telomeres and centromeres.

### 7.4 Shortcomings of the present study

The entire set of results described in **Chapter 3** is based on the assumption that the presence of a plasmid on yeast chromosome spreads indicates its association with chromosomes. Admittedly, the spreads contain nuclear entities other than chromosomes, notably nuclear membrane fragments, membrane associated proteins, nuclear pore proteins and potentially components of the nuclear scaffold where chromosome domains are anchored. However, the majority of these concerns regarding potential artifacts of the spread assays is allayed by experiments that uncouple an *STB* plasmid from the nuclear membrane but fail to uncouple it from chromosomes (**Chapter 4**). The experiments in **Chapter 5** demonstrating the requirement of plasmid replication for equal segregation ties in with previous findings that the renewal of the partitioning complex at *STB* during each cell cycle is cued by DNA replication. It would be desirable to test by biochemical assays how the functional association and dissociation of protein factors at *STB* are affected in the absence of replication. These experiments, expanding on a few preliminary findings, will be performed in the near future. Finally, while the mammalian cell system has provided support to the hitchhiking model by revealing the Rep proteins to be co-localized on sister chromatids in a symmetric pattern (**Chapter 6**; thereby eliminating the limitations of the yeast chromosome spreads to a large extent), we have not succeed in obtaining more direct proof for the model by visualizing the symmetric localization of *STB* plasmids on sister chromatids along with the Rep proteins. If construction of stable cell lines expressing Rep1 and Rep2 (currently being attempted) is

successful, it should be possible to critically test whether sister *STB* plasmids hitchhike on sister chromatids in the reconstituted 2 micron circle partitioning system.

Nearly all of the conclusions from the present studies are based on results obtained with single copy reporter plasmids. The native 2 micron plasmid, by contrast, is a multi-copy plasmid. The criticism that the behavior of the single copy reporter cannot be extrapolated to the multi-copy plasmid is well taken. However, since the organization of the latter and the number of individual molecules comprising the limited number of plasmid foci observed using fluorescent reporters are unknown, the single copy system provides the only rational approach to analyzing plasmid segregation. The results presented in this thesis at least permit us to make meaningful interpretations on how a single copy 2 micron plasmid segregates during mitotic cell divisions. At least for now, we propose that the individual plasmid molecules present in each of the multi-copy foci follows the behavior of the single copy reporter plasmids.

**Final thought**

In spite of the deficiencies of the experimental approaches employed in these studies, the totality of the results they have provided favors the hitchhiking mode of segregation for the 2 micron plasmid. For the present, it looks as though the yeast plasmid functionally mimics mammalian viral episomes (and in particular EBV episomes) in sharing the same logic of chromosome coupled long-term propagation.

**Publications from this study**

***1**. Huang, C. C., **Chang, K. M**., Cui, H., and Jayaram, M. (**2011**). Histone H3-variant Cse4-induced positive supercoiling in the yeast plasmid has implications for a possible plasmid origin of a chromosome centromere. **Proc Natl Acad Sci (USA): 108**: 13671-13676

*The work summarized in the above paper is not included in this thesis. The major portion of it was described as part of the PhD thesis of a previous graduate student, Dr. Chu-Chun Huang.

**2**. **Chang, K. M**., Liu, Y.T. and Jayaram, M. Replication of the 2 micron circle is dispensable in alleviating its mother bias but is essential for its equal segregation (under preparation).

**The title and list of authors are tentative at this point. The manuscript will be based principally on the results from **Chapters 3, 4 and 5**.

**Additional publications**

**1**. **Chang, 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.

**2**. Jayaram, M., **Chang, K. M**., Ma, C. H., Huang, C. C., Liu, Y. T. and Sau, S. (**2013**). Topological similarity between the 2 μm plasmid partitioning locus and the budding yeast centromere: evidence for a common evolutionary origin? **Biochem Soc Trans** 41: 501-507.

**3**. Liu, Y. T., Sau, S., Ma, C. H., Kachroo, A. H., Rowley, P. A., **Chang, K. M.**, Fan, H. F. and Jayaram, M. (2014) the Partitioning and copy number control systems of the yeast plasmid: an optimized molecular design for stable persistence in host cells (chapter for Plasmids: Biology and Impact in Biotechnology and Discovery; ASM Press, Washington DC).

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