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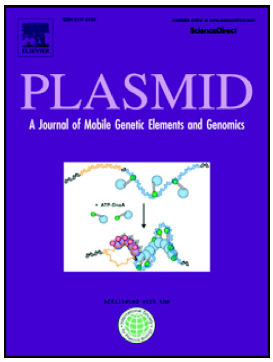

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Hitchhiking on Chromosomes: A Persistence Strategy Shared by Diverse Selfish DNA Elements

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Abstract

An underlying theme in the segregation of low-copy bacterial plasmids is the assembly of a 'segrosome' by DNA-protein and protein-protein interactions, followed by energy-driven directed movement. Analogous partitioning mechanisms drive the segregation of host chromosomes as well. Eukaryotic extra-chromosomal elements, exemplified by budding yeast plasmids and episomes of certain mammalian viruses, harbor partitioning systems that promote their physical association with chromosomes. In doing so, they indirectly take advantage of the spindle force that directs chromosome movement to opposite cell poles. Molecular-genetic, biochemical and cell biological studies have revealed several unsuspected aspects of 'chromosome hitchhiking' by the yeast 2-micron plasmid, including the ability of plasmid sisters to associate symmetrically with sister chromatids. As a result, the plasmid overcomes the 'mother bias' experienced by

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plasmids lacking a partitioning system, and elevates itself to near chromosome status in equal segregation. Chromosome association for stable propagation, without direct energy expenditure, may also be utilized by a small minority of bacterial plasmids--at least one case has been reported. Given the near perfect accuracy of chromosome segregation, it is not surprising that elements residing in evolutionarily distant host organisms have converged upon the common strategy of gaining passage to daughter cells as passengers on chromosomes.

Keywords: selfish elements; yeast plasmid; viral episomes; chromosome tethering; equal segregation; copy number control

Introduction

A decisive attribute in the evolutionary success of organisms, and of selfish or commensal genetic elements that depend on them, is the ability to faithfully replicate and segregate their genomes during cell division. While some elements harbor mechanisms for autonomous replication, others exploit their hosts for this purpose, either by integrating into the host chromosome(s) or by appropriating the host replication machinery. Extrachromosomal elements have evolved a number of alternative molecular strategies for ensuring that daughter cells (or mother and daughter cells) receive equal—or more or less equal—numbers of the replicated

genomes. The unifying rationale among these strategies is to accomplish energy driven directed movement either autonomously or by relying on the force of chromosome segregation by physically attaching to chromosomes. In this review, we highlight the yeast 2-micron plasmid paradigm for stable propagation by 'chromosome hitchhiking', a strategy utilized by other selfish DNA elements that reside in eukaryotic nuclei as well. Several aspects of the plasmid lifestyle are reminiscent of those of mammalian viruses of the papilloma family and gammaherpes subfamily, whose episomes also hitchhike on chromosomes in latently infected cells. Carefully orchestrated mechanisms for coordination between chromosome associated segregation and copy number control, epitomized by the yeast plasmid, likely have broad relevance to how selfish genomes—whose survival is inexorably linked to their hosts' fitness— minimize conflicts with host genomes by keeping in check the extra genetic load posed by them.

Chromosome segregation

Formation of viable and functional descendent cells from a progenitor cell is crucially dependent on the faithful doubling of the genome and equal segregation of the duplicated copies. In eukaryotes, the mechanisms for the pairing of newly replicated sister chromatids, their bi-oriented attachment to the mitotic spindle, and their one-to-one separation to opposite cell poles by spindle force are fairly well elucidated (Duro and Marston, 2015; Freitag, 2016; Hirano, 2015; McIntosh, 2017; McKinley and Cheeseman, 2016; Oliferenko, 2018). Considerable progress has been made in understanding eubacterial chromosome segregation (Barilla, 2016; Oliva, 2016). In a generalized scheme, the occupancy of a bacterial 'centromere' locus (the '*par*' site) by a DNA binding protein (one of two partitioning or 'Par' proteins) is followed by the recruitment of the second Par protein that utilizes NTP hydrolysis to directionally translocate DNA. Remarkably, this mechanism of a 'segrosome' assembly and transport is also utilized by low-copy bacterial plasmids for efficient transmission (Austin and Abeles, 1983; Ogura and Hiraga, 1983). In variations of this central theme, the force generating Par proteins harbor actin-like

ATPases, deviant Walker-type ATPases or tubulin-like GTPases, and assemble dynamic filaments that drag plasmids along the nucleoid surface, or push sister plasmids away from each other, or segregate them by a tram- or treadmill-like movement (Ebersbach and Gerdes, 2005; Schumacher, 2012; Vecchiarelli et al., 2012). While the majority of bacteria have only one chromosome, a subset of them (~10%) contain supernumerary megaplasmids or mini-chromosomes, and have evolved strategies for coordinating the replication and segregation of individual chromosomes with cell division (Espinosa et al., 2017; Jha et al., 2012; Val et al., 2014) . Our present grasp of chromosome segregation in archaeal bacteria, with their rather diverse cell cycle patterns and strikingly wide-ranging chromosome numbers, is somewhat preliminary (Barilla, 2016). Nevertheless, it would seem almost axiomatic that fidelity of chromosome segregation is at the heart of forces that drive the evolutionary success of any organism. Chromosome missegregation is the major underlying cause of several developmental abnormalities in higher eukaryotes including a variety of human cancers (Bakhoum and Landau, 2017; Funk et al., 2016; Gronroos and Lopez-Garcia, 2018; Levine and Holland, 2018).

Selfish DNA elements that take advantage of host chromosome segregation

Selfish DNA elements—those that coexist with genomes but make little or no contribution to their hosts' reproductive success—are widespread among prokaryotes and eukaryotes (Rowley et al., 2013). Such elements are adept at channeling the genetic endowments of their hosts towards self-preservation, and at establishing long-term coexistence with the host genomes with minimal mutual conflicts. Chromosomally integrated elements abound in both prokaryotes and eukaryotes (Gasser, 2016; Hartl et al., 1984; Iranzo et al., 2016; Jangam et al., 2017). Extra-chromosomal elements are copious among prokaryotes (Harmer and Hall, 2015; Jain and Srivastava, 2013; Smalla et al., 2015), but are quite rare among eukaryotes (Broach and Volkert, 1991; Frappier, 2004, 2015; Hughes and Welker, 1999; McBride, 2008). Nuclear plasmids have been found in *Dictyostelium* and budding yeasts (Broach and Volkert, 1991;

Hughes and Welker, 1999), and mammalian viruses of the papilloma family and gammaherpes subfamily exist as episomes in the nuclei of infected cells (Frappier, 2004; McBride, 2008). Eukaryotic nuclei also contain extra-chromosomal circular DNA molecules—eccDNA—excised from chromosomes. Their formation, presumably associated with DNA replication/repair events, may be important in the evolutionary sizing and shaping of genomes (Cohen and Segal, 2009; Moller et al., 2015; Shibata et al., 2012). A subset of these circles has been associated with human diseases (Autiero et al., 2002; Cohen et al., 1997). In contrast to integrated elements, extra-chromosomal elements must engender special strategies for efficient replication and stable propagation in order to ensure their survival over evolutionary time. Regulatory mechanisms that limit the genetic load posed by these elements are important in preventing significant loss in host fitness, which would be indirectly deleterious to the elements themselves.

A potential strategy for efficient transmission to daughter cells, short of covalent integration into the chromosome, would be for a selfish DNA element to physically associate with chromosomes. Indeed, the 2-micron plasmid of the budding yeast *Saccharomyces cerevisiae* and the mammalian viral episomes tether to chromosomes, and segregate by a hitchhiking mechanism (Frappier, 2004; Ghosh et al., 2007; Liu et al., 2015; McBride, 2008; Rizvi et al., 2018). The plasmid and viral partitioning loci contain repeated sequence elements, and are bound by cognate partitioning proteins (Chang et al., 2013; Frappier, 2015; McBride et al., 2012). The viruses generally utilize a single self-encoded partitioning protein, while the yeast plasmid utilizes two such proteins that interact with each other. The partitioning proteins may then bridge the viral or plasmid DNA to tethering sites on host chromosomes either by binding to chromosomal DNA, to integral chromatin proteins or to chromatin associated host factors. Several host proteins that interact with the viral partitioning proteins have been characterized (Barbera et al., 2006; Kapoor et al., 2005; You et al., 2004; You et al., 2006). Although the association of the plasmid partitioning proteins with yeast chromosomes has been established, the molecular basis of this interaction is not understood.

The eukaryotic plasmid and viral partitioning systems have broad general similarities in organization with the prokaryotic partitioning systems, but share no apparent functional or mechanistic features (Fig. 1). While protein-mediated recognition of cognate DNA is fundamental to both systems, there is no evidence for the involvement of NTP hydrolysis or direct utilization of force among the eukaryotic systems. However, there is at least one example of a bacterial plasmid that deviates from the canonical '*par* site-Par proteins-segrosome' mechanisms (Guynet and de la Cruz, 2011). Here, the plasmid associates with the nucleoid assisted by a plasmid-coded Par protein, and segregates by hitchhiking—much like the eukaryotic extra-chromosomal elements.

The 2-micron plasmid partitioning system: interaction with the plasmid amplification system

The 2-micron plasmid partitioning system is comprised of the plasmid-coded Rep1 and Rep2 proteins together with the *cis*-acting locus *STB* (Chang et al., 2013; Liu et al., 2015). The Rep proteins—these are misnomers and ought to be corrected to Par proteins—have no apparent role in plasmid replication, which is carried out by the cellular replication machinery. Rep1 and Rep2 are associated with *STB in vivo*, and promote equal (or nearly equal) segregation of duplicated plasmid molecules into mother and daughter cells. The Rep-*STB* system interacts with several host factors that assist plasmid partitioning (Cui et al., 2009; Hajra et al., 2006; Huang et al., 2004; Ma et al., 2013; Mehta et al., 2002; Mehta et al., 2005; Prajapati et al., 2017; Wong et al., 2002). Current evidence for the 'hitchhiking' model, in which the plasmid utilizes chromosomes as a vehicle for segregation, is extensive but largely circumstantial or indirect (Ghosh et al., 2007; Liu et al., 2016; Liu et al., 2013).

The *STB* locus may be divided into two separate functional units—*STB*-proximal (with respect to the plasmid replication origin), and contiguous to it *STB*-distal (Murray and Cesareni, 1986). The organization of *STB*-proximal, which is critical for normal partitioning and contains

nearly six copies of a ~60 bp consensus repeat element (McQuaid et al., 2018), is reminiscent of the iterated sequence elements present in the partitioning loci of bacterial plasmids and viral episomes (Frappier, 2004, 2015; McBride, 2008; McBride et al., 2012; Schumacher, 2012). The role of *STB*-distal, which includes a strong transcription terminator and a 'silencing box', appears to be in maintaining *STB*-proximal in its fully active state. Within the 2-micron plasmid genome, *STB*-proximal occupies a transcription-free zone (Sutton and Broach, 1985).

The association of several chromosome segregation factors—the cohesin complex, the RSC2 chromatin remodeler, the Kip1 nuclear motor and the histone H3 variant Cse4—at both chromosome centromeres (*CEN*s) and *STB* (Cui et al., 2009; Hajra et al., 2006; Huang et al., 2004; Ma et al., 2013; Mehta et al., 2002) has led us to suggest that the unusually short and genetically defined budding yeast *CEN* and *STB* originated from an ancestral partitioning locus that once directed the segregation of both chromosomes and the plasmid. A similar proposition has been independently posited, based on the presence of 2-micron related plasmids only among budding yeasts and the catastrophic loss of most or all of the RNAi machinery (required for establishing epigenetic regional centromeres) in this lineage (Aravind et al., 2000; Malik and Henikoff, 2009). A potential evolutionary link between *CEN* and *STB* is also consistent with the unusual positive DNA writhe observed at these loci in their functional states (Furuyama and Henikoff, 2009; Huang et al., 2011). If the notion of a shared origin for *CEN* and *STB* is true, the present day plasmid and chromosome segregation pathways represent a mutually compatible resolution of the competition between chromosomes and the plasmid for components of the same segregation machinery.

The Rep1-Rep2 proteins transcend their partitioning function by also acting as a bipartite repressor in plasmid gene expression (Murray et al., 1987; Reynolds et al., 1987; Som et al., 1988). The repressor function is important in regulating the plasmid amplification system constituted by the plasmid coded Flp site-specific recombinase and its head-to-head oriented target sites (*FRT*s) present within a larger inverted repeat sequence of the plasmid (Fig. 2). The

plasmid-coded Raf1 protein positively regulates amplification by antagonizing the Rep1-Rep2 repressor (Murray et al., 1987; Rizvi et al., 2017; Som et al., 1988). The amplification system counteracts any reduction in plasmid copy number resulting from rare missegregation events, while Rep1-Rep2 protects against over-amplification of the plasmid. The Raf1 protein, through its interactions with Rep1 and Rep2, has also subtle effects on plasmid segregation (McQuaid et al., 2017; Rizvi et al., 2017).

Amplification is initiated at low copy numbers by a Flp-mediated DNA inversion event coupled to plasmid replication (Fig. 3A). As a result, the normal bidirectional replication fork is reorganized into a pair of uni-directional forks that chase each other along the circular plasmid template (Futcher, 1986; Volkert and Broach, 1986). The resulting 'pseudo-dual rolling circle' replication spins out a tandem array of plasmid copies, which may be resolved into monomer units by Flp itself or by the homologous recombination machinery of the host. Plasmid amplification may also be triggered by a Flp-mediated recombination event that resolves a replicating plasmid dimer into monomers (Petes and Williamson, 1994) (Fig. 3B). Here, the result of recombination is uni-directional replication in two interlinked plasmid circles (a pince-nez structure). The common feature of amplification by Flp-mediated DNA inversion or resolution is the anti-termination of replication by reconfiguring the forks with respect to each other. This mechanism overcomes the cell cycle constraint by which the replication origin fires only once in every plasmid molecule during a cell cycle—just as a chromosomal replication origin (Zakian et al., 1979).

The Rep1-Rep2 repressor acts on the *FLP*, *RAF1* and *REP1* genes but does not control *REP2*, which appears to be constitutively expressed. The negative regulation of *REP1* provides a mechanism for exquisitely controlling the levels of the Rep1-Rep2 repressor as a function of the plasmid copy number. The opposing regulatory effects of Rep1p-Rep2 versus Raf1 ensure a prompt amplification response when needed without causing a runaway increase in plasmid copy number (Murray et al., 1987; Reynolds et al., 1987; Som et al., 1988). Thus, self-imposed

moderation of selfishness is an integral element in the survival strategy of the yeast plasmid (Liu et al., 2015; Velmurugan et al., 2003).

The 2-micron plasmid-chromosome connection in segregation

The early evidence for the coupling between the 2-micron plasmid and chromosome segregation was revealed by using multi-copy reporter plasmids fluorescence-tagged by the association of GFP- or RFP-repressor with the cognate operator array inserted into them (Mehta et al., 2002; Velmurugan et al., 2000). The dynamics and kinetics of plasmid segregation during the cell cycle were found to closely follow those of chromosome segregation. Furthermore, conditional mutations that cause gross chromosome missegregation have a similar and linked effect on the plasmid (Mehta et al., 2002; Velmurugan et al., 2000). The majority of the plasmid foci, roughly 3 to 4 per nucleus, missegregate in tandem with the bulk of the chromosomes. Chromosome-coupled plasmid segregation requires the assembly of the cohesin complex at *STB* during early S phase, which is dependent on the integrity of the mitotic spindle (Mehta et al., 2005). The role of the spindle in plasmid segregation is almost certainly indirect, and not by spindle attachment of the plasmid. All evidence so far points against the assembly of a kinetochore complex at *STB*. When spindle assembly is delayed during a cell cycle until DNA replication has been completed, cohesin is not recruited to *STB*, while cohesin-mediated pairing of sister chromatids proceeds normally. Upon spindle restoration in G2/M, cohesin associates with *STB*, but this association is not functional. When cells complete the cell cycle by exiting G2/M, chromosomes segregate normally but the plasmid missegregates. Thus, replication-coupled and spindle-dependent cohesin assembly at *STB* is a central event in plasmid segregation. The spindle-associated nuclear motor Kip1 and the microtubule-associated proteins Bik1 and Bim1 are also required for normal plasmid partitioning (Cui et al., 2009; Prajapati et al., 2017). The motor and non-motor proteins may act collaboratively as mediators of spindle associated functions in plasmid segregation. The behavior of the 2-micron plasmid in

the *mtw1* mutant, in which a large subset of kinetochores gets detached from the spindle (Pinsky et al., 2003), suggests preferential association of the plasmid with spindle-attached chromosomes (Mehta et al., 2005). The role of the mitotic spindle in plasmid segregation differs from its well understood conventional role in chromosome segregation.

The above observations formed the basis of a model in which the mitotic spindle and spindle-associated host factors assist plasmid association with chromosomes, while the cohesin complex directs this association to paired sister chromatids and ensures equal distribution of plasmid molecules to each sister. One-to-one segregation of sister chromatids would then deliver equal number of the hitchhiking plasmid molecules to each daughter cell. This segregation scheme is consistent with the localization of the plasmid in mitotic chromosome spreads (explained in further detail below), which requires Rep1, Rep2 and spindle integrity but not cohesin (Mehta et al., 2002; Mehta et al., 2005). While the model is generally satisfactory in explaining the nearly chromosome-like stability of the 2-micron plasmid (a loss rate of ~10^{-4} per cell per generation), it is inadequate in accounting for how 40-60 plasmid molecules (per haploid chromosome content) are equally segregated. A complicating factor is the clustering of plasmid molecules into a much smaller number of foci (≤ 4 foci in 80-90% of the cells) (Velmurugan et al., 2000). This reduction in 'apparent' copy number is not an experimental artefact, as fluorescence-tagged reporter plasmids and the native 2-micron plasmid probed by FISH yield similar results (Heun et al., 2001; Velmurugan et al., 2000). Cell biological observations suggest that each focus is an independent entity in segregation. For the hitchhiking model to work, there must be a high degree of organization within each plasmid cluster prior to and following replication. Notwithstanding the uncertainties regarding how multiple plasmid copies are counted and grouped for segregation, the hitchhiking model would seem to be most compatible with the available genetic, cell biological and biochemical data.

The problem of clustering and the questions it raises regarding replication and segregation are not unique to the yeast plasmid. Clustering has been observed in bacterial

plasmids as well, both low- and high-copy (Ebersbach and Gerdes, 2005; Gordon et al., 2004; Nordstrom and Gerdes, 2003; Pogliano et al., 2001; Weitao et al., 2000). The measured loss rates of low-copy plasmids lacking a *par* locus would be more consistent with each plasmid molecule, rather than the cluster itself, being the unit of segregation (Nordstrom and Gerdes, 2003). A potential resolution to this paradox is the recruitment of individual plasmid molecules one at a time to the replication machine for their duplication, which precedes segregation. An alternative possibility, as suggested for the yeast plasmid, is that the high degree of organization within the cluster enables both the cluster and the individual molecules to function as equivalent units of segregation.

A recent tracking study suggests that exclusion by the nucleoid is responsible for the cell pole-proximal clustering of multi-copy plasmids that segregate by random diffusion. The plasmid molecules can be mobile in the nucleoid-free space, and replication of individual molecules occur stochastically and independently. The biased localization near the pole promotes efficient random segregation of plasmid molecules to both daughter cells (Reyes-Lamothe et al., 2014). Quantitative super-resolution localization microscopy, in conjunction with single molecule fluorescence in situ hybridization (smFISH), is in general conformity with the above analysis (Wang et al., 2016). Plasmids do form large clusters, although random distribution throughout the bacterial cell within the extra-nucleoid space is quite frequent. Furthermore, exclusion of plasmids from the nucleoid is not total. The hybrid distribution, comprised of clustered and individual plasmid molecules localized primarily in the nucleoid-free space, is consistent with efficient segregation without the need for an active partitioning system.

2-micron plasmid segregation analyzed by single-copy reporter plasmids

The problem of plasmid clustering, combined with unknown copy numbers within individual clusters, makes segregation analyses using multi-copy reporter plasmids semi-quantitative at best. To circumvent this impediment, they have been replaced by single copy reporters (Ghosh

et al., 2007; Liu et al., 2013). In the original design, the copy number is kept close to one by including in the reporter a conditional '*CEN*', which can be inactivated by *GAL* promoter-driven high-level transcription through it (Ghosh et al., 2007). These 'nearly single-copy' reporters have not only upheld the inferences from multi-copy reporters, but have also suggested that plasmid sisters formed by replication become tethered to sister chromatids during segregation (Ghosh et al., 2007). The topological trapping of plasmid sisters by *STB*-associated cohesin (Ghosh et al., 2010) may, by proximity effect, encourage their positioning at symmetric 'tethering sites' located on sister chromatids—which are also bridged by cohesin.

The above results have been further authenticated and extended by using 'precisely single-copy' reporters excised in G1-arrested cells from their integrated state on a chromosome by site-specific recombination (Liu et al., 2013). In cells released from G1, the excised single-coy plasmid doubles during S phase, and segregation of the two plasmid sisters is scored in late anaphase cells with well separated mother and daughter nuclei (Fig. 4) (Liu et al., 2016; Liu et al., 2013). The pattern is clean and unambiguous: 1:1 (equal); 2:0 (missegregation to the mother); 0:2 (missegregation to the daughter). Several lines of evidence with this improved single-copy reporter system vindicate the predictions of the hitchhiking model, as outlined below.

First, when the entire set of paired sister chromatids are confined to either the mother or the daughter nucleus of anaphase cells by continuously expressing a non-cleavable version of the cohesin complex, the *STB*-plasmid sisters are almost perfectly correlated with chromosomes in their localization (Liu et al., 2016). This correlation holds both for the mother and the daughter nucleus. For a plasmid lacking *STB*, sisters are predominantly confined to the mother, regardless of chromosome localization in the mother or the daughter. This 'mother bias' of plasmids lacking a partitioning system arises from the constricted nuclear geometry at the yeast bud neck and the relatively short duration of mitosis, leaving insufficient time for

equilibration of plasmid molecules between mother and daughter compartments before nuclear division (Gehlen et al., 2011; Khmelinskii et al., 2011; Murray and Szostak, 1983).

Second, during a mitotic cell cycle under inappropriate expression of the meiosis-specific monopolin complex—which is primarily responsible for the co-orientation of sister kinetochores during the first meiotic division (Marston and Amon, 2004)—the percentage of co-segregation for sister chromatids and *STB*-plasmid sisters is nearly identical (Liu et al., 2013; Monje-Casas et al., 2007). The co-segregation frequency is increased for both plasmid and chromosomes when the meiosis-specific Spo13 is co-expressed along with monopolin, although the rise is slightly higher for the chromosomes. Furthermore, missegregation (or co-segregation) induced by monopolin or monopolin plus Spo13 is nearly bias-free (with respect to mother or daughter) for both the *STB*-plasmid and chromosomes. When monopolin-directed mitosis is performed in the *ipl1* mutant background, chromosome missegregation shows a clear daughter bias (Monje-Casas et al., 2007), which is also observed for the *STB*-plasmid (Liu et al., 2013). In addition, the bias is quantitatively similar for the plasmid and chromosomes.

Finally, when spindle assembly is postponed until G2/M, or the cohesin complex is depleted during the monopolin-directed cell cycle, plasmid-chromosome correlation in co-segregation breaks down. The monopolin complex clamps down sister kinetochores (Corbett et al., 2010) even when chromosome arms remain unpaired in the absence of cohesin. By contrast, monopolin does not associate with *STB* (Liu et al., 2013), and therefore cannot hold together plasmid sisters. The loss of proximity effects from the lack of replication-dependent cohesin assembly at *STB*—due to the absence of the spindle or of cohesin itself—would impede plasmid sisters from attaching to sister chromatids symmetrically. However, chromosome association of plasmid sisters in a random fashion is still possible, as suggested by the extent of their equal segregation (see also the results using non-replicating *STB*-circles described below).

Physical association of the 2-micron plasmid with yeast chromosomes

Single-copy reporters have verified conclusively the Rep1-Rep2 dependent association of an *STB*-plasmid with mitotic yeast chromosome spreads, first observed with a multi-copy *STB*-plasmid (Liu et al., 2016; Mehta et al., 2002). Rep1, Rep2 and the *STB*-plasmid are all colocalized in these spreads (Fig. 5). Furthermore, the level of chromosome association is only slightly lower than that of a *CEN*-plasmid. Similarly, the extent of co-association with chromosome spreads of two single-copy *STB*-plasmids present in the same nucleus is almost equal to that of each individual plasmid association (Liu et al., 2016). Chromosome spreads from meiotic yeast cells at the pachytene stage, with higher resolution than mitotic spreads, reveal co-localization of a multi-copy *STB*-plasmid foci with a subset of Rep protein foci (Sau et al., 2014) (Fig. 6A). The plasmid foci are preferentially located near chromosome tips, at telomeres or sub-telomeric regions (Fig. 6B). Despite the inability to visualize individual chromosomes in yeast, the spread data offer the most direct, and reasonably persuasive, evidence for plasmid and chromosomes being physically linked. They provide the underpinning rationale for a unified interpretation of 2-micron plasmid segregation in distinct genetic backgrounds and under altered cell cycle conditions.

Chromosome association of Rep1-Rep2 and their colocalization has also been demonstrated in mammalian cells expressing these proteins (Liu et al., 2016) (Fig. 7A, B). In individually resolved chromosomes of mammalian mitotic spreads, the Rep1-Rep2 foci are symmetrically distributed along the arms of sister chromatids paired at their centromeres (Fig. 7C). This positional symmetry seen in a heterologous system, viewed in the context of Rep1-Rep2-*STB*-plasmid colocalization in chromosome spreads from the native system, would be consistent with the symmetric, one-to-one hitchhiking of 2-micron plasmid sisters on sister chromatids (Fig. 8A). Indeed, when two differentially fluorescence-tagged *STB*-plasmids (one green and the other red) segregate equally (in ~70% of the cell population compared to ~85% for a *CEN*-plasmid), each of the two daughter cells almost always receives one green and one red plasmid (Ghosh et al., 2007).

Role of replication in 2-micron plasmid segregation

Current evidence suggests that the plasmid partitioning clock is reset at the G1-S transition stage during each cell cycle, and this event appears to coincide with (or immediately precede) the initiation of plasmid replication (Ma et al., 2013). Time-resolved chromatin immunoprecipitation (ChIP) assays suggest that assembly and disassembly of host coded partitioning factors at *STB* follow a sequential pattern, while the Rep proteins remain associated with *STB* nearly throughout the cell cycle (except during the G1-S window) (Ma et al., 2013). The single-copy plasmid excision design has helped shed additional light on how replication comes into play in the segregation process.

In order to mimic plasmid segregation in the absence of replication, two identical copies of an integrant, containing *STB* but lacking a replication origin, are excised from two separate chromosomal locations (Liu et al., 2016). These *ORI*-minus circles are designated as pseudo-sisters, and their segregation is followed in mother and daughter nuclei of anaphase cells. The pseudo-sisters are as capable of overcoming mother bias as plasmid sisters in the presence of Rep1 and Rep2, but their equal segregation frequency is only 40-50% (in contrast to ~70% for authentic *STB*-sisters) (Fig. 8B). A single-copy *STB*-circle *sans ORI* also distributes itself to mother or daughter without bias. These results further fortify the notion that replication assisted proximity of plasmid sisters and sister chromatids is responsible for symmetric chromosome attachment and hitchhiking by plasmid sisters. Furthermore, a pair of plasmid sisters may be oriented within the segrosome for one-to-one association with sister chromatids in *trans*, rather than *cis*-association with the just one sister (which would be antithetical to equal segregation). This replication-induced directed pairing is likely facilitated by the Rep proteins in conjunction with cohesin, and perhaps other host factors. In the absence of replication, the Rep-*STB* system still promotes random association between the plasmid and chromosomes—likely by its interaction with chromatin associated host protein(s). Such association is capable of overcoming

mother bias fully, but effects only 50% equal segregation, in accordance with the independent assortment of chromosomes (Fig. 8B). Coupled with replication, equal segregation is raised well above this value (70%) to nearly the same as a *CEN*-plasmid (~85%), which represents chromosomes in the segregation assays (Fig. 8A).

2-micron plasmid segregation during meiosis

The 2-micron plasmid is not only stably propagated during mitosis, but is also transmitted efficiently to all four spores during meiosis. Due to technical challenges, it has not been possible to follow meiotic plasmid segregation using the single-copy reporter system. Nevertheless, a fluorescence-tagged multi-copy *STB*-plasmid has yielded valuable mechanistic information on plasmid localization and dynamics during the highly specialized germ-line cell division (Sau et al., 2014). There is a steady relocalization of the plasmid from the nuclear interior to the periphery as mitotic cells switch to the meiotic program, and progress from early meiosis to the pachytene stage of meiosis I. Stable plasmid segregation requires Ndj1 and Csm4 belonging to the meiotic 'bouquet' proteins (Sau et al., 2014), with critical roles in anchoring telomeres to the nuclear envelope, their bouquet organization and their characteristic dynamics (Conrad et al., 2008; Conrad et al., 2007; Koszul et al., 2008; Scherthan et al., 2007; Wanat et al., 2008). The preferential telomere-proximal localization of *STB*-plasmid foci seen in meiotic chromosome spreads (Fig. 6B) is substantiated by quantitative measurements of plasmid dynamics during prophase, when rapid chromosome movements are triggered by a specialized nuclear envelope motor in preparation for homologue pairing and the subsequent events of meiotic recombination (Sau et al., 2014). As the motor engages the chromosomes by association with telomeres, the movements observed by *in situ* through-focus time-lapse imaging are fastest at chromosome tips, and slow down along the arms towards centromeres. The *STB*-plasmid reporter closely follows telomeres in their mean speed, maximum speed and in bias (which measures overall displacement from a starting location), while this is not the case for a plasmid lacking *STB* (Sau

et al., 2014). Both the *STB*-plasmid and telomeres are slowed down to similar extents by mutations that differentially affect power transfer from the motor to chromosomes. The absence of Csm4 (the force transducer) brings the plasmid and telomeres to a crawl, while the absence of Ndj1 (required for proper motor-telomere positioning) has a more modest slowing effect. The similarities beween fluorescence-taggged telomere and *STB*-plasmid foci in speeds, step-sizes and the range of their excursions are seen in the time traces shown in Fig. 9. In sum, these findings suggest that the 2-micron plasmid engages a meiosis-specifc motor that orchestates telomere-led prophase chromsome movements for its preferential telomere-associated segregation durng meiosis I. The germ-line transmission of the plasmid signifies a mechanistic variation within the shared theme of chromosome coupled segregation during both mitosis and meiosis.

Host-mediated moderation of the selfishness of the 2-micron plasmid

Although the 2-micron plasmid has minimal effect on its host's fitness at the normal steady state copy number, higher copy numbers are deleterious (Chen et al., 2005; Dobson et al., 2005; Holm, 1982; Xiong et al., 2009). Aberrant amplification of the plasmid leads to cell cycle abnormalities, early onset of senescence and a decrease in replicative life-span. The accumulation of autonomously replicating extra-chromosomal circles, rDNA circles or *ARS*-plasmids, in mother cells due to lack of equal partitioning is one of the reasons for accelerated aging of mothers and rejuvenation of nascent daughters among cells within a pedigree (Defossez et al., 1998; Shcheprova et al., 2008). In addition to transcriptional regulation of the *FLP* gene by Rep1-Rep2, the Flp protein is also post-translationally regulated by the host's SUMO and ubiquitin conjugation machineries (Chen et al., 2005; Xiong et al., 2009). Current evidence suggests that sumoylation of Flp triggers its ubiquitination by a SUMO-targeted ubiquitin ligase, followed by proteasome-mediated turnover. The steady state levels of Flp are thus kept below the threshold that would trigger inopportune plasmid amplification. Whether

sumoylation of Flp directly affects its function by attenuating target DNA recognition, allosteric interactions between Flp subunits and/or the strand cleavage/strand joining activities of the Flp active site is under current investigation. Rep-*STB*-mediated equal segregation of the plasmid also helps prevent the accumulation of the plasmid at high copy numbers due to missegregation. Plasmid-free cells resulting from elevated missegregation have a growth advantage, and tend to rise in the population with time. Without an efficient equal partitioning machinery, long-term persistence of the plasmid would be impossible. The Rep1 and Rep2 proteins are also the targets for SUMO modification, which assists plasmid segregation by promoting functional Rep-*STB* association (Pinder et al., 2013). The 2-micron plasmid exemplifies self-imposed and host-instituted regulatory mechanisms by which a selfish DNA element and its host genome establish almost conflict-free coexistence over evolutionary time.

Chromosome tethering: a survival strategy shared by budding yeast plasmids and mammalian viruses

Based on the evidence from the 2-micron plasmid, related plasmids present in budding yeasts with similar genetic organization (Blaisonneau et al., 1997) are almost certain to follow the hitchhiking mode of segregation. As already noted, episomes of infectious mammalian viruses also resort to a similar strategy (Barbera et al., 2006; Frappier, 2004; Ilves et al., 1999; McBride, 2008; You et al., 2004; You et al., 2006). Although yeast plasmids and mammalian viruses reside in hosts that diverged ~1.5 billion years ago, they share key physiological features designed for long-term persistence. These include cell cycle controlled duplicative replication of each plasmid/episome molecule, efficient chromosome-associated segregation during cell division, and the capacity for iterative replication in response to context-dependent demands on copy number (Barbera et al., 2006; Frappier, 2004, 2015; Liu et al., 2016; McKenzie and El-Guindy, 2015; Volkert and Broach, 1986; You et al., 2004; You et al., 2006; Zakian et al., 1979). The underlying mechanism for plasmid and viral segregation is the bridging of a specific extra-

chromosomal DNA sequence and chromosomal sequences via DNA-protein and protein-protein interactions (Barbera et al., 2006; Frappier, 2015; Liu et al., 2015; McBride, 2008; You et al., 2004; You et al., 2006). Bridging requires just one or two proteins expressed from the extra-chromosomal element, but may involve more than one—perhaps several—host coded proteins. Multiple protein-protein interactions have the potential to expand the range of chromosome sites with which the element may associate, with a given type of interaction likely specifying a subset of tethering sites that share common molecular features.

A common survival strategy among apparently unrelated selfish genomes suggests that molecular mechanisms for exploiting chromosome segregation machineries as a means for stable propagation evolved independently in the plasmid and viral lineages. However, the alternative possibility that the plasmid and the viruses represent the evolutionary divergence of a primordial chromosome-associated segregation mechanism cannot be ruled out entirely. While chromosome tethering helps the yeast plasmid escape mother bias, it protects the viral episomes against the danger of being excluded from the nucleus during the breakdown and reassembly of the nuclear membrane associated with mammalian mitosis. Chromosome hitchhiking highlights the parsimony of evolution in applying the same solution to distinct physiological challenges—nuclear geometry and mother bias or nuclear dynamics and cytoplasmic exile—posed by the idiosyncrasies of closed and open mitosis in lower and higher eukaryotes, respectively.

Summary and Perspectives

With multiple lines of evidence supporting chromosome-associated segregation of the 2-micron plasmid, the next step forward would be to identify and characterize plasmid tethering sites on chromosomes. There is no one particular chromosome, or a single chromosome locus, that the plasmid utilizes for hitchhiking. However, the distribution of potential plasmid tethering sites need not be uniform among individual chromosomes. When yeast chromosomes are forced to

missegregate one at a time during individual cell cycles by conditionally inactivating their centromeres, the equal segregation frequency of the 2-micron plasmid is not altered dramatically by the missegregation of any one chromosome. By contrast, during chromosome missegregation in bulk (or *in toto*), plasmid missegregation in tandem with chromosomes becomes evident (Liu et al., 2016; Liu et al., 2013; Mehta et al., 2002; Velmurugan et al., 2000). Furthermore, the Rep protein and plasmid foci distribution in meiotic chromosome spreads indicate that plasmid tethering sites are likely discrete, and not randomly distributed throughout chromosomes. Non-randomness of tethering sites is also consistent with the observed pattern of Rep1-Rep2 foci along sister chromatid arms in mammalian mitotic spreads. ChIP-seq analyses of the Rep proteins and of their chromatin associated host protein partners should, in principle, provide a genome-wide map of plasmid tethering sites. These results can be further verified by probing direct interactions between the plasmid and chromosome locales by 4C-seq and related chromosome conformation capture methods. Together, these high throughput analyses are expected to provide unequivocal verification of the hitchhiking model. Additionally, they are likely to reveal DNA sequence motifs, high-order DNA organization and/or epigenetic marks that designate chromosome locales as plasmid tethering sites.

The Epstein-Barr virus (EBV) and human papilloma virus (HPV) partitioning systems have been reconstituted by transforming reporter plasmids harboring the respective *par* sites in yeast strains engineered to express the requisite Par proteins (Brannon et al., 2005; Kapoor et al., 2001). The 2-micron plasmid partitioning system has not yet been fully reconstituted in mammalian cells. However, chromosome association of Rep1-Rep2 in these cells and the symmetry of this association between sister chromatids (Liu et al., 2016) suggest that the Rep-*STB* system is likely to be functional in these cells as well. The 2-micron-related pSR1 plasmid from *Zygosaccharomyces rouxii* can be propagated with high stability in *S. cerevisiae* by the plasmid partitioning system comprised of proteins P and S (orthologues of Rep1 and Rep2, respectively) and the partitioning locus Z (Jearnpipatkul et al., 1987). Partitioning systems

operating in native and non-native hosts provide the opportunity to potentially unveil common molecular rules followed by plasmids from different budding yeast species, and by yeast plasmids and viral episomes, for hitchhiking on chromosomes. They also offer the possibility of addressing evolutionary questions regarding shared persistence mechanisms utilized by the same selfish element placed in highly diverged eukaryotic hosts and by diverse selfish elements introduced into a given eukaryotic host.

Analogous segregation mechanisms utilized by the 2-micron plasmid and mammalian viral episomes signify a paradigm shift in our perception of these elements by demonstrating their common survival strategies despite the long evolutionary divergence between their hosts. Advancing our understanding of these mechanisms may help design extra-chromosomal strategies to correct genetic deficiencies or to manipulate gene expression in research and therapeutic contexts. Genetic engineering using extra-chromosomal vectors does not cause local rearrangements of the native genomic state, and is generally more amenable to reversal than that mediated by integrating vectors. These features enhance the desirability of non-integrating vectors in a variety of applications in biotechnology and medicine.

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Fig. 1. Plasmid/episome segregation mechanisms in prokaryotes and eukaryotes. A common feature underlying the different segregation systems is the association of a self-coded partitioning protein or a pair of such proteins (generalized as Par protein-1 here) with a *cis*-acting DNA locus (labeled as the *par* site). The *par* site includes a set of repeated elements (or iterons) shown as filled circles. Par protein-1 stands for ParB and ParR in the P1 and R1 plasmid systems, respectively. The *par* site for ParB is *par*S, and that for ParR is *par*C. In the case of the yeast 2-micron plasmid, Par protein-1 is comprised of two plasmid coded proteins (Rep1 and Rep2), which localize to their cognate *par* site *STB in vivo*. In the Epstein Barr virus (EBV) and human papilloma virus (HPV) systems, Par proein-1 is represented by the viral EBNA1 and E2 proteins, respectively. They bind to their respective *par* sites, present as repeated elements on the viral genome (called FR for family of repeats in the case of EBV and E2BS for E2 binding sites in the case of HPV). The Par protein-2, harboring NTPase activity, is unique to bacterial plasmid segregation. In the P1 system it is named ParA; the corresponding

protein in the R1 system is ParM. Nearly all of the well characterized bacterial partitioning systems involve active plasmid transportation. By contrast, the yeast and mammalian systems resort to non-covalent plasmid/episome association with chromosomes (tethering). A similar mode of nucleoid association may be utilized in rare instances by bacterial plasmids as well (see text). In the tubulin based segregation machinery utilized by certain bacterial virulence plasmids, the *par* site (*tub*C) is bound by TubR (Par protein-1), and the force for segregation is supplied by TubZ (Par protein-2; a GTPase).

Fig. 2. Gene organization and transcriptional regulation in the 2-micron plasmid. **A**. In the schematic representation of the yeast plasmid, the coding regions for the four plasmid proteins are demarcated, with the arrowheads indicating the direction of transcription. The *cis*-acting sites central to plasmid physiology are the replication origin (*ORI*; recognized by the host replication machinery), the plasmid partitioning locus *STB* with which the Rep1-Rep2 proteins associate, and the Flp recombinase target sites (*FRT*s) located within a large (599 bp) inverted repeat region (shown as the handle in the plasmid's dumbbell representation). **B**. Expression of the plasmid genes (except for *REP2*) is regulated by the putative Rep1-Rep2 repressor, which is antagonized by Raf1. The positive and negative controls delicately balance Rep1 levels—and, in turn, the Rep1-Rep2 repressor levels—as a function of plasmid copy number. When the plasmid population drops below the steady state value (Rep1-Rep2 repressor < threshold concentration), *FLP*, *RAF1* and *REP1* genes are turned on. Upon restoration of copy number (Rep1-Rep2 repressor ≥ threshold concentration), these genes are turned off. This regulatory circuit prevents unchecked rise in the plasmid population.

Fig. 3. Mechanisms for plasmid amplification by Flp-mediated recombination between *FRT* sites. Two possible modes of plasmid amplification are illustrated in **A**, **B**. In **A**, site-specific recombination between the head-to-head Flp target sites (*FRT*s; thin arrows) of a replicating

plasmid monomer sets up a pair of replication forks (thick arrowheads) that chase each other along the circular template. In **B**, site-specific recombination between two head-to-tail target sites in a replicating plasmid dimer generates two interconnected uni-directionally replicating circular monomers. Formation of amplified plasmid concatemers by the schemes in **A** and **B** may be regulated by a second recombination event to restore replication termination.

Fig. 4. Single-copy reporter plasmids for quantitative analysis of 2-micron plasmid segregation. **A**. A fluorescence-tagged reporter plasmid containing the 2-micron plasmid replication origin (*ORI*) and *STB* is integrated into a chromosome--at the *HIS3* locus on chromosome XV (shown here) or at the *TRP1* locus on chromosome IV. The reporter is bordered by two head-to-tail copies of the target site (*RRT*) for the R site-specific recombinase, which is engineered into the host strain under the control of the *GAL* promoter. The strain is also designed to express Rep1 and Rep2. Variations of the reporters included those lacking *STB* or *ORI* or both. For a subset of the experiments, two integrated copies of an *ORI*-minus reporter are utilized—one at the *HIS3* locus and the other at the *TRP1* locus. For excision of the reporters, cells arrested in G1 with α-factor are transferred from raffinose to galactose for 2 hr for optimal induction of the R recombinase. **B**. Plasmid segregation is quantitated precisely in cells released from arrest and permitted to complete the cell cycle.

Fig. 5. Localization of Rep1, Rep2 and an *STB*-reporter plasmid in chromosome spreads from mitotic yeast cells. **A**. Rep1 and Rep2, probed by immunostaining in mitotic spreads, are seen to be colocalized within the DAPI-stained chromosome mass. The resolution of the yeast spreads is poor. However, the Rep1-Rep2 pattern suggests that they are not randomly distributed over the entire chromosomal region. **B**. An *STB*-reporter plasmid colocalizes with Rep1 (and Rep2; not shown) in these spreads. The plasmid, harboring an operator array, is

visualized indirectly by immunostaining the bound GFP-repressor. Localization of the *STB*-plasmid in spreads is dependent on both Rep1 and Rep2 (not shown here).

Fig. 6. Localization or Rep1 (and Rep2; not shown here) foci in meiotic chromosome spreads from the pachytene stage. **A**. In meiotic chromosome spreads, with considerably higher resolution than mitotic spreads, Rep1 and a multi-copy *STB*-reporter plasmid are seen as well separated individual foci. The plasmid foci (fewer in number) are coincident with a subset of the Rep1 foci. Rep2 forms colocalized foci with Rep1 in these spreads (not shown). In the rightmost panel showing plasmid-Rep1 merger, the chromosomes stained with DAPI are also included. **B**. The paired homologues are visualized in meiotic spreads by immunostaining Zip1 (red), which marks the axial filaments of the synaptonemal complex. *STB*-plasmid foci (green) show preferential localization at or near chromosome tips. The enlarged view of selected regions of the spread (panels at the right) better illustrate this characteristic localization.

Fig. 7. Localization of co-expressed Rep1 and Rep2 on mammalian chromosomes. **A**. Rep1 and Rep2 colocalize on mitotic chromosomes when expressed together in mammalian cells. **B**. Colocalized Rep1-Rep2 segregate to daughter cells in a chromosome-associated fashion. The panels from left to right represent pre-anaphase, anaphase and telophase cells. **C**. In mitotic chromosome spreads, the vast majority of Rep1-Rep2 foci are symmetrically distributed along sister chromatid arms. The distribution of these foci does not cover entire chromosomes (stained with Hoechst). Thus, poorly resolved yeast spreads and the well resolved mammalian spreads suggest Rep1-Rep2 association with chromosomes to be discrete.

Fig. 8. Replication-dependent and replication-independent segregation of single-copy *STB*-reporters. **A**. Authentic sister copies of a single-copy *STB*-plasmid formed by replication tether symmetrically to sister chromatids. In theory, their equal segregation is 100%. The observed

value of ~70% for *STB*-plasmids is in reasonable agreement with the prediction, with *CEN*-plasmids yielding ~85% equal segregation. **B**. Two identical single-copy *STB*-circles lacking *ORI*, excised in G1 (Fig. 4) from two separate chromosome locales, can tether randomly to chromosomes with the assistance of Rep1-Rep2. The predicted equal segregation of these pseudo-sisters (colored dark and light green) is 50% (observed value = 40-50%).

Fig. 9. Rapid prophase movements of a multi-copy *STB*-plasmid in synchrony with telomeres. Time-lapse video-microscopy reveals the foci of a multi-copy *STB*-plasmid to move with similar speeds and step-sizes over similar ranges as the telomeres of chromosome IV homologues during rapid prophase chromosome movements. Mutations (*csm4Δ*; *ndj1Δ*) that affect force transfer from the motor to different extents cause similar speed reductions in the telomeres and the plasmid. The traces for individual plasmid foci are shown in separate colors (n = the number of plasmid foci). The red and green traces for chromosome IV (right panels) distinguish the telomeres of homologues. The dashed outlines represent the nuclear periphery.

Highlights

- The yeast 2-micron plasmid partitioning system promotes equal plasmid segregation via ‘chromosome hitchhiking’.
- Chromosome hitchhiking in the absence of replication helps the plasmid overcome ‘mother bias’.
- In the absence of replication, random plasmid attachment to chromosomes yields 50% equal segregation.
- Replication directed symmetric attachment of plasmid sisters to sister chromatids yields chromosome-like plasmid segregation.

- Diverse eukaryotic selfish genomes—plasmids and viral episomes—utilize chromosome hitchhiking for stable persistence.

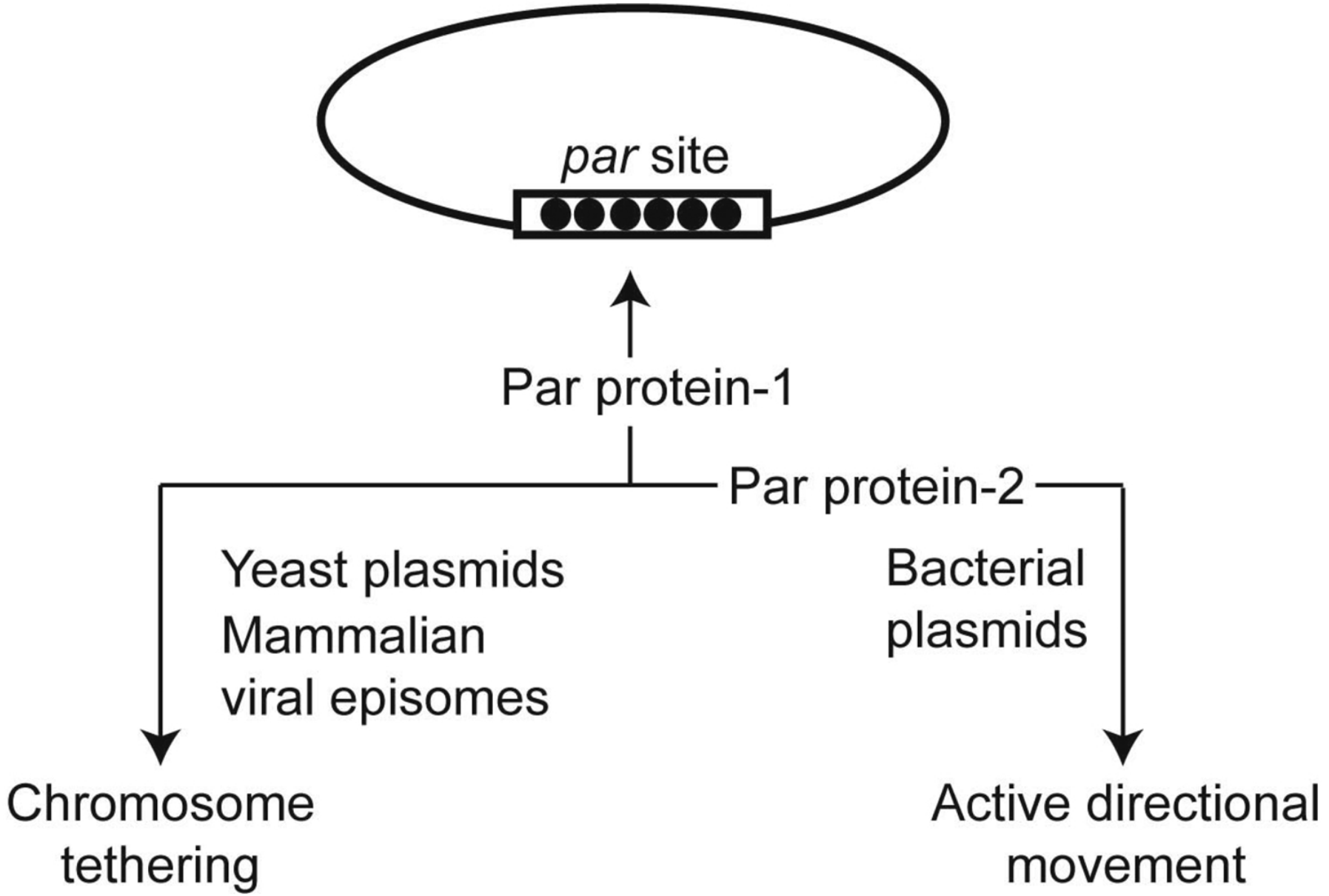

par site
Par protein-1
Par protein-2
Yeast plasmids
Mammalian
viral episomes
Bacterial
plasmids
Chromosome
tethering
Active directional
movement

Figure 1

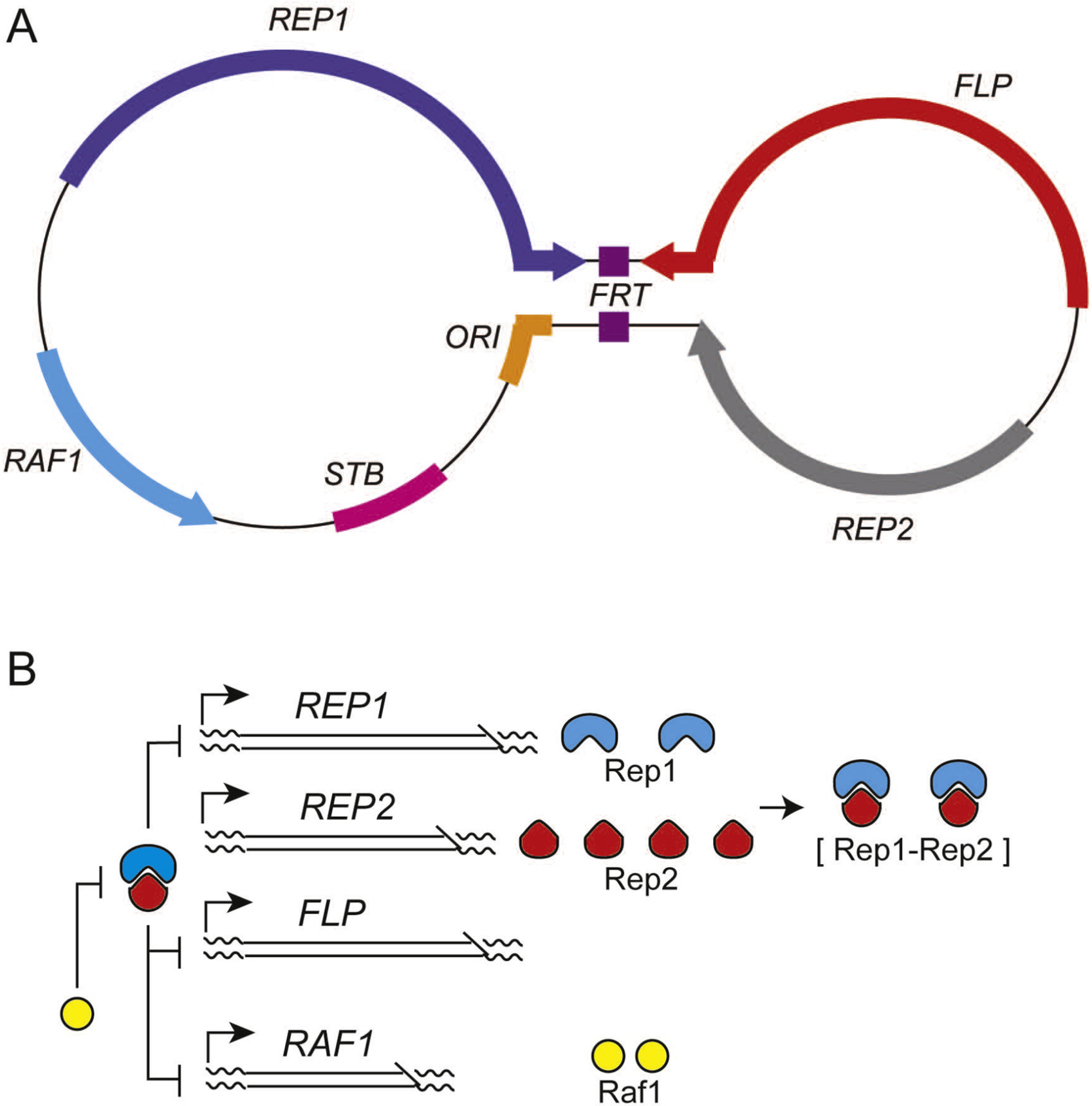

A
REP1
FLP
FRT
ORI
RAF1
STB
REP2
B
REP1
Rep1
REP2
Rep2
[Rep1-Rep2]
FLP
RAF1
Raf1

Figure 2

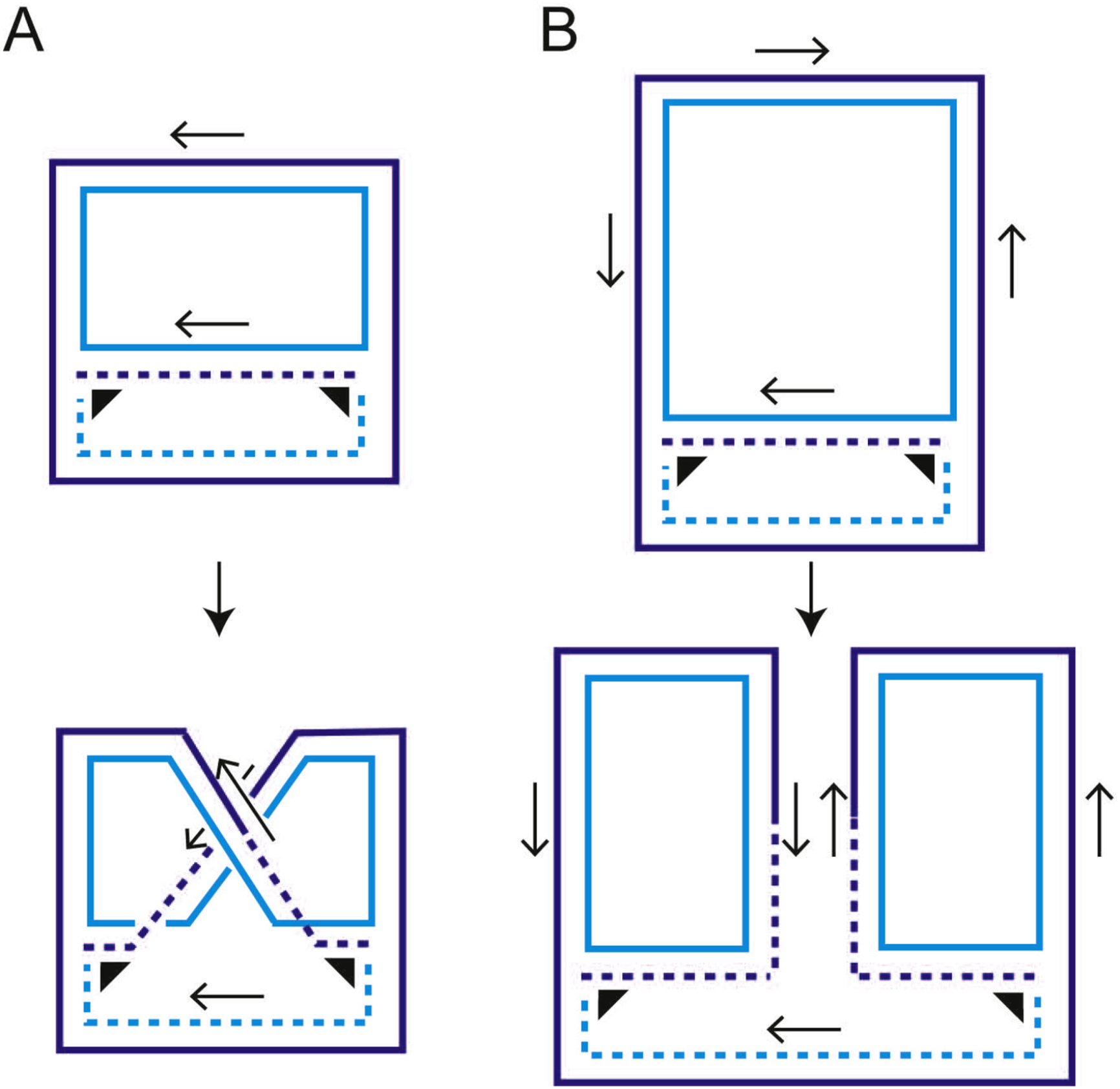


Figure 3

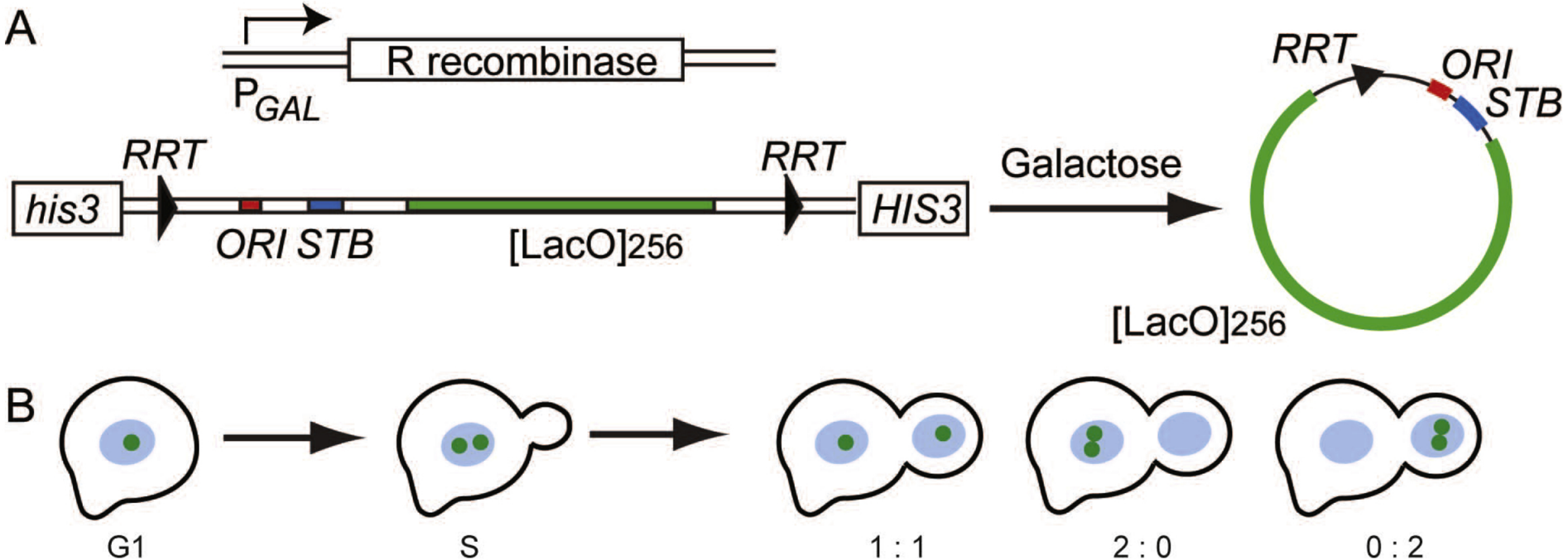
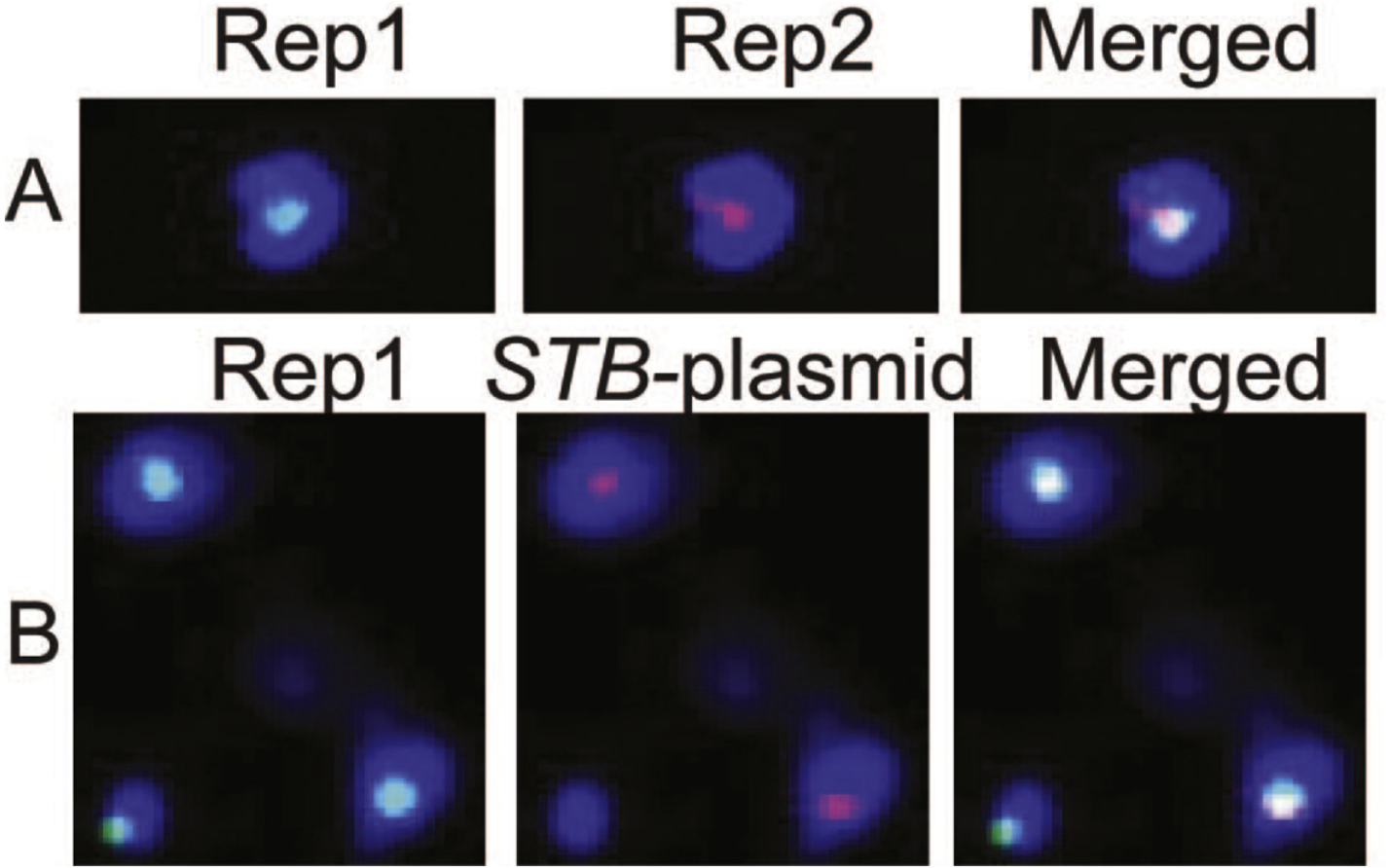

A
R recombinase
P_{GAL}
RRT
RRT
his3
HIS3
ORI STB
[LacO]256
Galactose
RRT
ORI
STB
[LacO]256
B
G1
S
1 : 1
2 : 0
0 : 2

Figure 4



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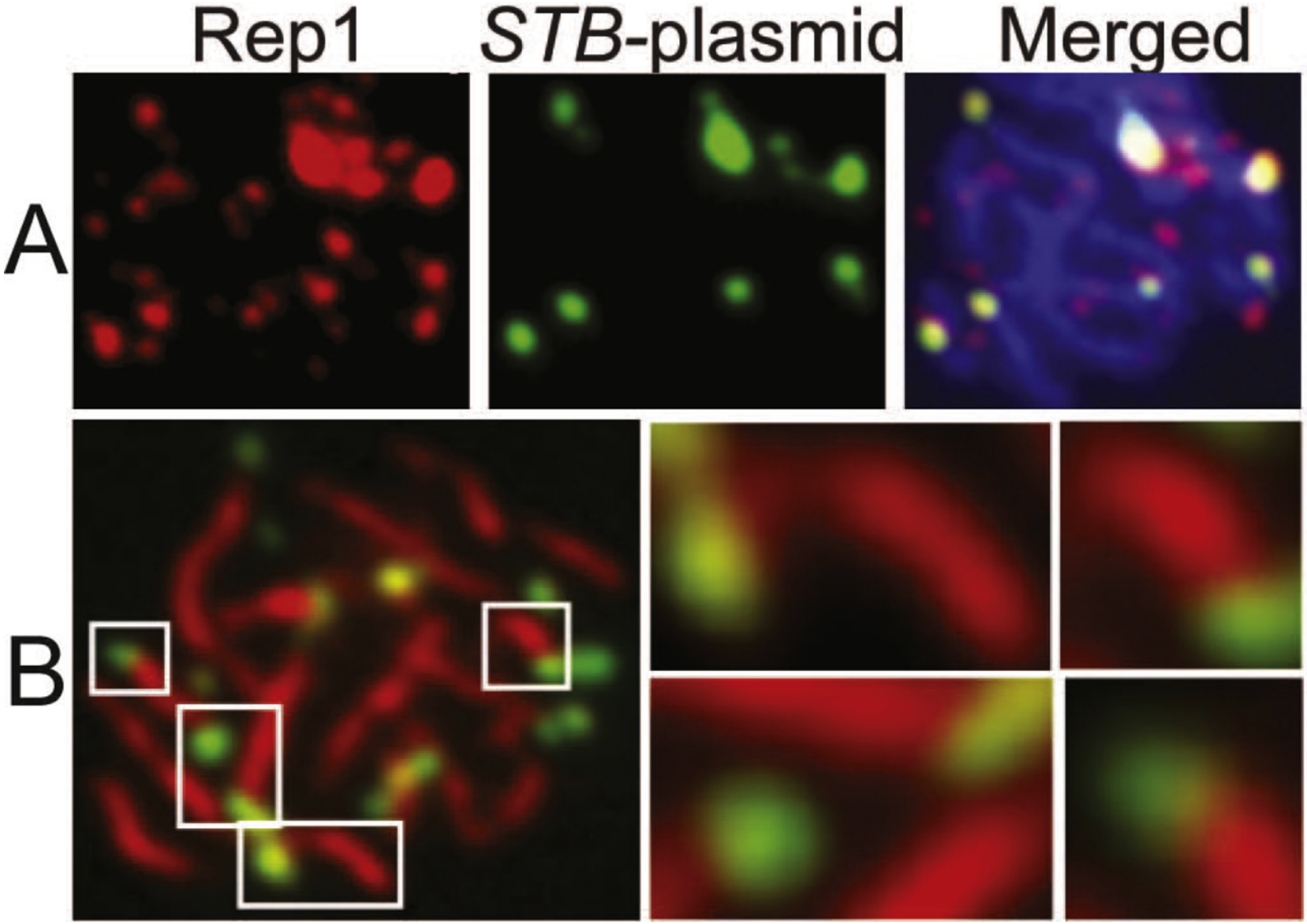

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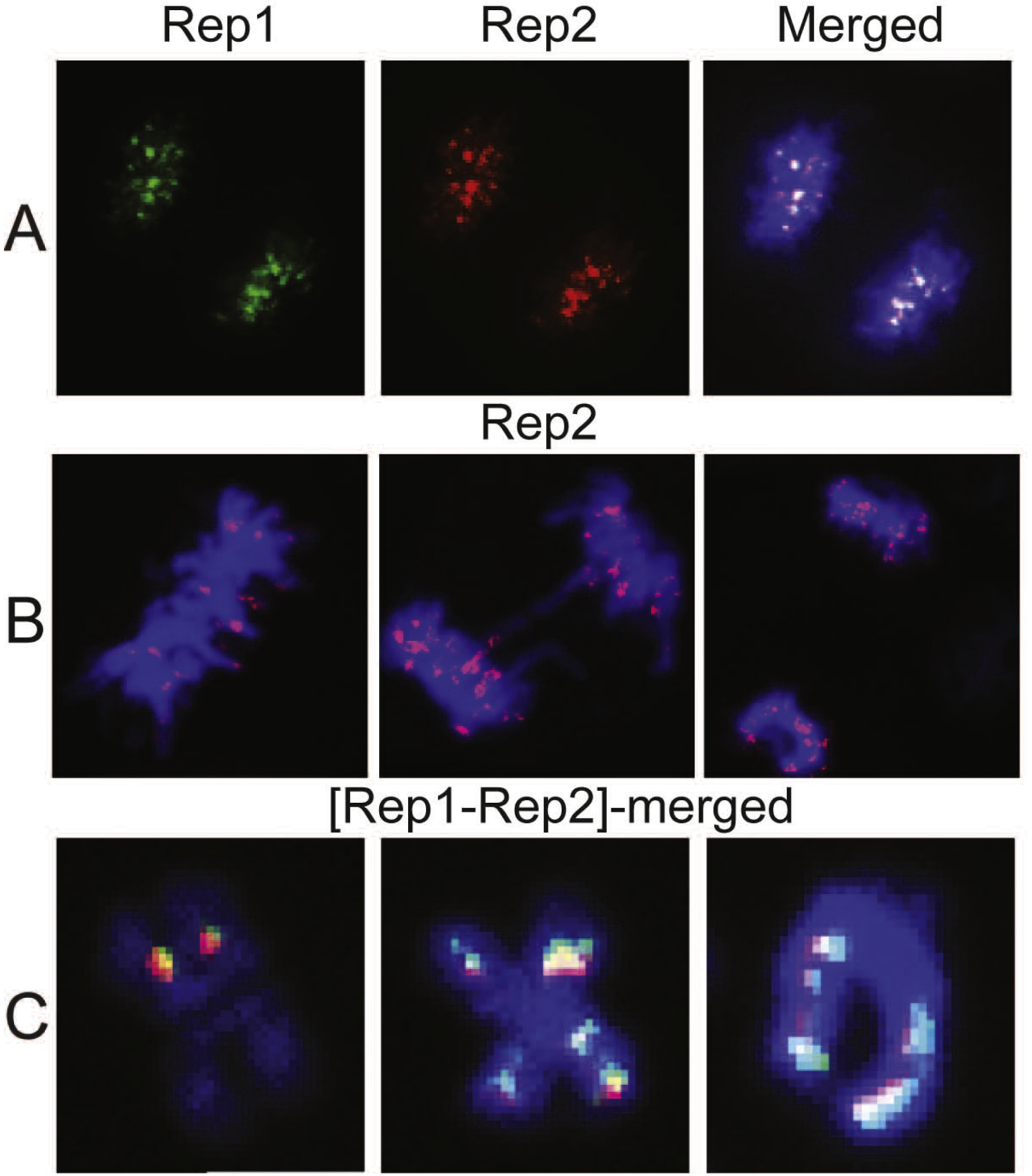

Figure 7

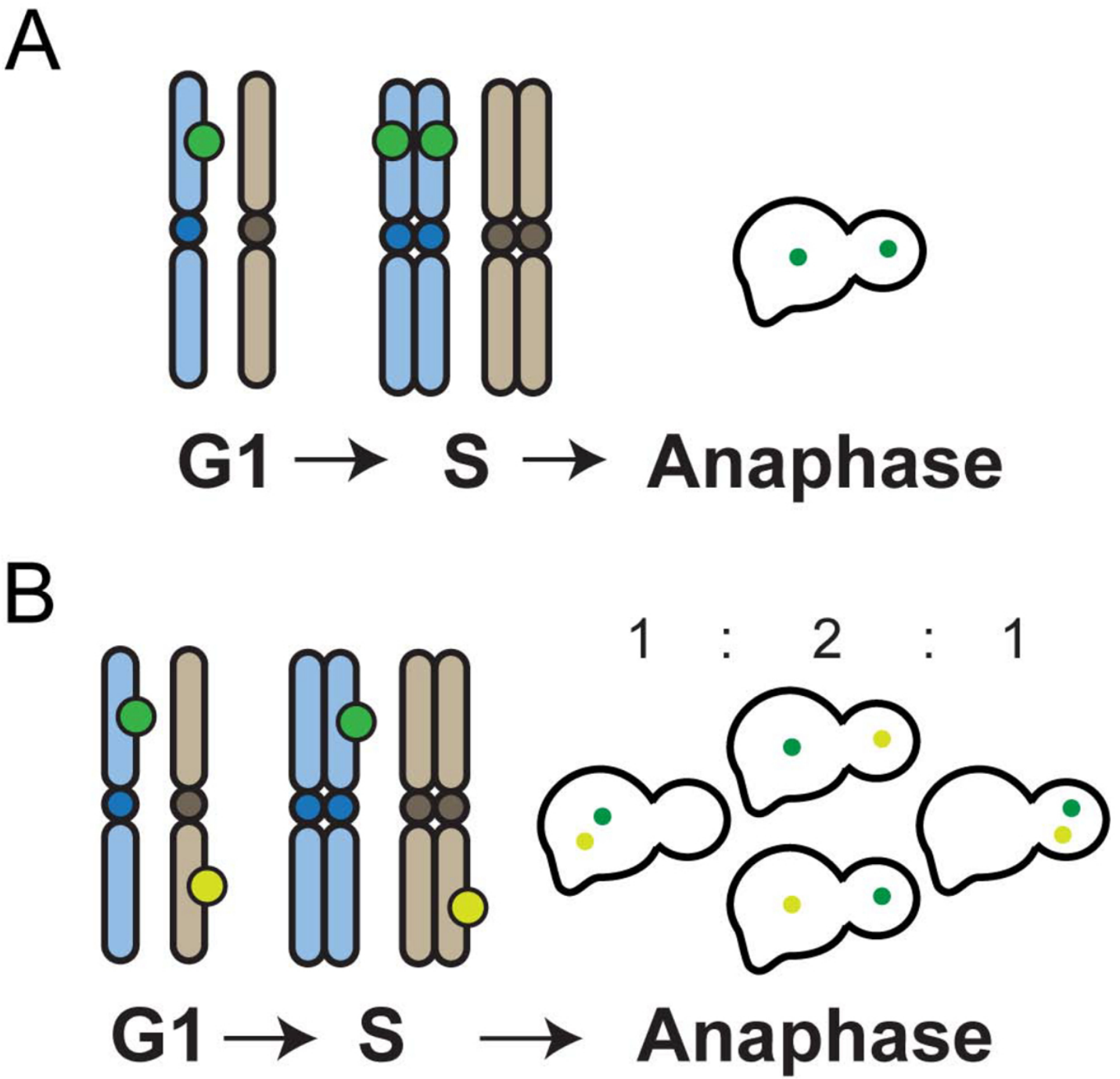

Figure 8

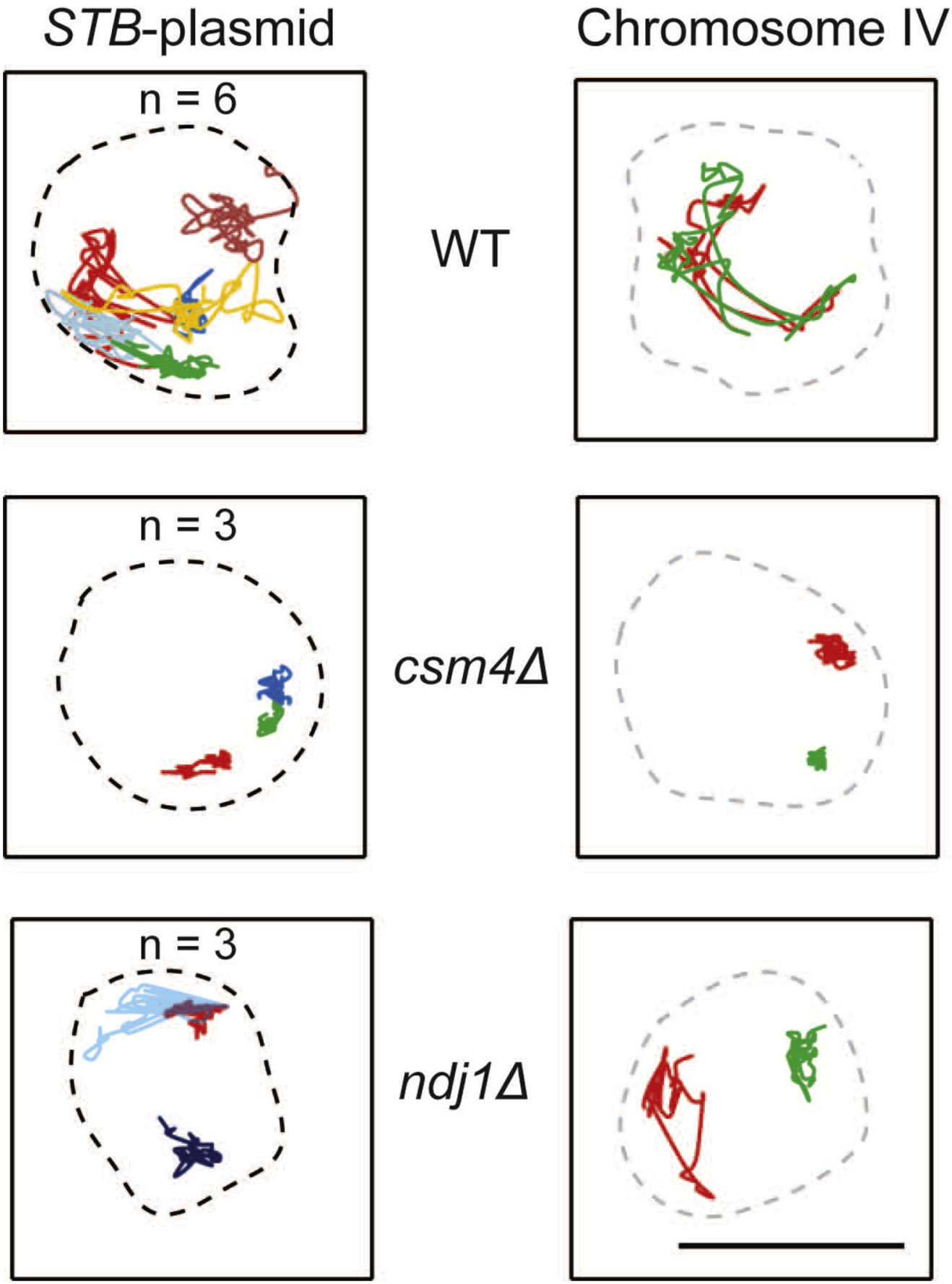

Figure 9