Co-segregation of yeast plasmid sisters under monopolin-directed mitosis suggests association of plasmid sisters with sister chromatids

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ABSTRACT

The 2-micron plasmid, a high copy extrachromosomal element in *Saccharomyces cerevisiae*, propagates itself with nearly the same stability as the chromosomes of its host. Plasmid stability is conferred by a partitioning system consisting of the plasmid-coded proteins Rep1 and Rep2 and a *cis*-acting locus *STB*. Circumstantial evidence suggests that the partitioning system couples plasmid segregation to chromosome segregation during mitosis. However, the coupling mechanism has not been elucidated. In order to probe into this question more incisively, we have characterized the segregation of a single-copy *STB* reporter plasmid by manipulating mitosis to force sister chromatids to co-segregate either without mother–daughter bias or with a finite daughter bias. We find that the *STB* plasmid sisters are tightly correlated to sister chromatids in the extents of co-segregation as well as the bias in co-segregation under these conditions. Furthermore, this correlation is abolished by delaying spindle organization or preventing cohesin assembly during a cell cycle. Normal segregation of the 2-micron plasmid has been shown to require spindle integrity and the cohesin complex. Our results are accommodated by a model in which spindle- and cohesin-dependent association of plasmid sisters with sister chromatids promotes their segregation by a hitchhiking mechanism.

INTRODUCTION

The precise molecular mechanisms underlying the highly efficient segregation of the 2-micron plasmid of *Saccharomyces cerevisiae* have remained elusive. The mitotic stability of the plasmid, which resides in the nucleus at a copy number of 40–60 molecules per cell, approaches that of the chromosomes of its host (1,2).

The partitioning system responsible for this remarkable stability consists of two plasmid-coded proteins Rep1 and Rep2 and a *cis*-acting locus *STB* (for plasmid stability). A decrease in copy number due to rare missegregation events is corrected by the Flp (plasmid flipping) site-specific recombination system of the plasmid by a DNA amplification mechanism (3,4). Positive and negative regulatory circuits acting on plasmid gene expression ensure a quick amplification response, when required, without the danger of runaway increase in plasmid copy number (5–7).

The 2-micron plasmid may best be viewed as a highly optimized selfish DNA element that confers no obvious selective advantage to its host and, at its normal copy number, poses no serious disadvantage either. In this regard, the plasmid resembles episomes of the papilloma and gammaherpes families of mammalian viruses during their long latent phase of infection. Latency is characterized by the maintenance replication of viral episomes followed by their efficient segregation to daughter cells with little adverse effect on normal cell functions (8–10). The basic mechanism for virus stability is the tethering of episomes to chromosomes by a viral protein that binds to a partitioning locus on the viral genome and to a host protein that binds to chromatin (11–13). Direct association of the viral partitioning protein to AT-rich regions of chromosomes through its AT-hook motifs has also been reported (14). In contrast to latency, the marked amplification of the virus during the reproductive phase is associated with cell cycle deregulation and checkpoint breakdown. In the case of a subset of 'high-risk' viruses, these events can lead to genetic instabilities and malignancies. In a rather analogous manner, an increased copy number of the 2-micron plasmid due to aberrant amplification results in cell cycle abnormalities and premature cell death in *S. cerevisiae* (15–17).

The 2-micron plasmid segregation during mitosis appears to be coupled to that of chromosomes. Mutations that conditionally missegregate chromosomes, *ipl1* and *ndc10*, for example, cause the plasmid to missegregate in tandem with the bulk of the chromosomes (18,19). So far, attempts to force chromosome

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missegregation while maintaining normal plasmid segregation have been unsuccessful, suggesting that the plasmid segregates by tethering to chromosomes or utilizes critical components of the chromosome segregation machinery for its own segregation. Furthermore, *STB* reporter plasmids with a copy number of one (or nearly one) segregate in a sister-to-sister fashion with high efficiency (20). In the tethering model, this would be equivalent to the association of sister plasmids with sister chromatids.

Chromosome segregation factors that play important functional roles at the centromere also associate with the *STB* locus and contribute to plasmid stability. They include components of the RSC2 chromatin remodeling complex, the spindle-associated Kip1 nuclear motor, the cohesin complex and the centromere-specific histone H3 variant Cse4 (18,21–25). Furthermore, the chromatin topology at *STB* and *CEN* in their functional states engenders non-standard positive DNA supercoiling, each locus contributing between +1 and +2 Lk (linking number) units (26,27). As chromosome segregation and plasmid segregation are coupled events, several of the aforementioned host factors may impact plasmid segregation indirectly through their effects on chromosome segregation. Based on circumstantial evidence, it has been suggested that the genetically defined point centromere of the budding yeast, which differs starkly from the epigenetically specified regional centromeres of most eukaryotes, is potentially a domesticated version of the partitioning locus of an ancestral 2-micron plasmid (28). Under this scenario, association of shared protein factors with *CEN* and *STB* might signify vestiges of their common ancestry prior to the divergence of extant chromosome and plasmid partitioning machineries.

The *S. cerevisiae* monopolin complex, consisting of Lrs4, Csm1, Mam1 and the protein kinase Hrr25, serves the critical function of clamping sister kinetochores together to ensure their co-segregation during meiosis I (29–32). An analogous function is served by the fission yeast Pcs1/Mde4 complex, which is the functional counterpart of the Csm1/Lrs4 subcomplex (33,34). Lrs4 and Csm1, but not Mam1 or Hrr25, also associate with kinetochores in response to the mitotic exit network (MEN) signaling pathway (35). Lrs4 and Csm1 are normally localized in the nucleolus and their release from this locale and association with kinetochores is dependent on the polokinase Cdc5 (36,37). Their distinct functions at the kinetochore during mitosis and meiosis I appear to be controlled by the difference in the timings of these associations, at the onset of anaphase in mitosis and during prophase in meiosis I as well as the absence of Mam1 during mitosis (32). However, by artificially assembling the MamI complex through the expression of *MAM1* and *CDC5* early during mitosis, sister chromosomes can be forced to missegregate to the same cell pole, as if they are going through meiosis I segregation (38).

We have now exploited monopolin-mediated co-segregation of sister chromatids during mitosis as a potential means to unveiling the basis for the chromosome-coupled segregation of the 2-micron plasmid. Chromosome missegregation caused by monopolin displays no bias between mother and daughter cells (38).

However, when the monopolin effect is combined with the *ipl1-321* mutation, the co-segregation of sisters becomes skewed toward the daughter (38,39). In contrast, an autonomously replicating yeast plasmid (*ARS* plasmid) or a single-copy DNA circle lacking a partitioning system, or an *STB* plasmid whose partitioning system is inactivated, has a strong tendency to be retained by the mother (40–42) (also results from this study). Fundamental mechanistic information on 2-micron plasmid segregation may thus be gleaned from how distortion of chromosome segregation in an unbiased or biased manner affects plasmid partitioning. Furthermore, if plasmid segregation is functionally correlated to chromosome segregation, such correlation should be lost in the absence of an active Rep–*STB* system. The extents and patterns of missegregation of a single-copy *STB* reporter plasmid under these different conditions are most easily explained by the tethering of plasmid sisters to sister chromatids. These findings have potential implications for the mechanisms involved in the meiotic segregation of the 2-micron plasmid as well.

MATERIALS AND METHODS

Strains and plasmids

Strains.

The genotypes of yeast strains used in this study are listed in Supplementary Table S1. In addition, the particular experiments that they were employed in as well as the figures depicting the relevant results are summarized.

Plasmids

A list of plasmids employed for this study follows the strain list in Supplementary Table S1. The multicopy 2-micron reporter plasmid pSV1 containing the $[LacO]_{256}$ array for fluorescence tagging by GFP-LacI has been described previously (19). The single-copy 2-micron reporter plasmid was derived from plasmid pKM1 described previously (27). Plasmid pKM1 contains the 2-micron circle replication origin and the *STB* locus flanked by two head-to-tail copies of the R recombinase target sites inserted into the commercial vector pRS403 (Stratagene) harboring the *HIS3* marker. The $[LacO]_{256}$ array, excised from pSV1 by SmaI plus Sbf1 digestion, was introduced into pKM1 between its Sbf1 and filled-in XbaI sites to generate pCM218. A derivative of pCM218 lacking the *STB* locus, constructed by digesting with SnaB1 and HpaI followed by self-ligation was named pTL29. The linearized forms of pCM218 and pTL29, obtained by restriction enzyme cutting within *HIS3*, were integrated separately at the *HIS3* chromosomal locus of the experimental strains. The excision products formed from the pCM218 and pTL29 integrants by R recombination provided the single-copy reporter plasmids *pSTB* and *pARS*, respectively.

Plasmid segregation assays

Single-copy reporter plasmid excised from the chromosome

The yeast strains containing the integrated forms of the single-copy reporter plasmids were grown in raffinose

media to an $OD_{600} = \sim0.2$ before arresting in G1 using α-factor. Following a 2- or 2.5-h treatment with α-factor, depending on the conditions of the assay, >90% of the cells showed the typical arrest phenotype. Cells were then shifted to 2% galactose in the presence of α-factor to induce the production of R recombinase. After 3 h in the presence of galactose, by which time the reporter plasmids were nearly quantitatively excised from their integrated states, cells were released into the cell cycle in glucose- or galactose-containing medium as desired. Fluorescence signals from the reporter plasmid were scored in anaphase/post-anaphase cells displaying two separated DAPI-staining zones in the mother and bud compartments (18,19).

Multicopy reporter plasmid

Yeast strains harboring the multicopy *STB* reporter plasmid, grown in selective media for plasmid maintenance to an $OD_{600} = \sim0.2$, were treated with α factor for 2 h to arrest >90% of the cells in G1. In assays where induction of *CDC5* and *MAM1* from the *GAL* promoter was required, the arrested cells were shifted to 2% galactose-containing medium with α-factor present for 1 h (38). After releasing cells from arrest in glucose- or galactose-containing medium as desired, plasmid distributions in the mother and bud compartments were scored in anaphase/post-anaphase cells.

Plasmid segregation during a cohesin-depleted cell cycle

In the experimental strain for the cohesin depletion assays, the native promoter of the gene for the kleisin subunit of cohesin (Mcd1/Scc1) was replaced by the methionine repressible promoter P_{MET}. During the 3-h period of galactose induction for excising the single-copy reporter plasmids in G1-arrested cells, 8 mM methionine was included in the medium. Furthermore, the medium in which the released cells resumed cell cycle also contained 8 mM methionine (38).

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) analyses were performed by following the protocols described previously (22) in a strain containing the native *MAM1* gene modified by 3HA epitope tagging. Metaphase cells were treated with 1% formaldehyde for 30 min at room temperature prior to DNA fragmentation and subsequent immunoprecipitation using antibodies to the HA epitope (Covance). Primer pairs employed to probe for *STB*, *CEN3* and chromosome arm sequences have been described previously (22,31). The linear range of amplification in the PCR reactions was standardized by serial dilutions of the template DNA. The template DNA in the 'input' reactions was diluted 1:200 with respect to the immunoprecipitated samples. The ChIP signal for a DNA locus was corrected by subtracting the corresponding signal from the mock-immunoprecipitated (no antibody) control. The corrected signal was normalized to that from the input reaction to obtain the ChIP efficiency of a locus.

Fluorescence microscopy

Observations were performed using an Olympus BX-60 microscope. Images captured at room temperature at ×100 magnification (oil NA 1.30 objective) using a Photometrics Quantix camera (Roper Scientific) were processed by MetaMorph (Universal Imaging Corporation) and PhotoShop CS (Adobe Systems, Inc.). Z-series sectioning of the yeast nucleus, deconvolution of the stacks and their 2D projections were performed as detailed previously (19,43).

In order to measure the fluorescence intensity of a plasmid focus, the signal intensities of individual pixels comprising that focus were measured from 2D projections using the Metamorph software. The maximum intensity, after subtracting the mean background intensity of a pixel obtained from neighboring pixels, was taken as the intensity of the plasmid focus.

DNA analysis by Southern blotting

Southern blotting was performed to assay the efficiency with which the single-copy reporter plasmids were excised from their integrated states. Approximately equal amounts of total yeast DNA were digested with diagnostic restriction enzymes (NcoI and HpaI) and were fractionated by electrophoresis in 1% agarose. After transferring the DNA 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).

Quantification of the degree of correlation between a reporter plasmid and a chromosome in segregation

The three parameters used for estimating correlations between a reporter plasmid and a reporter chromosome were the frequencies of equal (1:1) segregation (Ve), missegregation (2:0), biased toward mother (Vm) and missegregation (0:2), biased toward the daughter. In any given assay, the sum of Ve, Vm and Vd for a plasmid or chromosome would be 100%. We estimated ΔVe, ΔVm and ΔVd as the algebraic differences between a V-value for an experimental condition and the corresponding V-value for a reference condition. An increase or a decrease in V under a given condition was denoted by a + or – sign, respectively, for ΔV. As a change in equal segregation would be quantitatively balanced by a change in missegregation in the opposite direction, ΔVe + ΔVm + ΔVd = 0. We then normalized the ΔV-values to correct for the differences in the segregation frequencies for a reporter plasmid and a reporter chromosome during normal mitosis. Note that the values of Ve for chromosome IV, the *STB* reporter plasmid and the *ARS* plasmid during normal mitosis were 100, 63.7 and 23.4%, respectively (Figure 2). The normalized ΔVs = $\Delta V'$s, obtained by dividing ΔV-values by the corresponding Ve-values, were employed in deriving the relevant correlation coefficients.
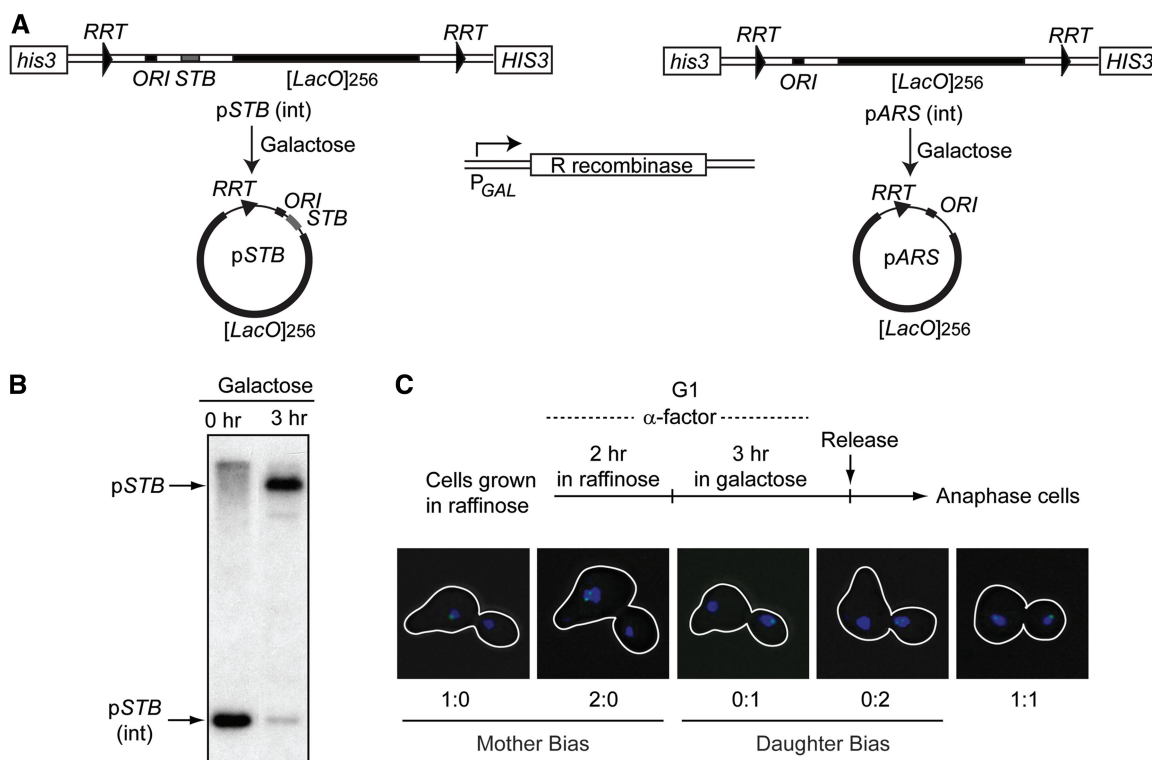
Figure 1. (**A**) Generation of single-copy reporter plasmids and analysis of the segregation of sister plasmids. The experimental set up for obtaining precisely one copy of the reporter plasmid, p*STB* or p*ARS*, by R recombinase-mediated excision from chromosome XV is schematically diagramed. (**B**) A 3 h induction of the R recombinase resulted in nearly complete excision of p*STB*. (**C**) Plasmid excision was performed in G1-arrested cells and plasmid segregation was assayed at the anaphase stage in cells released from G1 arrest. In the ratios depicting plasmid segregation, the number at the left refers to the mother; that at the right refers to the daughter.

Other protocols

Treatment of cells with nocodazole for G2/M arrest and subsequent release were done according to previously published procedures (19,24). Yeast and bacterial transformations, yeast DNA and plasmid DNA preparation, curing of the 2-micron plasmid from [Cir⁺] strains, culturing of yeast and bacteria and other general procedures were performed by following protocols listed on the Jayaram laboratory web page (http://www.sbs.utexas.edu/jayaram/jayaramlab_files/Protocols.htm).

RESULTS

Single-copy fluorescence-tagged *STB* and *ARS* reporter plasmid systems for assaying the effects of monopolin on sister plasmid segregation

As pointed out earlier, previous analyses suggest that sister copies of the 2-micron plasmid segregate from each other in a one-to-one fashion (20). Perhaps, the cohesin complex, which associates with *STB* (18,44), is involved in distinguishing plasmid sisters from plasmid 'homologs'. Such a role for cohesin would be analogous to the critical function it serves during chromosome segregation. The central strategy in the following analyses is to characterize the behavior of a pair of sister plasmids *vis a vis* that of a pair of sister chromatids during normal mitosis and a deviant 'meiosis I-like' version of mitosis

induced by monopolin, when sister chromatids missegregate to the same cell pole.

The general experimental designs to generate precisely two sister copies of a reporter plasmid and to establish monopolin-directed chromosome segregation are schematically illustrated in Figure 1 and Supplementary Figure S1. The two reporter plasmids employed (Figure 1A), containing the 2-micron circle replication origin and a [LacO]$_{256}$ array, were excised from their chromosomally integrated states during G1 by expressing the R recombinase from *Zygosaccharomyces rouxii* (45) (Figure 1B). They differed from each other in that, one contained the *STB* sequence (referred to as the *STB* reporter plasmid) and the other lacked this sequence (referred to as the *ARS* reporter plasmid).

The individual reporters were placed in strains containing the native 2-micron plasmid (serving as a source of the Rep1 and Rep2 proteins; [Cir⁺]) or lacking it ([Cir⁰]; no supply of Rep1 and Rep2). GFP-LacI expressed from the *HIS3* promoter in the host strains conferred green fluorescence on the reporter plasmids that they harbored. The positive control for segregation of sisters during normal mitosis and co-segregation of sisters during mitosis contrived by monopolin was provided by a strain in which chromosome IV (or chromosome V) harbored a [TetO]$_{224}$ array proximal to the centromere and TetR-GFP was expressed from the *URA3* promoter (38).

The patterns of a reporter plasmid or chromosome were assayed in anaphase cells for equal segregation (1:1) and
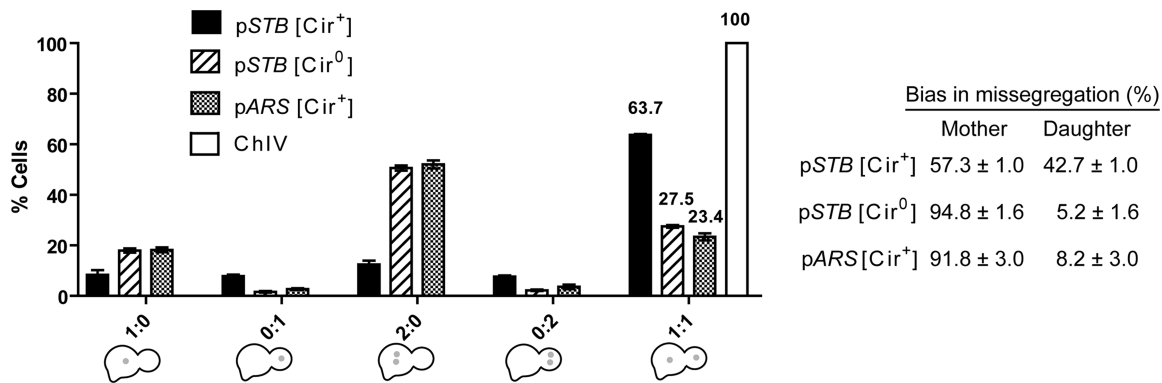
Figure 2. Plasmid and chromosome segregation patterns during normal mitosis. Plasmid and chromosome segregation patterns were analyzed in anaphase cells by counting the number of fluorescent foci in the well-separated daughter nuclei. Results represented by individual histograms were obtained from scoring 100–200 cells in each case. In this figure and Figures 3–6, panels (**A–C**) represent the *STB* reporter plasmid, the reporter chromosome and the *ARS* reporter plasmid, respectively. Similarly, the table in panel (**D**) summarizes the mother or daughter bias within the populations showing chromosome or plasmid missegregation. In A–C, the histograms are plotted as the mean ± SEM. (standard error of mean). (**D**) The values are expressed as the mean with the indicated 95% confidence interval.

unequal segregation (2:0) (Figure 1C). In order to indicate the mother–daughter bias during plasmid missegregation, we have assigned the number at the left in these ratios to the mother and that at the right to the daughter. Thus, 2:0 denotes missegregation biased toward the mother, and 0:2 denotes that biased toward the daughter.

We noticed a small but significant fraction of anaphase cells that revealed only a single fluorescent plasmid focus. In principle, this could represent the segregation of an unreplicated plasmid or a missegregated pair of sisters that happened to be co-localized. Fluorescence intensity estimations revealed that a single focus from this unexpected subset of anaphase cells was almost always twice (>1.7-fold) as intense as a single focus from G1 cells (Supplementary Figure S2). We conclude that the 1:0 and 0:1 patterns denote missegregation of plasmid sisters rather than a failure of plasmid replication.

For the *STB* reporter plasmid, equal segregation during normal mitosis (absence of monopolin) was 63.7% in the presence of the Rep proteins [Cir$^+$] and 27.5% in their absence [Cir^0] (Figure 2). The *ARS* reporter plasmid followed the 1:1 pattern in 23.4% of the [Cir$^+$] cells and was unaffected by the absence of the Rep proteins [Cir^0] (data not shown). Chromosome IV, as expected, segregated 1:1 in every case. The missegregation of the *ARS* reporter plasmid was biased strongly toward the mother (91.8%), and so was that of the *STB* reporter plasmid in the [Cir^0] host strain (94.8%). These values provide a reference frame for the interpretation of the segregation results obtained in cells undergoing mitosis in the presence of monopolin.
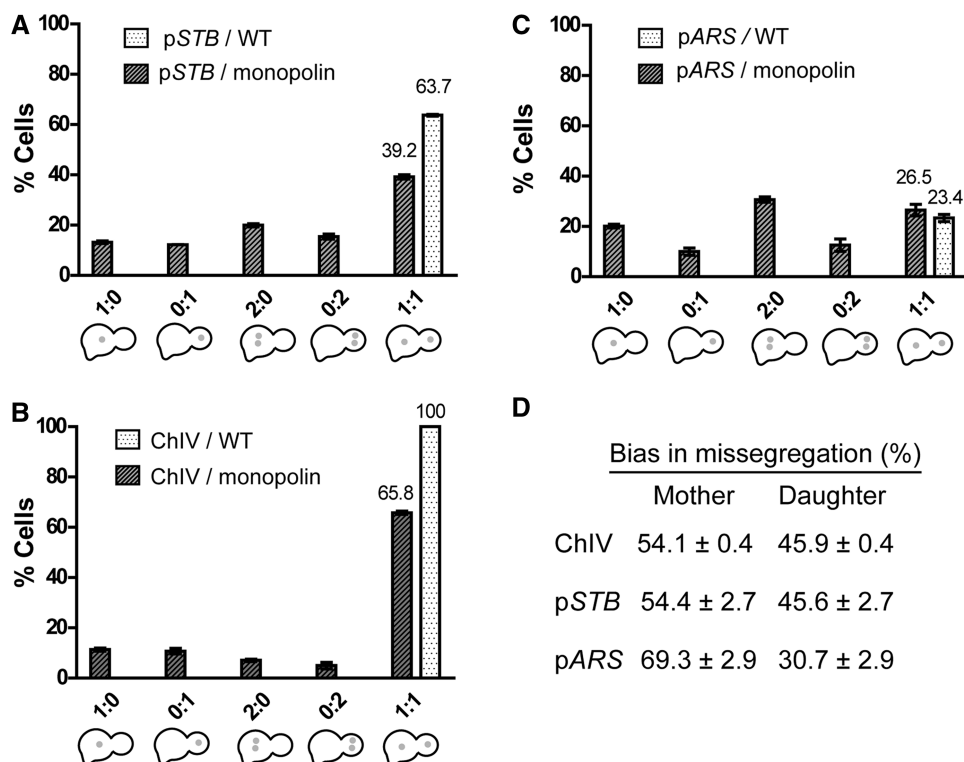
Expression of the monopolin complex during mitosis causes missegregation of the 2-micron plasmid

In order to accomplish monopolin assembly at kinetochores during mitosis, *CDC5* and *MAM1* genes were placed under the control of the *GAL* promoter, *MAM1* at its native genomic locale and *CDC5* as an extra copy integrated at the *URA3* locus (Supplementary Figure S1). These genes, along with the R recombinase gene, were expressed in G1-arrested cells by galactose induction prior to their release into the cell cycle in galactose containing medium. Although Cdc5 can mediate the early release of Lrs4 and Csm1 from the nucleolus, they are recruited to the kinetochore along with Mam1 in an interdependent manner (31,37). The mitotic cell cycle is not perturbed significantly by monopolin, except for a brief delay in the degradation of the anaphase inhibitor securin due, presumably, to a transient activation of the spindle checkpoint (38). All of the assays for probing the effect of monopolin on plasmid segregation were carried out in [Cir$^+$] host strains containing the excised single-copy *STB* and *ARS* plasmids as reporters or chromosome IV as the reference reporter.

During mitosis in the presence of monopolin, equal segregation of the *STB* reporter plasmid dropped to 39.2% (Figure 3A) (from 63.7% in the absence of monopolin; Figure 2) and that of chromosome IV to 65.8% (Figure 3B) (from 100% in the absence of monopolin; Figure 2). The relative decrease in equal segregation, normalized to mitosis without monopolin expression was 38.5% for the *STB* plasmid, nearly identical to that of chromosome IV (34.2%). In contrast, monopolin did not make a difference in the equal segregation frequency of the *ARS* reporter plasmid (Figure 3C). The frequency of monopolin-induced chromosome missegregation observed in our assays agreed well with that reported previously (38).

The missegregation of the *STB* plasmid sisters as a consequence of monopolin expression showed only a very small mother bias (54.4%; Figure 3D). This near lack of bias was strikingly different from the strong mother bias (94.8%; Figure 2) for missegregation in a [Cir^0] strain. Plasmid missegregation due to a lack of the Rep proteins and that due to monopolin are therefore mechanistically distinct. The *ARS* plasmid sisters missegregated with a clear-cut mother bias (69.3%; Figure 3D), although the magnitude of the bias was smaller than that observed during normal mitosis. Reasons for this modulation in segregation bias are not known. In agreement with a

Figure 3. Segregation of reporter plasmids and chromosome IV during a mitotic cell cycle with the assembly of the monopolin complex. The scheme for the assembly of the monopolin complex by inducing the expression of *MAM1* and *CDC5* is schematically outlined in Supplementary Figure S1. All segregation assays were performed in [cir$^+$] strains. The segregation results and the mother or daughter bias during missegregation are displayed as under Figure 2. Each segregation value is derived from 100–200 cells.

previous report (38), chromosome IV missegregation due to monopolin was essentially unbiased (54.1%; Figure 3D) in our assays as well.

Taken together, our data suggest that the tendency of a pair of *STB* sister plasmids and that of a pair of sister chromatids to co-segregate under the influence of monopolin without mother–daughter bias are well correlated.

Co-segregation of *STB* sister plasmids in the presence of monopolin plus *SPO13*

The *SPO13* gene, expressed exclusively during meiosis (46), is required for the maintenance of centromeric cohesion and kinetochore co-orientation during meiosis I and for progression through meiosis II without an additional round of DNA synthesis (47,48). Overexpression of *SPO13* during meiosis and mitosis causes a transient delay in securin destruction and leads to metaphase cell cycle arrest by inhibiting the cleavage of the Rec8 and Mcd1 subunits of cohesin, respectively (49,50). When *SPO13* is overexpressed along with *CDC5* and *MAM1* during mitosis, no cell cycle arrest occurs; however, sister chromatid missegregation is elevated dramatically (38). This mitotic effect of Spo13 appears not to be mediated through the monopolin complex, but rather by interfering with kinetochore function and/or cohesin disassembly at centromeres (38). Nevertheless, the strikingly high incidence of sister chromatid co-orientation brought about

by the two together provides the opportunity to test whether the co-segregation of the *STB* plasmid sisters is also elevated by their combined action. We induced the expression of *SPO13*, controlled by the *GAL* promoter, along with that of *CDC5* and *MAM1* in G1-arrested cells, released them in presence of galactose and scored plasmid segregation in anaphase cells.

The 1:1 segregation of the *STB* plasmid sisters was reduced to 17.8% under monopolin plus Spo13 (Figure 4A), an additional drop of 21.4% from that observed under monopolin alone (39.2%; Figure 3A). Chromosome IV segregated 1:1 in only 2.2% of the cells during the monopolin plus Spo13 mitosis (Figure 4B), signifying a 63.6% decrease from that conferred by monopolin alone (Figure 3B). When normalized to the data from regular mitosis, monopolin, together with Spo13, decreased the equal segregation frequency of the *STB* plasmid by 72.1% and that of chromosome IV by 97.8%. Unlike monopolin alone, monopolin plus Spo13 caused a decrease in the 1:1 segregation of the *ARS* plasmid sisters as well (from 23.4 to 11%; Figure 4C). However, in contrast to the *STB* plasmid and chromosome IV, whose missegregation showed little or no bias (56.8% for the *STB* plasmid; 51.7% for chromosome IV; Figure 4D), the missegregation of the *ARS* plasmid was still strongly biased toward the mother (82.2%; Figure 4D).

The co-segregation patterns of sister *STB* plasmids and sister chromatids due to monopolin plus Spo13 are consistent with those due to monopolin alone. Although the
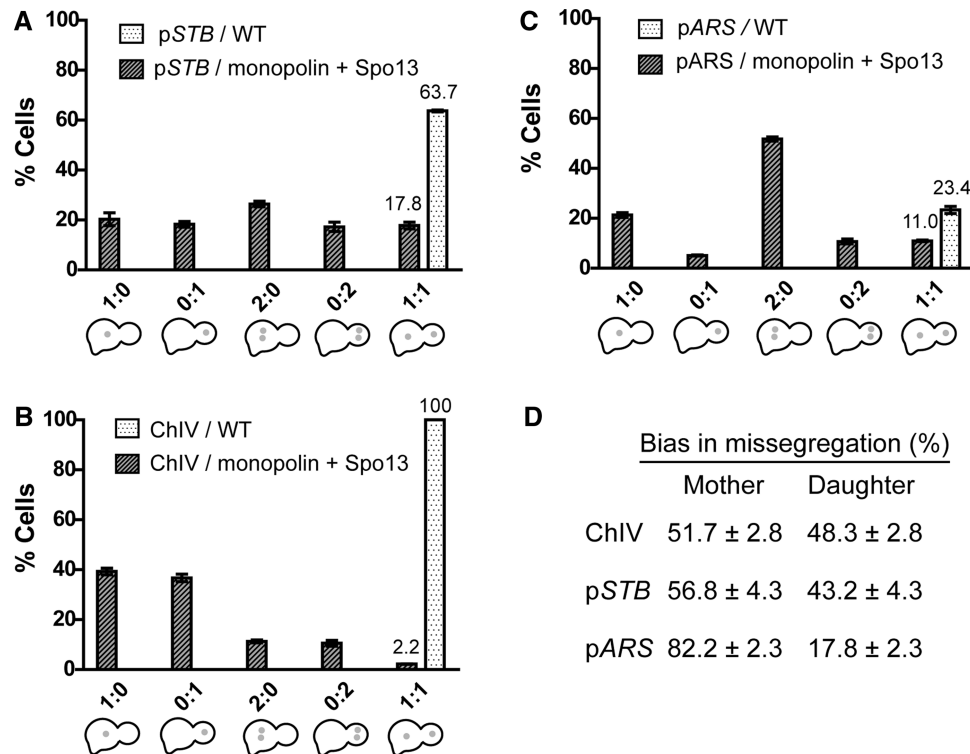
Figure 4. Mitotic segregation of reporter plasmids and chromosome IV under the influence of monopolin plus Spo13. The only difference in the experimental protocol from that described under Figure 3 is that *SPO13*, *CDC5* and *MAM1* were co-induced in the G1-arrested cells prior to their release.

additional effect of Spo13 was more pronounced for sister chromatids than the plasmid, they both missegregated without bias.

The combined effects of monopolin and *ipl1-321* on plasmid segregation

Ipl1 (Aurora B kinase), in association with Sli15 (INCENP) and Bir1 (Survivin), regulates several aspects of chromosome segregation during mitosis and meiosis in *S. cerevisiae* (51–53). These include kinetochore microtubule attachment and orientation (38,39), spindle assembly and stability (54) and coordination of chromosome segregation and cytokinesis (55). In the absence of Ipl1 function during the mitotic cell cycle, chromosomes missegregate because co-oriented sister kinetochores fail to detach from the spindle and establish bi-orientation. Furthermore, these kinetochores preferentially attach to microtubules emanating from the old spindle body, which migrates to the bud compartment (39). As a result, sister chromatid missegregation caused by the lack of Ipl1 function has a distinct daughter bias (Supplementary Figure S3). If the *STB* plasmid does hitchhike on chromosomes, the daughter bias imparted by the absence of Ipl1 on chromosomes should also apply to the plasmid. We tested this prediction by following plasmid segregation in *ipl1-321* (T^s) mutant cells going through mitosis at the semi-permissive temperature (34°C) in presence (Figure 5) and absence (Supplementary Figure S3) of the monopolin complex.

The relative decrease in the 1:1 segregation frequency due to monopolin plus the *ipl1-321* mutation was similar between the *STB* plasmid (58.7%) (Figure 5A) and

chromosome IV (51.3%) (Figure 5B) and so was the daughter bias in missegregation (64.0% for chromosome IV and 60.0% for the *STB* plasmid) (Figure 5D). The *ARS* plasmid also showed an increase in missegregation under these conditions (Figure 5C). However, unlike the *STB* plasmid, the direction of the bias was opposite to that of chromosome IV (65.8% toward mother; Figure 5D). Similar values for the decrease in equal segregation frequencies (54.1 and 54% for chromosome IV and the *STB* plasmid, respectively) and segregation bias toward the daughter (61% for chromosome IV; 58.2% for the *STB* plasmid) were observed due to the *ipl1*-321 mutation even without the induction of *CDC5* and *MAM1* (Supplementary Figure S3). The more dominant effect of *ipl1-321* over the Mam1 complex with respect to the magnitude of chromosome missegregation and the direction of its bias were also observed previously (38).

Thus, the quantitative effects of the *ipl1-321* mutation (with or without monopolin) on *STB* plasmid missegregation are analogous, though qualitatively distinct with respect to bias, to those of monopolin or monopolin plus Spo13. They further highlight the correlation between the *STB* plasmid and a chromosome, and the lack of it between an *ARS* plasmid and a chromosome when sister chromatids are forced to missegregate.

Effect of microtubule depolymerization on the monopolin-induced missegregation of the *STB* reporter plasmid

The mitotic spindle and the spindle-associated Kip1 motor promote equal segregation of the 2-micron plasmid,
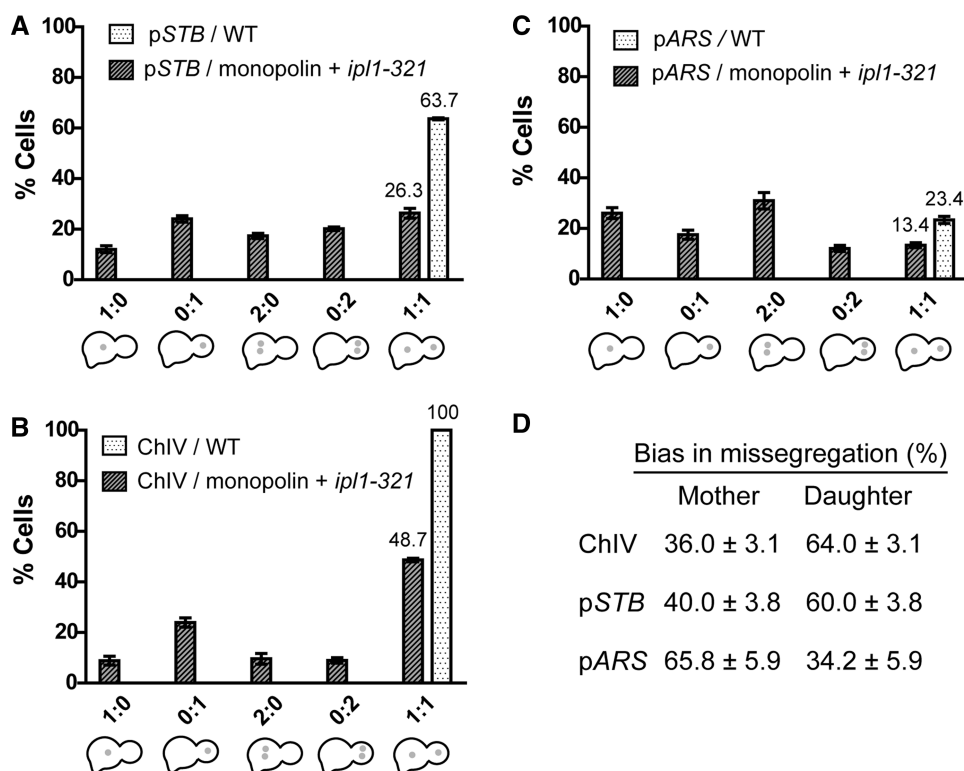
Figure 5. Segregation of reporter plasmids and chromosome IV under monopolin assembly combined with partial inactivation of Ipl1. The analysis was performed at 34°C, which is semipermissive for Ipl1 function.

although the mechanism remains unclear (21,43). Spindle disassembly disrupts plasmid association with chromosome spreads and renders the normally substoichiometric interaction of cohesin and Cse4 with *STB* undetectable (22,43). When cells treated with nocodazole and arrested in G2/M are allowed to reassemble the spindle and resume the cell cycle, chromosomes segregate normally. However, a multi-copy *STB* reporter plasmid missegregates (43). We wished to test the prediction then that spindle disassembly and reassembly would impact sister chromatid segregation and *STB* plasmid segregation distinctly during mitosis in the presence of monopolin.

During a cell cycle that progressed up to G2/M in the absence of the spindle and then continued with a restored spindle, the *STB* sister plasmids showed 39% equal segregation (Figure 6A), which is low but somewhat better than the 27.5% observed in the absence of Rep1 and Rep2 (Figure 2). Unlike the strong mother bias in segregation induced by the lack of the Rep-*STB* system, the mother bias was absent or quite weak (53.1%) under the spindle disassembly and reassembly regimen (Figure 6D). Clearly, the mechanisms by which the *STB* plasmid is uncoupled from chromosomes due to the absence of the partitioning system or due to the pre-G2/ M absence of the spindle are not the same. Spindle restoration in G2/M supported 1:1 segregation of sister chromatids (Figure 6B). Manipulation of spindle integrity did not affect the segregation of the *ARS* reporter plasmid (Figure 6C) or its pronounced mother bias (90.7%; Figure 6D).

When monopolin expression was superposed on spindle disassembly–reassembly, the co-segregation of chromosome IV increased dramatically. The relative decrease in 1:1 segregation was 72.4% (Figure 6B). The reason why the monopolin effect is exacerbated by delaying spindle assembly until G2/M is not clear. Perhaps, the extra time increases the fraction of sister kinetochores stably clamped by monopolin before they are captured by the spindle, facilitating their co-orientation. The significant result is that, in comparison with chromosome IV, the *STB* plasmid showed only a modest increase in co-segregation of sisters by monopolin when a functional spindle was absent until G2/M. The relative reduction in 1:1 plasmid segregation was 32.1%.

Thus, the correlation between sister chromatids and *STB* plasmid sisters in their monopolin-imposed co-segregation, is strongly diminished when the spindle contribution toward plasmid segregation (but not chromosome segregation) is ablated.

Segregation of chromosome V sisters and *STB* plasmid sisters during cohesin-depleted or cohesin-depleted but monopolin-supplemented mitosis

The bridging of sister chromatids by the cohesin complex during S phase, the biorientation of paired sister kinetochores on the mitotic spindle and the disassembly of cohesin during anaphase by cleavage of the cohesin subunit Mcd1 provide the basis for the 1: 1 segregation of sister chromatids (56,57). The cohesin complex also associates with *STB*, with similar timings of assembly and
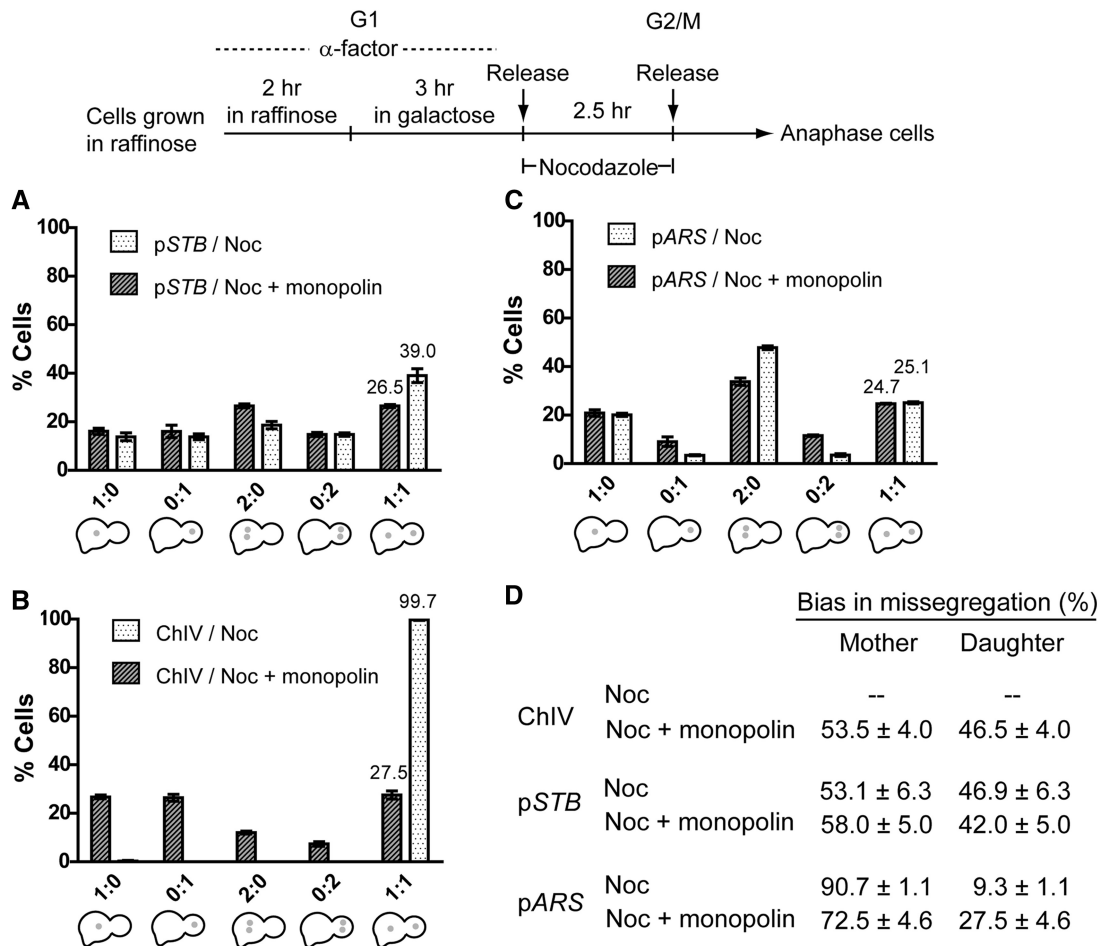
Figure 6. Effects of delaying spindle assembly or delaying spindle assembly in the presence of monopolin on the segregation of reporter plasmids and chromosome IV. The experimental scheme is schematically outlined. The cells were released from G1 in the presence of nocodazole so as to implement cell cycle progression till G2/M without the mitotic spindle. Removal of nocodazole resulted in restoration of the spindle and resumption of the cell cycle.

disassembly as those at chromosomal loci (18,23). Even though cohesin–*STB* association appears to be substoichiometric (44), it is apparently required for the chromosome-coupled segregation of the 2-micron plasmid. If this is true, the lack of cohesin assembly during a cell cycle should dissociate the segregation of *STB* plasmid sisters from sister chromatid co-segregation promoted by monopolin. The test of this prediction is summarized below. The reference chromosome in these assays was chromosome V and not IV as in previous experiemnts.

In order to deplete cohesin during the mitotic cell cycle, cells from the experimental strain expressing the *MCD1* gene from the *MET* promoter were arrested in G1, conditioned with methionine and released into medium supplemented with methionine (Supplementary Figure S4A). Under these conditions, chromosome V showed 65.4% 1:1 segregation (Figure 7A). In the absence of cohesin, but with monopolin expressed, the 1:1 segregation of chromosome V was further decreased to 49% (Figure 7A) or an additional relative drop of 25.1% imposed by monopolin. The 1:1 segregation frequencies of the *STB* plasmid sisters were almost identical (36.2 and 36.6%) in the absence of

cohesin and in the absence of cohesin but in the presence of monopolin, respectively (Figure 7B). The corresponding values for the *ARS* plasmid were 26.8 and 26.2%, respectively (Figure 7C).

The correlated co-segregation of *STB* plasmid sisters and a pair of sister chromatids that occurs during mitosis in the presence of monopolin is terminated if this mitosis is also cohesin deprived. The missegregation of sister chromatids carrying a centromere proximal fluorescence tag in the absence of cohesin is frequently signified by two separated fluorescent dots; however, superposition of monopolin upon cohesin depletion raises the incidence of coalesced single fluorescent dots (38). Based on this distinction, it is argued that monopolin functions independently of cohesin to promote sister chromatid co-segregation. In this case, uncoupling of plasmid segregation, due to lack of cohesin, from monopolin-induced sister chromatid co-segregation can only occur through cohesin's effect on the plasmid (see also 'Discussion' section). Note that the results assembled in Figure 3 have demonstrated that monopolin has very similar effects on sister chromatids and *STB* plasmid sisters.
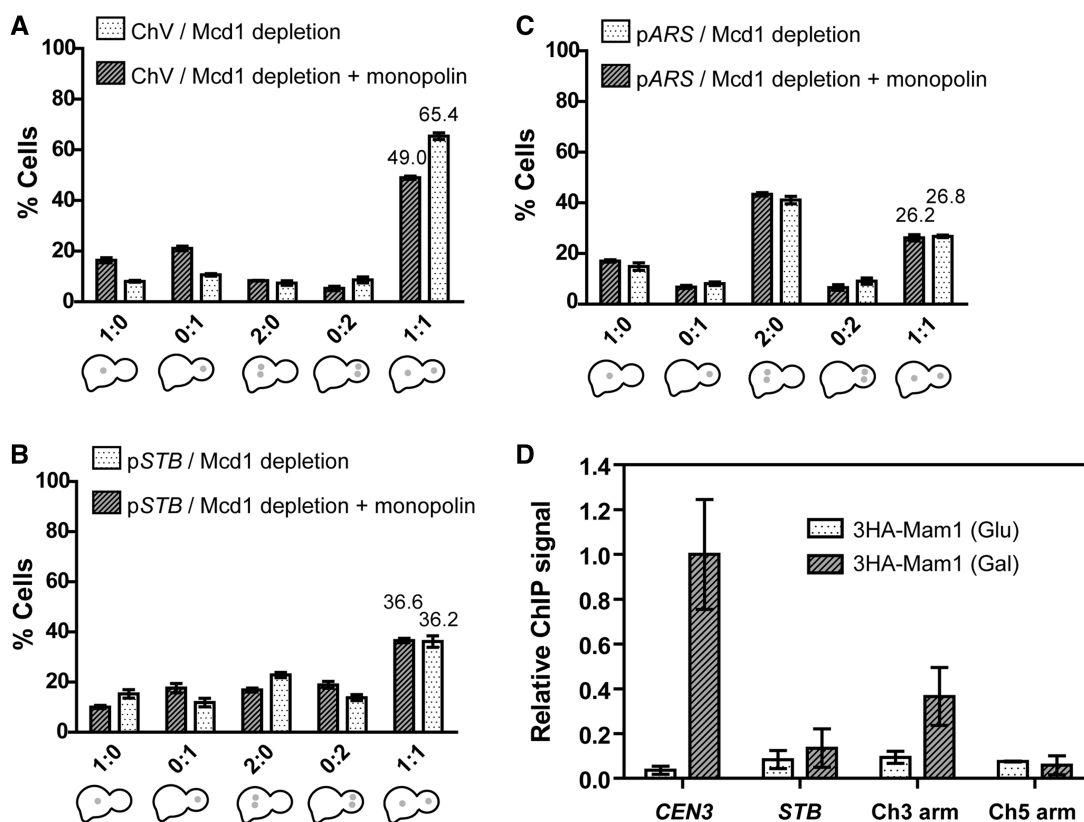
Figure 7. Effects of cohesin depletion or cohesin depletion and monopolin assembly on reporter plasmid and chromosome segregation; lack of monopolin interaction with *STB*. Cohesin was depleted during the cell cycle by turning off the *MET* promoter from which *MCD1*-18Myc was expressed (Supplementary Figure S4). In this set of experiments, the reference chromosome was V (and not IV utilized in all the other analyses). This change was dictated by the markers available in the parental strains used to generate the requisite haploid strain. This chromosome was also fluorescencetagged near the centromere by TetR-GFP and [TetO]$_{224}$. The segregation data are depicted in (**A–C**). The associations of Mam1, tagged by 3-HA, with *CEN3* and *STB* was probed by ChIP using an antibody to the HA epitope (**D**). *MAM1*, placed under the control of the *GAL* promoter, was not expressed in glucose-grown cells. Two loci from chromosomes III and V were utilized as controls. The relative ChIP signals plotted here are based on the intensities of the PCR-amplified DNA bands after normalizing them to the input signals (Supplementary Figure S4). The *CEN3* signal was assigned a value of 1.

The effect of monopolin on *STB* plasmid sisters in promoting their co-segregation with sister chromatids is indirect

Mam1 associates with kinetochores, as part of the monopolin complex, from late prophase I to the onset of anaphase I during meiosis (31,32). However, Mam1 is not expressed during mitosis, and its partners Csm1 and Lrs4 are sequestered in the nucleolus until G2. When *MAM1* and *CDC5* are overexpressed early during mitosis, Csm1 and Lrs4 are released prematurely into the nucleus. In association with Mam1, they are then assembled into monopolin, which is recruited to kineto-chores (38). As several centromere-associated proteins also interact with *STB*, Kip1 and cohesin, for example, it is formally possible that monopolin affects *STB* plasmid segregation directly rather than through chromo-some segregation. For example, analogous to clamping down sister kinetochores (29), monopolin might also hand-cuff sister *STB*s by interacting with the plasmid par-titioning complex. Under this scenario, the mitotic co-segregation of sister *STB* plasmids in presence of monopolin need not necessarily be dependent on sister

chromatid co-segregation. In order to address the possi-bility of direct monopolin action on the plasmid, we queried the presence of Mam1 at *STB* by ChIP.

An antibody to HA-tagged Mam1 immunoprecipitated centromere DNA from cells induced for the expression of 3HA-*MAM1* and *CDC5* from the *GAL* promoter (Figure 7D and Supplementary Figure S4B). No *CEN* DNA was detectable in the immunoprecipitate from uninduced cells. The signals for *STB* were within the range of those for two-arm loci from chromosomes III and V, with no significant difference between cells induced or uninduced for *MAM1* expression (Figure 7D and Supplementary Figure S4B). We conclude that Mam1 and monopolin by inference, is not recruited at *STB*.

As far as we know, the action of monopolin is restricted to centromeres, where it promotes the co-orientation of sister kinetochores and, consequently, co-segregation of sister chromatids. Monopolin brings about a similar effect on *STB* sister plasmid segregation, apparently vicari-ously. This seeming anomaly is resolved if the plasmid sisters are tethered to a pair of sister chromatids whose kinetochores are conjoined by monopolin, in a one-to-one fashion.

A quantitative summary of the coupled segregation of the *STB* reporter plasmid and chromosomes

The general picture that emerges from the cumulative data presented in Figures 2–6 and Supplementary Figure S3 is the strong coupling in segregation between the *STB* reporter plasmid and chromosomes as well as their uncoupling under certain conditions such as the absence of cohesin assembly or delayed spindle organization during a mitotic cell cycle. We have further analyzed these data to appraise more critically their quantitative significance.

The segregation behavior of a reporter plasmid or a chromosome could be described by three variables, Ve, Vm and Vd, denoting equal segregation, missegregation biased toward the mother and missegregation biased toward the daughter, respectively. In expressing the quantitative correlations between a plasmid and a chromosome, the normalized differences in their respective V-values (ΔV's) between a given experimental condition (say, mitosis with monopolin assembly) and the reference condition (normal mitosis) were employed (see 'Materials and Methods' section). This normalization compensates for the differences between the reporter plasmids and the reporter chromosomes in their segregation patterns during normal mitosis.

In the radar plots in Figure 8A and B, there was nearly perfect overlap between the *STB* plasmid (blue) and chromosome (green), whereas the *ARS* plasmid (red) was clearly distinct. When the data set from Figure 8A and B was reorganized as an *X–Y* scatter plot (Figure 8C), the strong positive correlation between the *STB* plasmid and chromosome IV was signified by $r = 0.969$ ($r^2 = 0.939$). The slope of the linear regression line was 0.885. The biological meaning of this slope is that within the 63.7% *STB* plasmid population that segregated equally during normal mitosis (against which the ΔV-values were normalized), 88.5% showed sister plasmid co-segregation coupled to sister chromatid co-segregation when conditions of mitosis were altered to intentionally missegregate chromosomes. The scatter plot for the *ARS* plasmid and chromosome IV showed poor correlation ($r = 0.416$ or $r^2 = 0.173$). The uncoupling of *STB* plasmid segregation from chromosome segregation by perturbing spindle integrity or by depleting cohesin during mitosis was revealed in the plots in Figure 8D by the dissimilar blue (*STB* plasmid) and red (chromosome IV or V) triangles.

DISCUSSION

Integration into the host DNA confers on a selfish element the same stability as its host genome. In the absence of integration, non-covalent association with chromosomes provides the element a reliable alternative means for stable propagation. Papilloma and gammaherpes family viruses exemplify the utility of tethering to chromosomes in the long-term maintenance of viral episomes in latently infected cells (58–61). The 2-micron plasmid resembles the viral episomes in that its stable segregation is apparently coupled to that of the host chromosomes, raising the prospect of the plasmid hitchhiking on chromosomes (18,19). The one-to-one segregation behavior of single-copy *STB* reporter plasmids observed previously (20) constrains the hitchhiking model by implying the association of sister plasmids with sister chromatids. The present study challenges the model by characterizing the mitotic segregation of *STB* plasmid sisters under forced co-segregation of sister chromatids during mitosis and fails to falsify it.

**STB plasmid sisters co-segregate when co-segregation is imposed on sister chromatids**

The nearly bias-free relative increases in the co-segregation of *STB* plasmid sisters and of sister chromatids promoted by monopolin and monopolin plus Spo13 are tightly correlated and stand apart from the biased segregation patterns of the *ARS* reporter plasmid (Figure 8A and C). The correlation is consistent with sister plasmids being tethered to sister chromatids in a one-to-one fashion. This mode of plasmid association with chromosomes may be referred to as 'symmetric tethering' to distinguish it from the association of plasmid sisters to the same chromosome (asymmetric tethering) or to distinct chromosomes (random tethering). Asymmetric tethering will always missegregate plasmid sisters (2:0), whereas random tethering can only achieve 50% equal segregation (1:1) due to independent assortment of individual chromosomes. Either mechanism alone is inconsistent with the equal segregation frequency of ∼64% during normal mitosis under a functional Rep-*STB* system.

The lack of perfect correlation between plasmid and chromosome in the co-segregation of sisters under programmed atypical mitosis may indicate an innate upper limit to the efficiency of the plasmid partitioning system. In general, fluorescence-tagged single-copy *STB* reporter plasmids show 70–80% equal segregation in the single-cell-cycle assay. The stabilities of 2-micron plasmid derived vectors are well below that of the native plasmid (lower by two to three orders of magnitude). Disrupting the genetic organization of the plasmid or insertion of extraneous sequences may have a significant deleterious effect on plasmid stability.

Imparting daughter bias to *STB* plasmid missegregation by inducing sister chromatids to co-segregate preferentially to the daughter

Unlike the bias-free missegregation induced by monopolin, missegregation of sister chromatids induced by *ipl1-321* or by monopolin plus *ipl1-321* has a distinct daughter bias. The bias neutrality or the very small mother bias in *STB* plasmid missegregation during normal mitosis is reversed toward the daughter by the same regimens. This correspondence between sister chromatids and *STB* plasmid sisters in the relative extents of missegregation and bias (Figure 8B and C) is accommodated by the physical association between plasmid sisters and sister chromatids.
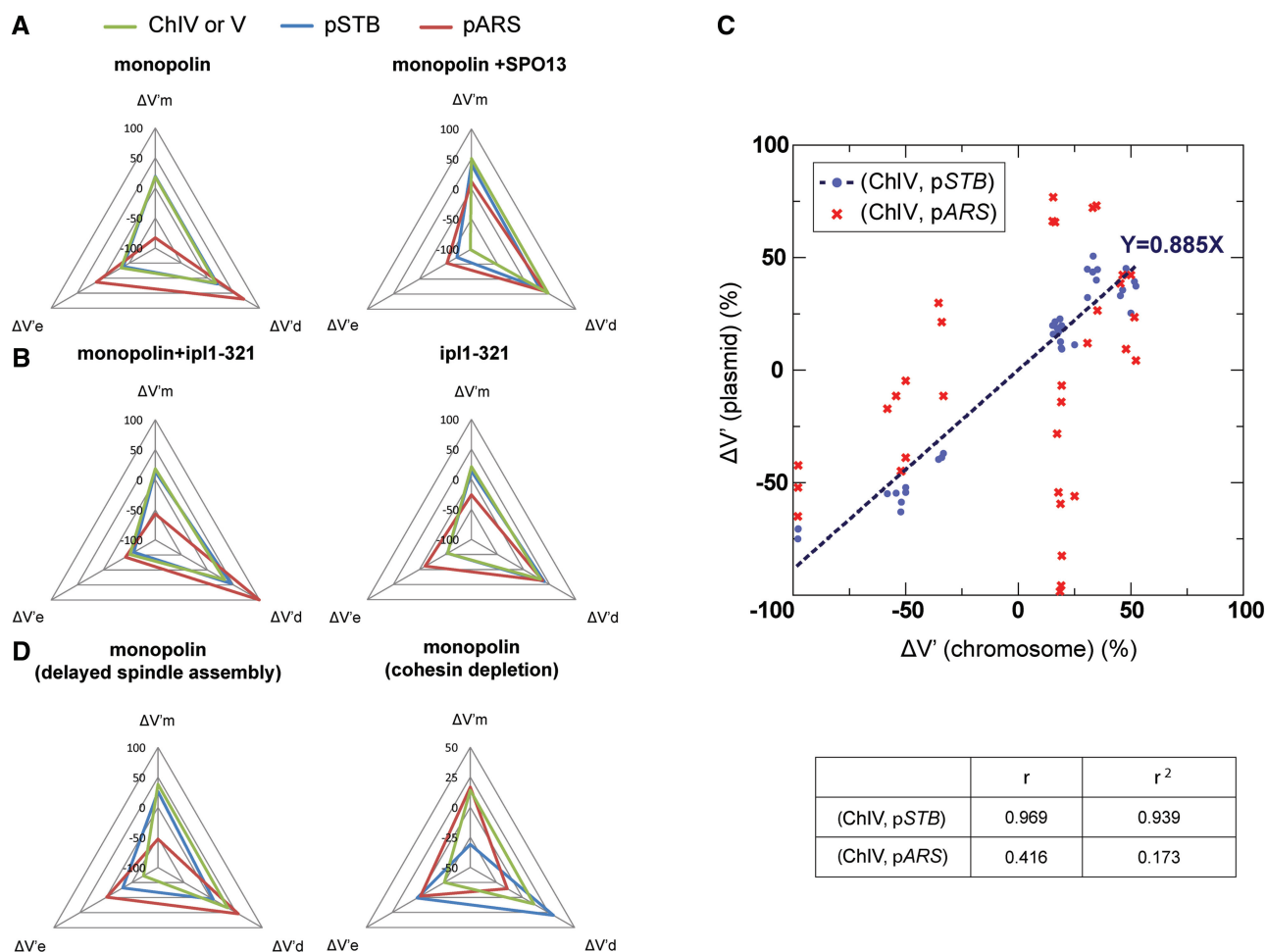
Figure 8. Correlations between *STB* plasmid sisters and sister chromatids under different mitotic programs. (**A**, **B**). The correlations among the *STB* plasmid, the *ARS* plasmid and a chromosome (Chr IV) under four different mitotic regimens are diagramed as radar plots. The depictions in (A) are based on the data from Figures 3 and 4; those in B are derived from the results summarized in Figure 5 and Supplementary Figure S3. (**C**). The $\Delta V'$-values for the reporter plasmids and chromosome IV are displayed as a scatter plot. The blue dots represent the *STB* plasmid and the red dots denote the *ARS* plasmid. The estimated coefficients of correlation (r) and coefficients of determination (r^2) are tabulated. (**D**). These radar plots pictorialize plasmid–chromosome correlations signified by the data from Figures 6 and 7.

Uncoupling 2-micron plasmid segregation from monopolin induced sister chromatid co-segregation

Segregation of the *STB* plasmid sisters can be unlinked from monopolin-induced co-segregation of sister chromatids either by delaying the assembly of the mitotic spindle until G2/M or by running the cell cycle under depletion of the cohesin complex (Figure 8D). Association of the 2-micron plasmid with chromosome spreads, suggestive of potential plasmid–chromosome tethering and recruitment of cohesin at *STB* are dependent on the integrity of the mitotic spindle (43). Furthermore, the cell cycle timing of cohesin–*STB* association is critical in the equal segregation of the 2-micron plasmid (18). When the spindle is assembled in G2/M, following a spindleless cell cycle up to that point, the plasmid localizes in chromosome spreads and cohesin associates with *STB*. Yet, these associations are futile with respect to plasmid partitioning.

There is a plausible common explanation for how cohesin depletion or lack of the spindle till late in the cell cycle might uncouple *STB* plasmid segregation from

chromosome segregation. The spindle-dependent assembly of cohesin at *STB*, concomitant with plasmid replication, may spatially confine plasmid sisters to promote their symmetric tethering to similarly confined sister chromatids. In the absence of sister plasmid containment by cohesin, plasmid tethering to chromosomes would be random, reducing the theoretical efficiency of equal segregation of plasmid sisters to 50%. Constraining chromosome sisters at their centromeres by monopolin will not help symmetric tethering, unless monopolin exerts a similar effect on plasmids as well. Indeed, Mam1 is not detected at *STB* but associates with *CEN*, as expected.

Summary and perspectives

In principle, the remarkable stability of the 2-micron plasmid may be accomplished by a combination of its high copy number, random segregation and correction of copy number by the Flp amplification system. In this model, the function of the plasmid partitioning system is

merely to free plasmid molecules from some barrier to their free diffusion (mother bias). However, there are serious limitations to this model. Fluorescence-tagged reporter plasmids suggest the organization of plasmid molecules into groups or foci (19), effectively reducing their copy number to a low value. Second, during steady-state growth, nearly all plasmid molecules replicate once, and only once, per cell cycle (62); that is, there is little or no amplification. Significantly, the mother bias of *ARS* plasmids appears to result not from their limited mobility per se. Rather, the barrier to their diffusion into the daughter cell is posed by the geometries of the mother and bud compartments, the constricted neck bridging the two and too short a duration of mitosis to permit equilibration of plasmid molecules between mother and daughter (41). Tethering of an *ARS* plasmid artificially to the nuclear envelope can partially overcome the diffusion barrier and improve its segregation efficiency (41,42).

The present study utilizing single-copy reporter plasmids, in conjunction with previous observations, suggests that mother bias is overcome by the symmetric attachment of plasmid sisters to sister chromatids. How does the segregation behavior of a single-copy reporter plasmid translate to that of the native multicopy 2-micron plasmid? Previous observations suggested that a fluorescence-tagged multicopy *STB* reporter plasmid segregates as a single cluster composed of three to five dynamic foci during mitosis (19). However, this is likely a misimpression conveyed by the small size of the yeast nucleus and the resolution limits of fluorescence microscopy assays. This reporter plasmid missegregates during mitosis under monopolin assembly along with Spo13 expression, as expected, but all plasmid foci do not missegregate in unison (Supplementary Figure S5). Each focus, likely harboring several plasmid copies, appears to be an independent unit in segregation.

In principle, a set of plasmid molecules paired with their sister molecules by cohesin could nucleate the organization of two sister foci that could then be tethered to sister chromatids. Such a role for cohesin would seem to be at odds with its substoichiometric presence at *STB* (44). The association of Cse4 with *STB* is also highly substoichiometric (63). Perhaps the organization of multiple *STB*s into a single segregation unit may confer functional competence to all of these *STB*s through the acquisition of Cse4 (and cohesin) by one or a few among them. Alternatively, these host factors may execute their function at all *STB*s within a unit in a catalytic fashion by shuttling from one *STB* to another; or, they may dissociate from the majority of *STB*s, once their function is completed. Proximity assays by the analytical methods of chromosome conformation capture has revealed the clustered organization of *STB*s in the native 2-micron plasmid (64).

The 2-micron plasmid is propagated efficiently to all four spores during meiosis (65). The mechanisms by which plasmid molecules segregate during the reductional and equational divisions of meiosis are unknown. If mitosis under monopolin expression mimics meiosis I, the prediction is that sister plasmids would co-segregate with sister chromatids during meiosis I. Single-copy *STB*

reporter plasmids would be valuable in characterizing the meiotic segregation of the 2-micron plasmid.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–5.

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