SI Appendix

Construction of the Reporter Plasmids pSG1 and pSG2. The reporter plasmids pSG1 and pSG2 were designed to investigate the mechanism of 2-μm plasmid segregation at or close to single-copy level.

The plasmid pSG1 was constructed as follows: A 321-bp BamHI–SalI fragment carrying *CEN3* was cloned into the BamHI–SalI backbone of pESC-*URA* (Stratagene, La Jolla, CA) to construct pESC-*CEN-URA*-1. The unique HindIII site of pESC-*CEN-URA*-1 was destroyed by digestion with HindIII and end-filling by Klenow polymerase. The blunt linear fragment was self-ligated to obtain pESC-*CEN-URA*-2. A 1,371-bp fragment harboring *CEN3* flanked by the *GAL1* promoter and *CYC1* terminator and engineered to carry HindIII and PstI sites near the ends was then prepared by PCR from the pESC-*CEN-URA*-2 template. This DNA piece was digested with HindIII plus PstI and was cloned into similarly digested pSV5 (1) to obtain pSG1.

The plasmid pSG2 was assembled as follows: A 1,415-bp *GAL1* promoter-*CEN3-CYC1* terminator DNA fragment with BglII and XbaI recognition sequences near the termini was obtained by PCR from pESC-*CEN-URA*-1. This DNA was digested with BglII and XbaI and cloned into YCplac22 (2) that was digested with the same enzymes. The resulting plasmid was called YCplac22-*GALCEN*. An XbaI–SphI fragment carrying *STB* and the 2-μm circle replication origin was obtained by restricting digestion of an appropriate DNA fragment amplified by PCR from the native plasmid. This DNA was cloned into XbaI- plus SphI-digested YCplac22-*GALCEN* to obtain YCplac22-*GALCEN-STB-ORI*. The *GALCEN-STB-ORI* DNA was then excised as an SphI–SpeI fragment and ligated to similarly digested pRS306 × 112TetO (3) to yield pSG2. The ligation mixture was directly introduced into appropriate yeast cells expressing red fluorescent protein fused to the Tet repressor (obtained from R. Rothstein, Columbia University, New York). The resulting Ura^+ transformants were scored for fluorescent foci, and a subset of the positives was analyzed by PCR to identify those that had acquired the correct plasmid construct.

Relative Copy Numbers of *STB*, *CEN*, and *CEN–STB* plasmids. The copy numbers of the *CEN–STB* plasmids pSG1 and pSG2 were compared to those of an *STB* plasmid (pSV5) or a *CEN* plasmid (pSV33) described in ref. 1. These plasmids are related in their overall organization. Whereas PSV5, pSV33, and pSG1 contain $LacO_{256}$ arrays, pSG2 contains a $TetO_{112}$ array instead. The results shown below in SI Fig. 7 are for pSG1; those for pSG2 were quite similar (data not shown).

In cells expressing the GFP-LacI hybrid repressor, pSV5 was organized into a cluster containing roughly between three and five fluorescent foci (Fig. 7*A*); by contrast, pSV33 displayed a single fluorescent dot in the majority of cells. The pSG1 pattern closely resembled that of pSV33, suggesting that the introduction of a centromere effectively brings down the average copy number of an *STB* plasmid to 1 or close to 1. This inference was verified by a more direct estimate of plasmid copy numbers. DNA samples obtained from log-phase cultures of appropriate strains were digested with *Eco*RI, fractioned by electrophoresis in agarose, transferred to Hybond-XL membrane (Amersham Pharmacia Bioscience, Little Chalfont, Buckinghamshire, UK), and hybridized to a radiolabeled probe specific to *TRP1*, harbored by all three plasmids. A *TRP1*-containing fragment, obtained by PCR amplification from yeast DNA, provided the template for randomly primed DNA synthesis in the presence of α-^{32}P dexoynucleoside triphosphates. The hybridization bands were visualized by using a phosphorimager (Molecular Imager FX; Bio-Rad Laboratories, Hercules, CA), and were quantitated by using Quantity One software (Bio-Rad Laboratories). From the ratios of the intensity of a plasmid band (Pl) to the corresponding chromosomal band (Ch), we deduced a copy number of close to 1 and 2 per cell for pSG1 and pSV33, respectively (SI Fig. 7*B*). Our estimate of roughly 10 copies per cell for pSV5 (SI Fig. 7*B*) is in close agreement with an average value of ≈10–12 molecules per cell derived in previous work from fluorescence intensity measurements (4).

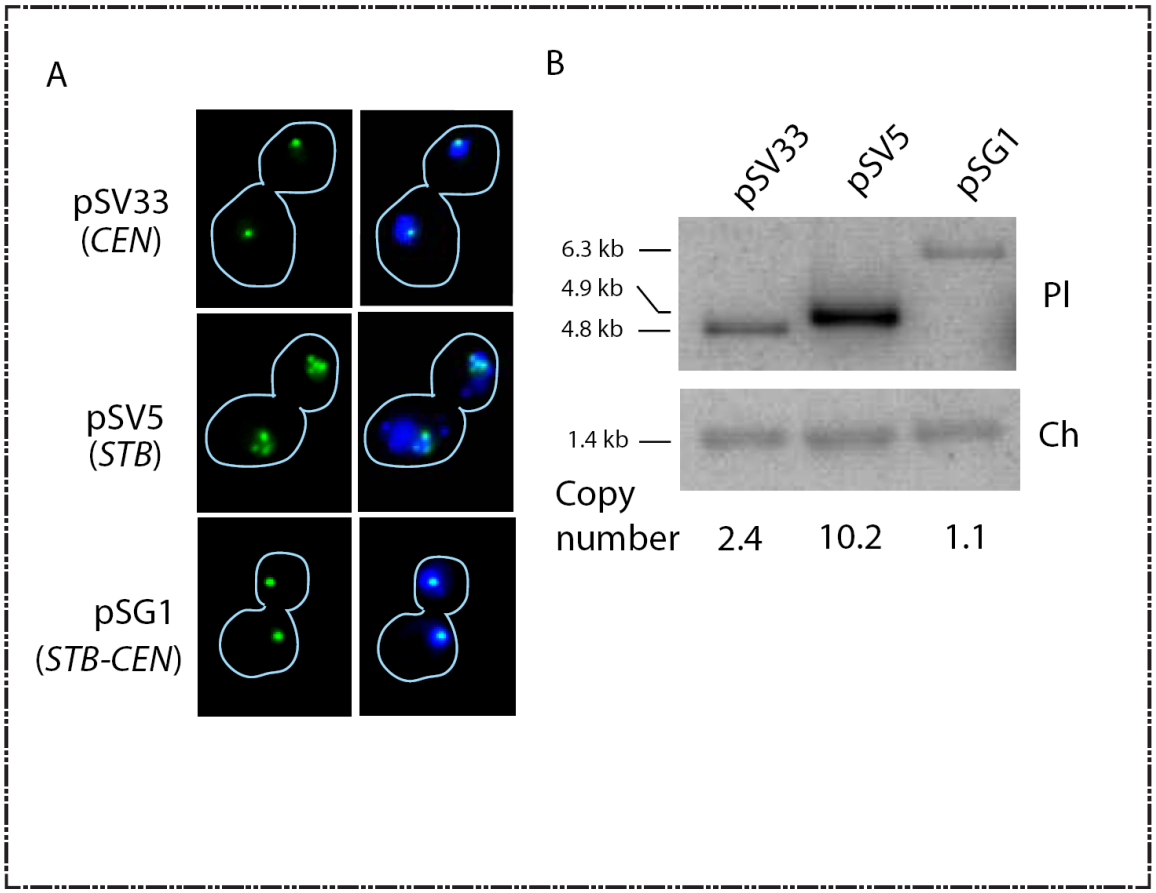

Fig. 7. The copy number of an *STB* plasmid can be reduced to nearly 1 by the presence of a *CEN* sequence in cis.

Segregation of Single Copy Reporter Plasmids in the *cse4-1* Mutant Strain. The *cse4-1* mutation induces the mitotic checkpoint, causing cells to stall in metaphase for an extended period (5). When a log-phase culture of the *cse4-1* strain, grown in galactose to inactivate the plasmid borne *CEN*, was shifted to 37°C for 3 h, ≈30% of the cells entered anaphase. They displayed chromosome segregation at a gross level, as indicated by well separated DAPI masses in the two cell compartments. However, because of the effect of *cse4-1* on chromosome segregation, nearly three quarters of the cells showed conspicuous inequality of DAPI between the two cell compartments. Consistent with the notion that plasmid cohesion is a central step in equal plasmid segregation, these cells showed a much larger proportion of 2:0 segregation of the fluorescent plasmid dots (class II plus class III; roughly 70%) relative to 1:1 segregation (class I) (SI Fig. 8). Late anaphase

cells from the 26°C population showed well separated and apparently equal DAPI masses in the two cell compartments. The principal mode of plasmid segregation in such cells was 1:1 (≈80%).

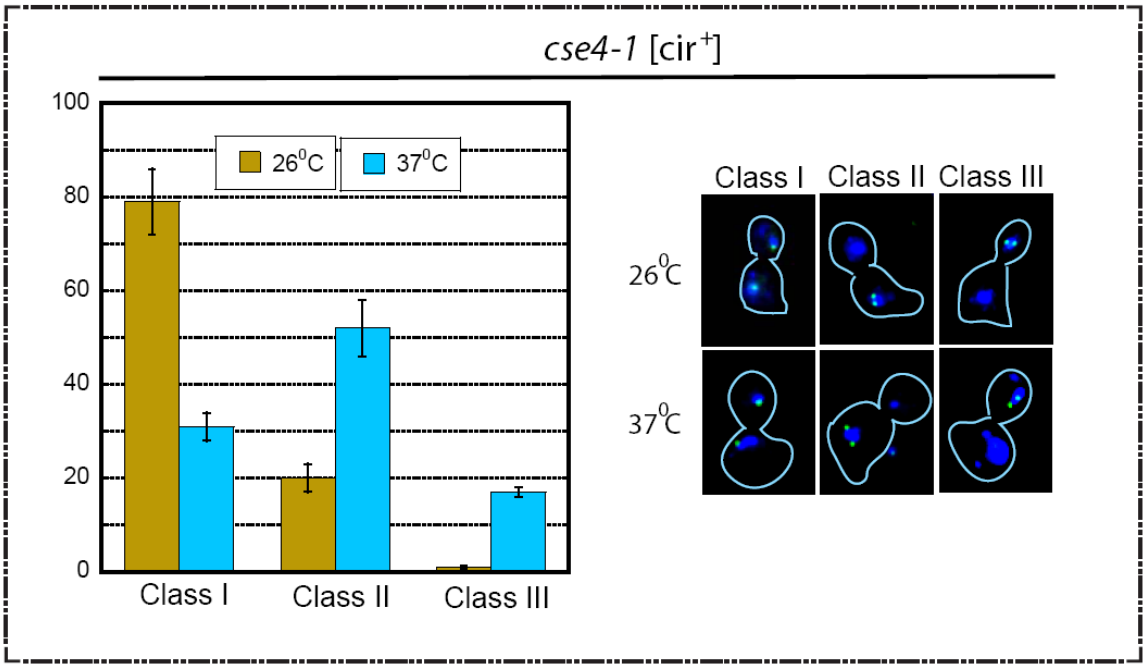

Fig. 8. Lack of functional Cse4p impairs the Rep-*STB*-mediated partitioning pathway, presumably because the cohesin complex cannot be assembled at *STB*.

Cohesin Loading Factor Scc2p Is Associated with *STB*. The requirement of the chromosomal cohesin loading factors Scc2p and Scc4p for cohesin assembly on the 2-μm circle suggests that one or both may associate with *STB*. The results of a chromatin immunoprecipitation assay carried out with HA-tagged Scc2p in a [cir$^+$] strain (SI Fig. 9*A*) are consistent with this notion. The association was specific to *STB*, and was not detected at other loci on the plasmid genome (data not shown). Furthermore, a similar ChIP analysis in an isogenic [cir^0] strain failed to reveal Scc2p at *STB* (SI Fig. 9*B*). These results imply that the interaction between Scc2p and *STB* is likely mediated through the Rep1 and Rep2 proteins.

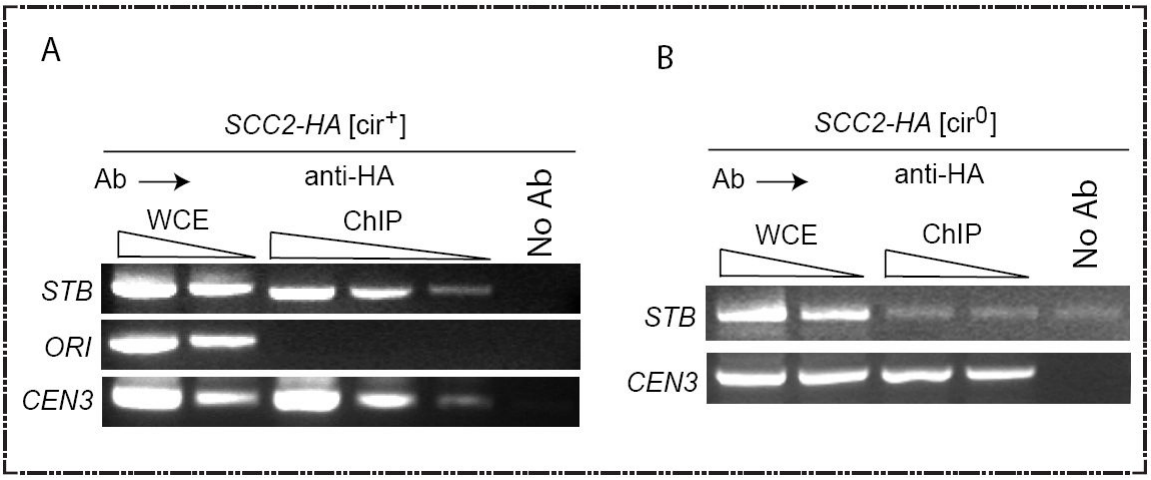

Fig. 9. Cohesin loading factor Scc2p is localized at *STB* in a [cir$^+$] strain.

Cohesin–*STB* Association Is Necessary but not Sufficient for the Establishment/Maintenance of Cohesion. The *ctf7*-203 [cir$^+$] strain containing a single-copy reporter plasmid was arrested in G1 and then released, after inactivation of the plasmid centromere, into the cell cycle at 26°C (permissive temperature) or 37°C (nonpermissive temperature). Plasmid fluorescence was assayed in metaphase cells at 100 min and 75 min into the cell cycles at 26°C and 37°C, respectively. Cohesion was normal at 26°C; it was severely impaired at 37°C (SI Fig. 10*A*). A similar analysis was then carried out in an isogenic [cir^0] strain in glucose medium, so as to inactivate *STB* without compromising centromere function. Metaphase cells from the 26°C and 37°C populations, scored at 70 min and 45 min, respectively, after release from G1, revealed roughly 75% cohesion at 26°C as opposed to only 10% at 37°C (SI Fig. 10*B*). In each experiment, we verified by FACS analysis that the predominant fraction of cells in the assayed population (>90%) contained 2N DNA, indicating completion of DNA replication (data not shown).

Thus, recruitment of cohesin at *STB*, which is unaffected by the *ctf7* mutation (S. Mehta and M.J., unpublished data), is necessary but not sufficient for establishing and/or maintaining cohesion between replicated plasmids. As is the case with cohesion at centromeres (and chromosome arms), cohesion at *STB* also appears to depend on the

establishment factor Ctf7p. Furthermore, observation of anaphase cells revealed that lack of plasmid cohesion caused by inactivation of Ctf7 results in high rates of plasmid missegregation (data not shown) (1).

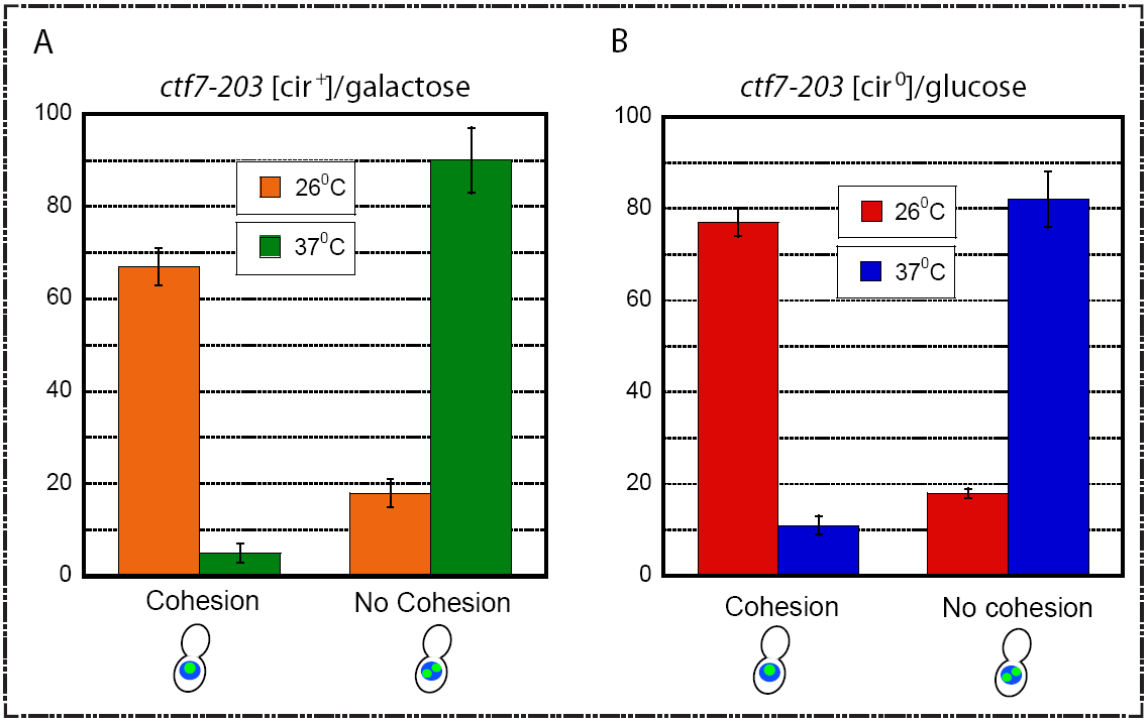

Fig. 10. When Ctf7p function is inactivated, plasmid cohesion does not occur even though cohesin recruitment is unaffected.

Plasmid Cohesion Assayed in Cells Harboring Two Single-Copy *STB–CEN* Plasmids as they Traverse the Cell Cycle. The plasmids pSG1 (tagged by green fluorescence; $[LacO]_{256}$-GFP-LacI) and pSG2 (tagged by red fluorescence; $[TetO]_{112}$-RFP-TetR) were simultaneously present in isogenic [cir^+] and [cir^0] strains. Cells were arrested in G1 by α factor and then allowed to proceed into the cell cycle with both *STB* and *CEN* on the plasmids active (glucose, [cir^+]), *STB* alone active (galactose, [cir^+]), *CEN* alone active (glucose, [cir^0]), or neither *STB* nor *CEN* active (galactose, [cir^0]). Cohesion was followed in metaphase cells. In SI Fig. 11, class I represents cohesion of both plasmids; classes II–IV represent lack of cohesion of one or both plasmids. Consistent with the results from other experiments (see main text), a functional *STB* or *CEN* was sufficient to

mediate plasmid cohesion. It was only in the galactose [cir^0] situation that metaphase cells exhibited a lack of plasmid cohesion.

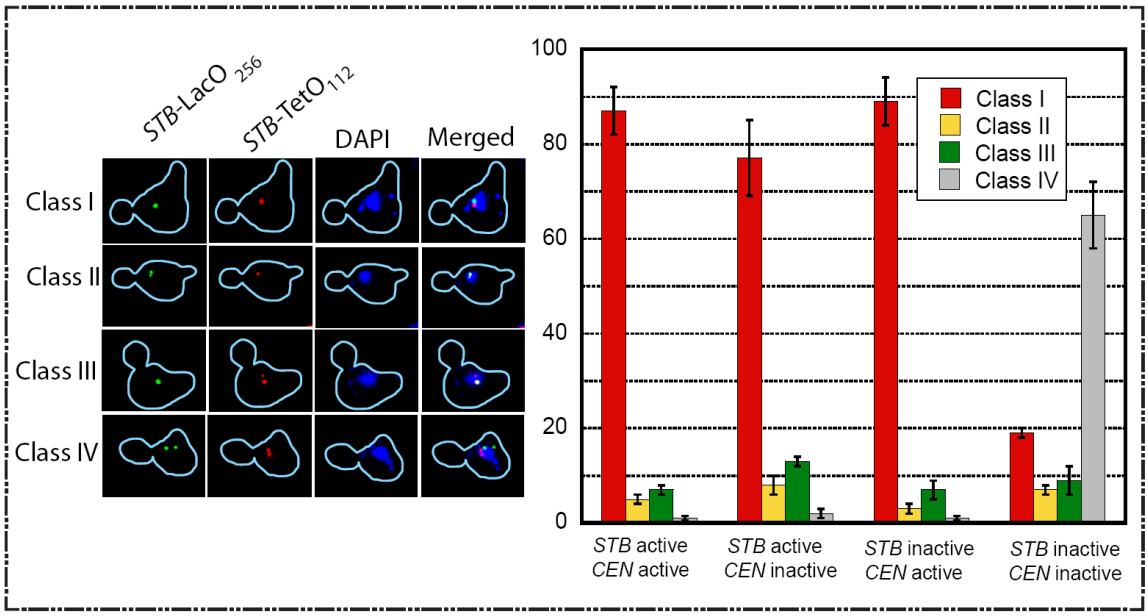

Fig. 11. Metaphase cells reveal cohesion of two unit-copy *STB* plasmids, one tagged by green and the other by red fluorescence.

Time Lapse Analysis of Plasmid Segregation Under *STB*, *CEN*, or *CEN–STB* control. Results from time-lapse analysis of plasmid segregation in individual cells (SI Fig. 12) were consistent with those obtained from population assays (Fig. 6*B*). Each of the experimental strains harbored two unit-copy plasmids, one tagged by green fluorescence ([LacO]$_{256}$-GFP-LacI) and the other by red fluorescence ([TetO]$_{112}$-RFP-TetR). SI Fig. 12 and the corresponding movies depict segregation patterns for two plasmids under *STB* control (pSG1 and pSG2 with galactose-inactivated *CEN*; SI Fig. 12*A* and Movie1), one plasmid under *STB* control and the other under *CEN* control (pSG2 with galactose-inactivated *CEN* and pSV33; SI Fig. 12*B* and Movie 2), and both plasmids operating under an active *CEN* (pSG1 and pSG2 in glucose to maintain *CEN* function; SI Fig. 12*C* and Movie 3). Note that pSV33 did not harbor *STB*, and its *CEN* was functional in galactose. The fluorescence tag on pSG1 and pSV33 was green and that on pSG2 was red.

For inactivation of *CEN* in the *STB–CEN* plasmids (pSG1 and pSG2), log-phase cells grown under selection in raffinose were arrested in G1 by treating them with α factor, first in raffinose for 1 h and then in galactose for 2 h. After extensive washing, cells were released into selection medium containing galactose and maintained for 60 min. They were then transferred on to slides layered with galactose-complete medium containing 25% (wt/vol) gelatin (6). For assays in which *CEN* function was maintained, glucose replaced galactose during the steps for G1 arrest, release, and transfer to slides. However, the maintenance time in glucose-containing selection medium before transferring cells to slides was only 30 min. Images were captured at 7 min intervals with an Olympus BX-60 microscope and a Photometrics Quantix camera (Roper Scientific, Tucson, AZ). They were processed using MetaMorph (Photometrics, Buckinghamshire, U.K.), and movies were generated by using Jasc Animation Shop (version 3.05; Jasc Software, Eden Prairie, MN) at 1 frame per sec.

Although the data set is limited, the red-to-red and green-to-green segregation was the predominant pattern observed. The numbers were 9 out of 13 for the *STB*/*STB* (SI Fig. 12*A*), 8 out of 10 for *STB*/*CEN* (SI Fig. 12*B*), and 11 out of 11 for *CEN*/*CEN* (SI Fig. 12*C*). During *STB*/*STB* segregation, out of a total 13, unequal partitioning was observed four times, 3:1 three times, and 4:0 one time. In the two *STB/CEN* cases (out of 10) showing 3:1 segregation, the *CEN* plasmid (green) segregated 1:1 and the *STB* plasmid (red) segregated 2:0. These results are indicative of a common mechanism by which sister chromatids and sister plasmid clusters (in their native multicopy state) are dispatched to daughter cells. Hitchhiking on sister chromatids by the duplicated plasmid clusters therefore appears to be a strong possibility.

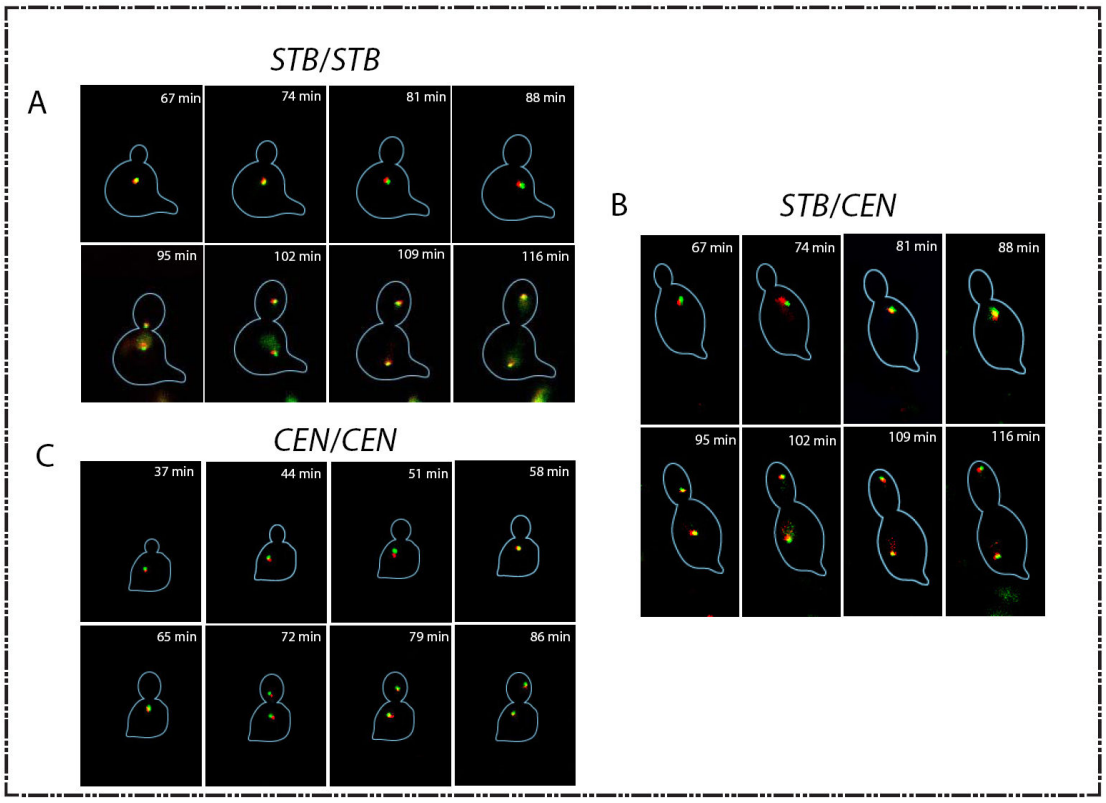

Fig. 12. Time-lapse fluorescence microscopy reveals similar segregation dynamics for unit-copy *STB* and *CEN* reporter plasmids.

Sister-to-Sister Segregation of the 2-μm Plasmid Is Independent of Its Multicopy Clustered State. The data establishing the sister-to-sister segregation of the 2-μm plasmid (Fig. 6) were obtained in the [cir$^+$] background, the fluorescence tagged reporter plasmids being resident within the native plasmid cluster. To examine the segregation pattern when the two reporters were the only plasmids present in the nucleus, we introduced pSG1 (tagged by green fluorescence) and pSG2 (tagged by red fluorescence) into an isogenic [cir^0] strain and supplied them with Rep1 and Rep2 proteins expressed from the bidirectional *GAL1-GAL10* promoter. A separate plasmid stability assay was performed to verify the inducible expression of the Rep proteins. As predicted, a test plasmid harboring the 2-μm circle origin and *STB* showed high stability in galactose but poor stability in glucose (data not shown). The results of cohesion assayed in metaphase cells and segregation assayed in postanaphase cells are assembled in SI Fig. 13 *A* and *B*, respectively. In these experiments, galactose caused the induction of Rep proteins

keeping *STB* active while, at the same time, inactivating *CEN*. Glucose, on the other hand, repressed the expression of the Rep proteins and inactivated *STB* but permitted *CEN* function. Maintaining either the *STB* or *CEN* function was sufficient to establish cohesion of both plasmids during metaphase (class I, SI Fig. 13*A*) and their sister-to-sister segregation during anaphase (class I, SI Fig. 13*B*). Classes II–IV in Fig. 13*A*, signifying a lack of cohesion, and classes II–V in Fig. 13*B*, indicating deviations from sister-to-sister segregation, constituted a small minority. We conclude that the normal mechanism of 2-μm circle segregation is preserved irrespective of the plasmid copy number. This result further strengthens the idea that the segregation behavior of the

plasmid cluster as a whole reflects the sum of the behaviors of its individual members.

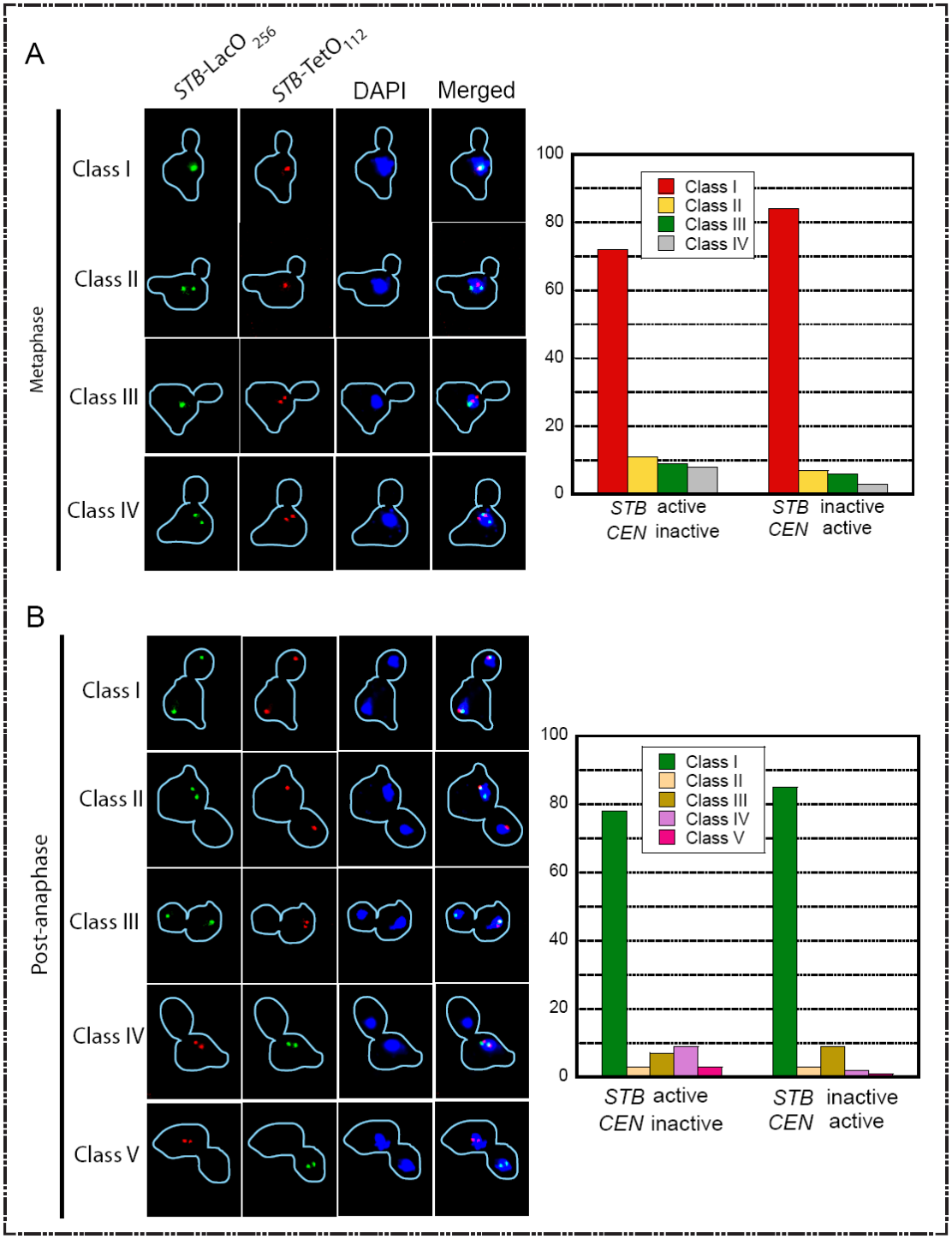

Fig. 13. Two single-copy *STB* reporter plasmids follow sister-to-sister segregation in a [cir^0] strain when supplied with Rep1 and Rep2 proteins.

Table 1. Yeast strains and plasmids used in this study

Strain or plasmid	Genotype or salient features	Source/ref.
MJY124	*MAT***a** *ade2-101 his3-11 leu2-3, 112 trp1 ura3-1* [cir$^+$]	1
MJY125	*MAT***a** *ade2-101 his3-11 leu2-3, 112 trp1 ura3-1* [cir^0]	1
YBS514	*MATα ura3-52 lys2-801ade2-101 his3Δ200 trp1Δ63 leu2Δ1 ctf7Δ1::HIS3 ctf7-203::LEU2* [cir$^+$]	7
MJY3015	*MATα ura3-52 lys2-801ade2::GFP-LacI::ADE2 his3Δ200 trp1Δ63 leu2Δ1 ctf7Δ1::HIS3 ctf7-203::LEU2* [cir$^+$]	This study
MJY3016	*MAT***a** *ade2::GFP-LacI::ADE2 his3-11 leu2-3, 112 trp1 ura3-1* [cir$^+$]	This study
MJY3017	*MAT***a** *ade2::GFP-LacI::ADE2 his3-11 leu2-3, 112 trp1 ura3-1* [cir^0]	This study
4aAS247	*MAT***a** *leu2-3, 112 trp1-63 ura3-52 smc1-2* [cir$^+$]	8
MJY3018	*MAT***a** *leu2-3, 112 trp1-63 ura3::GFP-LacI::URA3 smc1-2* [cir$^+$]	This study
MJY3019	*MAT***a** *leu2-3, 112 trp1 ura3-42 ade2::GFP-LacI::ADE2 cse4-1* [cir$^+$]	This study
MJY3020	*MAT***a** *his3-11 leu2::GFP-LacI::LEU2 trp1 ura3-1 RFP-TetR* [cir$^+$]	This study
MJY3021	*MAT***a** *his3-11 leu2::GFP-LacI::LEU2 trp1 ura3-1 RFP-TetR* [cir^0]	This study
MJY3022	*MAT***a** *leu2::GFP-LacI::LEU2 trp1 ura3 his3::GAL1p-REP1-GAL10p-REP2::HIS3 RFP-TetR* [cir^0]	This study
MJY3023	*MAT***a** *ade2-101 his3::SCC2-3HA::HIS3 leu2-3, 112 trp1 ura3-1* [cir$^+$]	This study
MJY3024	*MAT***a** *ade2-101 his3 leu2-3, 112 trp1 ura3-1 MCD1-3HA::KanMX* [cir$^+$]	This study
pSV5	$LacO_{256}$ cloned in YEpLac112 (*TRP1*)	1
pESC-*URA*	2-μm circle-derived plasmid carrying *GAL1-GAL10* bidirectional promoter	Stratagene
pSG1	*GAL-CEN3-STB-ORI* cloned in pSV5	This study
pRS306X112TetO	$TetO_{112}$ cloned in pRS306 (*URA3*)	3
pSG2	*GAL-CEN3-STB-ORI* cloned in pRS306X112TetO	This study
pSV30	pRS402-GFPLacI (*ADE2*)	M.J. laboratory
pSV31	pRS406-GFPLacI (*URA3*)	M.J. laboratory
pSV32	pRS405-GFPLacI (*LEU2*)	M.J. laboratory
pSV33	$LacO_{256}$ cloned in YCpLac22 (*TRP1*)	M.J. laboratory
pSG3	pRS403 expressing galactose-inducible Rep1p and Rep2p	This study

Plasmids and yeast strains constructed in-house or obtained from outside sources for this study are tabulated. Their relevant attributes are briefly summarized.

1. Mehta S, Yang XM, Chan CS, Dobson MJ, Jayaram M, Velmurugan S (2002) *J Cell Biol* 158:625–637.

2. Gietz RD, Sugino A (1988) *Gene* 74:527-534.

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