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Stable Kinetochore-Microtubule Attachment Constrains Centromere Positioning in Metaphase

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Summary

With a single microtubule attachment, budding-yeast kinetochores provide an excellent system for understanding the coordinated linkage to dynamic microtubule plus ends for chromosome oscillation and positioning. Fluorescent tagging of kinetochore proteins indicates that, on average, all centromeres are clustered, distinctly separated from their sisters, and positioned equidistant from their respective spindle poles during metaphase. However, individual fluorescent chromosome markers near the centromere transiently reassociate with their sisters and oscillate from one spindle half to the other. To reconcile the apparent disparity between the average centromere position and individual centromere proximal markers, we utilized fluorescence recovery after photobleaching to measure stability of the histone-H3 variant Cse4p/CENP-A. Newly synthesized Cse4p replaces old protein during DNA replication. Once assembled, Cse4-GFP is a physically stable component of centromeres during mitosis. This allowed us to follow centromere dynamics within each spindle half. Kinetochores remain stably attached to dynamic microtubules and exhibit a low incidence of switching orientation or position between the spindle halves. Switching of sister chromatid attachment may be contemporaneous with Cse4p exchange and early kinetochore assembly during S phase; this would promote mixing of chromosome attachment to each spindle pole. Once biorientation is attained, centromeres rarely make excursions beyond their proximal half spindle.

Results and Discussion

The assembly of nucleosomes containing a centromere-specific histone-H3 variant (CENP-A/Cse4p/Cid/Hcp3/Cnp1) provides an essential platform for kinetochore assembly and subsequent chromosome segregation [1, 2]. The selective incorporation of Cse4p at centromeres is therefore diagnostic of early steps in kinetochore assembly.

Assembled kinetochores attach to a single microtubule, and biorientation occurs with each sister kinetochore coupled to and facing the opposite spindle pole (Figure 1A) [3]. Improper attachments with both kinetochores linked to the same pole are corrected by attachment and detachment cycles. Biorientation stretches chromatin between sisters (Figure 1A), producing tension that signals bipolar attachment to the spindle assembly checkpoint [4, 5]. Sister kinetochores congress to a metaphase configuration (Figure 1A); however, the transient reassociation of DNA markers proximal to centromeres suggests continual attachment/detachment cycles during mitosis (Figure 1B) [6, 7]. Alternatively, coupled to microtubule growth, active movement of one centromere to its sister in the opposing spindle half may also result in the transient reassociations (Figure 1C). The position and dynamics of individual centromeres have been inferred from measurements with green fluorescent protein (GFP) markers to label chromosome arms near centromeres [6–11]. Kinetochores labeled by centromere histone-H3 Cse4-GFP localize into two clusters on either side of the spindle equator at metaphase [10, 12]. We examined the positional stability of all kinetochores with Cse4p-GFP and fluorescence recovery after photobleaching (FRAP) to determine whether kinetochore attachment and position persist through metaphase (Figure 1).

Cse4p Is a Stable Centromere Component Deposited at DNA Replication

We utilized a photobleaching assay to measure protein exchange through the cell cycle. Cse4-GFP fluorescence was photobleached in telophase cells (0.0 min), and recovery was measured at various time points into the next cell cycle. A low level of recovery was observed at the first time point (10 min) after photobleaching (average = 17.8% ± 12.5%; n = 7), indicative of a low level of Cse4p turnover during telophase and G1. However, significant Cse4-GFP fluorescence recovery occurred approximately 40 min later, near the time (within 10 min) of bud emergence (Figure 2A; n = 8). In single-time-point experiments, Cse4-GFP fluorescence recovered to an average of 62.7% ± 38.0% (n = 23) after bud emergence. Fluorescence recovery to 100% or 200% was expected for conservative/semiconservative deposition or complete replacement with new Cse4-GFP, respectively. However, the laser photobleaching technique possibly bleached a significant fraction of the free Cse4-GFP, resulting in bleached Cse4-GFP incorporation and decreased recovery levels.

Cse4-GFP fluorescence exhibited very low recovery after the deposition at bud emergence, indicating that Cse4p is not dynamic at metaphase kinetochores (see below; Figure 3A). To ensure that the low protein turnover was not a result of significant photobleaching of the non-kinetochore-associated Cse4-GFP, we measured turnover in cells with excess Cse4-GFP expression controlled by the galactose promoter. Cse4-GFP fluorescence at centromeres in G2/M (budded cells) did not recover after photobleaching, indicating that the low recovery was not a result of limited fluorescent protein

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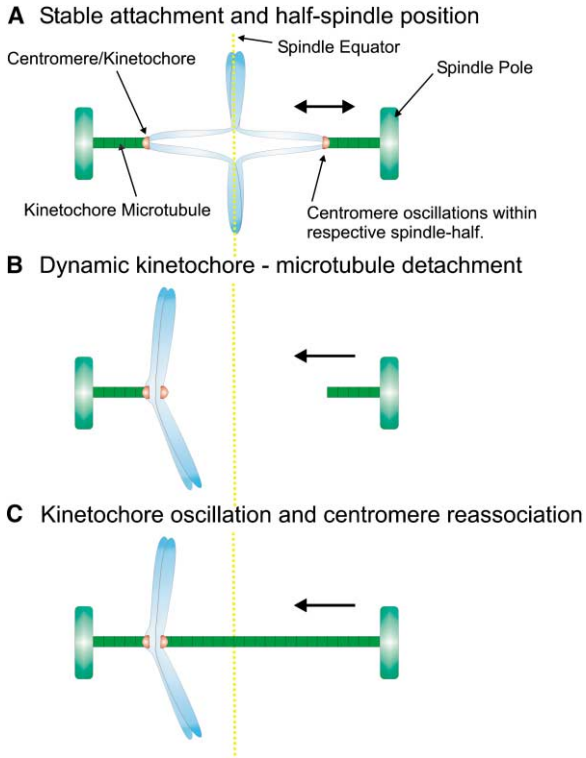

Figure 1. Models for Metaphase Kinetochore Movements and Microtubule Attachment

(A) At metaphase, kinetochores remain attached to their respective spindle poles and are dynamically positioned within their proximally attached spindle halves. These centromeres do not cross the spindle equator to move into the opposite spindle half.

(B) Dynamic kinetochore-microtubule attachment leading to centromere movements across the spindle equator into the opposite spindle half. Loss in kinetochore attachment to single microtubules results in centromere reassociation with a sister centromere crossing into the opposite spindle half.

(C) Kinetochore oscillations from one spindle half to the opposite. Reduced tension between sister centromeres by microtubule growth and movement of the attached centromere from one spindle half to the distal half are shown.

levels (n = 3; data not shown). The stability of kinetochore bound Cse4p has also been shown by S. Biggins and K. Collins ([13], this issue of *Current Biology*).

Daughter cell formation, as demarcated by bud emergence, occurs at approximately the time of centromere replication during the beginning of S phase [14–16]. Cse4p remains stable at kinetochores until the onset of DNA synthesis. The significant fluorescence recovery near the time of bud emergence reflects new Cse4-GFP deposition. After deposition, Cse4p remains stable at metaphase kinetochores.

To determine if new Cse4-GFP is exchanged for old protein, we monitored differentially labeled copies of *CSE4*. A wild-type strain (EYY3030) was constructed with a second copy of *CSE4-GFP* under the control of the *GAL1* promoter. We induced Cse4-GFP expression for 2–3 hr to label all centromeres and then repressed it to monitor replacement of the Cse4-GFP with an endogenous cellular level of unlabeled Cse4p. If the Cse4-GFP was stable at the time of replication, then we expected to see persistence of Cse4-GFP fluorescence. Galactose-induced Cse4-GFP expression resulted in fluorescent protein incorporation at the centromeres (Figure 2B). Asynchronous cells were then shifted to glucose medium, and telophase cells with Cse4-GFP labeled centromeres were followed in the next cell cycle (Figure 2B). Within 10 min of bud emergence, mother cell Cse4-GFP fluorescence could not be detected at kinetochores over background fluorescence (Figure 2B; 19/22 cells). The unbudded daughter cell retained Cse4-GFP fluorescence until bud emergence, when it lost fluorescence signal (Figure 2B; 6/6 cells). The advanced cell cycle timing of mother relative to daughter cells illustrates that loss of fluorescence at bud emergence was not the result of photobleaching during image acquisition. Rather, fluorescence did not significantly decrease until bud emergence from these daughter cells. Fluorescence loss in the mother and daughter cells at approximately the time of bud emergence and early DNA replication indicated that Cse4-GFP at the original centromere exchanges with unlabeled Cse4p.

The loss and complete exchange of Cse4p may render kinetochores nonfunctional until Cse4p deposition and reassembly occurs. The predicted loss in kinetochore function during Cse4p exchange provides a model for microtubule detachment during a specific phase of the cell cycle: DNA replication. Alternatively, Cse4p may be dispersed for a limited time in the cell cycle without disrupting kinetochore function. However, if Cse4p is essential for kinetochore establishment and maintenance [17–20], this detachment mechanism during early DNA replication may ensure that chromosomes randomize their polar orientation and segregate DNA of the same replicative age to mother and daughter cells [21].

Stable Kinetochore Attachment and Positioning within the Metaphase Half Spindle

The fact that Cse4p is physically stable at the centromere in metaphase allowed us to test the frequency of kinetochore detachment (Figure 1B) or reassociation, each coupled to polymerization dynamics of kinetochore microtubules (Figure 1C). One half-spindle cluster of Cse4-GFP was photobleached, and fluorescence recovery was measured for 12 min after the bleach event (Figure 3A). Fluorescence recovery of the bleached Cse4-GFP cluster was expected if (1) Cse4p protein is exchangeable, (2) kinetochores detach (Figure 1B), or (3) kinetochores move into the opposite half spindle via microtubule assembly (Figure 1C). Cse4-GFP recovered an average of 5.4% ± 4.6% (range 0.0%–19.3%) (Figure 3A; n = 9), indicating that Cse4-GFP was a physically stable component of budding-yeast centromeres during metaphase. To further minimize the contribution of photobleaching (during acquisition) to the low fluorescence recovery, we performed single-time-point experiments 10 or 20 min after photobleaching. The bleached fluorescence recovered an average of 4.5% ± 7.3% (range 0.0%–19.8%) and 2.2% ± 4.4% (range 0.0%–13.4%) after an average time of 10.6 and 20.3 min, respectively (n = 9 for each condition). This low fluorescence recovery indicated that (1) Cse4p is a stable protein component at metaphase kinetochores, (2) centromeres remain

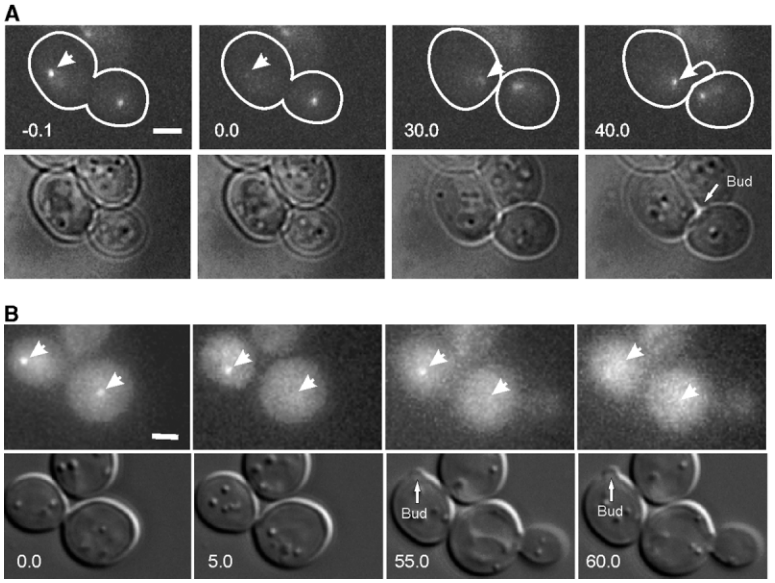

Figure 2. Cse4p Deposition with Bud Emergence

(A) Wild-type Cse4p fused to GFP was photobleached in telophase cells (0.0 min), and fluorescence recovery was followed in the next cell cycle. Coincidently with bud emergence (within 10 min), significant recovery of the bleached Cse4-GFP occurred (40.0 min). Arrowheads in the fluorescence images indicate the centromere clusters.

(B) When grown in galactose, a strain (EYY3030) with a single copy of *GAL1-CSE4-GFP* and the endogenous *CSE4* gene intact incorporated Cse4-GFP at centromeres (0.0 min). Cse4-GFP expression was repressed by shifting cells to glucose. Telophase cells with Cse4-GFP at the centromeres (0.0 min) were followed into the next cell cycle. Fluorescence was lost from the mother cell prior to bud emergence (5.0 min). Fluorescence was not lost from the daughter until daughter cell bud emergence (55.0–60.0 min). All Cse4-GFP fluorescence was replaced by endogenous, unlabeled Cse4p. The scale bar represents 2 μm. Time is in minutes.

attached to their microtubule, and (3) centromeres oscillate without crossing into the opposing spindle half during metaphase (Figure 1A).

The stability of centromere association with its respective pole at metaphase was also measured with a centromere-proximal lacI-GFP and lacO (1.8 kb from *CEN15*) marker [8]. Once the sister centromeres were separated during metaphase, one sister centromere marker was photobleached, resulting in a "bleached" and "unbleached" sister centromere (Figure 3B). The bleached mark exhibited partial recovery (81.6% $\pm$ 7.7%) after 20 min (n = 13), and it was distinctly dimmer than the unbleached mark. The orientation of the "dim" lacO, defined as the bleached sister centromere, toward the bud was monitored in a way similar to the timelapse imaging described above (data not shown) and 20 min after photobleaching (Figure 3B). We found that sister centromeres were stably oriented to their respective bud or mother cell (12 of 13 cells). The single example of centromere switching in orientation may have been the result of kinetochore detachment and reattachment, but it was most likely produced by spindle reorientation, which occurs infrequently in yeast cells [22, 23]. The low frequency of centromere reorientation further demonstrated that centromeres persist in their attachment to a single spindle pole during metaphase (Figures 1A and 3B).

Modeling Centromere Switching and Positional Changes

To quantify dynamic centromere movements from one spindle half to the other and to estimate the sensitivity of our FRAP assay, we utilized computational modeling adapted from Sprague et al. [24]. Experimental Cse4-GFP FRAP was simulated on the assumption that an unbleached kinetochore oscillating from one spindle half to the other contributed to the recovery of bleached fluorescence. The frequency of simulated kinetochore movement between spindle halves was matched to experimentally observed recovery rates (Figure 4A). The model parameters were defined such that centromeres did not frequently oscillate into the opposite spindle half, and the mean simulated recovery was 4.6% $\pm$ 4.3% (n = 30), which compares favorably with the experimental recovery of 5.4% $\pm$ 4.6%. During this simulation, on average 1.1 $\pm$ 0.2 centromeres of 32 were present in the opposite spindle half at any given time (Figure 4A, inset). On average, each kinetochore spent 3.5% $\pm$ 0.6% of the simulation time ($\sim$6.6 min) in the "wrong" or opposite spindle half.

The model parameters were adjusted until there was a significant fraction of centromeres on the opposite spindle half and a substantial fluorescence recovery statistically greater than that observed experimentally (Figure 4B; mean simulated recovery 22.1% $\pm$ 10.7%; n = 30 simulated runs). Under these adjusted parameters, 7.9 $\pm$ 0.6 centromeres of 32 were present in the spindle half opposite their attached spindle pole at any given time, distinct from the experimental data. Each kinetochore spent 24.8% $\pm$ 2.7% of the simulation time ($\sim$6.6 min) in the opposite spindle half (Figure 4B, inset). Because significant Cse4-GFP fluorescence recovery could be seen in the model with only a small fraction ($<$25%) of centromeres moving transiently into the incorrect spindle half, the Cse4-GFP FRAP experiment is predicted to be highly sensitive to centromere positioning within the correct spindle half. Thus, the low recovery observed in Cse4-GFP FRAP experiments is indicative of a small fraction of centromeres (approximately 1 of 32) moving into the opposing spindle half.

Using a combination of FRAP and computational modeling experiments, we found that upon metaphase congression in budding yeast, centromeres form stable microtubule attachments and maintain their position in one half of the mitotic spindle (Figure 1A). We also defined limits for the range of kinetochore oscillations (Figure 4A). Thus, biorientation with tension between sister centromeres is a stable configuration during metaphase.

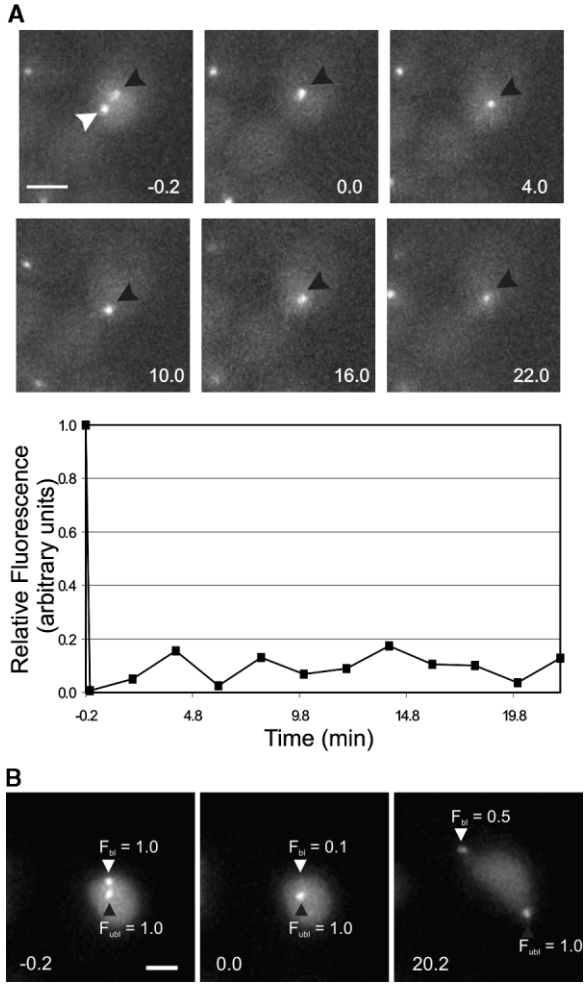

Figure 3. Stable Centromere Attachment and Position during Metaphase

(A) Cse4-GFP-labeled centromeres were photobleached (0.0 min) during metaphase. Fluorescence recovery was followed at 2 min intervals for 20 min. The lower panel describes a single example of fluorescence recovery. Average fluorescence recovery was low (5.4% ± 4.6% after 12 min), indicating that Cse4p was a stable protein component of the kinetochore and that centromeres remained positioned within their respective spindle half after metaphase congression. The white arrowhead indicates the bleached Cse4-GFP cluster, and the black arrowhead indicates the unbleached Cse4-GFP cluster.

(B) The persistent centromere orientation between spindle poles was confirmed by FRAP of a single centromere proximal marker. A separated lacI-GFP and lacO spot proximal to the centromere (1.8 kb from *CEN15*) was photobleached (0.0 min), and the orientation of the bleached spot along the mother-bud axis was monitored after 20 min to measure the frequency of switching orientation. Substantial fluorescence recovery was observed for lacI-GFP; however, the differential fluorescence allowed us to distinguish the bleached (F_{bl}; dim; white arrowhead) from the unbleached (F_{ubl}; bright; black arrowhead) centromere spot. The relative fluorescence intensity was determined for each chromosome marker. Orientation of sister centromeres was persistent in 12 of 13 experiments. These results demonstrated that centromeres did not frequently switch their orientation or position to the opposing spindle half upon metaphase biorientation. The scale bar represents 2 μm. Time is in minutes.

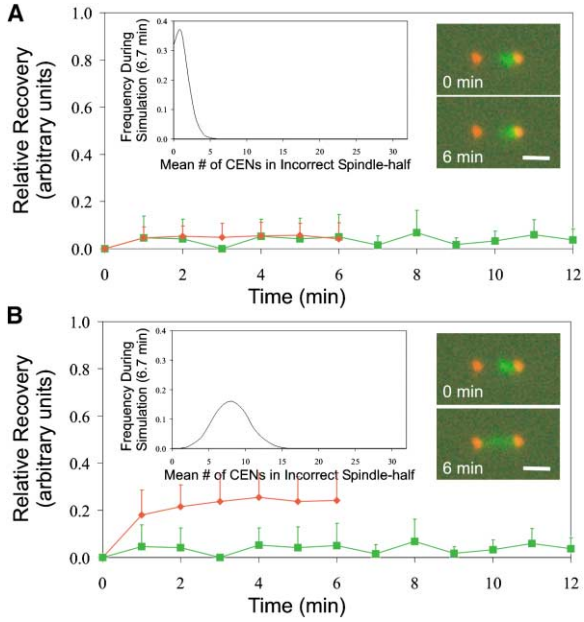

Figure 4. Computational Modeling Predicts Low-Frequency Half Spindle Switching Events

The low FRAP observed for Cse4-GFP was modeled to define the frequency of half spindle crossover events by generating a FRAP simulation of dynamic centromeres.

(A) Simulated average fluorescence recovery (red) was matched to the average experimental recovery (green) to determine the number of centromeres that were found in the opposing spindle half. The inset shows simulated fluorescence images that display fluorescence of Cse4-GFP (green) relative to spindle poles (red) immediately after photobleaching (0 min) and at the final simulation time point (6 min). The low fluorescence recovery was similar to that observed experimentally (Figure 3A). The inset distribution plot shows the average number of centromeres moving to the opposite spindle half (1.1 ± 0.2 of 32) at any given time.

(B) For modeling high-fluorescence recovery, the frequency of centromere movements to the opposite spindle half was increased in the computational model. Increased fluorescence recovery (red plot and fluorescence inset) was generated under these modeling conditions and compared to the experimental results (green plot and [A]). This model, which does not match the low experimental fluorescence recovery, predicted an average of 7.9 ± 0.6 of 32 centromeres moving into the opposite half spindle at any given time. Thus, centromeres in metaphase exhibited a limited number of translocations into the opposing half spindle, approximately one at any given time. The scale bar represents 1 μm. Time is in minutes.

This tension state may be analogous to the stabilization of kinetochore microtubule attachment that kinetochores experience in tissue cells [25].

Conclusions

We developed a FRAP-based assay to define the dynamics of kinetochore assembly, attachment, and position during budding-yeast mitosis. The budding-yeast centromere histone-H3, Cse4p, is assembled at kinetochores specifically during early DNA replication. Upon deposition, Cse4p remained relatively stable through the cell cycle. Cse4p replaced old protein at the centromere, and we propose this as a mechanism for kinetochore detachment and chromosome randomization. With stable bioriented kinetochore-microtubule attachment, ki-

netochores congress and persist in their respective half spindles without detaching from their microtubule. Thus, centromere dynamics are limited by stable attachment and regulated microtubule dynamics to remain in a metaphase state.

Experimental Procedures

Yeast Strains and Cell Culture

All cell culture and imaging media preparations were performed as described [10, 26]. Cse4-GFP was followed with KBY2010 (MATa *trp1-63*, *leu2-1*, *ura3-52*, *his3Δ200*, *lys2-801*, *cse4::HB* [hygromycin resistance], and pKK1) [10]. Single centromere movements were measured with CEN15 (1.8)-GFP (MATa *ade2*, *his3*, *trp1*, *ura3*, *leu2*, *can1*, *LacI-NLS-GFP:HIS3*, and *LacO::URA3* [1.8 Kb from *CEN15*]) [8]. For *CSE4-GFP* overexpression followed by gene promotor shut-off experiments, cells (EYY3030: MATa *ura3-1*, *leu2-3,112*, *his3-11*, *trp1-1*, *can1-100*, and *ade2-1* pJ4-1) were grown for approximately 2–3 hr in galactose media (to overexpress Cse4-GFP), washed, and resuspended in glucose media for approximately 2 hr before imaging. Live-cell imaging was performed on either galactose or glucose media. Plasmids pKK1 (*CSE4-GFP* fusion on *CEN* plasmid, pRS314) and pJ4-1 (p*GAL1-CSE4-GFP-URA3*) were used to label Cse4p with GFP [26].

Imaging Techniques

Cse4-GFP FRAP experiments were performed in a way similar to that of experiments with GFP-Tub1 in mitotic spindles [26, 28]. One cluster of Cse4-GFP fluorescence was photobleached in metaphase cells with a short 35 ms exposure of focused 488 nm laser light. A five-plane fluorescence Z series (0.5 μm steps at 2, 10, or 20 min intervals) was acquired immediately after laser exposure for the determination of the fluorescence photobleaching and recovery. Time-lapse imaging and recovery techniques were previously described [10, 12, 27]. Cse4-GFP fluorescence intensities were measured by the placement of a 5 by 5 pixel region around the localized fluorescence, and the integrated intensity was collected. Intracellular background levels were then subtracted to determine the total Cse4-GFP fluorescence intensity.

Simulation Methods

A MATLAB (version 6.0, The Math Works, Natick, MA)-based computer program was used for simulating the Cse4-GFP FRAP experiment. The dynamics of kinetochore microtubules and subsequent positions of kinetochores attached to the microtubule plus ends were simulated as previously described [24]. The microtubule parameters of dynamics were modified for each simulation to investigate the effect of kinetochore movements between spindle halves on the fluorescence recovery of Cse4-GFP after photobleaching (see Supplemental Experimental Procedures available with this article online).

Supplemental Data

Supplemental Experimental Procedures for this article are available at http://www.current-biology.com/cgi/content/full/14/21/1962/DC1/.

Acknowledgments

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