

# Global Chromosome Positions Are Transmitted through Mitosis in Mammalian Cells

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## Summary

We investigated positioning of chromosomes during the cell cycle in live mammalian cells with a combined experimental and computational approach. By non-invasive labeling of chromosome subsets and tracking by 4D imaging, we could show that no global rearrangements occurred in interphase. Using the same assay, we also observed a striking order of chromosomes throughout mitosis. By contrast, our computer simulation based on stochastic movements of individual chromosomes predicted randomization of chromosome order in mitosis. In vivo, a quantitative assay for single chromosome positioning during mitosis revealed strong similarities between daughter and mother cells. These results demonstrate that global chromosome positions are heritable through the cell cycle in mammalian cells. Based on tracking of labeled chromosomes and centromeres during chromosome segregation and experimental perturbations of chromosomal order, we propose that chromosome specific timing of sister chromatid separation transmits chromosomal positions from one cell generation to the next.

## Introduction

A global scheme of interphase chromosome organization has been observed in a variety of species including trypanosomes (Chung et al., 1990), fission yeast (Funabiki et al., 1993), *Drosophila* (Agard and Sedat, 1983; Marshall et al., 1996) and plants (Franklin and Cande, 1999). In cells from these organisms, interphase chromosomes are distributed in a polar manner inside the nucleus according to their centromere-telomere axis during chromosome segregation in mitosis. As a consequence, the chromosome arrangement in a given cell

resembles that of the previous cell generation. Such a configuration was initially proposed in 1885 based on transmitted light microscopy of salamander cells and is referred to as the Rabl configuration (Rabl, 1885).

Mammalian cells do not show such a simple polar organization of interphase chromosomes. Instead, their chromosomes occupy mutually exclusive, globular volumes referred to as chromosome territories (Cremer and Cremer, 2001). Centromeres and telomeres can be found distributed throughout the nucleus (Luderus et al., 1996; Shelby et al., 1996) with some preference for peripheral and perinucleolar positions (Shelby et al., 1996). It is also clear that interphase mammalian chromosomes do generally not undergo long-range movements exceeding  $\sim 1 \mu\text{m}$  (Abney et al., 1997; Kimura and Cook, 2001) but are constrained to local diffusional dynamics (Chubb et al., 2002; Marshall et al., 1997; Vazquez et al., 2001). Accumulating evidence obtained by chromosome-specific fluorescence in situ hybridization (FISH) supports the notion that also in mammalian nuclei, chromosomes are arranged in a non-random fashion (Parada and Misteli, 2002). However, models for such arrangements have remained very controversial (Allison and Nestor, 1999; Croft et al., 1999; Nagele et al., 1995).

More recent studies, again employing FISH in fixed cells, have focused on two modes of chromosome positions. The first is radial location within the nucleus measured from the center to the nuclear envelope. There is an emerging consensus that gene rich chromosomes are preferentially found in the nuclear interior, while gene poor chromosomes mostly localize closer to the nuclear envelope (Boyle et al., 2001; Croft et al., 1999; Sun et al., 2000; Tanabe et al., 2002). Radial organization was found to be evolutionarily conserved in primates (Tanabe et al., 2002) and the radial arrangement of early and late replicating chromatin domains could be shown to persist over two successive cell generations (Sadoni et al., 1999). The second mode of chromosome positions that has been documented is neighborhood relationships between two specific chromosomes. This is especially relevant for the question of homolog pairing and the proximity of chromosomes frequently undergoing translocations (Parada and Misteli, 2002; Sachs et al., 1997). It was found that certain neighborhoods occur more frequently than expected from a random arrangement of chromosomes (Parada et al., 2002). Most interestingly, the physical proximity of chromosomes undergoing translocations in mouse lymphoma cells was found to be conserved also in the closely related normal splenocytes (Parada et al., 2002).

Given that chromosomal positions correlate with gene expression and potentially the frequency of translocations, it is important to ask when and how chromosomes are positioned non-randomly in mammalian cells. Since chromosomes as a whole do not seem to undergo long-range movements in interphase, it seems likely that their positions are established in mitosis, after the constraints of the nuclear envelope are broken down and chromosomes are aligned and segregated by the mitotic spindle apparatus. Capturing of chromosomes by spindle microtu-

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bules is believed to be random (Alexander and Rieder, 1991), and massive, seemingly chaotic rearrangements of chromosomes occur in prometaphase (Kanda et al., 1998; Manders et al., 1999) leading to the flat arrangement of chromosomes on the metaphase plate that lacks most of the spatial information present in the three-dimensional interphase nucleus. It has been widely assumed that chromosome positions are randomized in mitosis. Preferred, non-random positions of chromosomes thus would have to be actively created after chromosome segregation. Interactions with the nuclear envelope after mitosis have been proposed as a possible mechanism for establishing preferred positions (Bridger et al., 2000).

In this study, we took an unbiased approach to determine how and when specific topological arrangements of chromosomes are established during the cell cycle. In particular, we were interested in whether this order can be transmitted through cell division or alternatively has to be actively restored during interphase in each cell cycle. Using mathematical modeling and computer simulation, we predicted chromosome dynamics in mitosis based on their known physical parameters and our own *in vivo* measurements. These predictions were then tested experimentally, using a novel non-invasive labeling method that allowed us to label any subnuclear set of chromatin in living cells. Our results demonstrate that global chromosome arrangements are preserved throughout the cell cycle in mammalian cells. Based on analysis of mitotic centromere dynamics and drug perturbations, we propose a mechanism that can transmit chromosomal positions from one cell generation to the next.

## Results

### Labeling Chromosome Subsets *In Vivo*

Mammalian cell lines stably expressing core histones tagged with green fluorescent protein (GFP) provide a convenient means to study chromosome dynamics in living cells by fluorescence microscopy (Kanda et al., 1998). However, the uniform labeling of decondensed chromatin in interphase nuclei makes it impossible to track the position of individual chromosomes. To analyze chromosome dynamics globally through the cell cycle without labeling specific chromosomes by FISH or chromosomal elements by replication labeling, we devised a non-invasive imaging approach that allowed us to mark any subnuclear region of chromatin. Chromatin was double-labeled in normal rat kidney (NRK) cells stably expressing histone 2B (H2B) tagged with cyan fluorescent protein (CFP) by co-expressing H2B tagged with yellow fluorescent protein (H2B-YFP). By selectively photobleaching only YFP in part of the nucleus, combinatorial labeling with defined geometrical patterns could be achieved (Figure 1A). Due to the very low dissociation rate of H2B from chromatin (Kimura and Cook, 2001), the photobleached mark remained detectable for many hours, allowing imaging of the dynamics of labeled chromosomes by three-dimensional confocal time lapse microscopy (4D imaging) and quantitative image analysis (Gerlich et al., 2001; Figure 1B; Supplemental Figure S1 available at <http://www.cell.com/cgi/content/full/112/6/751/DC1>). Transient expression of H2B-YFP as

well as photobleaching and fluorescence microscopy did not significantly perturb cellular functions, as ~90% of the bleach-labeled cells went through mitosis without morphological defects.

### Global Chromosome Positions Are Stable throughout Interphase

In mammalian cells, both G1 and G2 phase of the cell cycle have been described as times when individual chromosomal loci are less constrained in their motion (Bridger et al., 2000; Chubb et al., 2002). Therefore, we were interested to determine to which extent global chromosome positions are affected by chromatin motion in G1, S phase, and G2. First, we investigated maintenance of chromosome neighborhoods in G1 nuclei by bleach labeling nuclear halves of two daughter cells in early G1 nuclei (~30 min after metaphase, Figure 1C,  $n = 28$  nuclei). The labeling boundary in both daughter nuclei was oriented perpendicularly to each other to allow detection of positional exchange along both the long and the short axis of the flat ellipsoid nuclei. Four-dimensional imaging showed that the labeling boundary was still clearly visible and similar to initial labeling after more than 5 hr (Figure 1C, 312 min). Gradual loss of contrast between bleached and unbleached regions due to incorporation of fluorescent H2B-YFP in the bleached regions (Kimura and Cook, 2001) and overall photobleaching of the YFP signal did not allow imaging of labeled chromosomes for longer periods (Figures S2 and S3 available at above website). We therefore investigated large-scale chromatin reorganization during S phase and G2 in separate sets of experiments. Cells were arrested at the G1/S transition by aphidicolin and bleach-labeled on nuclear halves immediately after release to allow cell cycle progression. Time lapse imaging did not reveal any significant long-range movements of chromosomes during S phase (Figure 1D,  $n = 24$  nuclei), G2, or early prophase (Figure 1E, 0–234 min;  $n = 20$  nuclei). This lack of interphase dynamics was not due to cell damage, since the bleach-labeled and synchronized cells still entered mitosis without obvious delay or morphological defects (Figure 1E, 228–249 min, Supplemental Movie S1E available at above website). In conclusion, these data show that no significant large-scale reorganization of relative chromosome positions occurred during interphase.

### Computer Simulation of Chromosome Dynamics during Mitosis

The observation that chromosome positions do not change on a large scale from early G1 to early prophase strongly suggested that positioning of chromosomes is determined in mitosis. Mammalian cells expressing GFP-tagged histones have already been used to analyze the dynamics of chromosome condensation (Beaudouin et al., 2002; Manders et al., 1999), mitotic movements, and postmitotic expansion (Kanda et al., 1998; Manders et al., 1999) (Figure 2A). However, it has not been possible to follow single chromosomes because of the high compaction of all chromosomes during mitosis (Figure 2A, 31 min). To understand the mechanism underlying mitotic positioning of individual chromosomes, we therefore decided to simulate their behavior based on

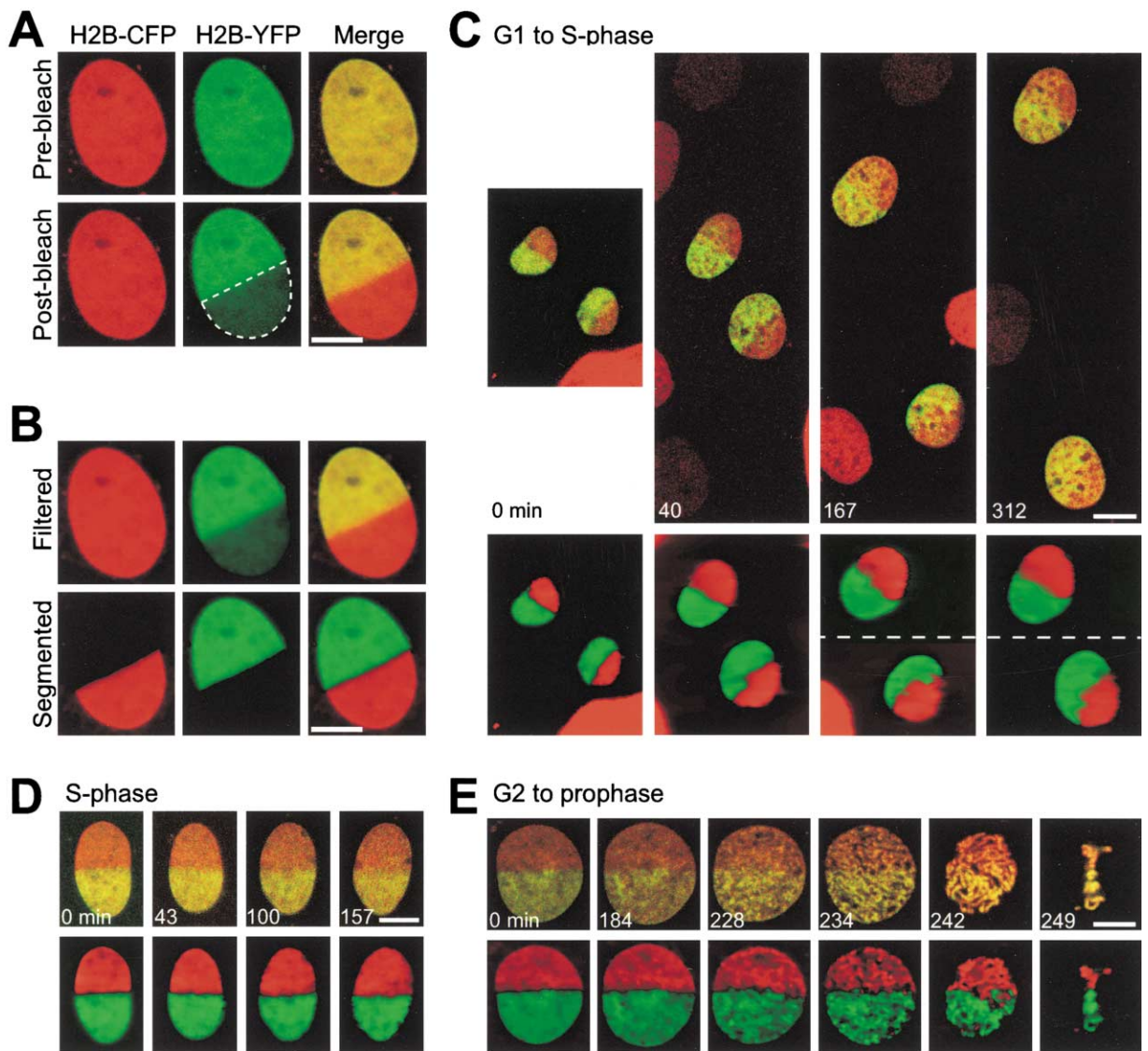


Figure 1. Global Chromosomal Order Is Stable throughout Interphase

(A–B) In vivo combinatorial labeling of nuclear subregions using pattern bleaching.

(A) NRK cell co-expressing H2B-CFP (red) and H2B-YFP (green) photobleached in the outlined region in the YFP channel.

(B) Segmentation of differentially labeled regions. (Filtered) For noise reduction, images were first processed by anisotropic diffusion filtering. (Segmented) Iterative subtraction of the CFP and YFP channels then resolved bleached and unbleached chromatin areas into separate color channels.

(C) Chromatin dynamics in G1. NRK cells bleach labeled  $\sim 30$  min after metaphase. Upper row shows a projection of 2 z-slices, lower row shows segmentations of the projections with the empty area between the cells not shown (dashed line).

(D) Chromatin dynamics in S phase. NRK cell bleach labeled immediately after release from aphidicolin block at the G1/S transition.

(E) Chromatin dynamics in G2 and prophase. NRK cell bleach labeled 3.5 hr after release from aphidicolin block. Bars are equal to 10  $\mu\text{m}$ . See also Supplemental Movie S1E available at <http://www.cell.com/cgi/content/full/112/6/751/DC1>.

known physical parameters and our own measurements of chromatin dynamics in NRK<sub>H2B-CFP</sub> cells (Figure 2A) (Beaudouin et al., 2002). The computer model is based on four basic assumptions (Figure 2B, Supplemental Material available at above website): (1) chromosomes occupy mutually exclusive volumes (and are modeled as spheres proportional to their DNA content) (Cremer and Cremer, 2001); (2) they condense and decondense isometrically without exchanging positions (Manders et al., 1999; Shelby et al., 1996); (3) their motion is directed toward the metaphase plate parallel to the axis of the

mitotic spindle during prometaphase and toward the spindle poles during anaphase; and (4) individual chromosomes move at random times in respect to others (Figure 2A, 13 min) (Alexander and Rieder, 1991). When the division of such a virtual nucleus containing 42 replicated chromosomes (corresponding to the NRK karyotype) was simulated, the predicted global chromosome dynamics were similar to the experimental observations (compare Figures 2A and 2C). The simulation predicted that positions of individual chromosomes should be randomized with respect to the mother nucleus (Figure 2B).

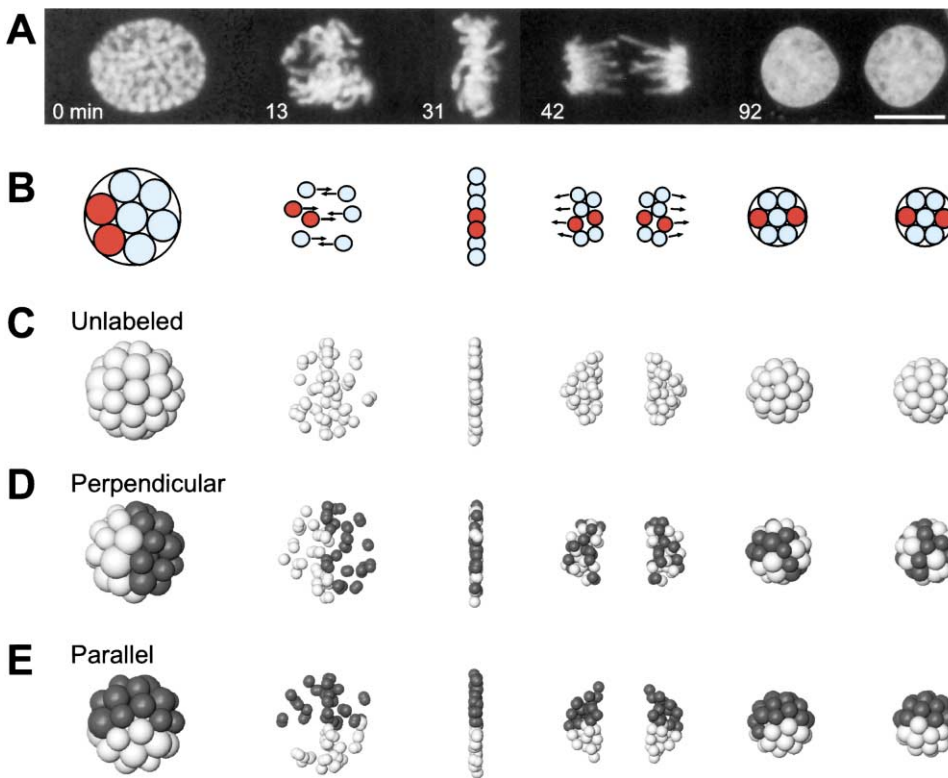


Figure 2. Computer Simulation of Chromosome Dynamics during Mitosis

(A) Projection of 3D time lapse of chromosomes during mitosis in an NRK cell stably expressing H2B-CFP. Long-range movements occur during congression to the metaphase plate (13 min) and after sister chromatid separation in anaphase (42 min). (B) Schematic illustration of the computer simulation. Two chromosomes are highlighted in red. After prophase condensation, chromosomes move toward the metaphase plate along linear trajectories (arrows) and sister chromatids move toward the spindle poles (arrows) in anaphase. (C–E) Simulated chromosome dynamics of a NRK karyotype with 42 chromosomes. (C) Overall dynamics are similar to *in vivo* observations in (A). (D) Simulation where chromosomes in opposite nuclear halves were assigned light and dark gray color with the labeling boundary oriented perpendicular to the spindle axis. (E) As before but parallel to the spindle axis. Bar is equal to 10  $\mu$ m. See also Supplemental Movies S2D and S2E available at <http://www.cell.com/cgi/content/full/112/6/751/DC1>.

The arrangement in the two daughter cells however, was predicted to be mirror symmetric as a result of the synchronous anaphase movements of sister chromatids (Figure 2B).

We decided to test whether the simulation made valid predictions, using our *in vivo* labeling approach in live mitotic cells (Figure 1A). Although bleach marks can create arbitrary patterns noninvasively (Beaudouin et al., 2002), labeling of entire single chromosomes remained difficult even in mitosis because of the poor z-discrimination of the bleach by single photon excitation. The computer simulation predicted however, that already a simple labeling geometry, i.e., bleaching one entire half of the nucleus, would be suitable to report position changes of chromosomes, provided the nuclear halves are labeled perpendicular to the axis of the mitotic spindle (Figure 2D, Supplemental Movie S2D available at <http://www.cell.com/cgi/content/full/112/6/751/DC1>). Labeling two nuclear halves in parallel to the spindle (Figure 2E, Supplemental Movie S2E available at above website) however, should not detect position exchanges, because almost no chromosome movements occur perpendicular to this axis (Figure 2A).

### Global Patterns of Chromosome Positions Are Inherited through Mitosis

We followed chromosome positions from early prophase to G1 by 4D imaging after labeling half of the nuclear volume by bleaching (Figure 3). The labeling boundary was oriented randomly at the start of the experiment. After division was completed, experiments were classified as being either parallel ( $\pm 45^\circ$ , Figure 3A) or perpendicular ( $\pm 45^\circ$  Figure 3B, Supplemental Movie S3B available at above website) to the mitotic axis and in some cases precisely diagonal labeling was produced (Figure 3C). Surprisingly, regardless whether cells had been labeled parallel ( $n = 14$ ) or perpendicular ( $n = 12$ ), 4D imaging showed that the global pattern of the mother cell was transmitted to the two daughter nuclei in G1 in a mirror symmetric fashion (representative examples shown in Figures 3A–3C). This was expected from the simulation for parallel labeling (compare Figure 2E), and the experiments showed that the relative position of the chromosomes perpendicular to the mitotic axis is preserved both during prometaphase congression as well as in anaphase and telophase (Figure 3A). For perpendicular labeling however, transmission

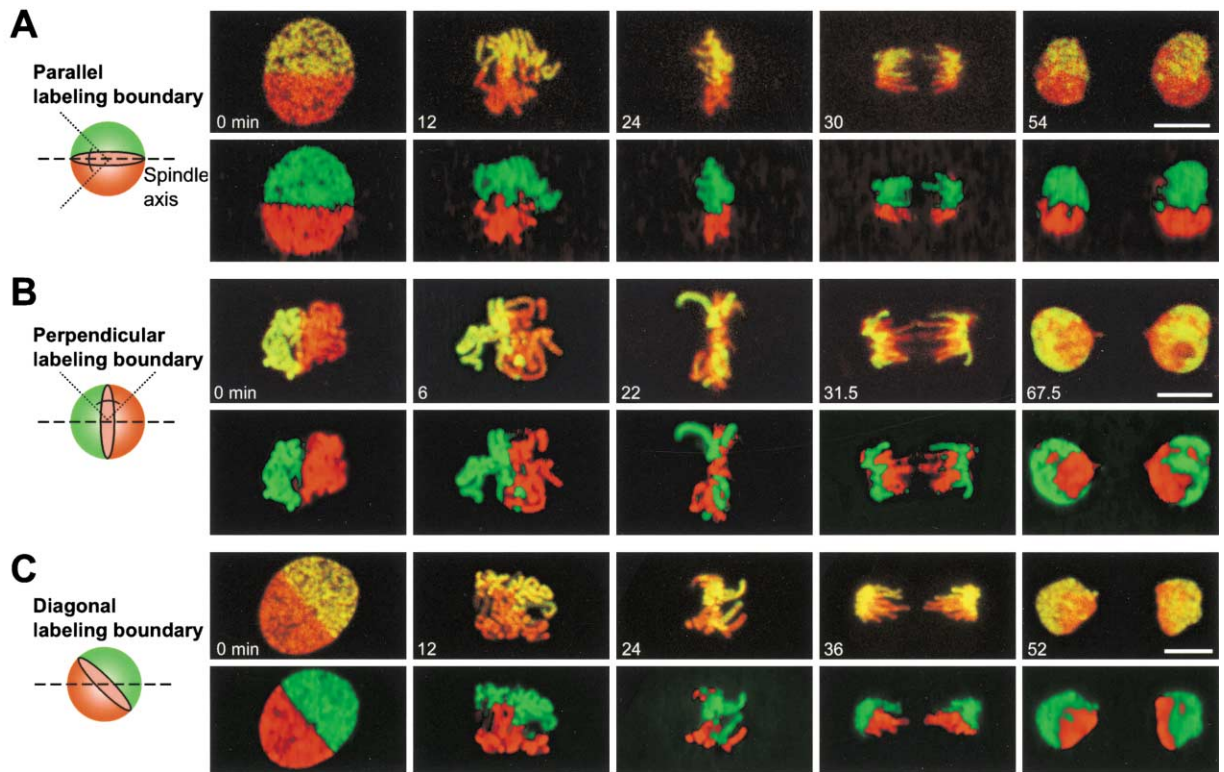


Figure 3. Positioning of Labeled Chromosome Subsets in Mitotic Cells

NRK cells were bleach-labeled in prophase and imaged in 4D through mitosis. The orientation of the labeling boundary in the prophase nucleus was classified after division by the orientation of the metaphase plate.

(A) Labeling boundary oriented parallel to the spindle axis. Projections of 5–10 z-slices (upper row) and segmentation of the projections (lower row; red = bleached regions, green = unbleached regions) are shown.

(B) Perpendicular orientation.

(C) Diagonal orientation. Bars are equal to 10  $\mu\text{m}$ . See also Supplemental Movie S3B available at <http://www.cell.com/cgi/content/full/112/6/751/DC1>.

of chromosomal order was completely unexpected and inconsistent with random positioning of chromosomes in mitosis (compare Figure 2D). From prophase to metaphase, the labeled chromosomes intermixed, as predicted by the simulation (compare Figures 3B and 2D). In anaphase and telophase however, the original global pattern of the mother cell was restored, leading to mirror symmetric half-labeled daughter nuclei (Figure 3B). Thus, relative chromosome positions were transmitted also along the mitotic spindle. Diagonally labeled cells exhibited intermediate behavior, with some intermixing in prometaphase (Figure 3C) and again restoration of mirror symmetric daughter nuclei that resembled the mother nucleus. We concluded that global chromosome positions are not randomized either perpendicular or parallel to the spindle axis throughout mitosis.

#### Positions of Individually Labeled Chromosomes Are Transmitted through Mitosis

While labeling half the nuclear volume could demonstrate transmission of global chromosomal order, some intermixing of labeled regions after mitosis was also observed. This could be caused by movements of individual chromosomes (Bridger et al., 2000; Chubb et al., 2002) or repositioning of chromosome arms of partially

bleached chromosomes. To investigate the behavior of single chromosomes directly, we therefore restricted the labeling to small peripheral spots, which at the nuclear rim marked on average only a single chromosome (Figure 4A, 33 min). Similar to the half-labeled nuclei, the position of the spots in the daughter nuclei of NRK cells was similar in a mirror symmetric fashion and resembled the arrangement in the mother nucleus (Figure 4A). As already observed in the half bleach experiments, the accuracy of repositioning had some variation both parallel as well as perpendicular to the spindle axis. The left daughter nucleus in Figure 4A shows slight randomization perpendicular to the spindle axis (the rightmost spot is shifted down) while the right daughter nucleus shows minor randomization parallel to it (the topmost spot is shifted to the left). To quantitate positioning accuracy of single chromosomes, we analyzed 69 daughter cells whose mothers had been labeled with two small spots on opposite sides of the nucleus. The two spots were oriented either parallel ( $\pm 45^\circ$ ) or perpendicular ( $\pm 45^\circ$ ) to the spindle axis (purple line in Figure 4B). In the daughter nuclei, the distance of the spot center to nuclear rim was then measured either parallel or perpendicular to the spindle corresponding to the orientation of the spots before mitosis (Figure 4B). Confirming the qualitative observation in Figure 4A, we found that posi-

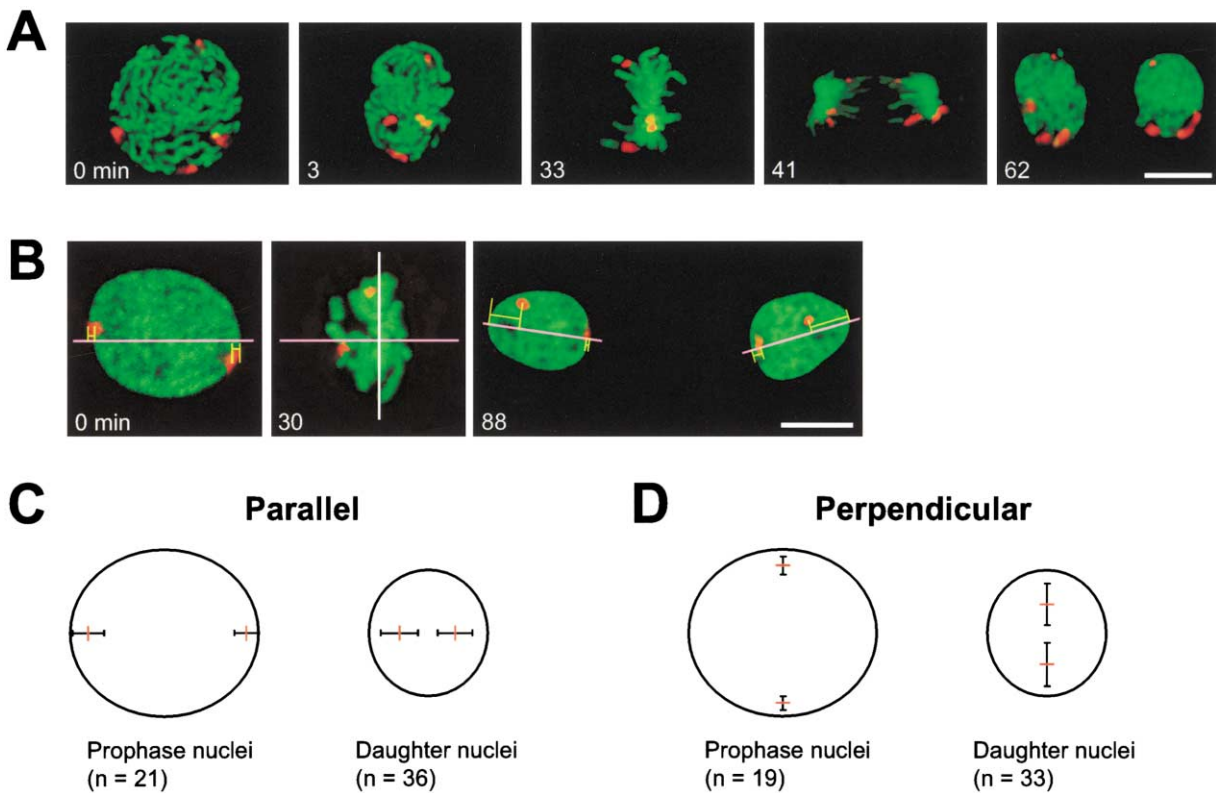


Figure 4. Positioning of Single Chromosomes in Mitotic Cells

(A) Segmented projections of a 4D recording of NRK chromosomes labeled in prophase on 4 chromosomes by spot bleaching. (B) Quantitative assay for repositioning of single chromosomes. Two NRK chromosomes were labeled with spots on opposite sides of the nucleus in prophase. Cells were classified as perpendicular or parallel according to whether the spots in prophase were on a line parallel or perpendicular ( $\pm 45^\circ$ ) to the spindle axis (purple line), which was defined perpendicularly centered on the metaphase plate (solid white line). Slight nuclear rotations (median angle:  $15.7^\circ$ ) were corrected according to the morphology of daughter nuclei. Bars are equal to  $10 \mu\text{m}$ . (C–D) Schematic representation of positioning accuracy in 69 daughter cells (the number of daughters is slightly lower than twice the mothers, because of low signal in some daughter cells). Median distance (measurement indicated in yellow in B) and standard deviation of the spot center from the nuclear rim on a centered axis in the daughter nucleus parallel or perpendicular to the spindle axis (purple line in B) is plotted. The distributions in parallel and perpendicular daughters were evaluated for significant differences using a Mann-Whitney U test.

tioning accuracy is not significantly different either parallel or perpendicular to the spindle axis ( $p = 0.27$ ). In both directions, more than 90% of the spots were positioned on the correct side of the nucleus, but proximity to the rim had some variation (Figures 4C and 4D). We therefore concluded that the positions of single chromosomes are similar before and after mitosis and that they are positioned with the same accuracy along or perpendicular to the spindle.

#### Chromosome Positions Are Projected on the Metaphase Plate during Congression

Our experimental data clearly refuted the prediction of the computer simulation that chromosomes are randomized along the mitotic axis. Since chromosome motion along the spindle axis occurs mainly during prometaphase congression and anaphase segregation, we focused first on chromosome congression. Both perpendicularly and parallel-labeled nuclei were analyzed in x-z projections viewed from the spindle pole from prophase to metaphase. In parallel-labeled cells, the metaphase plate was clearly divided into two differen-

tially labeled halves (Figure 5A). This was expected from the simulation and demonstrated that most chromosomal movements are directed linearly toward the metaphase plate and result in a two-dimensional projection of their interphase positions onto the plate (Figure 5A). For perpendicularly labeled cells, we found that the two sets of labeled chromosomes completely intermixed in the metaphase plate with no detectable preference of bleached or unbleached chromosomes for either side of the plate (Figure 5B). To ensure that the global labeling approach of nuclear halves did not fail to detect changes in the positioning of individual chromosomes, we also investigated the behavior of centromeres directly. Centromeres are the points where kinetochore microtubules exert force on the chromosome (Alexander and Rieder, 1991) and are thus a good reference for single chromosome mitotic positioning. In cells co-expressing H2B tagged with a dimeric red fluorescent protein (diHcRed see Experimental Procedures) and the centromere-specific variant of histone H3 (CENP-A) fused to EGFP, we tracked centromere positions during congression by 4D imaging with maximal spatial and temporal resolution to resolve the more than 40 paired points in a mammalian

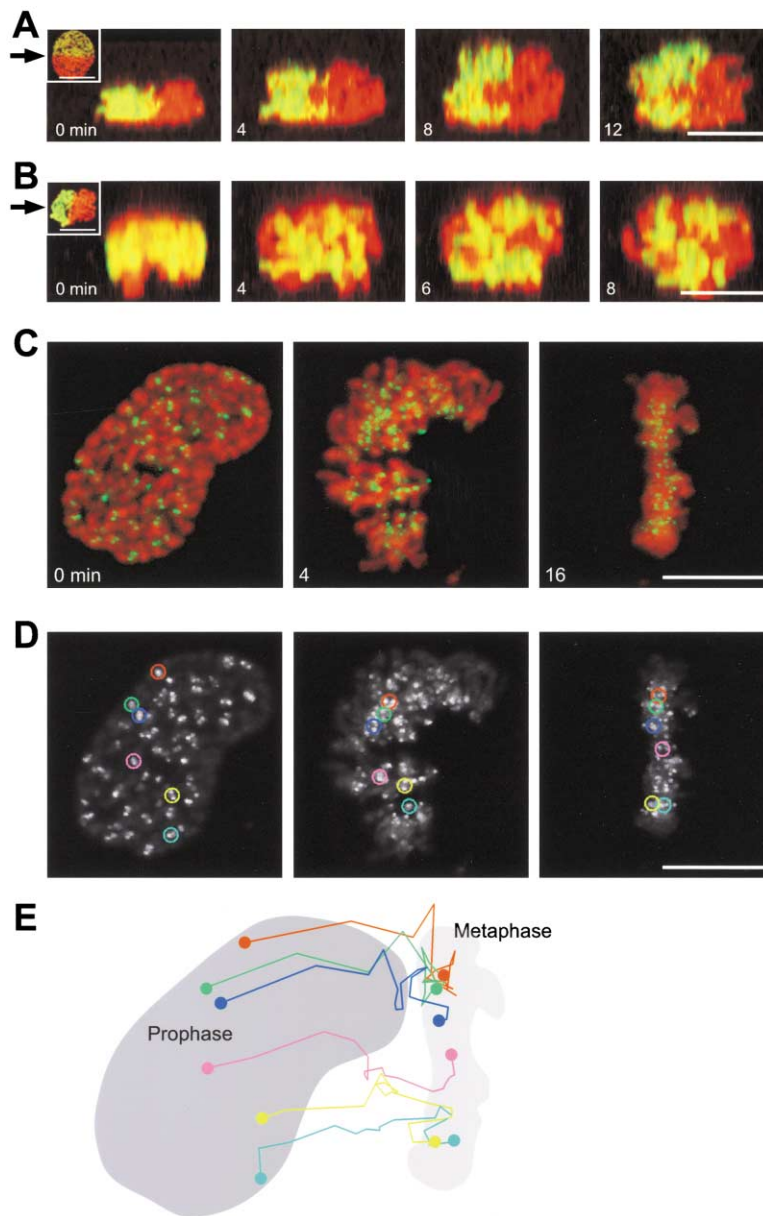


Figure 5. Chromosome Dynamics during Congression

(A–B) The same NRK cells shown in Figures 3A and 3B viewed as x-z projections of 16 z-slices acquired with 1  $\mu\text{m}$  z-step, viewed from the position of the spindle pole are shown from prophase to metaphase. Insets show x-y projections, arrow indicates the direction of view for the x-z projection.

(A) Parallel labeled nucleus.

(B) Perpendicular labeled nucleus.

(C–E) Tracking of centromeres from early prophase to metaphase. CENP-A-EGFP (green) and H2B-diHcRed (red) were co-expressed in HeLa cells and imaged in 4D with a time lapse of 1 min.

(C) Projections of 16 z-sections at selected time points are displayed.

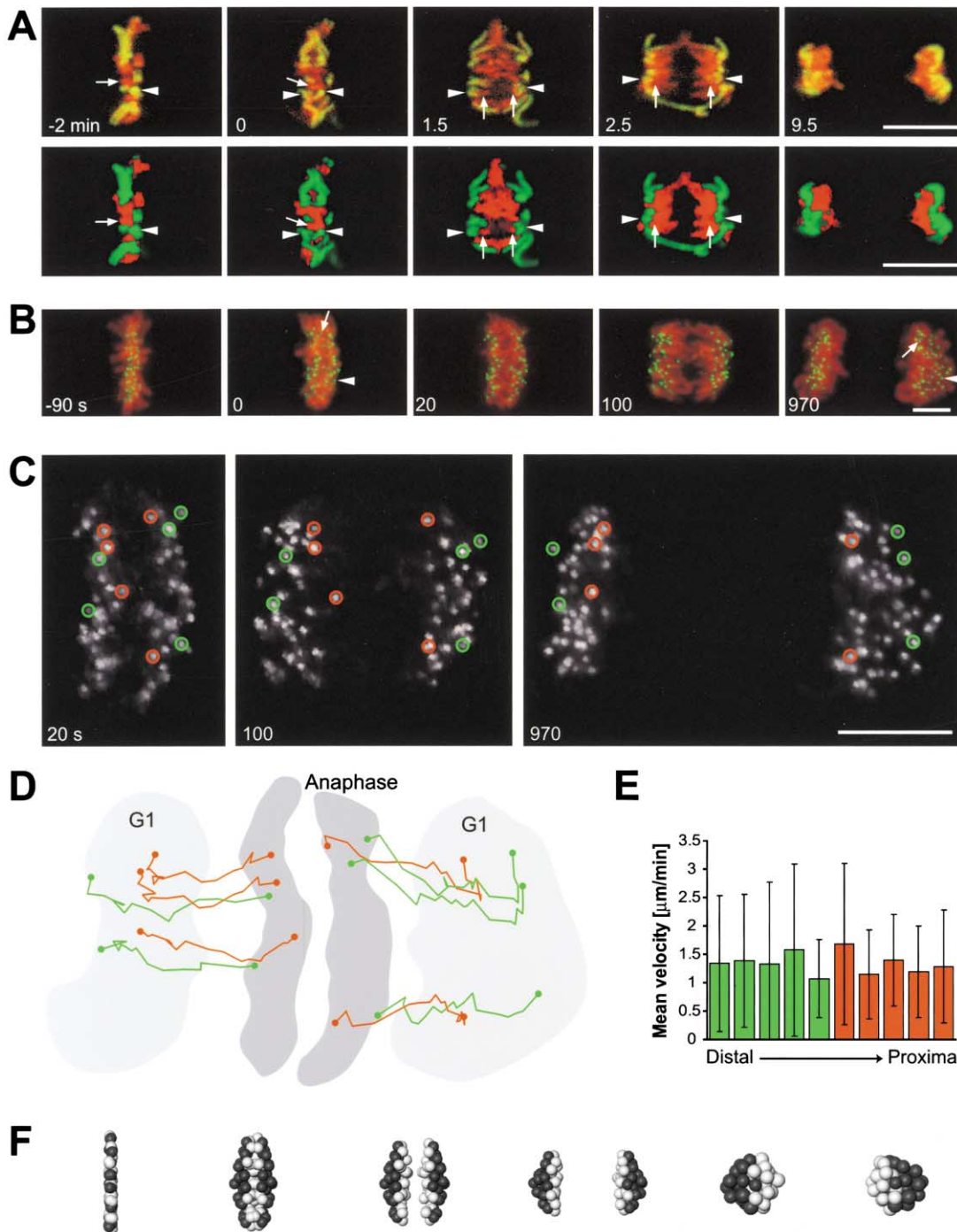
(D) 4D tracking of selected centromeres.

(E) x-y projections of the trajectories from the centromeres highlighted in (D), superimposed on the segmented prophase (dark gray) and metaphase chromatin regions (light gray). To facilitate visual interpretation, the metaphase contour was displaced about 15  $\mu\text{m}$  to the right side. Bars are equal to 10  $\mu\text{m}$ . See also Supplemental Movie S5D available at <http://www.cell.com/cgi/content/full/112/6/751/DC1>.

nucleus (Figure 5C). In the complete set of 3D stacks, we could typically track about six centromeres unambiguously from early prophase to their final positions in metaphase (Figure 5D, Supplemental Movie S5D available at <http://www.cell.com/cgi/content/full/112/6/751/DC1>). Centromere trajectories were superimposed on a projection of segmented early prophase nucleus and the metaphase plate based on the H2B-diHcRed labeling (Figure 5E). Consistent with the half nuclear labeling, centromere order perpendicular to the spindle axis was preserved in the metaphase plate, while their relative positions along this axis were lost in the flat metaphase plate (Figures 5D and 5E). This data showed that chromosome congression results in a simple linear projection of prophase chromosome positions onto the metaphase plate, which no longer contains spatial information about their original positions along the spindle axis.

### Chromosome Positions along the Spindle Are Re-Established at Anaphase Onset

Our results strongly suggested that a non-random mechanism must exist that restores the mother cell configuration from metaphase to anaphase. To investigate this, we examined metaphase to telophase dynamics of chromosomes in more detail in perpendicularly labeled cells. Single confocal sections in such datasets revealed that sister chromatids destined for a more poleward position in the daughter nucleus separated prior to chromosomes that would remain closer to the cleavage furrow (Figure 6A, Supplemental Movie S6A available at above website). This difference in timing was independent of the bleached/unbleached status of the chromosomes (compare Figure 7A) but correlated with the position of the chromosome along the spindle axis in the daughter nuclei.



**Figure 6. Transmission of Order along the Mitotic Spindle**

(A) A thin confocal section from the same NRK cell shown in Figure 3B bleach-labeled on nuclear halves with perpendicular labeling boundary, inspected from metaphase to G1. An early (arrowheads) and a late (arrows) separating pair of sister chromatids is shown as a projection of two z-slices (upper row) and segmentation of the projection (lower row).

(B–D) Tracking of centromeres from early anaphase to G1. CENP-A-EGFP (green) and H2B-diHcRed (red) were co-expressed in HeLa cells and imaged in 4D with variable time lapse (20 s in anaphase to 120 s in G1, 10 z-sections with 1  $\mu\text{m}$  step). To allow fast imaging at high spatial resolution, only the lower half of the cell was recorded.

(B) Projections of selected time points are displayed. The arrowhead points to an early separated centromere ( $t = 0$  s), which localizes to the distal side in the daughter nucleus ( $t = 970$  s); the arrow highlights a late separating centromere, which localizes to a proximal part of the daughter nucleus.

(C) 4D tracking of selected centromeres from early anaphase to G1. Distal centromeres are highlighted on the projection of the CENP-A-EGFP channel by green circles, proximal centromeres by red circles.

(D) x-y projections of the trajectories from the centromeres highlighted in (C), superimposed on the segmented anaphase chromatin regions (dark gray) and G1 daughter nuclei (light gray).

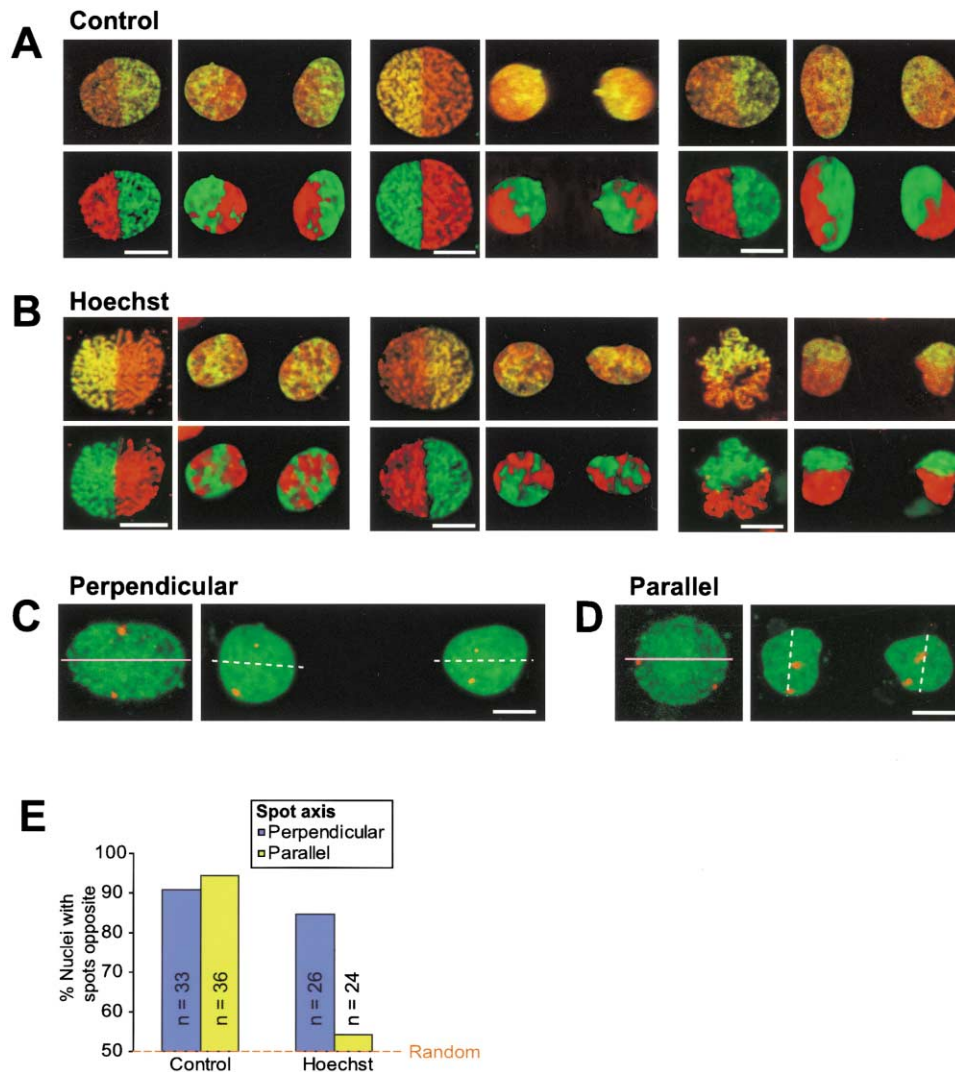


Figure 7. Randomization of Chromosome Positioning along the Spindle Axis

(A) Perpendicularly labeled control cells before and after mitosis.

(B) As in (A) for cells treated for 20 hr with 10  $\mu$ g/ml Hoechst 33258. Right images show a parallel-labeled cell.

(C–D) Single chromosome positioning in Hoechst-treated cells. Cells were classified as in Figure 4B. Daughter nuclei were scored as ordered if the two spots were found on opposite sides of a central axis (dashed white lines) parallel (C) or perpendicular (D) to the spindle axis (purple line, determined as in Figure 4B). Slight nuclear rotations (median angle: 15.7°) were corrected according to the morphology of daughter nuclei. Bars are equal to 10  $\mu$ m.

(E) Statistical analysis of 69 control and 50 Hoechst-treated daughter nuclei. Dashed red line indicates random positioning. Differences from a random distribution in Hoechst and control cells were evaluated using a binomial test.

To follow individual chromosomes from anaphase onset to G1, we again imaged individual centromeres directly in cells co-expressing CENP-A-EGFP and H2B-diHcRed (Figure 6B). Typically, about 10 centromeres could be tracked unambiguously and were analyzed by superimposition of their trajectories from metaphase to

G1 on chromatin regions (Figures 6C and 6D, Supplemental Movie S6C available at above website). Confirming our observations in perpendicularly half-labeled nuclei (Figure 6A), we found that centromeres that separate early in anaphase moved to parts of the daughter nuclei distal from the cleavage furrow (green in Figures 6C

(E) Mean velocities of anaphase centromeres. Quantitation of centromere trajectories shown in (D) was from  $t = 20$  s, where all sister chromatids have separated until  $t = 490$  s, where spindle poleward movement ceased. Each column represents mean velocity ( $\pm$  stddev.) of an individual trajectory. Columns were sorted according to proximal (red)/distal (green) position of centromeres in daughter nuclei relative to the cleavage furrow. (F) Computer simulation of chromosome dynamics with chromosome specific time of sister separation. Virtual nuclei were labeled perpendicular to the spindle axis in prophase. The position of each chromosome along the spindle axis in prophase determined its time of anaphase onset. Only metaphase to G1 is shown for comparison with (A). Bars are equal to 10  $\mu$ m. Time 0 corresponds to anaphase onset. See also Supplemental Movies S6A, S6C, and S6F available at <http://www.cell.com/cgi/content/full/112/6/751/DC1>.

and 6D). Quantitation of anaphase mean velocity after complete separation of all sister chromatids showed no significant differences between centromeres that moved to proximal or distal parts of daughter nuclei (Figure 6E). Subsequent expansion of daughter nuclei occurred isometrically without long-range centromere movements, thus preserving their relative neighborhood (Figures 6C and 6D; better appreciated in Supplemental Movie S6C available at <http://www.cell.com/cgi/content/full/112/6/751/DC1>).

We conclude that chromosome positions along the spindle axis are re-established by timing differences of sister separation at anaphase onset and maintained during spindle-poleward movements of chromosomes and expansion of daughter nuclei. To show that such a mechanism can operate in principle based on known physical properties of chromosomes, assumption (iv) of the computer model was modified such that chromosome dynamics were simulated with a chromosome specific time of anaphase onset according to its position along the spindle axis in the mother nucleus (Figure 6F, Supplemental Movie S6F available at above website). This modified simulation corresponded well to the experimental results from bleach-labeled cells (compare Figure 6F, 6A, and Supplemental Movies available at above website). Chromosomes that separated first in early anaphase were predicted to move to distal parts of the daughter nuclei (Figures 6B–6E) and relative neighborhoods would be preserved by subsequent uniform isometric expansion (Figures 6B–6D, Supplemental Movie S6C available at above website) and the lack of global rearrangements throughout interphase (Figure 1).

#### **Perturbation of Chromosome Positions along the Spindle Axis**

Our experimental and computational results suggested that chromosome specific difference in the timing of sister chromatid separation at anaphase onset coupled with the absence of whole chromosome movement in interphase are sufficient to explain the mechanism of position inheritance from one cell generation to the next. To gain insight into the timing mechanism, we tested if position inheritance can be perturbed by agents that interfere with the condensation of constitutive heterochromatin because it is required to form fully functional centromeres (Bernard et al., 2001; Taddei et al., 2001), the site where sister chromatid separation is initiated. Therefore, we carried out 4D imaging experiments of half-bleached nuclei undergoing mitosis after incubating the cells 20 hr with the DNA minor groove binder Hoechst 33258 that prevents formation of constitutive heterochromatin (Haaf and Schmid, 2000; Vig and Willcourt, 1998). Metaphase spreads of Hoechst-treated NRK cells showed that their centromeres were less condensed and their chromosomes generally less compacted compared to untreated cells (data not shown). In drug-treated cells that had been labeled perpendicular to the spindle axis, the positions of chromosomes were frequently disturbed ( $n = 9$ ) compared to highly ordered control cells ( $n = 12$ , compare Figures 7A and 7B). The perturbed G1 arrangement was quite similar to what was predicted by the computer simulation for a random distribution of chromosomes along the spindle

axis (compare Figures 7B and 2D). As expected, such randomization was not detected when chromosomes were labeled parallel to the spindle axis ( $n = 9$ ) as very little movement occurs perpendicular to the spindle axis in a normal mitosis (Figure 7B, right). To quantitate the effect of Hoechst on single chromosome positioning, we again labeled nuclei with two spots on opposite sides of the nucleus to measure order perpendicular ( $\pm 45^\circ$ ) (Figure 7C) or parallel (Figure 7D) ( $\pm 45^\circ$ ) to the spindle. Because in Hoechst-treated cells both spots were frequently on the same half after mitosis (Figure 7D), we could not use distance to the nuclear rim as a measure of order (compare Figure 4B). Rather, we measured if the two spots were positioned on opposite or the same side of the daughter nuclei corresponding to the prophase orientation of the spots. Confirming the results of the half bleach experiments (Figure 7B), we found that Hoechst strongly affected positioning parallel to the spindle axis but had no effect on perpendicular spots (Figures 7C and 7D). Whereas in control cells 94% of the daughter nuclei whose mothers had parallel spots ( $n = 36$ ) were ordered, only 54% of the Hoechst-treated daughters ( $n = 26$ ) with this orientation also had spots on opposite sides (Figure 7E). This was not significantly different ( $p = 0.15$ ) from randomly distributed chromosomes where 50% of the nuclei should remain ordered (Figure 7E, random), while the control cells were highly unlikely to result from a random distribution ( $p = 9 \times 10^{-9}$ ). By contrast, daughter nuclei whose mothers had perpendicular spots were highly ordered in control (91%,  $n = 33$ ,  $p = 6 \times 10^{-7}$ ) as well as in Hoechst-treated cells (85%,  $n = 26$ ,  $p = 2 \times 10^{-4}$ ). These results suggested that condensation of constitutive heterochromatin is required to restore chromosome positions in anaphase and demonstrate quantitatively that interference with chromosome structure can randomize global position inheritance of chromosomes.

#### **Discussion**

##### **Relative Chromosome Topology Is Stable during Interphase**

In this study, we addressed the question when and how non-random chromosome positions are established in mammalian cells. Exploiting a noninvasive labeling strategy of arbitrary chromatin domains, we examined live cells throughout the cell cycle by 4D imaging. Our initial experiments demonstrated that during interphase, in G1, S, and G2 phase of the cell cycle, global chromosome arrangements did not undergo significant changes. Previous studies that examined the dynamics of individually labeled loci have described constrained motion of small domains in interphase. In yeast and *Drosophila*, chromosome dynamics are characterized by slow constrained diffusional motion (Marshall et al., 1997; Vazquez et al., 2001) and appear to be regulated by the cell cycle stage and to depend on metabolic energy (Heun et al., 2001; Vazquez et al., 2001). Similarly, also in mammalian cells chromatin has been shown to be largely immobile. Individual loci or centromeric/photobleached regions undergo only slow diffusional motion confined to a radius of less than 1  $\mu\text{m}$  (Abney et al., 1997; Chubb et al., 2002; Kimura and Cook, 2001; Shelby et al., 1996)

and only rarely were single domains found to move 1–3  $\mu\text{m}$  (Shelby et al., 1996; Tumber and Belmont, 2001; Zink et al., 1998). These data are consistent with our findings that long-range movements of chromatin are not seen in interphase if chromosomes are labeled globally. In special cases, however, long-range movements of entire chromosome territories have been reported. Radial position changes in G1 have been documented by FISH after quiescent cells re-entered the cell cycle and required passage through S, G2, and M phase. We did not observe such long-range chromosome reorganization in any stage of G1 in proliferating cultures. Our conclusion that global positioning occurs in mitosis provides an explanation for the M phase requirement for genome rearrangement in these studies (Bridger et al., 2000). In addition, several reports using highly amplified reporter genes (lac operator or MMTV arrays) labeled by DNA binding protein:GFP-fusions have documented dramatic changes in their condensation status. These changes were seen during replication (Li et al., 1998) or as a result of transcriptional activation (e.g., Muller et al., 2001; Tsukamoto et al., 2000; Tumber and Belmont, 2001) and were sometimes accompanied by repositioning of the reporter locus (Li et al., 1998; Tumber and Belmont, 2001). However, it is unclear if such behavior is common for endogenous single copy genes and to what extent it could reorganize the topology of the whole nucleus. Our results strongly indicate that for the entire genome, repositioning by replication and transcription is minor and does not lead to massive changes of global chromosome arrangements.

#### **Global Chromosome Positions Are Transmitted through Mitosis**

When we examined the arrangement of labeled chromosomes in dividing mammalian cells we found that the global arrangement of chromosomes was transmitted from one cell generation to the next. Although most of our experiments have been performed in NRK and HeLa cells, we have obtained similar results in other cell lines such as human HCT116 and kangaroo rat PtK2 cells (Supplemental Figure S4 available at <http://www.cell.com/cgi/content/full/112/6/751/DC1>) suggesting that many mammalian cells inherit global chromosome positions. Our data is consistent with previous observations that the nuclear and even subchromosomal position of single chromosomal elements can be clonally inherited (Dietzel and Belmont, 2001; Robinett et al., 1996). It is also in line with data showing that radial organization of late and early replicating domains can be observed over two cell generations (Sadoni et al., 1999) and that global patterns of replication labeling persist for several divisions (Ferreira et al., 1997; Sadoni et al., 1999). These similarities, however, were assumed to reflect a radial nuclear organization determined by preferential attachment of silent chromosomes to the nuclear envelope in telophase and G1 (Boyle et al., 2001). The inheritance of order we observed here was more precise than expected for a simple radial organization of chromosomes according to their gene density, because such a mechanism should not result in preferred positions relative to the spindle axis but just to the center of the nucleus. Our data is consistent with such a radial organization and

does not argue against nuclear envelope attachments to function in stabilizing the postmitotic chromosome arrangement in telophase and interphase. However, our results on the dynamics of individual centromeres and sister chromatids in anaphase strongly argue that the interphase arrangement is already established within minutes after sister chromatid separation in early anaphase, before the nuclear envelope is present. Except for decondensation, this arrangement underwent little global changes from telophase to the end of G2. Thus, mitotic positioning preceded a possible anchoring by the nuclear envelope and it will be interesting to dissect the contributions of these two mechanisms to the spatial organization of the genome in future studies.

#### **Simulation and Experiment: Chromosome-Specific Anaphase Onset**

Given the complexity of rodent and human karyotypes and the seemingly chaotic mixing of more than 40 chromosomes during mitosis, the transmission of global positions was very striking. To predict chromosome dynamics in mitosis, we built a mathematical model based on known parameters of chromosome behavior. The model correctly simulated chromosome dynamics when compared to microscopic observations of uniformly labeled chromosomes. When used to simulate the outcome of pattern bleaching experiments, the simulation predicted random patterns of daughter nuclei relative to the mother nucleus that should be detectable in nuclei labeled perpendicular to the spindle axis. This was clearly refuted by the live cell observations that daughter nuclei strongly resembled the mother nucleus. The experimental finding that differences in sister separation during anaphase quickly re-established chromosome positions along the spindle axis, led us to introduce an additional chromosome specific parameter that would determine when a particular chromosome entered anaphase in each cell cycle. This revised model precisely reproduced the experimental data suggesting that a single chromosome specific property could in principle be sufficient *in vivo*.

#### **A Possible Molecular Basis of Chromosomal Position Information**

Which chromosomal feature could determine a specific onset of anaphase? A clue came from earlier studies on chromosome spreads from colcemid-arrested mammalian cells. In those experiments, different chromosomes were seen to reproducibly separate sister chromatids at different times in anaphase (Vig, 1983). This sequence of chromosome separation could be altered in cells treated with Hoechst 33258 (Vig and Willcourt, 1998) but not by spindle poisons and correlated with the amount of dense centromeric chromatin (Vig, 1983). Our observation that chromosome positioning along the mitotic axis is randomized upon application of Hoechst 33258 in live cells shows that transmission of order is not simply a “mechanical default” but rather a specific mechanism that is sensitive to drug treatment. This suggests that a chromosome specific and Hoechst sensitive feature determines position inheritance and the amount of pericentric heterochromatin is a good candidate, because it fulfills both criteria (Haaf and Schmid, 2000; Vig and

Willcourt, 1998). We propose that a higher amount of pericentric heterochromatin, which represents the major binding site for cohesins in human metaphase cells (Waizenegger et al., 2000), would result in stronger sister cohesion and delay sister separation. Consistent with this hypothesis, it is known that increases in centromere DNA length can delay sister separation of minichromosomes in yeast (Tanaka et al., 1999). It will be interesting to test whether structural differences between centromeres determine transmission of chromosome position in mammalian cells.

### Biological Relevance of Defined Chromosome Positions

What could be the biological function of positioning chromosomes in mitosis? It is conceivable that inheritance of chromosome order could function as an epigenetic mechanism. Higher order chromatin structure has emerged as an important factor that can influence gene expression (reviewed in Gasser, 2001) and there are indications that the nucleus is organized in transcriptionally preferentially silent or active nuclear subcompartments (Baxter et al., 2002; Galy et al., 2000; Pombo et al., 1999), which may increase the efficiency of gene expression or silencing events within them (Francastel et al., 2000). Positioning chromosomes in similar subnuclear regions from one cell generation to the next could thus facilitate the maintenance of global patterns of gene expression in mammalian cells. Daughters would by default resemble mother cells, but could then most likely change chromatin structure within that context to allow variation. Consistent with this idea, our data show that the accuracy of transmission is not 100% but that some intermixing of chromatin domains does occur already within one cell generation. We predict that strict transmission of order will be diluted further over several cell cycles. It will be very interesting to investigate mitotic chromosome positioning during embryonic development or in *in vitro* differentiation systems where large scale changes in gene expression and chromatin organization are expected to occur in a controlled manner (Borden and Manuelidis, 1988; Francastel et al., 2000; Martou and De Boni, 2000).

### Experimental Procedures

#### DNA Constructs, Cell Lines, and Cell Culture

H2B-diHcRed, was generated from a Xho1/BamH1 fragment containing the coding sequence for H2B from pH2B-CFP (Ellenberg et al., 1999), which was ligated into those sites pdiHcRed1-N1. pdiHcRed1-N1 is a modification of pHcRed1-N1 (Clontech Laboratories, Palo Alto, California) in which HcRed is replaced by a HcRed tandem spaced by a 17-mer glycine/alanine linker to favor an intramolecular dimer. H2B-YFP contains a Kpn1/BamH1 fragment encoding full-length H2B ligated into those sites in pEYFP-N1 (Clontech). For CENP-A-EGFP (a kind gift of Kevin Sullivan), full-length human CENP-A HA-tagged at its 3' end was inserted into pEGFP-C1 into Sma1/Xba1 sites.

For pattern bleaching experiments, NRK cells stably expressing H2B-CFP (Beaudouin et al., 2002) were transfected with H2B-YFP using FuGene 6 (Roche, Mannheim, Germany). For microscopy, cells were cultured in #1 LabTekII chambered cover glasses (LabTek, Naperville, IL) and maintained at 37°C on the microscope stage as described (Gerlich et al., 2001). Where indicated, 10 µg/ml Hoechst 33258 was added to the medium 20 hr prior to imaging. Synchronization was by arresting cells at the G1/S boundary 48 hr posttransfec-

tion for 15 hr in 0.5 µg/ml Aphidicolin, followed by a 7 hr release. For centromere imaging, HeLa cells were cotransfected with H2B-diHcRed and CENP-A-EGFP.

#### 4D Live Cell Microscopy and Noninvasive Chromatin Labeling

The 4D imaging system consisted of a Zeiss LSM 510 confocal microscope (Carl Zeiss, Göttingen, Germany) customized for fluorescent protein imaging and has been described in detail elsewhere (Gerlich et al., 2001). A typical z-stack was 512 × 512 × 15 with a 1.3 µm z-step, acquired within ~5 s using a 63× PlanApochromat 1.4 NA oil immersion objective. Nuclei were bleached in early prophase, to minimize phototoxic effects and loss of signal before entry into mitosis. Time lapse was typically set to 2–4 min, except for centromere imaging where stacks were acquired up to every 20 s. To label specific chromatin regions, half the nuclear volume or single spots were photobleached by two scans with 100% transmission of the 514 nm Ar laser line. This photobleached YFP fluorescence while signal from CFP remained unchanged. Maximum intensity projections were carried out using the LSM 2.8 software (Carl Zeiss). Because the identity of chromosomes cannot be determined in live cells, this approach can label single, but not specific chromosomes (e.g., discriminate chromosome #19 from #18). Anisotropic diffusion filters (Tvaruskó et al., 1999) or median filters were occasionally used to reduce background noise. To display image sequences, global translational and rotational movements were eliminated (Gerlich et al., 2001). Manual 4D tracking was carried out using the LSM 2.8 Software (Zeiss) and trajectories were visualized with Excel 2000 (Microsoft). For details of the image processing, see Supplemental Figures S1–S2 available at <http://www.cell.com/cgi/content/full/112/6/751/DC1>.

#### Computer Simulation of Mitotic Chromosome Dynamics

Chromosome movements from early prophase to G1 were simulated in a geometric force model. The model included cellular structures that affect distribution and relative movements of chromosomes, i.e., chromosomes, the nuclear rim, centrosomes, and spindle microtubules. Chromosome movements depended on microtubule forces, volume exclusion forces between chromosomes, forces exerted by the nuclear boundary, and Brownian motion. A detailed description of the model is provided as Supplemental Material available at above website.

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