Chromosomes Can Congress To The Metaphase Plate Prior To Bi-Orientation

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Abstract

The stable propagation of genetic material during cell division depends on the congression of chromosomes to the spindle equator before the cell initiates anaphase. It is generally assumed that congression requires that chromosomes are connected to the opposite poles of the bipolar spindle (i.e., “bi-oriented”). We found that chromosomes can congress before becoming bi-oriented. By combining the use of reversible chemical inhibitors, live-cell light microscopy and correlative electron microscopy, we found that mono-oriented chromosomes could glide towards the spindle equator alongside kinetochore fibers attached to other already bi-oriented chromosomes. This congression mechanism depended on the kinetochore-associated plus end-directed microtubule motor CENP-E (kinesin-7).
Successful cell division requires proper “bi-orientation” of chromosomes whereby microtubule bundles (K-fibers) connect sister kinetochores of each chromosome to opposite spindle poles (1). Bi-orientation errors are linked to chromosome loss and cancers (2). Formation of sister K-fibers occurs asynchronously (3), and once a kinetochore captures microtubules growing from a spindle pole, the chromosome is transported toward this pole becoming “mono-oriented” (4). Mono-oriented chromosomes remain near the spindle pole for variable times (3, 4) until they suddenly “congress” to the spindle equator. Current models of mitotic spindle formation (5, 6) postulate that chromosome congression occurs as the result of bi-orientation (7).

We followed movements of individual chromosomes in mammalian cells by Differential Interference Contrast (DIC) time-lapse microscopy (8). In addition to the chromosome oscillations that occur towards and away from spindle poles, we frequently observed mono-oriented chromosomes making direct movements to the metaphase plate as if they were attempting to congress (Fig.S1). Centromeres on these ‘congressing’ chromosomes were frequently stretched indicating force generation by the leading kinetochore (Movie S1). However, these movements did not always result in a stable alignment on the metaphase plate because chromosomes often returned to the spindle pole after a 3-4 µm excursion. This chromosome behavior was observed in essentially every cell we imaged and has also been previously reported (9-12). To determine whether these chromosomes were bi-oriented we followed mitotic cells by DIC microscopy until one of the chromosomes exhibited an extended linear movement toward the metaphase plate and fixed the cell when the chromosome had almost reached the metaphase plate (~5-7 µm from the proximal spindle pole; Fig.1, Movie S2). 3 of 5 chromosomes analyzed by electron microscopy (EM) (8) were already bi-oriented, as expected for congressing chromosomes (7). However, in the other 2 cases no microtubules emanated from the leading
kinetochore plate on the congressing chromosome. Instead, this kinetochore laterally interacted with microtubules of a mature K-fiber attached to a kinetochore of another bi-oriented chromosome positioned on the metaphase plate (Fig. 1D). The trailing kinetochore was attached to the proximal spindle pole via a mature K-fiber. This unexpected type of kinetochore-microtubule interaction suggested that chromosomes may not need to be bi-oriented during congression.

Because individual K-fibers are not resolved by DIC microscopy, we could not correlate the trajectory of an individual chromosome moving toward the spindle equator with the positions of surrounding K-fibers. To overcome this limitation we simultaneously imaged both microtubules and kinetochores by live-cell dual-channel fluorescence microscopy. PtK₁ cells were co-injected with fluorescein-conjugated non-function-blocking antibody against the kinetochore protein CENP-F (to label kinetochores) and X-rhodamine-conjugated α-tubulin (to label microtubules) (13). In 12 of the 49 cells analyzed, we found one mono-oriented chromosome whose trajectory, during the movement toward the spindle equator, precisely followed K-fibers of other, already bi-oriented chromosomes (Fig.2, Movie S3). This pattern indicated that such congressing chromosomes were not simply ejected away from the pole by the spindle ejection force acting on the entire chromosome (11, 14) but rather glided on the microtubules of mature K-fibers.

The time a mono-oriented chromosome spends at a spindle pole is variable, and the number of attempts it makes before finally achieving stable positioning on the metaphase plate is unpredictable (15). To examine the state of kinetochore-microtubule interaction during the first congression attempt, we established an experimental system in which several chromosomes congressed in a single cell within a narrow time window (Fig.3A). We combined high-resolution
imaging and chemical inhibitors to manipulate chromosome positions in dividing cells. Cells were treated with monastrol, a small-molecule inhibitor of the kinesin Eg5 (kinesin 5). This treatment blocked cells in monopolar mitosis with high incidence of syntelic (both sister kinetochores attached to the same spindle pole) chromosomes (16). Then, cells were released from monastrol into an Aurora kinase inhibitor. Under these conditions spindles bipolarized while many chromosomes remained syntelic (17). Relief from Aurora kinase inhibition resulted in the transport of syntelic chromosome to spindle poles from where they congressed to the metaphase plate (17). This assay allowed us to accumulate mono-oriented chromosomes whose congression was temporally controlled through wash-out of cell-permeable chemical inhibitors.

Using this assay, individual cells were imaged by time-lapse DIC and spinning-disk confocal microscopy. Once several mono-oriented chromosomes initiated their movement toward the metaphase plate, the cell was fixed for correlative serial-section EM analysis. On 6 out of 7 congressing chromosomes analyzed by this approach, the leading kinetochore was laterally associated with a mature K-fiber that extended from a different bi-oriented chromosome toward the proximal spindle pole (Figs. 3B and S2). By contrast, the trailing kinetochore was attached in typical tip-on fashion to a K-fiber connected to the proximal spindle pole (Figs.3B and S2). Thus, ~85% of chromosomes lacked microtubule attachments to the distal spindle pole (i.e., remained mono-oriented) during congression in our experimental system. Importantly, centromeres on the congressing chromosomes were stretched (>2 µm, Figs.3B and S2) indicating a force acting at the leading kinetochore.

Chromosomes fixed before initiating congression, were either syntelic (5 out of 6; Fig.S3) or mono-oriented (1 of 6; Fig.S4). In the latter case, one of the kinetochores was connected to the pole via a K-fiber, while its sister was laterally associated with a bundle of microtubules
bypassing the kinetochore and extending toward the spindle equator (Fig.S4). This configuration, once again, suggests that chromosome congression can be initiated via sliding of the unattached kinetochores alongside mature K-fibers.

We next considered the molecular mechanisms responsible for congression of mono-oriented chromosomes. Because microtubule polarity within a K-fiber is uniform (18), this movement is likely to depend on a motor protein that transports cargo towards microtubule plus-ends. Further, this motor must be concentrated at kinetochores during pro-metaphase. CENP-E (a member of the kinesin-7 family) is the only plus end-directed motor that meets both criteria (19, 20). Depleting CENP-E in human cells results in a mitotic arrest with significant numbers of mono-oriented chromosomes positioned very close to the spindle pole (21, 22). In addition, recombinant CENP-E binds the sides of microtubule bundles in vitro (23). We used our chemical inhibitor-based assay and siRNA to determine if CENP-E was responsible for the congression mechanism observed for mono-oriented chromosomes. Because the rat kangaroo (Potorous tridactylis) CENP-E is not yet cloned, we used human cells for these experiments.

Multiple syntelic chromosomes were observed after spindle bipolarization in the presence of an Aurora kinase inhibitor in control and CENP-E-depleted HeLa cells (Fig.4A, B). Thus, kinetochores remained capable of capturing microtubules in the absence of CENP-E. Within 1 hr after Aurora kinase activation by inhibitor removal, all chromosomes were positioned at the metaphase plate in 73±2% of control but only in 15±7% of CENP-E-depleted cells (4 experiments, >80 cells/experiment; Fig.4C-E). At the same time the number of polar chromosomes in CENP-E depleted cells increased dramatically (from 3.4±0.7 to 10.5±1.3) after Aurora activation (Fig. 4F). Thus, syntelic chromosomes that reside at a significant distance from their poles move to the pole and become mono-oriented after activation of Aurora kinase in
CENP-E-depleted cells. However, these mono-oriented chromosomes are not subsequently transported to the metaphase plate in the absence of CENP-E. Hence, depletion of CENP-E does not affect chromosome attachment to the proximal spindle pole. Instead, it diminishes the probability for mono-oriented chromosomes to be transported from the spindle pole toward the spindle equator, where they can acquire connections to the distal pole and become bi-oriented. Our EM data revealed that sliding of kinetochores toward the plus ends of mature K-fibers is a major mechanism for aligning mono-oriented chromosomes positioned near a pole, and this mechanism is missing in the absence of CENP-E. This defect explains why persistent mono-oriented chromosomes positioned very close to the spindle pole have been found consistently in CENP-E-deficient cells (19, 21, 22, 24).

Our findings address a long-standing question in cell division. It was unclear how chromosome accumulation at spindle poles in prometaphase and during correction of syntelic attachments leads to bi-orientation (25). Our data reveal that at spindle poles, mono-oriented chromosomes are likely to find mature K-fibers that are attached to other, already bi-oriented chromosomes, and congress alongside these K-fibers via a CENP-E-dependent mechanism. In this congression mechanism the probability of a mono-oriented chromosome to be transported toward the spindle equator progressively increases as more and more chromosomes become bi-oriented, increasing the density of K-fibers in the spindle. Thus, chromosome congression is a cooperative process which depends on chromosome positions relative to the mitotic spindle and is promoted for those chromosomes that remain mono-oriented as the spindle fully assembles and establishes a metaphase plate. Abrogating this cooperativity would mostly affect chromosomes that congress late during spindle formation, thereby inducing loss of one or two chromosomes, as has been observed after CENP-E depletion in murine cells (26).
References and Notes


8. Materials and methods are available as supporting material on Science Online.


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**Supporting Online Material**

[www.sciencemag.org](http://www.sciencemag.org)

Materials and Methods

Figs. S1, S2, S3, S4, S5

Movies S1, S2, S3
Figure 1. Leading kinetochores are not properly attached to microtubules during a chromosome’s attempt to congress. (A) Selected frames from a DIC time lapse-recording (also see Movie S2). The cell was fixed as one chromosome (arrows) moved toward the spindle equator (1304 s). (B) Distance vs. time plot confirmed that the chromosome’s movement (red curve) was typical for chromosome congression (cf. with blue and yellow curves which represent movements of chromosomes shown in Fig.S1 and Fig.3 respectively). (C) Lower-magnification EM image of the cell showing the position of the chromosome of interest (arrow) with respect to the spindle pole (arrowhead). (D) Selected 100-nm EM section from a full series through the centromere region of the chromosome. Note the prominent bundle of microtubules (highlighted red) connecting the trailing kinetochore (white arrow in Sect.14) to the proximal spindle pole. These microtubules approached the kinetochore at ~90° angle and terminated within the tri-
laminar kinetochore plate. By contrast the leading kinetochore (white arrow, Sect. 17) lacked attached microtubules but was laterally associated with a mature kinetochore fiber (highlighted yellow) which was attached to the kinetochore of a bi-oriented chromosome (black arrowheads in Sect.14-15) positioned on the metaphase plate.

Figure 2. Mono-oriented chromosomes are transported toward the spindle equator along kinetochore fibers of other chromosomes. (A) 2-color fluorescence image of a live PtK₁ cell in which kinetochores were labeled with CENP-F/Alexa488 (red) and microtubules with tubulin/Rhodamine (green). Area marked with white brackets is enlarged in (B-F). (B-F) selected frames from the 2-color time-lapse recording. In each frame CENP-F/Alexa488 fluorescence (kinetochores) is shown alone (top) and overlaid in red on microtubules (bottom). Arrows mark the kinetochore that moved toward the spindle equator. Note that trajectory of this kinetochore coincided with a prominent kinetochore fiber that extended from the spindle pole to a kinetochore on a bi-oriented chromosomes already positioned on the metaphase plate (arrowhead). Time in seconds. Scale bars: (A) 5 µm, (F) 2.5 µm. (G) Schematic illustrating the sequence of events presented in (B-F).
**Figure 3.** Leading kinetochores are laterally associated with kinetochore fibers of other chromosomes during chromosome congression. (A) Protocol for inducing synchronous chromosome congression. Cells were arrested in mitosis with monastrol to accumulate monopolar mitosis with high incidence of syntelic chromosomes (green – microtubules, red – chromosomes). Then monastrol was removed and Hesperadin was added with MG132 for 1 hr. This resulted in spindle bipolarization although many chromosomes remained syntelic. After 1 hr Hesperadin was removed, and cells were imaged live until fixation. Removal of Hesperadin resulted in simultaneous correction of syntelic attachments. Syntelic chromosomes moved to the pole, became mono-oriented and then attempted to congress. (B) Selected 100-nm EM sections from a full series through the centromere of a congressing chromosome. Note that similarly to non-treated cells (Fig.1) chromosomes congressed with their leading kinetochores unattached (black arrows) but sliding alongside mature kinetochore fibers of other chromosomes. By contrast, trailing kinetochores (white arrow) were always attached to prominent kinetochore fibers that terminated within the tri-laminar plate.
Figure 4. CENP-E is required for congression of mono-oriented chromosomes. 24 hrs after transfection, with mock (control) or CENP-E siRNA, synchronous chromosome congression was induced using the chemical inhibition/activation approach described in Fig.3. Cells were fixed either after 1 hr in Hesperadin (A-B), or 1 hr after removal of Hesperadin (C-F) and processed for immunostaining (tubulin, green; kinetochores [CREST], red). In the presence of Hesperadin, syntelic attachments were observed in both control (A) and CENP-E-depleted cells (B). After removal of Hesperadin chromosomes congressed to the metaphase plate in control cells (C), but mono-oriented chromosomes were observed near spindle poles in CENP-E-depleted cells (D, insets show CREST staining). For cells fixed 1 hr after removal of Hesperadin, bipolar spindles were counted and classified as fully aligned or containing polar chromosome(s) (E, average of 4 experiments). To quantify the number of chromosomes at the pole, the number of kinetochore pairs with no detectable K-fiber was counted in 3-D confocal images (F, averages from 19 CENP-E-depleted or 7 control cells for each condition, 2 experiments). All images presented as maximal-intensity projections. Insets (A,B) show optical sections at 2X magnification.