AN ABSTRACT OF THE THESIS OF

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Dahong Zhang

Abstract

Successful cell division requires proper chromosome movement, including accurate congression to the metaphase plate and proper segregation to spindle poles. Errors that occur in either event can cause chromosome aneuploidy in daughter cells, which may lead to cell death, genetic diseases and cancers (Matson and Stukenberg,2012, Compton, 2000). The force powering chromosome movement is mainly generated by the polymerization and depolymerization of kinetochore microtubules, and their interaction with the attached molecular motor proteins (Barton and Goldstein, 1996). Microtubule dynamics and motor activities have been studied extensively to understand how they move chromosomes, yet there are many questions remain to be answered. Our goal is to understand the impact of kinetochore microtubule numbers on chromosome movement. We hypothesized that chromosome segregation to the spindle pole requires synchronized shorting of kinetochore microtubules that attach the chromosome to the pole. Therefore, a higher number of kinetochore microtubules in a kinetochore fiber leads to a lower rate of chromosome movement to the pole in

anaphase. To test this hypothesis, we used micro-techniques to alter the number of microtubules at the kinetochore and measured its impact on the rate of chromosome segregation using polarization microscopy and computer-assisted tracking technology (Skibbens*et al.*,1993). We found that in single-chromosome cells created via micromanipulation, the chromosome captured more kinetochore microtubules but had slower rate of segregation than that of the control cells. If however, the chromosome failed to capture more kinetochore microtubules, it segregated at almost the same rate as that of the control cells. To minimize the potential impact of micromanipulation on the rate of chromosome segregation, we directly compared the segregation rates of homologues with altered number of kinetochore microtubules in the same cell. Again, the chromosome with more kinetochore microtubules always segregated slower than its homologue. We conclude that the rate of chromosome segregation in anaphase is inversely proportional to the number of kinetochore microtubules that attach the chromosome to the spindle pole.

The Impact of the Number of Kinetochore Microtubules on the Rate of Chromosome Segregation

by
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Major Professor, representing Zoology

Chair of the Department of Zoology

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Yunhan Duan, Author

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The Impact of the Number of Kinetochore Microtubules on the Rate of Chromosome Segregation

Introduction

Equal partition of genetic material is one of the most crucial steps in cell division, as errors may lead to chromosome loss, birth defects, diseases and cancer (Taylor *et al.*, 2004; Rao *et al.* 2009; Thompson and Compton 2011). This equal distribution of duplicated chromosomes into two daughter cells is mediated by a complex protein supersturcture called spindle apparatus. It is known that the forces that move chromosomes to the spindle equator (congression) and later to the spindle poles (segregation) are generated by spindle microtubules interacting with their motor proteins on chromosomes (mainly at their kinetochores). The spindle is primarily composed of three types of microtubules, including kinetochore microtubules, interpolar microtubules, and astral microtubules (Fig.1). The microtubules in the spindle possess uniform polarity: the more dynamic plus ends extend towards the chromosomes or cell cortex and the less dynamic minus ends focus at or near the spindle poles (Heidemann and McIntosh, 1980; McIntosh and Euteneuer, 1984). Both ends can polymerize and depolymerize, but at different rates (Margolis and Wilson, 1981).

Kinetochore microtubules either directly connect the chromosomes to the spindle poles, or are tightly bundled to other microtubules that contact the poles. Microtubules that attach to kinetochores are either laterally or directly embedded in the kinetochore disk (Compton, 2000). These microtubules provide major forces for chromosome movement. Interpolar microtubules extend from spindle poles into the central spindle, with a preference for antiparallel over parallel interactions (Mastronarde, 1993). The sliding movement between antiparallel microtubules may generate outward pushing forces, which counteract the inward pulling forces powered by kinetochore microtubules and motors (Mchedlishvili *et al.*, 2012). The outward

forces not only help maintain spindle bipolar structure, but also push the spindle poles apart after chromosome segregation (Masuda *et al.,* 1988). Astral microtubules extend from the spindle pole toward the cell periphery. The interaction between their plus ends and the cell cortex helps anchor the spindle in the cell as well as position the cleavage furrow for cytokinesis (Rappaport, 1996, and Tseng *et al.,* 2012).

Chromosomes segregate to spindle poles as kinetochore microtubules shorten during anaphase (Chen and Zhang, 2004). Three prevailing models have been proposed to explain the potential mechanisms of chromosome segregation, including the spindle matrix model (Scholey *et al.* 2001, Johansen and Johansen 2002, Fuge *et al.* 1985, and Fuge 1989), the PacMan model (Mitchison and Salmon 2001), and the traction fiber model (Ostergren1951; Hays and Salmon, 1990).

The spindle matrix model claims that both kinetochore and interpolar microtubules are constantly depolymerized at the minus ends along with the flux of spindle matrix. The three required components for the flux are plus end polymerization, minus end depolymerization, and poleward translocation of the microtubule polymers (Susan and Walczak, 2004). Fluorescent speckle microscopy (FSM) was employed to observe chromosome movement and kinetochore microtubules at the same time (Maddox *et al.,*2002; Danuser and Waterman-Storer, 2003). The FSM data suggested that spindle matrix flux facilitates microtubule minus end disassembly during anaphase chromosome movement (Desai *et al.,*1998; Wilson ,1994, and Forer and Wilson, 2000). FSM studies also showed that spindle flux can coexist with PacMan activity (Maddox *et al.,*2003).

The PacMan activity was first observed from EM studies of the loss of biotinylated-tubulins incorporated at the plus ends of kinetochore microtubules (Fig. 2) (Mitchison *et al.,*1986). The PacMan model claims that the depolymerization at the plus ends of kinetochore fiber, powered by motor proteins, converts the chemical energy of ATP hydrolysis into mechanical force at the kinetochore, which drives chromosomes towards the spindle pole (Inoue and Salmon, 1995; Rieder and Salmon, 1998;

Mitchison *et al.*, 2001; Scholey *et al.*, 2003; Rieder and Salmon, 1994). The *in vitro* study of chromosome movement and microtubule dynamics conducted by Mitchison's lab has suggested that chromosome segregation during anaphase is powered and regulated by microtubule depolymerization at the kinetochore, and the segregation rate depends on the microtubule's tendency to depolymerize (Koshland *et al.*,1988).

Unlike the PacMan model, the traction fiber model, initially developed by Ostergren (1951), envisions that the depolymerization occurs at the minus ends of the kinetochore fibers at the spindle pole (Fig. 2). The shortening of the fibers pulls chromosomes poleward. McIntosh's lab demonstrated that neither dynein nor ATP was needed for chromosome movement *in vitro*. Tubulin depolymerization from the kinetochore microtubule ends is sufficient to drive poleward chromosome movement (Coue *et al.*,1991). In addition, the photoactivation experiments in Salmon's lab supported the traction fiber model (Desai *et al.*, 1998). A laser-microbeam experiment in grasshopper spermatocytes has shown that the severed kinetochore fibers remain a constant length in anaphase. Consequently, the chromosome cannot move poleward unless the severed fiber reattaches to the pole via microtubules (Chen and Zhang, 2004). They conclude that the depolymerization of the kinetochore microtubules at the minus ends drives the net shortening of the kinetochore fiber (Chen and Zhang, 2004). Microtubule-severing protein Katanin (McNally *et al.*,1996) and microtubule-destabilizing kinesin KLP10A (Rogers *et al.*,2004) have been localized at the minus ends during microtubule disassembly in the spindle, which may shorten kinetochore fibers to generate poleward forces during anaphase (Chen and Zhang, 2004; Rogers *et al.*,2004). Hays and Salmon tested the traction model in chromosome movement, and postulated that the poleward force (F) at the kinetochore is proportional to the length (L) and number of its kinetochore microtubules (N), i.e., $F=kLN$, (k is a constant, identical for all kinetochores (Hays and Salmon, 1990). Based on this model, the pulling force that controls the poleward movement of a chromosome is proportional to both the number and length of kinetochore microtubules. However, classic UV-microbeam experiments suggest otherwise, as chromosomes with a completely

severed kinetochore fiber in the spindle may move or even accelerate poleward. Consequentially, the authors attributed the poleward pulling force to the actomyosin-driven system (Forer, 1965; Forer et al., 2003). Laser-ablation studies in grasshopper spermacytes (Chen and Zhang, 2004) revealed that the accelerated movement of the severed chromosomes was in fact driven by newly attached microtubules linking the severed chromosomes to the spindle pole. Notably, a fewer number of kinetochore microtubules resulted in a faster rate of poleward chromosome movement (Fig. 3; Chen and Zhang, 2004). These findings led to our further investigation on the impact of the number of kinetochore microtubules on the rate of chromosome segregation.

Materials and Methods

Cell Culture

The spermatocytes of the grasshopper *Melanopus femurrubrum* were used for experiments. Live grasshopper spermatocytes were obtained from primary cell cultures that were spread on the coverslip under a thin layer of halocarbon oil in a custom-made chamber slide (Chen and Zhang, 2004). The slides were kept at room temperature for further treatment and observation.

Microscopy and Imaging

Cells were observed with a digital-enhanced polarization microscope home-modified from an inverted Zeiss Axiovert-100 microscope equipped with a 1.4 NA/63x Plan-Apochromat objective and a 1.4 NA achromatic-aplanatic condenser. The modification was adapted primarily from the designs of Shinya Inoué (1986, 1997) as well as Nicklas and Ward (1994), and was with generous help of R. Bruce Nicklas and Edward D. Salmon.

Live cell images were recorded using a cooled-CCD digital camera (ORCA-100, Model C4742-95, Hamamatsu) and SimplePCI software, and were processed using SimplePCI software (C-imaging Systems, Cranberry Township, PA).

Micromanipulation and Distance Measurement

Micromanipulations for chromosome removal and kinetochore microtubule detachment were carried out using a glass miconeedle with a tip diameter of ~0.1 μm. The microneedle was maneuvered with a Burleigh MIS-5000 series piezoelectric micromanipulator (Chen and Zhang, 2004). The rate of chromosome movement was measured as distance changes over time between the chromosome and the spindle pole. Specifically, images of a micrometer recorded using the same digital-enhanced polarization microscope were superimposed over the digital sequences of chromosome movement. The distance from the chromosome to the pole was determined using the measurement module of SimplePCI software.

Determination of Kinetochore Microtubule Numbers

We determined the number of kinetochore microtubules by measuring the volume-birefringence (BR_{volume}) of a kinetochore fiber (Marek, 1978; Zhang and Nicklas, 1995). The BR_{volume} reflects the total mass of aligned birefringent material of a kinetochore fiber, i.e., the bundled kinetochore microtubules (Marek, 1978; Zhang and Nicklas, 1995). We determined the BR_{volume} of a kinetochore fiber via its retardation to the polarized light, which is the difference in image brightness between the background and the fiber. The reading was then calibrated using the corresponding retardation from the standard curve of image brightness versus retardation obtained from mica chips with known values (Salmon and Wolniak, 1984; Zhang and Nicklas, 1995). The number of kinetochore microtubules was calculated as a function of the measured BR_{volume} of the fiber, the molecular weight of the subunit (110,000 Daltons), and the coefficient of birefringence per unit volume of tubulin subunit (1.8×10^{-2}) (Cassim et al., 1968; Marek, 1978; Salmon and Wolniak, 1984).

Results

We compared the rate of chromosome segregation between the manipulated cell and the control cells on the same slide, from the same primary cell culture. Ten out of eleven bivalent chromosomes in metaphase I spermatocytes were removed via micromanipulation to increase the pool of microtubules to the remained bivalent. As expected, the remaining single bivalent gradually captured a larger number of kinetochore microtubules, resulting in a kinetochore fiber ~2 times thicker than the controls (Fig. 4a). Images were acquired sequentially once every 30 seconds as soon as the cells entered anaphase (Fig.4a, 0s). The rate of chromosome segregation in the manipulated cell was compared to the non-manipulated control cells nearby. The chromosomes in the control cell successfully segregated to regions near spindle poles in 720s, while the single-chromosome in the manipulated cell took about 1140s to travel through a similar distance (Fig.4a). Statistically, the chromosomes in control cells segregated nearly 2 times faster than that of the manipulated cells. The average rate of segregation in single-chromosome cells was 0.393 ± S.D. μm/min (n=20). In control cells, the rate of chromosome segregation was 0.634 ± S.D. μm/min (n=20).

In rare cases, the single chromosome in the manipulated cells segregated almost as fast as that of the controls, if the micromanipulation was performed too close to the onset of anaphase (Fig.5). In the first 300 seconds, the chromosome in the manipulated cell segregated at a rate of 0.7 μm/min, which was close to 0.77 μm/min for that of the control (Fig.5, 0-300s). Not surprisingly, little difference was found in their numbers of kinetochore microtubules. The kinetochore fiber in the manipulated cell had only ~5% more microtubules than that of the control (Fig. 5, 0-300s). Interestingly, as the chromosomes approach the spindle poles, differences in both segregation rate and kinetochore fiber thickness started to appear between the two cells: the chromosome in the manipulated cell slowed down to ~0.5 μm/min while in the control segregated at 0.625 μm/min. The kinetochore fiber in the manipulated cell became ~25.8% thicker than that in the control (Fig. 5, 300-540s).

In order to eliminate any deviation resulted from the micromanipulation to the experimental cells, we compared the segregation rates of the homologous chromosomes bearing different amount of kinetochore microtubules. To do so, ten out of eleven bivalent chromosomes were removed via micromanipulation from metaphase I cells. Upon anaphase onset, part of the kinetochore fiber attached to one segregating chromosome was quickly detached via micromanipulation, while the kinetochore fiber on its homologous partner remained untouched as the control (Fig. 6a). Such operations have created various numbers of kinetochore microtubules among twenty manipulated cells, from which a trend line was generated for the rates of segregating homologues bearing different amount of kinetochore microtubules. Again, we found that the rate of chromosome segregation is inversely proportional to the number of kinetochore microtubules (Fig. 6b, n=20).

Discussion

We altered the number of kinetochore microtubules by micromanipulation and examined its impact on the rate of chromosome segregation in grasshopper spermatocytes. We first increased the number of kinetochore microtubules on the only remaining bivalent chromosome by mechanically removing ten out of eleven bivalents, which allowed the remaining chromosome to capture more microtubules. We demonstrated that in the single-chromosome cells, the segregation rate of the chromosome is significantly slower than that in control cells (11 bivalent chromosomes).

We then directly compared the segregation rate of chromosome homologues bearing different number of kinetochore microtubules resulted from micromanipulation in the same cell. Such experiments eliminated the potential negative impact of micromanipulation on the manipulated cells, resulting in a slower rate of chromosome movement. Again, the chromosome with fewer kinetochore microtubules always segregated faster than its homologue. This system avoided the unpredictable potential differences between the manipulated and control cells, providing a comparison with an internal control in the same cell.

Our real-time observation data showed a consistent trend that higher numbers of kinetochore microtubules tend to slow down chromosome movement, suggesting that the number of kinetochore microtubules is inversely proportional to the rate of chromosome segregation.. Since the lengths of these kinetochore microtubules remain unaltered in both manipulated and control cells, it is the number, not the length of kinetochore microtubules, that affect the rate of segregation. We therefore propose that $F=kL/N$, which contradicts Salmon and Hayes' equation of $F=kLN$.

We conclude that chromosome segregation to the spindle pole requires synchronized shortening of kinetochore microtubules that attach the chromosome to the pole.

Therefore, a higher number of kinetochore microtubules in a kinetochore fiber leads to a lower rate of chromosome movement to the pole in anaphase. One explanation is that since the pulling force is produced by the net depolymerization of the kinetochore microtubules, the microtubule dynamic instability may actually cause them to interfere with one another. Thus, more kinetochore microtubules lead to a slower rate for their net disassembly, and consequently the slower rate of chromosome movement.

Due to the nature of volume-birefringence, our measurements on the number of kinetochore microtubules are not precise, but reliable enough to produce a trend line with its R^2 value greater than 75%. Further studies may use electron microscopy (EM) for accurate measurement of kinetochore microtubule measurement. However, since EM can only operate on fixed cells, it is not practical for real-time observations. One possible solution is to have two sets of the same experiment, with one set used for segregation measurement and the other set, after being fixed, used for EM. The advantage of this method is that by gaining accurate numbers of kinetochore microtubules, we can mathematically correlate the number of microtubules to the rate of segregation, instead of just seeing the trend.

Figures

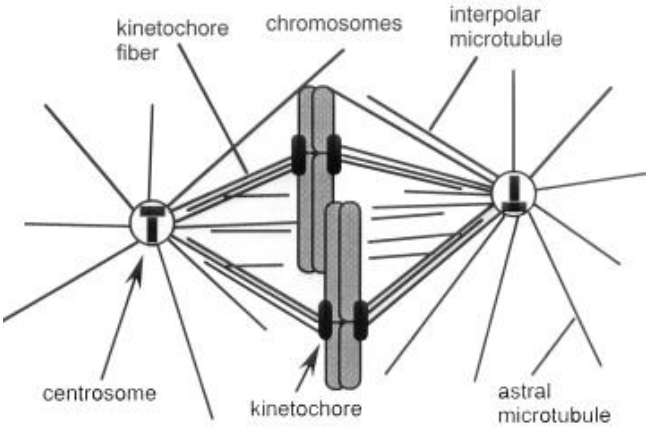

Fig. 1 Typical spindle structure during metaphase. Besides the three types of microtubules, centrosomes, chromosomes, and kinetochores are also essential components of the structure (Compton 2000)

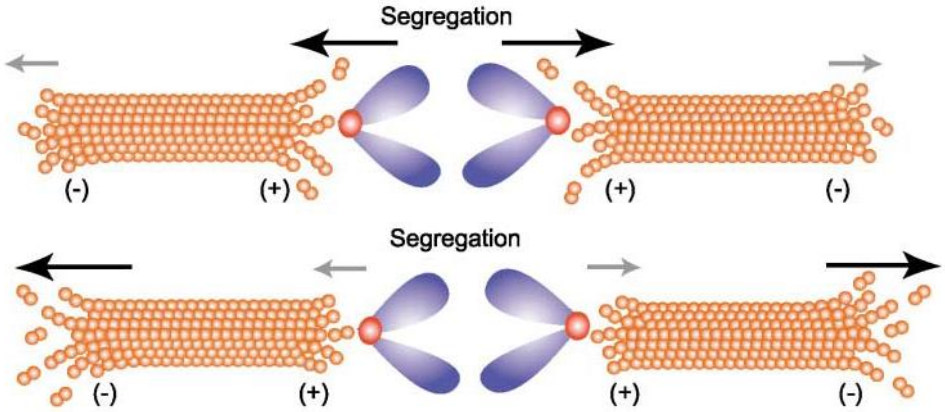

Fig. 2 Demonstration of PacMan model (upper) and traction model (lower). PacMan model states the force for chromosome segregation is generated by microtubule depolymerization at the plus end, and traction model argues that the force is generated at the minus end near the spindle pole(Susan *et al.* 2004)

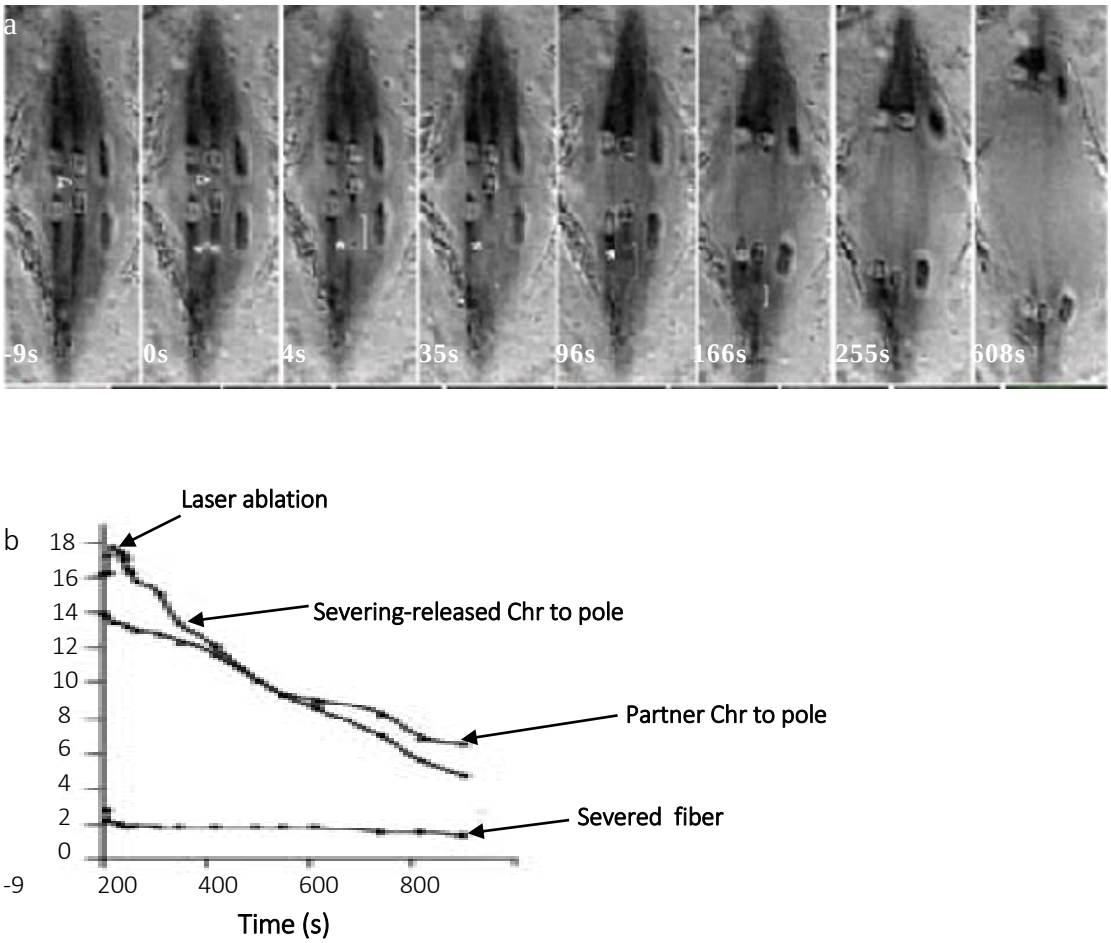

Fig. 3 During anaphase, the k-fiber of one chromosome was severed using laser microbeam. Upon laser ablation, the target chromosome retracted rapidly due to the stick chromatin bridge (0-4s). The location ofthe chromosome remained constant while other chromosomes kept moving towards the spindle poles (a 0-35s). Once the severed fiber regained attachment to the pole, it accelerated rapidly (a 35- 96s), passing nearby chromosomes (96- 166s), and reaching the pole (a 255- 608s). The summarizing chart showed the differences in chromosome location (b). (Chen and Zhang 2004)

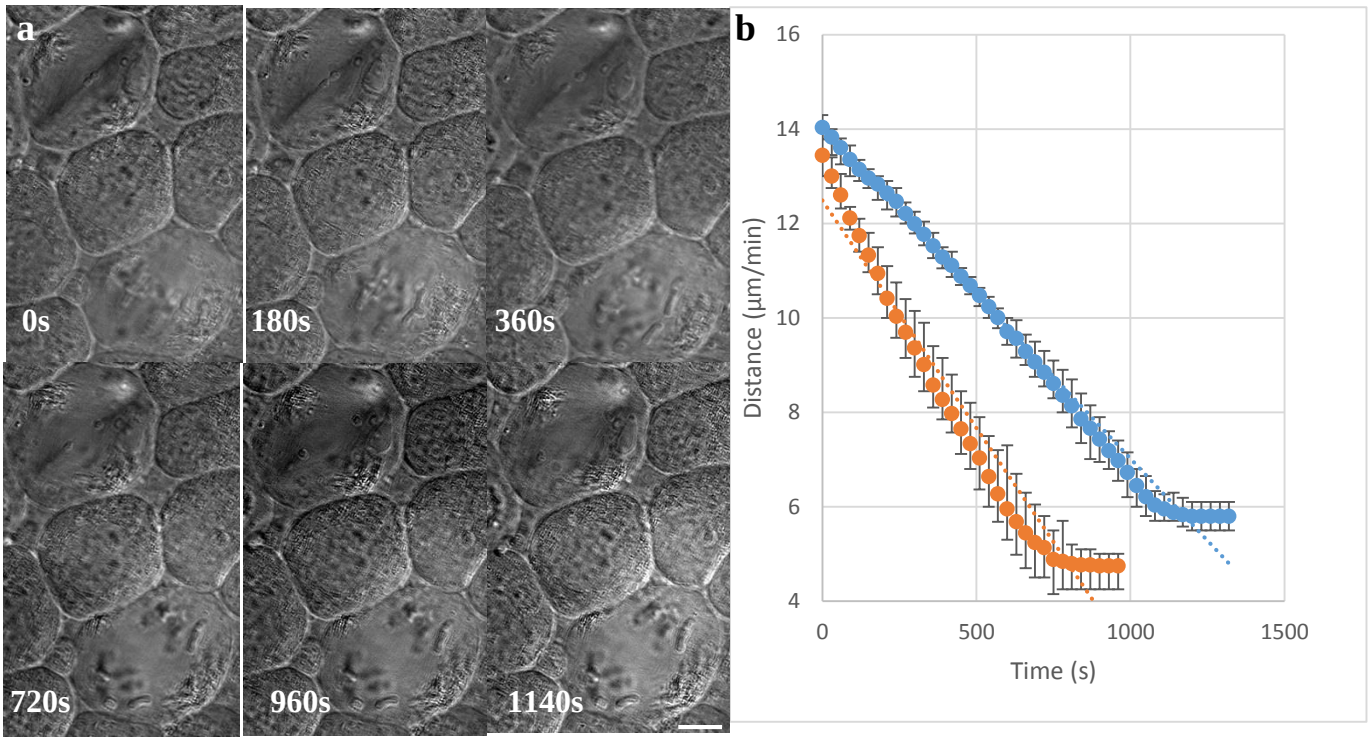

Fig.4 We compared the rate of chromosome segregation in single-chromosome cells and normal 12-chromosome cells (sex chromosome does not participate in segregation thus has no impact on this measurement). (**a**) When entering anaphase at the same time, the chromosomes in the normal cell (lower right) successfully segregated to regions near spindle poles within 720s, while the single-chromosome cell (upper left) used about 1140s to travel a similar distance (`8 µm). (**b**) When comparing the single-chromosome cells (blue dots) and normal cells (orange dots), the average rate of segregation in normal cells was 0.63 ± S.D. µm/min (n=20), while the average rate of segregation in single-chromosome cells was 0.39 ± S.D. µm/min (n=20). The values at each time point are the average rate ± the standard deviation from the 20 cells. The dotted blue and orange lines indicate the trend of the movement in single-chromosome and normal cells, respectively, with a *p* value of 0.07. Scale bar represents 10 µm.

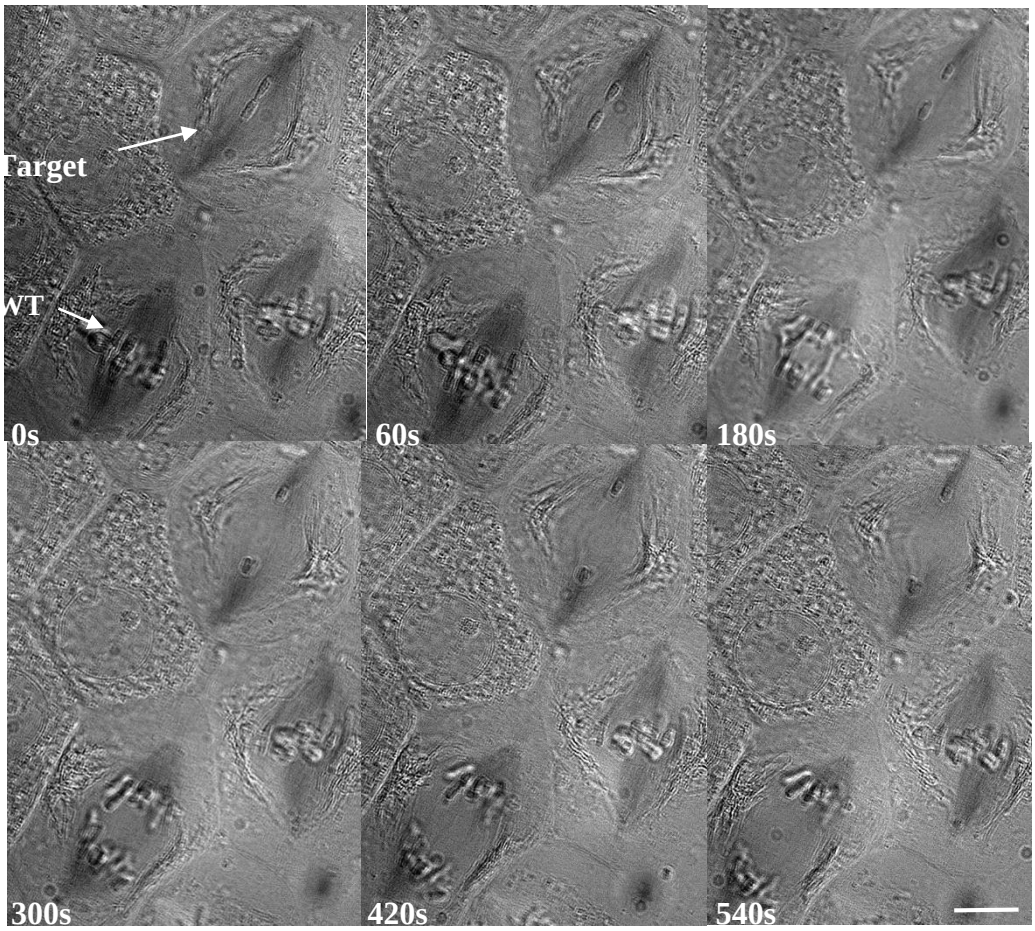

Fig. 5 Enhanced images of one special case in experiment I. The single-chromosome cell (upper right) and the WT (lower left) started with very similar rates of segregation in the first 300s, with the target moving 0.7 μm/min and the WT moving 0.77μm/min. Volume-birefringence results showed they had almost close numbers of microtubules (the target cell had ~ 5% more kinetochore microtubules than the WT). As the chromosomes segregated, the k-fiber on the single chromosome slowly became thicker than the WT (~26% more). Meanwhile, the rate of segregation in the target cell reduced to 0.5 μm/min, and the WT segregated at 0.625 μm/min (300-540s). Scale bar represents 10 μm.

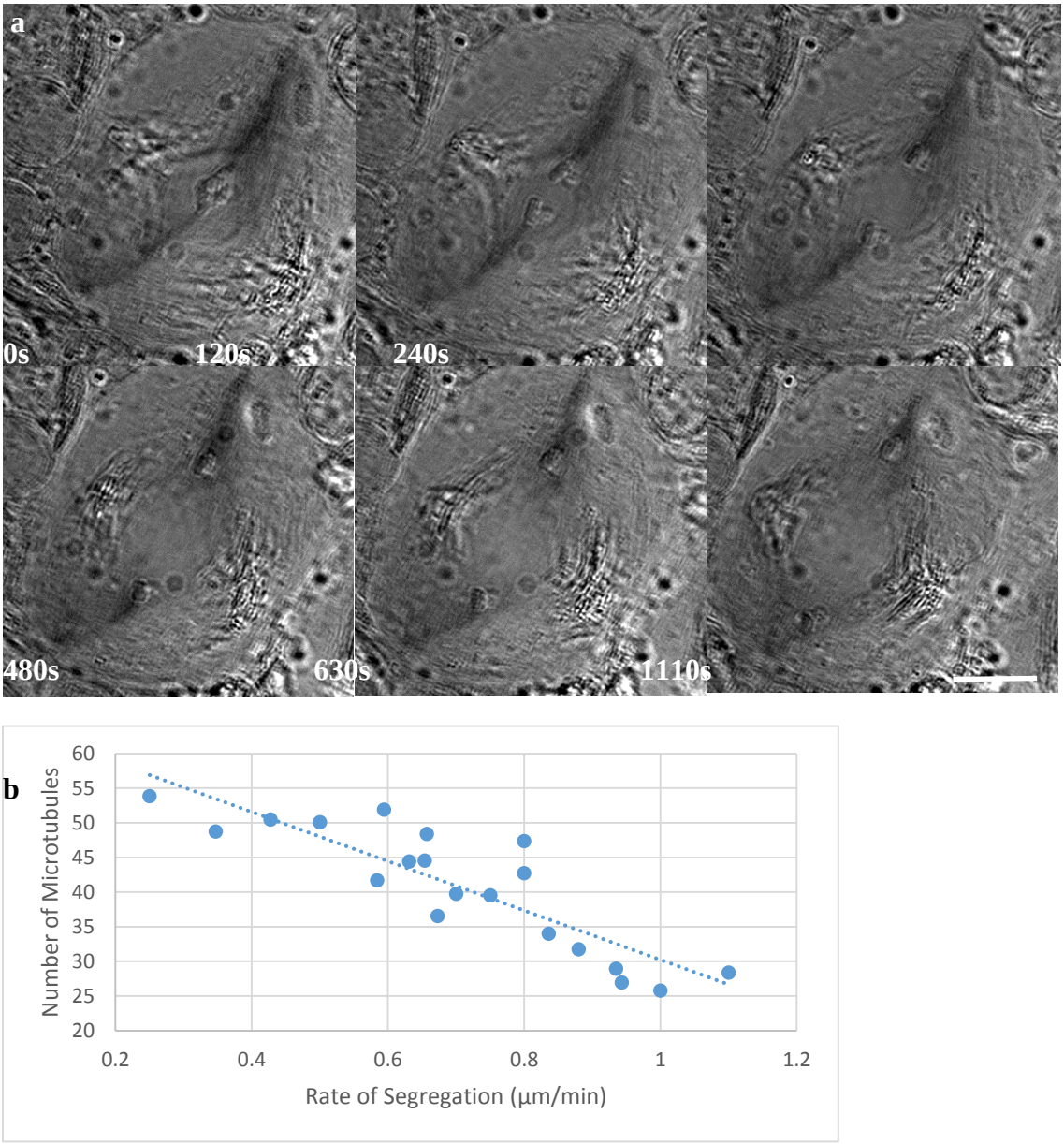

Fig.6 Upon anaphase onset, a random amount of kinetochore microtubules attached to the lower left chromosome was mechanically removed via micromanipulation. The position of the chromosome homologues was not affected due to the yet still fairly strong attachment between the two (a, 0s). As shown in a, in 480s the lower chromosome had almost reached the spindle pole, while the upper chromosome with a much thicker k-fiber used about 1110s. The segregation rate of the lower chromosome was `2 times faster than its homologue. (b) The statistical results of 20 measurements showed a trend suggesting the inverse correlation between the rate of segregation and the number of microtubules (n=20).Scale bar represents 10 µm.

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