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Elastic Tethers Between Separating Anaphase Chromosomes in Crane-Fly Spermatocytes Coordinate Chromosome Movements to the Two Poles

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Separating anaphase chromosomes in crane-fly spermatocytes are connected by elastic tethers, as originally described by LaFountain et al. (2002): telomere-containing arm fragments severed from the arms move backwards to the partner telomeres. We have tested whether the tethers coordinate the movements of separating partner chromosomes. In other cell types anaphase chromosomes move faster, temporarily, when their kinetochore microtubules are severed. However, in crane-fly spermatocytes the chromosomes move at their usual speed when their kinetochore microtubules are severed. To test whether the absence of increased velocity is because tethers link the separating chromosomes and coordinate their movements, we cut tethers with a laser microbeam and then cut the kinetochore microtubules. After this procedure, the associated chromosome sped up, as in other cells. These results indicate that the movements of partner anaphase chromosomes in crane-fly spermatocytes are coordinated by elastic tethers connecting the two chromosomes and confirm that chromosomes speed up in anaphase when their kinetochore microtubules are severed. © 2016 Wiley Periodicals, Inc.

Key Words: tethers; spindle function; anaphase chromosomes

Additional Supporting Information may be found in the online version of this article.

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Introduction

Separating anaphase chromosomes are not independent in crane-fly spermatocytes. For example, ultraviolet light (UV) microbeam irradiation of a single kinetochore spindle fibre in anaphase with some wavelengths of UV temporarily stops the movements of the associated chromosome but also stops the movement of the partner chromosome moving to the opposite pole [Forer, 1966; Sillers and Forer, 1981; Hughes et al., 1988], the *partner* chromosome being that chromosome with which the chromosome in question was conjoined in metaphase. As another example, treatments of anaphase spermatocytes with various drugs often affect different chromosomes in the cell differently, but the effects on partner chromosomes are always the same: both partner chromosomes either stop, slow, or move normally, independent of any effects on other chromosome pairs [Fabian and Forer 2005; Sheykhani et al., 2013a,b].

Another possible example of coordinated movements between partner chromosomes may occur after kinetochore microtubules are severed and the associated chromosome continues to move. When kinetochore fibre microtubules are cut with a laser or UV microbeam in anaphase grasshopper spermatocytes, newt fibroblasts, or PtK cells, the associated anaphase chromosomes increase velocity after the microtubules are cut and then shortly afterwards slow down to the original speed (reviewed in Forer et al. [2015]). When kinetochore fibre microtubules of anaphase crane-fly spermatocytes are cut by UV microbeam irradiation (wavelength 260–290 nm), however, the movements of the chromosomes remain unaffected: chromosome velocity remains the same as before the irradiation even though the kinetochore microtubules are severed (see Forer et al. [2015]). The difference in response between crane-fly spermatocytes and the other cells might be because of coordination between partner chromosomes: the chromosome with severed microtubules is prevented from moving faster because

its movement is coordinated with its partner chromosome moving to the other pole.

How Is the Coordination between Partner Chromosomes Achieved?

The linked movements of partner chromosomes seem to be mediated across the interzonal region between separating chromosomes: after UV microbeam irradiations of the interzonal region between separating chromosomes only the associated chromosome stops moving after irradiation of its kinetochore fibre [Yin and Forer, 1996; Wong and Forer, 2003]. Thus irradiating the interzonal region between separating partners unlinked their movements. Ilagan et al. (1997) suggested that the coordination between partners might be because separating (partner) chromosomes are physically connected. LaFountain et al. (2002) showed that this is the case, that partner chromosomes in anaphase crane-fly spermatocytes are physically connected by elastic connections that he called 'tethers'. Tethers were identified experimentally using laser microbeam irradiation: when LaFountain et al. [2002] cut the terminal portion of an arm from an anaphase chromosome, that arm fragment immediately moved across the equator at high speed until it reached the telomere of its partner chromosome. Not all arms were connected, however: in each half-bivalent only two of the four arms were connected by tethers. As anaphase progressed the fragments crossed the equator but did not reach the partner telomere, then were not transported across the equator, and eventually not transported at all. Thus LaFountain et al. [2002] concluded that either the elasticity of the tethers waned as anaphase proceeded or the tethers became disconnected from the telomeres. When telomeres were ablated, arm fragments did not move, further indicating that the tethers were elastic connections between separating telomeres [LaFountain et al. 2002]. LaFountain et al. [2002] suggested several possible roles for the tethers, including that they might act as 'conduits' for signals between partners. Our experiments have tested this suggestion.

In this article we test the possible role of tethers in coordinating movements between separating partner chromosomes in anaphase, by testing whether tethers prevent the expected increased chromosome velocity in crane-fly spermatocytes when kinetochore microtubules are severed. If the tethers indeed prevent increased velocity of the associated chromosome, cutting the tethers prior to cutting the kinetochore microtubules should allow chromosomes to increase in speed after their kinetochore microtubules are cut. Our experiments confirm this prediction: without tethers, chromosomes with severed KT fibre microtubules temporarily increase their speed while the movements of their partners are unchanged. This strongly suggests that tethers are responsible for linking movements of separating anaphase partner chromosomes in crane-fly spermatocytes.

Materials and Methods

Living Cells Preparation

Crane flies (*Nephrotoma suturalis* Loew) were reared in the laboratory essentially as described earlier [Forer, 1982], and preparations of living crane-fly spermatocytes were obtained as described by Forer and Pickett-Heaps [2005]. In brief, 4th instar larvae at the proper stage were covered with halocarbon oil, testes were removed and placed in a drop of Halocarbon oil, the oil was rinsed off by passing the testes through three drops of insect Ringer's solution (0.13 M NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 3 mM phosphate buffer, pH 6.8.), and then each testis was broken open and the cells spread out in a small (~2.5 μl) drop of insect Ringers solution that contained fibrinogen. Thrombin was added to form a fibrin clot to embed the cells, and the cells were placed in a perfusion chamber and perfused with insect Ringers solution. These cells were then studied using phase-contrast microscopy. To study cells using confocal microscopy that were previously followed while living, we marked a circle on the coverslip to indicate the region where the cell was, and after the experiment we lysed and fixed the cells and treated the preparation as described in Fabian et al. [2007]. In this way we were able to locate the irradiated cell after it was stained with fluorescently labelled antibodies.

Microscopy and Analysis

We studied the preparations using phase-contrast microscopy with a Zeiss Plan-Apochromat 63x NA 1.40 objective in a laser microbeam apparatus described in detail elsewhere [Shi et al., 2012; Harsono et al., 2013]. Most experiments were with the 740 nm 200-fs laser (described in Ferraro-Gideon et al. [2013]), but some were with the 532 nm wavelength 12-ps laser described in Sheykhani et al. [2013b]. We recorded images every 3–4 seconds, and cut the region of interest sometimes in one plane of focus, but usually in three planes of focus displaced along the Z-axis by about 0.4 μm. Images from each experiment were cropped and date-and-time stamped using Irfan View (www.irfanview.com), and compiled into movies using VirtualDub (www.virtualdub.org), as described previously [Ferraro-Gideon et al., 2013]. We used an in-house program (WinImage) to analyse chromosome movements [Wong and Forer, 2003].

Immunostaining and Confocal Microscopy

Cells were prepared for immunofluorescence basically using the staining protocol used by Fabian and Forer [2005]. Preparations of crane-fly spermatocytes were lysed for 20–40 min in a lysis buffer (100 mM piperazine N,N-bis(2-ethanesulfonic acid) [PIPES]; 10 mM EGTA; 5 mM MgSO4; 5% DMSO; 1% Nonidet P-40; pH 6.9). Lysed cells were fixed for 3–6 min in 0.25% glutaraldehyde in phosphate-buffered saline (PBS), rinsed in PBS (two times

for 5 min each), kept for 10 min in sodium borohydride (1 mg/ml) to neutralize free aldehyde groups, rinsed again with PBS (two times for 5 min each) and stored in PBS–glycerol 1:1 (v/v) at 4°C. To stain the preparation, the coverslips-cells were rinsed with PBS to remove the PBS/glycerol, cells were stained with YL1/2 rat monoclonal antibody specific for tyrosinated α-tubulin, diluted (1:1000), followed by Alexa 594 or Alexa 488 goat anti-rat immunoglobulin IgG (Invitrogen, Burlington, ON, Canada) diluted 1:100. The incubation time for each antibody was 60 min at room temperature. After each incubation period, the cells were rinsed two times for 5 min each with PBS, and then, to facilitate spreading of the antibody, they were rinsed with PBS containing 0.1% Triton X-100 prior to adding the antibodies. Preparations were kept in the dark during the incubation periods to prevent light inactivation of the fluorochromes. All dilution of antibodies was done in PBS. Coverslips were mounted in Mowiol (Calbiochem, Billerica, MA, USA) solution [Osborn and Weber, 1982] containing paraphenylene diamine as antifading agent [Fabian and Forer, 2005], and stored at 4°C in the dark until viewed in the confocal microscope.

Results

Experimental Overview

Our overall experimental procedure is illustrated in Figure 1. After the start of anaphase (Fig. 1A) we cut a fragment from an arm (Fig. 1B). The fragment moved rapidly toward the arm of the partner chromosome (Fig. 1C,D) and before it reached the partner we irradiated between the arm fragment and the partner (Fig. 1D). The arm fragment stopped moving after the second cut (Fig. 1E,F), showing that the laser cut the tether. Once we were sure the tethers were cut, we cut kinetochore spindle fibres associated with one chromosome of the pair whose tethers were cut (Fig. 1F) and monitored subsequent chromosome movement.

Anaphase Chromosome Arms are Connected by Tethers

We cut trailing arms of anaphase chromosomes using the laser microbeam. Upon cutting the arm, the arm fragment moved toward the partner chromosome, often, but not always, reaching the partner (Fig 2A and B; Supporting Information video 1), as described by LaFountain et al. [2002]. The initial velocities of the arm fragments were on average 4.8 μm/min ($n = 13$), with a range 1.1–18 μm/min., consistent with the values given by LaFountain et al. [2002]. Arm fragment velocities were considerably faster than anaphase chromosome movement (~ 0.5 μm/min), as described in detail by LaFountain et al. [2002]. The movements toward the partner chromosomes were at constant speed initially, but sometimes slowed as the fragment neared the partner chromosome (Fig. 2B).

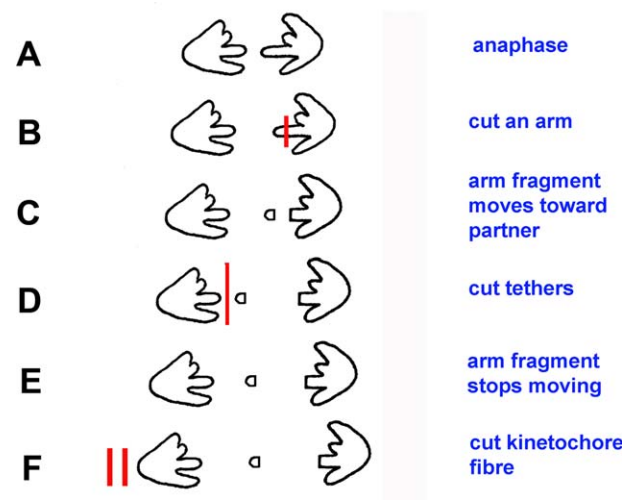

Fig. 1. Overview of experimental approach. One chromosome pair entering anaphase, moving to poles to the left and right (**A**), with one trailing arm in each chromosome. The trailing arm of the right chromosomes is cut with a laser at the position indicated by the line **(B).** The arm fragment that is formed moves toward the left chromosome **(C and D).** The tether between the fragment and the trailing arm of the left chromosome is cut **(D),** as indicated by the line. The arm fragment stops moving to the left chromosome **(D, E, F**), indicating that the laser cut the tether. The kinetochore fibre of the left chromosome is cut in two places **(F),** indicated by the lines. [Color figure can be viewed at wileyonlinelibrary.com]

LaFountain et al. [2002] found that only two of the four arms in a separating half bivalent have tethers. We have not tested this in detail, but in our experiments not all chromosome arm fragments moved toward the partner, either when single fragments were produced or when fragments were produced from several arms of one chromosome (Fig. 3). While this might imply a 50:50 chance of finding tethers when cutting single arms, we were able to identify arms that were connected with tethers in a much higher percentage than that. It is often the case in anaphase crane-fly spermatocytes that one or two arms are stretched behind while the others are angled more toward the side or even are forward from the kinetochore [Adames and Forer, 1996]; LaFountain et al. [2002] showed that when they cut one of the 'trailing' arms, the arm fragments moved toward the partner in almost all cases. In our experiments, by cutting mostly the 'trailing' arms, we were able to identify tethers (backward movement of arm fragments) in ~80% of the cuts (47/58). We could not detect any change in velocity of chromosomes whose arms were cut, so it would seem that either the force produced by the tether is small compared to the force on the kinetochore acting in the other direction, or the force on the chromosome adjusts for a reduced load, that velocity is independent of load, as described by Nicklas [1965].

The Laser Microbeam Cuts the Tethers

We cannot identify tethers morphologically, but we hoped that cutting between the arm fragment and the partner

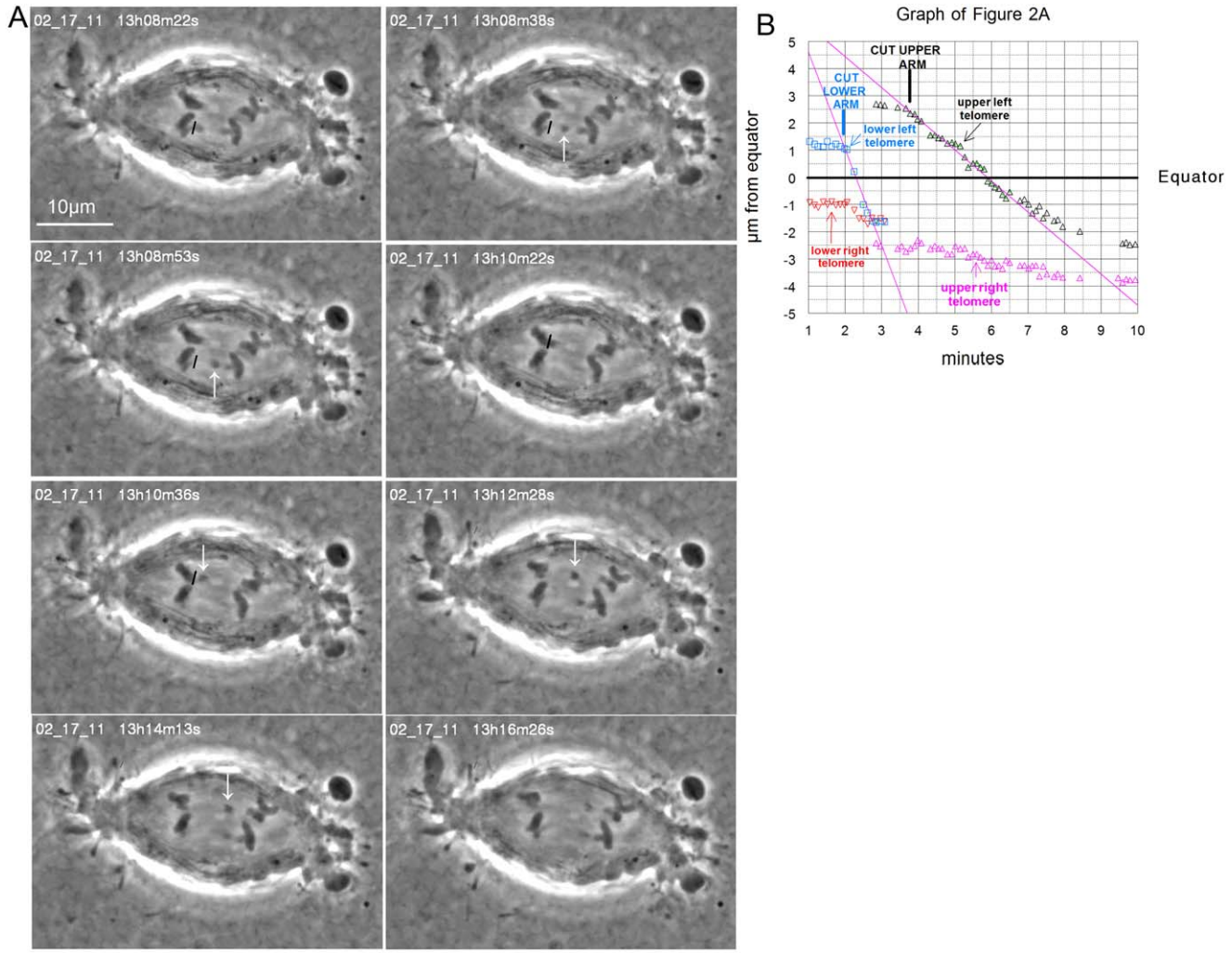

Fig. 2. Movements of arm fragments across the equator. **(A)** Illustrates movement of arm fragments across the equator. The black line across the trailing chromosome arm of the lower chromosome (13:08:22) indicates the position that the laser would cut. After the cut (in multiple planes), the separated arm fragment (arrows in 13:08:38 and 13:08:53) moves across the equator to the partner chromosome. The trailing arm of the upper chromosome was cut at the position of the black line (13:10:22 and 13:10:36) and the resultant arm fragment (arrows in 13:10:36, 13:12:28 and 13:14:13) moves across the equator toward its partner. The length of the line in the first image represents 10µm in the cell. **(B)** Graphically represents the movements of the two arm fragments shown in Fig. 2A. The telomeres of the lower of the half-bivalent pairs are represented by circles (blue for the left telomere and red for the right, partner, telomere). The telomeres of the upper of the half-bivalent pairs are represented by triangles (black for the left telomere and magenta for the right, partner, telomere). The positions are plotted against a fixed point chosen (separately for each pair) at the equator. The first (lower) arm fragment moved to its partner with velocity 3.6 µm/min and the second (upper) moved with initial velocity 1.1 µm/min but never reached its partner. The lines are the least-mean-squares fits to the indicated points. [Color figure can be viewed at wileyonlinelibrary.com]

telomere when the arm fragment was moving backward toward the partner would stop the movement of the arm fragment, indicating that the laser cut the tether. We cut across the entire region between the partner chromosomes, and because we could not identify where the tethers were, irradiations were in at least three focal planes. In most but not all cells [34/42] the irradiations stopped the backward motion of the arm fragments (Fig. 4). In those cells in which movement was not stopped the laser presumably did not cut the tethers.

Cutting the tethers caused the trailing arm to contract. In most cells the configurations of the arms were not seen clearly in the time-lapsed images, but in a few cells the images were especially clear. In these especially clear cells we saw that when the tether was cut, not only did the fragment stop moving but the extended arm of the partner chromosome retracted (Fig. 5; Supporting Information video 2). Also in especially clear cells we saw that the extended arm of the partner chromosome contracted as the arm fragment approached it, as seen with the lower chromosome in Figs. 2A and B and Supporting Information video 1. We could not detect any change in chromosome movement velocities when the chromosome tethers were cut.

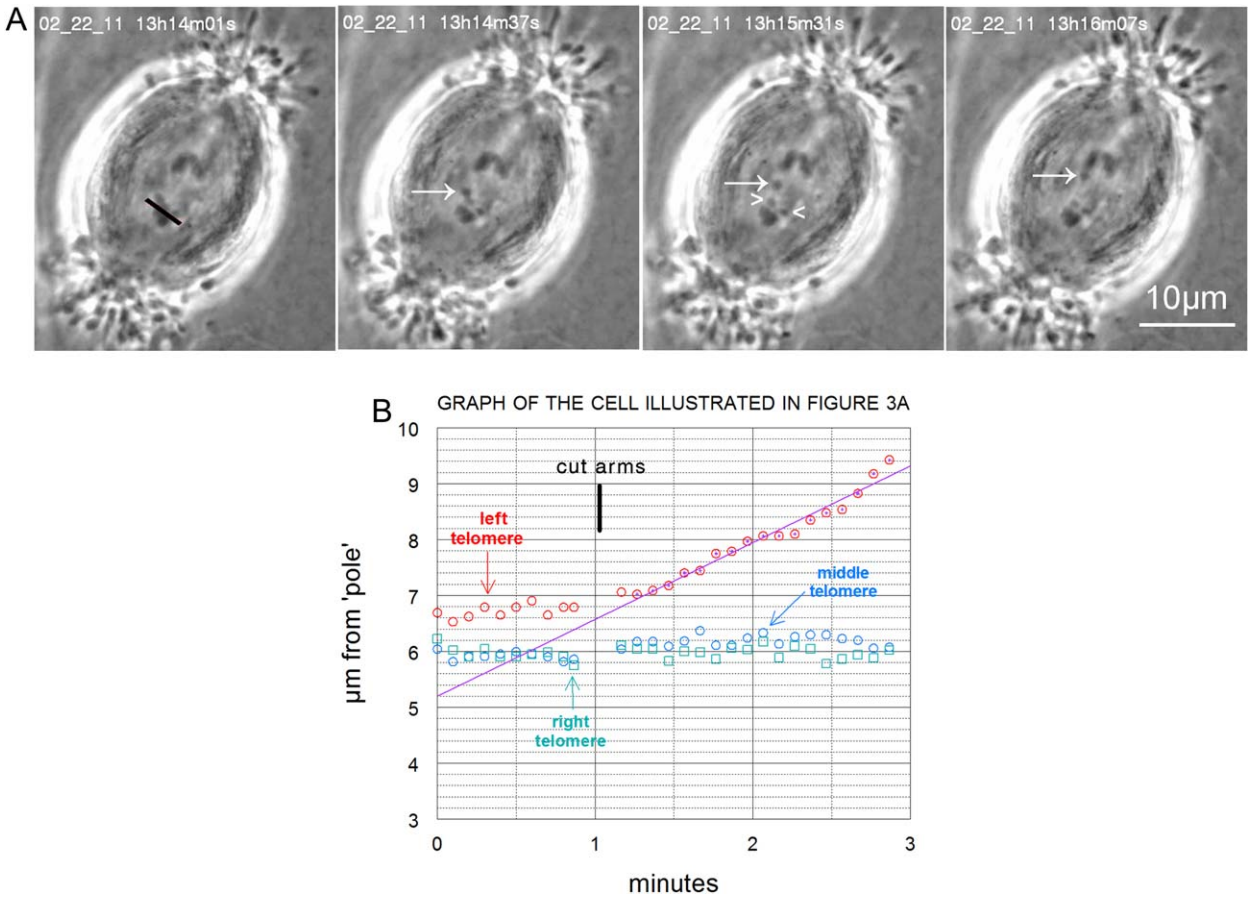

Fig. 3. Only some arms are connected with tethers. **(A)** Illustrates a cell in which three arms were cut but only one of the arm fragments moved to its partner. The position that the laser will subsequently cut is indicated by the black line (13:14:01). Three arm fragments were formed but only the one indicated by the white arrow (13:14:37; 13:15:31; 13:16:07) moves to its partner. The other two (indicated by < and > in 13:15:31) do not move. The line indicates 10 µm in the cell. **(B)** Graphically represents the movements of the three arm fragments illustrated in Fig. 2A. Two of them do not move, but the third moves across the equator to its partner (with velocity 1.4 µm/min). The positions are plotted against a fixed point chosen at the 'pole'. For comparison with the images in Fig. 3A, time zero in this graph is 13:13:09. The line is the least-mean-squares fit to the indicated points. [Color figure can be viewed at wileyonlinelibrary.com]

After cutting tethers, the backward motion of the arm fragment generally stopped. In most cells the arm fragment remained stationary after tethers were cut, but in some cells the fragment rapidly reversed direction and moved back to the arm-stub from whence it came (Fig. 5; Supporting Information video 2). Presumably, this occurred because the arms were not completely severed and some elastic component that was of less strength than the tether connected the arm-stub and the moving fragment.

Anaphase Chromosome Velocities Increase after Tethers are Cut Followed by Cutting Kinetochore Fibres

Cutting kinetochore fibres of anaphase chromosomes with the laser microbeam did not cause changes in the velocity of chromosome movements toward their poles, similar to when the kinetochore microtubules were cut by UV microbeam irradiations [Spurck et al., 1997]. The laser cuts kinetochore microtubules in these cells, e.g., Forer et al. [2013], as it does in other cells (reviewed in Forer et al. [2015]), and to test whether the presence of tethers prevents chromosomes from accelerating when their kinetochore microtubules are cut, we cut the tethers before irradiating the kinetochore fibres. We usually irradiated the kinetochore fibres in two separate positions along the length of the fibre.

When tethers were cut prior to cutting the kinetochore fibres, chromosomes associated with the cut kinetochore fibres increased their velocities, but only in some cells (Fig. 6). The results were not consistent, presumably because the laser irradiation did not always cut the tethers: the tethers are from two arms and probably exist in multiple planes, and though the laser cut in several planes we could not be certain that the laser cut either or both tethers. To be certain that tethers were cut prior to cutting kinetochore spindle fibres we followed the procedure outlined in Fig. 1. We first cut a trailing chromosome arm. As the arm fragment moved backwards we aimed the laser between the two

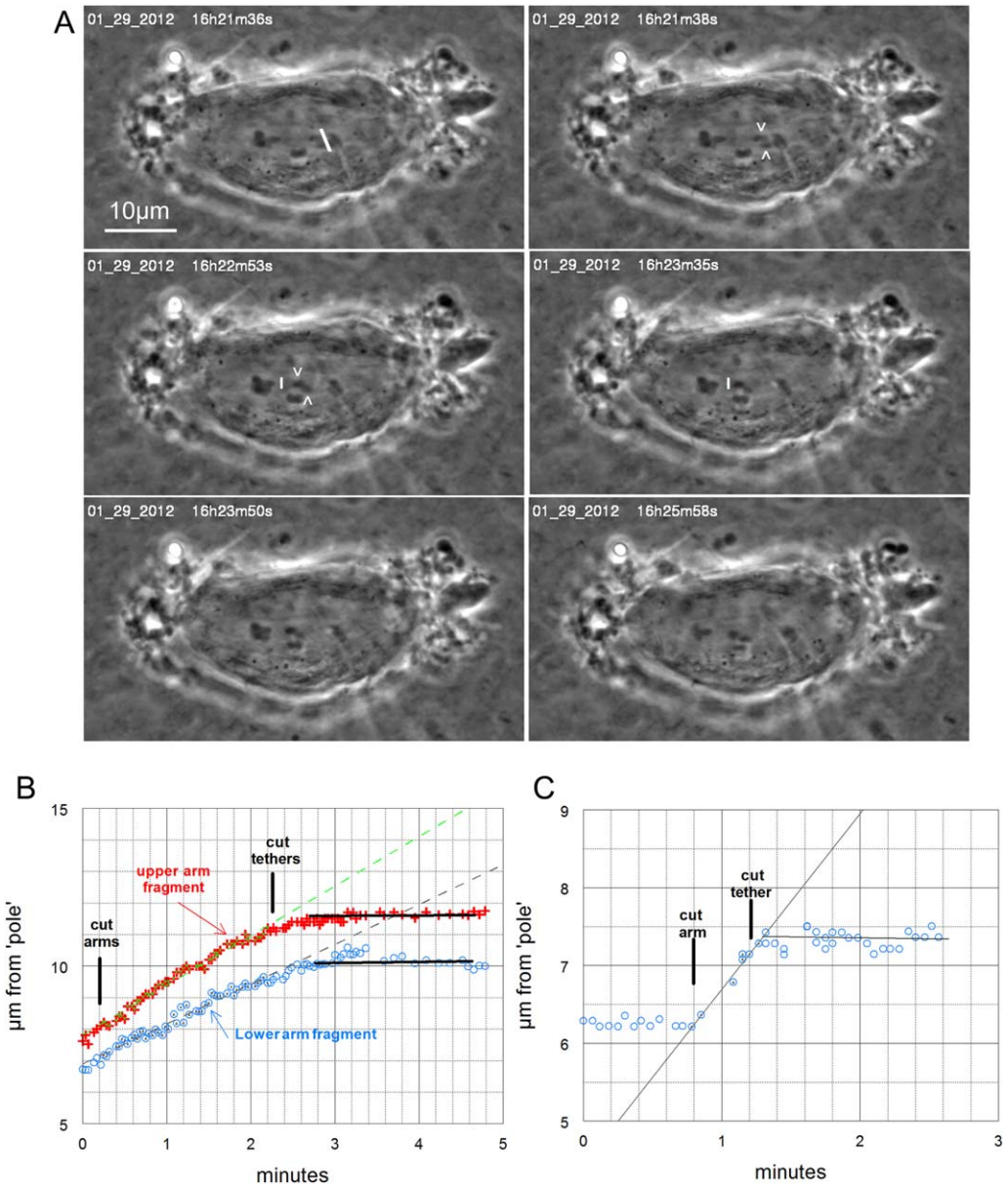

Fig. 4. Cutting tethers stops backwards movement of arm fragments. **(A)** Illustrates a cell in which arm fragment movements were stopped by cutting tethers. The position of the laser cut (at the time of the cut) is indicated by the white line (16:21:36). The two arm fragments formed (arrowheads in 16:21:38 and 16:22:53) move backwards toward their partners. The white line in 16:23:35 indicates the position of the laser as the tethers were cut. The line in the image at 16:21:36 indicates 10 µm in the cell. **(B)** Graphically represents the movements of the two arm fragments illustrated in Fig. 4A. The upper fragment moved with velocity 1.6 µm/min, the lower with velocity 1.3 µm/min. Both stopped moving when the tethers were cut. For comparison with the illustrations, time zero in this graph is 16:21:19. The distances were plotted against a fixed point chosen at a 'pole'. **(C)** Graphically illustrates how cutting tethers stops the backward movement of an arm fragment in another cell (that cell illustrated in *Fig. 7A*). The arm fragment moved toward its partner (with velocity 2.3µm/min) but stopped once the tether was cut. [For comparison with *Figure 7A*, time zero in the graph is 15:41:36.] The distances were plotted against a fixed point chosen at a 'pole'. [Color figure can be viewed at wileyonlinelibrary.com]

telomeres (and included the interzone along the width of the chromosome), to cut all tethers associated with the chromosome. Stopping the backward movement of the fragment indicated that its tethers were cut. We then cut the kinetochore fibre associated with that chromosome (Fig. 7; Supporting Information video 3). Since we generally monitored only one arm, it is possible that the laser did not cut the tethers associated with the other arm; nonetheless, chromosome velocities increased for almost 80% of the chromosomes whose kinetochore fibres were irradiated, 27/35. The increased velocity lasted 1–3 min, during which chromosome velocities were on average about

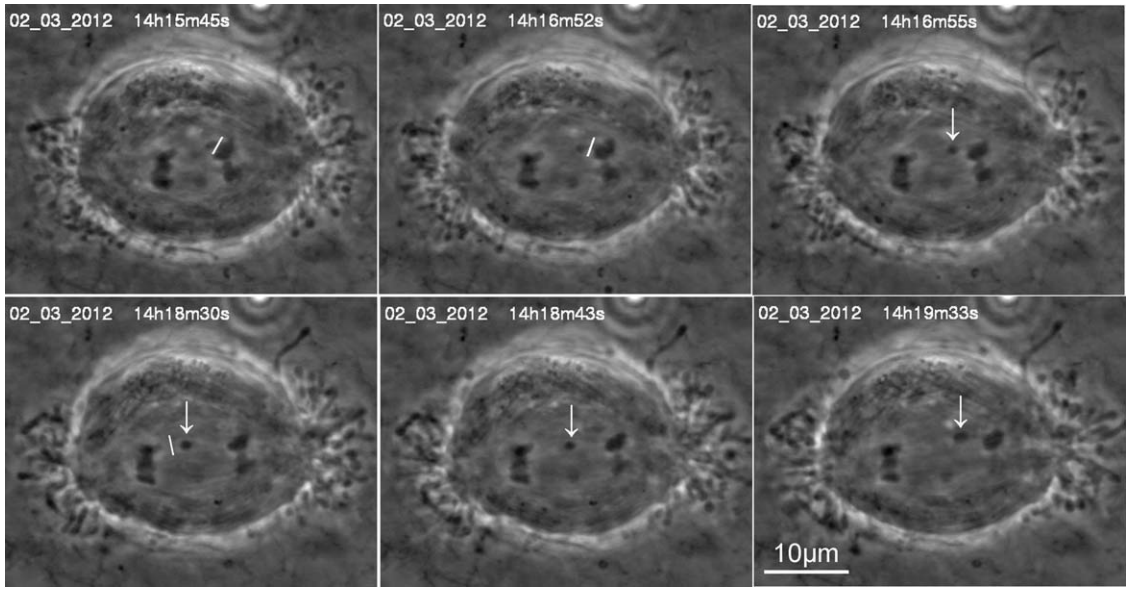

Fig. 5. Incomplete cutting of arms. Illustrates a cell in which the arm fragment reversed direction and moved back toward its original arm when its tether was cut. The white line in 14:15:45 indicates the position the laser will cut and the line in 14:16:52 is at the time of the cut. The arm fragment is indicated by white arrows in 14:16:55 and subsequent images. The tether is cut at the position of the white line in 14:18:30, after which the arm fragment moves back toward its original arm stub. The line in the image at 14:19:33 indicates 10μm in the cell

double their pre-irradiation velocities (Table I, Figs. 6, 7B). After the increased-velocity time period the chromosomes returned to their original velocities.

In some experiments the order of the cuts was reversed, cutting the kinetochore spindle fibre first, and then after a minute or two, cutting the arms and then the tethers: chromosomes did not change velocity in these cells.

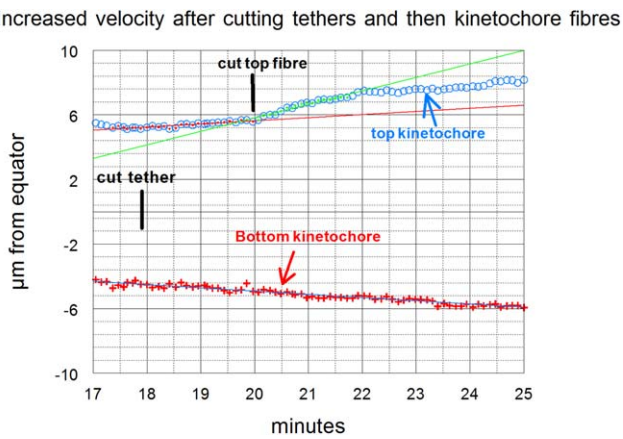

Fig. 6. Increased velocity after cutting kinetochore fibres. Graphically represents the increased velocity that occurs after first cutting the tethers and then cutting the kinetochore spindle fibre, plotted against a fixed point ('equator') between the partner chromosomes. Neither cutting the tether nor cutting the top kinetochore fibre altered the movement of the lower, partner, chromosome, which moved to the pole with velocity 0.2 μm/min, the same as the upper chromosome. The upper chromosome temporarily moved with increased velocity (of 0.84 μm/min) after its kinetochore fibre was cut, and after 2 min returned to its original velocity. The lines are least-mean-squares lines through the indicated points; the slopes of the lines are the velocities. [Color figure can be viewed at wileyonlinelibrary.com]

Immunostaining Confirms that the Laser Cut the Kinetochore Microtubules Associated with the Increased-Velocity Chromosomes

We assume that the temporary increased velocity of the chromosomes was because of cutting the kinetochore microtubules, since this is what occurs in other cells (review in Forer et al. [2015]). To confirm this, we stained irradiated cells with anti-tubulin antibody. In these experiments the laser cut the kinetochore fibres associated with anaphase chromosomes in multiple focal planes, as in the live cell analysis. Cells were lysed very shortly thereafter in preparation for immunofluorescence staining. Microtubules were absent from the cut regions of all six irradiated spindles that were studied using confocal microscopy (e.g., Fig. 8A, B). In some cells lysis took place before we could determine (from the images) whether chromosome velocity increased, but in other cells half-bivalents speed up after kinetochore fibre irradiation, before the cells were lysed (Fig. 8C). The irradiated region often can be seen in phase contrast microscopy images of the lysed cells, appearing as breaks in the otherwise phase-dark kinetochore spindle fibres (Fig. 8A).

Discussion

Our experiments show that when kinetochore microtubules in anaphase crane-fly spermatocytes are cut in the *absence* of tethers between partner chromosomes, the associated

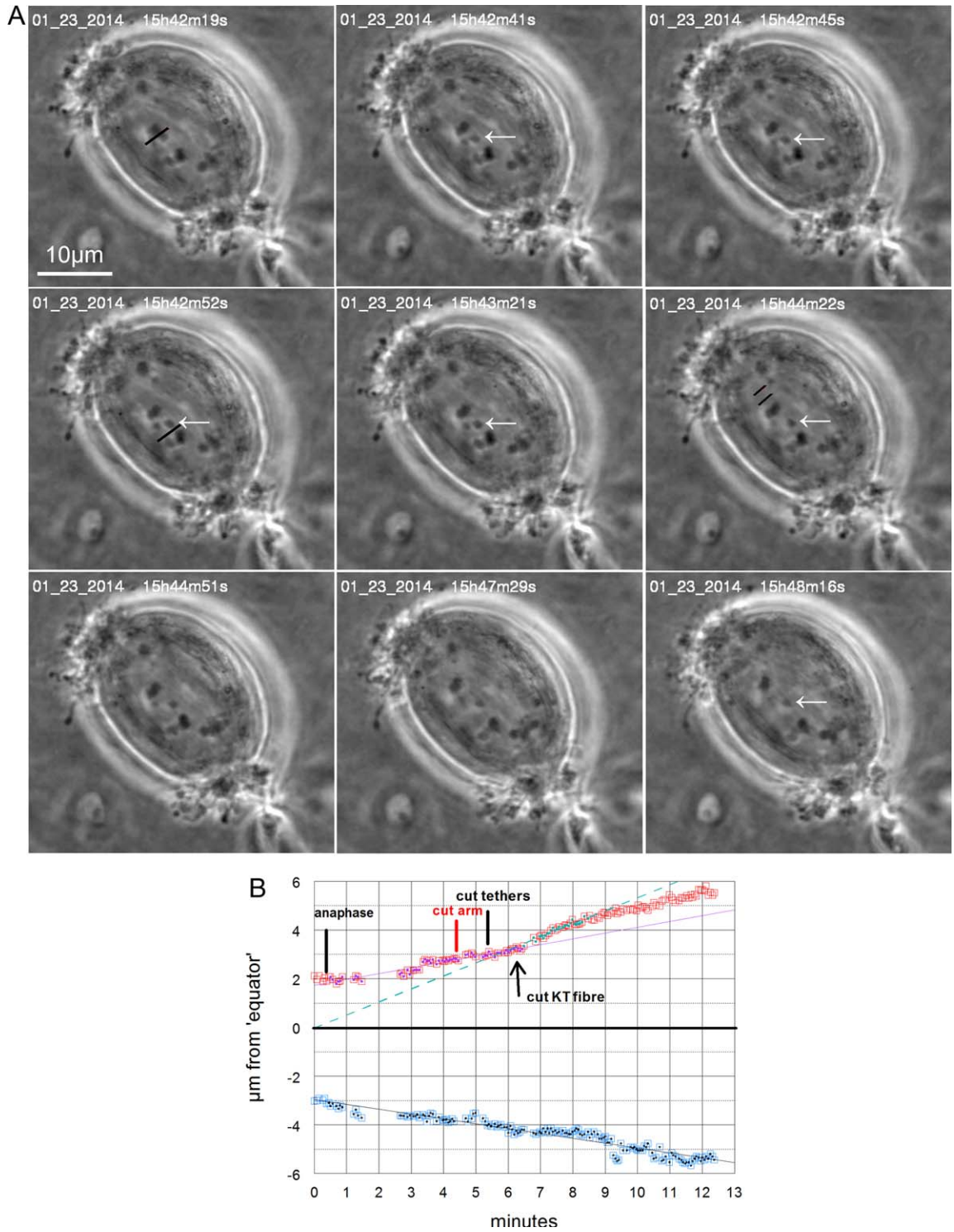

Fig. 7. Increased velocity after cutting arms, then tethers, then kinetochore fibres. **(A)** Illustrates a cell in which cutting tethers stopped the backwards movement of two arm fragments, after which the chromosome temporarily increased in velocity after its kinetochore spindle fibre was cut. The black line in 15:42:19 indicates the position of the laser as it was cutting two chromosome arms. The right arm-fragment is indicated by the white arrow in the subsequent five images. The black line in 15:42:52 indicates the position cut by the laser at the time the tether was cut, and the two black lines in 15:44:22 are the positions of the laser cuts on the kinetochore spindle fibre at the time it was being cut. The white line in the first image indicates 10µm in the cell. **(B)** Graphically represents chromosome movement in the cell illustrated in *Fig. 7A*. The kinetochore positions of the two partner chromosomes were measured against a fixed point ('equator') between them. Both moved poleward with velocities of 0.2 µm/min (magenta and black lines). The upper chromosome temporarily moved faster (0.54 µm/min, dashed green line) when the kinetochore fibre microtubules were cut, but returned to original speed after about 2 min. The lines are least-mean-squares lines through the indicated points. For comparison with the images of the cell in Fig. 7A, time zero on the graph is 15:38:00. [Color figure can be viewed at wileyonlinelibrary.com]

Table I. Increase of Chromosome Velocity after Cutting Arms, then Tethers, and then Kinetochore Microtubules

Number of chromosomes measured	Pre-irradiation velocity Average ± s.d	Post-irradiation velocity Average ± s.d	Time with increased velocity Average ± s.d	Ratio of individual velocities: post-irradiation/ pre-irradiation Average ± s.d
19	0.47 ± 0.21 μm/min	0.96 ± 0.53 μm/min	125 ± 61 seconds	2.4 ± 1.3

s.d. = standard deviation.

chromosome temporarily speeds up. In previous experiments (described in Forer et al. [2015]), when kinetochore microtubules are cut in anaphase crane-fly spermatocytes in the *presence* of tethers between partner chromosomes, the associated chromosome moves at the original speed. Our results suggest therefore that tethers coordinate the movements of separating partner chromosomes: severing the tethers unlinks their movements and allows the acceleration. To confirm that the laser cut the tethers we monitored behaviour of arm fragments: the arm fragments stopped moving to their partner when the tethers were severed. To show that the kinetochore microtubules were severed by the laser we studied cut kinetochore fibres using confocal immunofluorescence microscopy: kinetochore microtubules were severed at the sites of irradiation.

Crane-fly spermatocytes are different from other cells with respect to effects of cutting kinetochore microtubules. Whereas the associated chromosomes temporarily speed up after kinetochore microtubules are severed in anaphase in a variety of cells (described in the Introduction and reviewed in Forer et al. [2015]), chromosomes in crane-fly spermatocytes did not speed up when their kinetochore microtubules were severed. Crane-fly spermatocytes stood out as the single exception. Our results indicate that the reason the results in crane-fly spermatocytes were unique is because of tethers between separating anaphase chromosomes.

These data are consistent with the conclusion that tethers coordinate movements of partner chromosomes. However, while there is a correlation between cutting tethers and speeding up chromosome movement, we cannot be sure that the laser has not severed other components of the interzone region as well as severing tethers. Therefore, we cannot be completely sure that the coordination is because of tethers rather than something else coincidentally cut. Nor are there markers or stains to identify tethers in order to monitor them directly to see when they are cut. Nonetheless, we think the data strongly suggest that tethers between separating chromosomes coordinate the anaphase movements of separating chromosomes. The data also suggest that tethers are responsible for the coordinated stopping of chromosome pairs after UV microbeam irradiation of single kinetochore spindle fibres, and for the coordinated movements by pairs after drug treatment.

Several issues arise from these conclusions. One is how the tethers might influence the movements of the separating chromosomes. Since elastic elements would produce tension, it is reasonable to expect that tension produced in both partner chromosomes could act as a modulating signal so that both behave the same way, much as tension is thought to be involved in other chromosome activities. For example, proper attachment of chromosomes to the spindle applies tension between partners' kinetochores which stabilizes the correct attachment [Nicklas and Koch, 1969; Nicklas and Ward, 1994; Musacchio and Salmon, 2007].

Another issue is: what are tethers made of? Cytologically one cannot see tethers in crane-fly spermatocytes using phase-contrast microscopy, or interference microscopy [Muller, 1970], or after Feulgen or Giemsa staining [Janicke and LaFountain, 1982, 1984; LaFountain, 1985; Ladrach and LaFountain, 1986], or in most electron microscopy [e.g., Fuge, 1971]. One electron microscopic study of crane-fly spermatocytes, however, noted that in two favourably oriented anaphase cells there were filamentous structures about 5nm thick that extended between chromatid arms of three of the six separating pairs of anaphase chromosomes in the cells [Fuge, 1978], and an electron microscopic study of male meiosis-I in cockroaches found similar connections between separating anaphase chromosomes [Krishan and Buck, 1965]. Fuge [1978] suggested that these interzonal 'filaments' were from stretched chromatin, but without further information one cannot really deduce chemical composition from images in electron microscope sections: those 'filamentous' connections could be composed of anything. In other cell types studied light microscopically, interzonal connections between separating chromosomes have been detected by passing a micromanipulation needle between separating chromosomes in grasshopper neuroblasts [Carlson, 1952], and in fixed cells Feulgen-negative interzonal connections are regularly seen between anaphase chromosomes (e.g., [Schrader, 1953], pp. 43 et seq.), but there is no information on whether these could act as tethers. Feulgen positive (chromatin) bridges also have been seen between anaphase chromosomes, but they generally are associated with holding back the separating chromosomes, not functioning as tethers, though in some cells chromatin bridges are regularly seen in early

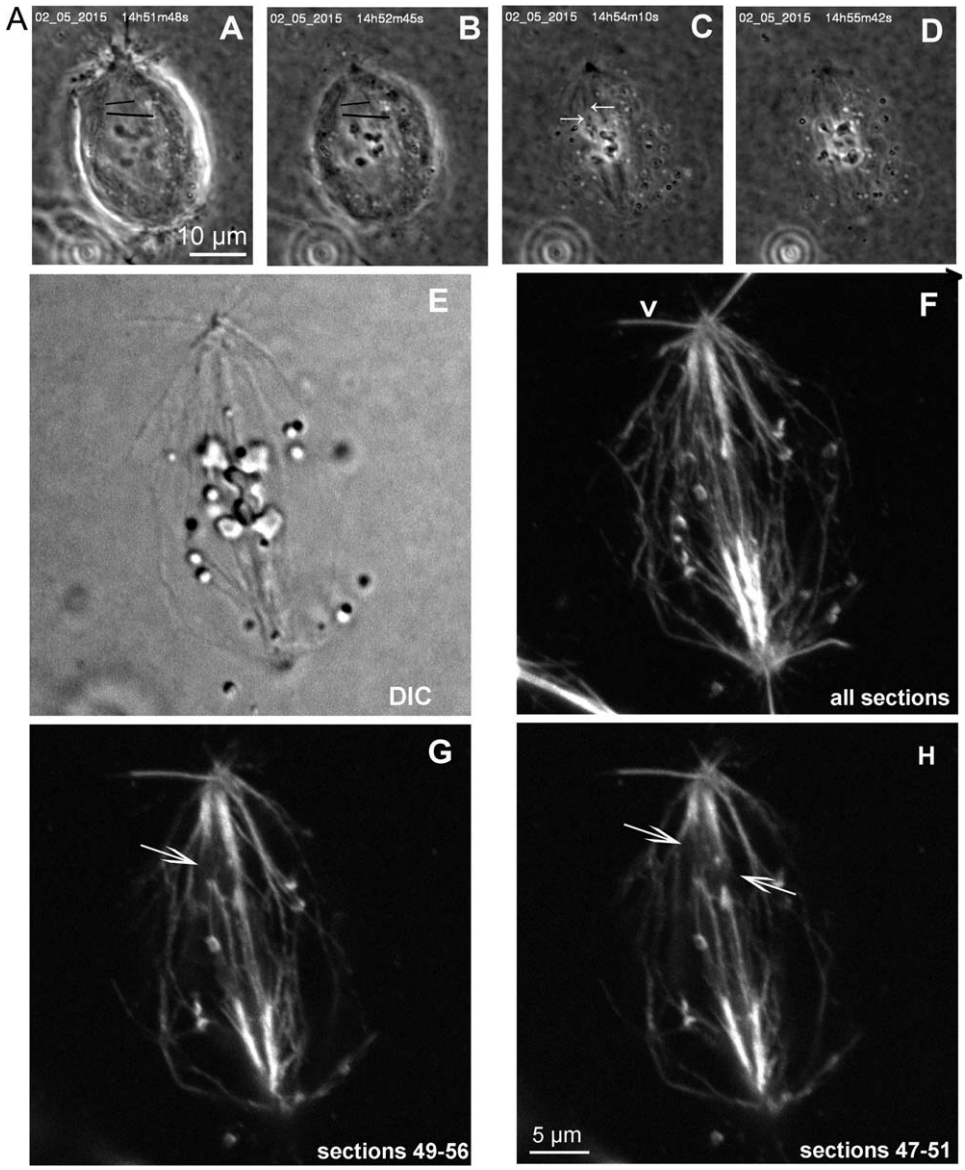

Fig. 8. Confocal microscopy images of cut kinetochore fibres. **(A)** Illustrates confocal microscopy images of cells stained for tubulin after kinetochore fibres in the cells were cut with laser irradiation. Images in panels *A* through *D* were taken using phase-contrast microscopy, and *E* to *H* are images of the same cell taken using confocal microscopy. The black lines in *A* show the position of the laser cuts on spindle fibres in the live cell prior to cutting the spindle fibres, and *B* is at the start of perfusion with lysis buffer. Images *C* and *D* are in lysis buffer after the cell was lysed. The arrows in panel *C* point to the kinetochore and pole ends of a cut in a kinetochore fibre. The ends no longer are aligned: the 'stubs' are at a slight angle to each other. The white line in *A* represents 10µm in the cell. The image in *E* is from DIC imaging that was recorded simultaneously with the fluorescence images. The image in *F* includes all confocal sections; the images in *G* and *H* include only select sections to illustrate the regions with severed microtubules (arrows). The arrowheads in *F* point to two precocious sperm tails that are found at each pole in these primary spermatocytes. The line in *H* represents 5 µm in the confocal images. **(B)** Illustrates confocal microscopy images of cells stained for tubulin after the spindles in the cells were cut with laser irradiation. Panels *A* through *F* are images taken with phase contrast microscopy and *G* through *J* are images of the same cell taken using confocal microscopy. Images *A* through *D* are of the live cell and the images in *E* and *F* are after lysis buffer was perfused into the sample. The black line in *A* indicates the laser position just after arms of the downward moving chromosome were cut. The black line in *B* indicates the laser position just as the tethers were cut. The black lines in *C* and *D* indicate the laser positions as the spindle fibres were cut, and the black lines in *E* (after cell lysis) are the same positions as in *C* and *D*. The white line in *A* represents 10µm in the cell. The image in *G* is from DIC imaging that was recorded simultaneously with the fluorescence images. The image in *H* includes all confocal sections; images in *I* and *J* include only select sections to illustrate the severed kinetochore microtubules (arrows) and that the remaining kinetochore stubs are slightly at an angle from the original orientation. The line in *G* represents 5 µm in the confocal images. **(C)** Graphically represents chromosome movement in the cell illustrated in Figure 8B as measured from a fixed point ('equator'). The positions of one kinetochore moving to the upper pole are indicated by red crosses. The black line is the least-mean-squares line through the indicated points, from which one obtains the velocity of movement before cutting the kinetochore fibre (0.47 µm/min); the blue line represents the movement after cutting the kinetochore fibre (velocity = 2.3 µm/min). KT = kinetochore.

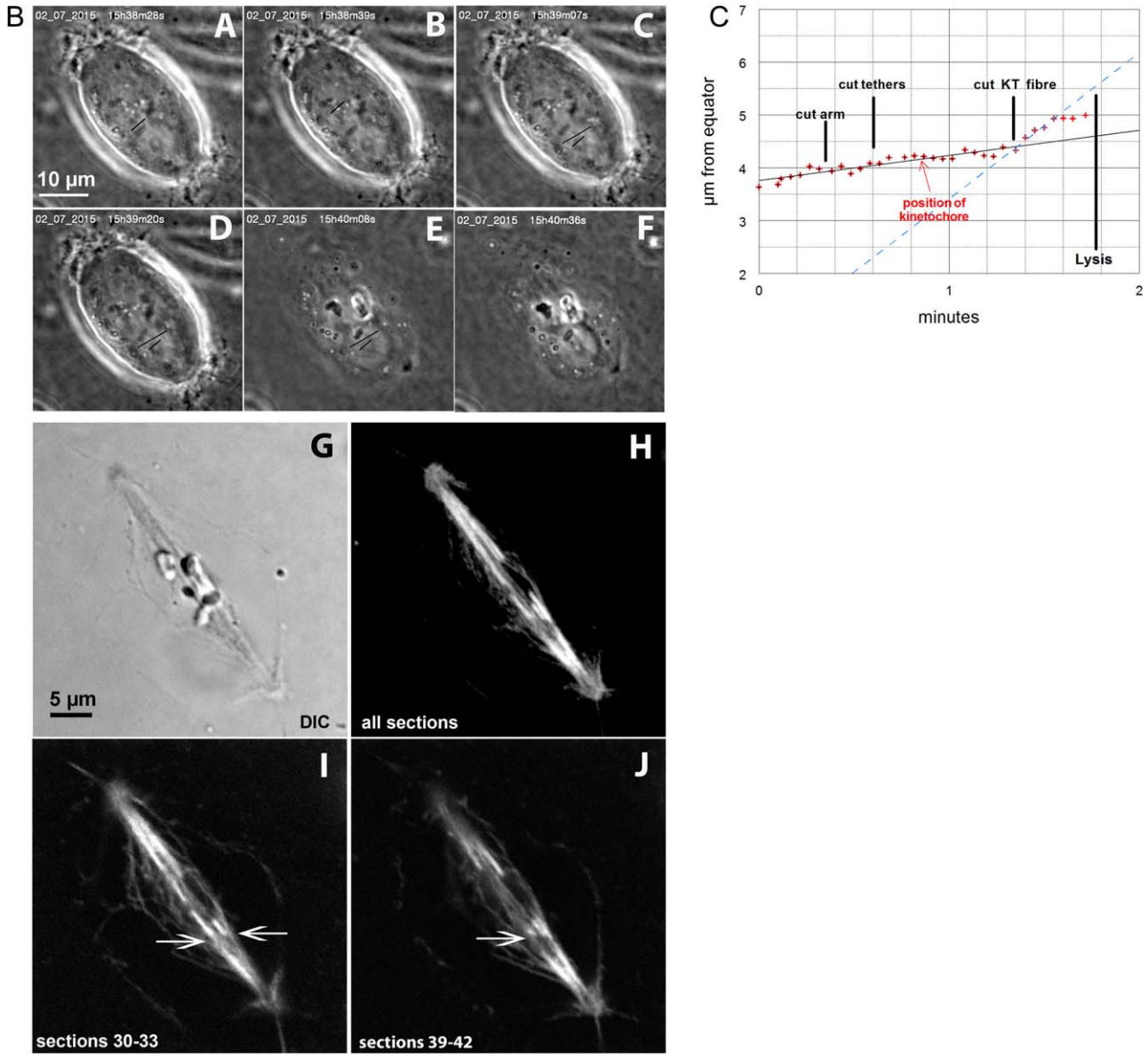

Fig. 8. (Continued)

anaphase (e.g., [Ris, 1942]) and these conceivably could act as tethers. We really have no data on what tethers are composed of, however, but whatever their composition, tethers must spin out between separating telomeres, lengthen as the telomeres separate, and until later anaphase must have elasticity enough to pull arm fragments to the attached partner with a force that is less than the poleward force acting on the kinetochores. One component that could satisfy these criteria actually extends between separating arms in anaphase crane-fly spermatocytes, and that is the protein titin [Fabian et al., 2007]. Fabian et al. [2007] identified titin in spindles of crane-fly spermatocytes, extending between the telomeres of separating anaphase chromosomes, exactly where the tethers are. The giant protein titin is the third most abundant protein in skeletal muscle and individual titin molecules extend from the Z-disk to the M-line of the sarcomere. Titin produces passive force as the sarcomere stretches [Miller et al., 2004; Granzier and Labeit, 2006] and is responsible for most of muscle elasticity. Because titin is present between separating telomeres in anaphase and because titin in muscle acts as sensor of tension [Miller et al., 2004; Tskhovrebova and Trinick, 2003], it might do the same in the spindle and act as a modulating signal in the movements of separating chromosomes. While we do not know what tethers are composed of, we speculate that titin might be one of the components. One could test this speculation by studying the effects of the laser microbeam on the titin extending between separating chromosomes.

Other issues arising from our data are what causes the chromosomes to move at all when kinetochore microtubules are severed, and why they speed up, and why the

speed up is only temporary. These issues are discussed in detail in Forer et al. [2015]. We think that both the continued movement and the temporary increase in velocity indicate that the force for chromosome movement arises from a spindle matrix [Johansen and Johansen 2007; Pickett-Heaps and Forer, 2009; Johansen et al., 2011; Sheyhkani et al., 2013a,b], and that the force does not arise from other microtubules interacting with the kinetochore stub, as [Elting et al., 2014] have suggested. We think that in control cells the speed of anaphase chromosome movement is slowed by microtubules extending between kinetochore and pole (the microtubules are 'governors' of the speed), that with the microtubules severed the governors are gone and the force from the spindle matrix causes increased speed, and that the speed increase is temporary because the kinetochore microtubule stub eventually encounters barriers (e.g., microtubules extending from the pole). Regardless, a main contribution of the data presented here is to remove crane-fly spermatocytes as an exception to chromosomes speeding up when kinetochore microtubules are cut.

A final issue is whether the existence of tethers is a more general phenomenon, or whether tethers occur only in crane-fly spermatocytes. We know of no other studies that have tested this in other cell types, so there is no evidence one way or another.

In sum, anaphase chromosomes in primary crane-fly spermatocytes move with unaltered speed when their kinetochore microtubules are severed, but when the tethers between separating chromosomes are severed first, they temporarily move faster when their kinetochore microtubules are severed. They return to their normal speed after a few minutes. Thus the tethers seem to modulate the movements of the separating chromosomes. The composition and structure of tethers is unknown, but we speculate that they might contain the giant protein titin.

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References

Adames KA, Forer A. 1996. Evidence for polewards forces on chromosome arms during anaphase. Cell Motility Cytoskel 34:13–25.

Carlson JG. 1952. Microdissection studied of the dividing neuroblast of the grasshopper, *Chortophaga viridifasciata* (De Geer). Chromosoma 5:199–220.

Elting MW, Hueschen CL, Udy DB, Dumont S. 2014. Force on spindle microtubule minus ends moves chromosomes. J Cell Biol 206:245–256.

Fabian L, Forer A. 2005. Redundant mechanisms for anaphase chromosome movements: crane-fly spermatocyte spindles normally use actin filaments but also can function without them. Protoplasma 225:169–184.

Fabian L, Xia X, Venkitaramani DV, Johansen KM, Johansen J, Andrew DJ, Forer A. 2007. Titin in insect spermatocyte spindle fibers associates with microtubules, actin, myosin and the matrix proteins skeletor, megator and chromator. J Cell Sci 120:2190–2204.

Ferraro-Gideon J, Sheykhani R, Zhu Q, Duquette ML, Berns MW, Forer A. 2013. Measurements of forces produced by the mitotic spindle using optical tweezers. Mol Biol Cell 24:1375–1386.

Forer A. 1966. Characterization of the mitotic traction system, and evidence that birefringent spindle fibers neither produce nor transmit force for chromosome movement. Chromosoma (Berl) 19:44–98.

Forer A. 1982. Crane fly spermatocytes and spermatids: a system for studying cytoskeletal components. In: Wilson L, editor. The Cytoskeleton. Methods in Cell Biology. Vol 25. New York: Academic Press. pp. 227–252.

Forer A, Pickett-Heaps J. 2005. Fibrin clots keep non-adhering living cells in place on glass for perfusion or fixation. Cell Biol Int 29: 721–730.

Forer A, Ferraro-Gideon J, Berns M. 2013. Distance segregation of sex chromosomes in crane-fly spermatocytes studied using laser microbeam irradiations. Protoplasma 250:1045–1055.

Forer A, Johansen KM, Johansen J. 2015. Movement of chromosomes with severed kinetochore microtubules. Protoplasma 252: 775–781.

Fuge H. 1971. Spindelbau, Mikrotubuliverteilung und Chromosomenstruktur während der I. meiotische Teilung der Spermatocyten von *Pales ferruginea*. Eine elektronenmikroskopische Analyse. Z Zellforsch 120:579–599.

Fuge H. 1978. Fine structure of anaphase bridges in meiotic chromosomes of the crane fly Pales. Chromosoma 65:241–246.

Granzier HL, Labeit S. 2006. The giant muscle protein titin is an adjustable molecular spring. Exerc Sport Sci Rev 34:50–53.

Harsono MS, Zhu Q, Shi LZ, Duquette M, Berns MW. 2013. Development of a dual joystick-controlled laser trapping and cutting system for optical micromanipulation of chromosomes inside living cells. J Biophotonics 6:197–204.

Hughes K, Forer A, Wilson P, and Leggiadro C. 1988. Ultraviolet microbeam irradiation of microtubules *in vitro*: the action spectrum for local depolymerization of marginal band microtubules *in vitro* matches that for reducing birefringence of chromosomal spindle fibres *in vivo*. J Cell Sci 91:469–478.

Ilagan A, Forer A, Spurck T. 1997. Backward chromosome movement in anaphase after irradiation of kinetochores or kinetochore fibres. Protoplasma 198:20–26.

Janicke MA, LaFountain JR. 1982. Chromosome segregation in crane-fly spermatocytes: cold treatment and cold recovery induce anaphase lag. Chromosoma 85:619–631.

Janicke MA, LaFountain JR. 1984. Malorientation in half-bivalents at anaphase: analysis of autosomal laggards in untreated, cold-treated, and cold-recovering crane fly spermatocytes. J Cell Biol 98: 859–869.

Johansen KM, Johansen J. 2007. Cell and molecular biology of the spindle matrix. Int Rev Cytol 263:155–210.

Johansen KM, Forer A, Yao C, Girton J, Johansen J. 2011. Do nuclear envelope and intranuclear proteins reorganize during mitosis to form an elastic hydrogel-like spindle matrix?. Chromosome Res 19:345–365.

Krishan A, Buck RC. 1965. Ultrastructure of cell division in insect spermatogenesis. J Ultrastruct Res 13:444–458.

Ladrach KS, LaFountain JR. 1986. Malorientatin and abnormal segregation of chromosomes during recovery from colcemid and nocodazole. Cell Motil Cytoskeleton 6:419–427.

LaFountain JR. 1985. Chromosome segregation and spindle structure in crane fly spermatocytes following colcemid treatment. Chromosoma 91:329–336.

LaFountain JR, Cole RW, Reider CL. 2002. Partner telomeres during anaphase in crane fly spermatocytes are connected by an elastic tether that exerts a backward force and resists poleward movement. J Cell Sci 115:1541–1549.

Miller MK, Granzier H, Ehler E, Gregorio CC. 2004. The sensitive giant: the role of titin-based stretch sensing complexes in the heart. Trends Cell Biol 14:119–126.

Müller W. 1970. Interferenzmikroskopische Untersuchungen der Trockenmassenkonzentration in isolierten Mitoseapparaten und lebenden Spermatocyten von *Pales ferruginea* (*Nematocera*). Chromosoma 30:305–316.

Musacchio A, Salmon ED. 2007. The spindle-assembly checkpoint in space and time. Nat Rev Mol Cell Biol 8:379–393.

Nicklas RB. 1965. Chromosome velocity during mitosis as a function of chromosome size and position. J Cell Biol 25: 119–135.

Nicklas RB, Koch CA. 1969. Chromosome micromanipulation. III. Spindle fiber tension and the reorientation of mal-oriented chromosomes. J Cell Biol 43:40–50.

Nicklas RB, Ward SC. 1994. Elements of error correction in mitosis: microtubule capture, release, and tension. J Cell Biol 126: 1241–1253.

Osborn M, Weber K. 1982. Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin-containing structures. Methods Cell Biol 24:97–132.

Pickett-Heaps J, Forer A. 2009. Mitosis: spindle evolution and the matrix model. Protoplasma 235:91–99.

Ris H. 1942. A cytological and experimental analysis of the meiotic behavior of the univalent chromosome in the bearberry aphid Tamalia (=Phyllaphis) coweni (CKLL). J Exp Zool 90:267–330.

Schrader F. 1953. Mitosis: The Movements of Chromosomes in Cell Division. New York, Columbia: University Press.

Sheykhani R, Shirodkar PV, Forer A. 2013a. The role of myosin phosphorylation in anaphase chromosome movement. Eur J Cell Biol 92:175–186.

Sheykhani R, Baker N, Gomez-Godinez V, Liaw L-H, Shah J, Berns MW, Forer A. 2013b. The role of actin and myosin in PtK2 spindle length changes induced by laser microbeam irradiations across the spindle. Cytoskeleton 70:241–259.

Shi LZ, Zhu Q, Wu T, Duquette ML, Gomez V, Chandsawangbhuwana C, Harsono MS, Hyun N, Baker N, Nascimento J, You Z, Botvinick EB, and Berns MW. 2012. Integrated optical systems for laser nanosurgery and optical trapping to study cell structure and function. In: A. Mendez-Vilas, editor. Current Microscopy, Contributions to Advances in Science and Technology, Badajoz, Spain: Formatex. pp. 685–695.

Sillers PJ, Forer A. 1981. Analysis of chromosome movement in crane fly spermatocytes by ultraviolet microbeam irradiation of individual chromosomal spindle fibres. I. General results. Can J Biochem 59:770–776.

Spurck T, Forer A, Pickett-Heaps JD. 1997. Ultraviolet microbeam irradiations of epithelial and spermatocyte spindles suggest that forces act on the kinetochore fibre and are not generated by its disassembly. Cell Motil Cytoskel 36:136–148.

Tskhovrebova L, Trinick J. 2003. Titin: properties and family relationships. Nat Rev Mol Cell Biol 4:679–689.

Wong R, Forer A. 2003. 'Signalling' between chromosomes in crane-fly spermatocytes studied using ultraviolet microbeam irradiation. Chromosome Res 11:771–786.

Yin B, Forer A. 1996. Coordinated movements between autosomal half-bivalents in in crane fly spermatocytes: evidence that 'stop' signals are sent between partner half bivalents. J Cell Sci 109: 155–163.