NUCLEUS
2020, VOL. 11, NO. 1, 19–31
https://doi.org/10.1080/19491034.2019.1710329

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

OPEN ACCESS Check for updates

# The role of phosphorylation in the elasticity of the tethers that connect telomeres of separating anaphase chromosomes

Emma Kite and Arthur Forer

Biology Department, York University, Toronto, Ontario, Canada

**ABSTRACT**

Elastic tethers, connecting telomeres of all separating anaphase chromosome pairs, lose elasticity when they lengthen during anaphase. Treatment with phosphatase inhibitor CalyculinA causes anaphase chromosomes to move backwards after they reach the poles, suggesting that dephosphorylation causes loss of tether elasticity. We added 50nM CalyculinA to living anaphase crane-fly spermatocytes with different length tethers. When tethers were short, almost all partner chromosomes moved backwards after nearing the poles. When tethers were longer, fewer chromosomes moved backwards. With yet longer tethers none moved backward. This is consistent with tether elasticity being lost by dephosphorylation. 50nM CalyculinA blocks both PP1 and PP2A. To distinguish between PP1 and PP2A we treated cells with short tethers with 50nM okadaic acid which blocks solely PP2A, or with 1μM okadaic acid which blocks both PP1 and PP2A. Only 1μM okadaic acid caused chromosomes to move backward. Thus, tether elasticity is lost because of dephosphorylation by PP1.

**ARTICLE HISTORY**
Received 30 September 2019
Revised 14 December 2019
Accepted 23 December 2019

**KEYWORDS**
Anaphase tethers; protein phosphatase 1; protein phosphatase 2A; Calyculin A; okadaic acid; crane-fly spermatocytes; anaphase forces; titin

## Introduction

Our experiments deal with tethers, elastic connections between the telomeres of all separating anaphase chromosome pairs. Tethers were initially described in crane-fly spermatocytes [1]. They also are present in a broad range of animal cells, including those from flatworms, insects, arachnids, flatworms, marsupials, and humans [2], connecting each separating pair of anaphase chromosomes. There were earlier suggestions that separating anaphase chromosomes in crane-fly spermatocytes were physically connected, because irradiation of kinetochore spindle fibers with some wavelengths of ultraviolet light (UV) caused both separating partner chromosomes to stop moving [3], because UV irradiation of the interzonal region between the separating chromosomes unlinked the movements of the partners [4], and because sometimes partner chromosomes moved backward after irradiation of kinetochores themselves [5]. While suggestive, these experiments did not prove that partner chromosomes were physically connected, especially since no connections can be seen in living cells using standard phase-contrast or DIC microscopy.

Elastic connections between separating partner chromosomes were demonstrated in crane-fly spermatocytes by LaFountain et al. [1]. When the telomere-containing portion of a chromosome arm was severed by a visible-light laser microbeam in early anaphase, when the inter-telomere distances (tether lengths) were short, the telomere-containing arm fragment traveled fast in the anti-poleward direction toward the partner chromosome. When telomere-containing arm fragments were produced in later anaphase, when the tethers were longer, the arm fragments moved slower and did not travel as far in the anti-poleward direction as the fragments that were formed in early anaphase. When the telomere-containing fragments were produced in yet later anaphase, when the tethers were even longer, the extent of the fragments' backward movement decreased (Figure 1(a)), which also holds true for arm fragments produced in PtK cells (Figure 1(b)). This suggests either that tether elasticity decreases as anaphase progresses, or that the tethers disconnect from the telomeres in later anaphase [1]. This ambiguity was subsequently resolved by cutting tethers directly. In both anaphase crane-fly spermatocytes and anaphase PtK

**CONTACT** Arthur Forer aforer@yorku.ca Biology Department, York University, Toronto, Ontario M3J 1P3, Canada
Supplemental data for this article can be accessed here.

cells the trailing arms of separating chromosomes shrunk by around 10% when the tethers were cut, indicating that the arms were being stretched by the tethers [2]. The arms were stretched even at tether lengths at which arm fragments did not move toward the partner telomere, indicating that tethers persist throughout anaphase [2]. Therefore, as anaphase progresses and the tethers elongate, the separating chromosomes remain attached but the tethers become more and more inelastic.

Movements of arm fragments to the partner telomere are due to 'tethers', not to other spindle components such as microtubules or ultra-fine DNA strands. Microtubules are ruled out by several experiments. For example, telomeres are required

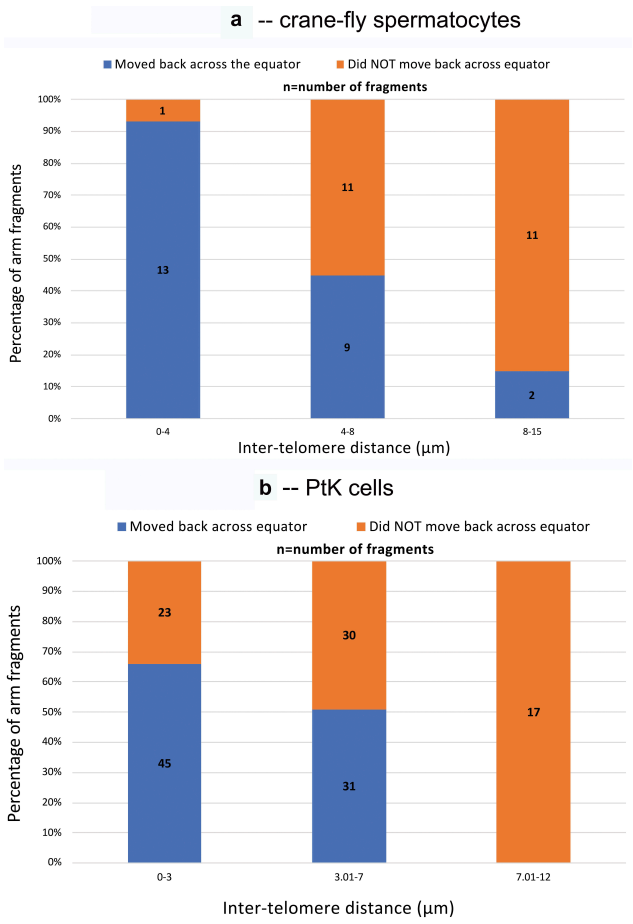


**Figure 1.** Movement of arm fragments created at different tether lengths, (a) for crane-fly spermatocytes and (b) for PtK cells. The data for crane-fly spermatocytes, plotted from values given in LaFountain et al. [1], are of telomere-containing arm fragments in crane-fly spermatocytes that move backward across the equator, or not, as a function of the length of the tether (i.e., the inter-telomere distance) when the arm was severed. The data for PtK cells, for the same parameters, were adapted from data in Forer et al. [2].

for the arm fragment to move to the partner telomere, because if either of the two telomeres is ablated the fragment stops moving [1]; further, if the moving arm fragment is cut in half, only the piece with the telomere moves [1]. Thus, while microtubules *can* indeed propel akinetic chromosome pieces, i.e., pieces of chromosomes that do not contain kinetochores, movements of the arm fragments across the equator require both telomeres. Further, treatment with taxol stabilizes spindle microtubules, stops microtubule-associated movements of akinetic fragments in the spindle [6], and slows or stops anaphase chromosome movements, but fragments from severed chromosome arms move at the same high speeds in taxol-treated cells that they do in control cells [7]. Thus, microtubule forces do not move the arm fragment across the equator to the telomere of the partner chromosome. Nor are these movements due to ultra-fine DNA strands. Tethers, identified by motion of arm fragments or loss of tension between arms, connect each set of separating anaphase chromosomes, though not necessarily each arm – for example, they connect the telomeres of only two of the four arms of each separating partner chromosome in crane-fly spermatocytes [1, 8, 9]. Ultrafine DNA strands, on the other hand, sometimes are found at telomeres, but many are found interstitially in the chromosomes and those that connect telomeres are seen in only a small fraction of anaphase chromosomes, not the 100% required (e.g., 10; 11; 12). Further, Su et al. [13], showed that induced ultra-fine DNA strands slow down anaphase chromosomes, whereas tethers do not, because cutting tethers with a laser during anaphase does not affect the velocities of the associated anaphase chromosomes [8]. Thus, tethers seem to be so-far-unidentified structures that extend between the telomeres of separating anaphase chromosomes, and the elasticity of the tethers decreases as the tethers get longer during anaphase.

Tether elasticity may be moderated by phosphorylation. Late-anaphase partner chromosomes in crane-fly spermatocytes often moved backward toward the cell equator after 50nM Calyculin A (CalA), an inhibitor of Protein Phosphatase 1 and Protein Phosphatase 2A (PP1 and PP2A), was added earlier in anaphase; the backward movements were led by the chromosomes' telomeres, the telomeres moving toward telomeres of the

partner chromosome [14]. It is reasonable to assume that the backward movements observed after treatment with CalA are due to tethers, since the movements are directed telomere to telomere. If so, this means that the tethers that ordinarily become inelastic as they lengthen have maintained their elasticity after treatment with CalA; therefore, phosphatase activity of PP1 and/or PP2A prevents loss of tether elasticity. Experiments using okadaic acid, another phosphatase inhibitor, suggest that blocking PP1 is the cause of maintaining tether elasticity, as follows.

Both CalA and okadaic acid affect the serine/threonine protein phosphatases PP1 and PP2A [15,16], but with different relative effectiveness, as indicated by their $IC_{50}$ (50% inhibitory concentration) values, shown in Table 1, and their activity curves (Figure 2). In the concentration range of 10–100 nM, CalA inhibits both PP1 and PP2A whereas okadaic acid inhibits only PP2A, not PP1. Okadaic acid in the μM range is needed to achieve the same effects on PP1 as 10nM of CalA (Figure 2; and [15]). Thus, 50nM CalA would be expected to affect both PP1 and PP2A while 50nM okadaic acid would affect solely PP2A. When crane-fly spermatocytes were treated with 50nM okadaic acid, there were no backward movements [14]; when HeLa cells were treated with 1-μM okadaic acid, on the other hand, separating partner chromosomes moved backward [13]. These data suggest, as concluded by Fabian et al. [14], that the backward chromosome movements are caused by inhibiting PP1.

**Table 1.** $IC_{50}$ values of CalA and okadaic acid. These values represent the concentration of compound need to inhibit 50% of protein phosphatase 1 and 2A activity (measured *in vitro* as specified in the cited articles).

| $IC_{50}$ (50% Inhibitory Concentration) values | | | |
|---|---|---|---|
| Compound | PP1 | PP2A | Source |
| Calyculin A | 0.4 nM | 0.25 nM | Swingle et al. [35] |
| | 0.4 nM | 0.25–0.3 nM | Honkanen et al. [33] |
| | 2 nM | 0.5–1 nM | Ishihara et al. [34] |
| Okadaic acid | 15–50 nM | 0.1–0.3 nM | Swingle et al. [35] |
| | 49–51 nM | 0.28–0.3 nM | Honkanen et al. [33] |
| | 60–500 nM | 0.5–1 nM | Ishihara et al. [34] |

One can test the role of phosphatases in tether elasticity by studying the effects of PP1 and PP2A inhibitors at different tether lengths, since tethers lose elasticity as they get longer. If the loss of elasticity as tethers lengthen is due to dephosphorylation, then preventing dephosphorylation by adding CalA at longer and longer tether lengths should cause less and less backward chromosome movement: at the longer tether lengths, the tethers would have been completely dephosphorylated and CalA would not produce any backward movement. We added 50nM CalA to crane-fly spermatocytes at different times in anaphase when the tethers were different lengths. Our hypothesis is that CalA prevents dephosphorylation of tethers and thereby preserves their elasticity, which in turn gives rise to backward movement of chromosomes (Figure 3). The results of the experiments reported here fit the prediction: when we

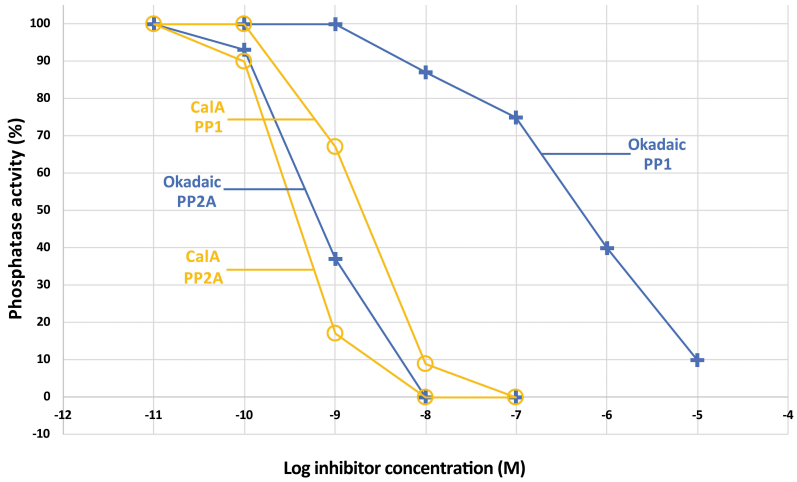


**Figure 2.** Phosphatase activity curves for CalA and okadaic acid. Adapted from data presented in Ishihara et al. [34]. The labels on the graph indicate which phosphatase inhibitor (PP1 or PP2A) was tested. The substrate was phosphorylated phosphorylase a.

added CalA to crane-fly spermatocytes when tethers were of different lengths, fewer half-bivalents moved backward when the CalA was added at longer tether lengths than when it was added at shorter tether lengths. To test whether the backward movements were due to inhibiting PP1 or PP2A we treated cells that had short tethers with low (50 nM) and with high (1 μM) concentrations of okadaic acid; the low concentration did not cause backward movements, but the high concentration did, indicating that the effects are due to blocking PP1.

## Materials and methods

Crane flies (*Nephrotoma suturalis* Loew) were reared in the laboratory as previously described [17]. Preparations of living cells were obtained as follows. IV-instar larvae of the proper stage were dissected under a drop of halocarbon oil. The testes were removed and placed in a drop of halocarbon oil to prevent cell dehydration. Before preparing a slide of each testis the oil was washed off by passing it 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). Each testis was then transferred to a coverslip into a 2.3 μl drop of insect Ringer's solution containing fibrinogen [18], then broken open and the cells spread out. Thrombin (2.3 μl) was added to form a fibrin clot that embeds the cells. The coverslip containing the fibrin clot was inverted over a small drop (~0.5 ml) of insect Ringer's solution in a perfusion chamber [18] that then was sealed with a thin coating of Vaseline followed by

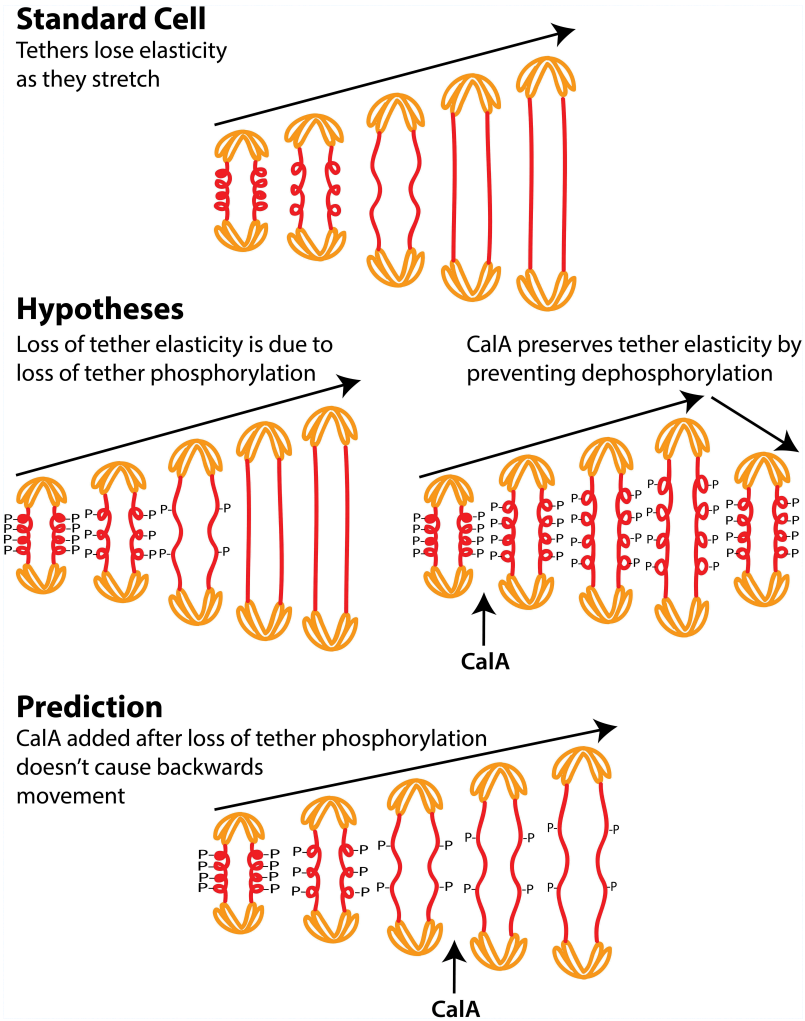


**Figure 3.** Our hypotheses and predictions. Cartoon of a *standard cell* in which tether elasticity decreases throughout anaphase. Cartoons of *our hypotheses* that tether elasticity is due to tether phosphorylation, and that CalA maintains tether elasticity by preventing dephosphorylation of tethers. Cartoon of our *prediction* that adding CalA in later anaphase when there is less phosphorylation results in less backward movement. P indicates phosphorylation.

a molten 1:1:1 mixture of Vaseline, lanolin, and paraffin. The cells in the chamber were immediately thereafter perfused with insect Ringer's solution.

The live cells were studied using phase-contrast microscopy. Cells in the proper stage of division were found, and real-time video images were recorded on DVDs while using a 100x, 1.4 NA phase-contrast oil immersion lens. At different times after the start of anaphase, we perfused anaphase cells with insect Ringer's solution containing either 50nM CalA (LC Laboratories, Woburn, MA) or okadaic acid (50nM or 1μM) (LC Laboratories, Woburn, MA). The stock solutions were prepared in DMSO and diluted by 1000 when added to the cells. Division proceeds normally in 0.1% DMSO and there is no effect on anaphase movement [6,19, 20, 21].

Video sequences were converted into time-lapse videos using freeware VirtualDub2. Single frames of chromosome movements were tracked and analyzed using an in-house program, Winimage [22], and graphs of inter-telomere distances (tether lengths) or distances from a fixed point at a pole were plotted using Excel. In this way we measured cells that we experimented on and recorded. We also measured cells recorded by Fabian et al. [14], which they had not analyzed to take into consideration the tether lengths at which the CalA was added. (The videos were kindly given to us by Dr Fabian.)

## Results

### *Control cells*

In a standard crane-fly spermatocyte undergoing anaphase, we observe the following, as illustrated in Figure 4. At the onset of anaphase, the three paired bivalent chromosomes that are aligned at the cell's equator disjoin and the half-bivalents move toward opposite poles. They generally reach the poles in 20–30 min. During this time the univalent sex chromosomes remain at the equator without moving [36; 23], as shown in Figure 4. Once the autosomes reach the poles, the poles elongate and the two univalent sex chromosomes that stayed at the equator begin to segregate at speeds of about 0.2 μm/min [23] and, more-or-less simultaneously, the cleavage furrow ingresses.

### *Calyculin A*

We treated individual anaphase spermatocytes with 50nM CalA at different inter-telomere distances. Figure 5 illustrates a cell treated with 50nM CalA when tethers were short: all half-bivalents that moved to the lower pole subsequently moved backward toward the upper pole. The backward movements were led by the telomeres, with the kinetochores trailing, as indicated by arrows in Figure 5, as seen in supplemental videos 1 and 2, and as described previously by Fabian et al. [14]. In addition to the backward movement after the half-bivalents neared the poles, one can see in Figure 6 and supplemental video 1 that, as described in detail in Fabian et al. [14], poleward movement for all half-bivalents rapidly speed up after addition of CalA. This acceleration has been attributed to hyper-phosphorylation of the myosin that is associated with kinetochore fibers [14,24].

When half-bivalents of all three pairs of half-bivalents moved backward, sometimes the partners met in the middle of the cell (Figure 5), occasionally closer to one pole, and sometimes at the other pole (e.g., supplemental video 2). Once met, the half-bivalents from both poles grouped together as described by Fabian et al. [14], sometimes moving around in the middle of the spindle or moving toward one pole or the other. Sometimes half bivalents of only 1 or 2 pairs moved backward, while the others stayed at the poles (e.g., supplemental video 1), similarly sometimes meeting in the middle of the cell and sometimes meeting toward or at one pole. Those half-bivalents that remained at the poles (while other half bivalents moved backward) made short back and forth movements at the pole, small but readily visible movements in the direction of the pole and then in the direction of the equator of the cell (e.g., Supplementary video 1). These movements also were described by Fabian et al. [14]. Finally, in our sample of 30 cells, and in others not reported on here, backward movements sometimes, albeit rarely, occurred near mid-anaphase, before the chromosomes neared the poles.

CalA did not always cause backward movements in our experiments, which we attribute to CalA being added at different tether lengths. When CalA was added at short tether lengths (< 2 μm), nearly all

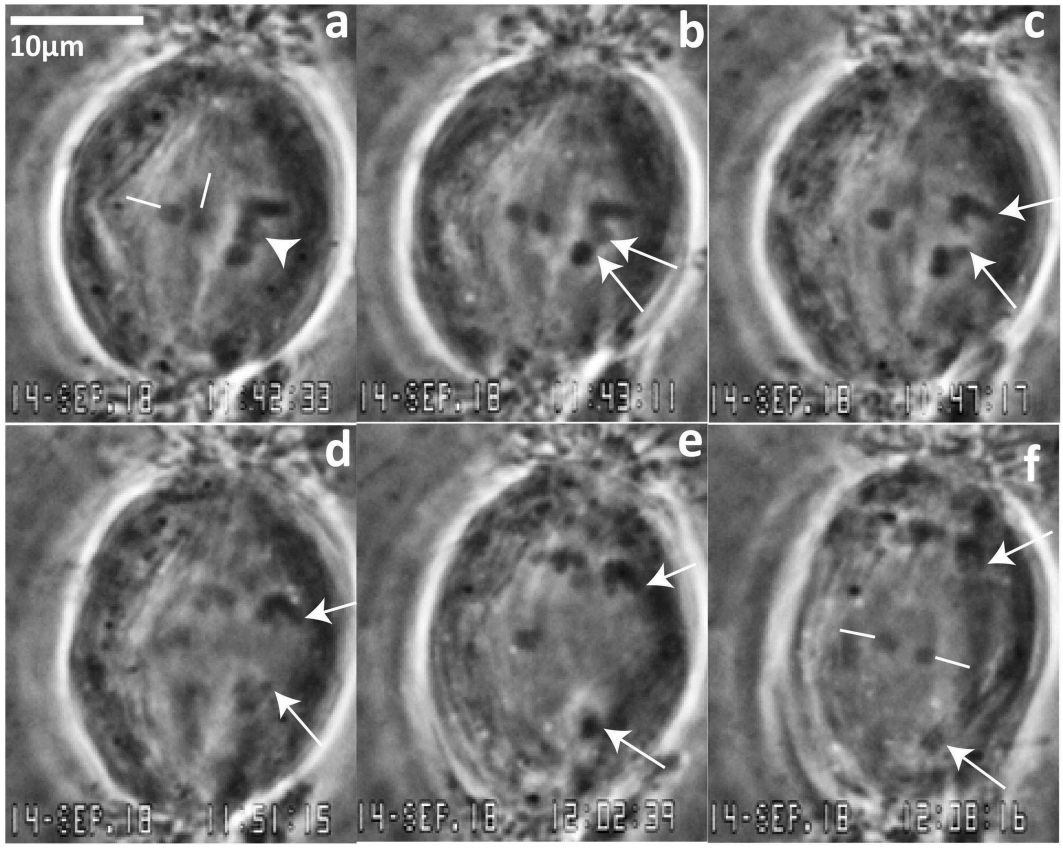


**Figure 4.** Normal cell division in a crane-fly spermatocyte. The time stamp in all images indicates hrs:min:sec. The arrowhead in (a) indicates a bivalent in metaphase, and the arrows in (b–f) indicate the positions of separating half-bivalents as they move apart from each other and travel to the poles. Lines indicate the position of sex chromosomes, which are stationary throughout the time when the autosomes move to the poles.

separating half-bivalents moved backward after first reaching (or nearing) the poles. When CalA was added at increasingly longer tether lengths the number of half-bivalents that moved backward decreased, and at tether lengths >11 µm no half-bivalents moved backwards (Figure 7(a)). This conclusion was strengthened by adding to our sample of chromosomes from 30 cells (Figure 7(a)) those cells from the experiments of Fabian et al. [14] that we measured from the videos, cells that were not previously analyzed with respect to tether lengths at which CalA was added. The same conclusions are reached from the additional cells, and putting the two sets together gives a larger sample size and more robust data (Figure 7(b)).

With respect to our measurements of inter-telomere distances (tether lengths) at which we added CalA, the three half-bivalent pairs in a cell are often not in the same focal plane when CalA is added, so we could not get direct inter-telomere distance measurements from all half-bivalents in a cell. For tether lengths <3 µm all three half-bivalents were plotted using the distance measurements taken from one or two half-bivalent pairs in the cell that were in the same focal plane. This is because in early anaphase there is very little variation in the inter-telomere distances of the three half-bivalent pairs. Differences in inter-telomere distances for chromosome pairs in the same cell manifest themselves later in anaphase; consequently, for all tether lengths ≥3 µm we plotted only the half-bivalent pairs that were directly measured.

The increase of poleward speeds of the half-bivalents took place after the addition of CalA (Figure 6) independent of tether length when CalA was added and independent of whether or not the chromosomes subsequently moved backward. Acceleration was visible in 26/30 cells. We did not measure the accelerated speeds in all of them, since this was described in detail in Fabian et al. [14], but we measured the acceleration in

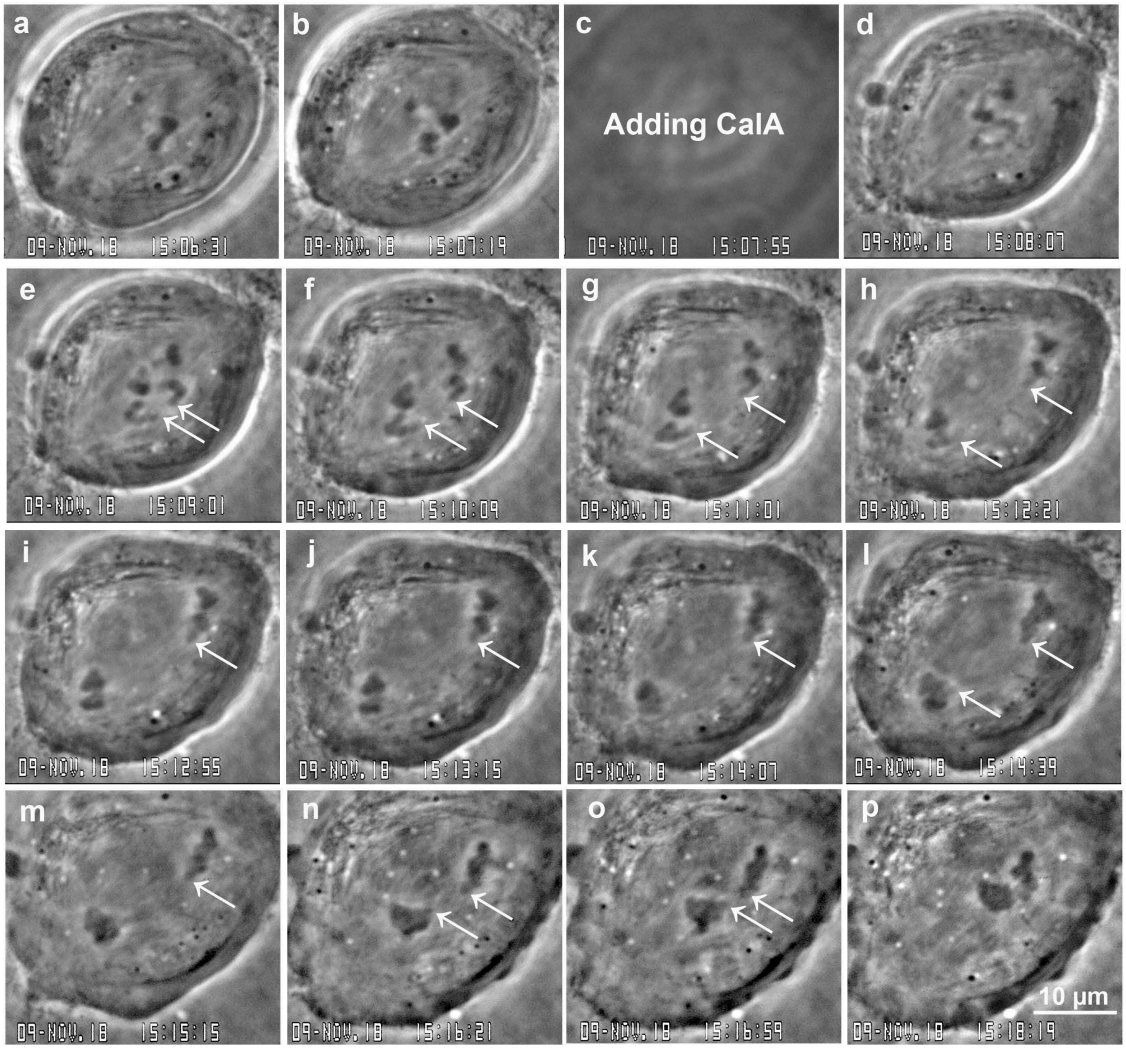


**Figure 5.** Backward movement after CalA treatment. Photo montage showing the progression of anaphase in a crane-fly spermatocyte that was treated with 50 nM CalA. The time stamp in all images indicates hrs:min:sec. (a): Metaphase, showing one bivalent. (b): Anaphase. That bivalent has disjoined into two half bivalents. (c): CalA was added. (d–j): The half-bivalents move poleward. The arrows point to the trailing telomeres on separating arms. (k–p): the bottom set of half-bivalents moves toward the top set, as the top set moves down somewhat (cf. distance from the top pole), both sets led by a telomere, perhaps more readily seen in the top set than the bottom. The line in (p) indicates 10 µm.

a sample of six cells; in these cells chromosomes accelerated to a speed roughly twice the pre-CalA speed, about the same as reported by Fabian et al. [14].

The sex chromosomes also were affected by CalA. In control cells, throughout autosomal anaphase the sex chromosomes stay at the equator without moving. After CalA was added, in all cells but one each of the two univalents moved rapidly, irregularly, and independently up and down the spindle instead of remaining at the equator; this behavior is described by Fabian et al. [14], is illustrated in Figure 8(a), and is clearly seen in Supplemental video 2. After CalA addition the sex chromosomes moved at velocities of up to 2 µm/min (Figure 8(b)), 10x faster than their anaphase movements in control cells and faster than autosomes move during anaphase in control cells. The sex-chromosome excursions up and back along the spindle occurred at all half-bivalent tether lengths at which CalA was added, up to 15µm, the longest tether length for which we have those data; they were independent of whether the half-bivalents moved backward. Thus, in addition to CalA causing half-bivalents at the pole to move backward, other effects of CalA were poleward acceleration of half-bivalents, and rapid irregular excursions of the sex chromosomes along the

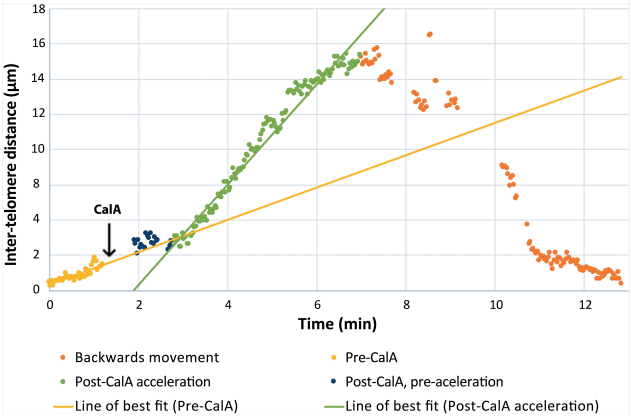


**Figure 6.** CalA caused first acceleration and then backward movement of chromosomes. The chromosome graphed is from the cell illustrated in Figure 5. 50 nM CalA was added in early anaphase (as indicated on the graph) at 1min14s after anaphase onset, at an inter-telomere distance of 1.3 µm. Lines of best fit (to the similarly colored points) show the increased separation velocity of the separating half-bivalents starting about 1 min and a half after CalA was added.

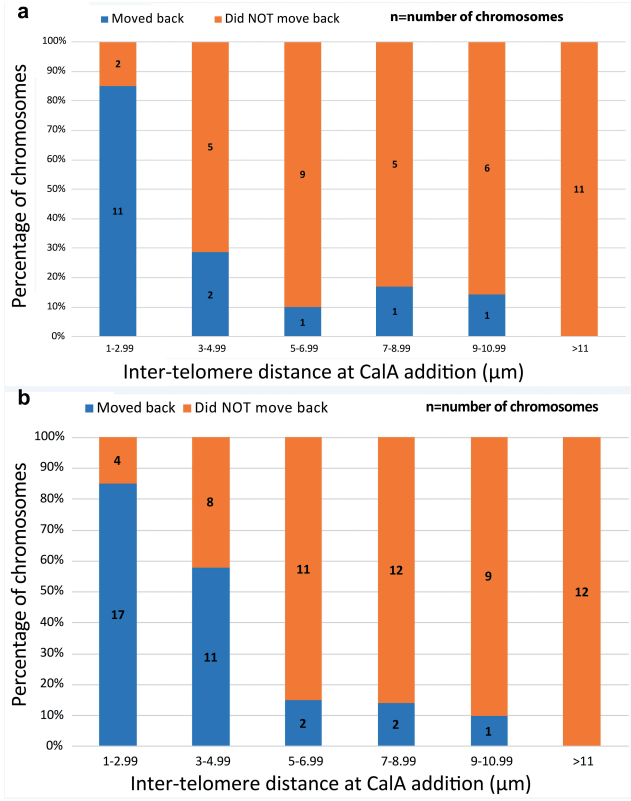


**Figure 7.** Effects of CalA added to crane-fly spermatocytes at various tether lengths. Percentage of backward movement of crane-fly spermatocyte chromosomes after 50 nM CalA was added at various tether lengths. Figure 7(a) are data from our experiments. Figure 7(b) are the data from Figure 7(a) to which were added data from videos of Fabian et al. [14], which were not analyzed by them with respect to tether length when CalA was added. Lacramioara Fabian kindly provided us with the .avi files of the time-lapsed cells; we analyzed the videos to determine backward movement with regards to tether lengths at the time CalA was added. Those values were added to those of Figure 7(a) to obtain Figure 7(b). The conclusions are the same, but with more robust numbers in Figure 7(b).

spindle axis. The latter two effects always occurred, whether or not the half-bivalents moved backward.

## *Okadaic acid*

We studied the effects of okadaic acid to distinguish between effects on PP1 versus on PP2A. When a low concentration (50nM) of okadaic acid was added to 6 cells at short tether lengths (0.4–1.7 µm), there was no backward movements. Fabian et al. [14] also saw no backward movements after adding 20 or 50nM okadaic acid, but they did not measure tether lengths at the time they added the okadaic acid. When we added a high concentration (1 µM) of okadaic acid to 6 cells at short tether lengths (0.8–1.7 µm), there was backward movement in more than half the chromosome pairs (Figure 9). There were varying responses to high concentrations of okadaic acid in single cells – some half-bivalent pairs moved others did not, as seen in Supplementary video 3. These data indicate that blocking PP1, not PP2A, causes the chromosomes to move backward at the end of anaphase.

We also looked at whether okadaic acid caused acceleration of autosomes, and/or caused rapid excursions of sex chromosomes. No half-bivalents accelerated after treatment with either high or low concentrations of okadaic acid, unlike the universal acceleration caused by CalA. Sex-chromosome excursions took place after treatment with both low and high concentrations of okadaic acid treatment: the sex chromosomes moved up and back along the spindle in 3 of the 6 cells treated with 50nM okadaic acid and in all 6 of the cells treated with 1-µM okadaic acid.

Okadaic acid also often altered normal chromosome movements. After treatment with 50nM okadaic acid at short tether lengths, in all six cells the separating half-bivalents slowed down immediately after drug addition, but recovered shortly thereafter. After treatment with 1-µM okadaic acid at short tether lengths, the separating half-bivalents slowed in 2/6 cells, independent of effects

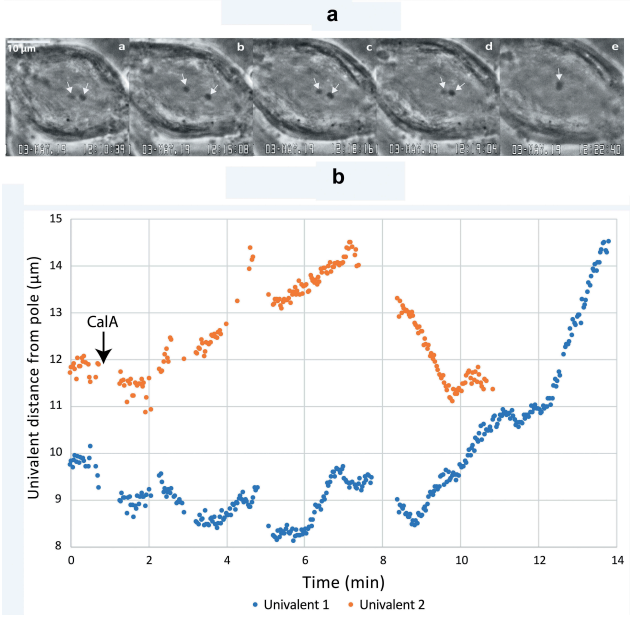


**Figure 8.** (a). Movement of sex chromosomes after CalA treatment. In cells treated with 50nM CalA the sex chromosomes (indicated by white arrows) move up and back along the spindle Figure 8(a). The movements of the pair seen in Figure 8(a) are shown graphically in Figure 8(b). Sex chromosomes move at speeds of up to 2 μm/min after treatment with CalA. Arrow in Figure 8(b) indicates when CalA was added, about 46 s after anaphase onset.

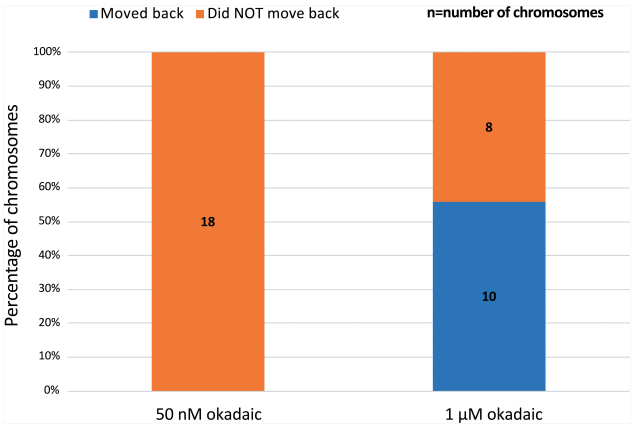


**Figure 9.** Effects of different concentrations of okadaic acid on backward movement of chromosomes. In all cells okadaic acid was added when tether lengths were short (<2 μm).

on backward movement, and also recovered shortly thereafter.

## Discussion

The main conclusion from our experiments is that tether elasticity is regulated by phosphorylation. Whereas tethers ordinarily become inelastic as anaphase proceeds, tether elasticity is maintained throughout anaphase when dephosphorylation is inhibited early in anaphase (Figure 7) when tethers are short and elastic, which we interpret to mean that elastic tethers are phosphorylated (Figure 3). Fully elastic tethers then cause anti-poleward movement of separated half-bivalents after they reach the poles. Inhibiting dephosphorylation later in anaphase when the tethers have already become less elastic (partially dephosphorylated) does not have the same effect: in mid-anaphase, only a small number of chromosomes move back after separating. By late anaphase, when the tethers are fully inelastic (fully dephosphorylated), there is no longer any backward movement: inhibiting dephosphorylation when the tethers are already dephosphorylated does not have any effect (Figure 7). In reaching these conclusions we assume that the backward (anti-polar) movements of the half-bivalents are due to the action of tethers, and we assume that the phosphorylation that controls elasticity is of the tethers themselves and not some indirect effect of phosphorylating another component. While we have no direct proof that the backward movements of the half-bivalents are due to tethers, the backward movements are led by the telomeres and are directed toward each other, as the kinetochores trail (Figure 5, supplemental videos 1 and 2), strongly suggesting that tethers cause the movements. Further, the movement of arm fragments versus tether lengths in crane-fly spermatocytes and PtK cells (Figure 1) matches very well the backward movement of half-bivalents treated with CalA at various tether lengths (Figure 7), suggesting very strongly that tethers produce the backward (anti-polar) forces on the chromosomes. More direct proof might arise from severing arms at long tether lengths in cells treated with CalA at short tether lengths.

Our interpretation is that elastic tethers are phosphorylated and inelastic tethers are not (Figure 3). But we don't really know that this is true. Our data show that dephosphorylation of *some* component(s) causes tethers to become inelastic, and it is possible that the effects on tethers are some steps removed from the primary phosphorylation/dephosphorylation events that are affected by CalA. Our data do not distinguish between the two interpretations. However, our

interpretation is straightforward and is consistent with several other lines of evidence. One is the location of PP1 in the cell: we would be able to negate our interpretation if PP1 is not present in the interzone between separating anaphase chromosomes because our interpretation says the dephosphorylation occurs on the tethers themselves. But PP1 is active during anaphase and is present in the interzone, the same area that contains the tethers [25]. Further data show that PP1 associates with both spindle microtubules and chromosomes [26]. These data thus are consistent with our interpretation that tethers are phosphorylated and lose elasticity during anaphase due to dephosphorylation by PP1.

Other data also make it plausible that phosphorylation/dephosphorylation are of tethers themselves. These data hinge on what tethers are composed of. Tethers cannot be seen in living cells but linear structures between the telomeres of separating anaphase chromosomes were described in fixed and stained cells in the early 1900s and presumably are tethers [37]. Their composition is unknown. Spindle proteins myosin and actin were considered as potential candidates for tethers because in crane-fly spermatocytes they extend between telomeres during anaphase [27]. However, when crane-fly spermatocytes treated with CalA were then treated with the actin inhibitor Latrunculin B (LatB), or myosin inhibitors 2,3-butanedione monoxime (BDM) or Y-27632, the backward half-bivalent movements were not altered [14], indicating that the backward movements are not due to actin or myosin. Having eliminated actin and myosin as producing the backward force, Fabian et al. [14,27] then suggested that titin, also present between separating telomeres, might be a component of tethers. Single titin molecules, MW ~3 MDaltons, extend the more than 1 μm distance between the Z-line and the middle of the A-band in skeletal muscle. Titin is responsible for passive elasticity in muscle [28], so it is not unreasonable that it might also be responsible for tether elasticity. The elasticity of titin depends on its phosphorylation state: titin increases in elasticity ('stiffness') when phosphorylated in its PEVK region (a region common to titin in both heart and skeletal muscle) and loses elasticity when it is dephosphorylated [29–32]. Further, this phosphorylation is removed by addition of PP1 [38]. Thus, while we have not eliminated the counter-hypothesis that the phosphorylation/dephosphorylation effect on tether elasticity is indirect rather than being on the tethers themselves, we think there is a reasonable (albeit circumstantial) case that the tethers themselves are phosphorylated when elastic and not phosphorylated when they are inelastic, and that titin may be a key component of tethers.

Our interpretation (or any interpretation) needs to deal with the general loss of tether elasticity. The loss of elasticity is gradual both in PtK cells and crane-fly spermatocytes. The speeds of arm-fragment movements decrease as the arms are severed later in anaphase, as do the distances the arm fragments move (Figure 1; also, [1, 2]). This implies that dephosphorylation occurs along the tether gradually throughout anaphase; in our interpretation dephosphorylation could proceed from the ends of the tethers, from the middle, or evenly along the length of the tether (Figure 10). We have no data on this issue, or indeed have no data that the tethers themselves are phosphorylated. If we could identify tethers chemically and morphologically, we could test for their phosphorylation, and test where the dephosphorylation occurs.

We have argued above that PP1 activity causes the tethers to become inelastic. This conclusion arises because CalA prevents dephosphorylation by inhibiting two different serine/threonine phosphatases, PP1 and PP2A. At a concentration of 50nM, CalA causes separating half-bivalents to move backward when added at short to intermediate tether lengths. At that concentration CalA greatly reduces the activity of both PP1 and PP2A (Figure 2). Okadaic acid, on the other hand, affects only PP2A at the low concentration of 50nM, and at that concentration okadaic acid does not cause backward movements. At a higher concentration, 1μM, okadaic acid inhibits PP1 as well as PP2A (Figure 2) and causes backward movements. Thus, the backward movements of autosomes at the end of anaphase seem to be caused by blocking PP1. Though the activity curves of CalA and okadaic acid can vary based on substrate [33–35], the activity curves using different substrates are similar, and the conclusions hold that much higher concentrations of okadaic acid are needed to block PP1 than to block PP2A. There are other serine/

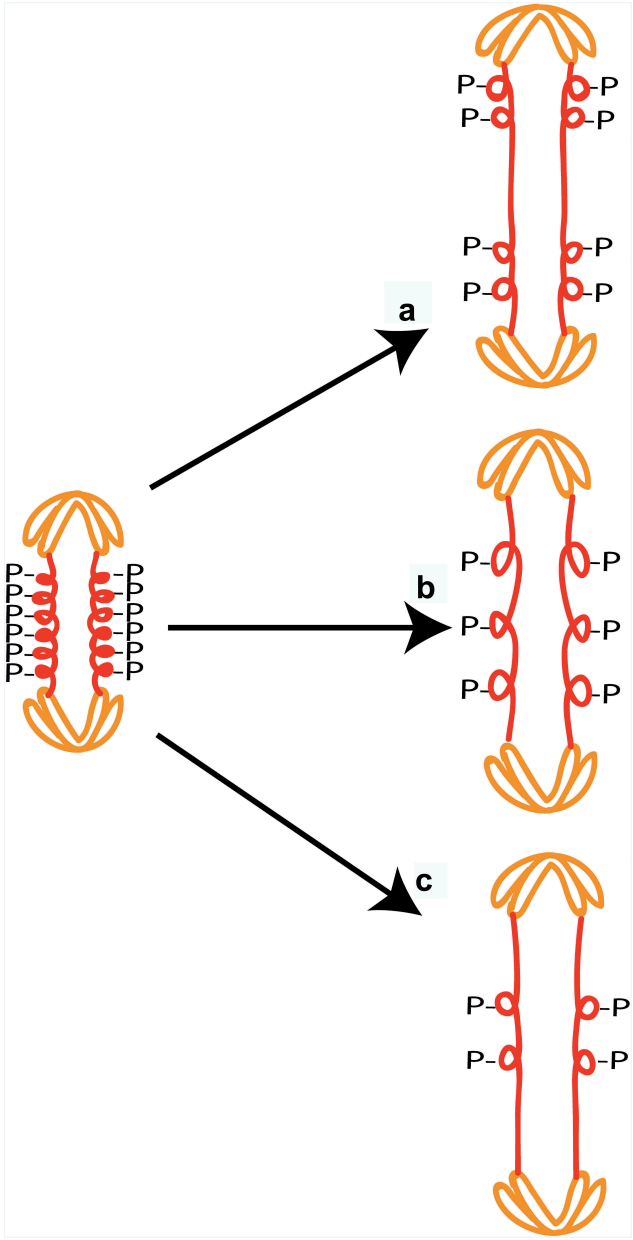


**Figure 10.** Possible patterns of dephosphorylation of tethers. Tethers may be dephosphorylated starting at the center and moving outwards, leaving the ends elastic for a longer time than the middle (a); they may be dephosphorylated evenly along the tether (b); or they may be dephosphorylated from the ends, moving toward the middle, leaving the middle elastic for a longer time than the ends (c).

threonine protein phosphatases, however [39], PP1 through PP7; could any of these be involved in the backward movements of half-bivalents that we observed? Honkanen and Golden [39] and Swingle et al. [35] summarize the $IC_{50}$ values for all of these phosphatases. By comparing the $IC_{50}$ values with the requirement that nanomolar concentrations of CalA cause backward movements, the only phosphatases that are affected by nM Calyculin are PP1, PP2A and PP4. We have eliminated PP2A, as above. We also eliminate PP4 because nM okadaic concentrations inhibit PP4 yet do not cause backward movements of half-bivalents. Thus, our data point to PP1 as the phosphatase that causes tethers to become inelastic.

That chromosomes move backwards at the end of anaphase suggests that there is a change in the balance of forces at that time. Tethers do not slow anaphase movements in control cells. We know this because anaphase chromosome velocities do not change when the associated tethers are severed [8]. Thus, during normal anaphase tethers produce much less backward force than do the spindle fibers propelling the chromosomes polewards. Tethers that have remained elastic throughout anaphase (because of CalA or okadaic acid addition) cause anti-poleward movement at the end of anaphase; this indicates that the anti-poleward forces from the tethers were stronger at that time than the poleward forces from the spindle fibers and thus that the poleward forces weaken as the chromosomes near the poles. In some cells not all half-bivalents moved backward; the half-bivalents that did not move backward moved somewhat up and back along the spindle axis, as if there were a 'tug-of-war' between nearly equal polar forces and anti-polar tether forces.

While high concentrations of okadaic acid added in early anaphase caused backward movements of half-bivalents after they reached the poles, okadaic acid seems less potent in inducing backward movement than is CalA (Figure 7 vs. Figure 9): CalA caused backward movement of 81% of the half-bivalents and okadaic acid of only 55%. We are not certain why the difference. The graph of phosphatase activity versus okadaic acid concentration, Figure 2, indicates that 1μM okadaic acid inhibits 60% of PP1 activity, whereas 50nM CalA inhibits over 90% of PP1 activity. It may be that there is enough PP1 activity remaining after treatment with 1μM okadaic acid to sometimes cause tethers to become inelastic. Perhaps if one used higher concentration of okadaic acid the effects on backward movement might be the same as when using CalA.

Though not directly related to our experiments and conclusions concerning tethers, our data show that preventing dephosphorylation by CalA and okadaic

acid affects other chromosomal behaviors as well as tether elasticity, namely speeding up anaphase chromosomes and causing sex chromosomes to have irregular excursions off the equator. The two inhibitors had somewhat different effects, though. *CalA* in most cells caused acceleration of anaphase movements to the pole (Figure 6), and caused irregular, rapid movements of sex chromosomes (Figure 8). The effects on chromosome speed and sex chromosome movements occurred in all cells treated with CalA, independent of effects on backward movements. Even in cells where CalA was added in late anaphase and the chromosomes did not undergo backward movement the autosomes accelerated toward the poles and the sex chromosomes took rapid excursions up and back along the spindle. The effects of *okadaic acid* were somewhat different. Neither 50nM nor 1 μM okadaic acid added to early anaphase cells caused the separating anaphase half-bivalents to speed up in their movements to the pole. However, rapid sex chromosome excursions took place in half of the cells treated with 50nM okadiac acid and in all cells treated with 1 μM okadaic acid. This suggests perhaps that effects on both PP1 and PP2A are needed to cause sex chromosome excursions.

In conclusion, our data demonstrate that preventing dephosphorylation in early anaphase by inhibiting PP1 causes backward movement in late anaphase of separated crane-fly half-bivalents. There is less and less backward movement when the phosphatase inhibitor is added at longer and longer tether lengths. This matches the elasticity of tethers deduced from the movement of arm fragments severed from arms at different tether lengths, substantiating that the backward movements are due to elastic tethers, and strongly suggests that loss of tether elasticity is due to dephosphorylation. Because of the different effects of CalA and okadaic acid, the phosphatase involved would seem to be PP1. Our working interpretation is that tethers that are elastic are phosphorylated and that tethers become inelastic when dephosphorylated.

## Acknowledgments

We are grateful to Lacramoiara Fabian for giving us her video sequences to analyse. This work was supported by a grant to AF from the Natural Sciences and Engineering Research Council of Canada.

## Disclosure statement

No potential conflict of interest was reported by the authors.

## Funding

This work was supported by the Natural Sciences and Engineering Research Council of Canada [RGPIN-2019-06299].

## ORCID

Arthur Forer http://orcid.org/0000-0001-8747-0037

## References

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

[2] Forer A, Duquette ML, Paliulis LV, et al. Elastic 'tethers' connect separating anaphase chromosomes in a broad range of animal cells. Eur J Cell Biol. 2017;96:504–514.

[3] Forer A. Characterization of the mitotic traction system and evidence that birefringent spindle fibers neither produce nor transmit force for chromosome movement. Chromosoma. 1966;19:44–98.

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

[5] Ilagan AB, Forer A, Spurck T. Backward chromosome movement in anaphase, after irradiation of kinetochores and kinetochore fibers. Protoplasma. 1997;198:20–26.

[6] LaFountain JR, Oldenbourg R, Cole RW, Rieder CL. Microtubule Flux Mediates Poleward Motion ofAcentric Chromosome Fragments during Meiosis in Insect Spermatocytes. Molecular Biology Of The Cell Vol. 2001;12:4054–4065.

[7] Forer A, Sheykhani R, Berns MW. Anaphase chromosomes in crane-fly spermatocytes treated with taxol (paclitaxel) accelerate when their kinetochore microtubules are cut: evidence for spindle matrix involvement with spindle forces. Front Cell Dev Biol. 2018;6:77. doi:10.3389/fcell.2018.00077

[8] Sheykhani R, Berns M, Forer A. Elastic tethers between separating anaphase chromosomes in crane-fly spermatocytes coordinate chromosome movements to the two poles. Cytoskeleton. 2017;74(2):91–103.

[9] Adames KA, Forer A. Evidence for polewards forces on chromosome arms during anaphase cell motility and the cytoskeleton. 1996;34:13-25. doi:10.1002/(ISSN)1097-0169

[10] Chan K-L, North PS, Hickson ID. Blm is required for faithful chromosome segregation and its localization defines a class of ultrafine anaphase bridges. Embo J. 2007;26:3397–3409.

[11] Barefield C, Karlseder J. The BLM helicase contributes to telomere maintenance through processing of late-replicating intermediate structures. Nucleic Acid Res. 2012;40:7358–7367.
[12] Gemble S, Ahuja A, Buhagiar-Labarchède G, Onclercq-Delic R, Dairou J, Biard DS, Lambert S, Lopes M, Amor-Guéret M.Genet PLoS. 2015; http://dx.doi.org/10.1371/journal.pgen.1005384.
[13] Su K-C, Barry Z, Schweizer N, et al. A regulatory switch alters chromosome motions at the metaphase to anaphase transition. Cell Rep. 2016;17((7)):1728–1738.
[14] Fabian L, Troscianczuk J, Forer A. Calyculin A, an enhancer of myosin, speeds up anaphase chromosome movement. Cell Chromos. 2007;6:1 doi:10.1186/1475-9268-6-1. Available from: http://www.cellandchromosome.com/content/6/1/1.
[15] Favre B, Turowski P, Hemmings BA. Differential inhibition and posttranslational modification of protein phosphatase 1 and 2A in MCF7 cells treated with calyculin-A, okadaic acid, and tautomycin. J Biol Chem. 1997;272(21):13856–13863.
[16] MacKintosh C, MacKintosh RW. Inhibitors of protein kinases and phosphatases. Trends Biochem Sci. 1994;19 (11):444–448.
[17] Forer A. Crane fly spermatocytes and spermatids: a system for studying cytoskeletal components. Methods Cell Biol. 1982;25:227–252.
[18] Forer A, Pickett-Heaps J. Fibrin clots keep non-adhering living cells in place on glass for perfusion or fixation. Cell Biol Int. 2005;29:721–730.
[19] Forer A, Pickett-Heaps JD. Cytochalasin D and latrunculin affect chromosome behaviour during meiosis in crane-fly spermatocytes. Chr Res. 1998;6(7):533–550.
[20] Forer A, Pickett-Heaps JD. Checkpoint control in crane-fly spermatocytes: unattached chromosomes induced by cytochalasin D or latrunculin treatment do not prevent or delay the start of anaphase. Protoplasma. 1998;203(1–2):100–111.
[21] Silverman-Gavrila RV, Forer A. Chromosome attachment to the spindle in crane-fly spermatocytes requires actin and is necessary to initiate the anaphase-onset checkpoint. Protoplasma. 2000;212(1–2):56–71.
[22] Wong R, Forer A. 'Signalling' between chromosomes in crane-fly spermatocytes studied using ultraviolet microbeam irradiation. Chr Res. 2003;11(8):771.
[23] Forer A, Ferraro-Gideon J, Berns MW. Distance segregation of sex chromosomes in crane-fly spermatocytes studied using laser microbeam irradiations. Protoplasma. 2013;250(5):1045–1055.
[24] Sheykhani R, Shirodkar PV, Forer A. The role of myosin phosphorylation in anaphase chromosome movement. Eur J Cell Biol. 2013;92:175–186.
[25] Trinkle-Mulcahy L, Andrews PD, Wickramasinghe S, et al. Time-lapse imaging reveals dynamic relocalization of PP1γ throughout the mammalian cell cycle. Mol Biol Cell. 2003;14(1):107–117.
[26] Andreassen PR, Lacroix FB, Villa-Moruzzi E, et al. Differential subcellular localization of protein phosphatase-1 α, γ1, and δ isoforms during both interphase and mitosis in mammalian cells. J Cell Biol. 1998;141 (5):1207–1215.
[27] Fabian L, Xia X, Venkitaramani DV, et al. Titin in insect spermatocyte spindle fibers associates with microtubules, actin, myosin and the matrix proteins skeletor, megator and chromator. J Cell Sci. 2007;120(13):2190–2204.
[28] Tskhovrebova L, Trinick J. Titin: properties and family relationships. Nat Rev Mol Cell Biol. 2003;4:679–689.
[29] Hamdani N, Herwig M, Linke WA. Tampering with springs: phosphorylation of titin affecting the mechanical function of cardiomyocytes. Biophys Rev. 2017;9 (3):225–237.
[30] Hidalgo C, Granzier H. Tuning the molecular giant titin through phosphorylation: role in health and disease. Trends Cardiovasc Med. 2013;23(5):165–171.
[31] Kötter S, Gout L, vFreiling-Salewsky M, et al. Differential changes in titin domain phosphorylation increase myofilament stiffness in failing human hearts. Cardiovasc Res. 2013;99:648–656.
[32] Krüger M, Linke WA. Protein kinase-A phosphorylates titin in human heart muscle and reduces myofibrillar passive tension. J Muscle Res Cell Motil. 2006;27 (5–7):435–444.
[33] Honkanan RE, Codispoti BA, Tse K, et al. Characterization of natural toxins with inhibitory activity against serine/threonine protein phosphatases. Toxicon. 1994;32(3):339–350.
[34] Ishihara H, Martin BL, Brautigan DL, et al. Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. Biochem Biophys Res Commun. 1989;159(3):871–877.
[35] Swingle M, Ni L, Honkanen RE. Small-molecule inhibitors of Ser/Thr protein phosphatases. Methods Mol Biol. 2007;365:23–28.
[36] Forer A. Chromosome movements in the meiosis of insects, especially crane-fly spermatocytes. In: Blackman RL, Hewitt GM, Ashburner M, editors. Insect cytogenetics. Symposia of the Royal Entomological Society of London: Number Ten; 1980.
[37] Paliulis LV, Forer A. A review of "tethers": elastic connections between separating partner chromosomes in anaphase. Protoplasma. 2018;255:733–740.
[38] Hidalgo C, Hudson B, Bogomolovas J, Zhu Y, Anderson B, Greaser M, Labeit S, Granzier H. Pkc phosphorylation of titin's pevk element- a novel and conserved pathway for modulating myocardial stiffness circulation res. 2009;105(7): 631–638
[39] Honkanen RE, Golden T. Regulators of serine/threonine protein phosphatases at the dawn of a clinical era? Curr Med Chem. 2002;9(22):2055–2075.
[40] Wong R, Forer A. Backward chromosome movement in crane-fly spermatocytes after UV microbeam irradiation of the interzone and a kinetochore. Cell Biol Int. 2004;28:293–298.