Examining the role of phosphorylation in the gain and loss of the elasticity of the tethers that connect separating anaphase chromosomes in crane-fly spermatocytes

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ABSTRACT

This thesis deals with elasticity of the tethers that connect anaphase chromosomes in crane-fly spermatocytes. Chromosomes move backwards after reaching the poles when Calyculin A (CalA), an inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A), is added in early anaphase. CalA added in later anaphase causes increasingly less backwards movement. Inhibiting only PP2A with low concentrations of okadaic acid does not result in backwards movement. I suggest that as anaphase progresses tethers are dephosphorylated by PP1. I next studied phosphorylation by inhibiting protein kinase C (PKC), PKA and general kinase activity, followed by (or simultaneous with) treatment with CalA in early anaphase. Chromosomes still moved backwards in these experiments. The main findings of this thesis are that the loss of tether elasticity during anaphase is due to dephosphorylation, presumably by PP1, that neither PKC nor PKA act during anaphase, and that tether phosphorylation likely occurs prior to anaphase.

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Chapter One

General Introduction

THE CELL CYCLE AND CELLULAR DIVISION

Cellular division is the process by which a single parent cell divides to give rise to at least two identical daughter cells. In many multicellular organisms the cells are diploid, meaning that they contain two complete sets of chromosomes in their nucleus. In the cell cycle prior to the onset of division, cells are in **interphase**. Interphase consists of three different stages: G_1 (gap, as the cell prepares for DNA synthesis), S (synthesis, in which DNA is replicated), and G_2 (gap, as the cell prepares for division). In the S stage of interphase, the genetic material within the nucleus is replicated such that each cell contains four complete sets of chromosomes. The replicated chromosomes are called sister chromatids and they are connected to each other by a DNA sequence called the centromere. Following interphase, the cell will enter either mitosis or meiosis. Mitosis gives rise to somatic, or body, cells after a single round of division. In this case, the parent cell gives rise to two identical diploid daughter cells. In meiosis, however, there are two rounds of division resulting in four gametes, or reproductive cells. Because there are two back-to-back divisions without the cell entering interphase and duplicating its genetic material the resulting daughter cells are haploid, meaning that they each contain only one complete set of chromosomes.

Both mitosis and meiosis generally consist of five stages. These stages are **prophase, prometaphase** (first described by Schrader, 1953)**, metaphase, anaphase, and telophase**. Although there is significant variation in cellular division in different cell types (many reviewed in White, 1977), I will describe the general events that occur in each stage.

Prophase: During prophase the genetic material that is stored in the nucleus as long strands of chromatin begins to condense and shorten into individual chromosomes. As the chromosomes condense the spindle apparatus outside the nucleus begins to form. The spindle is part of the mechanical scaffolding necessary for coordinating and directing the movements of the chromosomes as division progresses. The spindle itself is organized by structures in the cell known as microtubule organizing centers (MTOCs), which produce microtubules, dynamic tubulin polymers that are involved with moving the chromosomes throughout division. In many cell types, though not all, an organelle called the centrosome functions as the MTOC. The centrosome duplicates in interphase and become the poles in the dividing cell.

Prometaphase: Dissolution of the nuclear envelope that encapsulates the genetic material within the cell marks the onset of prometaphase. This allows the condensed

chromosomes to interact with the bipolar spindle microtubules. In most cases the spindle microtubules attach to the sister kinetochores, a protein complex that forms around the centromere of the sister chromatids (Rieder and Salmon, 1998). As the microtubules from both poles begin to connect to the kinetochores, the pulling force on the chromosomes increases in one direction and then the other, moving them parallel along the spindle. This balance of forces shifts as more microtubules from a pole are attached to the kinetochore. The chromosome will always move in the direction of the greatest pulling force. Once the chromosome is fully attached to each of the opposite poles through both sister kinetochores the forces begin to balance, aligning the chromosome at the center of the cell. This process is called chromosome congression.

Metaphase: The defining feature of metaphase is the alignment of the chromosomes equidistant from both poles at the midline of the cell, also called the metaphase plate. This process is not necessarily coordinated for all chromosomes in a cell at once, such that some may still be in late prometaphase while others have entered metaphase. Once at the metaphase plate, the partner chromosomes stay connected, held together by protein complexes called cohesins. The transition from metaphase into anaphase depends on the formation of kinetochore microtubules, microtubule attachment to kinetochores, and tension at the kinetochore from both poles (Shah and Cleveland, 2000; Zhou et al., 2002).

Anaphase: As the cell makes the transition into anaphase the cohesin proteins that bind the partner chromosomes together are broken down by proteins recruited by the anaphase promoting complex, which plays a central role in marking proteins for degradation (Kotani et al., 1998). Once the cohesion breaks down, the partner chromosomes can separate and move towards the opposite poles. The classic understanding of chromosome movement in anaphase is that the kinetochore microtubules are the primary force producer and that they pull each partner chromosome, or half-bivalent, towards the pole through depolymerization of tubulin either from the kinetochore or from the pole. Studies with yeast, however, have shown that chromosomes are still capable of segregating in the absence of microtubules (Castagnetti et al., 2010). This suggests that microtubules are not the only force producing component of the spindle. It has been proposed that there is a gel-like elastic spindle matrix which produces forces that act on the microtubules to move the anaphase chromosomes towards the poles (Johansen et al., 2011). As the chromosomes move towards the poles the spindle fibers lengthen and the cell elongates.

Telophase: The final step of mitosis and meiosis is telophase, when the cell begins to split into the two daughter cells. A cleavage furrow is formed as a contractile ring containing the proteins actin and myosin tightens creating an indent in the cell which gradually deepens (Guertin et al., 2002). Once the half-bivalents have reached their respective poles the new nuclear envelope will often begin to form to create the new nucleus. In some meiotic cells, however, the nuclear envelope does not reform, allowing for immediate entry into the second division led by prophase II.

Lastly, the cell enters the final event of the cell cycle, cytokinesis. Cytokinesis occurs when the cleavage furrow has deepened enough that it pinches off completely and the cytoplasm finally splits, resulting in two distinct daughter cells. In mitosis this would be the final stage until the daughter cells were ready to divide and enter the entire cell cycle again. In meiosis, however, the resultant daughter cells will enter meiosis II and begin the cycle once more starting with prophase II. The experiments described in this thesis deal with chromosome movements during metaphase I and anaphase I.

PHOSPHORYLATION AND DEPHOSPHORYLATION

The cell cycle is a highly regulated process and as the cell moves through the stages of division the progress is closely monitored and controlled. Phosphorylation and dephosphorylation play a large role in regulating this process and act as a sort of molecular switch to propagate positive and negative signals to advance or inhibit the progression of the cell through the cell cycle. These signals work to activate or deactivate various proteins in the cell. Protein phosphorylation is performed by enzymes called kinases, and dephosphorylation is performed by protein phosphatases. The target protein can be directly phosphorylated by a kinase or can be indirectly phosphorylated due to a kinase cascade, wherein one kinase activates another kinase, which then activates yet another, and so on until the target protein is reached. This cascade method can also amplify a phosphorylation signal, as each activated kinase can in turn activate multiple others leading to an exponential amplification of the original signal.

When a kinase phosphorylates a protein it covalently bonds a phosphoryl group to the target protein at the hydroxyl (-OH) group of one of three amino acid residues: serine, threonine, or tyrosine. To dephosphorylate a protein the phosphoryl group is removed by phosphatases. The majority of the kinases (and phosphatases) in the human genome act on serine/threonine residues

(Manning et al., 2002a). Other kinases phosphorylate only tyrosine residues, whereas yet others can act on all three amino acid residues (Miller et al., 2008).

When a phosphoryl group is added to a protein the properties of the protein are modified, thereby regulating its function. A classic example of this is the phosphorylation of histones. In a cell nucleus the decondensed DNA, or chromatin, is wound around proteins called histones. Occasionally the DNA can become damaged and DNA repair enzymes need to be recruited to correct the damage before the cell can divide. When the histone is phosphorylated in the area around the DNA damage the phosphoryl groups act as a recruitment signal for enzymes involved in chromatin relaxation and DNA repair (Rossetto et al., 2012). The chromatin relaxation and molecular signaling result in the chromatin being separated from the histone which allows room for the DNA repair enzymes to bind to the chromatin and repair the damage. Once the DNA is repaired the histone can be dephosphorylated which stops the signal and allows the chromatin to once again coil tightly around the histone. This is, of course, only one example of many, as kinases and phosphatases work together in the cell in a multitude of ways to orchestrate a coordinated progression through the cell cycle by activating or deactivating the target protein.

Kinases

The human genome contains more than 500 known kinases (Manning et al., 2002a) which are categorized into several families. Phylogenetic analyses suggest that most of these kinase families are conserved between humans, roundworms (*Caenorhabditis elegans*), fruit flies (*Drosophila melanogaster*), and yeast (*Saccharomyces cerevisiae*) (Manning et al., 2002b). My experiments are performed on crane flies whose genome has not yet been sequenced. Many drugs that work in *Drosophila*, however, have similar effects in crane flies. For example, *Drosophila* antibodies for titin and tropomyosin used in crane-fly spermatocytes have the expected effects and appropriately stain the target protein (Fabian et al., 2007b; DiSalvo, 2017). Therefore, it is possible that crane flies have many kinases and phosphatases in common with *Drosophila*, and that in many circumstances *Drosophila* can act as an analog organism for crane flies.

Kinases that regulate various aspects of the cell cycle and division can be found in multiple families. For example, the CMGC family (named for **C**yclin dependent kinases (Cdk), **M**itogen-activated protein kinases (MAPK), **G**lycogen synthase kinase 1 (GSK1), and **C**DC-like

kinase (CLK)) includes Cdks and MAP kinases, both of which are prominent regulators of the cell cycle. The STE family, for **ste**rile kinases, and the AGC family, named for protein kinases A, G, and C, both contain kinases implicated in activating Cdks and MAP kinase cascades (Ardito et al., 2017; Poli et al., 2014; Seger and Krebs, 1995). Kinases in the AGC family, along with kinases from the Polo family, regulate the activity of the anaphase promoting complex. I will now discuss in greater detail how specific kinases from the families mentioned above contribute to cell cycle regulation and control.

There are several regulatory checkpoints in the cell cycle. If a cell fails to meet certain conditions at any of these checkpoints then division will be aborted and the cell may enter apoptosis, or programmed cell death, to avoid aberrant division leading to cancer or damaged cells. If the cell successfully passes through each checkpoint it will proceed with division as normal. Before the onset of mitosis or meiosis, as a cell moves through interphase, it goes through three different stages: G_1, S, and G_2. At the transition point between each stage, as well as the transition into mitosis or meiosis, there is a checkpoint. These checkpoints are tightly controlled by different cyclins and Cdks. The cyclin acts as a regulatory subunit and the Cdk as the catalytic subunit. Once bound, the two subunits can act on protein targets as a kinase which regulates protein activity. During the transition into mitosis or meiosis from interphase, the kinase Cdk1, the most prominent and well-known mitotic kinase, acts with cyclins B1/B2, B3, and A (Kreutzer et al., 1995). Although there are other kinases that can be involved in mitotic entry and exit, Cdk1 activation and deactivation is sufficient for that transition to occur (O'Farrell, 2001; Deibler and Kirschner, 2010).

Once a cell has successfully passed the interphase checkpoints and has entered prophase, the first stage of division, there are kinases from several subfamilies, including the Cdk subfamily, the Aurora subfamily, the Polo subfamily, and kinases from the ACG family (Nigg, 2001) that play a role in regulating the events of division. Key kinases from these subfamilies include Cdk1 with cyclins A and B, Aurora kinase A and B, Polo-like kinase (Plk) 1, and protein kinases (PK) A and C (Kim et al., 2016). As division progresses some of these kinases become less active while others become more active.

The Cdk1-cyclin complex is involved in many steps of the cell cycle including nuclear envelope breakdown, spindle assembly, and further regulation of the anaphase-promoting complex (Nigg, 2001). Once the Cdk1-cyclin complex has been activated it can go on to

phosphorylate many other substrates. Cdk1 with cyclin A is active through the end of prophase, and Cdk1 with cyclin B is active through metaphase.

Kinases in the Aurora subfamily are active for most of division. Aurora kinase A is active through prophase and metaphase. In *Drosophila*, Aurora kinase A is involved in the separation of the centrosome in early prophase (Glover et al., 1995), and in vertebrates it is seen localized at the spindle poles and microtubules (Bischoff and Plowman, 1999). Activity of Aurora kinase B peaks later than Aurora kinase A. Aurora kinase B activity is seen in cells starting in metaphase and continuing through the end of anaphase. This kinase localizes at the spindle midzone in anaphase (Giet and Prigent, 1999; Mora-Bermúdez et al., 2007) and is important for chromosome alignment and partner chromosome separation (Adams et al., 2001).

Plk1 is also active during anaphase. Plk1 is thought to regulate the activity of micro-tubule associated proteins in *Drosophila* (do Carmo-Avides and Glover, 1999) and to positively regulate the anaphase promoting complex (Kotani et al., 1998). Negative regulation of the anaphase-promoting complex, which is important for the metaphase/anaphase transition as well as exiting mitosis, is due in part to PKA which is active throughout mitosis (Kotani et al., 1998).

PKA plays many roles throughout division. In HeLa cells PKA activity has been shown to be high near centrosomes during prophase and prometaphase, and high near the chromosomes during metaphase and anaphase (Vandame et al., 2014). Specifically, PKA is required for chromosome condensation and is responsible in part for phosphorylation of Aurora kinase A (Collas et al., 1999; Vandame et al., 2014). Another kinase from the ACG family that plays a role in division is PKC. PKC is involved in the stability of the metaphase spindle (Kalive et al., 2011), and microtubule reorganization following PKC activation has been observed (Kiley and Parker, 1997). In addition to spindle stability, PKC is important for the transition from metaphase to anaphase (Viveiros et al., 2004). My experiments deal specifically with inhibiting or activating protein kinase C and inhibiting protein kinase A.

Phosphatases

In addition to the kinases that regulate the cell cycle through phosphorylation, there are phosphatases that regulate it through dephosphorylation. There are just over 100 protein phosphatases in *Drosophila*, far fewer than there are kinases (Chen et al., 2007). Therefore, phosphatases are less specific in regard to the proteins that they can dephosphorylate with a

single phosphatase potentially dephosphorylating many different proteins. Like kinases, phosphatases are also grouped into different families. The three phosphatase families are phosphoprotein phosphatases (PPPs), metallo-dependant protein phosphatases (PPM), and protein-tyrosine phosphatases (PTPs) (Jin and Pawson, 2012). The PPP and PPM families both dephosphorylate phosphoserine/phosphothreonines. I will discuss PP1 and PP2A from the PPP family in Chapter Two. The PTP family, as the name suggests, dephosphorylates phosphotyrosine. This family also contains dual-specificity phosphatases such as cell division cycle (Cdc) phosphatases that can dephosphorylate phosphoserine, phosphothreonine, or phosphotyrosine (Alonso and Pulido, 2016).

There are 22 phosphatases that play a role in regulating the cell cycle and division that have been identified in *Drosophila* (Chen et al., 2007). During division several protein phosphatases (PPs) from the PPP family are active, such as PP1, 2A, and 4 (Ardito et al., 2017; Kim et al., 2016). PP1 is essential for mitotic progression in *Drosophila* (Axton et al., 1990) and is active during prophase and anaphase, and inactive during prometaphase and metaphase. PP4 is required for centrosome maturation (Sumiyoshi et al., 2002) and is therefore active in prophase before becoming inactive in prometaphase and metaphase. PP2A remains present throughout all stages of division and is important for many mitotic events in *Drosophila* including spindle polarity, proper kinetochore function, and progression through the metaphase-anaphase checkpoint (Chen et al., 2007).

Phosphatases from the PTP family are active in division as well, including Cdc phosphatases, which regulate Cdks. Cdc25 B/C is active from prophase until metaphase, and Cdc14 A/B becomes active in anaphase and is important for mitotic exit in yeast (Jaspersen and Morgan, 2000). In animal cells, however, it is PP1 and PP2A from the PPP family of phosphatases that are important for mitotic exit (Wurzenberger and Gerlich, 2011).

Although knowledge on phosphatases lags behind that of kinases, their importance in the cell cycle is recognized. Some studies have identified interactions between some of the kinases and phosphatases discussed above during the different stages of division (Table 1.1). For example, Cdk1 can be dephosphorylated by many phosphatases, including PP1, PP2A, PP4, and Cdc14 (reviewed in Mochida and Hunt, 2012). Aurora A and Aurora B can both be dephosphorylated by PP1 (Kim et al., 2010), and Plk1 can be dephosphorylated by PP2A (Hauf et al., 2005).

Table 1.1. A summary of the kinases and phosphatases discussed in this chapter and the stages of division in which they have been reported to be active. My work is focused on the kinases PKA and PKC and the phosphatases PP1 and PP2A during metaphase and anaphase (bolded in the table below)

Table 1.1	**Prophase**	**Prometaphase**	**Metaphase**	**Anaphase**	**Telophase**
Kinases	Cdk1-cyclin A and B, Aurora kinase A, PKA	CDK1-cyclin B, Aurora kinase A, PKA	Cdk1-cyclin B, Aurora kinase A, **PKA, PKC**	Aurora kinase B, Plk1, **PKA, PKC**	PKA
Phosphatases	PP1, PP4, PP2A, Cdc25	PP2A, Cdc25	**PP2A**, Cdc25	**PP1, PP2A**, Cdc14	PP2A, Cdc14

Understanding the role that different kinases and phosphatases play can be a challenging endeavor as the process of phosphorylation is complex. Kinases and phosphatases can each have multiple substrates and can each function in multiple pathways. Further, activation of a kinase can lead to a phosphorylation cascade resulting in numerous downstream effects that are not immediately attributable to the initial enzyme. However, there are some key methods for studying the role of specific kinases and phosphatases in cells and cellular division. The first method is to inhibit their function and observe what changes occur. This inhibition can be accomplished either with the use of drugs that enter the cell and prevent the enzyme from properly binding with its substrate or through genetic manipulation whereby the gene responsible for the enzyme is knocked out. The second method is to amplify the effect of kinases and phosphatases in the cell either through drugs that enhance their activity or through genetic editing in which the gene that produces the enzyme is duplicated or upregulated. The third method is to study where the enzymes are expressed in a cell using immunostaining and fluorescence. All three methods help to produce a more comprehensive understanding of the roles of various kinases and enzymes. In my experiments I focus on utilizing cell-permeable drugs added to living cells to either inhibit or enhance the activity of kinases and phosphatases.

TETHERS

For this thesis I performed experiments to further understand the role that phosphorylation and dephosphorylation play in the elasticity of the tethers that are found between separating anaphase chromosomes. During anaphase the chromosomes in a cell begin to move apart from their partner and move towards the spindle pole. Although the partner

chromosomes are moving apart from one another they are not yet independent of each other because they are tethered together by a physical cellular structure. This structure is called a "tether". I will now review the history of these tethers, including how they were first noted in the literature, how micromanipulation and UV microbeam studies pointed towards a "tethering" function, and how they were definitively shown to be elastic and connect separating partner chromosomes by LaFountain et al. (2002).

Historical reports regarding tethers

When chromosomes in division were initially being studied in the late 1800s and early 1900s the standard procedure was to fix and stain the chromosomes from cells at many points throughout division, and to then draw the state of the chromosomes within the cells. The drawings were made using a technique called *camera lucida* projections in which the image being viewed is optically superimposed onto the drawing surface. This technique allows for an accurate representation of the subject, in this case chromosomes in division. In many of the figures from the literature of this time, connections were indicated in the interzone of the cell between separating chromosomes of many different cell types of plant and animal origin in anaphase (e.g. Wilson, 1905; Stevens, 1905 and 1909, others reviewed in Paliulis and Forer, 2018).

The interzonal connections were also discussed in the text of some of the papers cited above and there was speculation on their composition and endurance throughout anaphase. A study of meiosis and mitosis in stink bugs (*Pentatomidae*) described the connections as connective fibers likely derived from the chromatin that is contained in chromosomes (Montgomery, 1898). Later, these connections were described in studies of the first meiosis in primitive coccids (*Llaveia*) (Hughes-Schrader, 1931), salamaders (*Amphiuma*) (Schrader, 1935), and the broad-headed bug (*Protenor*) (Schrader, 1935). In coccids Hughes-Schrader described the interzonal structure as an "interchromosomal tube" that extends between and connects anaphase chromosomes. The tubes are then described as stretching as the chromosomes separate before eventually breaking as the cytoplasm divides (Hughes-Schrader, 1931). Schrader also discussed the connections and allowed that they may be a tube or sheath, which would confer differing viscosities and elasticities (Schrader, 1935). In primary grasshopper (*Acrididae*) the interzonal connections between anaphase chromosomes were reported as being morphologically

distinct from the spindle fibers. Rather, it was suggested that they connected the "free" ends of the separating chromosomes (Carothers, 1936). Though there were many interpretations of these connections at that time, there was not a definitive description and no function was known.

Micromanipulation studies of tethers

The first evidence of a function for these connections was put forward in 1953. Micromanipulation experiments performed on anaphase grasshopper neuroblasts indicated that the interzonal connections were playing a tethering role between two separating half-bivalents (Carlson, 1953). When a microneedle was moved within the interzonal region of the anaphase cell along the spindle from one pole to another, the chromosomes were not displaced, only the mitochondria moved around the needle as it passed through. However, when the microneedle was moved *across* the interzone of the cell crosswise along the spindle axis, the trailing arms of *both* separating partner chromosomes moved even though the microneedle didn't come into contact with either of them. Further, these connections were reported as being very tough because they stretched, rather than broke, to accommodate the pulling of the needle (Carlson, 1953). That separating chromosomes experienced coordinated movements after manipulation of the interzonal region of the cell indicated that they are somehow tethered together.

Similar results were seen in micromanipulation experiments in crane-fly spermatocytes (Forer and Koch, 1973). When an anaphase half-bivalent was pushed with a needle perpendicular to the spindle axis, the partner chromosome moved as well. Further evidence that the separating partner chromosomes are mechanically connected came from experiments done by Paliulis and Nicklas (2004). Primary metaphase and anaphase chromosomes were studied using grasshopper spermatocytes. They used a micromanipulation needle to pull on separating chromosomes. One experiment showed that during anaphase when they pulled on one arm of a half-bivalent it resulted in movement of the arm of the partner chromosome, again showing an apparent tethering between the partner chromosomes. Taken together, these micromanipulation experiments showed that despite there being no visible connections between the distal ends of separating anaphase half-bivalents they responded in tandem to the manipulation of either the space between them or of the partner chromosome and were therefore mechanically tethered.

UV microbeam studies of tethers

In addition to micromanipulation studies, experiments using ultraviolet (UV) microbeam irradiation, a technique first proposed by Tchakhotine (1912) that targets specific parts of a chromosome, continued to support the growing evidence that separating anaphase chromosomes are tethered together. For example, the spindle pole of silkworm (*Bombyx mori*) spermatocytes in telophase was irradiated using a UV microbeam (Nakanishi and Kato, 1965), which resulted in the chromosomes at that pole moving across the cell towards the opposite pole. In some cases, the movements of the chromosomes coming from the irradiated pole were impeded by swollen mitochondria and they did not fully reach the other pole, but in other cases the chromosomes were able to reach the other pole. Most importantly, though, chromosomes from the ablated pole moved towards the opposite pole in all cells.

This was followed by various experiments in crane-fly spermatocytes. A UV microbeam was used to irradiate the kinetochore fibers that connect the kinetochore of one separating half-bivalent to the pole to which it was traveling (Forer, 1966). When these fibers were irradiated with certain wavelengths of UV light not only did the half-bivalent stop moving towards the pole, but its partner chromosome across the cell stopped moving as well. Irradiation of the spindle interzone itself either before or after the irradiation of the kinetochore fibers resulted in the unlinking of partner chromosomes (Yin and Forer, 1996). This suggested that the connections between separating chromosomes were responsible for the coordinated movements that were seen in earlier experiments. Furthermore, after kinetochore irradiation the chromosome would sometimes move backwards across the cell towards its partner chromosome (Ilagan and Forer, 1997). These micromanipulation and UV microbeam irradiation experiments were highly indicative that there was a physical connection between separating partner chromosomes.

Tethers are proven to exist

Although there was a growing body of indirect evidence that separating partner chromosomes are physically tethered together, there was not definitive experimental proof. Micromanipulation and UV microbeam studies did not rule out other interzonal structures, such as microtubules, and backwards movement induced by UV microbeam experiments were not regularly occurring (Ilagan and Forer, 1977). However, experiments with crane-fly

spermatocytes by LaFountain et al. (2002) finally demonstrated that it is in fact tethers that link partner chromosomes, and that tethers are elastic in early anaphase.

LaFountain et al. (2002) used a laser microbeam to physically disconnect fragments of the trailing arms of chromosomes during anaphase (Figure 1.1). When the telomere-containing fragment of a chromosome arm was produced in early anaphase, it moved rapidly away from its chromosome of origin and towards the partner chromosome. This rapid backwards movement of the arm fragment provided clear evidence that tethers link separating anaphase chromosomes and that they are elastic in nature in early anaphase. When these fragments were produced later in anaphase, the fragments moved less rapidly and would only move to the spindle midzone, rather than all the way to the partner chromosome. When these fragments were produced yet later, they would sometimes not move at all, or would begin to move towards the midzone before returning to their chromosome of origin. These results suggested that the elasticity of the tethers is not constant throughout anaphase, but rather that it is either reduced during the process or that the tethers disconnect from the chromosomes as anaphase progresses.

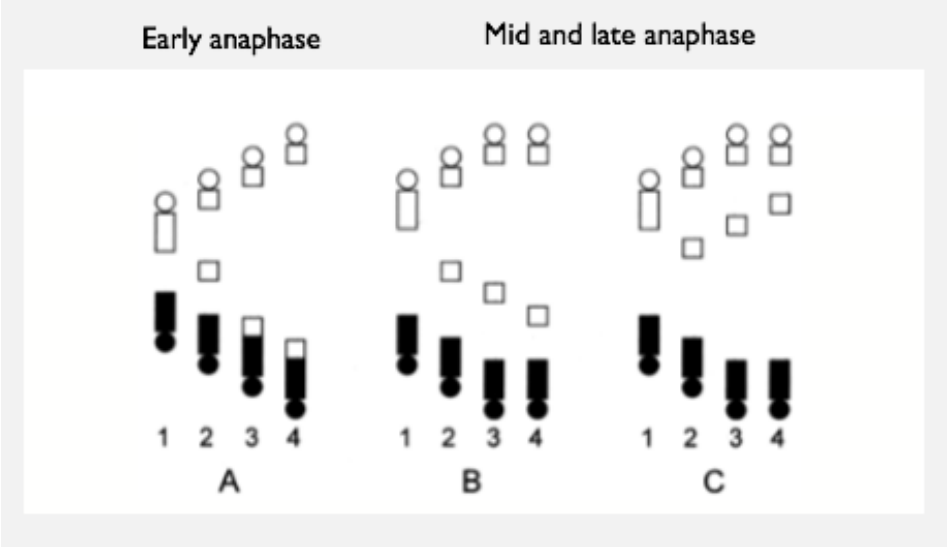


Figure 1.1. Modified from LaFountain et al. 2002. The rectangles represent the chromosome arm and the circles represent the kinetochore. The squares represent the cut telomere-containing fragments. Panel A depicts the backwards movement of a telomere-containing arm fragment created in early anaphase. The fragment moves backwards rapidly (A2) and then makes contact and moves with the partner chromosome (A3, A4). Panels B and C depict two patterns of backwards movement that can occur when the fragment is created in mid or late anaphase. The fragment either moves towards the opposite chromosome (B2, B3) but does not make contact (B4), or it may begin to move towards the opposite chromosome (C2) before reversing movement and moving towards the original pole (C3, C4).

LaFountain et al. (2002) also demonstrated that the telomere of the chromosome is the critical structure for tether activity. The first way this was demonstrated was through a double UV microbeam cut. First, the telomere-containing end of the chromosome was cut, as was done in the initial experiment. Then, a second cut was made to separate the telomere end of the fragment from the rest of the fragment. After this cut was made, the fragment without the telomere ceased its movement towards the partner chromosome while the telomere-containing fragment continued toward the partner chromosome.

The second way this was demonstrated was through the irradiation of the telomere itself, either on the arm fragment or on the partner chromosome. Telomere irradiation at either of these locations resulted in the arm fragment ceasing its movement towards the partner chromosome. These two experiments also ruled out the possibility that the fragment movements were due to spindle microtubules. If microtubules were responsible for the observed fragment movement, then irradiating the telomeres would have had no effect on backwards movement, because microtubules do not attach to the telomere. Taken together, these experiments definitively demonstrated that there are elastic tethers that extend between the telomeres of separating partner chromosomes.

Characterization of tethers

There are several known characteristics of elastic tethers beyond what has been described above. First, **they extend between only two of the four arms** of separating chromosomes (Figure 1.2). Following treatments that slow chromosome speeds in anaphase such as UV microbeam irradiation or anti-actin drugs, one or two of the trailing arms will sometimes move ahead of the kinetochore towards the pole (Adames and Forer, 1996; Forer and Pickett-Heaps, 1998a).

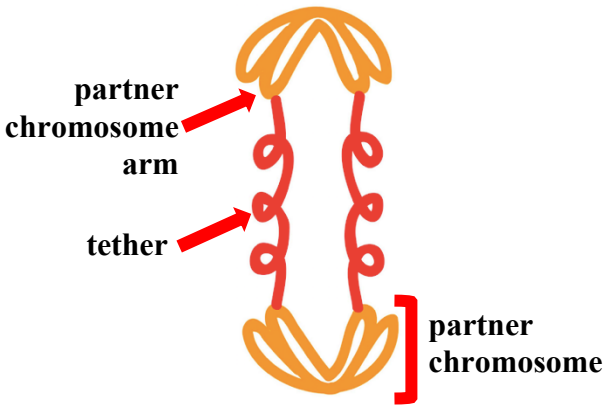


Figure 1.2. A graphic showing two separating partner chromosomes. Two tethers extend between two arms of each chromosome partner. The other two arms are not connected.

However, since it is never more than two arms that move ahead, it was suggested that the other two arms are linked to each other (Adames and Forer, 1996). This was corroborated by the experiments done by LaFountain et al. (2002) because the results described in the section above did not occur after cutting more than two of the arms.

Another characteristic of tethers is that **they persist throughout anaphase**. LaFountain et al. (2002) concluded that tethers were elastic due to the backwards movements produced by cutting a telomere-containing fragment from a chromosome arm in early anaphase. However, when this same cut was made later in anaphase there was decreased backwards movement of the telomere-containing arm fragments. La Fountain et al. posited that either tethers lose their elasticity over the course of anaphase, or that tethers simply detach or degrade by the end of anaphase. To test this, tethers were cut directly in anaphase (Sheykhani et al., 2017; Forer et al., 2017). In both crane-fly spermatocytes and *Potorous tridactylus* kidney (PtK) cells, these cuts resulted in the tethered chromosome arm shrinking ~90% of their pre-cut length. These retractions occurred even when the tethers were cut at a length that would not have resulted in backwards movement, as determined by La Fountain et al. (2002). This suggested that the tethers are still present as anaphase progresses, and indicated tethers are elastic and produce a pulling force on the chromosomes.

Lastly, **they are ubiquitous**. I have discussed experiments that used many different cell types containing tethers. Tether ubiquity was tested directly by Forer et al. (2017) by cutting telomere-containing fragments from chromosome arms in a wide range of cell types, including cells from insects, flatworms, arachnids, marsupials, and humans. In each of these cell types the resulting arm fragments moved backward, indicating the presence of tethers. The ubiquity of tethers suggests that they are an important component of faithful cell division. Understanding this highly conserved cellular structure is necessary for understanding how chromosomes communicate as they divide and how errors may arise and lead to cell death, birth defects, and cancers.

STUDY SYSTEM

I studied elastic tethers during the first meiotic metaphase and anaphase in crane-fly (*Nephrotoma suturalis* Loew) spermatocytes. Although the crane fly is not a standard model organism, their spermatocytes are an excellent study system for cell division because of multiple

benefits that they offer. These benefits include 1) the relative ease with which an animal of the proper developmental stage can be selected and 2) their multiple favorable cytological characteristics, such as their small number of chromosomes and their large cell size (Forer, 1982).

For my experiments I dissected the testes of IV-instar crane fly larvae to observe their spermatocytes. Crane-fly testes are highly synchronous, and all the cells within a testis are roughly the same stage of spermatogenesis (Forer, 1982). As such it is important to be able to identify larvae in the correct stage, otherwise one may end up dissecting an animal that has long since completed spermatogenesis, or an animal that has not yet begun any meiotic cell division. As the larvae grow, they put on white fat that can be seen through their skin and the spiracles on their distal end grow larger and darker. By the IV-instar phase the fat in many of the larvae will partially occlude the GI tract and the spiracles are large and dark and can be clearly seen with the naked eye. These external indicators of life-stage allow for higher chances of finding dividing cells upon dissection and slide preparation.

Another benefit to studying cell division using crane-fly spermatocytes is their cytological characteristics that make the cells highly conducive to observing and following the movements of individual chromosomes. Crane-fly spermatocytes each contain three autosomal chromosomes and one pair of sex chromosomes, making their diploid number eight. This low number of chromosomes means that when following a specific chromosome, it will not get lost amongst the others. Further, the spermatocyte itself is quite large with an average spindle size of 20-30 μm (Forer, 1982). A large spindle allows plenty of room for the chromosomes and makes following an individual chromosome throughout cell division an easy task. Both of these features were important for my research, as I needed to be able to track the movements of individual chromosomes from the moment they first began to separate from their partner chromosome at the onset of meiotic anaphase.

STUDY PLAN

Although some key characteristics of tethers have been studied, such as their presence between two of the four chromosome arms, their persistence and decreasing elasticity throughout anaphase, and their ubiquity, it was unknown how tethers developed their elasticity and how that elasticity was lost over the course of anaphase. A first hint to answering these questions came

from experiments done by Fabian et al. (2007a) in which partner chromosomes would sometimes move back towards each other after reaching the poles when the cell was treated in anaphase with the phosphatase inhibitor Calyculin A (CalA). This backwards movement was interpreted as being due to maintained tether elasticity throughout anaphase, as it was a similar response to what was observed by LaFountain et al. (2002) when he severed telomere-containing arm fragments. These backwards movements following treatment with a phosphatase inhibitor suggested that tether elasticity may be due to phosphorylation and that the loss of elasticity is due to dephosphorylation. Therefore, by inhibiting dephosphorylation with CalA the tethers are able to maintain their initial level of elasticity throughout anaphase. In my first set of experiments I tested the hypothesis that tethers lose elasticity over the course of anaphase due to dephosphorylation.

The results from these experiments (Chapter Two, published in *Nucleus*) shed some light on the question of how and when tethers lose their elasticity, as well as the roles of some potentially associated phosphatases. This led then to my next hypothesis, that tethers become phosphorylated prior to anaphase by kinases that have been shown to phosphorylate the giant protein titin, a potential component of tethers (Chapter Three). In all of my experiments I explored these questions by treating living crane-fly spermatocytes at various points throughout metaphase I and anaphase I with different phosphatase inhibitors, kinase inhibitors, and a kinase activator, and then observing and analyzing the resulting chromosome movement.

Chapter Two

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

Kite E, & Forer A. (2020). The role of phosphorylation in the elasticity of the tethers that connect telomeres of separating anaphase chromosomes. *Nucleus*, *11*(1), 19-31.

SUMMARY

Elastic tethers connect telomeres of all separating partner chromosomes during anaphase in animal cells. As anaphase progresses the tethers lengthen and lose their elasticity. Our experiments look at the possible role of phosphorylation in regulating tether elasticity. When anaphase crane-fly spermatocytes were treated with Calyculin A (CalA), an inhibitor of serine/threonine phosphatases PP1 and PP2A, some chromosomes moved backwards after they reached the poles (Fabian et al., 2007a). We propose that the backwards movements are due to maintained tether elasticity. To test this hypothesis, we added 50 nM CalA to living crane-fly spermatocytes at various points in anaphase when the tethers were of different lengths. When CalA was added to cells in early anaphase, when tethers were short, almost all partner chromosome pairs moved backwards after nearing the poles. When added at increased tether lengths (and decreased elasticities), fewer chromosomes moved backwards. When added in late anaphase, when the tethers were >11 μm long, none moved backward. This indicates that tether elasticity is lost when there is dephosphorylation by PP1 or PP2A. To distinguish between the two phosphatases, we treated cells with okadaic acid. 50 nM CalA blocks both PP1 and PP2A, but 50 nM okadaic acid blocks solely PP2A. Much higher concentrations of okadaic acid are needed to block PP1. We added high or low concentrations of okadaic acid to anaphase cells when tethers were short. Only the higher concentration of okadaic acid caused chromosomes to move backward. Thus, tether elasticity is lost because of phosphatase activity of PP1.

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 (LaFountain et al., 2002). They also are present in a broad range of animal cells, including those from flatworms, insects, arachnids, flatworms, marsupials, and humans (Forer at al., 2017), 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 (Forer, 1966), because UV irradiation of the interzonal region between the separating chromosomes unlinked the movements of the partners (Yin and Forer, 1996), and because sometimes partner chromosomes moved backwards after irradiation of kinetochores themselves (Ilagan et al., 1997). While

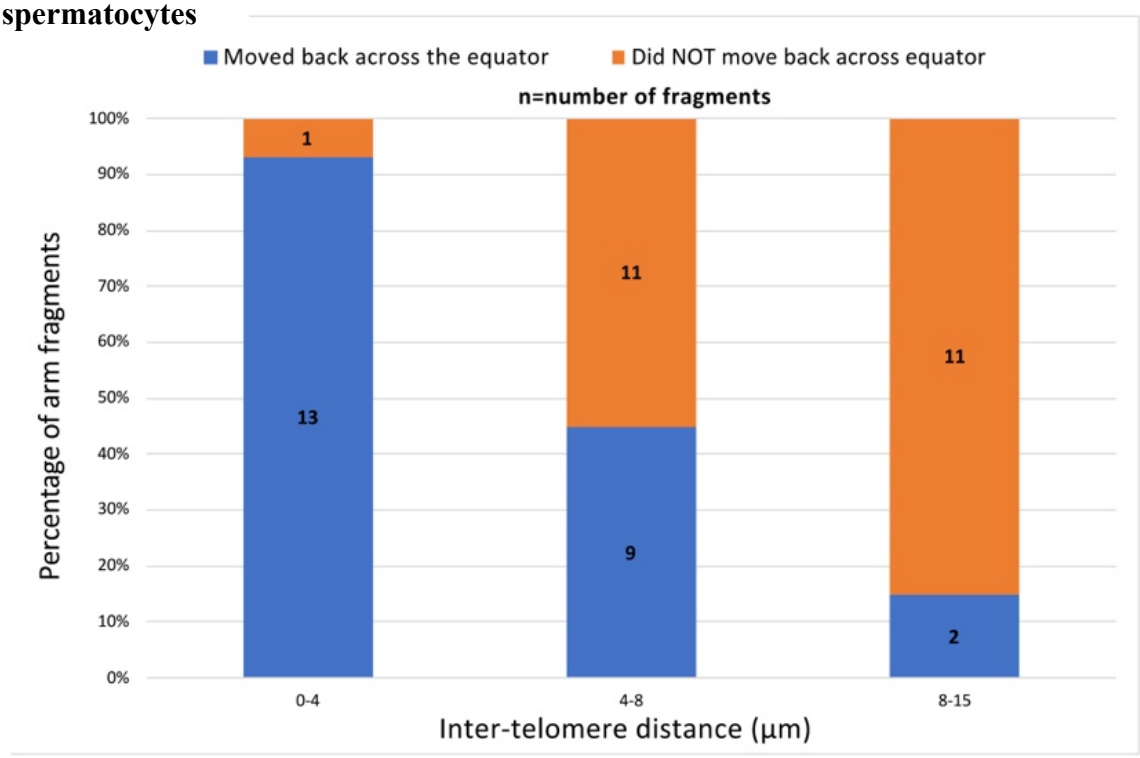
suggestive, these experiments did not prove that partner chromosomes were physically connected, especially since no connections can be seen in living cells using standard phase-contrast or DIC microscopy.

Elastic connections between separating partner chromosomes were demonstrated in crane-fly spermatocytes by LaFountain et al. (2002). 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 towards 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' backwards movement decreased (Figure 2.1A), which also holds true for arm fragments produced in PtK cells (Figure 2.1B). This suggests either that tether elasticity decreases as anaphase progresses, or that the tethers disconnect from the telomeres in later anaphase (LaFountain et al., 2002). This ambiguity was subsequently resolved by cutting tethers directly. In both anaphase crane-fly spermatocytes and anaphase PtK 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 (Forer et al., 2017). 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 (Forer et al., 2017). 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 for the arm fragment to move to the partner telomere, because if either of the two telomeres is ablated the fragment stops moving (LaFountain et al., 2002); further, if the moving arm fragment is cut in half, only the piece with the telomere moves (LaFountain et al., 2002). 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 (LaFountain et al., 2001), 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 (Forer et al., 2018). 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 (LaFountain et al., 2002; Sheykhani et al., 2017; Adames and Forer, 1996). 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., Chan et al., 2007; Barefield and Karlseder, 2012; Gemble et al., 2015). Further, Su et al. (2016) showed that induced ultra-fine DNA strands slow down anaphase chromosomes, whereas tethers do not, because cutting them with a laser during anaphase does not affect the velocities of the associated anaphase chromosomes (Sheykhani et al., 2017). 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.

2.1A – Crane-fly spermatocytes



2.1B – PtK cells

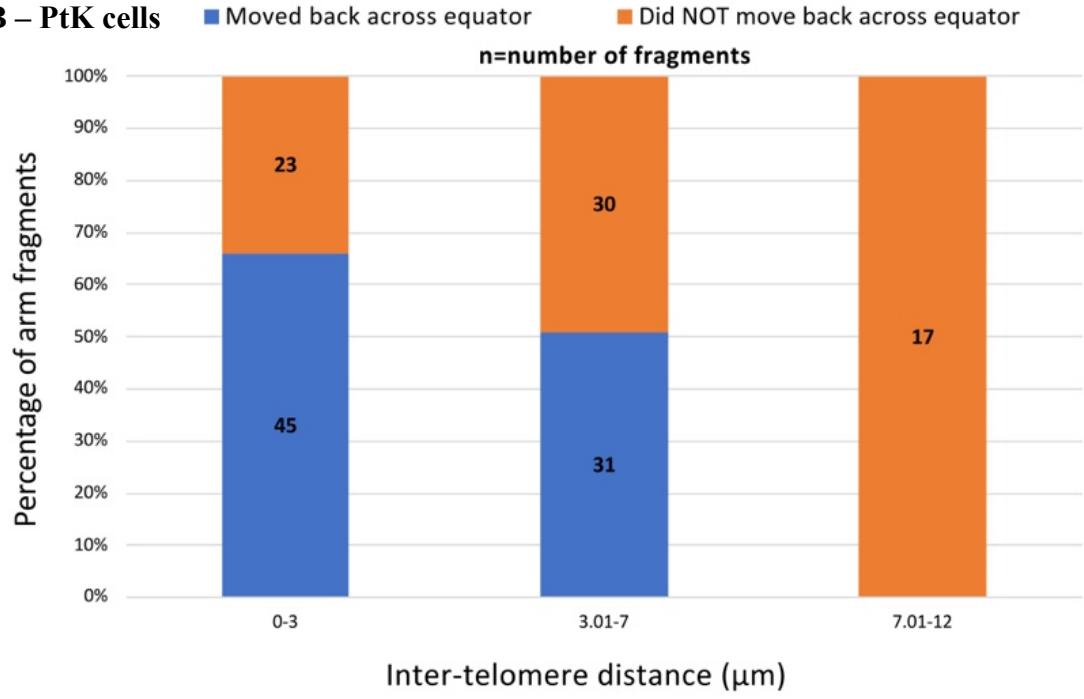


Figure 2.1A, 2.1B. **Movement of arm fragments created at different tether lengths, (A) for crane-fly spermatocytes and (B) for PtK cells**. The data for crane-fly spermatocytes, plotted from values given in LaFountain et al. (2002), are of telomere-containing arm fragments in crane-fly spermatocytes that move backwards 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. (2017).

Tether elasticity may be moderated by phosphorylation. Late-anaphase partner chromosomes in crane-fly spermatocytes often moved backwards towards the cell equator after 50 nM 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 (Fabian et al., 2007a). It is reasonable to assume that the backwards 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 (Favre et al., 1997; MacKintosh et al., 1994), but with different relative effectiveness, as indicated by their IC_{50} (50% inhibitory concentration) values, shown in Table 2.1, and their activity curves (Figure 2.2). 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 10 nM of CalA (Figure 2.2; and Favre et al., 1997). Thus, 50 nM CalA would be expected to affect both PP1 and PP2A while 50 nM okadaic acid would affect solely PP2A. When crane-fly spermatocytes were treated with 50 nM okadaic acid, there were no backwards movements (Fabian et al., 2007a); when HeLa cells were treated with 1 μM okadaic acid, on the other hand, separating partner chromosomes moved backwards (Su et al., 2016). These data suggest, as concluded by Fabian et al. (2007a), that the backwards chromosome movements are caused by inhibiting PP1.

Table 2.1. IC_{50} values of CalA and okadaic acid. These values represent the concentration of compound needed 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. 2007
	0.4 nM	0.25-0.3 nM	Honkanen et al., 1994
	2 nM	0.5-1 nM	Ishihara et al., 1989
Okadaic acid	15-50 nM	0.1-0.3 nM	Swingle et al. 2007
	49-51 nM	0.28-0.3 nM	Honkanen et al., 1994
	60-500 nM	0.5-1 nM	Ishihara et al., 1989

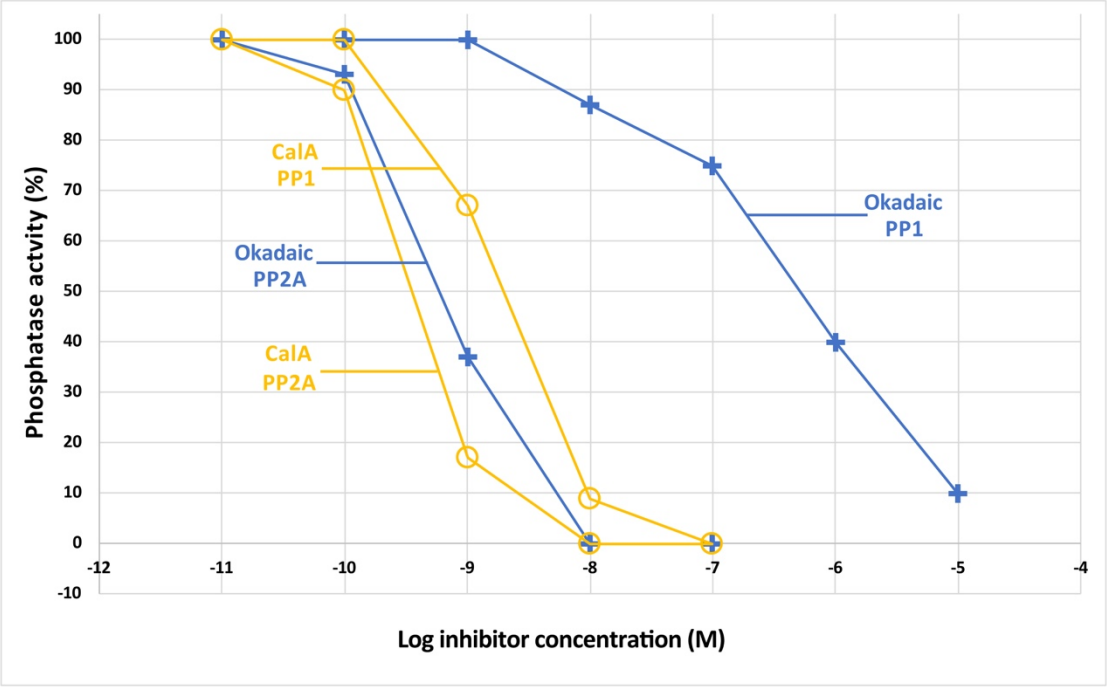


Figure 2.2. Phosphatase activity curves for CalA and okadaic acid. Adapted from data presented in Ishihara et al. (1989). The labels on the graph indicate which phosphatase inhibitor (PP1 or PP2A) was tested. The substrate was phosphorylated phosphorylase a.

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 backwards chromosome movement: at the longer tether lengths, the tethers would have been completely dephosphorylated, and CalA would not produce any backward movement. We added 50 nM 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 backwards movement of chromosomes (Figure 2.3). The results of the experiments reported here fit the prediction: when we added CalA to crane-fly spermatocytes when tethers were of different lengths, fewer half-bivalents moved backwards 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 backwards movements, but the high concentration did, indicating that the effects are due to blocking PP1.

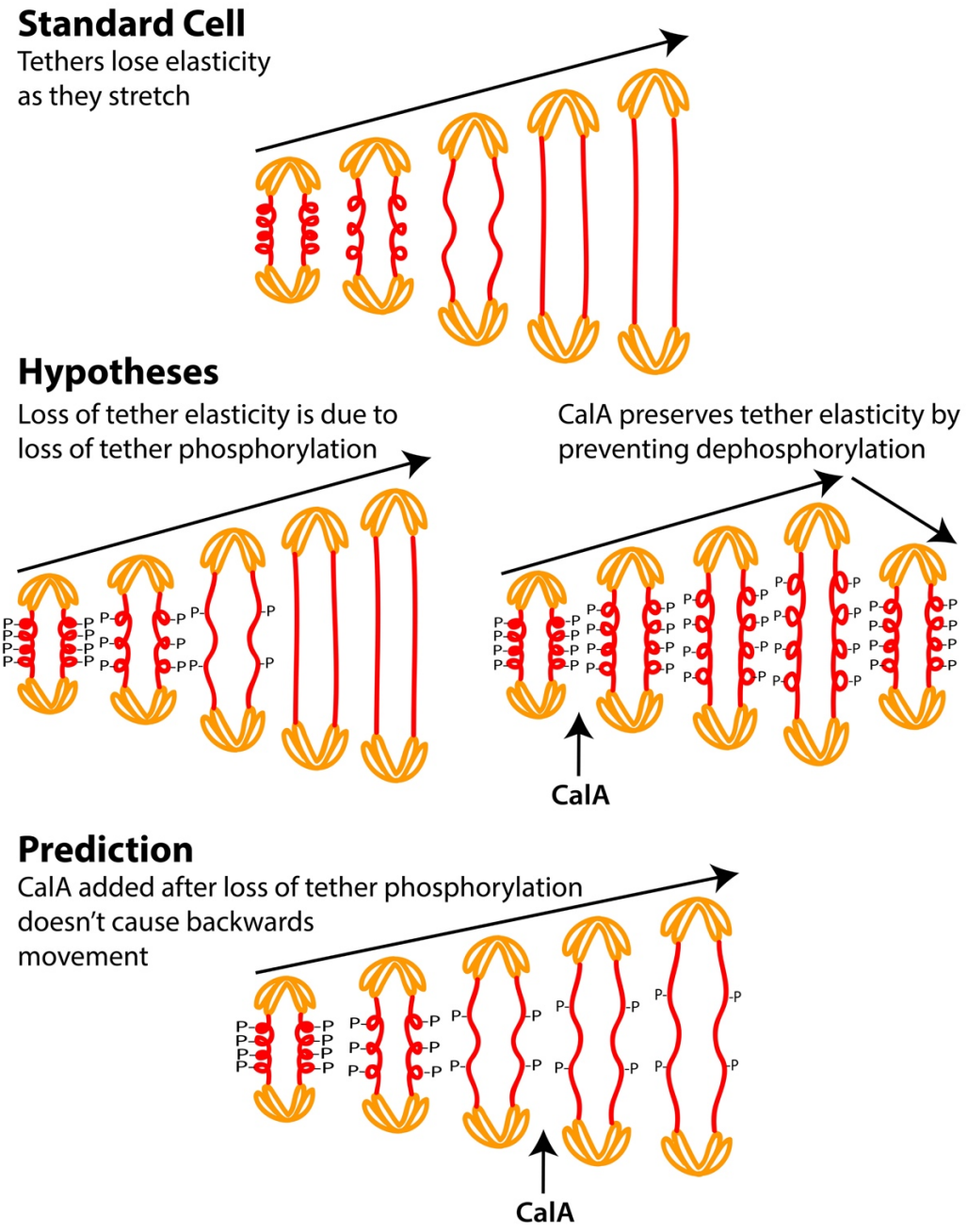


Figure 2.3. **Our hypotheses and predictions**. Cartoon of a *standard cell* in which tether elasticity decreases throughout anaphase. Cartoons of *our hypotheses* that tether elasticity is due to phosphorylation, and that CalA maintains tether elasticity by preventing dephosphorylation of tethers. Cartoon of our *prediction* that adding CalA in later anaphase when there is less phosphorylation results in less backwards movement.

MATERIALS AND METHODS

Crane flies (*Nephrotoma suturalis* Loew) were reared in the laboratory as previously described (Forer, 1982). 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 (Forer and Pickett-Heaps, 2005), 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 (Forer and Pickett-Heaps, 2005) that then was sealed with a thin coating of Vaseline followed by a molten 1:1:1 mixture of Vaseline, lanolin, and paraffin, and 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 50 nM CalA (LC Laboratories, Woburn, MA) or okadaic acid (50 nM or 1 µM) (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 (Forer and Pickett-Heaps, 1998a; Forer and Pickett-Heaps, 1998b; Silverman-Gavrila and Forer, 2000; La Fountain et al., 2001).

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 (Wong and Forer, 2003), 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 in that we experimented on and recorded, and we also measured cells recorded by Fabian et al. (2007a) which they had not analysed 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 2.4. At the onset of anaphase, the three paired bivalent chromosomes that are aligned at the cell's equator disjoin and the half-bivalents move towards opposite poles. They generally reach the poles in 20-30 minutes. During this time the univalent sex chromosomes remain at the equator without moving (Forer, 1980; Forer et al., 2013) as shown in Figure 2.4. 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 (Forer et al., 2013) and, more-or-less simultaneously, the cleavage furrow ingresses.

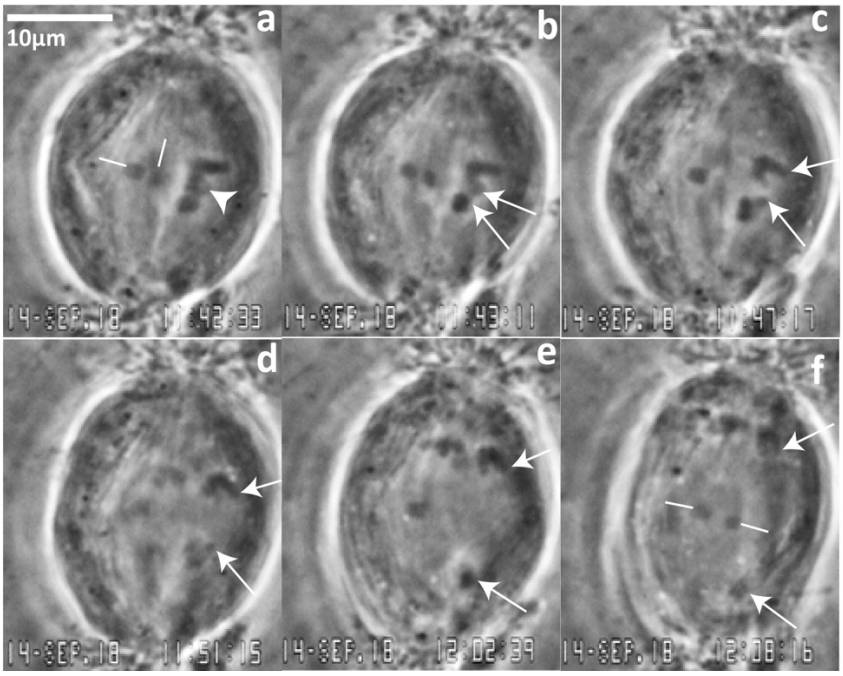


Figure 2.4. **Normal cell division in a crane-fly spermatocyte**. The time stamp in all images indicates hrs:min:sec. The arrowhead in (a) indicates a bivalent in metaphase, and the arrows in (b) – (f) indicate the positions of separating half-bivalents as they move apart from each other and travel to the poles. Lines indicate the position of sex chromosomes, which are stationary throughout the time when the autosomes move to the poles.

***Calyculin A*:** We treated individual anaphase spermatocytes with 50 nM CalA at different inter-telomere distances. Figure 2.5 illustrates a cell treated with 50 nM CalA when tethers were short: all half-bivalents that moved to the lower pole subsequently moved backwards toward the upper pole. The backwards movements were led by the telomeres, with the kinetochores trailing, as indicated by arrows in Figure 2.5, as seen in supplemental videos 1 and 2, and as described

previously by Fabian et al. (2007a). In addition to the backwards movement after the half-bivalents neared the poles, one can see in Figure 2.6 and supplemental video 1 that, as described in detail in Fabian et al. (2007a), 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 (Fabian et al., 2007a; Sheykhani et al., 2013).

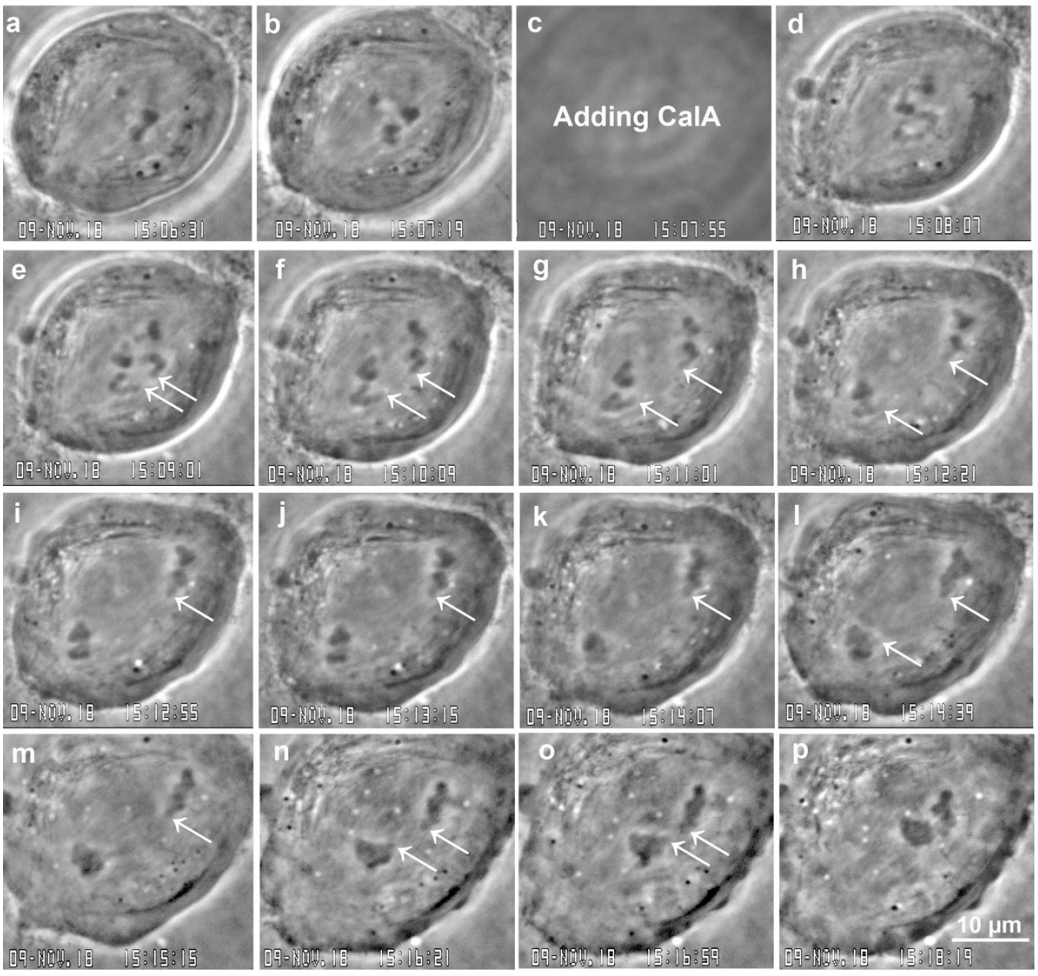


Figure 2.5. **Backward movement after CalA treatment**. Photo montage showing the progression of anaphase in a crane-fly spermatocyte that was treated with 50 nM CalA. The time stamp in all images indicates hrs:min:sec. (a): Metaphase, showing one bivalent. (b): Anaphase. That bivalent has disjoined 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.

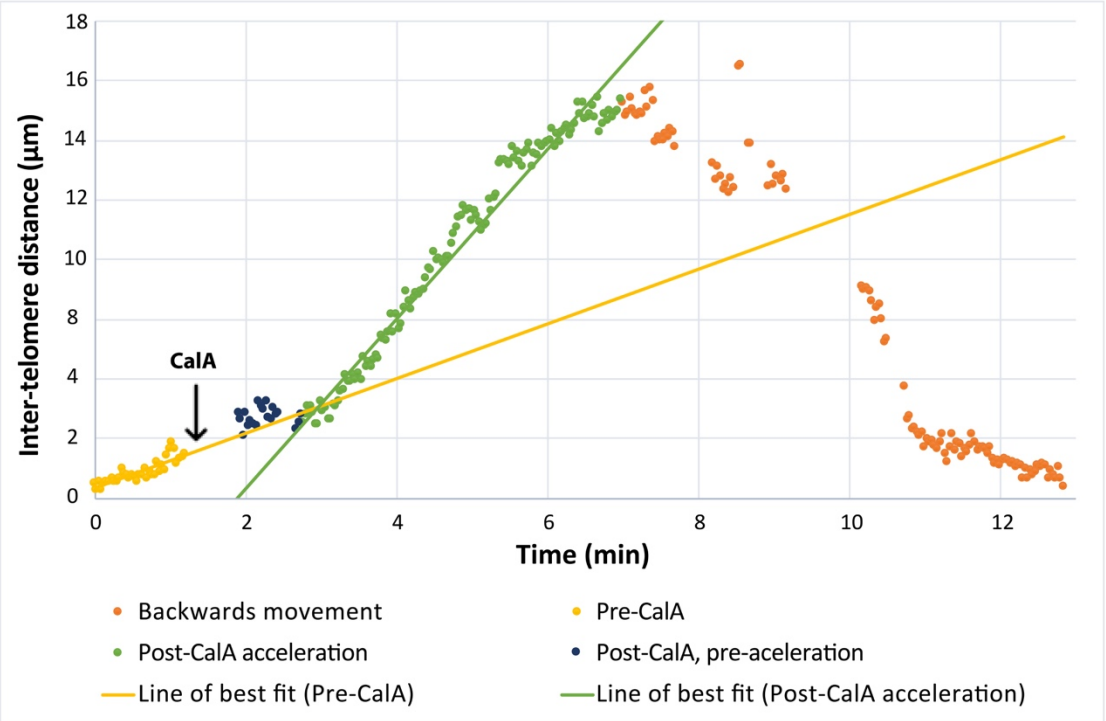
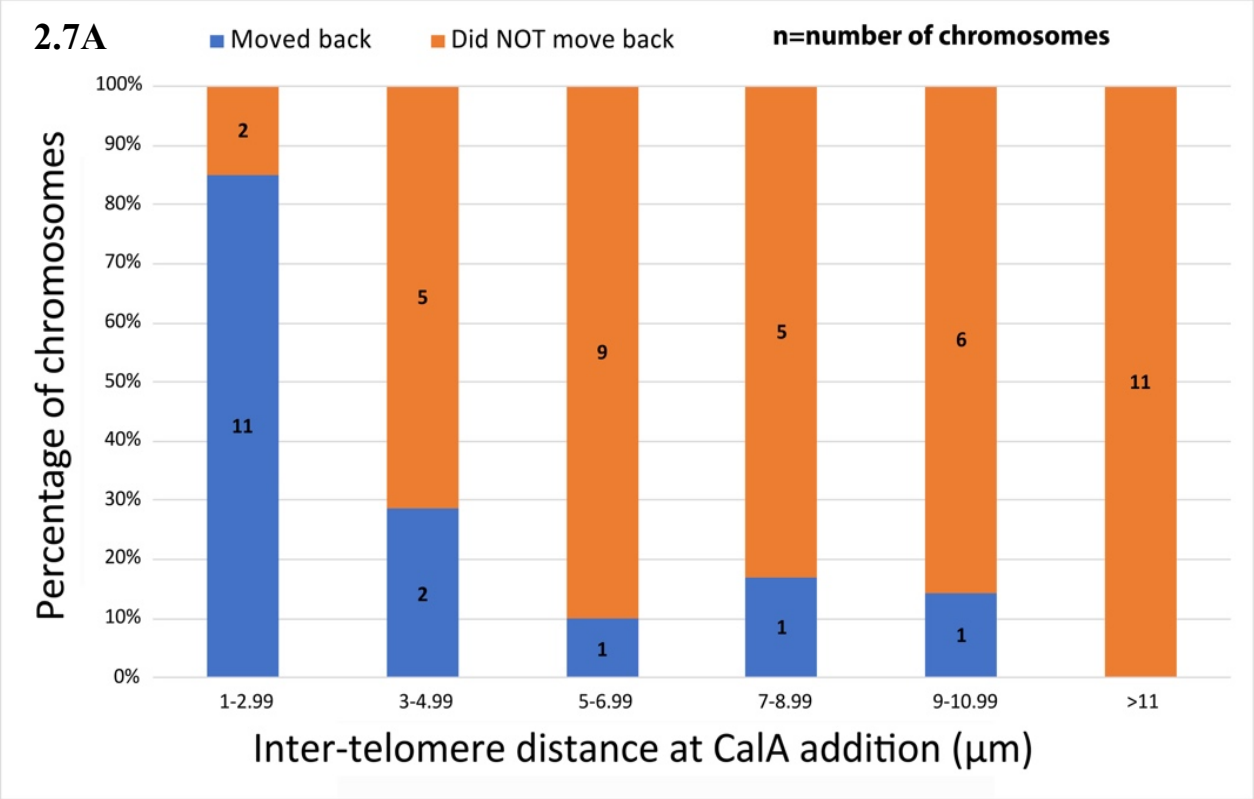


Figure 2.6. CalA caused first acceleration and then backwards movement of chromosomes. The chromosome graphed is from the cell illustrated in Figure 2.5. 50 nM CalA was added in early anaphase (as indicated on the graph) at 1min14s after anaphase onset, at an inter-telomere distance of 1.3 µm. Lines 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.

When half-bivalents of all three pairs of half-bivalents moved backwards, sometimes the partners met in the middle of the cell (Figure 2.5), 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. (2007a), sometimes moving around in the middle of the spindle or moving towards one pole or the other. Sometimes half bivalents of only one or two pairs moved backwards, 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 backwards) 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., supplemental video 1). These movements also were described by Fabian et al. (2007a). Finally, in our sample of 30 cells, and in others not reported on here, backwards movements sometimes, albeit rarely, occurred near mid-anaphase, before the chromosomes neared the poles.

CalA did not always cause backwards movements in our experiments, which seems to be attributed to CalA being added at different tether lengths. When CalA was added at short tether lengths (<2 μm), nearly all separating half-bivalents moved backwards after first reaching (or nearing) the poles. When CalA was added at increasingly longer tether lengths the number of half-bivalents that moved backwards decreased, and at tether lengths >11 μm no half-bivalents moved backwards. (Figure 2.7A). This conclusion was strengthened by adding to our sample of chromosomes from 30 cells (Figure 2.7A) those cells from the experiments of Fabian et al. (2007a) that we measured from the videos (Figure 2.7B), 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.



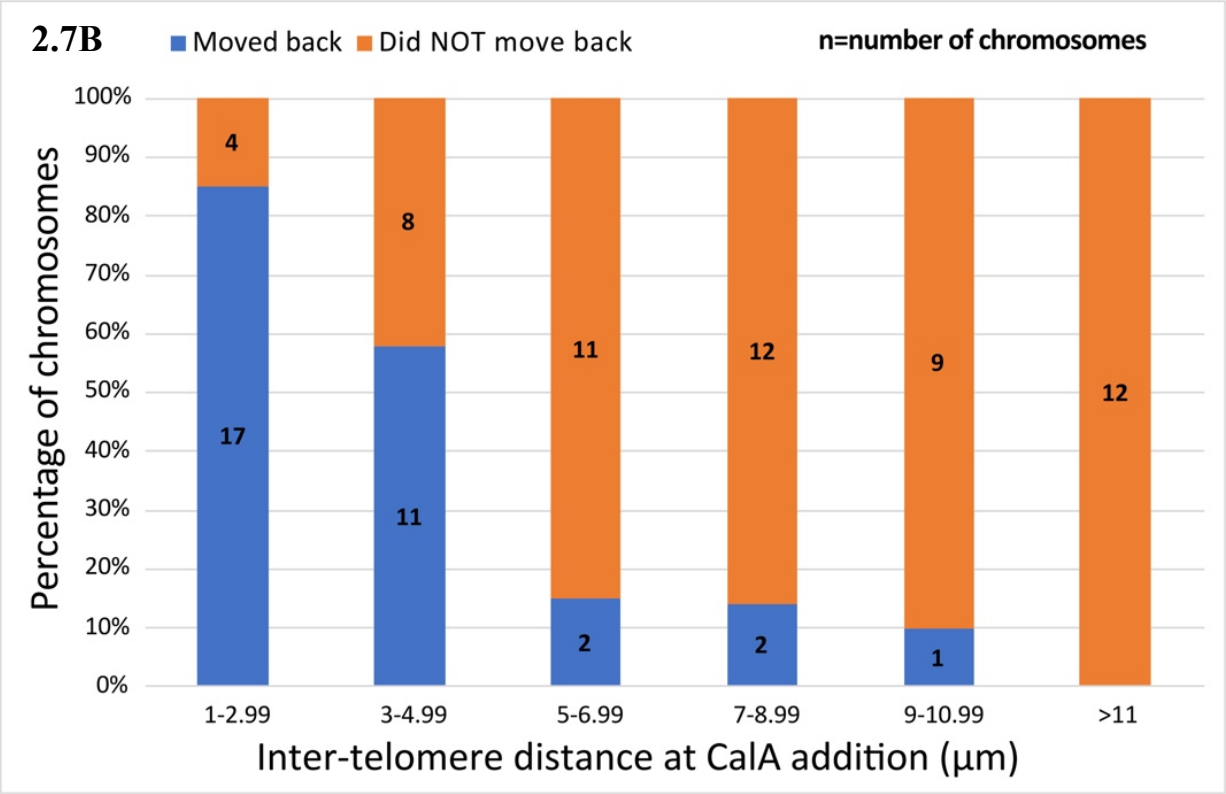


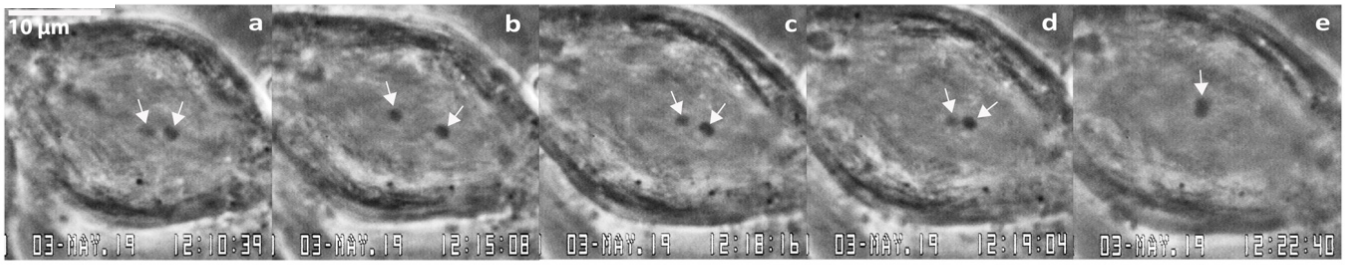
Figure 2.7A, 2.7B. **Effects of CalA added to crane-fly spermatocytes at various tether lengths**. Percentage of backwards movement of crane-fly spermatocyte chromosomes after 50nM CalA was added at various tether lengths. **Figure 2.7A** are data from our experiments. **Figure 2.7B** are the data from Figure 2.7A to which was added data from videos of Fabian et al (2007a) which were not analysed 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 backwards movement with regards to tether lengths at the time CalA was added. Those values were added to those of Figure 2.7A to obtain Figure 2.7B. The conclusions are the same, but with more robust numbers in Figure 2.7B.

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 2.6) independent of tether length when CalA was added and independent of whether or not the chromosomes subsequently moved backwards. 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. (2007a), but we measured the acceleration in a sample of 6 cells; in these cells chromosomes accelerated to a speed roughly twice the pre-CalA speed, about the same as reported by Fabian et al. (2007a).

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. (2007a), is illustrated in Figure 2.8A, and is clearly seen in supplemental video 2. After CalA addition the sex chromosomes moved at velocities of up to 2 μm/min (Figure 2.8B), 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 backwards. Thus, in addition to CalA causing half-bivalents at the pole to move backwards, other effects of CalA were poleward acceleration of half-bivalents, and rapid irregular excursions of the sex chromosomes along the spindle axis. The latter two effects always occurred, whether or not the half-bivalents moved backward.

2.8A



2.8B

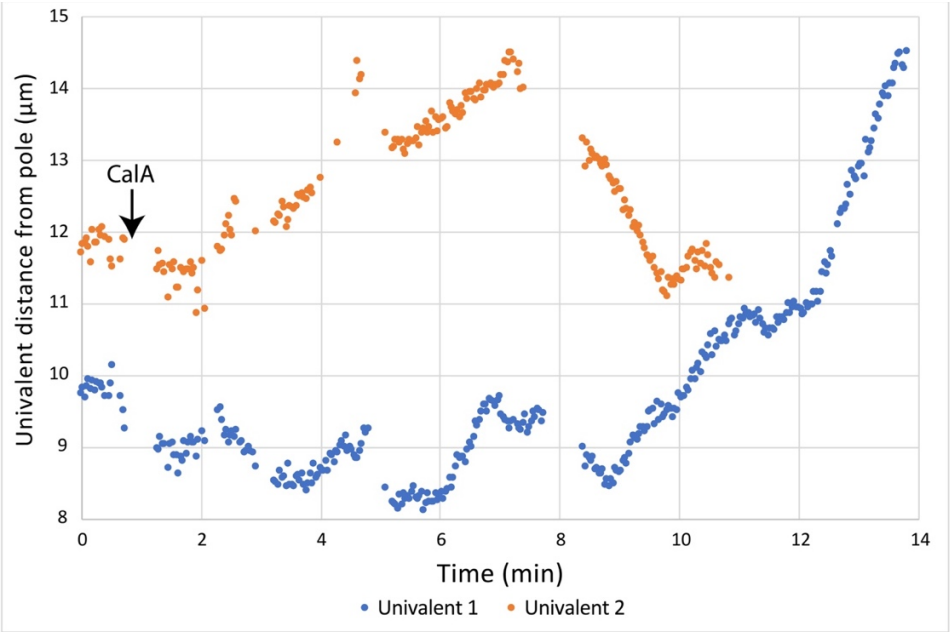


Figure 2.8A, 2.8B. Movement of sex chromosomes after CalA treatment. In cells treated with 50nM CalA the sex chromosomes (indicated by white arrows) move up and back along the spindle (Fig 2.8A). The movements of the pair seen in Figure 2.8A are shown graphically in Figure 2.8B. Sex chromosomes move at speeds of up to 2 µm/min after treatment with CalA. Arrow in (Fig 2.8B) indicates when CalA was added, about 46 seconds after anaphase onset.

***Okadaic Acid*:** We studied the effects of okadaic acid to distinguish between effects on PP1 versus on PP2A. When a low concentration (50 nM) of okadaic acid was added to 6 cells at short tether lengths (0.4-1.7 µm), there was no backwards movements. Fabian et al. (2007a) also saw no backwards movements after adding 20 or 50 nM 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 backwards movement in more than half the chromosome pairs (Figure 2.9). There were varying responses to high concentrations of okadaic acid in single cells – some half-bivalent pairs moved back whereas

others did not. Nonetheless, these data indicate that blocking PP1, not PP2A, causes the chromosomes to move backwards at the end of anaphase.

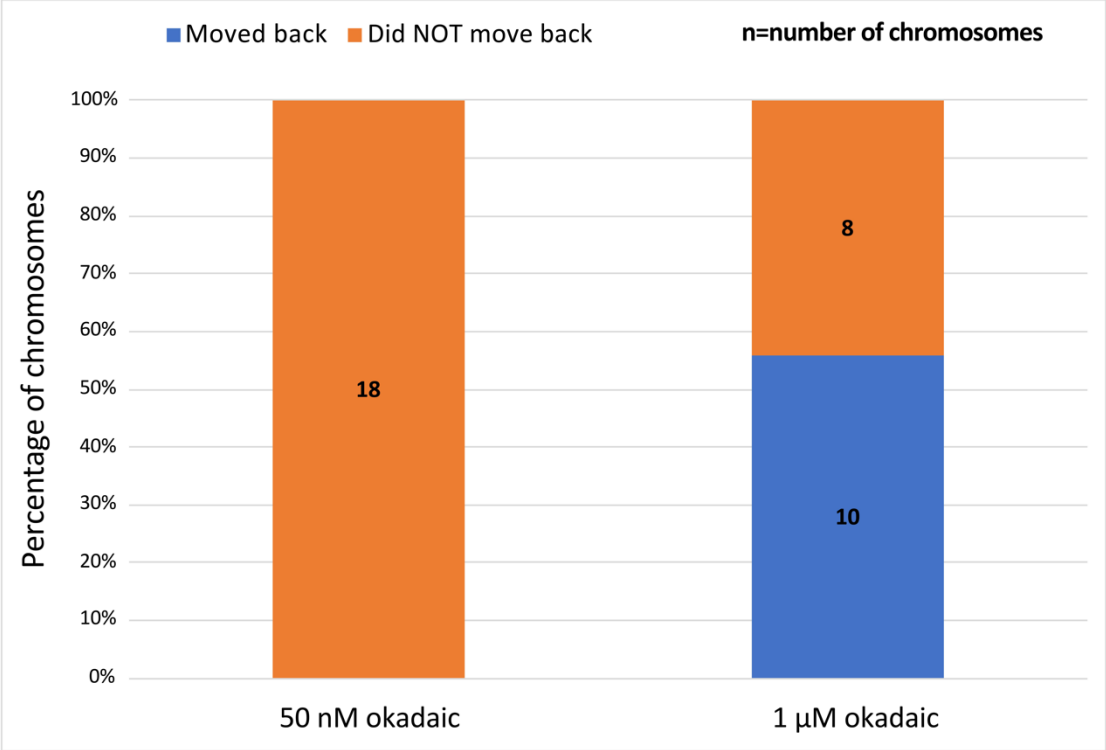


Figure 2.9. Effects of different concentrations of okadaic acid on backwards movement of chromosomes. In all cells okadaic acid was added when tether lengths were short (<2 µm).

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 half of the cells treated with 50 nM okadaic acid and in all of the cells treated with 1 µM okadaic acid.

Okadaic acid also often altered normal chromosome movements. After treatment with 50 nM 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 on backwards 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 2.7) when tethers are short and elastic, which we interpret to mean that elastic tethers are phosphorylated (Figure 2.3). 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 2.7). In reaching these conclusions we assume that the backwards (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 backwards movements of the half-bivalents are due to tethers, the backwards movements are led by the telomeres and are directed towards each other, as the kinetochores trail (Figure 2.5, 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 2.1) matches very well the backward movement of half-bivalents treated with CalA at various tether lengths (Figure 2.7) suggesting very strongly that tethers produce the backwards (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 2.3). 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. 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 (Trinkle-Mulcahy et al., 2003). Further data show that PP1 associates with both spindle microtubules and chromosomes (Andreassen et al., 1998). 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 (Paliulis and Forer, 2018). 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 (Fabian et al., 2007b). 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 backwards half-bivalent movements were not altered (Fabian et al., 2007a), indicating that the backwards movements are not due to actin or myosin. Having eliminated actin and myosin as producing the backwards force, Fabian et al. (2007a,b) 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 (Tskhovrebova and Trinick, 2003), 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, (Kötter et al., 2013; Hidalgo and Granzier, 2013; Krüger and Linke, 2006; Hamdani et al., 2017). Further, this phosphorylation is removed by addition of PP1 (Hidalgo et al., 2009). 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 2.1; also, LaFountain et al., 2002; Forer et al., 2017). 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 2.10). 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 50 nM, CalA causes separating half-bivalents to move backwards when added at short to intermediate tether lengths. At that concentration CalA greatly reduces the activity of both PP1 and PP2A (Figure 2.2). Okadaic acid, on the other hand, affects only PP2A at the low concentration of 50 nM, and at that concentration okadaic acid does not cause backwards movements. At a higher concentration, 1 μM, okadaic acid inhibits PP1 as well as PP2A (Figure 2.2) and causes backwards movements. Thus, the backwards 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 (Ishihara et al., 1989; Swingle et al., 2007; Honkanen et al., 1994), 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/threonine protein phosphatases, however (Honkanen and Golden, 2002), PP1 through PP7; could any of these be involved in the backwards movements of half-bivalents that we observed? Honkanen and Golden (2002) and Swingle et al. (2007) 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 backwards 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 does not cause backwards movements of half-bivalents. Thus, our data point to PP1 is the phosphatase that causes tethers to become inelastic.

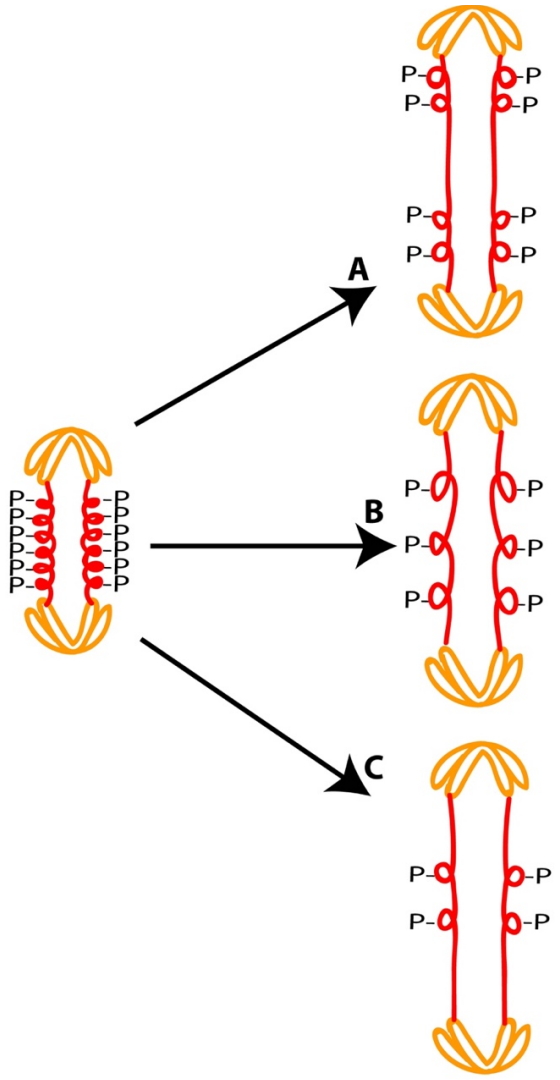


Figure 2.10. **Possible patterns of dephosphorylation of tethers**. Tethers may be dephosphorylated starting at the center and moving outwards, leaving the ends elastic for a longer time than the middle (A); they may be dephosphorylated evenly along the tether (B); or they may be dephosphorylated from the ends and moving towards the middle, leaving the middle elastic for a longer time than the ends (C).

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 (Sheykhani et al., 2017). 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 backwards; the half-bivalents that did not move backwards 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 backwards movements of half-bivalents after they reached the poles, the okadaic acid seems less potent in inducing backwards movement than is CalA (Figure 2.7 vs. Figure 2.9), CalA causing backwards 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 2.2, indicates that 1 μM okadaic acid inhibits only 60% of PP1 activity, whereas 50 nM CalA inhibits over 90% of PP1 activity. It may be that there is enough PP1 activity remaining to sometimes cause tethers to become inelastic. Perhaps if one used higher concentration of okadaic acid the effects on backwards 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 2.6), and caused irregular, rapid movements of sex chromosomes (Figure 2.8). The effects on chromosome speed and sex chromosome movements occurred in all cells treated with CalA, independent of effects on backwards movements. Even in cells where CalA was added in late anaphase and the chromosomes did not undergo backwards movement the autosomes accelerated towards the poles and the sex chromosomes took rapid excursions up and back along the spindle. The effects of *okadaic acid* were somewhat different. Neither 50 nM 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 50 nM 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 backwards movement in late anaphase of separated crane-fly half-bivalents. There is less and less backwards 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 backwards 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 they become inelastic when dephosphorylated.

Chapter Three

Exploring the timing and method of the phosphorylation of the elastic tethers in crane-fly spermatocytes

SUMMARY

The tethers that connect separating anaphase chromosomes are elastic, and their elasticity decreases as the partner chromosome move apart in anaphase. This decrease in elasticity is due to tether dephosphorylation. Further, the loss of elasticity can be prevented by inhibiting activity of protein phosphatase 1 (PP1) with Calyculin A (CalA) resulting in backwards movements of the chromosomes after they reached (or neared) the spindle poles. However, it is still untested at what point in division tether phosphorylation occurs, as well as which kinases are involved. The following experiments test the hypothesis that tethers are phosphorylated prior to anaphase and test whether two kinases that are known to phosphorylate the elastic regions of the giant protein titin are responsible or tether phosphorylation. I inhibited or activated protein kinases C and A in metaphase and anaphase, followed by the addition of CalA in early anaphase. If there is no backwards movement of chromosomes in these cells, tether phosphorylation was successfully blocked. Further, treating cells with a broad-spectrum kinase inhibitor in metaphase or in anaphase followed by CalA in early anaphase can indicate whether tether phosphorylation occurs prior to anaphase or if the tethers become phosphorylated as they extend in anaphase. The preliminary results suggest that tethers begin anaphase fully phosphorylated. Further, no significant change in backwards movement was observed in cells treated with PKC and PKA inhibitors or activators, suggesting that it is unlikely that either of these kinases are solely responsible for tether phosphorylation. All results are preliminary and lay the groundwork for future experimentation.

INTRODUCTION

Elastic tethers that connect the telomeres of anaphase chromosomes were first described in crane-fly spermatocytes by La Fountain et al. (2002) and have been identified in a wide variety of species including other insects, arachnids, marsupials, and humans (Forer et al., 2017). La Fountain et al. (2002) found that when the telomere-containing end of a half-bivalent arm was cut from the rest of the half-bivalent the resulting fragment moved backwards towards the partner half-bivalent instead of continuing towards the pole. However, the movements changed as anaphase progressed; when the fragment was severed from the rest of the half-bivalent in mid or late anaphase, the fragment moved back a smaller distance or not at all. Similar results were found in PtK cells (Forer et al., 2017).

The phosphatase inhibitor Calyculin A (CalA) preserves tether elasticity throughout anaphase when it is added to the cell in early anaphase (Kite and Forer, 2020). After CalA is added to a cell in early anaphase the chromosomes move back to their partners after moving to the poles (Fabian et al., 2007a; Kite and Forer, 2020). To test whether this is because blocking phosphatases blocks tether dephosphorylation, thereby preserving their elasticity, I added CalA to cells when the tethers were of different lengths. I found that as anaphase progressed, and the tether lengths increased, the chromosomes underwent less and less backwards movement (Figure 2.1). This suggests that at longer tether lengths the tethers had been dephosphorylated to a point that preventing dephosphorylation no longer preserved any elasticity and anaphase chromosomes did not move backward (Kite and Forer, 2020).

CalA inhibits dephosphorylation through inhibition of protein phosphatases (PP) 1 and 2A. I tested whether the effects on tethers were due to inhibition of PP1 or PP2A by using a second inhibitor. Okadaic acid inhibits both phosphatases at high concentrations but inhibits only PP2A at low concentrations (Favre et al., 1997). When early anaphase cells were treated with 50 nM okadaic acid, there was no backwards movement of the chromosomes (Fabian et al., 2007a; Kite and Forer, 2020), but there was backwards movement when they were treated with 1μM okadaic acid (Kite and Forer, 2020). This suggests that inhibition of PP1 is necessary to prevent tether dephosphorylation and preserve elasticity.

The experiments detailed above give us a better idea of how and when tether elasticity declines in anaphase, but we do not yet know how and when it is established. When CalA is added to a cell in early anaphase it prevents dephosphorylation from occurring, and the tethers remain elastic and phosphorylated. This indicates that tethers likely begin anaphase fully phosphorylated. Therefore, kinases are likely involved at some point in metaphase before the chromosomes begin to separate.

STUDY PLAN

The goal of the experiments discussed in this chapter is to test the hypothesis that tethers are phosphorylated prior to anaphase and to test whether two kinases that are known to phosphorylate the giant protein titin are responsible or tether phosphorylation. To study the effects of kinases on tether elasticity I added inhibited a kinase inhibitor in metaphase followed by the addition of CalA in early anaphase at tether lengths between 1 and 2.99 μm. When CalA

was added at these tether lengths in my previous experiments, 81% of chromosomes move backward (Figure 2.1, Kite and Forer, 2020). If tether phosphorylation was successfully inhibited, then treatment with CalA would not result in backwards movement of the chromosomes because there would not be any phosphorylation (elasticity) to preserve. I also enhanced the activity of one kinase in metaphase and mid-anaphase followed by treatment with CalA in mid-anaphase. If the kinase that was enhanced is the kinase that phosphorylates tethers, then they might retain their elasticity for longer into anaphase than normal. If the tethers stay elastic longer then there would be more backwards movement in mid to late anaphase. To study the time frame in which tethers are phosphorylated I treated cells with a broad-spectrum kinase inhibitor in early anaphase concurrent with CalA. If blocking tether phosphorylation in anaphase results in reduced backwards movement this would suggest that the tether phosphorylation occurs at least partly in anaphase rather than occurring entirely prior to anaphase.

For these experiments I altered general kinase activity in crane-fly spermatocytes with a broad-spectrum inhibitor, and I altered the activity of two specific kinases that are involved in moderating the elasticity of the giant protein titin. The composition of tethers is unknown, but it has been suggested that titin may be a component of tethers (Fabian et al., 2007b) because titin is found between separating anaphase chromosomes and is a well-known source of elasticity in muscle and heart cells. The first kinase is protein kinase C (PKC), which is involved in phosphorylation of the PEVK region of titin (Hidalgo et al., 2009). The PEVK region of titin, a region that is rich in proline, glutamate, valine, and lysine, seems to act as a spring when titin is stretched, and it unravels as stretch is applied to the molecule (Tskhovrebova et al., 1997). The second kinase is protein kinase A (PKA), which is involved in phosphorylation of the N2Bus region of a cardiac isoform of titin (Krüger et al., 2009). The N2Bus region of titin is also extensible (Trombitás et al., 1999). These two regions of titin behave differently when they are phosphorylated. Phosphorylation of N2Bus decreases titin stiffness whereas phosphorylation of PEVK increases titin stiffness (Kötter et al., 2013).

Drug Treatments

To study the effect of these kinases on tether phosphorylation I used various kinase inhibitors as well as one kinase activator. To examine the role of PKC on tethers I treated cells with a PKC inhibitor and a PKC activator. Bisindolylmaleimide IX (BIM IX) was used to **inhibit**

the kinase and phorbol 12-myristate 13-acetate (PMA) was used to **activate** it. BIM IX is a potent inhibitor of many isoforms of PKC, though does show a slight preference for PKCα (Wilkinson et al., 1993) which is the isoform that phosphorylates the PEVK region of titin (Hidalgo et al., 2009). This inhibitor is cell-permeable and has been used to reduce PKC activity in *Drosophila* both *in vivo* and *in vitro* by feeding the flies food supplemented with the drug or by incubating cells in medium enhanced with the drug (Shim et al., 2019). To enhance PKC activity, I treated cells with the phorbol ester PMA, which activates several PKC isoforms (Blumberg, 1988). PMA is cell permeable and after being applied to dissected *Drosophila* larvae a significant increase in a protein directly downstream of the target protein was observed (Aravamudan and Broadie, 2003).

The next kinase I studied was PKA. I inhibited this kinase through cell treatment with H89, a cell-permeable and potent PKA inhibitor. However, it is not selective and can also inhibit a handful of additional kinases (Davies et al., 2000). Despite this, H89 is commonly used as a disruptor of PKA activity. For example, after being bathed in a solution containing H89, signals dependent on PKA activity were reduced in *Drosophila* larvae, suggesting successful inhibition of PKA by H89 (Bhattacharya et al., 1999).

The final inhibitor I used was staurosporine. Staurosporine is a broad-spectrum inhibitor though it is also a potent inhibitor of PKC (Tamaoki et al., 1986; Rüegg and Gillian, 1989). This inhibitor is cell-permeable and affects phosphorylation in crane flies when perfused across anaphase spermatocytes (Sheykhani et al., 2013). Although staurosporine is the only drug used in my experiments that is known to alter phosphorylation in crane-fly spermatocytes (Sheykhani et al., 2013), the studies described above report effects in *Drosophila* of the other inhibitors and activators, which is generally a reliable guide for crane flies.

MATERIALS AND METHODS

Preparations of living cells were made as previously described (Kite and Forer, 2020). The cells were studied using phase-contrast microscopy. Cells in the proper stage of division were found in the prep and videos were recorded on DVDs in real-time using a 100x, 1.4 NA phase-contrast oil immersion lens. During metaphase the preps were perfused with insect Ringer's solution containing BIM IX (100 nM, 200 nM, or 1 μM), containing 200 nM H89, containing a combination of 100 nM BIM IX with 200 nM H89, containing staurosporine (1 μM

or 10 μM), or containing PMA (200 nM). Then, at the very start of anaphase, the prep was perfused again with insect Ringer's containing 50 nM CalA and the drug that was previously perfused across the cell in metaphase. In one set of experiments the prep was perfused with insect Ringer's solution containing both 1 μM staurosporine and 50 nM CalA in early anaphase. In another set of experiments the prep was perfused with insect Ringer's solution containing 200 nM PMA and 50 nM CalA in mid-anaphase.

Time-lapse movies were made from the DVD recordings using freeware VirtualDub2. Single frames of chromosome movements were analyzed for movement patterns and inter-telomere distances using an in-house program, Winimage (Wong and Forer, 2003).

RESULTS

Single kinase inhibition

I first tested the effect of inhibiting PKC in four metaphase cells using 100 nM to 1 μM BIM IX (IC_{50}=23 nM; Wilkinson et al., 1993). 50 nM CalA (with BIM IX) was added to all cells at the start of anaphase. If tether elasticity is due to PKC activity, then by inhibiting PKC we would expect no backwards movement even after CalA is added. However, I found that at all concentrations all three chromosomes in every cell underwent backwards movement (Table 3.1). In three cells treated with 100 nM-200 nM BIM IX in metaphase, division continued normally until the chromosomes reached the poles at which point they moved backwards towards their partner chromosome. I treated one cell with 1 μM BIM IX to see if a higher concentration would prevent backwards movement. This cell, like the other three, had backwards movement of all chromosomes. Experiments by A. Forer (2020, unpublished) supported these results, showing that neither BIM IX nor a second PKC inhibitor, Gö 6983, prevented any backwards movements.

I then tested the effects of inhibiting PKA in three metaphase cells using 200 nM H89 (IC_{50}=140 nM; Davies et al., 2000). 50 nM CalA (with H89) was added to all cells at the start of anaphase. In all cells all chromosomes moved back after they separated (Table 3.1). Thus, neither inhibitors of PKC nor PKA inhibited the backwards movements caused by CalA.

In addition to causing backwards movements of chromosomes, CalA has two other characteristic effects on cells. The first is that it causes the sex chromosomes to move along the spindle axis as the autosomal chromosomes divide rather than sitting at the midline of the cell (Fabian et al., 2007a; Kite and Forer, 2020). In all cells treated with H89 and BIM IX I saw the

sex chromosomes moving after addition of CalA. This suggests that neither PKA nor PKC play a role in moderating the sex chromosome excursions along the spindle pole. The second effect of CalA is that it causes the separating partner chromosomes to accelerate towards the poles at speeds nearly twice their normal rate (Fabian et al., 2007a; Kite and Forer, 2020). I did not see this result in any cells treated with H89 or BIM IX. Rather, some chromosomes appeared to slightly slow their speeds. These results are more characteristic of cells in which PP2A is inhibited through treatment with 50 nM okadaic acid (Kite and Forer, 2020).

Table 3.1. Breakdown of the single kinase inhibition experiments. The second column shows length of time the cell was in the kinase inhibitor prior to the very start of anaphase when CalA was added. The third column indicates whether or not all chromosomes in the cell underwent backwards movement.

Cell	Drug and concentration	Time in drug before anaphase	Backwards movement?
1	**100 nM BIM IX**	**17 min**	**Yes**
2	**100 nM BIM IX**	**50 min**	**Yes**
3	**200 nM BIM IX**	**43 min**	**Yes**
4	**1 µM BIM IX**	**14 min**	**Yes**
5	**200 nM H89**	**27 min**	**Yes**
6	**200 nM H89**	**24 min**	**Yes**
7	**200 nM H89**	**51 min**	**Yes**

Multiple kinase inhibition

The results of the previous experiments suggested that neither inhibiting PKC nor inhibiting PKA blocks phosphorylation of tethers in metaphase. However, there may be redundant pathways such that one pathway might compensate for the other if one is blocked. So, to test if there is such a redundancy with PKC and PKA, I added both 100 nM BIM IX and 200 nM H89 to two cells during metaphase. 50 nM CalA (with BIM IX and H89) was added to both cells in early anaphase. In each case all chromosomes moved backwards after reaching the poles (Table 3.2, Cells 1 and 2). Neither PKC nor PKA alone seem to be involved, and if there is a redundancy, there may be more kinases at work.

I then used the broad-spectrum kinase inhibitor, staurosporine (IC_{50}=3 nM; Tamaoki et al., 1986) at either 1 μM or 10 μM in metaphase. 10 μM staurosporine has known effects in crane-fly spermatocytes, albeit with sometimes slowed or stopped chromosome movement in anaphase (Sheykhani et al., 2013). Given this, I first used staurosporine in μM concentrations for my experiments to test whether we could prevent tether phosphorylation in metaphase. I treated one cell with 1 μM staurosporine in metaphase as a control (no CalA) and it successfully entered anaphase and the chromosomes moved to the poles. 50 nM CalA (with staurosporine) was added to all other cells at the start of anaphase. I treated two cells with 1 μM staurosporine and the chromosomes moved only a short distance apart and stopped moving towards the poles altogether, remaining "frozen" in their early anaphase position. The chromosomes then clumped together and moved as a group to one pole (Table 3.2). Next, I treated three cells with 10 μM staurosporine. The chromosomes in two of these cells "froze" and in one cell the chromosomes separated but did not move backward. Because cells completed anaphase normally in the staurosporine control cell it may be that staurosporine in metaphase followed by CalA in anaphase blocks proper chromosome movement in anaphase. However, it may also be that there were not enough controls performed to establish the full effects of adding staurosporine to metaphase cells, and that these results are due to the effects of staurosporine alone.

Next, to test if tether phosphorylation occurs in anaphase, I perfused three early anaphase cells with 1 μM staurosporine together with 50 nM CalA (Table 3.2, Cells 8-10). If tether phosphorylation occurs in anaphase and staurosporine blocks that phosphorylation, then the chromosomes won't move back even after CalA is added in early anaphase. However, there was backwards movement of all chromosomes after reaching the poles in two of the three cells. These are preliminary results; however, it seems that tethers are fully phosphorylated at the start of anaphase.

Table 3.2. Breakdown of the multiple kinase inhibition experiments. The second column shows length of time the cell was in the kinase inhibitor prior to the very start of anaphase when CalA was added. The third column indicates whether or not the cell underwent backwards movement.

Cell	Drug and concentration	Time in drug before anaphase	Backwards movement?
1	**100 nM BIM IX and 200 nM H89**	**36 min**	**Yes**
2	**100 nM BIM IX and 200 nM H89**	**77 min**	**Yes**
3	**1 µM staurosporine**	**13 min**	**N/A – didn't separate**
4	**1 µM staurosporine**	**10 min**	**N/A – didn't separate**
5	**10 µM staurosporine**	**6 min**	**N/A – didn't separate**
6	**10 µM staurosporine**	**8 min**	**N/A – didn't separate**
7	**10 µM staurosporine**	**8 min**	**No**
8	**1 µM staurosporine in ana**	**0 min**	**Yes**
9	**1 µM staurosporine in ana**	**0 min**	**Yes**
10	**1 µM staurosporine in ana**	**0 min**	**No**

Kinase activation

For this set of experiments, I tried the opposite approach: stimulating phosphorylation rather than inhibiting it. To do so, I used a PKC activator, the phorbol ester PMA (EC_{50}=11.7 nM; Xu et al., 2003) (Table 3.3). I added 200 nM PMA in either metaphase or mid-anaphase to five cells. 50 nM CalA (with PMA) was added to all cells in mid-anaphase. When cells are treated with CalA in mid-anaphase there is ~33% backwards movement of the chromosomes (Kite and Forer, 2020). By adding PMA in mid-anaphase, I can test whether it leads to greater rates of backwards movement than cells treated with CalA alone. I first added 200 nM PMA to three metaphase cells followed by 50 nM CalA in mid-anaphase. In one cell all chromosome pairs moved back after reaching the poles. The other two cells underwent no backwards movement (Table 3.3, Cells 1-3).

Adding PMA in metaphase might have no effect if the PP1 concentration in the cell is sufficient to remove the extra phosphoryl groups before CalA is added and prevents dephosphorylation. So, I added 200 nM PMA with 50 nM CalA at mid-anaphase to two cells

(Table 3.3, Cell 4 and 5). This allows PMA to act without having to fight activity by PP1. In these two cells one pair of chromosomes moved backwards, but no other chromosomes did.

These experiments tested the hypothesis that PKC is responsible for tether phosphorylation. If PKC is responsible for phosphorylating tethers, then adding PMA and enhancing PKC activity would enhance tether elasticity resulting in greater rates of backwards movements of chromosomes at longer tether lengths. However, the preliminary results indicate the opposite effect at tether lengths of 3-4.99 μm where I completed the greatest number of experimental trials (Figure 3.1). In contrast, when CalA was added at tether lengths between 5 and 6.99 μm two thirds of the chromosomes did move back, which looks like enhanced backwards movement. However, future experimentation with greater numbers of cells is needed to determine if these results are significantly different from control cells in which CalA is added by itself at these tether lengths.

Table 3.3. Breakdown of the kinase activation experiments including the length of time the cell was in PMA before the CalA (with PMA) was added, the tether length at time of CalA addition, and the number of chromosomes that underwent backwards movement. N/A indicates tether lengths that could not be measured at CalA addition due to focal plane differences.
* indicates which chromosome pair moved back.

Cell	Time in PMA before CalA	Tether length at CalA addition (measured from visible chromosome pairs)	Backwards movement?
1	**76 min**	**6.5 μm*, 5 μm*, N/A***	**(3/3)**
2	**22 min**	**3.6 μm, 3 μm, N/A**	**(0/3)**
3	**38 min**	**4.8 μm, N/A, N/A**	**(0/3)**
4	**0 min**	**5.3 μm, 8.5 μm, N/A***	**(1/3)**
5	**0 min**	**4.1 μm, 4.5 μm, N/A**	**(0/3)**

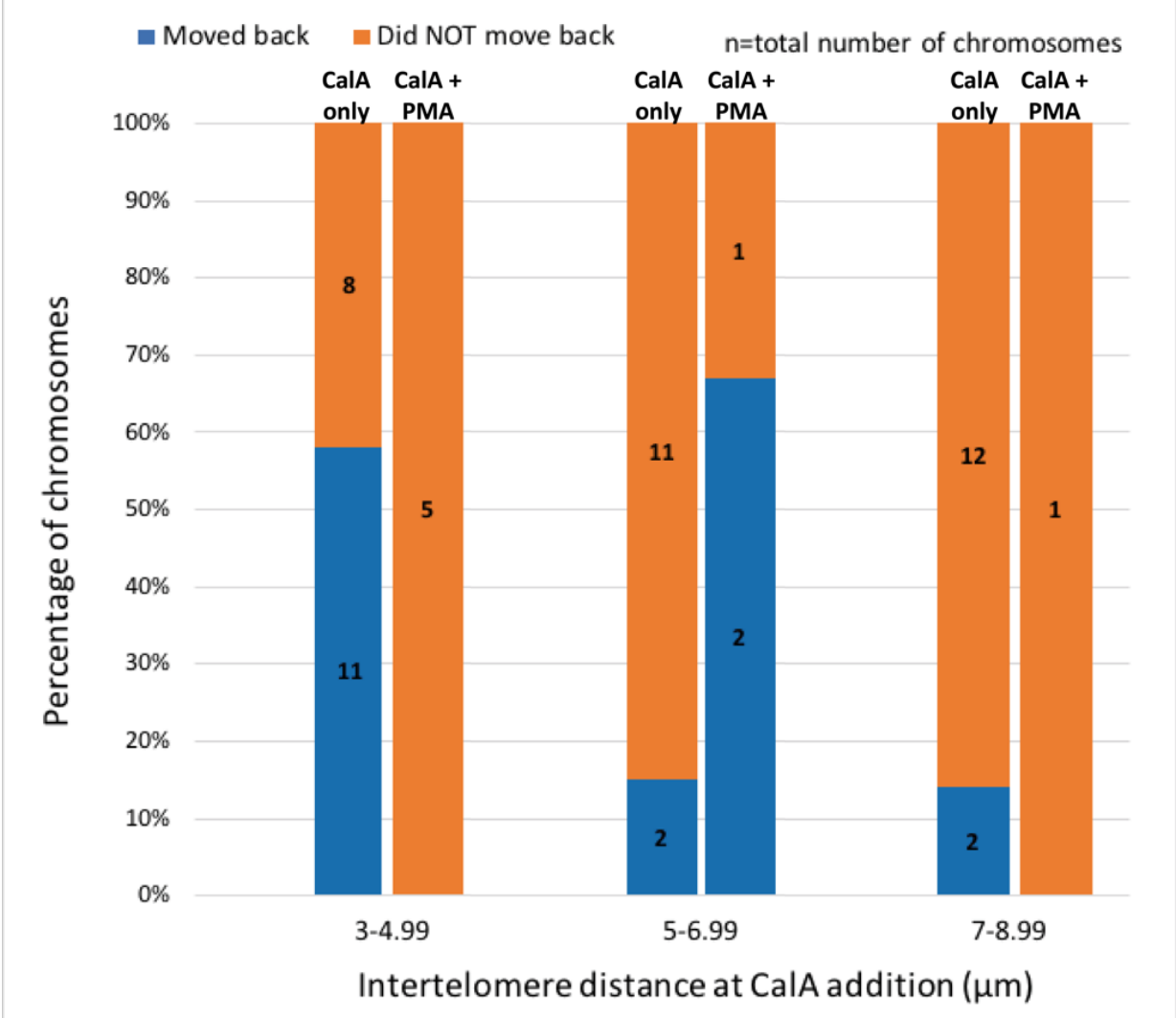


Figure 3.1. Data adapted from Kite and Forer, 2020. The first column at each intertelomere distance shows the percentage of chromosomes that move back after treatment with CalA at tether lengths spanning 3 µm to 8.99 µm. The second column labeled "PMA" shows the percentage of chromosomes that moved back after treatment in metaphase or mid-anaphase with 200 nM PMA and treatment with 50 nM CalA at tether lengths from 3 µm to 8.99 µm. All chromosomes whose tether lengths (intertelomere distances) could be measured at the time of CalA addition are included (Table 3.3)

DISCUSSION

All the drugs I used in these experiments are cell permeable and have effects in *Drosophila* (Shim et al., 2019; Aravamudan and Broadie, 2003; Bhattacharya et al., 1999), but only staurosporine has a known effect on phosphorylation in crane-fly spermatocytes (Sheykhani et al., 2013). Therefore, it is important to note that seeing backwards movement after treatment with a kinase inhibitor in metaphase followed by CalA in anaphase does not necessarily mean that the kinase inhibitor didn't inhibit the kinase acting on tethers. It may also mean that the

inhibitor wasn't able to get into the cell properly or that it didn't have the expected effect. All interpretations of the data must be made with that in mind.

These experiments tested the hypothesis that tethers are phosphorylated by either PKC or PKA, two kinases that phosphorylate the elastic regions of the giant protein titin, and that tether phosphorylation occurs prior to anaphase. The experiments were cut short due to lab closures in response to COVID-19, and there were not enough data points to come to any definitive conclusions, however, these initial experiments do point to preliminary conclusions regarding tether phosphorylation and may set the stage for future experimentation on this topic. The first preliminary conclusion is that it seems unlikely that PKC or PKA are responsible for tether phosphorylation. In each cell that was treated in metaphase with a PKC inhibitor, PKA inhibitor, or both inhibitors, the separated chromosome pairs moved back after CalA was added in early anaphase indicating that the tethers were phosphorylated in the presence of the inhibitor. Assuming the drug was able to enter the cell, if either BIM IX or H89 had blocked tether phosphorylation, then the tethers would not be phosphorylated and elastic when the CalA was added and the chromosomes would not move backwards. Tether elasticity cannot be maintained with CalA if they are not phosphorylated when it is added. If either PKC or PKA was involved in tether phosphorylation, then the maintained backwards movement of chromosomes after the inhibitor was added could have been due to compensation through redundant phosphorylation pathways. Redundancy in phosphorylation pathways has been shown with myosin phosphorylation in anaphase crane-fly spermatocytes (Sheykhani et al., 2013). However, it seems that PKC and PKA, if involved in tether phosphorylation, do not act as redundant pathways for each other as the chromosomes still moved backwards after the kinases were inhibited simultaneously.

Further indication that PKC is not responsible for tether phosphorylation comes from experiments with PMA. When PKC activity was enhanced with PMA treatment in metaphase followed by CalA treatment in mid-anaphase, one out of three cells had all three chromosomes move back. When PMA was added in mid-anaphase with CalA to two cells, there was one chromosome pair that moved back. If PKC was responsible for tether phosphorylation, then by activating it and stimulating phosphorylation we would expect to see more backwards movement of chromosomes at longer tether lengths. As it stands, however, the current data do not point to increased instances of backwards movement after treatment with PMA (Figure 3.2). There are

several possible interpretations of these preliminary data. It may be that the PMA was not in the cell for a long enough time to have an effect – there was backwards movement of all three chromosomes in the cell that was in PMA for the greatest amount of time. Or perhaps PMA may be having no effect because PKC is not the kinase acting on tethers. No conclusions can yet made. A possible next step would be to add PMA and CalA at tether lengths greater than 11 µm, when there would not typically be backwards movement of chromosomes in cells treated with CalA alone. Then, if there was any backwards movement at all, it would be highly suggestive of the involvement of PKC in tether phosphorylation.

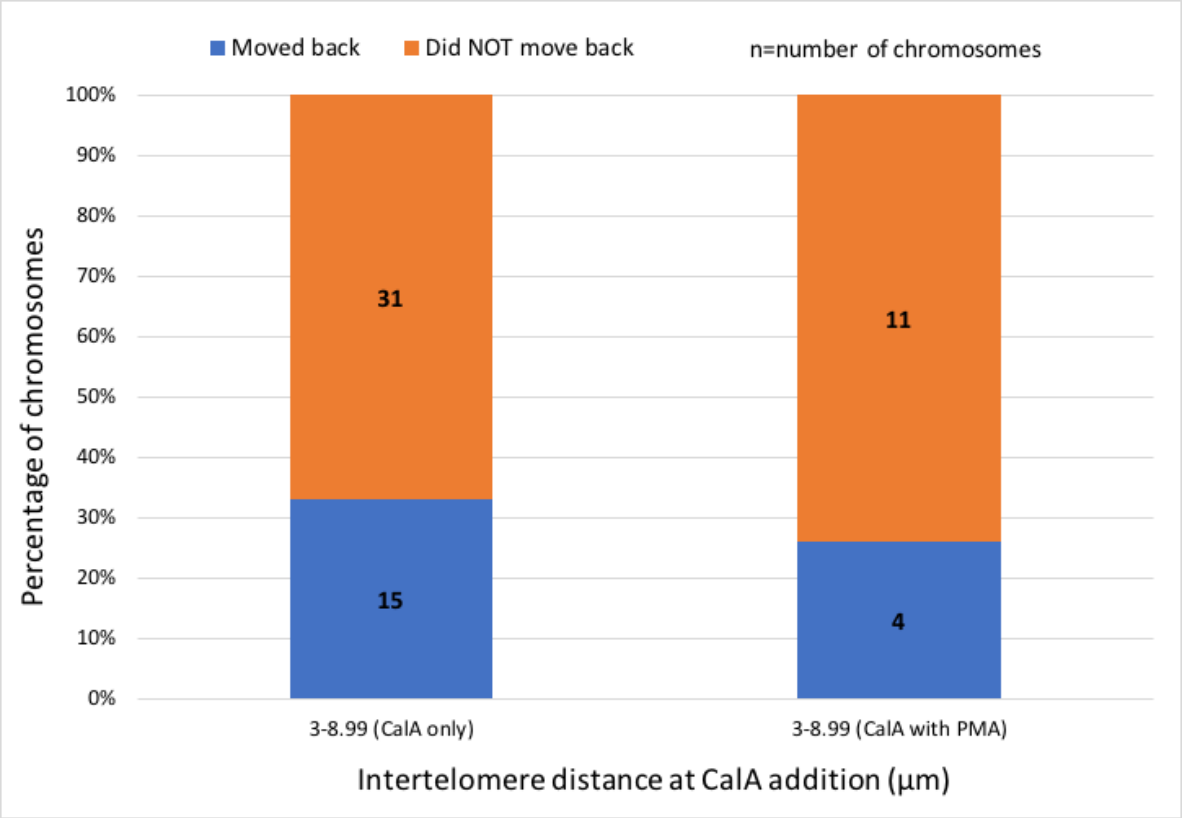


Figure 3.2. The percentage of backwards movement of chromosomes in cells treated with 50 nM CalA only (left column) and in cells treated with 200 nM PMA and 50 nM CalA (right column) at tether lengths (intertelomere distances) spanning 3-8.99 µm. All chromosomes from Table 3.3 are included, even if their intertelomere distance was not directly measured. The tether lengths here span nearly 6 µm, which likely encompasses the tether lengths of all chromosomes in the cells when CalA was added.

These experiments also aimed to identify the time frame in which tethers become phosphorylated. Cells that are treated with CalA alone in early anaphase often undergo

backwards movement and I interpreted this as indicating that tethers begin anaphase phosphorylated. However, that interpretation had not yet been specifically tested by my experiments. I found that when cells were treated with CalA and the broad-spectrum kinase inhibitor staurosporine in early anaphase, most chromosomes moved back. These results seem to support the interpretation that tethers begin anaphase phosphorylated. Adding a combination of a kinase inhibitor and a phosphatase inhibitor will "freeze" the phosphorylation state of the tethers at the time the drugs are added. If the phosphorylation of tethers at anaphase onset is sufficient for backwards movement of the chromosomes, then adding staurosporine will not inhibit it. Because chromosomes still moved back after this combination of drugs was added in early anaphase, it seems that tether phosphorylation occurs prior to anaphase.

To further test this, I added staurosporine in metaphase, presumably blocking tether phosphorylation. However, this resulted in abnormal cells which could not complete anaphase. Staurosporine is a broad-spectrum inhibitor and therefore likely blocked activity of kinases that play a role in facilitating the transition from metaphase into anaphase, so it is not entirely unexpected that the chromosomes could not complete anaphase. In the one cell that did continue successfully into anaphase the chromosomes did not move back. This is the expected result following broad-spectrum kinase inhibition because blocking all kinases will also block the kinase that phosphorylates the tethers, thereby preventing them from becoming elastic.

In conclusion, these data seem to suggest that neither PKC nor PKA are individually responsible for tether phosphorylation. Further experiments with PMA may provide a more conclusive answer as to whether PKC is involved in tether phosphorylation. The experiments with staurosporine and CalA added together in early anaphase point towards tethers starting anaphase phosphorylated, which suggests that metaphase is likely the crucial time for phosphorylation. To further test this hypothesis and to identify the kinase involved, a different method of study is required. A potentially useful direction could be to use gene editing technologies with which individual titin genes could be knocked out and individual amino acids could be replaced with a non-phosphorylatable version. An alternate possibility, more amenable to our lab's current capabilities, would be to test titin phosphorylation directly via immunostaining.

Chapter Four

General Discussion

The experiments discussed in this thesis deal with the elasticity of the tethers that connect separating anaphase chromosomes in crane-fly spermatocytes, and the role that phosphorylation and dephosphorylation play in regulating that elasticity. Earlier studies of tethers indicated that tethers are elastic in early anaphase and that they become less elastic as they elongate over the course of anaphase (LaFountain et al., 2002; Sheykhani et al., 2017; Forer et al., 2017). Additionally, there was evidence that tether elasticity may be moderated by phosphorylation, after experiments in which the partner chromosomes in anaphase crane-fly spermatocytes sometimes underwent backwards (anti-polar) movements after being treated with the phosphatase inhibitor, CalA (Fabian et al., 2007a).

My first set of experiments, discussed in Chapter Two, tested the role of phosphorylation in tether elasticity by using inhibitors that target phosphatases PP1 and PP2A together, or by inhibiting PP2A alone, at various tether lengths (Kite and Forer, 2020). I did this by treating crane-fly spermatocytes with either CalA, which effects both PP1 and PP2A at the test concentration of 50 nM, or by treating cells with two different concentrations of okadaic acid. Okadaic acid affects only PP2A at low concentrations but affects both PP1 and PP2A at high concentrations (Favre et al., 1997; Figure 2.2). The curves plotted in Figure 2.2 were from experiments using vertebrate cells, and my experiments did not test activity of PP1 or PP2A in the cell directly. However, previous experiments have confirmed that CalA has the expected effects in crane-fly spermatocytes. This was demonstrated by measuring levels of phosphorylated myosin both before and after treatment with CalA (Sheykhani et al., 2013). Further, the effects of okadaic acid on PP2A and PP1 activity have been tested directly in *Drosophila*, which is a reliable guide for crane-fly spermatocytes (Lu et al., 2009; Fang et al., 2007). The concentrations used in my experiments for both CalA and okadaic acid were selected because of their observed effects in crane-fly spermatocytes and HeLa cells (Fabian et al., 2007a; Su et al., 2016).

The main finding of my first set of experiments is that 1) tether elasticity is regulated by phosphorylation, and that 2) the loss of elasticity is due to dephosphorylation, likely due to activity by PP1. When CalA was added in early anaphase at tether lengths less than 3 μm about 81% of chromosomes moved back. However, when CalA was added later in anaphase at longer tether lengths there was increasingly reduced backwards movement until there was no backwards movement at all when CalA was added in late anaphase, when tether lengths exceeded 11 μm. Further, when cells were treated with a low concentration of okadaic acid in early anaphase,

which presumably only inhibits PP2A, there was no backwards movement. When cells were treated with a high concentration of okadaic acid in early anaphase I again saw backwards movement. These experiments demonstrate that inhibiting PP1 and blocking dephosphorylation in early anaphase results in the subsequent backwards movements of separated anaphase chromosomes.

The above experiments answered some questions regarding tether elasticity, such as how it decreases over the course of anaphase, and the likely phosphatase necessary for dephosphorylation. Next, I tested the hypothesis that tethers were phosphorylated prior to anaphase and that this is done by a kinase that acts on an elastic region of the giant protein titin. Experiments discussed in Chapter Three provide a preliminary framework for better understanding these aspects of tether elasticity. Firstly, when cells were treated with a broad-spectrum kinase inhibitor in combination with CalA in early anaphase, there was still backwards movement. This suggests that tethers begin anaphase fully phosphorylated and elastic, and do not require additional phosphorylation. Therefore, it may be that the crucial timeframe for tether phosphorylation is in metaphase. Next, when cells were treated with a PKC inhibitor or a PKA inhibitor in metaphase, potentially preventing tether phosphorylation, followed by treatment with CalA in early anaphase there was still backwards movement, which suggests that neither PKA nor PKC are solely responsible for tether phosphorylation. It is still unclear which kinase or kinases phosphorylate tethers.

The kinases tested in this thesis were selected because they phosphorylate elastic regions of titin, which has been put forward as a potential component of tethers (Fabian et al., 2007b). My preliminary experiments showed no effect on tether elasticity after inhibiting PKC or PKA, both of which phosphorylate titin, followed by treatment with the phosphatase inhibitor, CalA. However, this does not necessarily mean that tethers are not composed of titin. First, the negative results I obtained are from a very small sample size, and therefore may not represent the effects of the inhibitors accurately. Further experiments using the PKC activator PMA at long tether lengths could potentially answer this question for PKC. We normally do not see any backwards movement of chromosomes after treatment with CalA at long tether lengths (>11 μm) (Kite and Forer, 2020). Therefore, any increase in backwards movement at these tether lengths following PKC activation would be highly suggestive of PKC involvement in tether phosphorylation. If

these experiments still yield negative results, it may be that PKC does not phosphorylate tethers, in which case another kinase that acts on titin may be responsible.

An interesting way to address the question of whether or not titin is a component of tethers is to stain for phosphorylated titin directly. Previous studies have identified titin extending between separating anaphase chromosomes in crane-fly spermatocytes (Fabian et al., 2007b). However, staining for phosphotitin would be particularly revealing if we observed a decrease in phosphotitin as anaphase progressed, as this matches the pattern seen in the CalA experiments (Kite and Forer, 2020). This would be clear evidence of a temporal phosphorylation gradient on titin in the area of tethers.

Of course, it is also possible that tethers are composed of another molecule altogether, and future experiments should not exclude this possibility. Other kinases that are active during metaphase could be tested, either using a similar method to the experiments completed in this thesis or by alternate procedures such as immunostaining. I would suggest Aurora kinase B as a potential starting point, as we know it is active in metaphase, acts opposite the phosphatase PP1, and localizes in the spindle midzone during anaphase (Kim et al., 2016; Mora-Bermúdez et al., 2007; Kim et al., 2010).

In conclusion, these data and preliminary experiments provide a clearer picture of how the tethers that connect separating chromosomes in crane-fly spermatocytes gain and lose their elasticity. Tethers are likely phosphorylated in metaphase, although it is not yet clear which kinases moderate this, and future experiments are needed. Once a cell enters anaphase and the chromosomes begin to divide, the tethers lose their elasticity due to dephosphorylation likely done by the phosphatase PP1. If this dephosphorylation is inhibited, then the tethers will remain elastic resulting in backwards movement of the chromosomes after reaching the spindle poles.

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