Tethered to Telomeres: Characterizing the Form and Dynamic Elasticity of Tethers in Anaphase Crane-Fly Spermatocytes

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Abstract

Elastic tethers connect sister chromosomes during anaphase in a broad range of animal cells, likely aiding in communication and coordination of movement. Calyculin A prevents dephosphorylation of tethers by protein phosphatase 1 (PP1) and, consequently, causes them to remain elastic throughout anaphase, resulting in backward movement. In my thesis experiments, I further explore the composition and behaviour of tethers. I expand upon existing research with Calyculin A, elucidating an optimal working concentration of 10 nM for investigating the reversibility of this drug on backward movement when rinsed out at various stages. I show that PP1 is critical during the onset of anaphase and that backward movement is not readily reversible. I also support the existing notion that tethers are made of titin, disproving the hypothesis that tethers are made of DNA. These contributions further enhance our understanding of tether composition and regulation during a central mechanistic phase of cell division.

Acknowledgement

I am deeply humbled and honoured to share in this thesis the fascinating and exciting experiments I had the privilege of conducting in the Forer Lab. Art, as he insisted we call him, was a formidable force in the worlds of science, education, music and social justice activism. He was not only an incredibly accomplished scientist, but also an incredible mentor. His unwavering curiosity and passion for cell biology propelled him to steadfastly continue his research until his passing at the age of 89 years old. While he and I planned excitedly to conduct my PhD research in his lab and I am deeply saddened by his loss, I am forever grateful for the two years that I spent learning from him. He ignited in me a deep curiosity for cell biology and reminded me how much is not yet known about even the most basic principles in science. I will endeavour to keep my mind open and pursue knowledge as passionately as he did.

From the time that I joined the Forer lab, I worked hard in pursuit of my goal of transferring directly to the PhD program. This is what Art had always envisioned for me, and what I had envisioned for myself. I never imagined that I would instead be defending my Master's thesis without his comforting and supportive presence, or that I would celebrate my graduation without him. As these drastic changes unfolded and I was faced with navigating them without my supervisor, my mentors and peers at York University provided such heartwarming support. Dr. Steven Connor, my first professor as a master's student and my dedicated committee member, did not hesitate to take on the role of supervisor. He provided invaluable insights and advice, all the while assuring me that I had his unwavering support in whatever path I chose moving forward. My lab mates Maral, Aisha, Yuliya and Lames have always fostered a fun and supportive lab environment, and we all felt lucky to be able to

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I owe my success to a long life history of support, encouragement and learning from dedicated educators, mentors, friends and family. As I complete my master's studies and explore my next steps in the world of research and education, I will continue to honour Art's memory. I look forward to seeing what the future holds and will always remember those who have helped me along the way.

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CHAPTER 1: INTRODUCTION

Review of the Literature

General Cell Biology

Cell theory was formulated in the 19th century, though the emergence of the field of cell biology arguably took place two centuries prior with the invention of the microscope. In the years since, the field of cell biology has developed into a rich, expansive array of experimental approaches and findings, all centered around the unifying principle that the cell is the most fundamental unit of life. In fact, a broad range of subfields and inter-disciplinary connections have developed, including but not limited to cell metabolism, genetics, proteomics, cell signalling, cell organelles, cell cycles, cancer biology, evolution, phylogenetics, and more.

Critically, fully leveraging insights gained using advanced cell biology techniques requires applying these approaches in model organisms derived from Earth's many and diverse species. Species are categorized using a taxonomic system of increasingly specific ranks, rooted in the fields of cell, molecular and evolutionary biology. These ranks are Domain, Kingdom, Phylum, Class, Order, Family, Genus and Species. One of the first distinctions assigned to species, new and old, is phylogenetic Kingdom. Cells, and the organisms they make up, may be categorized as Archaebacteria, Eubacteria, Protista, Fungi, or Plantae. More broadly, this second highest order of classification unites life forms by the basic principles and structures governing their cells. Perhaps the only biological entity which does not possess cells is a virus, though this lack of cell structure generates debate of whether or not viruses should be considered alive, at all. That viruses may not be considered alive based on lack of cells, despite possessing genomes, reproducing and evolving, highlights the importance of cells as a keystone of life.

From bacteria, fungi and protists, to plants and animals, there is great diversity not only between these groups, but also within them. The extent to which cells are fundamentally alike, as well as incredibly diverse, is a testament to the importance of research in each of these Kingdoms. As I will highlight in later sections, even considering solely animals, there is a vast array of characteristics, including many exceptions to so-called 'rules' of cell structure and behaviour. Before exploring these unique cases, however, it is important to consider our foundational understanding of cell division.

Meiosis

Cell division can be divided into two main types: mitosis and meiosis (Cooper, 2000). While mitosis describes the division of somatic cells into two genetically identical daughter cells, meiosis describes the division of gametocytes, or immature sex cells, usually into four genetically unique haploid daughter cells. Despite some key differences, the general progression of the cell cycle in meiosis as described below largely applies to mitosis, as well. All cells exist in phases of the cell cycle. Interphase is generally the longest in duration, including the G1, S and G2 phases. In the G1 phase, cells grow and prepare for the S phase, during which chromosomes are duplicated. During G2, the cell finally prepares for the most eventful stage in the cell cycle: division. Meiosis in animals ultimately produces either sperm or ova gametes.

Meiosis is the first stage in sexual reproduction, preceding conjugation, or fertilization, after which two haploid gametes combine to form a diploid zygote. In some cases, fertilization may also occur during meiosis, as in prophase-arrested human oocytes, triggering the resumption of meiosis. Meiosis I follows the typical sequence of division events: prophase, metaphase, anaphase and telophase. Meiosis I is characterized by two key events: homologous chromosome pairing and

exchange of genetic material via crossing over, as well as subsequent separation to opposite poles and daughter cells. This division is typically reductional, meaning that chromosome number is reduced (from diploid (2n) to haploid (n) in humans and crane flies). During the stereotypical meiosis I, homologous chromosomes line up at the equator during metaphase before segregating to opposite poles in anaphase. Notably, recombination occurs only during meiosis I. Additionally, genetic material is only replicated prior to meiosis I. Meiosis II resembles mitosis in that it involves the separation of sister chromatids. Meiosis II is an equational division, meaning that chromosome number is not reduced. The end result is the production of four haploid daughter cells, each with a unique combination of genetic material (Cooper, 2000).

Cells enter prophase I with replicated DNA and after having passed through several checkpoints in place to detect genetic or chromosomal abnormalities (Cooper, 2000). DNA replication occurs only once in meiosis and does not occur again before prophase II. Typically, the nuclear envelope breaks down at the end of prophase, releasing the chromosomes contained within. Upon completion of prophase, the cell is prepared for the dramatic reorganization of cell structure triggered by activation of the maturation promoting factor (MPF) protein (Cooper, 2000). Nuclear envelope breakdown marks the boundary between prophase and prometaphase. Prometaphase, the transitional period between prophase and metaphase, entails formation of the meiotic spindle. The spindle is a complex of microtubules, including kinetochore microtubules, polar microtubules and astral microtubules, as well as other proteins (Figure 1). During prometaphase, chromosomes move between the equator and the poles. While kinetochore microtubules connect chromosome kinetochores to the centrosome, moving them poleward, polar microtubules elongate the cell, generating distance between the chromosome and the centrosome. Astral microtubules extend outward from the centrosomes at either end of the

cell and, like polar microtubules, help to orient the spindle by pushing spindle poles apart (Roostalu et al., 2010). Proper orientation of kinetochores and proper attachment of kinetochore microtubules are crucial outcomes of prometaphase necessary for successful metaphase and anaphase (Monda & Cheeseman, 2015). Cell cycle progression is halted in the event of improperly attached kinetochores, and the cell must resolve the issue before progressing to anaphase in order to ensure proper chromosome segregation and euploidy in the daughter cells (Monda & Cheeseman, 2015). Finger-like projections containing actin also extend outward from the two poles of the cell. They appear around the poles of the cell during prometaphase as bunched clusters, moving in wave-like, fluid motions in our crane-fly spermatocytes. After the rapid chromosome movement characteristic of prometaphase, chromosomes migrate to the center of the cell, forming chromosome pairs (McKee et al., 2012). Once bipolar bivalent orientation has been achieved in prometaphase, the cell transitions into metaphase as the chromosomes align at the equator (McKee et al., 2012).

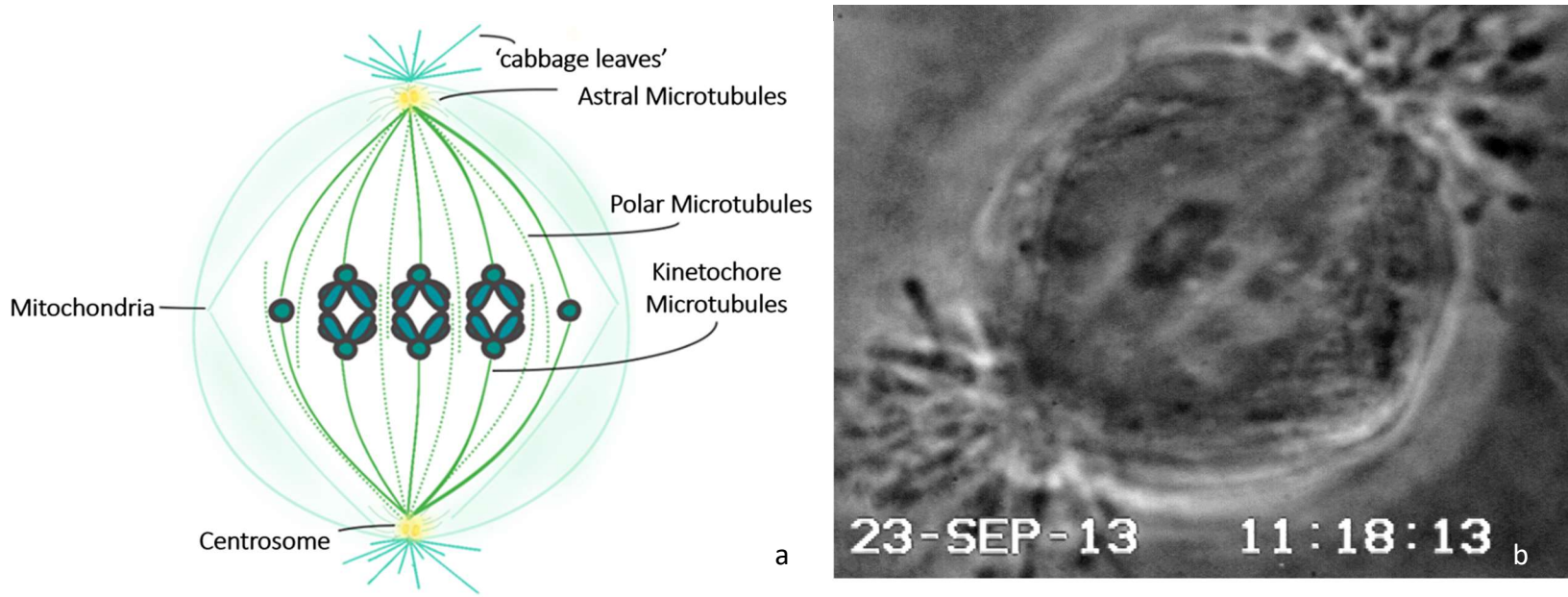


Figure 1: (a) Hand-drawn and (b) phase contrast image of a metaphase I crane-fly spermatocyte. Crane-fly spermatocytes are large cells with large and few chromosomes. They possess 3 bivalent autosome pairs, as well as 2 unpaired univalent sex chromosomes. During meiosis, the spindle is outlined by diamond-shaped mitochondria. Kinetochore microtubules attach the kinetochores of partner half-bivalents to opposite poles. The univalent sex chromosomes form microtubule attachments to both poles. During metaphase, all chromosomes line up at the equator.

Metaphase is initiated by MPF, which acts both indirectly in triggering downstream protein kinases, as well as directly by phosphorylating some of the proteins necessary for structural reorganization of the cell, such as microtubules (Cooper, 2000). A cell has reached metaphase when paired homologous chromosomes line up at the metaphase plate, with homologous chromosomes oriented toward opposite poles in Meiosis I and sister chromatids oriented toward opposite poles in Meiosis II. Each homologous chromosome in Meiosis I contains two sister chromatids, which orient to the same pole. Otherwise known as the equator, the metaphase plate exists halfway between opposite poles of the cell. In crane-fly spermatocytes, the metaphase plate runs along the short axis of a prominent diamond shape created by the mitochondria. Crane flies have three bivalent autosome pairs, as well as two univalent sex chromosomes. Bivalent chromosome pairs typically form stable, bipolar attachments to microtubules from opposite spindle poles. In contrast, univalent chromosomes, which are unpaired homologs, often form more unstable attachments to microtubules. Metaphase is pivotal from a genetic standpoint, as chromosomes must line up properly at the metaphase plate to ensure that each daughter cell receives one copy of each homologous pair. Upon passing through a cell cycle check point to ensure proper chromosome alignment, cells in metaphase may progress to anaphase (Cooper, 2000).

Anaphase begins as partner chromosomes separate and move toward opposite poles of the cell. While anaphase looks different in different cell types, the overarching regulatory mechanisms are highly conserved (Roostalu et al., 2010). Dephosphorylation of mitotic phosphoproteins is a key factor in spindle function and chromosome segregation in all eukaryotes. For example, phosphorylated Ase1/PrC1 (anaphase spindle elongation 1/protein regulating cytokinesis 1) prevents spindle elongation during metaphase, while dephosphorylated Ase1/PrC1 promotes spindle elongation in anaphase

(Roostalu et al., 2010). The anaphase-promoting complex, triggered by MPF, initiates the breakdown of several protein complexes important in metaphase, such as cohesins (Cooper, 2000). Cohesins maintain the connection between sister chromatids until anaphase, when the release of cohesion from the sister chromatids leads to separation and anaphase chromosome motion. In Anaphase I, cohesion is dissolved between the arms of sister chromatids as cohesins are disassociated from chromosomes by the conserved enzyme separin (Cohen-Fix, 2000). Centromeric cohesion is maintained until Anaphase II, when sister chromatids separate (Cohen-Fix, 2000). The anaphase-promoting complex also indirectly inactivates the MPF, triggering cytokinesis and allowing the new daughter cells to return to the interphase state. As anaphase concludes, in animal cells a cleavage furrow forms at the metaphase plate (Cooper, 2000).

During telophase and cytokinesis, respectively, the nuclear envelope is reformed around the chromosomes at each pole, while the cleavage furrow is pinched inward, parallel to the equator, by a contractile ring composed of actin and myosin filaments (Cooper, 2000; Thyberg & Moskalewski, 1998). The plasma membrane is consequently pulled inward until the cell becomes cleaved in two (Cooper, 2000). Interestingly, while the overall processes of mitosis and meiosis are comparable in eukaryotic organisms, plant cells employ a different method of telophase and cytokinesis. In higher plant cells, vesicles from the Golgi apparatus carry cell wall precursors to associate with spindle microtubules, accumulating at the equator. There, they form a cell plate which expands outward until reaching the plasma membrane. Finally, the plasma membrane fuses with the cell plate, dividing the cell into two. Regardless of cell type, both spatial and temporal control are crucial during cytokinesis to ensure proper partitioning of cell contents (Wolf, 1999). Telophase and cytokinesis represent the crucial

terminal phase of division, characterized by (usually) equal division of the cytoplasm, organelles and genetic material between two new daughter cells (Wolf, 1999).

Exceptions to the Norm: Unique Cases

Much of our scientific research is centered around so-called 'model' organisms. These organisms, mainly mice, rats, fruit flies, nematodes, zebrafish, frogs, budding yeast and thale cress, are recognized by the US National Institutes of Health (NIH) (Ankeny & Leonelli, 2020). These models offer unique experimental advantages which include similarity to humans, short generation times, and low maintenance requirements. Additionally, advances in genomic data generation confer further incentive to study these organisms.

Despite the experimental advantages that this select list of model organisms provides, there is a growing disparity between the knowledge of 'model organisms' and other species encompassed within the vast diversity of life (Ankeny & Leonelli, 2020). Out of ~4600 developmental papers published in 1965, 1975, 1985, 1995 and 2005, combined, two fifths were accounted for by the genera *Mus* and *Drosophila* (Davies, 2007). While the advantages outlined for these model organisms are valid and their research is undeniably warranted, the overwhelming focus on them can lead to a pin-hole glimpse of life on Earth that fails to reflect it's diversity. A deeper review of the literature on different cell types reveals great diversity and non-conformity to generic rules or norms of cell biology. Below, I will outline a couple of noteworthy examples explored in the Forer Lab that demonstrate this principle.

Distance (non-random) Segregation, Oscillations and Premature Cleavage Furrows* – Mesostoma *Flatworms

The Forer lab is one of the only labs to rear the hermaphroditic freshwater turbellarian flatworms formally known as *Mesostoma ehrenbergii*. *Mesostoma* spermatocytes display unique chromosome movement that occurs during meiosis. In fact, some characteristics of *Mesostoma* meiosis directly contradict the principles which are presented as fact, even in university-level cell biology lectures and publications. *Mesostoma* cells possess three bivalent autosomes, as well as four (two pairs of) univalent sex-chromosomes. Bivalent chromosomes exist in a homologous pair, whereas univalent chromosomes lack a homologous partner during meiosis. While conventional meiosis involves lining up of the chromosomes at the equator during metaphase, chromosomes in *Mesostoma* spermatocytes skip this step (Ferraro-Gideon et al., 2014). In these cells, prometaphase lasts up to several hours, during which time bivalent kinetochores oscillate rapidly, and a precocious cleavage furrow forms as soon as the spindle forms. Bivalent reorientation occurs during prometaphase and often results in sister kinetochores forming attachments to opposite poles, causing partner half-bivalents to exchange poles. There is no real metaphase plate, as half-bivalents simply separate and move poleward at some point during oscillation.

Univalent sex chromosomes also move periodically between the poles during meiosis I. Univalent segregation during meiosis I in *Mesostoma* is an example of distance segregation. Distance segregation describes segregation of chromosomes that are not physically linked during metaphase by chiasma or sister chromatid cohesion (Brady & Paliulis, 2015). Rather, movements of these unpaired chromosomes are coordinated at a distance. *Mesostoma*'s four univalents do not pair, yet always end up such that each daughter cell receives two univalents, one of each kind (Ferraro-Gideon et al., 2014).

Furthermore, univalents often exchange poles even after having achieved the correct 2:2 chromosome compliment, suggesting a distribution pattern that not only achieves euploidy, but also according to some pre-determined distribution pattern, perhaps requiring univalents from the same parent to segregate together, or to form some other combination (Oakley, 1985).

Additionally, after depolymerizing spindle microtubules in spermatocytes with the anti-mitotic drug nocodazole (NOC), all three bivalents detached selectively from one pole (Fegaras & Forer, 2018). Given that microtubules produce the force directing chromosome movement, quickly depolymerizing microtubules should have caused chromosomes to arrest at the equator, if their segregation to one pole or another were random. Non-random bivalent segregation may be driven by non-microtubule spindle components, in this case. Hence, *Mesostoma* divisions provide not only an example of distance segregation, but also evidence for non-random chromosome segregation. An interesting hypothesis formulated in the Forer lab is that elastic tethers, which have been shown in *Mesostoma,* might help mediate these unique segregation patterns (Forer et al., 2017).

Crane-fly Spermatocytes Have Delayed Sex Chromosome Segregation

Crane-fly spermatocytes (*Nephrotoma)* are the subject of my current research. Crane-fly spermatocytes have been foundational in laying the groundwork for tether research. Several key characteristics of crane-fly spermatocytes make them ideal candidates for tether research. In addition to standard lab organism advantages, such as short generation time and cost effectiveness, crane flies possess large spermatocytes with few and large chromosomes. Crane-fly spermatocyte meiotic divisions are fairly typical, with the exception of delayed sex chromosome segregation. During anaphase, three autosomal bivalents segregate to each pole of the cell. The univalent sex

chromosomes remain at the equator during this time and segregate to the two poles following autosomal anaphase.

In Crane-Fly Spermatocytes Tethers Connect the Telomeres of Separating Anaphase Chromosomes

Some of the early evidence suggesting a physical 'tether' between partner chromosomes was published in 1997 by Ilagan, Forer and Spruck, before the term had even been coined (Ilagan et al., 1997). In this experiment, irradiating crane-fly spermatocyte kinetochores/kinetochore areas inconsistently yielded backward chromosome movement at increased velocities. In the same year, Ilagan and Forer used a UV microbeam to irradiate a single kinetochore, spindle fibres or chromosome arms (Ilagan & Forer, 1997). The latter two experiments were controls in place to isolate the effect of a disabled kinetochore from any potential off-target effects of irradiation. All trials were conducted in anaphase-I crane-fly spermatocytes. Ilagan and Forer found that irradiating a single kinetochore caused all six half-bivalent autosomes to cease poleward movement for several minutes, after which poleward movement resumed. Conversely, only the targeted chromosomes and their homologous partners were affected by spindle fibre or chromosome arm irradiation (Ilagan & Forer, 1997). This corroborates similar findings reported by Sillers and Forer in 1981 and suggests the presence of a "stop signal" transmitted between kinetochores (Ilagan & Forer, 1997; Sillers & Forer, 1981). Based on the experiments of Yin and Forer just one year prior, these signals were suggested to be transmitted through the interzone between partner chromosomes (Yin & Forer, 1996). Spindle fibre irradiation during anaphase-I halted the movement of the associated half bivalent and its partner. In contrast, irradiation of the interzone either before or immediately after spindle fibre irradiation uncouples the partnered half bivalents, allowing the sister chromosome to resume poleward movement while the affected chromosome is halted (Yin & Forer, 1996). The findings of Forer, Sillers, Ilagan and Yin show

that partner chromosome movements are coordinated via a mechanism in the interzone, paving the way for an exciting and promising emerging area of research into tethers.

Tethers

Tethers are physical, elastic connections between two out of four arms of separating partner half-bivalents in anaphase I. We propose that they are made of the giant protein titin and are phosphorylated at the start of anaphase, becoming dephosphorylated and, consequently, losing their elasticity by the end of anaphase, before eventually becoming resolved. The existing literature on tethers suggests that they are a widespread structure, present in organisms from insects and flatworms to humans, that produce physical forces involved in the controlled and coordinated movement of chromosomes during cell division (Forer et al., 2017). Tethers were first described and named in 2002 by LaFountain and colleagues, who found that when they used a laser to sever the trailing arms of anaphase chromosomes the acentric fragments that were produced moved rapidly to the partner telomere in the opposite half-spindle, suggesting that tethers are elastic structures that connect separating telomeres (LaFountain et al., 2002). LaFountain and colleagues also cut the acentric, telomere-containing fragments, thereby generating two fragments, but the interstitial fragments ceased movement, while the telomere-containing fragments continued to move backward. This finding ruled out lateral interactions between the sides of chromosome arms or linear spindle elements as contributing factors to backward movement. Moreover, the acentric fragments halted backward movement when either telomere of the two was ablated, supporting the notion that tethers coordinate movement between sister chromosomes. Importantly, LaFountain and colleagues reported that tethers only connect two out of the four arms in a half-bivalent pair. Chromosome arms with tethers can often be identified as they trail behind during anaphase and appear slightly stretched toward their partner.

The collection of evidence described by LaFountain and colleagues supports the notion that tethers attach to telomeres, rather than other cellular structures such as microtubules, which attach to kinetochores (LaFountain et al., 2002).

In addition to studies involving physical cutting of chromosome arms, the interzonal space between chromosomes has also been irradiated using laser microbeams (Sheykhani et al., 2017). Sheykhani and colleagues investigated tethers as a possible explanation as to why crane-fly spermatocyte chromosomes, unlike grasshopper spermatocytes and newt fibroblasts, do not speed up during anaphase when microtubules are severed (Forer et al., 2015). Interestingly, the associated chromosomes did speed up when the interzonal region, (where the presumed tethers are located), was cut first (Sheykhani et al., 2017).

Sheykhani and colleagues employed an innovative approach to overcome the challenge of cutting something which can not be seen (Sheykhani et al., 2017). They first cut a stretched chromosome arm and then, as the telomere-containing fragment moved backward, cut the interzonal space between the backward-moving fragment and its partner. Halting of backward movement proved that the tether had, indeed been severed. The finding that tethers compensate for severed microtubules by maintaining normal chromosome movement speeds reinforces the idea that tethers play an important role in the connection and coordination between sister chromosomes (Sheykhani et al., 2017).

In addition to physical cutting by both UV and laser microbeams, tethers have also been manipulated chemically, for instance with the drug Calyculin A (Fabian, Troscianczuk, et al., 2007; Forer et al., 2021; Kite & Forer, 2020). Calyculin A is an inhibitor of PP1 and PP2A, two catalysts of

dephosphorylation (Forer et al., 2021). Both PP1 and PP2A are protein Ser/Thr phosphatases (Trinkle-Mulcahy & Lamond, 2006). PP1 is involved in variety of cellular processes, including but not limited to glycogen metabolism and muscle contraction (Bollen et al., 2010). PP2A is plays a key role in signalling pathways and regulation of mitosis, including the regulation of sister chromatid cohesion (Bollen et al., 2010). Fabian and colleagues added Calyculin A to anaphase cells and found that chromosomes moved backward at the end of anaphase (Fabian, Troscianczuk, et al., 2007). Fabian and colleagues describe the following three effects of Calyculin A on crane-fly spermatocytes: increased chromosome velocity during anaphase, some chromosomes randomly returning to the equator upon migrating to the poles, and sex chromosome oscillation. Actin and myosin inhibitors 2,3-butanedione monoxime (BDM) and Latrunculin B (LatB) did not stop backward movement when added to Calyculin A treated cells during backward movement, suggesting that actin and myosin do not drive this process. Additionally, Fabian and colleagues' observations do not support the alternative idea that chromosomes could be sliding backward along microtubules, as chromosomes travel back to the equator with their arms leading. If microtubules were responsible for backward movement, the chromosomes should have travelled with their arms trailing, as microtubules attach at the kinetochore (Fabian, Troscianczuk, et al., 2007). These studies lay a strong foundation for the existence of tethers.

More recently, Kite and Forer perfused Calyculin A into crane fly spermatocyte preps, while observing cells at different stages of anaphase (Kite & Forer, 2020). Kite and Forer observed backward chromosome movement in Calyculin A-treated cells. Interestingly, they further found that chromosomes were far more likely to exhibit backward movement if Calyculin A was perfused earlier in anaphase, when tethers are shorter. This supports the previous conclusion from movement of arm

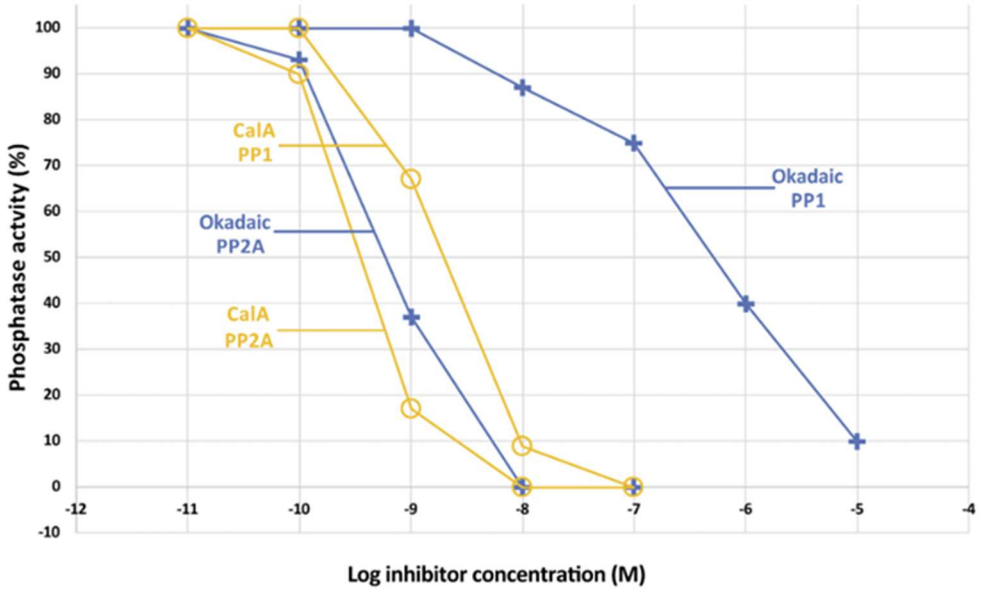
fragments that tethers lose elasticity (Forer et al., 2017; LaFountain et al., 2002), suggesting that the backward movements after Calyculin A are due to tethers.

PP1 and Calyculin A

Protein phosphatase 1 (PP1) is an evolutionarily ancient enzyme found in nearly all eukaryotic organisms, from yeast to humans (Heroes et al., 2013; Moorhead et al., 2007). In eukaryotic cells, PP1 catalyzes the majority of protein dephosphorylation events, highlighting its fundamental importance in cellular processes (Bollen et al., 2010). Furthermore, PP1 regulates conserved cellular pathways and functions, such as cell cycle progression, DNA damage repair and metabolism (Bollen et al., 2010; Heroes et al., 2013). PP1 plays a pivotal role in orchestrating the dynamic dephosphorylation events governing the progression of mitosis and meiosis (Bollen et al., 2010; Moorhead et al., 2007). During cell division, PP1 functions as a key regulator of spindle dynamics, chromosome segregation, and cytokinesis by targeting a myriad of proteins, including kinetochore proteins, microtubule-associated proteins, and elements of the contractile ring (Capalbo et al., 2019; Trinkle-Mulcahy et al., 2006). Moreover, dysregulation of PP1 activity has been implicated in various human diseases, including cancer, highlighting its significance in cellular homeostasis (Bollen et al., 2010). The intricate interplay between PP1 and other regulatory proteins indicates essential roles in coordinating the complex choreography of cell division processes, ultimately contributing to the maintenance of genomic stability and organismal development.

Calyculin A, a potent natural toxin derived from marine sponges, inhibits protein phosphatases 1 (PP1) and 2A (PP2A) (Wakimoto et al., 2002). Calyculin A inhibits PP1 by binding to its catalytic subunit, or active binding site, thereby disrupting its ability to dephosphorylate substrates (Wakimoto

et al., 2002). This inhibition leads to dysregulation of several key signaling pathways and cellular functions, including but not limited to tether elasticity (Fabian, Troscianczuk, et al., 2007). On a more general scale, inhibition of PP1 activity can result in cytotoxicity, causing altered cell cycle progression and impaired cytoskeletal dynamics (Bollen et al., 2010; Heroes et al., 2013, p. 1; Wakimoto et al., 2002). Overall, the interaction between Calyculin A and PP1 underscores the importance of PP1 in cellular homeostasis and highlights Calyculin A as a valuable tool for studying PP1-regulated processes. PP1 has been shown to be active during anaphase and present both on and between separating chromosomes (Sheykhani et al., 2024). While Calyculin A inhibits PP1 as well as PP2A, current evidence suggests that effects on PP1, alone, account for the retention of tether elasticity and consequent backward movement in treated cells (Kite & Forer, 2020). Specifically, Okadaic acid inhibits PP2A without affecting PP1, and application of Okadaic acid does not trigger backward movement. Furthermore, Kite and Forer investigated Calyculin A at a concentration of 50 nM, noting, as well, that the IC50 of Calyculin A on PP2A is lower than for PP1. The IC50 of Calyculin A on PP1 and PP2A, as well as Okadaic Acid, was determined using the substrate phosphorylated phosphorylase a (Figure 2) (Kite & Forer, 2020).



(From The role of phosphorylation in the elasticity of the tethers that connect telomeres of separating anaphase chromosomes. *Nucleus*, **© 2020** Kite & Forer, reprinted by permission of Informa UK Limited, trading as Taylor & Taylor & Francis Group, http://www.tandfonline.com)

Figure 2: IC50 curves of Calyculin A and Okadaic Acid on both PP1 and PP2A, modified from Ishihara and colleagues (Ishihara et al., 1989) by Kite and Forer (Kite and Forer, 2020). The effect of different concentrations of Calyculin A and Okadaic acid on the activity of protein phosphatases 1 (PP1) and 2A (PP2A) are shown. Phosphatase activity was assessed in vitro using the substrate phosphorylated phosphorylase a. The IC50 (50% inhibitory concentration) of Calyculin A as determined by Ishihara and colleagues is 2 nM.

Tethers are Made of the Protein Titin

Calyculin A-mediated dephosphorylation of tethers makes sense in light of the well-documented phosphorylation events affecting the giant protein titin. Recently, I had the opportunity to contribute to the article, “Titin is present in the elastic tethers that connect separating anaphase chromosomes in crane-fly spermatocytes” (Sheykhani et al., 2024). I used confocal microscopy to capture images and study fixed crane-fly spermatocytes and myofibrils stained with antibodies against titin. Titin typically stains in a punctate manner, as it is a large protein with different domains that may be targeted by primary antibodies. In our recent experiments, actin filaments were stained with 488-Alexa phalloidin, while titin was stained with 4 anti-titin antibodies. All 4 anti-titin antibodies stained

strands between telomeres of separating partner chromosomes in anaphase in crane fly spermatocytes. Moreover, these titin strands were severed by laser microbeam radiation, as are tethers. Combining these data with that of Fabian and colleagues, who found titin-stained strands using 3 other primary antibodies, tethers have been effectively stained with a total of 7 different titin antibodies (Fabian, Xia, et al., 2007). This collection of data drastically minimizes the risk of misinterpreting off-target stains, as it seems very unlikely that 7 stains targeting 7 different components of the titin protein would all bind to the same off-target cellular structure. Titin antibodies also stain chromatin, reinforcing the electron microscopy finding that tethers seem to emerge during anaphase from telomeres, themselves (Forer & Otsuka, 2023).

Titin is found in several cell types across several different species (Mikelsaar et al., 2012). Of particular significance is titin's role in skeletal and cardiac muscle. Phosphorylation of cardiac muscle in the PEVK region, a unique sequence in titin's elastic I-band, increases the passive tension of titin, and dephosphorylation in this region is mediated by PP1 (Linke et al., 1998). This is similar to the dynamic, phosphorylation-dependent elasticity observed in tethers (Sheykhani et al., 2024). Overall, there is a growing body of evidence suggesting that tethers are made of titin and that they are dephosphorylated by PP1 during anaphase, causing them to become inelastic.

Warecki et al.'s DNA Strands

In January of 2023, researchers from outside of the Forer Lab and its colleagues joined the conversation around tethers, citing several articles from chief tether researchers including Forer, Paliulis, and Berns in their publication, "Connections between sister and non-sister telomeres of segregating chromatids maintain euploidy" (Warecki et al., 2023). Warecki and colleagues present

tethers as a mechanism that maintains euploidy in the presence of lagging chromosomal fragments. Warecki and colleagues characterize these telomeric connections as DNA bridges which connect both sister and non-sister chromatids in some, but not all, chromosomes. While these bridges are typically resolved by mid-late anaphase, they are stabilized to recruit histones, kinases and the protein barrier-to-autointegration factor (BAF) in the presence of unrepaired DNA breaks. These findings led Warecki and colleagues to the conclusion that DNA bridges exist as a highly conserved evolutionary mechanism supporting euploidy in cell divisions wherein chromosomes have been fragmented and unrepaired. They further suggest that mitotic tethers might contain DNA, likely different from ultrafine DNA bridges as ultrafine bridges don't stain with DAPI and because they typically do not extend from telomeres. The functional significance of ultrafine bridges is not entirely clear, however they are a form of chromosome bridges that likely exist between separating sister chromatids in most divisions. They can extend from multiple locations along the chromosome, but are most commonly found between centromeres. Ultimately, Warecki and colleagues suggest that thin, telomeric DNA strands exist between separating chromosomes in anaphase, and that these strands are either resolved or, in the presence of chromosomal breaks, stabilized by tether proteins to aid lagging chromosome rescue.

Warecki and colleagues' methods differ from our own in several respects. Firstly, and most obviously, their model is different. While I study tethers in crane-fly spermatocytes and Forer and colleagues have examined them in a wide range of cell types, Warecki and colleagues are the first to investigate them in *Drosophila* neuroblasts. Accordingly, Warecki and colleagues also study mitosis, rather than meiosis. Warecki and colleagues also dissect their specimens in NaCl or PBS, whereas we dissect crane flies in an insect Ringer's solution. Notably, Warecki and colleagues also state that these

strands stain too faintly to be viewed under normal conditions. Instead, they view DAPI-stained inter-telomere bridges only with high gain.

Ringer's Solutions

In 1883, Ringer conducted a foundational study exposing live tissues to solutions with unbalanced and properly balanced ions (Lockwood, 1961). He found that proper ionic concentration and osmotic pressure is critical for prolonging the life and health-spans of cells in vitro. For example, a frog's heart in NaCl isotonic to the blood ceased beating and became unresponsive to stimulation within just 20-30 minutes. Adding $CaCl_2$ to the solution restored cardiac activity. Addition of KCl and $NaHCO_3$ further prolonged this activity, allowing the heart to beat for several hours. Lockwood goes on to review this work in 1961, outlining the basis for ionically-balanced saline solutions that mimic in-vivo conditions as closely as possible. Lockwood provides a table of Ringer's solutions for different species, and the Forer lab has relied on these tried-and-true solutions for decades of cell biology research.

Introduction to my Research in the Forer Lab

I conduct my experiments on crane-fly spermatocytes during meiosis I and meiosis II, with a focus on characterizing and defining tethers. Previous work in the Forer lab established that Calyculin A causes tethers to remain elastic throughout anaphase by inhibiting phosphorylation by PP1, ultimately resulting in backward autosome movement. I began my research with Calyculin A to characterize the phosphorylation events governing tether elasticity. More specifically, I wanted to determine the reversibility of Calyculin A and, consequently, the time period in anaphase during which PP1 dephosphorylates tethers.

As I concluded my live cell Calyculin A experiments, we became aware of Warecki and colleagues' publication about DNA bridges. This provided a research opportunity that would both contribute to the growing discourse about tethers, as well as provide an opportunity for me to learn immunohistochemistry and confocal microscopy. I will outline the methods and findings of both experiments in the following sections.

CHAPTER 2: PHOSPHATASE INHIBITOR CALYCULIN A IRREVERSIBLY PRODUCES BACKWARD CHROMOSOME MOVEMENT BY MAINTAINING TETHER ELASTICITY

Chapter Summary

Tethers are physical, elastic connections between the telomeres of separating sister chromosomes in a broad range of cell types. Tethers are phosphorylated and elastic at the onset of anaphase, and gradually become dephosphorylated and inelastic as they elongate between separating chromosomes. In this experiment, I use the phosphatase inhibitor Calyculin A to prevent dephosphorylation of tethers by protein phosphatase 1 (PP1) in crane-fly spermatocytes, thus causing them to remain elastic. Initially, I tested Calyculin A concentrations of 0.5 nM, 1 nM, 2.5 nM, 5 nM, 10 nM, 25 nM, 50 nM and 100 nM, finding that 10 nM is the lowest concentration at which Calyculin A reliably causes backward movement. I then proceeded with the rest of my experiments using this concentration, administering Calyculin A at the onset of anaphase and then rinsing it out with insect Ringer's solution at various stages. I found that the backward movement producing effect of Calyculin A is not readily reversible, showing that PP1 activity is critical at the onset of anaphase for tether dephosphorylation.

Introduction – Chapter 2

Experimental data suggest that tether elasticity is modulated by PP1. In 2020, Kite and Forer used the phosphatase inhibitor Calyculin A to inhibit dephosphorylation of tethers by PP1 in anaphase (Kite & Forer, 2020). The results suggest that tethers remain phosphorylated and, consequently, retain their elasticity. Cells treated with 50 nM Calyculin A at the beginning of anaphase exhibited 'backward movement,' wherein separating half-bivalents reverse direction and move back toward each other. They further discovered that PP1 is primarily responsible for dephosphorylation of tethers. Importantly, 50 nM Calyculin A inhibits both PP1 and PP2A, which suggests that the effects of Calyculin A may not be

solely through inhibition of PP1. To test the possibility that PP2A is also involved, Kite and Forer used 50 nM okadaic acid to inhibit PP2A exclusively. Cells treated with 50 nM okadaic acid did not exhibit backward movement, indicating that PP1 is the primary drive of tether dephosphorylation (Kite & Forer, 2020). Accordingly, our understanding of tethers is that they begin phosphorylated and elastic when anaphase starts, and are gradually dephosphorylated by PP1 as anaphase progresses, causing them to become inelastic (Sheykhani et al., 2024).

While the literature does provide a foundational understanding of tethers (Sheykhani et al., 2024), the details of their structure and regulation of their function remain unclear. Firstly, while 50 nM Calyculin A allowed Kite and Forer to produce backward movement, it also contributes to declining cell health. To limit effects on cell viability, optimal working concentrations should reliably produce backward movement while minimizing the risk of potential off-target effects. Importantly, the existing literature on backward movement also does not address the reversibility of Calyculin A on backward movement. My research aims to address these issues through establishing the lowest effective concentration of Calyculin A, administered at the onset of anaphase and rinsed out at various stages, to elucidate when in anaphase PP1 is active. In other words, I endeavor to explore if tethers are dephosphorylated early in anaphase, or if elasticity loss is a more gradual process.

Methods

Preparing living cells

I extract testes under halocarbon oil from crane-fly larvae in the fourth instar (last stage) of larval development when spermatocytes undergo meiosis. These testes are then rinsed in insect Ringer's solution and punctured on a flamed coverslip in 25 µl of fibrinogen. I quickly spread the cells in

a thin layer across the coverslip and add 25 µl of thrombin to form a clot. Finally, the coverslip is placed cell-side down into insect Ringer's within a perfusion chamber, and the chamber is sealed with wax. We make insect Ringer's with final concentrations of 0.13M NaCl, 5mM KCl, 1.5mM $CaCl_2$ and 3mM buffer (KH_2PO_4 and $Na_2HPO_4 \cdot 7H_2O$), and adjust pH to 6.8.

Observation, Recording and Analysis of Live Cells

Live cell preparations are observed using a Nikon Phase Contrast – 2 LWD 0.52 microscope. I scan preps initially using a Nikon 40x magnification objective lens and then use a Nikon or Zeiss 100x magnification lens. Finally, I observe and record cells at 100x magnification onto a DVD using a Panasonic TV camera.

Cell recordings are first saved to DVDs and then converted into avi files using VirtualDub2. I also use VitrualDub2 to improve contrast and clarity of the image, create a time-lapse by decimating (reducing) the frame rate to one frame per every two seconds, remove audio, merge multiple clips (if applicable), and compress files. These files are then opened in WinImage, our lab developed program, to plot x and y coordinates of poles (fixed coordinates) and chromosome kinetochores frame-by-frame. This generates values of distance between the moving chromosomes and the fixed pole over time. Finally, these data are transferred to SlideWrite, which is used to plot the points on a graph.

Treating cells with Calyculin A

I treated crane-fly spermatocytes with Calyculin A as a component of two different experiments: 1) determining the IC50 of Calyculin A and 2) determining the reversibility of Calyculin A on backward movement. In all cases, Calyculin A was administered at the onset of anaphase and cells were observed for a total of approximately 21 minutes to 3 hours and 3 minutes, with an average observation time of

about 1 hour and 13 minutes. For determining the IC50, cells were treated with Calyculin A concentrations of 0.5 nM, 1 nM, 2.5 nM, 5 nM, 10 nM, 25 nM, 50 nM and 100 nM. Calyculin A was dissolved first in DMSO to a concentration of 100 µM, and then further diluted in insect Ringer's. Cells in these experiments were observed and assessed on whether or not backward movement occurred, on whether sex chromosomes oscillated, and whether chromosome poleward speeds increased. Having determined that 10 nM was optimal, I treated spermatocytes with 10 nM Calyculin A at the onset of anaphase and then rinsed out the drug with insect Ringer's at various time intervals, and determined if there was backward movement at the end of anaphase. In all Calyculin A experiments (with the exception of 2 cells treated at the end of metaphase), I perfused Calyculin A at the very onset of anaphase. Tether lengths at the time of Calyculin A perfusion were typically within the range of 0.5-2 µm. I conducted Calyculin A experiments with insect Ringer's rinse in 23 cells and rinsed at various stages ranging from pre-anaphase, early anaphase, mid anaphase, late anaphase to post anaphase/after backward movement onset. Times between Calyculin A treatment and insect Ringer's rinse range from 42 seconds to 14 minutes and 10 seconds.

Results and Discussion

Control cells

There are several notable features of healthy control crane-fly spermatocyte divisions. One of the most easily recognizable signs of division are the finger-like projections we colloquially refer to as 'cabbage leaves.' Another key characteristic of metaphase and anaphase cells is a diamond-shaped spindle, outlined in this distinct shape by the mitochondria. These are the key features we look for when selecting a cell for any experiment, and they are retained throughout healthy control divisions.

We also typically look for cells in late metaphase, when all chromosomes are lined up at the equator. Selecting cells in metaphase affords us the time to set up a recording, prepare any perfusions, and ensure that the entirety of anaphase is captured. Additionally, flat, unobstructed cells are ideal for observing all chromosomes in the same focal plane. Control cells are observed in order to establish a 'normal' anaphase baseline with which to compare treated cells. Control cells are immersed in chambers of insect Ringer's solution and in some cases also perfused with approximately 10 mL of insect Ringer's solution. I observe control cells in both first and second meiotic divisions, until the conclusion of anaphase and often throughout telophase, as well. I have conducted control trials on six cells, with two to six half-bivalents visible in each. Control trials show chromosome movement that is consistent with what is described in the literature (Kite & Forer, 2020). Autosomes move from equator to pole at relatively constant speeds of around 0.35 µm per minute, and sex chromosomes segregate thereafter.

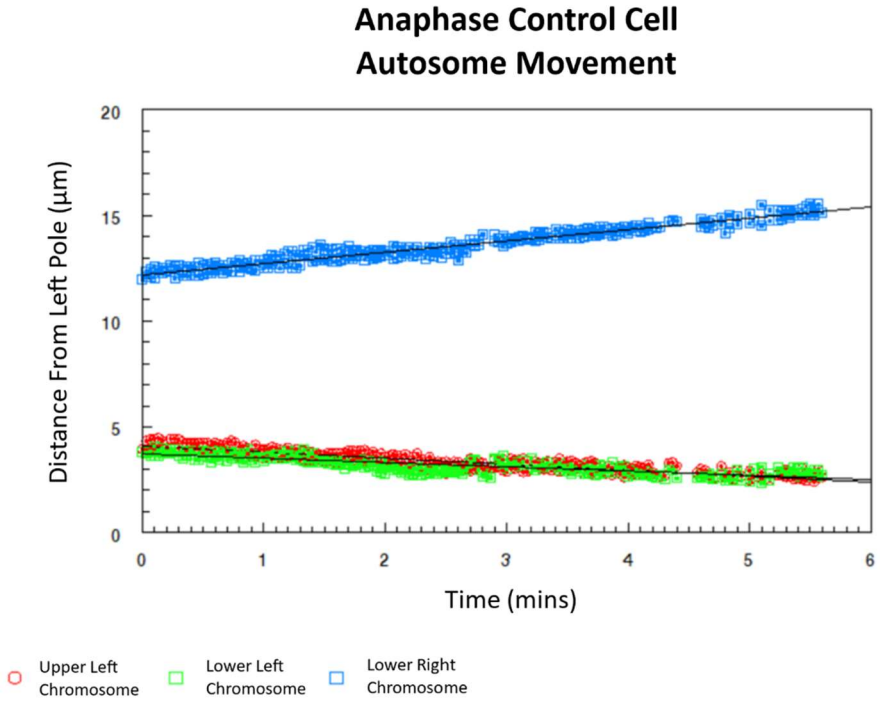


Figure 3: Distance/time movement graph of 3 autosomes during anaphase in a control cell. The green and blue squares represent partner half-bivalents, while the red circle represents a third half-bivalent. Each point on the graph represents the distance of an autosome from the left pole at a given time. The chromosome represented by the blue square is segregating toward the right pole of the cell, while the chromosomes represented by the green square and red circle are segregating toward the left pole. Autosome movement in control anaphase crane-fly spermatocytes occurs at constant speeds.

Calyculin A Cells With No Rinse – Determining the IC50 of Calyculin A on Backward Movement

Upon completion of the control portion of my experiments, which established an important baseline for healthy cell behaviour, chromosome dynamics, etc., I moved onto experiments with PP1-inhibitor Calyculin A. Before investigating the reversibility of Calyculin A, however, I first sought to establish the optimal working concentration. While Calyculin A has been used for the purpose of inducing backward movement in previous experiments, the IC50 curve had not been established. This was an important first step as although concentrations have been effective at preventing dephosphorylation of tethers, these concentrations also increase the possibility of off-target effects on cell health. The ideal working concentration for my experiments is the lowest concentration at which backward movement is caused reliably and consistently, while preserving cell viability. I tested concentrations of Calyculin A ranging from 0.25 nM to 100 nM, administering the drug at the onset of anaphase and looking for backward autosome movement. Higher concentrations induced a 'shrivelled' cell appearance, as demonstrated by a loss of cell smoothness. 'Cabbage leaves' also became thickened and lacked fluidity.

In addition to the problem of reduced cell health and off-target effects which, in turn, call into question the reliability of cause-and-effect conclusions, there is also the important consideration of time. Given that the goal of these experiments was to treat cells with Calyculin A at the onset of anaphase and rinse it out at various stages, it is important that backward movement occur only after anaphase has concluded. With very high concentrations chromosomes move backward before reaching the poles, which does not allow sufficient time for an insect Ringer's rinse. On the opposite end of the spectrum, concentrations of 5 nM and below yielded inconsistent results, suggesting that they were not effective enough at inhibiting dephosphorylation to reliably produce backward movement. My data

indicate that at a concentration of 10 nM, Calyculin A causes backward movement 100% of the time, with autosome velocity increase occurring within about 1.5 to 2 minutes (Figures 4 and 6a). Additionally, cells treated with this concentration maintained their structural integrity and in terms of morphology, resembled control cells.

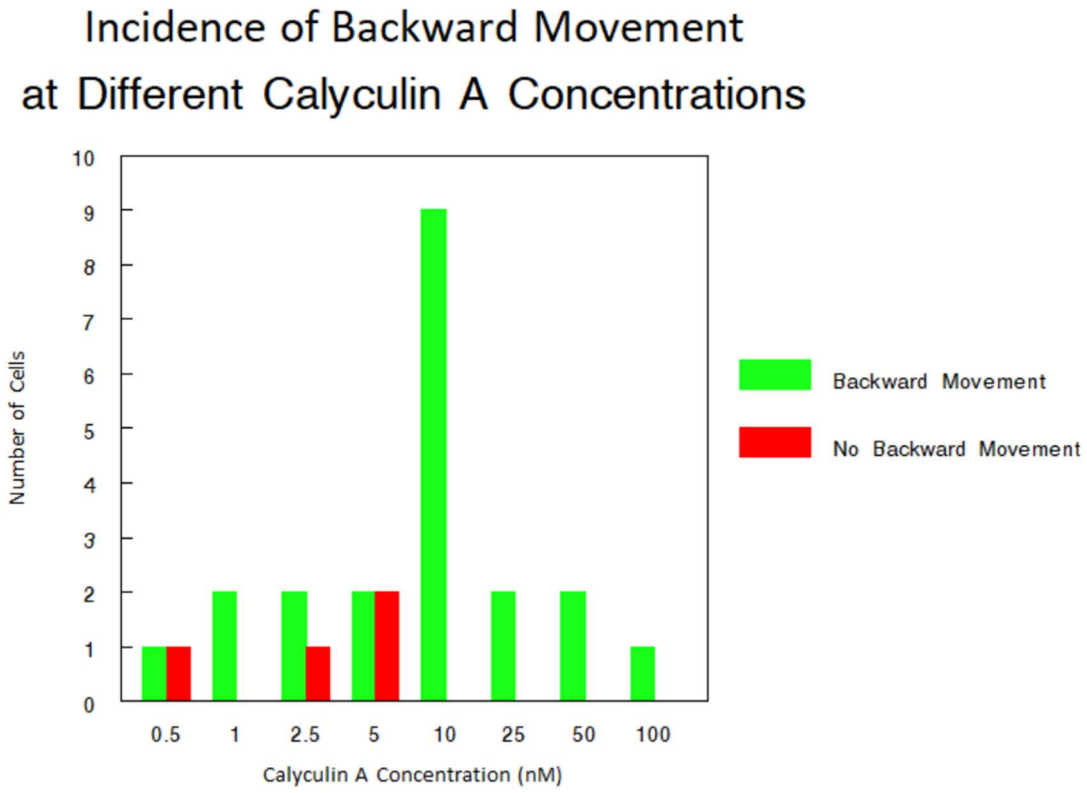


Figure 4: Graphical representation of IC50 data of Calyculin A on backward movement. Based on experiments conducted in a total of 25 cells, wherein Calyculin A was administered at concentrations ranging from 0.5 nM to 100 nM, the IC100 of Calyculin A on backward movement was determined to be 10 nM. Backward movement is produced 100% of the time at concentrations of 10 nM and above.

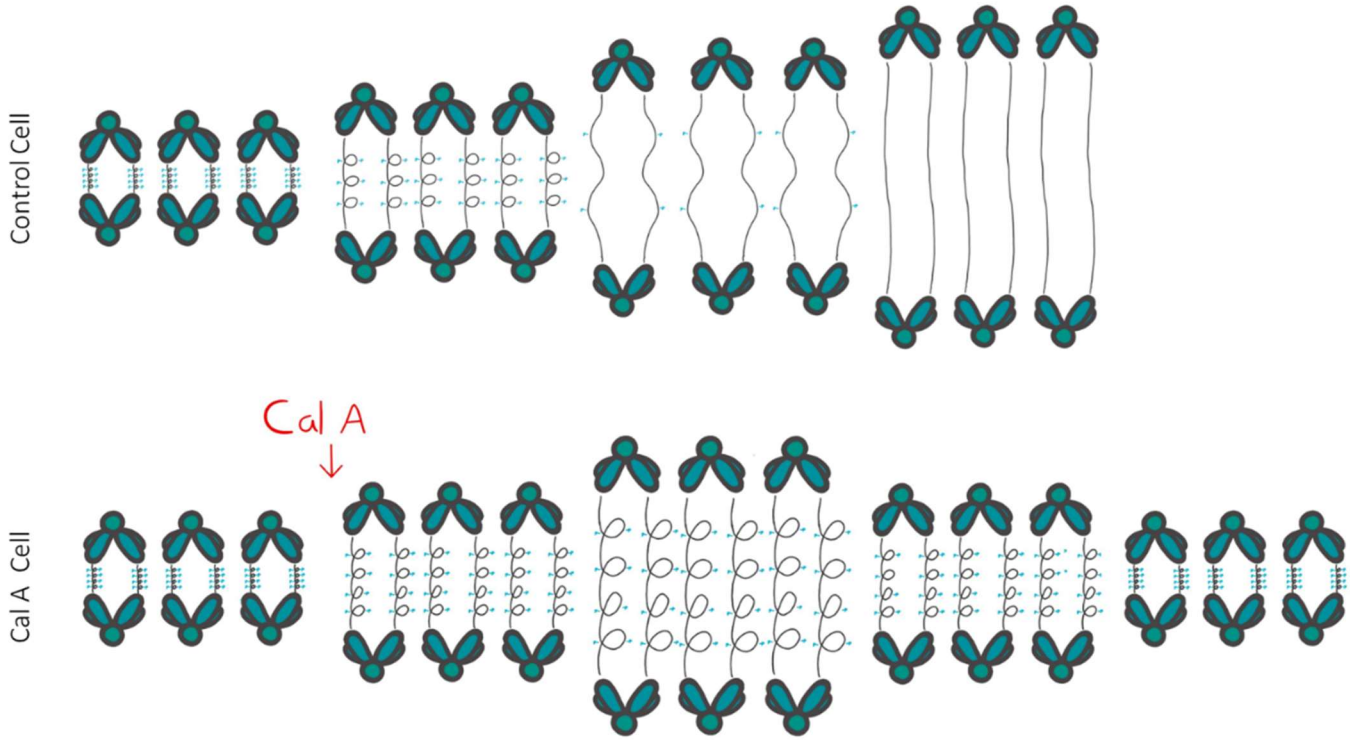


Figure 5: Hand-drawn illustration of control and Calyculin A experiments showing tether phosphorylation and elasticity, as well as anaphase chromosome movement. Coiled lines represent elastic tethers, while straight/wavy lines represent inelastic tethers. Small bright blue 'P's represent phosphate groups. Based on our current model, elastic tethers have more phosphate groups attached, and they become inelastic throughout anaphase in control cells as they are dephosphorylated by PP1. In experimental cells, Calyculin A is added at the onset of anaphase (when partner half-bivalents begin to separate), inhibiting PP1. In these cells, tethers fail to become dephosphorylated and remain elastic throughout anaphase. This results in backward autosome movement, or movement of partner half-bivalents toward each other.

Calyculin A and Insect Ringer's Rinse Cells **– Determining the Reversibility of Calyculin A on Backward Movement**

Having determined the IC50 data and optimal working concentration of Calyculin A on backward movement in crane-fly spermatocytes, I proceeded with the main focus of my experiments with Calyculin A: to determine the reversibility of Calyculin A on backward movement. Based on data collected in the IC50 experiments, I found that a concentration of 10 nM was the lowest concentration at which backward movement was produced consistently. In this next phase of experimentation, I administered 10 nM Calyculin A at the onset of anaphase and rinsed the drug out with insect Ringer's

at various measured stages. The times between Calyculin A perfusion and insect Ringer's perfusion ranged from 42 seconds to 14.1 minutes. Only 3 out of 23 cells did not display backward movement (Table 1). The first 2, which were exposed to Calyculin A for 42 seconds and 1 minute, respectively, cleaved normally. The third cell was exposed to Calyculin A for 2 minutes and, while not moving backward, did not cleave.

Data for Calyculin A experiments were collected by cell, rather than by bivalents. The reasoning for this method of analysis is that cells treated with Calyculin A tend to develop chromosome clumps. While chromosomes may separate to the poles in 3 distinct pairs, they typically form clumps at the poles. The chromosome pairs become indistinguishable, whether backward movement occurs or not. For this reason, the data is represented as number of cells, rather than number of pairs. Additionally, it is important to lay out the criteria for backward movement. Backward movement may be 'complete' in that all 6 autosomes revert to the equator and make contact with their partners. This is typically observed in cells perfused with higher concentrations of Calyculin A. Cells treated with 10 nM Calyculin A may exhibit less or partial backward movement. For the purposes of my experiments, any degree of backward autosome movement was considered indicative of backward movement. In some cases, one autosome moves all the way across the cell to reach its partner at the opposite pole. In other cases, partner autosomes do not make contact, but do move backward. In some cells, some but not all autosomes move backward. Each of these cases is considered backward movement. Variations in the extent of backward movement between chromosome pairs of the same cell are likely influenced by different segregation times. For example, our laboratory experience shows that monochiasmatic chromosomes tend to segregate slightly later than their bichiasmatic counterparts. Thus, tether lengths between partners of different pairs in the same cell may vary at the time of Calyculin A administration.

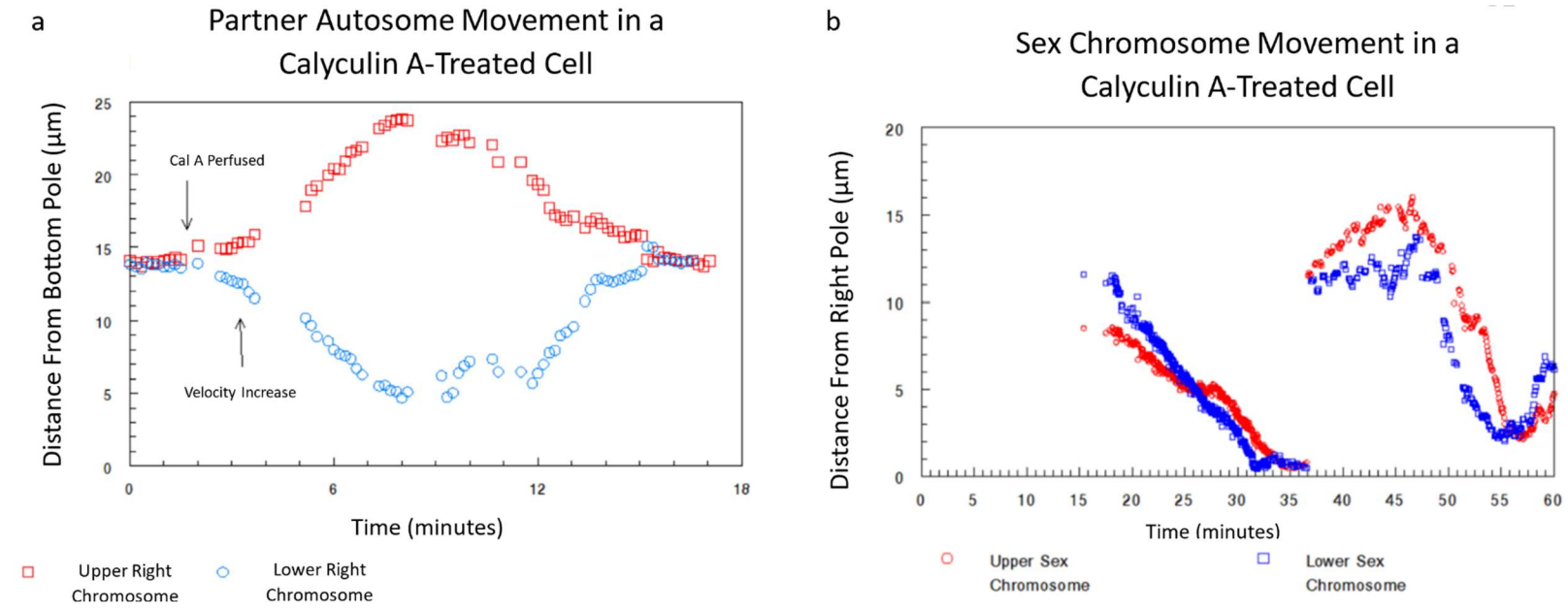


Figure 6: Distance/time movement graphs of (a) partner autosomes and (b) sex chromosomes in Calyculin A-treated anaphase crane-fly spermatocytes. Calyculin A causes an increase in autosome velocity and backward autosome movement (a), as well as sex chromosome oscillations (b).

The results of my experiments with Calyculin A show that its effects on backward movement are not readily reversible when administered at a concentration of 10 nM and washed out with insect Ringer's solution (Table 1). The IC50 data that I have established should provide a helpful framework for future experiments with Calyculin A, allowing researchers to reliably and consistently produce backward movement while minimizing the overall detriment to cell health. Moreover, my experiments with 10 nM Calyculin A and insect Ringer's rinse highlight the importance of PP1 at the very onset of anaphase for tether dephosphorylation. Rinsing Calyculin A out of the cell generally does not restore PP1 activity in time for tethers to become dephosphorylated and inelastic. Overall, these results serve to further characterize the dynamic nature of tether elasticity throughout anaphase.

Table 1: Results of Calyculin A and insect Ringer's rinse experiments. Out of 23 cells, 3 exhibited poleward movement without backward autosome movement. All 3 of these cells were exposed to Calyculin A very briefly, including one exposure of under a minute. Overall, these results suggest that Calyculin A-induced backward movement is not readily reversible.

Calyculin A Concentration	**Approximate Time of Insect Ringer's Rinse**	**Time between Calyculin A Treatment & Insect Ringer's Rinse (min)**	**Backward Movement?**
10 nM	Pre anaphase	6:00	Chromosomes did not separate
10 nM	Pre anaphase	6:21	Chromosomes did not separate
10 nM	Early anaphase	0:42	No (cleaved)
10 nM	Mid anaphase	1:00	No (cleaved)
10 nM	Early anaphase	1:00	Yes
5 nM	Early anaphase	1:30	Yes
10 nM	Early anaphase	1:30	Yes
10 nM	Early anaphase	2:00	No
10 nM	Mid anaphase	2:20	Yes
10 nM	Mid anaphase	2:30	Yes
10 nM	Mid anaphase	2:30	Yes
10 nM	Mid anaphase	2:32	Yes
10 nM	Mid anaphase	3:00	Yes
10 nM	Mid anaphase	3:00	Yes
10 nM	Mid anaphase	3:00	Yes
10 nM	Mid anaphase	4:00	Yes
10 nM	Late anaphase	5:04	Yes
10 nM	Late anaphase	5:06	Yes
10 nM	Late anaphase	6:25	Yes
10 nM	Post anaphase	8:23	Yes
10 nM	Late anaphase	9:11	Yes
10 nM	Post anaphase	12:12	Yes
10 nM	Post anaphase	14:10	Yes

CHAPTER 3: TETHERS ARE UNIQUE STRUCTURES DISTINCT FROM DNA BRIDGES AND DO NOT STAIN WITH DAPI UNDER 'NORMAL' CONDITIONS

Chapter Summary

Physical connections between separating chromosomes in anaphase have been observed in several species and cell types. As tethers gain traction within the scientific community, there is a growing effort to visualize and characterize their properties. One such experiment, described by Warecki and colleagues, revealed DAPI-stained ultrafine bridges between chromosomes, suggesting that tethers are made of DNA. Conversely, the Forer lab has demonstrated titin-specific staining of tethers using a variety of antibodies to effectively target the protein giant. A closer look into the methods of Warecki and colleagues reveals some key differences when compared to our own, potentially accounting for our contrasting results. Namely, Warecki and colleagues prepare *Drosophila* neuroblasts in either PBS or NaCl solutions, while the Forer lab uses crane-fly spermatocytes in insect Ringer's solution. Additionally, Warecki and colleagues' DAPI-stained tethers are only visible with elevated gain. To test whether our tethers and Warecki and colleagues' ultrafine bridges are the same cellular structure, and to elucidate whether procedure differences might impact these final results, we compared control cells prepared under our standard procedures to cells prepared using Warecki and colleagues' methods. Ultimately, we observed DAPI-stained bridges between chromosomes in some experimental cells prepared in NaCl or PBS viewed with high laser power and gain. Contrastingly, we did not observe DAPI-stained strands in control cells prepared in insect Ringer's solution, nor in any cells viewed with normal laser power and gain. We also note indicators of poor health under these experimental conditions, likely due to the lack of ionic balance and proper pH of NaCl and PBS. In summary, our results indicate that our tethers are not, in fact, composed of DNA.

Introduction – Chapter 3

With mounting evidence for the presence of tethers and growing characterization of their behaviour in a wide variety of cell types, tested and supported using a range of experimental methods, a current challenge lays in visualizing these structures. Our most recent article suggests that tethers are made of the giant protein titin (Sheykhani et al., 2024). Titin is responsible for elasticity in myofibrils, as well as skeletal and cardiac muscle. Furthermore, tension within titin is mediated by phosphorylation. Similar to tethers, cardiac titin in the PEVK region is made more elastic by phosphorylation, and this elasticity is reversed by dephosphorylation via the phosphatase enzyme PP1 (Sheykhani et al., 2024). In crane-fly spermatocytes, inhibition of PP1 by the drug Calyculin A results in retained tether elasticity throughout anaphase, exhibited by backward chromosome movement. To investigate whether tethers are composed of titin, we recently stained crane-fly anaphase spermatocytes for titin, using four different antibodies (Sheykhani et al., 2024). Since titin is a giant protein, it usually stains punctate, with primary antibodies targeting a specific portion of the protein. Using four antibodies ensures both comprehensive staining of the different components of titin, while increasing our confidence in on-target staining of titin (Sheykhani et al., 2024). Combined with results from a similar study published in 2007, inter-telomere connections between separating sister chromosomes were stained with 7 different anti-titin antibodies (Fabian, Xia, et al., 2007). Moreover, we also stained myofibrils, which we know for certain contain titin, as an added measure to confirm antibody specificity and reliability of our results and interpretations.

As we were in the process of preparing this article for publication, Warecki and colleagues published their own research on DNA bridges, which they compare to our tethers (Warecki et al.,

2023). Warecki and colleagues use the common DNA stain DAPI in *Drosophila* neuroblasts, viewing these stains with a confocal microscope. Importantly, Warecki and colleagues only observe connections, which were often faintly stained or incomplete, using high gain. Warecki and colleagues also employ different methods for their sample preparation, as they prepare their cells in either PBS or 0.7% NaCl (Warecki et al., 2023). In the Forer lab, we use the carefully balanced insect Ringer's solution to maintain proper tonicity and mimic in vivo conditions as closely as possible when preparing our cells. Importantly, deviations from typical ion concentrations can potentially impact protein stability and interactions, enzyme activity, electrical excitability, osmotic integrity, and membrane permeability (Lockwood, 1961).

Based on their experimental findings, Warecki and colleagues suggest that tethers are made of DNA and are typically resolved during anaphase (Warecki et al., 2023). In the presence of chromosomal breakage, however, Warecki and colleagues suggest that these DNA bridges can recruit tether proteins to stabilize connections between sister or non-sister chromosomes and prevent genome loss(Warecki et al., 2023). Through the following experiments, we sought to combine our own methods with those of Warecki and colleagues to investigate the possibility of DNA in tethers.

Methods

Preparing Living Cells and Observation, Recording and Analysis of Living Cells

Methods are repeated from Chapter 2: Phosphatase Inhibitor Calyculin A Irreversibly Produces Backward Chromosome Movement by Maintaining Tether Elasticity.

PBS and NaCl-Treated Cells

We followed our typical procedure for preparing crane-fly spermatocytes, except that we substituted NaCl or PBS for insect Ringer's solution. NaCl was dissolved into double-distilled water to a final concentration of 0.7%, while PBS was prepared with final concentrations of 131 mM NaCl, 5.1 mM Na_2HPO_4, 1.56 mM KH_2PO_4 and double distilled water, to an adjusted pH of 7.0. I dissected testes from larvae under halocarbon oil, rinsed them in drops of NaCl or PBS and immersed them in perfusion chambers containing NaCl or PBS. Fibrinogen was also made using NaCl or PBS in place of insect Ringer's solution. An additional portion of the experiment entailed perfusing approximately 10 mL of NaCl or PBS through the preparation. All of these steps involving NaCl or PBS were modified from our standard insect Ringer's procedure to create maximum exposure to the solutions NaCl or PBS.

Immunohistochemistry and Confocal Microscopy

For immunostaining, cells from control, NaCl and PBS experiments were lysed, fixed and stained for observation under confocal microscopy. I lysed cells by perfusing 10 mL of lysis buffer and, after 10-20 minutes, transferring coverslips to small petri dishes filled with lysis buffer. The lysis buffer stabilizes the cytoskeleton and the detergent causes the rest of the cell to be washed away. Lysis buffer is composed of 100 mM Pipes buffer (pH 6.9), 10 mM EGTA, 5 mM MgSO4, 5% DMSO, and 1% Igepal. After a minimum of 30 minutes, coverslips were removed from lysis buffer and fixed by adding several drops of glutaraldehyde to the coverslip, after 4 minutes rinsing the coverslip in PBS, immersing the coverslip in glycine (for 2 minutes, to neutralize any free aldehyde groups) and rinsing the coverslip again in PBS. Fixed preparations were stored in 50% glycerol in PBS and refrigerated until staining.

I stained fixed cells for total tubulin and for DNA. Total tubulin was stained using the primary antibody YL ½ at 1:500 or 1:1000, and then the secondary antibody Alexa 488 at 1:50 or 1:100. DNA was stained using 1:100 Sigma Aldrich Readymade Solution DAPI. All dilutions are made with PBS. The procedure for staining is as follows. Coverslips are rinsed cell-side up 2-3 times in petri dishes of PBS, for 15-30 minutes each. Coverslips are then squirted with PBS, PBS, and PBS/Triton X to prepare for staining. The coverslips are inverted over 100 µL of primary antibody solution and allowed to incubate for 1 hour. Coverslips are then rinsed in 3 petri dishes of PBS, for 5-15 minutes each. They are again washed with PBS, PBS and PBS/Triton X and are subsequently inverted over 100 µL of secondary antibody solution for 1 hour. Secondary antibodies and DAPI are prepared in the dark and preparations are incubated under a black sheet. After secondary antibody incubation, coverslips undergo 3 PBS rinses and PBS, PBS, PBS/Triton X washes again before being inverted over 100 µL of DAPI for 15 minutes to 1 hour. We detected no difference in the DAPI staining based on incubation periods within this time-frame. Finally, the coverslips were rinsed 3 times in PBS and placed in 1 drop of glycerol for 1-2 minutes. They were then placed cell-side down into 100 µL of Mowiol on a frosted slide. Stained preparations were placed in a fume hood under protection from light for 2 to 3 days to allow the Mowiol to harden, after which they were stored in the fridge.

Stained preparations were observed using a Zeiss Laser Scanning LMS 700 confocal microscope. Images were taken using the channels Alexa 488 (total tubulin, green), DAPI excited with a 405 nm laser (DNA, blue) and DIC. I performed two Z-stack experiments for each cell analyzed. The first Z-stack was performed under normal parameters. The second Z-stack was performed with drastically increased gain, laser power or both for the DAPI channel. Z-stacks were then analyzed using Fiji Image J.

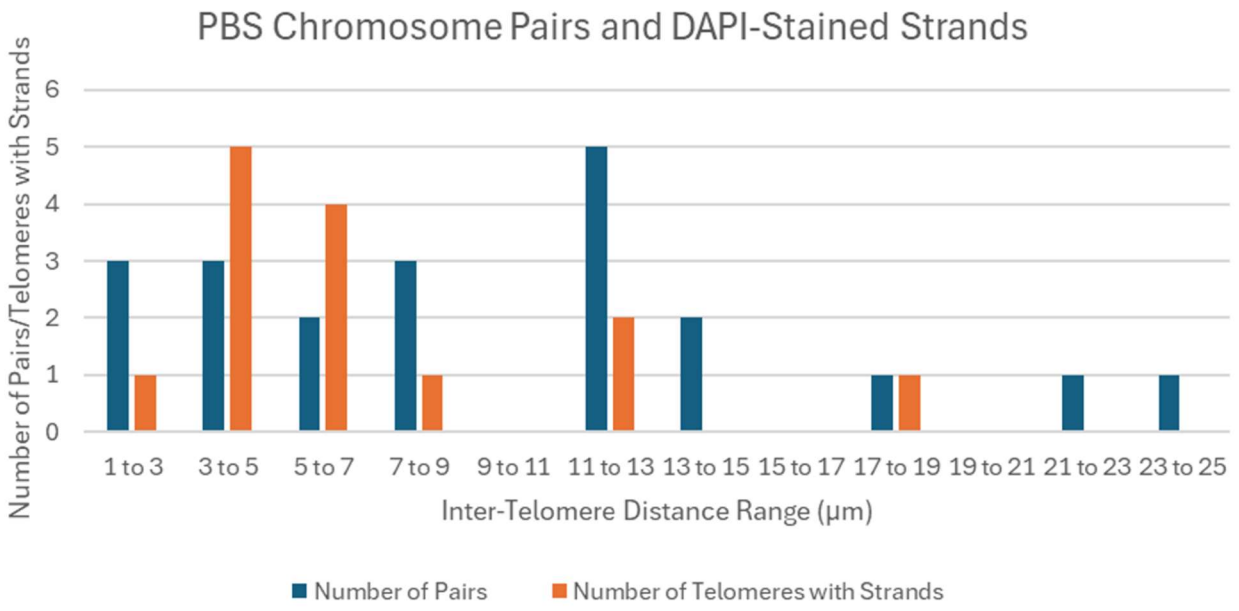
Results and Discussion

Live PBS and NaCl-Treated Cells

Several features of crane-fly spermatocytes prepared in NaCl or PBS distinguish them from healthy cells in insect Ringer's solution. For example, cells in PBS or NaCl tend to lack healthy-looking 'cabbage leaves', have clumped chromosomes and appearing 'shrivelled.' They also have stretched arms during anaphase. Arms are stretched to longer lengths and throughout all of anaphase. In normal cells, arms stretch less and if stretched they are stretched no later than mid anaphase.

DAPI-Stained Strands in PBS and NaCl-Treated Cells

A high proportion of telomeres were connected to DAPI-stained strands when viewed at high laser power and gain. Inter-telomere distances and potential strands were assessed for a total of 17 NaCl and 21 PBS chromosome pairs (Figure 7). Of these pairs, 4 strands were observed in NaCl cells and 14 in PBS cells. Like the results of Warecki and colleagues, not all inter-telomere connections stained similarly. Approximately 33% of DAPI-stained strands I observed were complete (Figure 8a). I noticed that complete strands tend to be stained more faintly in the middle, but nevertheless visibly connect two telomeres. The remaining 67% of strands were either incomplete or presented as very short extensions, or 'dimples' pulled out from the telomere (Figure 8c). Of the strands that Warecki and colleagues describe in their article, about 25% were complete (complete, uninterrupted) and 75% were incomplete (interrupted) or dimples (stretched/erstwhile). Additionally, Warecki and colleagues observed strands mostly in early anaphase cells. I observed strands more frequently in cells with inter-telomere distances of less than 10 µm. In contrast to these experimental results, I did not observe DAPI-stained strands in control (insect Ringer's) cells.



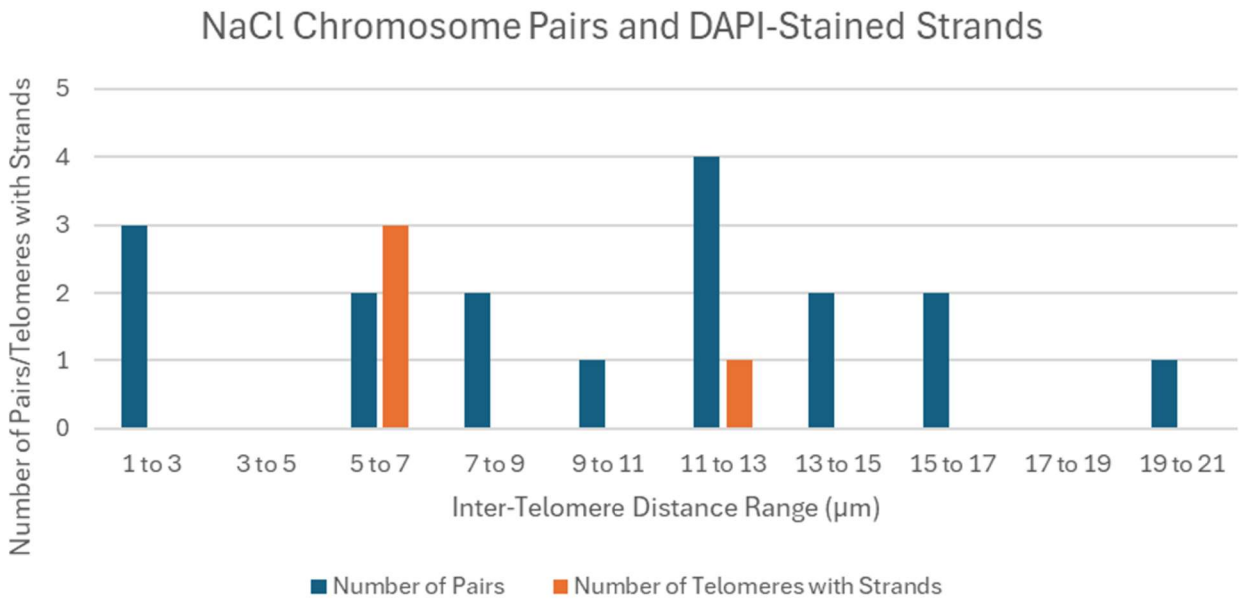


Figure 7: Bar charts comparing number of chromosome pairs analyzed to number of telomeres with strands observed, categorized by inter-telomere distances in 2 µm intervals. Inter-telomere DAPI-stained strands were observed more frequently in PBS-treated cells than in NaCl-treated cells. Strands were also observed more frequently in cells with inter-telomere distances of less than 10 µm.

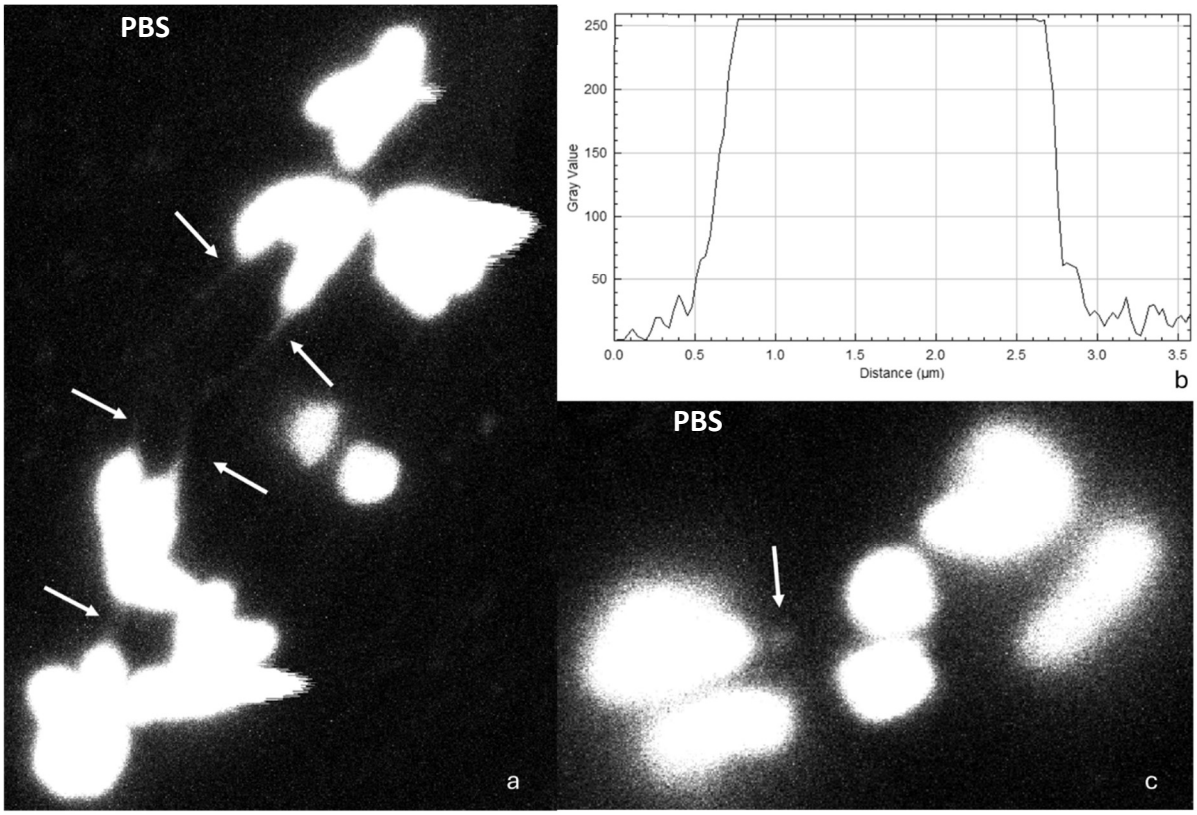


Figure 8: DAPI channel hyperstacks showing (a) a mid-late anaphase PBS-treated cell using high laser power/gain with strands emerging from 5 telomeres, indicated by white arrows. (b) A saturated intensity curve of the bottom chromosome in (a) illustrating high laser power/gain. (c) An early anaphase PBS-treated cell observed using high laser power/gain, with an incomplete strand/dimple emerging from a telomere, indicated by the white arrow.

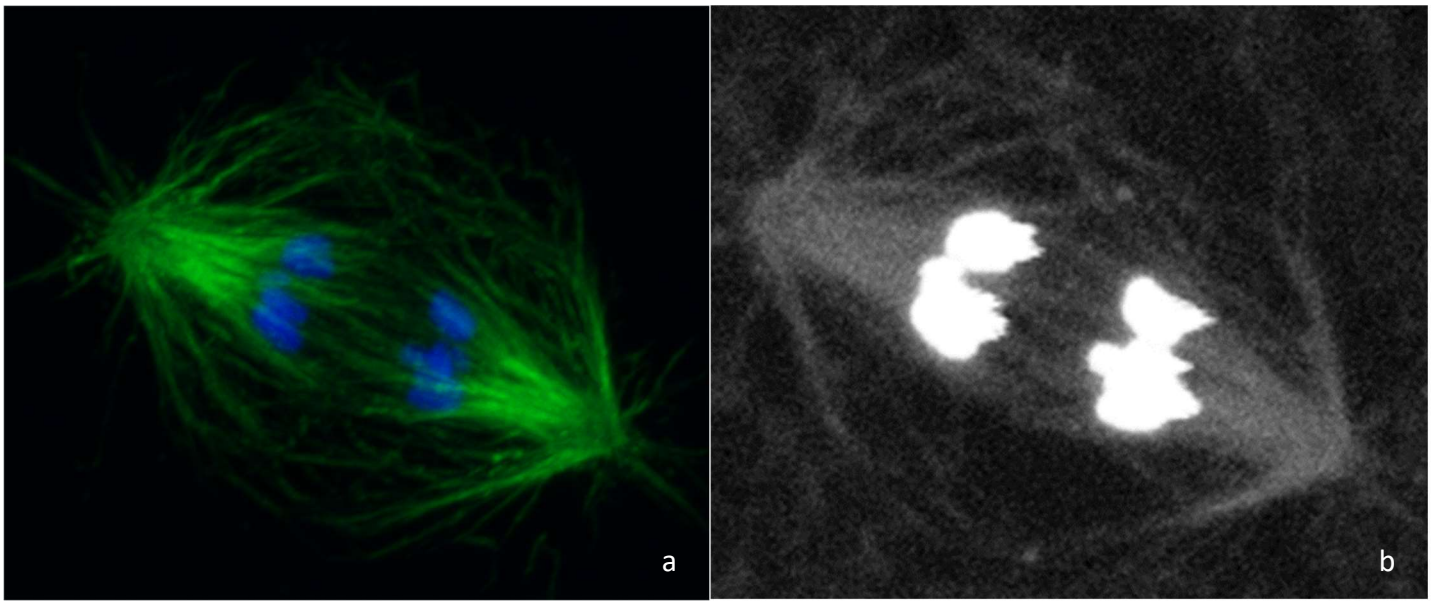


Figure 9: (a) Merged confocal image of a mid anaphase control cell stained for tubulin (Alexa 488, green) and DNA (DAPI, blue), with normal laser power/gain. (b) The same cell viewed with high laser power/gain in the DAPI channel.

Other Abnormalities in Stained Preps

In addition to these strands, several other abnormalities were observed (Figure 10). Firstly, many of the cytoskeletons appeared unhealthy. Some of these cells with abnormal microtubules and chromosome distortion were not interpretable for inter-telomere data collection. These cells were found in each NaCl or PBS DAPI-stained preparation and illustrate the detriment of using NaCl or PBS. For example, many cells showed stained clusters of microtubules in the interzonal space. This is abnormal, as healthy cells typically have microtubules concentrated around the interzonal space, with fewer, clearly defined kinetochore and polar microtubules interspersed between separating chromosomes. Contrastingly, some cells showed microtubules that were overall sparse and appeared to have distorted morphology.

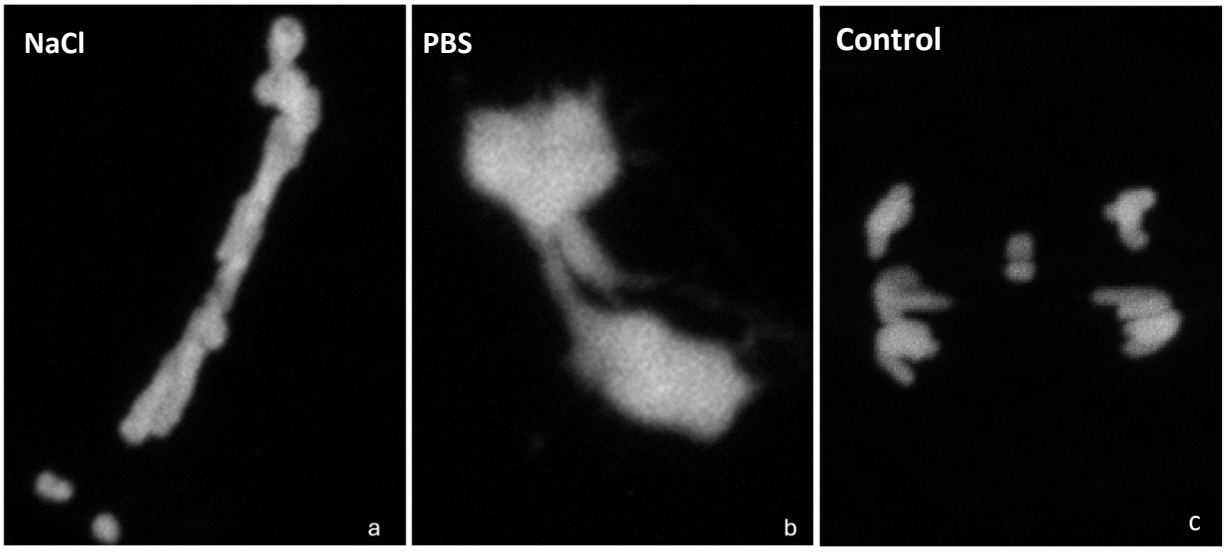


Figure 10: Confocal images (normal laser power/gain) of DAPI stained crane-fly spermatocytes treated with (a) NaCl, (b) PBS and (c) insect Ringer's solutions (control). The NaCl-treated cell (a) is unhealthy and may be in prometaphase, but is ultimately uninterpretable in terms of data collection. Dramatic chromosome stretching is visible. The PBS-treated cell shown in (b) is also uninterpretable. It is unhealthy, with abnormally stretched chromosomes. In comparison, the anaphase control cell shown in (c) has typical looking autosomes (segregating to the poles) and sex chromosomes (at the equator). All 6 half bivalents and both univalent sex chromosomes are clearly identifiable.

Off-Target Staining

Several lines of evidence, both from my research as well as articles in the literature, indicate that DAPI does not have the degree of specificity for DNA binding that many scientists believe. While DAPI is hailed as a gold-standard and 'go-to' DNA stain, several publications draw attention to the fact that DAPI is susceptible to off-target staining. For example, DAPI has a high affinity for protein aggregates and especially to amyloid fibrils (Mora et al., 2020).

My own research corroborates these findings. Firstly, I saw DAPI staining of the entire spindle at high laser power/gain (Figure 11a). I saw staining patterns that seem to overlap with spindle staining, calling into question the reliability of data collected under these conditions. Polar microtubules or sex chromosome kinetochore microtubules in many cases are passable as inter-telomere connections between separating chromosomes. In addition to microtubule staining, I also observed staining of the centromeres. Interestingly, this off-target staining differs from the microtubule staining, as the centromeres actually stained more strongly in the DAPI channel. In other words, the centromere staining is not a simple consequence of the high power 405 laser used for DAPI exciting 488 channel fluorophores. This finding may be due to the satellite DNA present in centromeres, however, the following staining experiment provides more compelling evidence that DAPI binds to more than just DNA.

As a part of my contribution to the manuscript, "Titin is present in the elastic tethers that connect separating anaphase chromosomes in crane-fly spermatocytes," I collected confocal microscopy images of crane-fly myofibrils stained for actin (Phalloidin Alexa Fluor 488), titin (Alexa 568 or DyLight 550) and DAPI. While not relevant for this publication, DAPI staining was done to obtain

further data regarding specificity. Myofibrils are the contractile unit in muscle fibres and are composed primarily of actin and myosin filaments, as well as proteins such as titin. Fascinatingly, myofibrils stained with DAPI, despite containing no DNA (Figure 11b). Furthermore, miscellaneous 'junk' in these preps was also visible in the DAPI channel. The observation of DAPI-stained myofibrils provides compelling evidence of DAPI's potential for off-target staining, and these results are strongly supported by the literature.

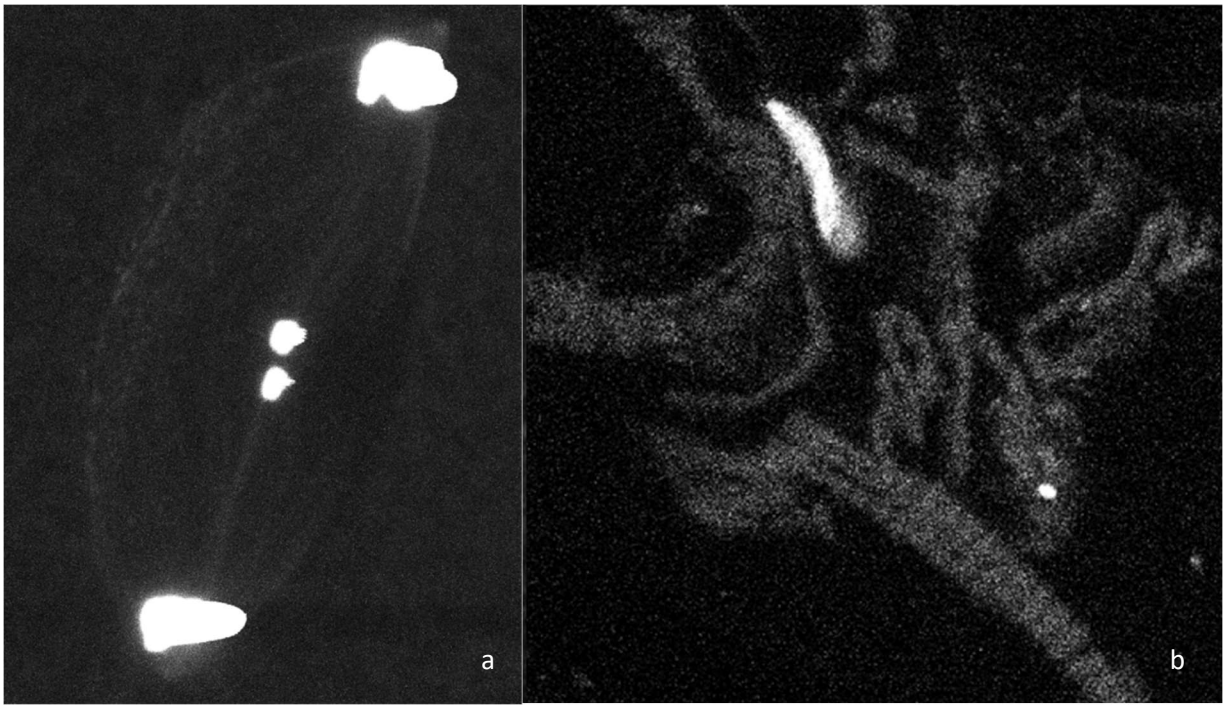


Figure 11: Confocal images with high laser power/gain DAPI staining of (a) a late anaphase control anaphase crane-fly spermatocyte and (b) rabbit myofibrils. There are no strands between chromosomes in the spermatocyte (a), but strong tubulin and background staining is evident. Note myofibril staining, as well as unidentified brightly stained objects in (b).

More specifically, the literature investigating DAPI's specificity, albeit limited, suggests that DAPI's binding affinity extends beyond DNA and varies significantly depending upon conditions. Most articles describing DAPI binding explore the different modes of DAPI binding to DNA, and do not

investigate potential non-DNA binding. Notably, a recent study published in 2020 by Mora and colleagues suggests that the reliability of DAPI assays for measuring cellular nucleic acid content in the presence of protein aggregates depends on several factors, including the specific experimental conditions and the nature of the protein aggregates. They found both false-positive signalling and background noise, suggesting that DAPI binds to protein aggregates, perhaps even more readily than to DNA (Mora et al., 2020). Additionally, other findings identified smooth muscle titin aggregates using electron microscopy, suggesting an amyloid nature based upon a distinct shift in absorption spectrum when stained with Congo red (Bobylev et al., 2016). Given the evidence suggesting that titin forms amyloid protein aggregates, it seems plausible that DAPI could bind off-target to titin and other proteins. An even more current article published in 2023 showed SiR-DNA/SiR–Hoechst-induced DNA damage and anaphase chromatin bridges (Rajendraprasad et al., 2023). Interestingly, this damage was only observed in SiR-DNA/SiR–Hoechst-stained cells under 640 nm laser-based imaging; not with SiR-DNA/SiR–Hoechst or laser-based imaging, alone. This bridge-causing interplay between DNA stain and imaging laser begs the question of whether the high laser-power technique of Warecki and colleagues impacts the stained chromatin, directly.

While DAPI remains a seemingly accessible and effective go-to for scientists practising techniques such as confocal microscopy, flow-cytometry and Fluorescence In Situ Hybridization (FISH), mounting evidence warrants a degree of caution when producing and interpreting DAPI-stained samples. The same may be said for the use of PBS and NaCl for cells. It is my hope through publishing this research into DAPI-stained DNA bridges that tethers not only become better understood, but also that readers will be encouraged to be mindful about the potential caveats of PBS/NaCl and DAPI.

CHAPTER 4: DISCUSSION

Future Work

Through my research and learning as a Master's degree student in the Forer lab, I have come to discover that many of the principles taught during my undergraduate studies are not, in fact, universal. Reevaluating what I do not know about cell biology has given me new perspective and drive to develop a deeper understanding of the structures and processes governing cell division. I have developed a passion for shedding light on a cellular structure that is not generally taught or recognized, even by fellow cell biologists. As tethers appear to aid in chromosome communication and coordination of movement, they will likely become relevant in a broad variety of research areas, including aneuploidy, cancer (especially in terms of telomere degradation), cell rescue, aging, non-random segregation and more. In addition to potential applications of tether research, I am interested in dispelling the common misconception that cells are uniform and divide in a clear-cut fashion. Building upon the findings of my master's research, future goals in the field may include developing a deeper understanding of the mechanisms underlying tether phosphorylation and its implications across a broad spectrum of species.

To date, tethers have only been shown in a small subset of animal cells (Forer et al., 2017). Tethers are present in all of these animal cells, but it is also unknown if tether elasticity is controlled by dephosphorylation in any cell type other than crane-fly spermatocytes (Forer et al., 2017). One of many exciting avenues for future research involves broadening the horizons of tether research to encompass more cell types. For example, tether research has never been conducted in plants. Given their important role in chromosome segregation and cell division, as well as the promising fact that most higher eukaryotic plants produce several forms of titin, this presents a very exciting opportunity to further characterize tethers in the context of evolution (Mohanta et al., 2019). If tethers exist in both plants and animals, we can likely trace their evolution further down the evolutionary tree of life. Another cell type that might provide valuable insight into the prevalence and function of tethers is

human cancer (U2OS) cells, which should be particularly interesting since tethers attach to telomeres and telomere abnormalities are characteristic of cancer cells. Showing tethers in diverse cell types should strengthen the case that they are present in all cells. Moreover, investigation of tethers in healthy versus cancerous, typical versus atypical, sex versus somatic, and animal versus plant cells will provide much needed data on this largely unrecognized cell structure.

Future experiments might also focus on further characterizing the composition and phosphorylation of tethers. While we have stained tethers with anti-titin antibodies, overlapping microtubules confound the images. Obtaining clear, unobstructed images of tethers is challenging, especially because titin is associated with multiple cellular structures, likely including but not limited to spindle fibres, centrioles, nuclear membranes and chromosomes. Future experiments might use NOC to disassemble interpolar microtubules. Interestingly, NOC also produces backward movement by eliminating the opposing (poleward) force to tethers. NOC-treated cells can be lysed, fixed and stained with anti-titin antibodies for microtubule-free tether imaging. Producing backward movement using NOC should corroborate the findings of my Calyculin A experiments by producing backward movement via a different pathway, without affecting tethers directly. Moreover, staining NOC-treated cells would also compliment my experiments with DAPI. While my experiments indicate that tethers are not made of DNA, our opposing hypothesis that tethers are made of titin would be strengthened by more convincing titin staining. To advance tether staining even further, these cells can also be stained with anti-phosphorylated serine/threonine antibodies or, even better, anti-phosphorylated titin antibodies. These experiments should provide clearer and more convincing images of tethers. Currently, it remains unclear whether tethers, themselves, are dephosphorylated, or if tethers lose elasticity as a

downstream consequence of PP1 inhibition. Staining with anti-phosphorylated titin antibodies should also help clarify the mechanism of phosphorylation mediated elasticity.

In addition to experiments with other chemical agents, our understanding of phosphorylation-mediated tether elasticity could also be strengthened by incorporating physical/mechanical methods. A caveat of working with a drug like Calyculin A is that the drug affects many cellular processes and structures apart from tethers, as PP1 has many substrates within the cell (Bollen et al., 2010). One way to test whether Calyculin A-induced backward movement is, in fact, due to the drug's effect on tethers would be to treat cells with Calyculin A and use a laser microbeam to sever tethers. In this experiment, the tether(s) between one of the three chromosome pairs could be ablated during backward autosome movement. If backward movement is halted by severing the tether, this would support the hypothesis that Calyculin A-induced backward movement is caused by elastic tethers. Overall, my experiments shed light upon the structure and function of tethers, suggesting that they are made of titin and that they are dephosphorylated by PP1 at the beginning of anaphase. These characterizations open the door for further research and exploration into this key cellular structure.

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