

***Mesostoma ehrenbergii* SPERMATOCYTES PROVIDE AN UNUSUAL AND EXCITING
MODEL TO INVESTIGATE CHROMOSOME MOVEMENT, CLEAVAGE FURROWS
AND TETHERS DURING CELL DIVISION**

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ABSTRACT

For my PhD thesis I studied and tracked chromosome movement in primary spermatocytes from a species of aquatic flatworm, the *Mesostoma ehrenbergii*, with the hope to better understand what determines the force behind these intricate and highly co-ordinated movements during meiosis. Canonical theories of chromosome movement state that microtubules are the main producers of force for chromosome movement in the meiotic spindle, however my research has shown chromosomes can not only move but can move even faster in the absence of microtubules. I also tracked the cleavage furrow and showed it was able to simultaneously move and ingress in the absence of microtubules. I worked on determining how the force is produced by using a laser microbeam to sever various components in the cell and by adding various myosin and actin inhibiting and enhancing drugs to see how chromosome movement may be altered. In addition, I discovered elastic tethers are present in *Mesostoma* during metaphase and anaphase and my research on functionally disabling tethers suggests they play a role in coordinating or maintaining non-random chromosome movement in these cells.

In sum, my PhD research has added evidence to the non-microtubule model of chromosome movement by showing actin and myosin is involved in movement and that tethers may play a role in coordinating or maintaining non-random chromosome movement in these cells.

For Alora

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GENERAL INTRODUCTION

My work focuses on observing chromosome movements, with the hope to better understand what determines the force behind these intricate and highly co-ordinated movements during meiosis. The currently accepted models of chromosome movement position microtubules as the main producers of force within the meiotic spindle. My research adds evidence to non-microtubule-based models of chromosome movement, the spindle matrix model and/or the actin-myosin models. In particular, I study the primary spermatocytes from *Mesostoma ehrenbergii*, a species of hermaphroditic flatworm. These cells undergo a very unusual meiotic division, the details of which have been reviewed (Ferraro-Gideon et al. 2014). Their unique cell division provides a useful tool to question the canonical theories of cell division. To start off with, I will first provide a background on the conventional models of meiosis and then describe meiosis in *Mesostoma* spermatocytes.

A. Conventional Meiosis

Meiosis is a specialized form of cell division. It is a form of sexual reproduction in eukaryotes and generates diversity within the population (Ohkura 2015), converting diploid cells into haploid cells. Meiosis occurs in the germ cell line and has two successive rounds of cell division, meiosis I and meiosis II. Meiosis I is generally a reductional division, where homologous chromosomes separate, and the cells become haploid. Meiosis II is generally an equational division, where the sister chromatids separate (Ohkura 2015). The result are four unique gametes; in the case of oogenesis, it is one egg plus three polar bodies, and in the case of spermatogenesis it is four sperm. The diploid number is restored when the gametes from the two sexes fuse to form the zygote (Champion and Hawley 2002). I will first discuss the features of a

typical meiosis, and then provide a comparison to animals that have unusual cell division strategies.

A. 1. Prophase I

The main outcome of prophase is homologous recombination. This is the first source of genetic variation in meiosis by creating new combinations of parental genes. Crossing over occurs between homologous non-sister chromatids at regions of sequence homology (Page and Hawley 2003). A structure called the synaptonemal complex forms to hold homologous chromosome pairs together along their entire lengths to promote crossing over. The DNA from one chromatid inserts into another, known as a single strand invasion, which then stabilizes to form a double Holliday junction (Schwacha and Kleckner 1995). This forms a lasting connection known as a chiasma. Every homolog pair creates at least one chiasma (Morgan 2007). By the end of prophase, the chromosomes form bivalents, which contains a set of two paternal chromatids (one homolog) physically linked together at the chiasma with two maternal chromatids (second homolog). This configuration is also referred to as a tetrad. Bivalents are held together not only at the chiasma but also the protein cohesin, which holds the bivalents together at the centromeres and along the lengths of the chromosome arms (Paliulis and Brady 2000). This bivalent geometry is crucial and helps homologs monitor their partner's position, ensuring proper chromosome alignment and segregation at later stages of division (Brady and Paliulis 2015).

A. 2. Prometaphase I

Bivalents are now visible in the cell, and the bipolar spindle which is made up of microtubules begins to form at either pole and captures chromosomes (Wieser and Pines 2015). The “search and capture model” describes how microtubules, which are constantly growing or shrinking, reach out in the cytoplasm and eventually contact a chromosome's kinetochore. This

process involves many different plus-end binding proteins. (Mimori-Kiyouse and Tsukita 2003). Microtubules attach to chromosomes at specific localized regions called the kinetochores (Cheeseman and Desai 2008). Some chromosomes do not have localized kinetochores and are instead holocentric (Maddox et al 2004). The proper set up and attachment of the spindle to the kinetochore of every chromosome is crucial to ensure successful segregation at later stages. Chromosome congression signals the end of prometaphase, which is when the chromosomes orient themselves near the metaphase plate.

A. 3. Metaphase I

Once the spindle is set up chromosomes begin to congress and align along the metaphase plate. The random assortment of chromosomes on the metaphase plate is the second major source of genetic variation in meiosis. According to Mendel's law of independent assortment the separation of alleles for one gene is independent to allele separation for another gene (Mendel 1865). Chromosomes must also align properly and achieve bi-polar orientation. The correct orientation for metaphase I is when sister kinetochores of the same homologue behave as one unit and face the same spindle pole, lying side by side (Paliulis and Nicklas 2000). Sister kinetochores behave as a single kinetochore, acting as though they are fused together (as reviewed in Moore and Orr-Weaver 1998). This is due to monopolin proteins which silence one kinetochore (as reviewed in Page and Hawley 2003, Toth et al 2000, Rabitsch et al 2003). Most bivalents achieve this orientation by the end of prometaphase, but some must go through a series of trial and error (Page and Hawley 2003). Misalignment is detected by a tension sensing mechanism within the cell. If one chromosome is misaligned it will delay anaphase onset (Rieder et al 1994). This was determined via a micromanipulation experiment where a needle applied tension to a misaligned chromosome, stimulating the cell to enter anaphase (Li and Nicklas

1995). Once the correct orientation is achieved, the increase in tension between the kinetochore and the spindle allows the cell to enter anaphase.

A. 4. Anaphase I

What marks the beginning of anaphase I is the removal of cohesion along the chromosome arms, but not at the centrosome (Paliulis and Nicklas 2000). This specific removal of cohesion ensures only homologous chromosomes and not chromatids separate from one another, a key difference between meiosis II and somatic mitosis (Morgan 2007). The key regulator for cohesin removal is the anaphase promoting complex, or cyclosome (APC/C) (Morgan 2007). Once cohesin is cleaved, anaphase commences (Yanagida 2015; Wieser and Pines 2015). Anaphase is separated into two processes. Anaphase A is when chromosomes move towards either pole due to the shortening of the spindle microtubules. Various motor proteins which act at either the kinetochore end (Pacman) and/or the poleward end (Flux) function to move the chromosomes (Morgan 2007). Anaphase B is when chromosomes separate because the spindle poles move apart (Morgan 2007). The result of anaphase I are two haploid cells with sister chromatids still linked together.

A. 5. Telophase

Once chromosomes have segregated towards their respective cell poles by the end of anaphase, telophase begins. The mitotic spindle disassembles, the chromosomes decondense, and the nuclear membrane reforms around each set of chromosomes, separating the two sets of DNA (Morgan 2007). Telophase is the last phase in the M phase of nuclear division. The next phase, cytokinesis, is separate and is when the cytoplasm of the two daughter cells divides.

A. 6. Cytokinesis

In animal cells the cleavage furrow is a contractile ring made of actin and myosin II as well as various other regulatory proteins (Eggert et al. 2006). Once anaphase begins the furrow ingresses at the midzone of the cell during telophase, eventually cleaving the progenitor cell into two equally sized daughter cells during cytokinesis (Barr 2007). Furrow position is determined before or during anaphase onset, usually during the metaphase-anaphase transition and is irreversible even if the mitotic apparatus is removed (Rappaport 1981). The three main furrow positioning models are the astral stimulation, astral relaxation and central spindle stimulation models (Barr 2007). These models all rely on the presence of microtubules to transmit a signal to the cell cortex, whether it be astral microtubules that produce either a positive or a negative furrow stimulus (Rappaport 1961, 1965, 1985), and interzonal (non-kinetochore) microtubules which transmit signaling proteins to the cortex by an unknown mechanism as shown in various cell types through addition of drugs that selectively target astral microtubules while conserving interzonal microtubules (von Dassow 2009) or the creation of asterless mutants (Bonaccorsi et al. 1998). Although there are several other theories that we have not mentioned, the two requirements all these theories share is the presence of microtubules and the commencement of anaphase.

A. 7. Meiosis in other organisms

So far, I have only described standard meiosis, the main goal of which is to produce four unique gametes with a random assortment of chromosomes. While the standard view is correct for some species there are a variety of species that employ drastically different mechanisms of cell division. For example, during spermatogenesis in *Sciara* there is no pairing of homologues and no bivalents form (Metz et al 1926). Instead, a monopolar spindle with one giant centriole

forms at one pole and only attaches to maternal chromosomes, segregating them towards that pole. (Esteban et al 1997; Fuge 1994, 1997; Kubai 1987). Similarly, the diploid unisexual female-only fish *Poeciliopsis monacha-lucida* (Schultz 1966, 1967; Hubbs and Hubbs, 1932, 1946; Kallman, 1962) completely excludes the paternal chromosomes from the genetic continuum during oogenesis. A unipolar spindle forms and only the maternal chromosomes align along the metaphase plate (Cimino 1972 A and B). And in the coccids, or mealybugs, an entire haploid set of chromosomes in the male embryo becomes heterochromatic and remains so throughout the animal's development (Schrader 1921). These chromosome(s) are genetically inactive during the animal's course of development and are excluded from the genetic continuum (Brown and Nur 1964). Thus, the standard picture of meiosis provides only one example of meiosis and is not utilized by all species.

B. *Mesostoma ehrenbergii*, a general overview

Mesostoma are a diverse species of predacious aquatic flatworms (Blaustein and Dumont, 1990; DeRoeck et al., 2005; Trochine et al., 2005; Trochine et al., 2006). They are in the order Rhabdocoela, class Turbellaria, and have been well-described anatomically by ecologists for decades (Ferguson and Hayes, 1941; Kolasa and Schwartz, 1988; Bedini and Lanfranchi, 1990; Kalita and Goswami, 2012). They are found in both fresh and saltwater systems (Woodworth, 1897; Graff, 1913), and have unique feeding behaviors (Schwartz and Herbert, 1981; Wrona and Koopowitz, 1998). They prey on a host of microscopic organisms such as mosquito larvae and can potentially offer pest control (Case and Washino, 1979; Kolasa and Mead, 1981; Kolasa, 1984; Kolasa et al., 1985; Blaustein and Dumont 1990; Tranchida et al., 2009). *Mesostoma* adults are hermaphroditic and can lay two different types of eggs depending on the environmental conditions (Bresslau, 1903; Fiore and Ioalè, 1973; Domenici

and Gremigni, 1977; Heitkamp, 1977). Animals that contain S-eggs, which are subitaneous eggs with a thin shell and little yolk, will give birth to live young when reared in favourable conditions (Fiore and Ioalè, 1973). In unfavourable conditions adults will produce D-eggs, which are diapausing eggs with a thick shell and are yolk-rich, also known as overwintering eggs (Fiore and Ioalè, 1973). These eggs need a period of several months rest in order for the young to hatch and can be stored for an indefinite period at temperatures below 0°C and in the dark (methods described in Hoang et al. 2013). D-eggs can be stored for an indefinite period of time in a typical refrigerator, making them conducive for rearing in the lab. Specifically, we work with and rear the *Mesostoma ehrenbergii* from a protocol developed in-lab that encourages the worms to produce live young (Hoang et al., 2013). We extract the testes material from an adult and study the primary spermatocytes. *Mesostoma ehrenbergii* spermatocytes chromosome number and morphology was first described by Husted and Ruebush (1940). These cells have three bivalents which span across the middle of the cell, four univalents, of two kinds, oriented at either pole, and a precocious cleavage furrow which gives these cells a characteristic dumbbell-like shape. These cells have several unusual features, the details of which will be fully described, but in summary the bivalents persistently oscillate, the univalents perform excursions and segregate at a distance, the furrow forms precociously and the cell is theorized to divide via non random segregation (as described in Ferraro-Gideon et al. 2013 A). These unusual features make this cell a very advantageous model to use to probe the mechanisms behind cell division.

B. 1. Prometaphase bivalent oscillations

Bivalent chromosomes in *Mesostoma* spermatocytes are relatively large and span across the midzone of the cell with their kinetochores stretching from pole to pole (Fuge 1987, 1989; Fuge and Falke, 1991). The main feature of bivalent movement is they constantly oscillate

towards and away from their respective poles. The three bivalents persistently oscillate towards and away from either pole, at an average speed of between 5-6 $\mu\text{m}/\text{min}$, without ever aligning at a metaphase plate (Ferraro-Gideon et al. 2014). Instead, bivalents spend upwards of 2 hours consistently oscillating in prometaphase before directly entering into anaphase. When bivalent movement is plotted in a movement graph, the oscillations follow a saw-tooth like pattern where bivalents slow down as they reach the pole and briefly pause for a couple of seconds at the pole before again moving away from the pole (Fuge 1987, 1989; Oakley 1983, 1985; Ferraro-Gideon et al. 2013 A and B, 2014). The mechanism behind the oscillations towards the pole may be different than the mechanism for away from the pole oscillation since these two speeds were found to be significantly different, 6.24 $\mu\text{m}/\text{min}$ and 5.23 $\mu\text{m}/\text{min}$ respectively (Ferraro-Gideon et al. 2014).

The cause of the persistent bivalent oscillations is unknown. The sister kinetochores on each bivalent will oscillate independent of one another but can sometimes oscillate in-phase or out of phase (Fugre 1989; Fuge and Falke 1991). Fuge (1989) suggested that oscillations were due to tension along the length of the bivalents. However, based on our data, oscillations cannot be solely due to tension in the bivalent since oscillations continued even when bivalents were severed in half (Chapter 4). Bivalent oscillations are not unique to *Mesostoma* and do occur in other cell types as well. For example, within the same spindle in PtK1 (rat kangaroo kidney epithelial cells) sister chromatids may either oscillate or stay still at the metaphase plate (Civelekoglu-Scholey et al. 2013). The chromosome positioning within the cell dictates this behaviour, as chromosomes closer to the centre of the midzone show increased oscillation velocities (Civelekoglu-Scholey et al. 2013). What is unique about *Mesostoma* bivalents is that bivalents can also perform bivalent reorientations (Ferraro-Gideon et al 2013, 2014). These are

often and regular occurrences and at least one bivalent in any one given cell will perform a reorientation. The mechanism for reorientation is not known although it is speculated to be caused by a malfunction in kinetochore-microtubule tension where the kinetochore-microtubule connections to one pole may be momentarily lost (Ferraro-Gideon et al. 2014). These reorientations may also be an indication these cells divide via non-random segregation. This topic will be discussed further in the upcoming section B.4.

B. 2. Univalent excursions

There are four univalent chromosomes in this cell, of two different types. The two larger univalents are metacentrics and the two smaller univalent are acrocentrics (Fuge 1989; Oakley 1983, 1985). Throughout prometaphase the four univalents reside at either spindle pole in various combinations but by the time anaphase commences the typical arrangements is one of each kind at either pole (Oakley 1985). Univalents frequently moved between spindle poles, sometimes up to 7 times in the same cell (Ferraro-Gideon et al 2014). Because univalents are constantly moving, at any one given time there can be different arrangements of the two types of univalents at either pole. For example, there may be a 2:2 distribution, a 3:1 distribution or even a 4:0 distribution of univalents at either pole. Univalents of the same kind are not physically paired to one another. During anaphase, univalents segregate via distance segregation, which is when partner chromosomes are unpaired and do not physically connect but still segregate to opposite poles (Hughes-Schrader 1969).

Univalents are also suspected to divide non-randomly. That is because univalent excursions occur too often to be due to chance, as though they are actively rearranging themselves. For example, Oakley reasoned that if pole-to-pole movements of univalents were required only to achieve distance segregation, then the cell would need at most 1 or 2 excursions,

however it is typical for there to be 3+ excursions in one cell. More convincingly, univalents of the same type will often switch places with one another, such as one metacentric oriented at the top pole will switch places with the metacentric oriented at the bottom pole (Oakley 1985, Ferraro-Gideon et al 2014). A mechanism such as this one would only be necessary if the cells required a X1Y1 at one pole and X2Y2 at the other.

B. 3. Precocious pre-anaphase cleavage furrow

Mesostoma spermatocytes have precocious furrow giving them their characteristic dumbbell-like shape (Forer and Pickett-Heaps 2010). The precocious furrow has the same dynamics as a regular cleavage furrow, and when anti-actin or anti-myosin drugs are added the furrow regresses and the cell does not enter into anaphase (Forer and Pickett-Heaps 2010). However, there are certain cell types that do not require anaphase commencement to have their cleavage furrows form. In various diatom species such as *Hantzschia*, *Nitzschia*, *Pinnularia*, *Cymatopleura* and *Surirella* (Pickett-Heaps et al. 1978, 1979; Pickett-Heaps, 1980; Pickett-Heaps, 1991), furrows appear at the start of prometaphase. Precocious cleavage furrows are also common in various green algae, such as *Spirogyra* (McIntosh et al., 1995). In these organisms, the precocious furrow will partially ingress and arrest until anaphase when it will then fully ingress and cleave the cell in two. Likewise, in primary spermatocytes of *C. elegans*, the spindle forms before segregation of homologous chromosomes (Wang et al 2021).

Although the formation of a precocious furrow is not unique to *Mesostoma*, what is unique is its ability to move in response to chromosome imbalances in the cell (Ferraro-Gideon et al. 2013 A, 2014). Throughout prometaphase, univalent excursions can result in temporary unequal numbers of univalents at either pole, such as 3 univalents at one pole and 1 at the other (Oakley 1984). When this occurs, the furrow shifts 2-3 μm towards the pole with fewer

univalents (Ferraro-Gideon et al. 2013 A, 2014; Forer and Pickett-Heaps 2010). This creates an asymmetrically positioned furrow, with one half cell being larger than the one with fewer chromosomes. It is important to note that the asymmetric furrow in *Mesostoma* is not the same as asymmetrically dividing cells such as *C. elegans* or *Drosophila* embryos. In these cells the aim is to generate cell-fate diversity and the spindle is positioned asymmetrically in the cell (Cytrynbaum et al. 2005; Grill et al. 2001; Gonczy 2008), whereas in *Mesostoma* the spindle remains symmetric throughout division to produce two equally sized daughter cells (Forer and Pickett-Heaps 2010; Ferraro-Gideon et al. 2013 A).

B. 4. Non-random segregation

Mesostoma spermatocytes are speculated to divide via non-random segregation. Non-random segregation goes against Mendel's law of independent assortment, which states the separation of alleles for one gene is independent to allele separation for another gene. Thus, the orientation of each homologous pair is *random* and is not affected by the orientation of any other homologous pair (Mendel 1865). Non-random segregation was first observed in the mole cricket *Gryllotalpa borealis* by Payne in 1912, and later confirmed in *Gryllotalpa* by Camenzind and Nicklas (1968). Since then it has been observed in a variety of species, such as liverworts (Lorbeer 1934), flea beetles (Wilson et al 2003; Kupfer and Wise 2000 A and B) and sciarid flies (Metz et al. 1926), as reviewed in Brady and Paliulis (2015). For example, in mealy bugs and *Sciara*, all male-derived autosomes go to one pole and all female-derived chromosomes go to the other pole (mealy bugs: Schrader 1921, 1923; Nur 1982. *Sciara*: Metz et al. 1926; Metz 1938; Fuge 1994, 1997).

There are two pieces of evidence that suggest that *Mesostoma* spermatocytes might divide via non-random segregation. The first is univalent excursions (Oakley 1983), as previously

described. Another line of evidence is from bivalent reorientations (Ferraro-Gideon et al. 2014). The bipolarly oriented bivalents in *Mesostoma* spermatocytes frequently reorient during prometaphase, and since in most reorientations the bivalent kinetochores switched poles, i.e., reversed their initial segregation, this might indicate that the bivalents, too, segregate non-randomly (Ferraro-Gideon et al. 2014, Brady and Paliulis 2015). In my experiments I show a third possible piece of evidence that *Mesostoma* may have non-random segregation since chromosomes consistently move to one pole after addition of NOC.

C. Tethers

For my thesis I was the first to show evidence that tethers are present in *Mesostoma ehrenbergii* spermatocytes during prometaphase and anaphase and I also performed experiments to further understand the role tethers may play in chromosome movements. Tethers are a recently discovered elastic component which extend between the telomeres of separating bivalents. Although they do not prevent the polewards motion of chromosomes, they do exert an anti-polewards or ‘backwards’ force on separating bivalent chromosomes. Tethers were first described in crane flies but have since then been found in a broad range of animal cells, including *Mesostoma*. I will now provide a background on how tethers were first characterised operationally in crane fly spermatocytes and describe how tethers can be elastic or inelastic depending on their phosphorylation state.

Preparations of cells undergoing division have pointed to the suggestion that a structure connecting the separating chromosomes, later termed tethers, was present in dividing cells since the early 1900’s (Carnoy 1885; Montgomery 1899; Wilson 1905; Stevens 1905; Stevens 1909; Andrews 1915; as reviewed in Paliulis and Forer 2018). Further evidence came from Carlson’s micromanipulation experiments. When a needle was swept through the interzone region of the

cell between the separating sister-chromatid arms, the two separating arms moved following movements of the needle, suggesting there was a physical connection between the arms (Carlson 1952). Similar results were later found in crane flies (Forer and Koch, 1973), and LaFountain et al. (2002) was the first to call the elastic physical connections between arms of separating chromosomes “tethers”.

The operational function of tethers has been well described in crane flies (LaFountain et al 2002, Sheykhan et al 2017). Tethers were first identified operationally by severing the telomere-containing tips of bivalent arms using a laser microbeam. These fragments did not remain stationary as one would expect, but rather moved backwards towards the partner telomere at the opposite cell pole (LaFountain et al., 2002). These cuts were also performed at various stages of anaphase and the backwards movements and speeds were described in detail. In sum, arm fragments that were cut in early anaphase moved relatively fast and a further distance in comparison to arm fragments cut in later anaphase which either moved slowly and a shorter distance or did not move at all (La Fountain et al 2002). They interpreted this movement to mean that tethers are elastic structures which appear to become less elastic as anaphase progresses.

The telomere containing tip is the critical structure for tether activity. That is because only the telomere containing portion of the arm fragment moves, and if either partner chromosome telomere is ablated, the backwards movement stops (La Fountain et al 2002). Also, when the backward moving arm fragment nears the partner telomere, that intact arm loses length (Sheykhan et al., 2017), it ‘shrinks’, further indicating that the connections, the ‘tethers’, are elastic. Thus, the forces for movement are applied specifically at the telomere, both telomeres need to be intact, and the forces seem to arise from elastic connections(‘tethers’) that extend between separating telomeres.

It was also determined the loss of tether elasticity is due to a dephosphorylation event. Tether elasticity can be preserved by blocking the dephosphorylation. Treatment of crane fly spermatocytes with the serine/threonine phosphatases PP1 and PP2A inhibitor CalyculinA (CalA) causes partner chromosomes to move backwards, but only when added in early anaphase (Fabian et al. 2007; Kite and Forer 2020). When added in late anaphase partner chromosomes either move backwards very little or do not move backwards at all, suggesting a dephosphorylation event occurs sometime during the cell's progression from early to late anaphase. We do not yet know when phosphorylation is originally established and what kinases are involved (Smith and Forer 2020), and there may be redundant phosphorylation pathways (Sheykhan et al 2013). The working hypothesis is that tethers that are elastic are phosphorylated and they become inelastic when dephosphorylated (Smith and Forer 2020).

We also do not know what tethers are comprised of, although they are speculated to contain titin (Sheykhan et al 2017). Titin has been visualized in the interzone region of the crane fly spindle where we suspect tethers are present (Fabian et al 2007). Titin has elastic properties which would rationalize the elastic like behaviour of tethers, and titin acts as a stretch and tension sensor in muscles (Miller et al 2004, Granzier and Labeit 2006, Tskhovrebova and Trinick 2003). Some suggest tethers might be an artifact in the cell, consist of microtubules, chromatin bridges, or ultra-fine (DNA) bridges (Chan et al., 2007; Barefield and Karlseder, 2012; Gemble et al., 2015; as discussed in Paliulis and Forer 2018). However, there are several lines of evidence which negate this as discussed in detail in Kite and Forer 2020.

Thus, tethers are previously unrecognised component of the mitotic spindle. Tethers may play a role in providing tension between separating chromosomes as tension is required for

anaphase commencement (Nicklas and Koch 1969), and tethers need to be accounted for in general models of mitosis in terms of forces on chromosomes.

D. Components of the mitotic spindle apparatus

The spindle apparatus is a cytoskeletal structure found in eukaryotic cells which forms during mitosis/meiosis (Bray 2001). It sets up the framework crucial for proper segregation of chromosomes. It is a complex network, primarily composed of microtubules that work harmoniously with many other cytoskeleton components including actin and many other proteins. The motor proteins associated with microtubules and actin filaments are responsible for almost all the movements that occur in eukaryotic cells (Bray, 2001).

D. 1. Microtubules and its associated motor proteins

Microtubules are the most abundant protein found in the spindle and they form three main arrangements. The first are astral microtubules, which emanate from the poles and extend toward the periphery of the cell. Secondly there are interpolar microtubules, which extend between separating chromosomes in the midzone of the spindle and overlap in an antiparallel fashion. Lastly are the kinetochore microtubules or fibres, which extend from the cell pole and insert into the kinetochores (Meunier and Vernos 2012; Karsenti and Vernos 2001). The widely accepted theories of chromosome movement heavily rely on microtubules and their associated motor proteins, so I will now further describe their structure and function.

Microtubules are present in plant and animal cells as tracks to direct the movement of vesicles, organelles and other components of the cell (Bray, 2001; Karp, 2003). Microtubules are long, rigid, hollow cylinders, mainly comprised of α - and β -tubulin subunits in a heterodimer (Nogales et al. 1998, 1999; Dutcher 2003), tubulin is present in every eukaryotic organism (Dutcher 2003), and a homologue is found in prokaryotes (Erickson 2017). Microtubules

undergo various post-translational modifications such as acetylation, detyrosination, phosphorylation, glutamylation, glycylation and amination (Yu et al. 2015; Verhey and Gaertig 2007; Vemu et al. 2016; Honda et al. 2017). γ -tubulin, forms the γ -tubulin ring complex (γ -TuRC) which forms what is known as the microtubule organizing centre, or MTOC. The MTOC is crucial for spindle formation during both mitosis and meiosis (Oakley 2000).

Microtubules, much like actin, exhibit polarity. In the case of microtubules, subunits are added at the (+) and removed at the (-) end (Howard 2001; Chretien and Wade 1991). The polarity of a microtubule influences the directed movement of cellular components throughout the cell because the microtubule-associated motor proteins, kinesin and cytoplasmic dynein, direct attached cargo, in general, towards the plus end of the microtubule and towards the minus end of the microtubule, respectively (Hyman and Mitchison, 1991). Cargo attachment is specific to the motor protein as each motor protein has a different tail and associated light chains that allow for the appropriate organelle to attach (Alberts et al., 2008). Microtubules provide an elaborate linear network that allows the directed transport of intracellular components via the motor proteins, kinesin and dynein, throughout the cytoskeleton of the eukaryotic cell.

Microtubules also exhibit dynamic instability, referring to a microtubule's overall ability to switch between elongation and shortening. Dynamic instability is a crucial feature of microtubules that form the bipolar mitotic spindle during cell division, allowing them to "probe" the cytoplasm to capture chromosomes at their kinetochore (Mitchison and Kirschner 1984). The growth or shrinkage of a microtubule is related to the gain or loss of the GTP cap, respectively (Nogales et al. 1998, 1999). If tubulin is added onto a filament faster than β -subunits hydrolyze the GTP into GDP, this creates a GTP-tubulin cap on the growing (+) end of the microtubule. Staining for EB1 which binds specifically to GTP-tubulin and thus is a plus end

tracking protein, shows that the cap is hundreds of subunits deep and the size of the cap is a direct measure of polymerization speed (Bieling et al. 2007). The event termed “catastrophe” occurs when the GTP cap is lost and is linked to a conformational change in tubulin (Alushin et al. 2014). This is followed by rapid depolymerization (Cassimeris et al 1988).

The major motor proteins of microtubules are dynein and kinesin. Dynein moves towards the (-) end of microtubules (Gibbons, 1963), and kinesin moves towards the (+) end (Vale 1999). These two motor proteins which move in opposite directions along microtubules act antagonistically to set up the spindle during prometaphase. During anaphase, Kinesin-13 depolymerizes microtubules and is partly responsible for chromosome movement at the kinetochore end. Kinesin and dynein also serve other functions in non-dividing cells. Due to their ability to “walk” along microtubules, kinesin is responsible for anterograde transport of vesicles or organelles towards the plasma membrane, and dynein for retrograde transport towards the interior of the cell (Lodish et al. 2000). Dynein is also a major component in cilia and flagella (Alberts et al. 2008).

D. 2. Actin

Actin microfilaments are also important filamentous cytoskeleton components that allow cells to undergo remarkable forms of motility. Actin was originally thought to be present only in eukaryotes (Ampe and Vandekerckhove 1999) but since then a homologue has been found in prokaryotes (Erickson 2017) and has various isoforms (Whalen et al. 1976; Vale 1999). Each actin subunit has a binding site for either ATP or ADP. Its monomeric form is referred to as G-actin which can polymerize to form filamentous actin (F-actin) (Howard 2001). The addition of each monomer is followed by ATP hydrolysis into ADP and the release of inorganic phosphate (Pi).

Although monomers are added at both ends, actin filaments have a polarity and monomers are added more quickly onto the faster growing (+) end and added more slowly onto the slower growing (-) end. The difference between the two ends can be visualized by decorating the filaments with heavy meromyosin, which forms an arrowhead with the arrows pointing towards the (-) end (Woodrum et al. 1975). Disassembly of microfilaments involves disassembly machinery such as actin depolymerizing factor ADF/cofilin which severs microfilaments (Reymann et al. 2011).

The dynamic remodelling of cellular actin, through disassembly then reassembly, makes it largely responsible for movements of the cell. In the cell actin localizes to the cortex which is right below the plasma membrane, and actin polymerization drives movement in lamellipodia, filopodia, and pseudopodia assembly. Actin nucleation occurs at the leading edge, with newly polymerized actin filaments pushing the plasma membrane forward (Blachoin et al. 2014). F-actin in association with its molecular motor protein myosin can also generate force in muscle contraction and in cleavage furrow ingression during cytokinesis.

D. 3. Myosin

Actin motility requires its actin-based motor, myosin. Myosin walks along actin filaments powered by ATP hydrolysis and is involved in muscle contraction, cell movement, cytokinesis and membrane transport (Lodish et al. 2000, Vale and Milligan, 2000). Myosin was first isolated in a complex with actin (Kuhne et al. 1864; as reviewed in Hartman and Spudich 2012), and then later isolated on its own (Straub 1942; Szent Gyorgyi 1941; as reviewed in Bugyi and Kellermayer 2019). The myosin superfamily contains at least 37 distinct myosin families, and myosins are present in most eukaryotes (Vale and Milligan, 2000). Myosin-2 (or conventional myosin) is known for producing muscle contractions in the sliding filament model (Silverthorn

2016), and non-muscle contractions like cytokinesis, cell adhesion and cell migration (Vincente-Mansanares et al 2009).

Myosin contraction occurs through a power stroke that is generated through the hydrolysis of ATP (Tyska and Warshaw, 2002). The direction of myosin movement is determined by the polarity of actin filaments. Because actin filaments are oppositely oriented in the sarcomere, myosin activation causes actin to slide past one another to generate muscle contraction in the sliding-filament model (Huxley 1957, Huxley and Simmons 1971, Vale 1999; Bray 2001). The activity of non-muscle myosin is regulated by phosphorylation of its light chain. In my thesis I studied whether myosin might be involved in NOC-induced chromosome movement using various myosin altering drugs. For that reason, I now describe different processes involved in myosin regulation

D. 4. Myosin regulation

Phosphorylation of the myosin regulatory light chain (MRLC) controls myosin filament assembly (Trybus, 1991) and is responsible for smooth muscle contraction (Vale 1999). MRLC phosphorylation is controlled by a variety of factors. Neuronal or hormonal stimulation causes the release of calcium which binds calmodulin (CaM) and induces conformational changes in myosin to have actin-activated myosin ATPase activity (Cremo and Hartshome, 2008; Bray, 2001). MRLC phosphorylation can also be monophosphorylated at serine 19 (Ser19) (Ikebe and Hartshome, 1985; Tan et al., 1992), or threonine 18 (Thr18), or biphosphorylated at both sites (Umemoto et al., 1989). Several kinases phosphorylate MRLC, such as myosin light chain kinase (MLCK), Rho-associated kinase (ROCK), citron kinase (CK), leucine zipper interacting kinase (ZIPK) and integrin-linked kinase (ILK) also can phosphorylate MRLC at both Ser19 and Thr18 (Amano et al., 1996; Yamashiro et al., 2003; Murata-Hori et al., 1999; Wilson et al., 2005).

Dephosphorylation of MRLC is due to the action of a single enzyme, myosin light chain phosphatase (MLCPase).

E. Mechanisms involved in anaphase chromosome movement

Proper segregation of chromosomes during cell division is a key event in the equal distribution of genetic material into two daughter cells. The mechanisms underlying chromosome-to-pole motion in anaphase of mitosis have been heavily debated in cell biology. Cell biologists originally believed that the spindle was structureless because spindle fibres could only be seen following fixation and could not be seen in living cells with a conventional microscope. It was not until the work of Inoue (1953) using the polarization microscope that cell biologists realized that the spindle was composed of weakly birefringent fibres. Inoue (1953) demonstrated that the birefringent spindle fibres he could see in living cells resembled the same structures that were observed by researchers in fixed and stained cells. This provided the first set of evidence that spindle fibres exist in living cells, however the exact mechanism(s) of how chromosome movement is achieved has remained elusive. The conventional and most popular mechanism thought to be involved relies solely on microtubules, microtubule dynamics and microtubule motor proteins (kinesin and dynein); however, there is strong evidence for the involvement of other cytoskeletal, non-microtubule, elements in propelling the chromosomes towards the poles. I now describe some of the two most well known microtubule-based theories for chromosome movement, PacMan and Flux.

E. 1. Pacman and Flux

In the Pac-Man model kinetochores behave much like the character it is named after in the Pac-Man computer game. The Pac-Man model describes chromosome-to-pole movement as occurring when the kinetochore "chews" its way towards the spindle pole with microtubule

depolymerization occurring at the kinetochore end (Rieder and Salmon, 1994, Cassimeris and Spittle 2001; Koshland et al., 1988; Nicklas, 1989). Several early experiments in favor of Pac-Man model were reviewed in Forer, 1988. For example, fluoresceinated or rhodamine-labelled tubulin was injected into porcine kidney cells to visualize microtubule spindle fibres (Gorbsky et al, 1987, 1988). The mitotic spindle was then photobleached using a laser microbeam to produce a narrow bar of fluorescence-loss. Any change in the position of fluorescence-loss, which looked like a gap along the length of the microtubule, was observed throughout cell division. As the chromosomes moved poleward during anaphase the microtubule gap did not move, indicating the kinetochores moved poleward along the length of the stationary microtubules (Gorbsky et al., 1987, 1988). Other experiments also point to similar conclusions. Using a micromanipulation needle, Nicklas (1989) severed all the microtubules located between the chromosomes and the pole and removed the spindle pole while leaving chromosomes and kinetochores intact. Chromosome movement continued normally during anaphase, indicating that the force generating agents are present at or near the kinetochore (Nicklas 1989).

The Pac-Man model also proposes that the force required to produce chromosome-to-pole motion is driven by minus end directed motors (e.g. dynein) at the kinetochore (Rieder and Salmon, 1994). Further studies pointed to the role of microtubule associated motor proteins, such as dynein and kinesin, as being fundamentally important for both the organization of the spindle and the movement of chromosomes during mitosis (Ahmad et al., 2000; Steuer et al., 1990; reviewed in Maney et al., 2000). The motor for chromosome motion was suggested to be dynein (Hildebrandt, and Hoyt, 2000) because immunofluorescence studies showed that dynein is localized at the kinetochore (Pfarr et al., 1990; Steuer et al., 1990; Sharp et al., 2000; Yeh et al., 2000), and because dynein is an ATPase known to move cargo along microtubules toward their

minus end (Echeverri et al., 1996; reviewed in Karki and Holzbaur, 1999). But other studies suggested that dynein might not be involved in anaphase, because it is completely depleted from the kinetochores and from the spindle fibres in anaphase (review in Howell et al., 2001; Fabian and Forer, 2005), and because inhibition of dynein/dynactin does not block metaphase congression, poleward anaphase movements, or cytokinesis (Howell et al, 2001). Overall, the Pac-Man model proposes that anaphase chromosome movement is due to force generated at or near the kinetochores.

Although the Pac-Man model has been widely accepted, there is data that refute this model. For example, in diatoms, very few microtubules insert at the kinetochore and instead attach to another structure termed a collar (Tippit and Pickett-Heaps 1977; Pickett-Heaps et al., 1978). Since the premise of the Pac-Man model is that the microtubules terminate at the kinetochore, this key piece of evidence refutes this model. In addition, in some cells once anaphase is complete chromosomes have been observed to continue moving past the end of the spindle pole (Zhang and Nicklas 1996). In one experiment, chromosomes and their associated kinetochores were surgically removed from the cell. Since microtubules disassembled at a speed consistent with normal anaphase, this suggests that the disassembly of microtubules does not require the presence of the kinetochore and may be accomplished by other proteins within the spindle (Zhang and Nicklas 1996).

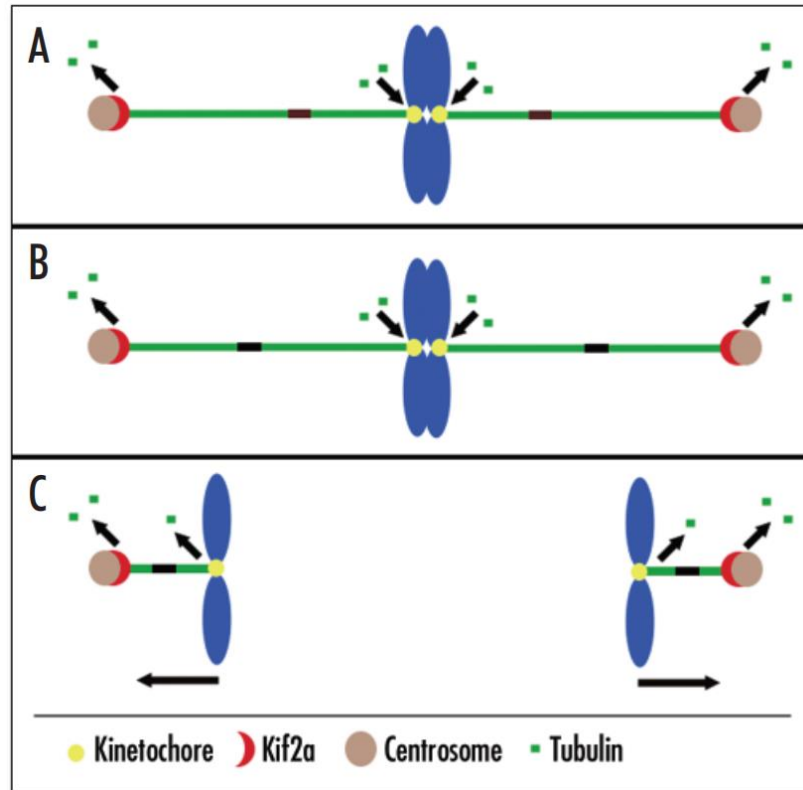


Figure 1 A-C. Poleward microtubule flux during metaphase (A and B) and anaphase (C). (A) During metaphase, microtubule depolymerization at the pole is balanced by tubulin addition at the kinetochores. (B) Throughout metaphase, individual tubulin subunits (as labelled with the black bars) move poleward along the length of the microtubule, however the length of microtubules remains relatively stable due to treadmilling. (C) During anaphase, microtubule depolymerize quicker at the poleward end, thereby decreasing the length of the microtubule. Poleward microtubule flux also contributes to poleward movement of the kinetochore. Figure modified from Ganem and Compton 2006.

The other microtubule-based model for chromosome movement is the Flux model, which in contrast to the PacMan model, is when chromosomes move towards the pole not via microtubule shortening at the kinetochore end but at the polewards end (Figure 1). To better describe this model, I will first describe the phenomenon of microtubule treadmilling. Microtubule treadmilling is the simultaneous assembly of tubulin subunits at microtubule plus ends and disassembly of these subunits at minus ends while the microtubules remain of constant length (Margolis and Wilson, 1978). Typically, during metaphase, the tubulin subunits polymerize and depolymerize at similar rates, therefore achieving a steady-state dynamic that

maintains a constant microtubule length. This process is known as treadmilling (Margolis and Wilson 1981). During anaphase the equilibrium shifts to favour the rate of depolymerization of microtubules at the polewards end by Kif2a (Ganem and Comptom 2006). This subsequently shortens the kinetochore fibre, thereby moving the chromosome toward the pole (Buster et al. 2007). This process is coupled with microtubule flux, a poleward force which helps pull the chromosome towards the pole (Ganem and Comptom 2006). Flux is the movement of monomers in the microtubule from one end to the other which occurs through sliding mechanisms (Khodjakov and Kapoor 2005). Overall, the Flux model proposes that poleward chromosome movement during anaphase is a summation of depolymerization of the microtubule coupled with microtubule flux (Rogers et al. 2005; Ganem and Comptom 2006).

The main evidence for the flux come from experiments using UV irradiation, photobleaching, photoactivation of tubulin, or fluorescent speckles of tubulin, all of which produce gaps along the length of microtubules which act as visual markers (Forer, 1965; Mitchison, 1989; Desai and Mitchison 1998; LaFountain et al., 2004). These experiments showed that the markers move poleward with the chromosomes, suggesting that microtubules depolymerize at the poles (review in Khodjakov and Kapoor, 2005; Mitchison, 2005; Rogers et al., 2005). To support the role of minus end depolymerisation at the pole in anaphase movement, KLP10A which belongs to the Kin I (kinesin-13) family has been identified in *Drosophila* localised at spindle poles; KLP10A induces depolymerisation of microtubules and inhibition of this protein significantly blocks flux (Rogers et al., 2004). Another component identified was the CLASP protein, Mast/Orbit, which is responsible for promoting polymerization at the plus-end and is downregulated during anaphase onset (Maiato et al. 2004). Also, kinesin-like proteins such as Eg5 which are embedded in the spindle matrix are known to be involved in sliding of

microtubules; inhibiting this protein led to reduced flux rate (Miyamoto et al., 2004). Thus, sliding of microtubules is also important in maintaining flux and moving chromosomes poleward.

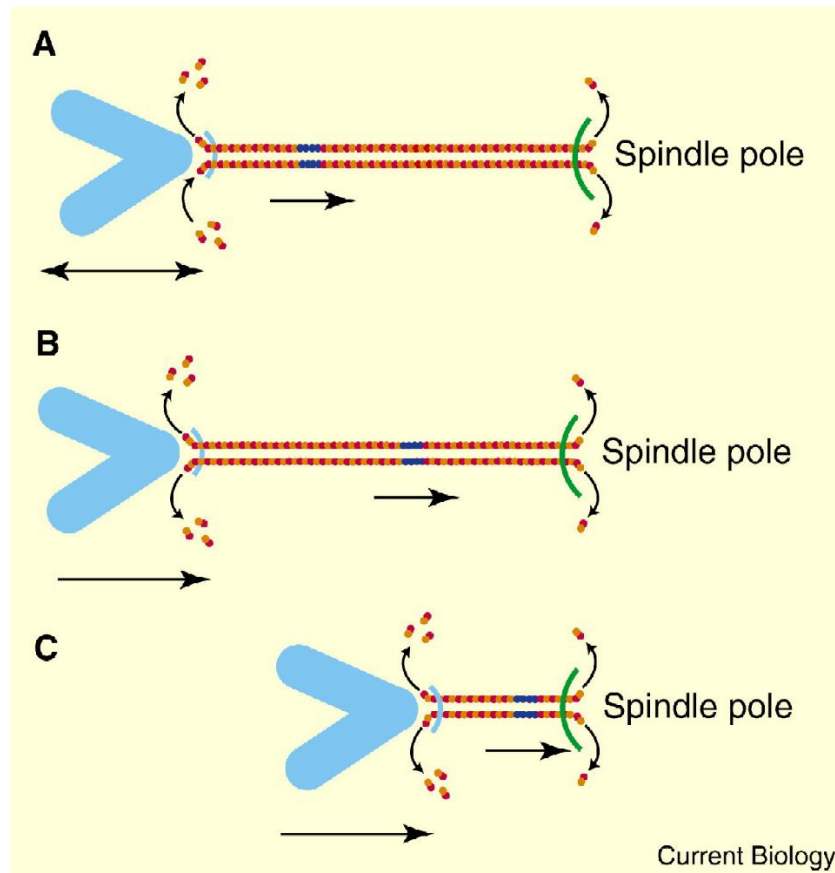


Figure 2 A-C. Microtubule flux dynamics of kinetochore microtubules during metaphase (A) anaphase onset (B) and anaphase (C). (A) During metaphase tubulin subunits are continuously removed from the minus ends of microtubules at the spindle pole, while kinetochores switch between periods of tubulin polymerization and removing tubulin subunits from microtubule plus ends. (B) At the onset of anaphase kinetochores switch to continuous microtubule depolymerization. (C) During anaphase chromosomes move poleward on microtubules at the kinetochore end (Pac-man model) while at the same time move poleward via microtubule depolymerization and flux at the pole (Flux model). Figure taken from Khodjakov and Kapoor 2005.

Although flux has an important role in moving chromosomes in anaphase, in various cell types the rate of flux is less than the speed of anaphase chromosomes (reviewed by Rogers et al., 2005). This is thought to be due to cooperative flux/Pac-Man or only a Pac-Man mechanism

which provides higher velocity for chromosome movement during anaphase than flux alone (Figure 2) (Rath et al., 2009). In crane-fly spermatocytes using fluorescent speckle microscopy, the rate of flux is twice as much as the velocity of chromosome movement during anaphase, ruling out the role of Pac-Man in anaphase movement of chromosomes in this cell (LaFountain et al., 2004). In sum, the take home message for both the Pac-Man model and Flux model assume that microtubules and their associated motor proteins are the only components responsible for chromosome-to-pole motion.

E. 2. Spindle Matrix Model

There is growing evidence that microtubules may not be the sole producer of force in the mitotic spindle. The spindle matrix model considers microtubules as rigid fibres, "governors", that limit the rate of movement and whose rate of depolymerisation, a consequence of force production, not its cause, governs the velocity of chromosome-to- pole motion (Forer et al., 2003; Forer et al., 2008; Pickett-Heaps and Forer, 2009). Rather, microtubules may play a more passive role acting as a scaffold for other cytoskeletal elements that produce the force for chromosome movement (Johansen and Johansen 2007; 2011, Pickett-Heaps and Forer 2009, Spurck et al. 1997). The existence of a spindle matrix was first described in the 1980's on experiments in diatoms. It was shown that diatoms display during mitosis a specific structure, distinct from the microtubules, a dense "collar" around microtubules, which extends between kinetochores and the poles and has elastic properties, and which was called "spindle matrix" (Pickett-Heaps et al., 1982; Pickett-Heaps et al., 1984).

There is a vast array of protein candidates suggested to form this elusive spindle matrix and with varying roles such as helping to form and maintain the mitotic spindle, organize and stabilize microtubules, actin and other spindle elements (Johansen and Johansen 2007; Forer and

Wilson, 1994; Pickett-Heaps et al., 1997). In order to be considered a feasible molecular candidate for the spindle matrix, the protein should exhibit some of the following criteria: 1) coalign with the microtubule spindle 2) remain in the absence of microtubules 3) when these proteins are targeted there should be some affect on spindle assembly or function and 4) they should interact with microtubules or their associated motor proteins (as discussed in Johansen and Johansen 2007).

Some of the most compelling spindle matrix candidates are the nuclear pore complex-associated proteins Skeletor, Chromator, Megator and EAST, which were first identified in *Drosophila* (Qi et al., 2004, 2005; Rath et al., 2004; Walker et al., 2000). The first to be identified in this complex was Skeletor (Walker et al., 2000). During interphase and prophase Skeletor is associated with the nuclear envelope and chromosomes and then redistributes into a spindle-like structure from prophase into anaphase (Walker et al., 2000). Skeletor is required for normal mitosis and remains intact in the spindles devoid of microtubules after nocodazole treatment (Walker et al., 2000). Chromator directly interacts with Skeletor. It contains a chromodomain which directly associates with chromosomes in a pattern that is very similar to and overlaps with Skeletor (Rath et al., 2004). Chromator mutation or RNAi depletion in *Drosophila* S2 cells caused various defects in spindle morphology and the chromosomes were unable to properly segregate (Rath et al., 2004; Ding et al., 2009). Megator is an essential coiled-coil protein, and unlike Skeletor and Chromator it has the ability to form polymers (Qi et al., 2004) allowing it to possible provide a more structural element to the spindle matrix (Johansen and Johansen 2007). Also unlike Skeletor and Chromator, during interphase Megator localizes to the intranuclear space instead of the chromosomes, and then during metaphase Megator localises to the spindle (Yao et al., 2012). Megator also persists in the absence of

microtubules brought about by addition of nocodazole and cold treatment (Qi et al. 2004).

Although Skeletor Chromator and EAST have no known mammalian homologs, Megator is similar to the mammalian nuclear pore complex Tpr protein (Zimowska et al., 1997) and to yeast nuclear pore complex-associated proteins Mlp1p and Mlp2p (Kosova et al., 2000; Strambio-de-Castillia et al., 1999). More recently, Skeletor Chromator and Megator have also found in crane-fly and locust spermatocytes with a similar localization patterns with the spindle and chromosomes (Silverman-Gavrila and Forer, 2003, Fabian et al., 2007 a and b). The final nuclear pore complex-associated protein in this complex is EAST. It interacts with Megator and is involved in spindle matrix formation during mitosis (Qi et al. 2005). During interphase, EAST is also able to interact with Megator and localize to the space surrounding the chromosomes, and then during metaphase it relocates with the other spindle proteins mentioned to the cytoplasm where it contributes to the formation of the spindle matrix.

There are many other potential spindle matrix protein candidates in addition to the main ones just mentioned (Johansen and Johansen 2007; Johansen et al., 2011). These include but are not limited too: nuclear mitotic apparatus protein (NuMA) (Lydersen and Pettijohn, 1980; Merdes et al., 1996; as reviewed in Scholey et al., 2001), Poly (ADP-ribose) or PAR (Chang et al. 2004, 2005), the intermediate filament Lamin B (Herrmann and Aebi, 2004; Ma et al., 2009), the kinesin-related motor protein Eg5 (Enos and Morris, 1990; Hagan and Yanagida, 1990), and tektins (Steffen and Linck, 1992; van Hemert et al., 2002).

The evidence for the existence of the spindle matrix model and its role in chromosome movement is as follows. Experiments using the ultraviolet (UV) microbeam severed kinetochore microtubules during anaphase. In grasshopper (Gordon and Inoue 1979) and newt fibroblasts (Spurck et al. 1997, Pickett-Heaps et al. 1996) chromosomes continued moving poleward, and

even moved faster than pre-cut speeds. Although crane-fly spermatocyte chromosomes do not change speed when their microtubules are severed, they do speed up when the tether that connects separating chromosomes is first cut with a laser (Sheykhani et al. 2017), giving the same end result. Following UV irradiation of all the microtubules across the entire half spindle of newt and PtK fibroblasts, the entire spindle pole associated with the irradiation moved towards the equator (Spurck et al., 1990; Snyder et al., 1991). This shows that interpolar forces persist without microtubule continuity between the spindle poles. When kinetochore microtubules were irradiated in metaphase in newt fibroblasts, the chromosome attached to the irradiated kinetochore fibre moved to the pole associated with the irradiation (Spurck et al., 1997), indicating that chromosomes can continue to move after their kinetochore microtubules are severed. Based on these experiments, Forer and his colleagues concluded that kinetochore microtubules are rigid structures that are pulled by the spindle matrix, and that the forces acting on the kinetochore microtubules cause them to disassemble (Forer et al., 2007, 2008).

Some have argued, however, that UV irradiations would destroy all components in that area and therefore, any other force producing system would be ablated along with microtubules (Scholey et al. 2001). This is not the case though, because Sillers and Forer (1983) showed that depending on the wavelength of the UV microbeam and dosage of irradiation, they were able to produce different results: at certain UV wavelengths, an area of reduced birefringence (ARB) could be produced without any effects on chromosome movements while at other wavelengths, chromosome movements could be stopped without the production of ARBs (Sillers and Forer 1983). There are related data from metaphase cells, also.

Chromosomes are at the equator at metaphase because they are positioned and held in place by kinetochore microtubules. Thus, when microtubules are depolymerized, it is expected

that chromosomes should not be able to move poleward and arrest at the equator; indeed this does occur in many cells (Reider and Salmon 1994). However, when kinetochore microtubules were depolymerized in diatoms, the bipolarly attached chromosomes detached and moved to either pole at random (Pickett-Heaps and Spurck 1982). Pickett-Heaps and Spurck (1982) argued that microtubules resisted force from another spindle element, the spindle matrix, and once microtubules were depolymerized due to the addition of colcemid and the resistance was gone, the matrix pulled the chromosomes towards either pole at random. This experiment in fact laid the groundwork for much of my research using NOC in *Mesostoma* (Chapter 1 and 2).

It is also theorized that these spindle matrix proteins may also work with actin and myosin to produce force (Forer et al., 2008). From an evolutionary point of view, it makes sense for the spindle to evolve with elements from interphase cells (i.e. matrix proteins) but also with components from other motility systems such as actin and myosin (Pickett-Heaps & Forer, 2009). These various systems would then come together to form the redundant reliable mitosis; however, it is also the reason it is so difficult to characterize and explain how the spindle functions. In the next section I will describe the actin and myosin in the spindle and how it may work alongside spindle matrix proteins and could be considered components in this model in a somewhat revised version of the original spindle matrix model.

E. 3. Actin and Myosin Model

Similar to the spindle matrix model, the actin and myosin model theorizes that microtubules are more of a structural component rather than the main producer of force in the spindle. Kinetochore fibres contain multiple other non-microtubule components that might be involved in force production and microtubule flux (Forer et al., 2007; Forer, 1966; Forer, 1969; Forer 1988). Using various visualizing techniques such as electron microscopy (Forer and

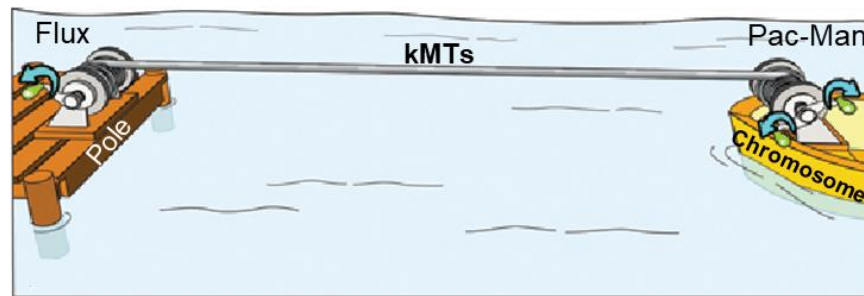
Jackson 1979) and phalloidin staining (Silverman-Gavrila and Forer 2003), actin has been found to co-localize with microtubules in various plant and animal cells (Forer et al. 2003, Behnke et al., 1971; Forer and Behnke, 1972; Forer. 1985; Forer, 1988). Actin is usually concentrated at the poles and extends from the pole to the equator, like microtubules (Silverman-Gavrila and Forer 2003, Schuh and Ellenberg 2008). Actin is found throughout the spindle in human, mouse, porcine and ovine eggs (Mogessie and Schuh 2017). Myosin was also identified in spindles (review in Forer et al, 2003), and myosin has also been observed to co-localize with microtubules at the spindle (Silverman-Gavrila and Forer 2003). Not only do actin and myosin localize to the microtubule spindle, but further evidence for actin and myosin involvement stems from treatments that target actin and myosin function to show their effect on microtubule dynamics and on chromosome motion.

Actin and myosin are also implicated in microtubule dynamics. For example, Silverman-Gavrila and Forer (2000) used an indirect measure of flux to study whether actin and myosin were involved in causing flux. It had been shown previously that when tubulin subunits are newly polymerised into kinetochore fibres they are not acetylated, but after a time lag, as these subunits flux to the poles, they become acetylated (Wilson et al., 1994). Thus, there is a gap in staining for acetylated tubulin that is at the kinetochore, and the gap represents flux.

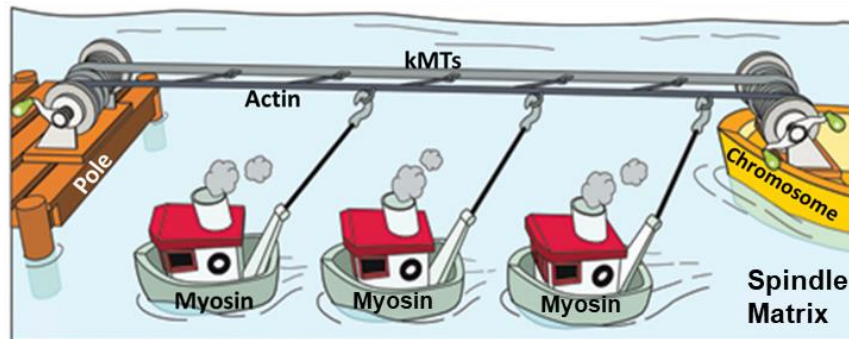
Cytochalasin D, latrunculin B, swinholide (actin inhibitors) and BDM (a myosin inhibitor) eliminate this gap (Silverman-Gavrila and Forer (2000). In another set of experiments, Forer et al. (2007) generated a kinetochore stub by UV microbeam irradiation of kinetochore fibres in metaphase crane-fly spermatocytes. According to the Pac-Man model, if a kinetochore chews its way towards the pole then the stub generated here should shorten and disappear. However, the kinetochore stub elongated because of the incorporation of tubulin at the kinetochore and fluxing

of tubulin subunits towards the cut end. To test whether actin and myosin are involved in generating flux and elongating the kinetochore stub, Forer et al. (2007) found that cytochalasin D, latrunculin A, and BDM blocked the elongation of the stub. It was also found in mouse oocytes that actin helps to drive formation of the kinetochore fibers by bundling and aligning them is essential for the alignment and segregation of chromosomes during anaphase (Mogessie and Schuh 2017). Based on these experiments, actin and myosin have been considered components of the spindle which may act along the length of the kinetochore fibre to aid in producing the force driving chromosome-to-pole motion (Pickett-Heaps et al., 1997).

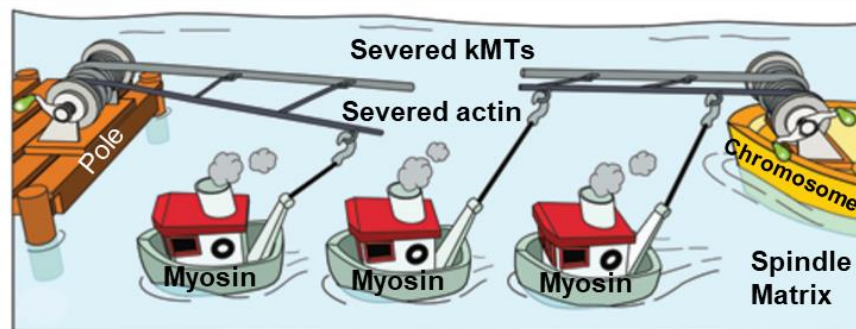
There are several ways in which actin and myosin might function in chromosome movement (Forer et al. 2008) We do not know enough about the arrangement of actin and myosin with respect to kinetochore microtubules to evaluate this model, but the analogy represented in Figure 3 A-C is one possible interpretation of actin and myosin's involvement in chromosome movement. In this analogy, the boat being pulling to the dock along a rope represents a chromosome moving poleward during anaphase along the path of a kinetochore microtubule. A represents the microtubule-based theories of Pac-Man and Flux, which rely on the presence of microtubules, or ropes, to move their cargo. Our proposed model is shown in B, where kinetochore microtubules are cross-linked to actin, to which the myosin motor protein acts indirectly to propel microtubules poleward and thereby drive poleward chromosome movement. In this case it is not the rope that is pulling the boat, but rather the tugboats that are attached to the rope that is shortening. The water current, which represents the surrounding spindle matrix, may also play a role in helping to move the tugboats and/or the boat.



A. Microtubule-based theories



B. Non-microtubule-based theories



C. Non-microtubule-based theories can explain how chromosomes can still move poleward even with severed kinetochore microtubules

Figure 3 A-C. Diagrammatical representation of microtubule-based vs. non-microtubule-based theories of chromosome movement. The analogy being used is a boat being pulled into dock is representative of a chromosome moved towards its pole during anaphase. Labels have been added to the original figure indicating the corresponding parts of the cell each item in this analogy represents, and kMT are kinetochore microtubules. (A) Is representative of the Flux and/or Pac-Man models where the kinetochore microtubule either depolymerises at the poleward end (at the winch on the dock) or at the kinetochore on the chromosomes (at the winch on the boat). (B) Is representative of the Spindle Matrix model in association with actin and myosin. (C) Illustrates how chromosomes can still move even if kinetochore microtubules are severed. The poleward force of the spindle matrix (shown by the water) and myosin (the tugboats) will continue to move the chromosome (boat) towards the pole. Figure modified from Forer et al, 2008.

F. Laser irradiation

Asides from using anti-microtubule drugs, there are various methods to get rid of spindle microtubules, such as laser irradiation. Various types of lasers can be used to sever microtubule connections to chromosomes (Berns 2020). The femtosecond laser microbeam, also known as “optical scissors” or an “optical cutting laser”, forces the photons emitted from a specific wavelength of light into a tightly bound range of phase, frequency, polarization, and direction (Berns and Greulich 2007). This allows for greater precision than earlier methods, allowing one to selectively irradiate only the spindle microtubules, while leaving other components in the spindle such as actin and myosin fully intact (Sheykhani et al 2013). The laser also allows for chromosomes to be cut in half or the kinetochore to be ablated (Harsono et al 2013). In the absence of microtubules, one can demonstrate the force responsible for chromosome movement does not arise from spindle microtubules, but another non-spindle component such as actin or myosin.

G. Study objective

In a previous experiment, NOC was added to a *Mesostoma* spermatocyte to depolymerize the microtubules, causing all 3 bivalent chromosomes to selectively detach from one pole and move towards the opposite pole (Forer and Pickett-Heaps 2010). This experiment was performed on only one cell, and so my current experiments started out by determining whether this was a one-time event or if it was repeatable, and if so, how often. My first set of experiments adding NOC to *Mesostoma* primary spermatocytes showed chromosomes detached from only one pole in the vast majority of cells studied, and that this movement was very fast, up to 200x quicker than control anaphase movements in this cell (Chapter 1). I also tracked the ingression and movement of the precocious cleavage furrow and showed it was able to simultaneously move

and ingress in the absence of microtubules (Chapter 2). After establishing the pattern of movement and speed in these NOC-treated cells, I then wanted to investigate reasons as to why chromosomes only detached from one pole and determine the possible mechanisms for the very fast chromosome movement.

According to several pieces of evidence, it has been suggested that *Mesostoma* may divide via non-random segregation (Oakley 1985; Ferraro-Gideon et al, 2013 A). Therefore, I hypothesized the selective detachment from one pole during NOC might be due to a physical trait in these cells. I sought out a way to predetermine which side would detach and to see if I could alter detachment from one pole. Tethers may play a role in co-ordinating chromosome movement in a broad range of animal cells, including *Mesostoma* (Chapter 3), so I investigated how chromosome movement might be altered in a cell with functionally disabled tethers using the femtosecond laser (Chapter 4). My work disabling tethers provided some insight in the sidedness, although in the Discussion section I outline several more experiments I suggest one to attempt.

I then wanted to investigate a possible mechanism for the very fast chromosome movement. Since chromosome movement could not be due to microtubules, I had to look to alternative theories of chromosome movement, such as the Spindle Matrix model. I hypothesized the very quick movement might be due to actin and myosin since they have both been found in various spindles (as reviewed in Forer et al, 2003), they can remain in the spindle in the absence of microtubules and targeting them perturbs chromosome movement (as reviewed in Johansen and Johansen 2007). I worked on determining how the force for chromosome movement is produced in a series of double-drug experiments adding various myosin and actin inhibiting and

enhancing drugs plus NOC to see how various parameters of chromosome movement in *Mesostoma* spermatocytes might be altered (Chapter 5).

Overall, the data presented in this thesis favors the spindle matrix model and actin and myosin producing forces for chromosome movement rather than microtubule-based models of chromosome movement, Pac-Man and Flux, and adds more evidence to the theory that *Mesostoma* divide via non-random segregation. I will now outline each of my experiments in the following chapters.

CHAPTER 1

CHROMOSOMES SELECTIVELY DETACH AT ONE POLE AND QUICKLY MOVE
TOWARDS THE OPPOSITE POLE WHEN KINETOCHORE MICROTUBULES ARE
DEPOLYMERIZED IN *Mesostoma ehrenbergii* SPERMATOCYTES

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Chromosomes selectively detach at one pole and quickly move towards the opposite pole when kinetochore microtubules are depolymerized in *Mesostoma ehrenbergii* spermatocytes

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Abstract

In a typical cell division, chromosomes align at the metaphase plate before anaphase commences. This is not the case in *Mesostoma* spermatocytes. Throughout prometaphase, the three bivalents persistently oscillate towards and away from either pole, at average speeds of 5–6 $\mu\text{m}/\text{min}$, without ever aligning at a metaphase plate. In our experiments, nocodazole (NOC) was added to prometaphase spermatocytes to depolymerize the microtubules. Traditional theories state that microtubules are the producers of force in the spindle, either by tubulin depolymerizing at the kinetochore (PacMan) or at the pole (Flux). Accordingly, if microtubules are quickly depolymerized, the chromosomes should arrest at the metaphase plate and not move. However, in 57/59 cells, at least one chromosome moved to a pole after NOC treatment, and in 52 of these cells, all three bivalents moved to the same pole. Thus, the movements are not random to one pole or other. After treatment with NOC, chromosome movement followed a consistent pattern. Bivalents stretched out towards both poles, paused, detached at one pole, and then the detached kinetochores quickly moved towards the other pole, reaching initial speeds up to more than 200 $\mu\text{m}/\text{min}$, much greater than anything previously recorded in this cell. As the NOC concentration increased, the average speeds increased and the microtubules disappeared faster. As the kinetochores approached the pole, they slowed down and eventually stopped. Similar results were obtained with colcemid treatment. Confocal immunofluorescence microscopy confirms that microtubules are not associated with moving chromosomes. Thus, these rapid chromosome movements may be due to non-microtubule spindle components such as actin-myosin or the spindle matrix.

Keywords Meiosis · Microtubules · Non-random segregation · Spindle matrix · Nocodazole

Introduction

The present work deals with what causes asymmetries in spindle behaviour and with what causes chromosome movements during cell division. Possible asymmetric spindle behaviour

occurred after nocodazole (NOC) was added to *Mesostoma* spermatocytes during prometaphase. In general, lower concentrations of NOC cause kinetochore fibres to lose their formerly tight connection to the kinetochore and higher concentrations produce microtubule fragments and cause microtubules to detach from the centrosomes (Vasquez et al. 1997; Yang et al. 2010). Similar effects are also seen with high concentrations of the microtubule depolymerizer colcemid (Yang et al. 2010). Therefore, after NOC is added to *Mesostoma* cells, one would expect the chromosomes to stop moving and stay at the equator, as they do in other cells (Cassimeris et al. 1990). Instead, however, all the chromosomes selectively moved towards only one pole, in the one cell that was described (Forer and Pickett-Heaps 2010). One focus of this work was to see if all chromosomes consistently moved to one pole. Previous work on *Mesostoma* spermatocytes raised the possibility that the chromosomes segregate non-randomly in meiosis-I (Ferraro-Gideon et al. 2013),

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which might be a possible explanation for why all chromosomes move to the same pole, if indeed that occurs consistently. In non-random segregation, contrary to Mendel's law of independent assortment, partners of different bivalents in meiotic cells always go to the same pole. Non-random segregation was first observed between two chromosomes in spermatocytes of the mole cricket *Gryllotalpa borealis* by Payne (1912), and later confirmed by Camenzind and Nicklas (1968). Since then it has been observed in a variety of species, such as liverworts (Lorbeer 1934), flea beetles (Wilson et al. 2003), and sciarid flies (Metz et al. 1926), as reviewed in Brady and Paliulis (2015). For example, in mealy bugs and *Sciara*, all male-derived autosomes go to one pole and all female-derived chromosomes go to the other pole (mealy bugs: Schrader 1921, 1923; Nur 1982. *Sciara*: Metz et al. 1926; Metz 1938; Fuge 1994, 1997).

There are two pieces of evidence that suggest that *Mesostoma* spermatocytes might divide via non-random segregation. The first is univalent excursions (Oakley 1983). In *Mesostoma* spermatocytes, bivalents are bipolarly oriented and the two univalent pairs are unipolarly oriented. By anaphase each univalent is present at a spindle pole (Fig. 1), one of each kind at each pole, but univalents often move between poles throughout prometaphase, presumably to correct errors in segregation. Since there are more excursions than necessary to correct improper segregation if the two partners of each univalent pair were assorting randomly, Oakley (1985) suggested that the univalents might segregate non-randomly. Another line of evidence is from bivalent reorientations (Ferraro-Gideon et al. 2014). The bipolarly oriented bivalents in *Mesostoma* spermatocytes are clearly under tension because the bivalents elongate as the bivalents oscillate along the spindle axis. They nonetheless frequently reorient during prometaphase, and since in most reorientations the bivalent kinetochores switched poles, i.e. reversed their initial segregation, this might suggest that the

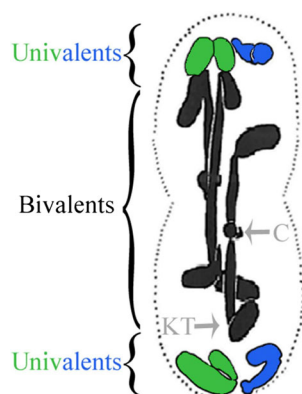


Fig. 1 Illustration of a *Mesostoma* spermatocyte modified from Husted and Ruebush (1940) and Ferraro-Gideon et al. (2013). The large bracket indicates the position of the three metacentric bivalents which are shown in black; the small brackets indicate the position of the univalents, with the two kinds indicated by blue and green. The arrow with a C points to a chiasma, and KT points to a kinetochore

bivalents, too, segregate non-randomly (Ferraro-Gideon et al. 2014; Brady and Paliulis 2015).

In this article, we present a third possible piece of evidence. When we depolymerized *Mesostoma* spermatocyte spindle microtubules using nocodazole (NOC), the three bivalents detached selectively from one pole in 52 out of 59 cells and all moved very quickly to the opposite pole. This supports the suggestion that non-random segregation occurs in these cells.

The second main issue from our work is what causes chromosome movement in the apparent absence of microtubules. Microtubules are considered the producers of forces that move chromosomes during cell division. According to the prevalent hypotheses, microtubules produce forces either because microtubules shorten at the poleward end (Cameron et al. 2006), the traction fibre or flux model, or because microtubules shorten at the kinetochore end, the PacMan model (Rieder and Salmon 1994). However, there is growing evidence suggesting that microtubules may play a more passive role as laid out in the spindle matrix model (Johansen and Johansen 2007; Johansen et al. 2011; Pickett-Heaps and Forer 2009; Spurck et al. 1997). In this model, microtubules impede rather than assist chromosome movement, and in their absence chromosomes are able to move quicker (Forer et al. 2015). Some of the evidence for this is as follows. When kinetochore fibres are cut during anaphase, chromosomes can continue polewards movement. In grasshopper (Gordon and Inoué 1979; Gordon 1980) and newt fibroblasts (Spurck et al. 1997; Pickett-Heaps et al. 1996) kinetochore fibres were severed with a UV microbeam and chromosomes continued moving and moved faster than previously. Crane-fly spermatocyte chromosomes do not change speed when their microtubules are severed, but do speed up when the “tether” that connects separating chromosomes is first cut with a laser (Sheykhan et al. 2017). These data suggest that microtubules limit the velocity of chromosome movements so that the chromosomes speed up when the ‘impeding’ microtubules are severed. There are related data from metaphase cells, also, as follows.

Chromosomes are at the equator at metaphase because forces on them to each pole are equal. Thus, when microtubules are depolymerized, it is expected that chromosomes should stay in the middle of the cell and arrest at the equator, which occurs in many cells (Cassimeris et al. 1990). However, when kinetochore fibres were depolymerized during metaphase in diatoms, the bipolarly attached chromosomes detached at random and moved to either pole at random (Pickett-Heaps and Spurck 1982). Pickett-Heaps and Spurck (1982) argued that microtubules resisted force from another spindle element, the spindle matrix, and that once colcemid was added and the resistance was gone, the matrix pulled the chromosomes towards either pole at random. In our experiments, immunofluorescence images show that after NOC treatment, *Mesostoma* spermatocyte microtubules are highly fragmented and are not associated with the moving

kinetochores when they move. Since the selective movement of chromosomes to one pole occurs in the absence of microtubules, this raises the issue of what causes their movement.

Materials and methods

Living cell preparations and drug treatment

Mesostoma were reared in the lab as described in detail by Hoang et al. (2013). To study live spermatocytes, their testes are sucked up using pulled 5, 10 or 15 μ L micropipettes (Fisher) and then expelled onto a cover slip into *Mesostoma* Ringers solution (61 mM NaCl, 2.3 mM KCL, 0.5 mM CaCl₂ and 2.3 mM phosphate buffer) that contains 0.2 mg/mL fibrinogen (Calbiochem). Thrombin is added, to cause formation of a fibrin clot, as previously described (Forer and Pickett-Heaps 2005, 2010; Ferraro-Gideon et al. 2014), and the cells then immersed in *Mesostoma* Ringers solution in a perfusion chamber. In order to ensure the cells were healthy, they were recorded for at least 10 min to track several cycles of bivalent oscillations. After concluding that the cells were healthy, the cells were perfused either with *Mesostoma* Ringers, as a control, or with drugs: 5, 10, or 20 μ M NOC in *Mesostoma* Ringers (Sigma) or 10, 20, 50, or 100 μ M colcemid in *Mesostoma* Ringers (Sigma). Both drugs were diluted with Ringers from a 1000 $\times$ concentrated stock solution in Dimethyl sulfoxide (DMSO). Addition of diluted DMSO by itself had no effect on oscillations (Forer and Pickett-Heaps 2010). The cells either were left in drug or rinsed out with Ringers solution 10–15 min later. The cells were followed on average for 45 min after drug addition. Live cells were viewed with phase-contrast microscopy using a Nikon 100 $\times$ oil immersion lens (NA 1.3) and recorded in real-time on a DVD recorder.

Data analyses

The free online program VirtualDub (www.virtualdub.com) was used to time-lapse the movies. We then imported the videos into our in-house program WinImage (Wong and Forer 2003). The position of each visible kinetochore in each frame was marked. The poles as seen electron microscopically are at the cell membrane at the ends of the spindle. They are not visible as discrete positions as viewed in phase-contrast microscopy, and if we were to measure kinetochore positions versus the pole, the pole positions we chose would invariably vary in different images since we cannot see discrete pole positions. The cells do not move when held in a clot so to overcome the variability in choosing pole position, in the first frame we analysed we chose a point that seemed to be ‘the pole’, and we used that same, fixed pixel position to measure against in all subsequent images. Thus, the movement graphs are in comparison to a fixed point at or near the spindle pole (see diagram in Fig. 2b); the values obtained may be slightly off in terms of actual distances

from the pole, depending on how close our fixed point is to the actual pole position, but they are close, and they are accurate in terms of speeds and distances that the kinetochores travelled. Distances were calculated by the WinImage program and were converted to movement graphs using the commercial program SlideWrite. Adobe Photoshop was used to construct diagrams representing different kinds of chromosome movement and to generate montages from a series of single images. Student’s *t* test was done to compare the different times and velocities after adding the different concentrations of drugs.

Immunostaining

Cells that were destined for immunostaining were not perfused with drug. After the prep was made and the cells were deemed healthy, the coverslip was directly submersed in a Petri dish with the desired concentration of drug for varying lengths of time (less than 5 and 30 sec, 1, 3, 4, 6, and 10 min) and then added to a lysis buffer with glutaraldehyde. Control cells that were not treated with drug were immediately placed into this lysis buffer. This lysis buffer, called NTSDc, contains 3% NP40, 2% Triton X-100, 2% Saponin, 5% DMSO, 0.5% Sodium Deoxycholate and 0.25% glutaraldehyde, all in PEM. (PEM is 100 mM PIPES, 10 mM EGTA, 5 mM MgSO₄ adjusted to pH 6.9.) After 2 min in NTSDc, the preps were then put for 1 to 2 hr into NTSDc without glutaraldehyde. Preps were then rinsed twice with phosphate-buffered saline (PBS) for 5 min each, then put in sodium borohydride (1 mg/mL) for up to 10 min in order to neutralise the free aldehyde groups, then rinsed twice again in PBS for 5 min each. The preps were then stored in a solution containing equal parts PBS:Glycerol at 4 °C for up to 2 weeks.

Both primary antibodies were added at the same time, and both secondary antibodies were added at the same time. The primary antibodies were anti-tubulin YL1/2 rat monoclonal antibody diluted 1:1000. The secondary antibodies were Alexa 568 or 594 goat anti-rat diluted 1:100. All antibodies were from Invitrogen, Burlington, ON, Canada, and all antibody dilutions were in PBS. The incubation time was 24 hr at room temperature in the dark for each double antibody stain, after which they were submersed in PBS twice for 5 min. Before adding the next double antibody, the preps were rinsed in PBS containing 0.1% Triton X-100 to aid in spreading the antibodies. Once the staining was complete, the preps were submersed in PBS twice for 5 min, then PBS: glycerol was added on top of the cells, to allow the glycerol in the Mowiol (described in the next step) to penetrate into the cell layer. Coverslips were then placed in Mowiol (Osborn and Weber 1982) which contained 0.2 g/L paraphenylene diamine (PPD) as anti-fading agent (described in Fabian and Forer 2005), put onto slides, and left to harden for 24–48 hr, after which they were stored at 4 °C until viewed with the confocal microscope. The cells were viewed using an Olympus Fluoview 300 confocal microscope using an

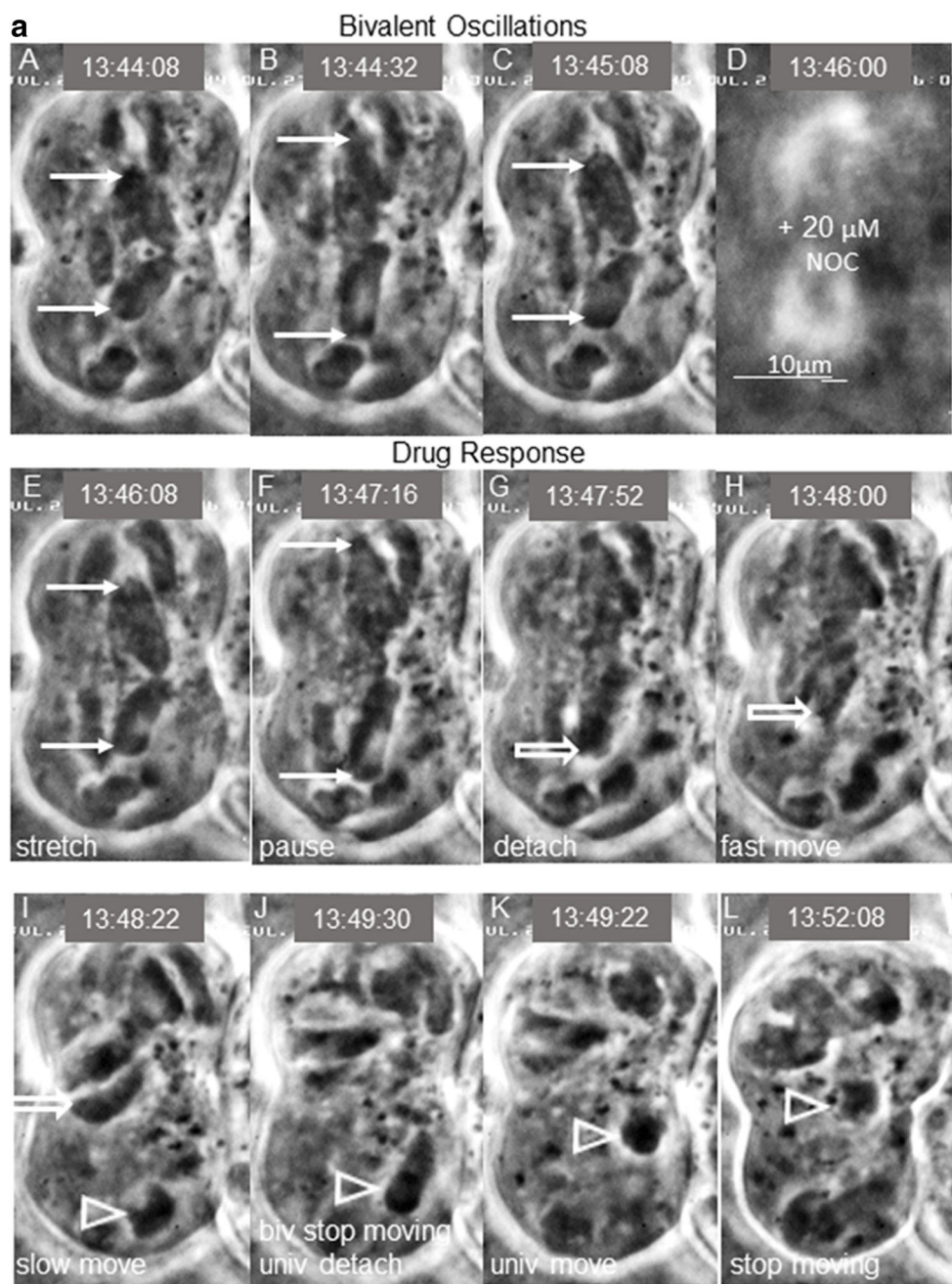


Fig. 2 **a** **b** **c** Image sequences and movement graphs of chromosome movement in a *Mesostoma* spermatocyte before and during treatment with 20 μ M NOC. **(a)** A-L. In images A-C the cell is immersed in Ringers solution. The white arrows point to the bottom and top kinetochores of one bivalent. They both oscillate towards their respective poles in A to B, then away from the poles in B to C. Image D is during the perfusion of 20 μ M NOC. In images E-L the cell is immersed in 20 μ M NOC. Bivalents begin stretching towards either pole in E, and once they reach both poles they pause for a period of time as shown in F. The open arrow in G shows the initial detachment of only the bivalents from the bottom pole. Images H and I are during the fast and slow movement of the bivalents towards the top pole. The kinetochores swing out towards the left side of the cell. Both univalents remain at the bottom pole during bivalent movement, but one univalent detaches in J, moves towards the

top pole in K, and stops in L. A video sequence of this cell is presented as supplemental video 3. In this and all other video figures the times are given in hours:minutes:seconds. **(b)** Graphical representation of chromosome movement in the same cell before and during 20 μ M NOC addition. Bivalent oscillations during prometaphase appear as sine or saw tooth waves. NOC addition is indicated by the solid black line. Chromosome speeds are determined from the lines of best fit, as indicated in grey. The diagram to the right of the graph shows how kinetochore positions were determined. Kinetochore positions, as indicated by the pink and green arrowheads, were plotted relative to their distance from a fixed point at the bottom edge of the cell membrane near the centrosome, as shown by the red X. **(c)** Close up of the movement of the bottom kinetochore (which is the green triangle in B). This pattern of movement is consistent among all the 52 cells in which detachment occurred at only one cell pole

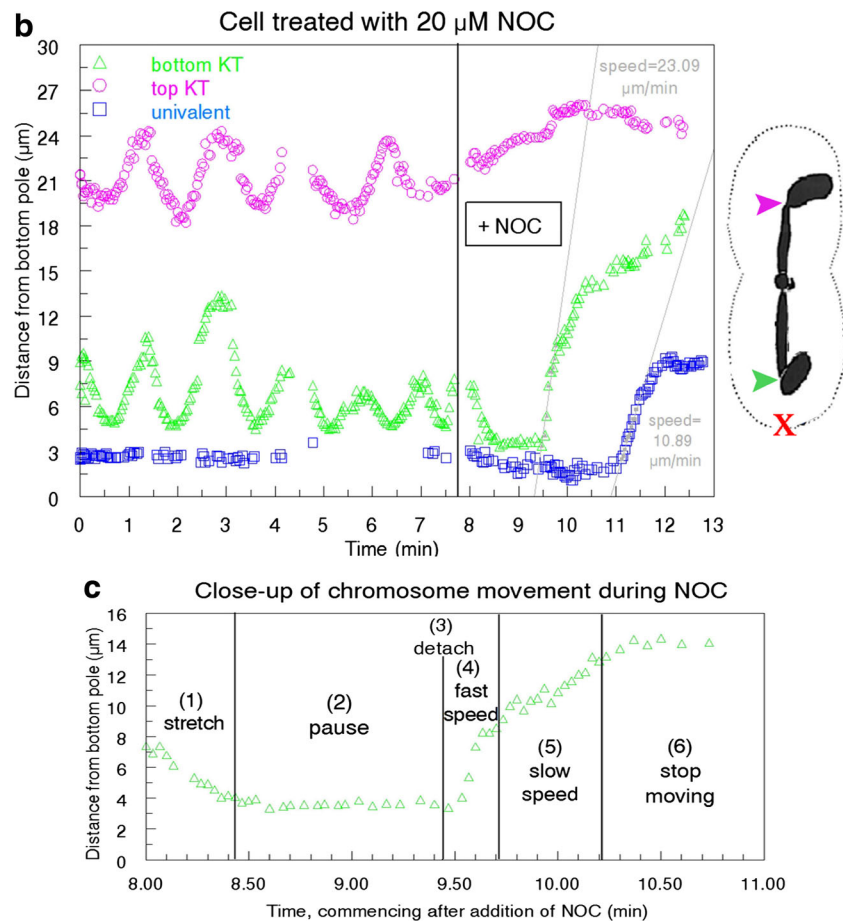


Fig. 2 (continued)

Olympus plan-apo 60× objective, NA 1.4. Images were taken using the Fluoview 300 program, and colour was added to Z-projected stacks using the free online program ImageJ. Tubulin is shown in green and DIC in black and white.

Results

Control cells

Bivalent oscillations

Bivalent chromosomes in *Mesostoma* spermatocytes constantly oscillate towards and away from their respective poles, from the time the bivalents are bipolarly oriented until anaphase (Fuge 1987, 1989; Oakley 1983, 1985; Ferraro-Gideon et al. 2013, 2014). An example of oscillation in one bivalent is illustrated in Fig. 2a, images A–C. The arrows indicate the position of the bottom and top kinetochores of an oscillating bivalent. Between A and B the bottom and top kinetochores of the bivalent both oscillate towards their respective poles, and from B to C they both oscillate away from their pole returning to their original positions. The oscillations of the two kinetochores of

one bivalent vary during prometaphase (Ferraro-Gideon et al. 2014): sometimes they both go towards the pole at the same time as in Fig. 2a, thereby stretching the chromosome, and sometimes one moves towards its pole while the other moves away, so the chromosome moves as a whole. Different bivalents oscillate independently, with different speeds and directions of their two kinetochores (Ferraro-Gideon et al. 2014). Chromosome movement is also shown graphically by plotting the relative distance of each bivalent's kinetochore to a fixed position (shown by the X) near the pole, seen in Fig. 2b. The oscillation speeds towards and away from the pole differ: movement away from the pole averages 5.2 $\mu\text{m}/\text{min}$ and towards the pole 6.2 $\mu\text{m}/\text{min}$ (Ferraro-Gideon et al. 2014). Since bivalents do not stop oscillating before anaphase, they never align along a metaphase plate. There is question whether the entire period should be called prometaphase or metaphase, or whether prometaphase lasts only until the univalents are properly oriented at the two poles after which the stage is metaphase (discussed in Fuge 1989); we will consider that the majority of meiosis-I is spent in prometaphase, which lasts up to 2 h, after which the cell goes directly into anaphase-I, with continuing oscillations throughout prometaphase but no stable intervening metaphase (Fig. 3 i).

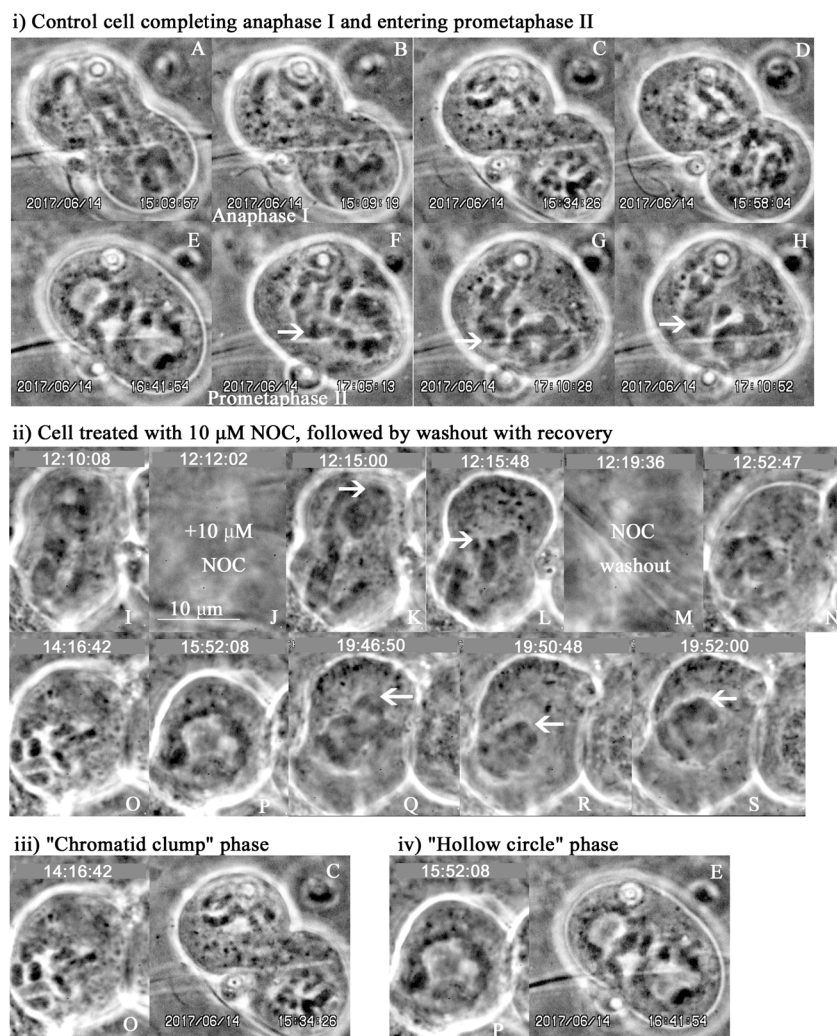


Fig. 3 i–iv Cells treated with NOC appear similar to control cells in-between meiosis I and II, and attempt to go into second division. Times are shown as hr:min:sec. **(i)** A control cell completes anaphase I and the two resultant nuclei enter into meiosis II. In C and D the bivalents have formed into two distinct groupings of chromatids and in E the chromosomes are arranged at the periphery of the nucleus, appearing as a hollow circle. In F–H the left nucleus enters second division and the chromatids begin oscillating along the spindle axis. Although not shown, the second nucleus enters second division sometime later and both cells complete anaphase II. **(ii)** After the addition of 10 μM NOC in figure J, the bivalents stretch out, detach and move to the bottom pole. NOC is washed out

with Ringers in figure M, after which the chromosomes in the cell move, a nucleus forms, and the chromosomes become arranged as in control cells between meiosis-I and meiosis-II. This is followed by an abortive attempt at second division (O–S). In O, the bivalents have moved down in the cell and formed into one distinct grouping of chromatids. In P the nucleus reforms and the chromatids arrange themselves in a hollow circle. In Q–S the cleavage furrow reforms and the chromosomes begin to oscillate but do so irregularly and the cell does not enter anaphase. The **(iii)** 'chromatid clump' and **(iv)** 'hollow circle' phases appear almost identical when comparing the NOC treated cell to the control cell

Second division

Meiosis II has not been studied in these cells, and we have been able to follow only 3 cells from meiosis-I to meiosis-II. After completing anaphase-I, the two resultant nuclei form two groupings of chromatids which then arrange themselves into a hollow circle in the newly reformed nucleus (Fig. 3i). These observations appear similar to Steven's early drawings of spermatogenesis (Stevens 1905, 1906). Although not pictured here, the nuclei and their chromatids rotate back and forth. Two or more hours after anaphase-I completion, the

cells enter meiosis-II as a spindle is set up and the chromatids begin oscillating up and back in the spindle at speeds similar to those recorded in prometaphase-I.

Precocious furrow

Another unusual feature to this cell's meiosis-I is that most *Mesostoma* spermatocytes have a precocious, pre-anaphase cleavage furrow, as shown in Figs. 2a and 3i). This furrow moves in response to chromosome imbalances in the two half spindles which can occur throughout prometaphase. When a

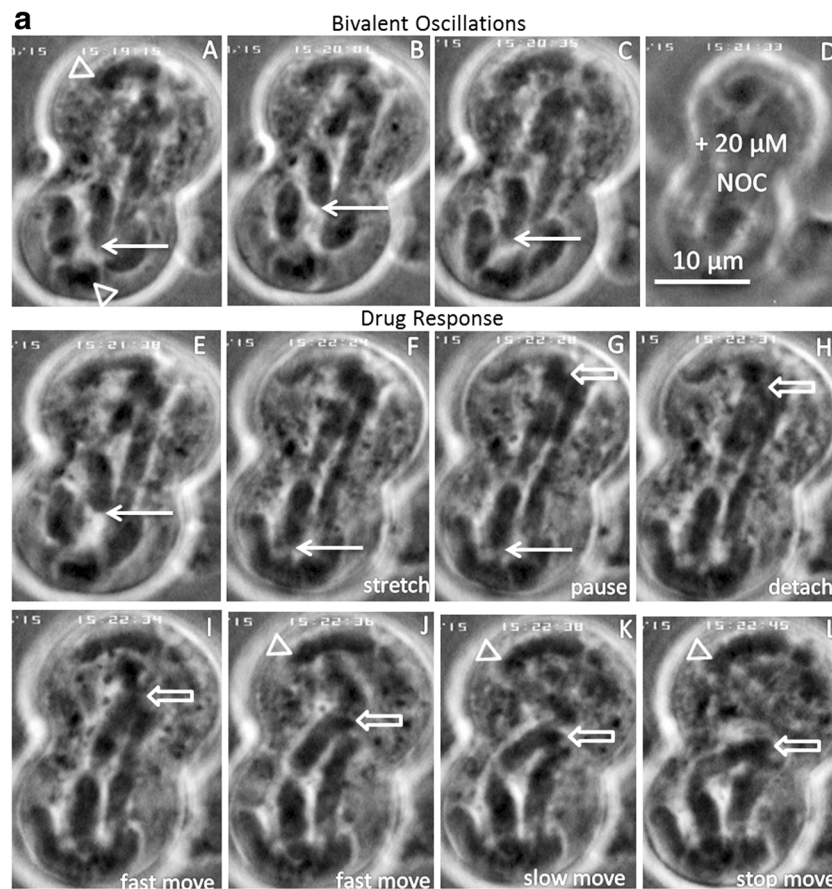


Fig. 4 **a, b** Image sequence of a *Mesostoma* spermatocyte treated with 20 μ M NOC. Times are shown as hr:min:sec. **(a)** A–L. In images A–C the cell is immersed in Ringers solution. In A, the triangles indicate the positions of the univalents at the two poles. The white arrow points to the bottom kinetochore which oscillates away from the pole in A to B, then towards the pole in B to C. Image D is during the perfusion of 20 μ M NOC. In images E–L, the cell is immersed in 20 μ M NOC. Image E–F shows bivalents stretching towards their poles. G shows they pause at both poles. H shows the initial detachment of only the bivalents from the top pole, as indicated by the open arrow. Images I and J are during the fast movement of the kinetochores towards the bottom pole, and the triangle indicates the univalents that stay behind. Figure K is during the slow movement, and L is after bivalents stop moving. **(b)** The same cell illustrated in A. Chromosomes do not move in a straight line during the addition of NOC. The image sequences in i–iii all use the same image frames. These images were originally at an angle but were tilted in order to line the cell axis vertically. This cell is shown in supplemental video 1. (i) The positions of the two kinetochores are shown by the two white dots in each frame. Each image frame is 1 s apart. An overlay of the kinetochore positions in images A–J shows that the kinetochores do not move in a straight line, but change orientation and move towards the right. [There appears to be one dot fewer associated with the right kinetochore than the left kinetochore because two of the dots superposed.] (ii) A vertical line

that includes the centrioles at both poles (as indicated by the directions the kinetochores were moving in) is drawn through the midline of the cell to show bivalents bend to the left side as the kinetochores move towards the right. (iii) A tracing of the bivalents was drawn on top of frames A–J, and the background was then subtracted. As the kinetochores move towards the bottom pole and to the right, the body of each bivalent bends towards the left side. The length of the bivalents also decreases (the length is the distance between the sister kinetochores of one bivalent). For stretched bivalents, we measured the straight-line lengths from kinetochore to kinetochore but as the bivalents started to bend, we estimated straight-line lengths from each kinetochore to the middle of the bend and then summed the two. The left bivalent decreased in length (from its maximum length during stretch) from 20.5 μ m to 16.4 μ m, i.e. to about 80% of its stretched length. The actual length of the right bivalent may be longer than we measured because the bivalent appears to bend backwards out of the plane of focus. (iv) Graphical representation of the fast and slow movement of the right kinetochore. The kinetochore position was plotted 30 times per second over a course of 12 s. Each second from 2 to 11 s lines up with the corresponding images in i–iii, as labelled. As seen by comparing the images in i–iii to the speeds in the graph, kinetochore movement to the right and bivalent bending towards the left occur during both the fast and slow movements

univalent excursion or a bivalent reorientation occurs, for example, the furrow responds by shifting an average of 1–2 μ m towards the half cell with fewer chromosomes (Forer and Pickett-Heaps 2010; Ferraro-Gideon et al. 2013, 2014). This creates a cell with one-half cell that is smaller than the other.

NOC-treated cells

Chromosome movement

There were three different responses after addition of nocodazole (NOC). Chromosomes (1) detached from only

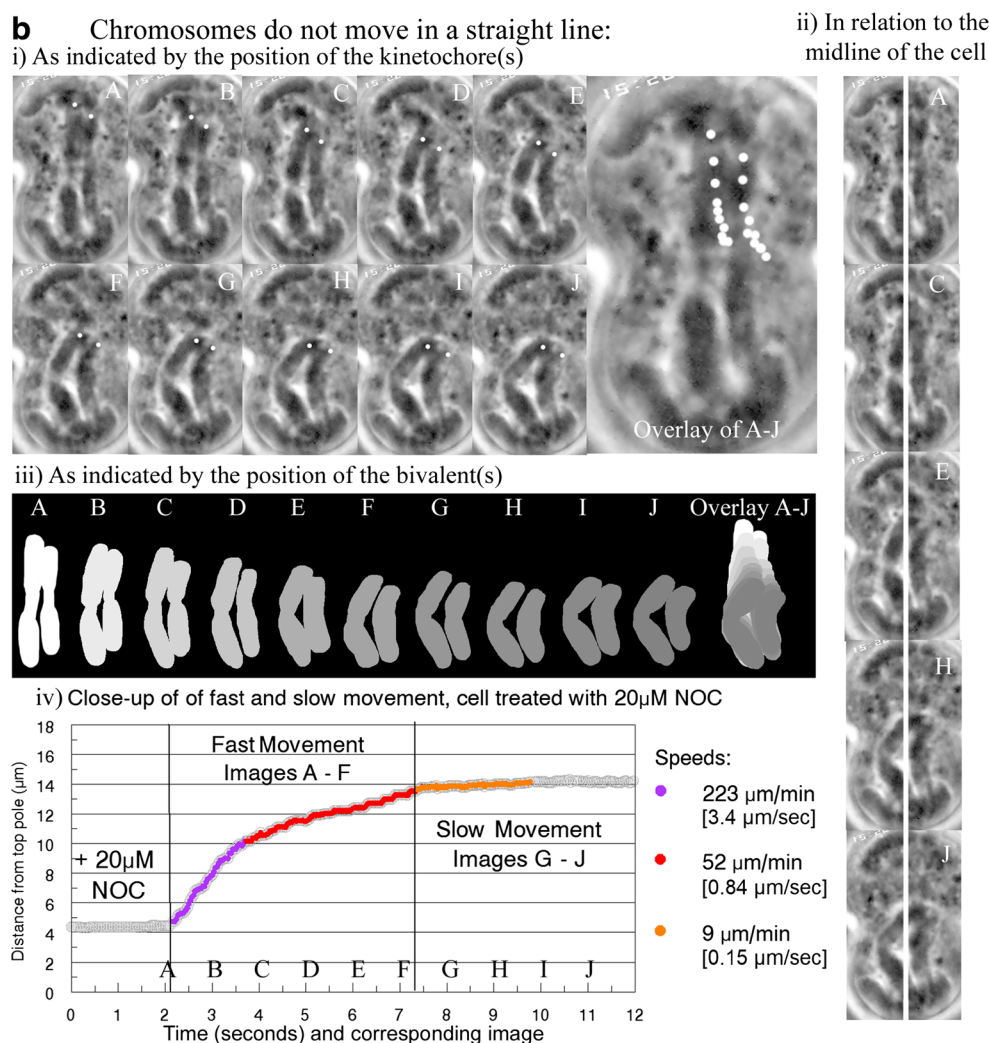


Fig. 4 (continued)

one pole, (2) detached from both poles, or (3) both poles collapsed. We describe these responses in turn.

Response (1), detached from only one pole. When chromosomes detached from only one pole (52/59 cells), they always followed a consistent 6-stage pattern of movement (Figs. 2b, c). After addition of drug, the chromosomes stretch towards both poles, pause for some period of time, detach at only one pole, and the detached kinetochores quickly move towards the opposite pole, slow down and then stop. The 'stretch' increases the length of the bivalent by about 25–30% from its length immediately prior to NOC, and the length decreases back to its original length immediately after detachment, in the first 10–15 s during the fast movement, after which the movements of the kinetochore take place at constant length. In most cells, only the bivalent kinetochores detached and moved, but in 14 cells both the bivalents and univalents detached and moved to the same pole. In 7 of these 14 cells, the univalents moved at the same time as the bivalents. In the other cells the univalents moved from times varying from 1 to 15 min later (e.g. Fig. 2a and

supplemental video 3). The univalents that moved at the same time as the bivalents moved with the same speed as the bivalents. The other 7 moved with average speed of $11.8 \pm 2.6 \mu$ m/min (s.d), range 8.7–16.5 μ m/min.

In order to understand the forces acting on the chromosome kinetochores, it is worth looking more carefully at the manner in which the chromosomes move, and the speeds of the movements, to see, for example, whether the movements could be due to recoil of the stretched chromosomes after release from a pole or whether other forces need be invoked. Immediately after detachment, the kinetochores move extremely rapidly towards the opposite pole, usually at an angle to the direction the kinetochore partners were aligned in, and not in a straight line (Fig. 4a, b). As the one kinetochore moves the bivalent bends at an angle to its original orientation, as illustrated in Fig. 4 b i–iii and supplemental video 1. Bivalents in any given cell often move at the same time, but each kinetochore and bivalent move independently, and not as a linked group: the chromosome arrangements change during movement and some move farther

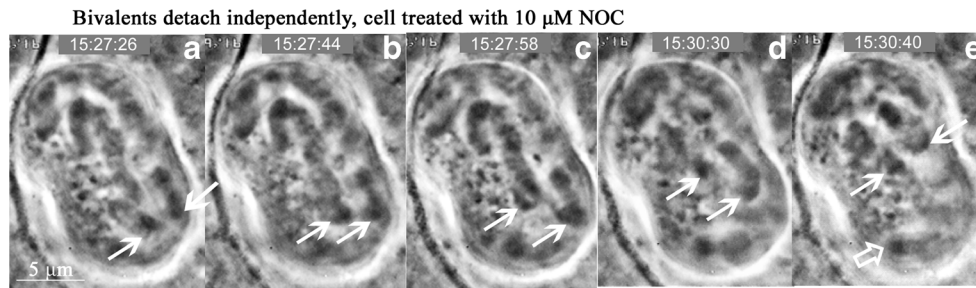


Fig. 5 **a–e** Bivalents detach at separate times within the same cell, during the addition of NOC. As seen in **a** white arrows indicate the position of two visible kinetochores of two separate bivalents that are in the process of stretching. In **b** the left kinetochore begins to detach before the right

one, and in **c** the left kinetochore is seen moving towards the top pole. In **d** the right kinetochore has detached and begins moving towards the top pole, and both bivalents reach the top pole in **e**. Times are shown as hr:min:sec

than others. Furthermore, in some cells, bivalents detach at separate times, though all the chromosomes nonetheless move towards one pole (Fig. 5). The drawings in Fig. 6 illustrate the configurations at the final ‘stop’ positions.

Response (2), kinetochores detached from both poles. In 5/59 cells one bivalent detached from either pole. [One such cell is shown in supplemental video 4.] This resulted in a mix of some bivalents moving toward one pole, and some moving toward the other pole. As illustrated in Fig. 6 (2), the left bivalent detached from the top pole, while the right bivalent detached from the bottom pole. Sometimes one of three bivalents did not detach and remained in the stretched configuration, such as the middle bivalent in Fig. 6 (2). Univalents always remained at the poles.

Response (3), chromosomes remain in the middle. Chromosomes stretched out towards either pole as in responses (1) and (2), paused at either pole, then the bivalents shortened slowly. There was no visible, abrupt detachment from either centrosome. Later, the univalents at both poles drifted slowly (< 3 μm/min) towards the cell equator and bivalents shortened

as the univalents and bivalents moved away from the poles as a clump, for a short distance, never reaching the equator [one example is shown in supplemental video 5]. This response occurred very rarely, in only 2/59 cells.

Washout

We assume that the chromosome movements that occur after NOC treatment reflect on the functioning and organisation of the force-producing apparatus in normal cells. Because treated cells change drastically after treatment with NOC, however, and do not look ‘normal’, we need to be sure that chromosome movements after NOC reflect the ordinary function of the spindle and are not abnormal, pathological movements, induced by imminent apoptosis. Several lines of evidence indicate that the cells do not die after NOC treatment. For one, in the continuing presence of NOC, the furrow ingresses and moves towards the pole with fewer chromosomes (Fegaras E., and Forer F., Precocious cleavage furrows simultaneously move and ingress when

Movement type:						
(1) Chromosomes detach from only <u>1</u> pole				(2) Chromo- somes detach from <u>2</u> poles	(3) Chromo- somes remain in the middle	
A) Univalents don't move, bivalents move to 1 pole		B) Bivalents and univalents move to 1 pole				
Past equator	Not past equator	Past equator	Not past equator			
						
5μM NOC	10	2	2	0	2	1
10μM NOC	5	8	5	2	1	0
20μM NOC	11	2	2	3	2	1
Cell Totals:				=52	=5	=2

Fig. 6 The 6 observed configurations of chromosomes in *Mesostoma* spermatocytes in response to the addition of NOC, and the numbers of each configuration that were seen. The univalents are seen at either pole, and the bivalents stretch across the middle of the cell. When kinetochores move, the chromosomes curl and continue moving, kinetochore first, either past or not past the equator. The equator of the

cell is the site where the precocious cleavage furrow pinches in, creating the dumbbell shape. The univalents may either travel with the bivalents or stay behind at their respective poles. Numbers of cells seen in each category are included below each diagram, arranged by drug concentration

kinetochore microtubules are depolymerized in *Mesostoma ehrenbergii* spermatocytes. Submitted to *Protoplasma*, in press), sometimes moving off the end of the cell but often forming two different sized cells, still attached, resembling somewhat the appearance of a “shmoo” (Capp 1948), seen in supplemental video 3. Further, univalents left behind at a pole sometimes move across the equator to the opposite pole, up to 15 min after addition of NOC. These all indicate that the cells may be wounded, but that they are alive and functioning.

To further study whether the cells are alive and functioning, we washed out the NOC after the kinetochores moved to the opposite

pole, to test whether normal cell activity resumes after removal of NOC. After washout, the spindle does not reform but the cells undergo changes reminiscent of the changes that occur between meiosis-I and meiosis-II in control cells, as illustrated in Fig. 3ii). In that particular cell, NOC was washed out 7.5 min after NOC was added, shortly after the kinetochores moved to one pole. Subsequently the cell developed morphological changes characteristic of spermatocytes between meiosis-I and meiosis-II, namely formation of an apparent nucleus, and then formation of the chromatid clump and hollow circle phases, and even developing what looks like a spindle. Although the chromosomes oscillate up

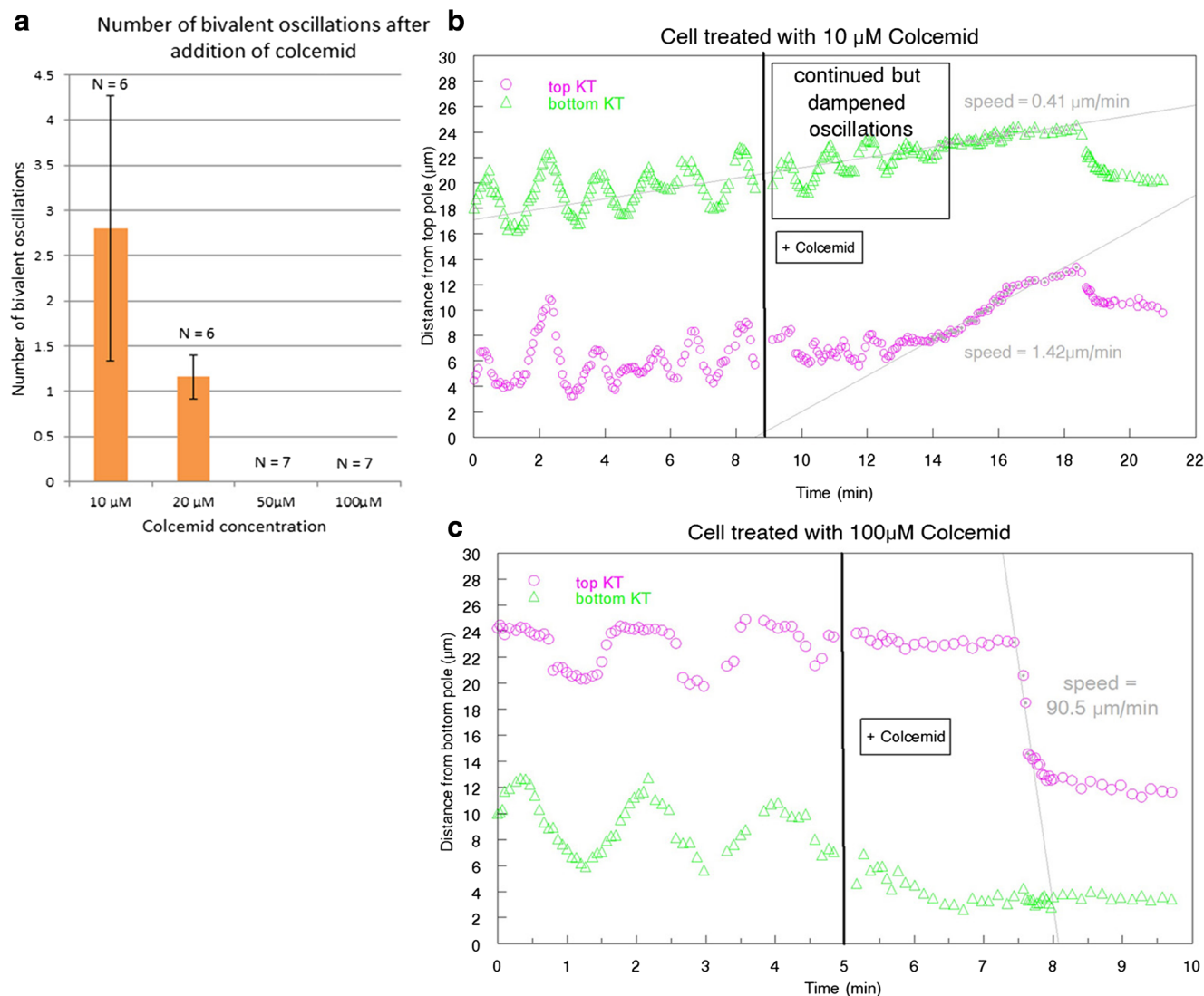


Fig. 7 **a–c** *Mesostoma* spermatocytes treated with varying concentrations of colcemid. At low concentrations of colcemid the bivalents continue to oscillate; there are fewer and fewer oscillations as the colcemid concentration is increased. At 50 and 100 μM colcemid there are no oscillations and the response is the same as in NOC treated cells. **a** The average number of bivalent oscillations in each cell during the addition of colcemid. One oscillation is when a kinetochore completes an entire wavelength in the graph. N = number of cells. The capped bars indicate the standard deviations. **b** Movement graph of a cell treated with 10 μM colcemid. The vertical black line indicates addition of colcemid, and the

black box indicates the oscillations which continue in the presence of drug. As visualised by the green triangles, the kinetochore moves towards the pole while oscillating but eventually stops oscillating and stays at that pole. The other kinetochore on the same bivalent, violet circles, also continues to oscillate, stops, then both kinetochores move towards the same pole. **c** Cell treated with 100 μM colcemid. The vertical black line indicates addition of colcemid. The chromosome responds in the same pattern as cells treated with NOC. In this cell the detached kinetochores moved at around 91 μm/min towards the opposite pole

and back in direction of the spindle axis, in what appears to be a meiotic-II spindle, the oscillations are irregular, are slow, the chromosomes remain in a clump, and it is hard to discern the position of the kinetochores. Figure 3iii, iv provides a comparison of the NOC treated cell and a control cell about to enter second division, showing that the phases between division are very similar in both circumstances. The NOC-treated cells take a prolonged period of time before attempting to enter second division, up to 7 and a half hours instead of the usual 2 h. We have not been able to follow all of the cells for which we washed out the NOC for the > 7 h to form a meiosis-II spindle, but we have seen chromatid clump and hollow circle phases in the majority of cells after NOC washout. We have not seen meiosis-II anaphase in cells after washing out the NOC, but all the changes that occur as described above indicate that the NOC itself was not lethal to the cells—chromosomes move, the cleavage furrow functions, and the cells differentiate towards meiosis-II—and therefore that the movements that occur as a result of NOC treatment are not pathological but are due to normal forces in the living cell.

Colcemid-treated cells

To test whether the results after treatment with NOC were due to depolymerization of microtubules and not to side effects of the NOC, we treated cells with colcemid, another agent that depolymerizes microtubules. NOC suppresses microtubule dynamics by decreasing the elongation and shortening rates, thereby increasing catastrophes. At high concentrations NOC causes microtubules to fragment and depolymerize (Vasquez et al. 1997;

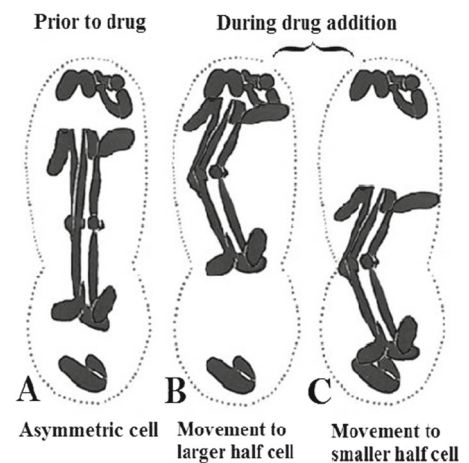
Jordan et al. 1992) and may also cause the centrosome to fragment (Jordan et al. 1992). In comparison, colcemid at low concentrations binds to microtubule plus-ends and can stabilise microtubules, and at higher concentrations, it binds to minus ends and interferes with microtubule dynamics, and may also promote microtubule detachment from the centrosome (Jordan and Wilson 2004; Yang et al. 2010).

The results after treatments with higher concentrations of colcemid are identical to those after treatments with NOC, but results with lower concentrations of colcemid are different than after NOC, perhaps due to their different mechanisms of action. At lower concentrations of colcemid (10 and 20 μM), bivalents continued oscillating for a short period of time with continually decreasing amplitude and period (Fig. 7b, and supplemental video 2), and bivalents did or did not detach abruptly. In all cells treated with 10 μM colcemid bivalents stretched and remained stretched in the middle of the cell (response 3 of Fig. 6). At 20 μM colcemid, the chromosomes detached from one pole in 3 out of 6 cells, and the detached chromosomes slowly moved towards the opposite pole. The number of oscillations that occurred in the presence of colcemid depended on the colcemid concentration (Fig. 7a). At the higher concentrations of 50 and 100 μM colcemid, all bivalents detached and moved to one pole (Fig. 7c) (response 1 of Fig. 6) similar to treatment with NOC. Thus, when depolymerisation of microtubules is rapid, the colcemid effects are the same as those after NOC, and thus, the effects we observe are due to depolymerisation of microtubules.

Table 1 Comparison of whether bivalents move into the larger or smaller half-cell, when the cell was asymmetric prior to addition of NOC or colcemid. All illustrations are of an asymmetric cell prior to drug addition in which there are three univalents at the top pole, and only one at the bottom. The imbalance in the number of univalents causes the furrow to shift towards the pole with fewer univalents, the

bottom pole. A The cell prior to addition of drugs. B, C Chromosome movement after addition of drug. In B, movement is towards the large half-cell, and in C movement is towards the smaller half-cell. The number totals indicate that there was no preference for movement to either the larger or the smaller side

Drug	Conc. (μM)	Prior to drug, furrow was asymmetric	During drug, bivalents moved into:	
		(Response A)	larger half cell (Response B)	smaller half cell (Response C)
Colcemid	10	1	0	1
	20	1	1	0
	50	0	0	0
	100	4	2	2
Nocodazole	5	4	2	2
	10	6	3	3
	20	2	1	1
Totals:		18	9	9



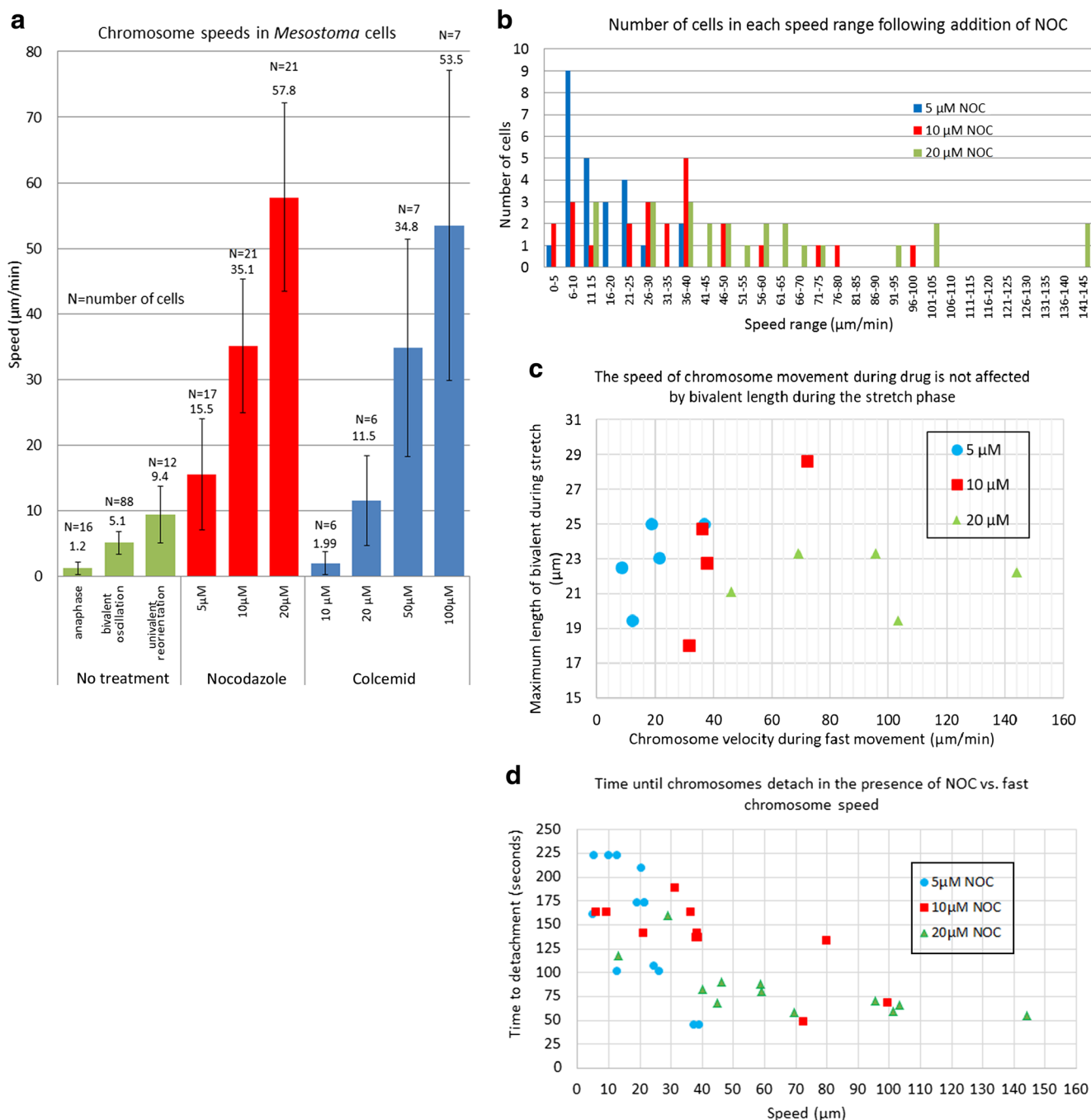


Fig. 8 **a–c** Speeds of chromosome movement in control cells or after addition of NOC or colcemid. **a** Speeds of chromosomes after detachment with NOC or colcemid increase with increasing concentration of drug, and are much larger than speeds in non-treated cells. [The data for non-treated cells is from Ferraro-Gideon et al. 2014.] Standard deviations are represented by the half-lengths of each bar. At the top of each bar is the average speed for that condition and above that is N, the number of cells. These values are also shown in Table 2. Using Student's *t* test, the speeds at the different concentrations of NOC are significantly different ($p < 0.05$), as are those for the different concentrations of colcemid ($p < 0.05$). **b** Distribution of fast chromosome

movement speeds after detachment with respect to concentration of NOC. **c** Length of stretched bivalents (at their maximum stretch before NOC was added) versus the fast speed of the kinetochore movement, at different concentrations of NOC. There does not seem to be any relation between speed and length. **d** Time until chromosomes detach in the presence of NOC vs. fast chromosome speed. Chromosomes that detach earlier are more likely to move at a faster speed, whereas chromosomes that take a longer time to detach are more likely to move at a slower speed. These data include only cells in which kinetochores detached from one pole and moved across the cell equator

Table 2 Average times and speeds for each stage of the chromosome response for the different concentrations of NOC. The fast chromosome movement speeds also are shown in Fig. 5. The $\pm$ standard deviations are included for each value, and the cell sample size is indicated by the n = value

NOC conc.	Stretch duration (min:sec)	Pause duration (min:sec)	Time from drug addition to detachment (min:sec)	Fast and slow movement duration (min:sec)	Fast speed chromosome movement ($\mu\text{m}/\text{min}$)
5 μM ($n = 17$)	01:11* $\pm$ 00:26	01:29* $\pm$ 00:54	02:40 $\pm$ 01:20	01:21 $\pm$ 00:26	15.5* $\pm$ 9.07
10 μM ($n = 21$)	01:00* $\pm$ 00:23	01:06* $\pm$ 00:27	02:06 $\pm$ 00:50	01:20 $\pm$ 00:51	35.1* $\pm$ 24.2
20 μM ($n = 21$)	00:46* $\pm$ 00:18	00:58* $\pm$ 00:36	01:44 $\pm$ 00:54	00:43 $\pm$ 00:17	57.8* $\pm$ 35.1

*Values that are significantly different using Student's t test at $p < 0.05$

Sidedness

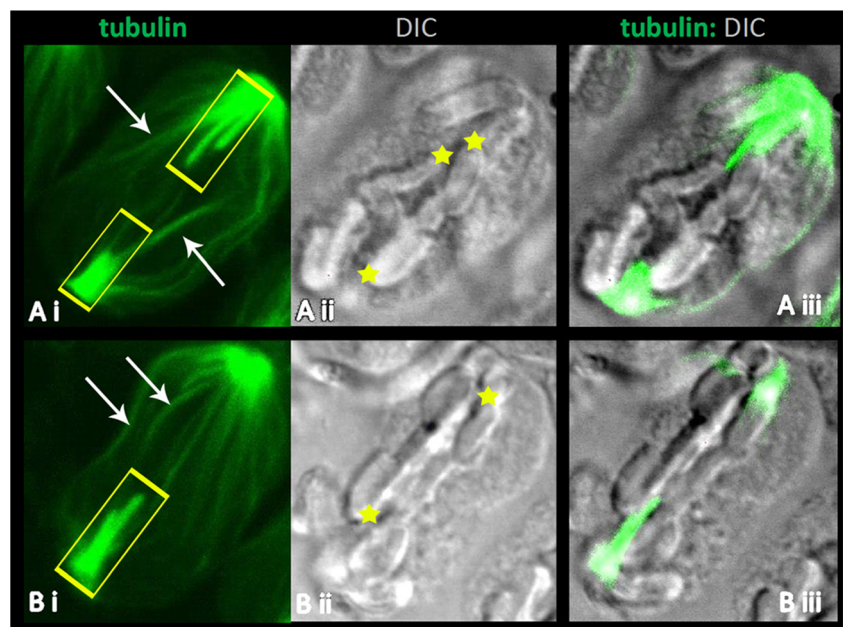
What is the difference between the two poles, such that all the chromosomes detach from one and move to the other? We have not been able to tell which pole the chromosomes will detach from by scrutinising the video sequences and graphs and looking at the behaviour of the bivalents, univalents, and the furrow before addition of drug. We considered various possibilities. For example, if bivalent kinetochores at one pole oscillated at an irregular speed that may be the pole from which bivalents detach. Or, if a univalent excursion recently occurs from one pole, perhaps that is the pole the bivalents will detach from. We also analysed kinetochore oscillation directions to see if they would indicate the pole to which the chromosomes would move after addition of NOC, but they gave no indication: when a bivalent was oscillating towards a pole before addition of drug, the kinetochore did not necessarily move towards that pole after NOC. None of these parameters allowed us to predict the sidedness. We then considered whether furrow asymmetry might indicate the sidedness of the response. Asymmetric cells occur when there is an unequal distribution of chromosomes, for example three univalents at one pole and

one at the other (Forer and Pickett-Heaps 2010). We treated asymmetric cells with NOC or colcemid to see if chromosomes would consistently move into either the larger or smaller half-cell (Table 1). If the response was due to the location of the furrow there would only be bivalent movement to the larger or smaller half-cell. However, chromosomes were equally likely to move into the larger half-cell or the smaller half-cell when treated with either NOC or colcemid (Table 1). Thus, the non-random movement of chromosomes, the sidedness of the spindle, is due to some factor we have not yet determined.

Chromosome speeds increase as drug concentration increases

The speeds that chromosomes move after they detach from one pole after addition of NOC or colcemid increase with increasing concentration of drug (Fig. 8a). The speeds for chromosome movement are significantly different at each drug concentration and are much larger than prometaphase oscillation speeds in non-treated *Mesostoma* spermatocytes, which average 5.1 $\mu\text{m}/\text{min}$ (Ferraro-Gideon et al. 2014). Cells treated with 10 μM NOC most often had speeds in the 36–40 $\mu\text{m}/\text{min}$ range, with

Fig. 9 A–B Tubulin immunofluorescence staining in control cells, showing the presence of non-kinetochore microtubules (some are indicated by white arrows), kinetochore fibres (enclosed in yellow boxes) and centrosomes at either pole. DIC image of the chromosomes are in grey. Kinetochore marked with the yellow asterisk have visible kinetochore fibre attachments as seen in the merged images. The tubulin images are of a Z-stack; the DIC images and tubulin:DIC images are of only one image in the stack



a maximum speed of 100 $\mu\text{m}/\text{min}$. Cells treated with 20 μM NOC had the fastest speeds; 4 cells had speeds over 100 $\mu\text{m}/\text{min}$, up to 145 $\mu\text{m}/\text{min}$ (Fig. 8b). [Chromosome speeds indicated in Fig. 8b and throughout are low estimates. They derive from graphs with data points 2 s apart. When 30 points were obtained per second, as in Fig. 4b (iv), speeds were above 200 $\mu\text{m}/\text{min}$]. In cells treated with lower concentrations of colcemid, the chromosomes did not detach and chromosome speeds were more in line with speeds in control cells, whereas in cells treated with higher concentrations of colcemid the chromosomes detached and moved with speeds in the same range as after NOC treatment (Fig. 8a). We tested whether the increased speeds were related to the length of the stretch period and the length of the stretch itself before chromosomes detached.

Chromosome speeds increase as the times to detachment decrease, but are independent of stretched length

The fast speeds of chromosome movement did not seem to be related to the length of the stretched chromosomes (Fig. 8c) but did seem related to NOC concentration. As the NOC concentration increased, the stretch duration and pause duration decreased, while the fast speed of chromosome movement increased (Table 2). We considered ‘the time to detachment’ as the stretch and pause duration lumped together. The relationship between the time to detachment and chromosome speeds is shown graphically in Fig. 8d. Based on the trend of the data, chromosomes that detach and begin movement earlier are more likely to move at a quicker speed, whereas chromosomes that take a longer time to detach move at a slower speed. Chromosomes in 5 μM -NOC-treated cells take the longest time to detach, and have the slowest speeds, whereas those in 20 μM -NOC-treated cells generally detach quickly and move the fastest (Fig. 8d). This suggests that the faster the spindle microtubules are lost the faster the chromosomes move.

But we need to test when microtubules are lost after the drugs are added.

Immunofluorescence

Control cells

Staining for tubulin in *Mesostoma* primary spermatocyte cells shows that the spindle consists of many thin non-kinetochore microtubules and a few thick kinetochore fibres (Fig. 9 and 10a). There are also brightly stained centrosomes at each pole. The kinetochore fibres radiate from the centrosome and terminate at the kinetochore of a bivalent. In the cell illustrated in Fig. 9, image Ai, there are two easily distinguishable kinetochore fibres at the top pole connecting to two separate bivalents near the top pole, and one very thick bundle at the bottom pole that is associated with one of the bivalents near that pole.

In Fig. 9, image Bi there are two kinetochore fibres at the bottom pole both of which connect to the bottom kinetochore.

NOC-treated cells

The microtubules of the meiotic spindle immediately began to fragment and depolymerize after NOC addition. This is illustrated in Fig. 10, a montage of single confocal microscope sections of spindles that were fixed after NOC treatment. After only 30 s in 10 μM NOC, the non-kinetochore microtubules have fragmented and have begun to depolymerize, and the effects are even stronger in 20 μM NOC (by studying adjacent confocal sections we confirmed that the microtubules

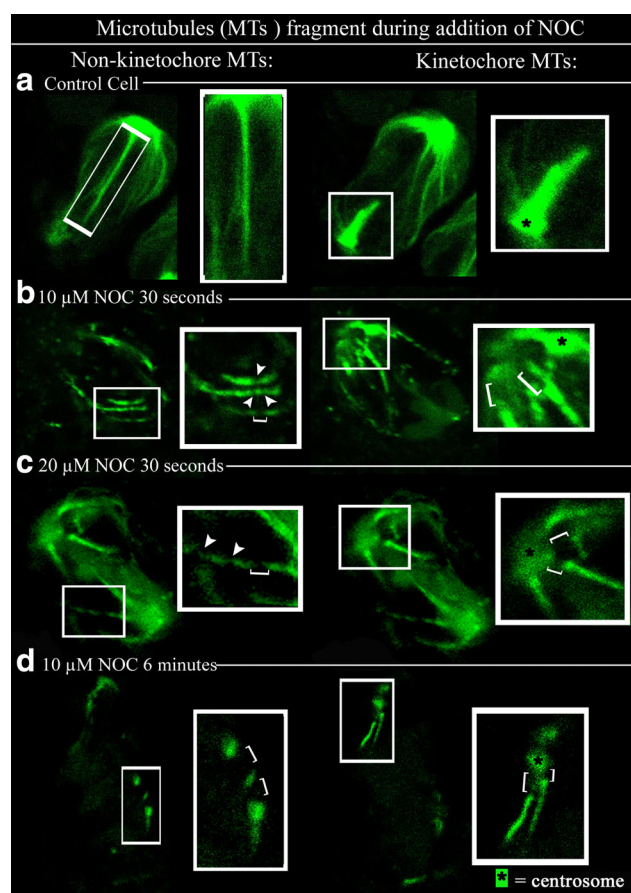


Fig. 10 a–d Tubulin immunofluorescence staining shows both non-kinetochore and kinetochore microtubules fragment upon the immediate addition of NOC. Each image is one layer taken from the Z-stack. The asterisk indicates the position of the centrosomes, arrowheads point to microtubule regions in the process of fragmenting, and brackets span across larger regions where microtubules have depolymerised. **a** A control cell showing a long non-kinetochore microtubule spanning across the middle of the cell, as well as brightly stained kinetochore microtubule oriented towards the bottom kinetochore of a bivalent. **b, c** After the addition of either 10 or 20 μM NOC, there are no continuous non-kinetochore microtubules and the kinetochore microtubules have broken away from the centrosome. As seen in **b**, the kinetochore microtubules have also angled away from the centrosome. **d** After the addition of 10 μM NOC for 6 min barely any microtubules remain, and both kinetochore and non-kinetochore microtubules are highly fragmented

indeed were fragmented). The kinetochore microtubules are also not as thick or bright as those in the control cell, are fragmented, and appear to be disconnected and/or angled away from the centrosome at the top pole in both cells. After 6 min in 10 μ M NOC, there are barely any microtubules remaining, and ones that remain have very large breaks and any remaining kinetochore fibres are disconnected from the centrosome. The progression of microtubule depolymerisation over time, relative to the concentration of NOC, in cells fixed after different times of treatment, is illustrated in Fig. 11, which is a montage of Z-stacks that include all images throughout the depth of the cell. Immediately upon addition of all concentrations of NOC non-kinetochore microtubules begin to fragment and contort. The non-kinetochore microtubules depolymerize quickest in 20 μ M NOC; they are gone between 3 and 4.5 min, whereas some are still detectable in 5 μ M NOC up to 6 min, albeit fragmented and with greatly reduced fluorescence. The kinetochore microtubules are more resistant to depolymerisation and although their fluorescence fades over time, some fragmented microtubules with reduced fluorescence are present in 5 μ M and 10 μ M NOC for up to 10 min. All kinetochore microtubules appear to be gone in 20 μ M NOC after 4.5 min.

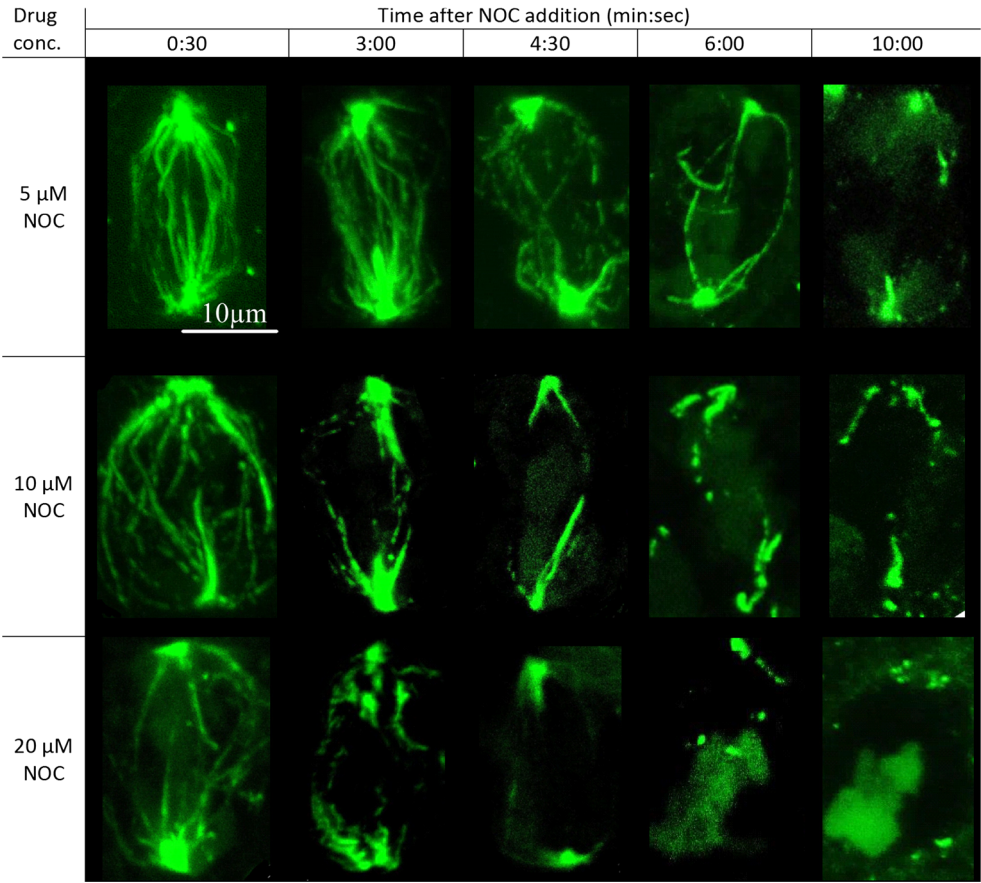
We have not seen microtubules attached to kinetochores that have moved, or to the chromosomes arms that have moved. Any microtubules present are either not in contact with the bivalents,

are fragmented or bent, or are not in the same plane of focus of the bivalents, as illustrated in Fig. 12, in which the Z-stacks include only those planes of focus that include chromosomes. For example, in Fig. 12Aiii, Ciii, the remaining kinetochore fibre(s) do not reach the kinetochore of the bivalents that have moved to the bottom pole. In Biii the microtubules radiating from the bottom pole lead to the top kinetochore of a bivalent, but they are fragmented into several pieces. In this cell the univalents remained at the top pole and appear to be held there by the centrosome and its associated microtubules. Centrosomes either move with the chromosomes or remain at their respective poles. This occurs irrespective of whether any univalents remain behind. As seen in Biii, the centrosome stayed behind with the univalent and did not move. In Diii, the centrosome moved with the bivalents and left the univalent behind at the pole.

Discussion

Our experiments deal with two issues, (1) why the ‘sidedness’ of the spindle such that detached chromosomes all move to the same pole, and (2) what causes movement of chromosomes (kinetochores) at very high speeds after treatment with drugs that remove microtubules.

Fig. 11 Tubulin immunofluorescence staining at various time intervals after the addition of 5, 10 or 20 μ M NOC, showing the progression of microtubule depolymerisation. All images are oriented so that chromosome movement (based on the DIC images of the same cells) was towards the bottom pole. Fifteen individual cells are illustrated. Images are of the entire Z-stack. In some images, there is some background fluorescence of the chromosomes (e.g. the cell at 10:00 min treated with 20 μ M NOC)



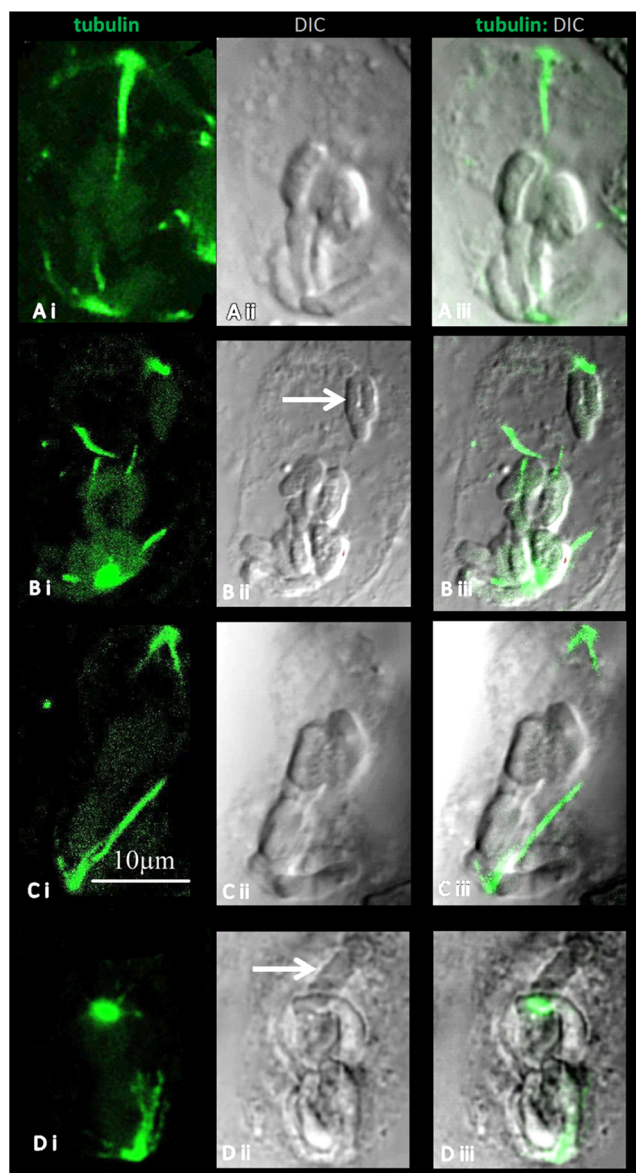


Fig. 12 A–D Tubulin immunofluorescence staining during or immediately after chromosomes have moved after the addition of NOC, as seen in 4 separate cells. White arrows point to univalents which remain at their respective poles. Although some microtubules have remained, many of the remaining microtubules are fragments and few of the remaining microtubules contact the kinetochores of the chromosomes that have moved, and at most are near only one of the moving kinetochores, or a chromosome itself. All images are oriented so that chromosome movement is towards the bottom pole. Tubulin images are of a Z-stack that includes only the 2 or 3 slices that include chromosomes, whereas the DIC images are of only one slice in the stack

Non-random movement to one pole

After the addition of NOC, chromosomes detach and move towards one pole. It is highly unlikely that the movement to only one pole was due to chance since this was observed in 52 out of 59 cells. The bivalent kinetochores move independently, in that they take different paths, change arrangements, move to the pole

to different extents, and while all move at roughly the same time, often they move at separate times (e.g. Fig. 5, and supplementary video 3). We think this is because their kinetochore microtubules attached at that pole all are detached at near the same time. This possibility could be tested using UV or laser microbeams to cut the kinetochore microtubules at only one pole.

We do not know what the difference is between the two poles, but we think that there is a difference, and that it may be related to possible non-random segregation in these cells suggested by several lines of evidence reviewed in the Introduction and in Brady and Paliulis (2015). It may be that the ‘sidedness’ of the spindle is a marker for which pole the paternal-derived or maternal-derived chromosomes segregate to. We recognise that this is speculative on our part, but it seems a distinct possibility when considered together with the other evidence for non-random segregation in these cells. While difficult to prove, we could negate it if we could find some predictor of the sidedness prior to addition of NOC. Nothing we looked at in chromosome behaviour enabled us to predict which direction the detached chromosomes would move, however. The position of asymmetric furrows can vary from one side of the spindle to the other, several times in the course of division, and seems to be due to chromosome ‘bulk’ imbalance (e.g. Forer and Pickett-Heaps 2010). If the ‘sidedness’ of chromosomes moving to a pole was associated with the asymmetry in numbers of univalent at a pole—e.g. the chromosomes always move to the pole with fewer univalents—then that would negate the hypothesis and mean that there is no inherent sidedness to the spindle itself. But asymmetry of the cleavage furrow also was not a predictor of sidedness of chromosome movement (Table 1). So far, we have not been able to negate the possibility that the movements to one pole of all bivalent kinetochores (and univalents, when they move) are because of non-random segregation, so we speculate that the sidedness may be related to non-random chromosome segregation.

We might be able to test some of the speculations by staining the centrioles. The mother centriole is implicated in asymmetric cell division in both *Drosophila* and budding yeast (Yamashita 2009) and may also be implicated in symmetrically dividing cells. In HeLa cells treated with low concentrations of NOC, it was found that mis-segregated chromosomes moved towards the pole with the older, mother centriole (Gasic et al. 2015). Perhaps in *Mesostoma*, bivalents detach and move towards (or away from) the pole containing the mother centriole.

Chromosome movement after addition of NOC or colcemid

After the addition of NOC, chromosomes stretched, paused, detached from one pole, and then the detached kinetochores moved towards the other pole. The stretch elongates bivalents by about 25–30% and after detachment bivalent length returns almost immediately to the pre-stretch value and it remains at that length during subsequent fast movements. Immunofluorescence

observations indicated that during the stretch and pause stages non-kinetochore microtubules depolymerised and fragmented and kinetochore microtubules fragmented, separated from the centrosome, changed angle and began to depolymerise (Figs. 10, 11, 12). By the time of the detachment and movement phases, most microtubules had depolymerised. An explanation for some of these events might be as follows. During the stretch phase, bivalents move towards both poles at a rate similar to normal prometaphase poleward oscillations, $6.2 \pm 1.8 \mu\text{m}/\text{min}$ (Ferraro-Gideon et al. 2014). If poleward oscillations of bivalents are caused by (or accompanied by) the disassembly of microtubules as suggested by Fuge (1989) and Ferraro-Gideon et al. (2013), movement of bivalents towards the two poles during the stretch phase may be caused by microtubules depolymerising in response to NOC. Microtubules and components of the brightly stained centrosome may be responsible for anchoring the chromosomes at the poles during the pause stage. Detachment occurs when there appear to be no kinetochore microtubules remaining. The detached kinetochores move extremely fast away from the pole of detachment towards the opposite pole, in an arc, not a straight line, with velocities up to $200 \mu\text{m}/\text{min}$ or more, at constant bivalent length. The moving kinetochores swing to one side and as they move they cause the chromosomes to bend (Fig. 4a, b).

What is responsible for this very rapid movement? We think it highly unlikely that the detached kinetochores are pushed by kinetochore microtubules, because those movements would be linear, not in an arc, and the microtubules would have to elongate at speeds over $200 \mu\text{m}/\text{min}$ while causing the kinetochore to swing out and then move towards the opposite pole. In any event, we saw no indication of kinetochore microtubule attachment to the detached kinetochores or chromosomes.

Chromosomes move faster when sliding along microtubules than when attached head on to them. Could the very rapid movement be due to the kinetochores sliding along non-kinetochore microtubules? There are non-kinetochore microtubules near kinetochores, as seen in electron micrographs of *Mesostoma* spermatocytes by Fuge (1987) and Fuge and Falke (1991), so sliding might be a possible mechanism. However, when anaphase chromosomes are attached to microtubules at kinetochores, the highest velocities in anaphase for chromosome movements are up to $6 \mu\text{m}/\text{min}$ (Wang et al. 2010), and in prometaphase are up to $10 \mu\text{m}/\text{min}$ (Ferraro-Gideon et al. 2013). When not-attached chromosomes slide along microtubules (Rieder and Alexander 1990), average speeds are up to $40 \mu\text{m}/\text{min}$, though instantaneous speeds can be up to $50 \mu\text{m}/\text{min}$ (Alexander and Reider 1991). The in vitro movement of microtubules along kinesin support speeds up to $30 \mu\text{m}/\text{min}$ (DeLuca et al. 2001). We know of no reports of sliding at the highest speeds at which the *Mesostoma* spermatocyte kinetochores move after NOC treatment. Our immunofluorescence data indicates that the non-kinetochore microtubules are fragmented very soon after addition of NOC, within 30 s, earlier

than the movements of the detached kinetochores, and none are present during the movements of the univalent chromosomes which may occur up to 15 min later. We see no indication in the immunofluorescence images that even fragments of microtubules are close to each detached kinetochore or moving chromosome arms, so we think it is highly unlikely that the detached kinetochores move by sliding against microtubules.

In sum, there are several reasons why we think it unlikely that microtubules produce the forces that move the detached kinetochores. One is that the kinetochores do not travel in a straight path but curl to one side; thus, the force has to change directions rather than act in a straight line in the direction of a linear microtubule. The force must allow for high speeds, and we know of no reports that microtubules and microtubule motors are able to produce speeds near or above $200 \mu\text{m}/\text{min}$. And lastly, the force must act on all kinetochores at the same time to produce the co-ordinated movement to only one pole, yet in the immunofluorescence images kinetochore microtubules are highly fragmented and any microtubules that do remain are not associated with the kinetochores (Figs. 10 and 12). Even if one microtubule-kinetochore association remained, that still could not be responsible for the co-ordinated movement of all three bivalents that was consistently observed in 52 out of the 59 cells. Since we have not seen attachment of microtubules to moving chromosomes, either direct or along the side of the chromosome, we conclude that the rapid kinetochore movements do not appear to be due to microtubules. There are other sources of forces, however, forces that do not arise from microtubules.

Could the movements be due to 'recoil', after tension is released on stretched chromosomes? Release in tension due to stretching could cause the shortening of the detached chromosome, and this undoubtedly occurs, release of tension causes some of the movement, but the fact that kinetochores swing to one side early in their movement indicates that the movement we observe is not due solely to a release in tension along the length of the chromosome. Recoil forces along the length of the chromosome would result in travel in a straight path to the opposite kinetochore but not at an angle to it as occurs in these cells (Fig. 4b). Further, were the movements arising solely from recoil from the stretch, the speeds would not vary with concentration of NOC or colcemid and would not vary depending on how long it took for the movements to occur after adding the drug (Fig. 8a). Nor do the speeds of movement seem to vary with length of stretched bivalent: chromosomes that are stretched to a longer inter kinetochore distance do not move any faster than shorter chromosomes (Fig. 8c). Finally, when kinetochore microtubules in untreated cells are severed using a laser, the associated kinetochores move quickly towards the other pole, likely from 'recoil' after being stretched; these movements are at speeds an order of magnitude slower than those seen after NOC treatment, maxima of $20 \mu\text{m}/\text{min}$ (Ferraro-Gideon 2013; Hoang 2013), instead of the $200 \mu\text{m}/\text{min}$ seen after NOC. Thus, we think that most of the movement we see is not due to 'recoil' from elastic stretch and

we therefore must look for outside forces acting on the chromosomes. Consistent with this proposition is that univalent chromosomes move towards the equator at high speeds, averaging $11.8 \mu\text{m}/\text{min}$ ($n = 7$), up to $16.5 \mu\text{m}/\text{min}$, often after the bivalents have moved, at times when there are no spindle microtubules remaining in the cells.

One potential force away from the pole is the elastic ‘tethers’ that connect chromosome arms. When arms of anaphase chromosomes are cut, the arm fragments move rapidly towards the partner chromosomes because of elastic tethers that extend between the arms (LaFountain et al. 2002). Tethers are present in most (or all) animal cell spindles, including *Mesostoma* (Forer et al. 2017), and probably are universally present (Paliulis and Forer 2017). In *Mesostoma* spermatocytes tethers also extend between the free arms of the prometaphase/metaphase bivalents so that when an arm is cut, the arm fragment moves rapidly to the other free arm (Forer et al. 2017). One could imagine that when a kinetochore detaches from the pole the tether would pull the attached telomere towards the telomere of the other arm to which it is attached. Forces from tethers may contribute to the force for the movement; they would only act between telomeres and depending on the geometry of the arms they might cause the twisting, but there would not be differences in speed depending on NOC or colcemid concentration or on how long the bivalents remain stretched (Fig. 8d). Thus, other forces must contribute, perhaps those from the spindle matrix or from actin-myosin.

The spindle matrix model, as discussed in Pickett-Heaps and Forer (2009), wherein a spindle matrix propels the chromosomes towards the poles, is consistent with the movements we have described. In the original descriptions of spindle matrix in diatoms, in which the matrix is identifiable using the electron microscope, the matrix is connected to kinetochores and after depolymerisation of microtubules using colchicine the chromosomes detached and moved at random to either pole (Pickett-Heaps and Spurck 1982). Our findings are the same, except that in *Mesostoma* spermatocytes, the chromosomes move towards only one pole, and a matrix is not visible using electron microscopy (e.g. Fuge and Falke 1991; Forer and Pickett-Heaps 2010). Data from other cells show that the spindle matrix remains after microtubules are depolymerized, but that the matrix is compressed and gradually loses shape after the microtubules are gone (Yao et al. 2012). This suggests that in our experiments the higher speeds for kinetochore movements might be due to the complete matrix remaining, because they occur when kinetochore movements are initiated at shortest times after drug treatment (Fig. 8); slower speeds, at later times after drug treatment, due to matrix that is partially gone or destabilised; and when microtubules are removed gradually (as with low concentrations of colcemid), the matrix is completely gone and the chromosomes are not able to move. We can extend this interpretation to explain the spindle sidedness: we suggest that the matrix may be polarised towards one pole. This could be tested by looking at the arrangements of spindle matrix components.

Another possibility for producing force in the absence of microtubules is actin and myosin. Actin and myosin have been identified as being present in large numbers of spindles (Forer et al. 2003, Table 1) and physiological data support actin and myosin being involved in spindle function (e.g. Mogessie and Schuh 2017; Sheykhan et al. 2013a, b). Another possibility is the ‘spindle matrix’ (e.g. Johansen and Johansen 2007; Pickett-Heaps and Forer 2009). Consideration of actin-myosin and spindle matrix are not mutually exclusive since, as discussed in detail in Johansen et al. (2011), the spindle matrix includes proteins such as the nuclear derived proteins Skeletor, Megator, Chromator and EAST and muscle proteins such as actin, myosin, and titin, that colocalize or align with the microtubule spindle in a variety of different cells types (Johansen and Johansen 2007; Johansen et al. 2011). In crane-fly spermatocytes, for example, actin, phosphorylated myosin, and titin are aligned along kinetochore microtubules (Fabian et al. 2007; Sheykhan et al. 2013b), in close proximity to spindle matrix proteins (Fabian et al. 2007). Just as the spindle matrix ‘framework’ of a spindle shape remains after removal of spindle microtubules (Johansen and Johansen 2007; Yao et al. 2012), and slowly disappears, the actin ‘spindle’ that remains after microtubules in mouse meiotic spindles are depolymerised with NOC also seems to slowly disappear (Mogessie and Schuh 2017, Fig. S2B). The slow disappearance of the putative force production mechanism that acts on *Mesostoma* chromosomes after microtubules are gone (after NOC treatment) could explain why chromosome movements are slower when time to detachment is longer. Thus, actin/myosin too might be possibilities for causing the rapid movements of chromosomes/kinetochores.

One issue that arises from these considerations is the directionality of the forces produced by the underlying spindle-matrix/actin-myosin: if, as argued elsewhere, these are the forces that propel chromosome poleward when kinetochore microtubules are severed during anaphase, or when microtubules are completely gone (Pickett-Heaps and Forer 2009; Forer et al. 2015), how might the same components act to propel *Mesostoma* bivalents and univalents in the opposite direction, from pole to equator, when the kinetochore microtubules are depolymerised? This is not necessarily a contradiction, however. As seen in *Drosophila* cells, the spindle matrix is not static: it changes organisation with time and depending on interactions with microtubules (e.g. Yao et al. 2012). Chromosome movements in the *Mesostoma* spermatocytes we have described herein are in cells prior to anaphase, in which the chromosomes move both towards and away from the poles. It is conceivable that the underlying matrix-actin-myosin network prior to anaphase is bidirectional and it reorganises so that by anaphase it is unidirectional. It also is conceivable that the matrix components are unidirectional towards one pole prior to anaphase, towards the pole which the chromosomes all move after NOC treatment. Experimental analysis of the presence and organisation of the matrix and actin-myosin components of *Mesostoma*

spermatocytes can test for the presence of the components and for whether their organisation changes at anaphase, and could help decide whether our interpretations and speculations have merit.

In conclusion, studies investigating possible differences between the two poles, and what is responsible for chromosomes movement in the absence of microtubules, in particular investigating possible involvement of the spindle matrix and actin-myosin, might provide insight into the normal functioning of these meiotic cells.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Alexander SP, Reider CL (1991) Chromosome motion during attachment to the vertebrate spindle: initial salutatory-like behavior of chromosomes and quantitative analysis of force production by nascent kinetochore fibers. *J Cell Biol* 113(4):805–815. <https://doi.org/10.1083/jcb.113.4.805>
- Brady M, Paliulis LV (2015) Chromosome interaction over a distance in meiosis. *R Soc Open Sci* 2:1–10
- Camenzind R, Nicklas BR (1968) The non-random chromosome segregation in spermatocytes of *Gryllotalpa hexadactyla*. A micromanipulation analysis. *Chromosoma* 24(3):324–335. <https://doi.org/10.1007/BF00336200>
- Cameron LA, Yang G, Cimini D, Canman JC, Kisurina-Evgenieva O, Khodjakov A, Danuser G, Salmon ED (2006) Kinesin 5-independent poleward flux of kinetochore microtubules in PtK1 cells. *J Cell Biol* 173(2):173–179. <https://doi.org/10.1083/jcb.200601075>
- Capp A (1948) The life and times of the Shmoo. Pocket Books, New York
- Cassimeris L, Rieder C, Rupp G, Salmon ED (1990) Stability of microtubule attachment to metaphase kinetochores in PtK1 cells. *J Cell Sci* 96:9–15
- DeLuca JG, Newton CN, Himes RH, Jordan MA, Wilson L (2001) Purification and characterization of native conventional kinesin, HSET, and CENP-E from mitotic HeLa cells. *J Biol Chem* 276(30):28014–28021. <https://doi.org/10.1074/jbc.M102801200>
- Fabian L, Forer A (2005) Redundant mechanisms for anaphase chromosome movements: crane-fly spermatocyte spindles use actin filaments but also can function without them. *Protoplasma* 225(3–4):169–184. <https://doi.org/10.1007/s00709-005-0094-6>
- Fabian L, Xia X, Venkitaramani DV, Johansen KM, Johansen J, Andrew DJ, Forer A (2007) Titin in insect spermatocyte spindle fibers associates with microtubules, actin, myosin and the matrix proteins skeleton, megator and chromator. *J Cell Sci* 120(13):2190–2204. <https://doi.org/10.1242/jcs.03465>
- Ferraro-Gideon J (2013) Rearing *Mesostoma ehrenbergii* and studying chromosome movements during meiosis in their spermatocytes. Ph D thesis, York University, Toronto
- Ferraro-Gideon J, Hoang C, Forer A (2013) *Mesostoma ehrenbergii* spermatocytes—a unique and advantageous cell for studying meiosis. *Cell Biol Int* 37(9):892–898. <https://doi.org/10.1002/cbin.10130>
- Ferraro-Gideon J, Hoang C, Forer A (2014) Meiosis-I in *Mesostoma ehrenbergii* spermatocytes includes distance segregation and inter-polar movements of the univalents, and vigorous oscillations of bivalents. *Protoplasma* 251(1):127–143. <https://doi.org/10.1007/s00709-013-0532-9>
- Forer A, Pickett-Heaps J (2005) Fibrin clots keep non-adhering living cells in place on glass for perfusion or fixation. *Cell Biol Int* 29(9):721–730. <https://doi.org/10.1016/j.cellbi.2005.04.010>
- Forer A, Pickett-Heaps J (2010) Precocious (pre-anaphase) cleavage furrows in *Mesostoma* spermatocytes. *Eur J Cell Biol* 89(8):607–618. <https://doi.org/10.1016/j.ejcb.2010.03.001>
- Forer A, Spurck T, Pickett-Heaps J, Wilson P (2003) Structure of kinetochore fibres in crane-fly spermatocytes after irradiation with an ultraviolet microbeam: neither microtubules nor actin filaments remain in the irradiated region. *Cell Motil Cytoskeleton* 56(3):173–192. <https://doi.org/10.1002/cm.10144>
- Forer A, Johansen K, Johansen J (2015) Movement of chromosomes with severed kinetochore microtubules. *Protoplasma* 252(3):775–781. <https://doi.org/10.1007/s00709-014-0752-7>
- Forer A, Duquette M, Paliulis L, Fegaras E, Ono M, Preece D, Berns MB (2017) Elastic 'tethers' connect separating anaphase chromosomes in a broad range of animal cells. *Eur J Cell Biol* 96(6):504–514. <https://doi.org/10.1016/j.ejcb.2017.07.001>
- Fuge H (1987) Oscillatory movements of bipolar-oriented bivalent kinetochores and spindle forces in male meiosis of *Mesostoma ehrenbergii*. *Eur J Cell Biol* 44:294–298
- Fuge H (1989) Rapid kinetochore movements in *Mesostoma ehrenbergii* spermatocytes: action of antagonistic chromosome fibres. *Cell Motil Cytoskeleton* 13(3):212–220. <https://doi.org/10.1002/cm.970130308>
- Fuge H (1994) Unorthodox male meiosis in *Trochosia pubescence* (Sciaridae). Chromosome elimination involves polar organelle degeneration and monocentric spindles in first and second division. *J Cell Sci* 107:299–312
- Fuge H (1997) Nonrandom chromosome segregation in male meiosis of a sciarid fly: elimination of paternal chromosomes in first division is mediated by non-kinetochore microtubules. *Cell Motil Cytoskeleton* 36(1):84–94. [https://doi.org/10.1002/\(SICI\)1097-0169\(1997\)36:1<84::AID-CM8>3.0.CO;2-H](https://doi.org/10.1002/(SICI)1097-0169(1997)36:1<84::AID-CM8>3.0.CO;2-H)
- Fuge H, Falke D (1991) Morphological aspects of chromosome spindle fibres in *Mesostoma*: “microtubular fir-tree” structures and microtubule association with kinetochores and chromatin. *Protoplasma* 160(2–3):39–48. <https://doi.org/10.1007/BF01539955>
- Gasic I, Nerurkar P, Meraldi P (2015) Centrosome age regulates kinetochore-microtubule stability and biases chromosome mis-segregation. *elife* 19:1–15
- Gordon GW (1980) The control of mitotic motility as influenced by ultraviolet microbeam irradiation of kinetochore fibers: a dissertation in biology. Ph D Thesis University of Pennsylvania
- Gordon GW, Inoué S (1979) Unexpected increase in poleward velocities of mitotic chromosomes after UV irradiation of their kinetochore fibers. *J Cell Biol* 83(2 part 2):376
- Hoang C (2013) *Mesostoma ehrenbergii* as a model organism for studying meiosis: a description on how to rear these animals in the lab, highlights of their unique chromosome behaviour and using them to study the spindle matrix model of chromosome movements. M Sc thesis, York University, Toronto
- Hoang C, Ferraro-Gideon J, Gauthier K, Forer A (2013) Methods for rearing *Mesostoma ehrenbergii* in the laboratory for cell biology experiments, including identification of factors that influence production of different egg types. *Cell Biol Int* 10:1089–1105
- Husted L, Ruebush TK (1940) A comparative cytological and morphological study of *Mesostoma ehrenbergii ehrenbergii* and *Mesostoma*

- ehrenbergii wardii*. J Morphol 67(3):387–410. <https://doi.org/10.1002/jmor.1050670302>
- Johansen KM, Johansen J (2007) Cell and molecular biology of the spindle matrix. Int Rev Cytol 263:155–210. [https://doi.org/10.1016/S0074-7696\(07\)63004-6](https://doi.org/10.1016/S0074-7696(07)63004-6)
- Johansen KM, Forer A, Yao C, Girton J, Johansen J (2011) Do nuclear envelope and intranuclear proteins reorganize during mitosis to form an elastic hydrogel-like spindle matrix? Chromosom Res 19(3):345–365. <https://doi.org/10.1007/s10577-011-9187-6>
- Jordan MA, Wilson L (2004) Microtubules as a target for anticancer drugs. Nat Rev Cancer 4(4):253–265. <https://doi.org/10.1038/nrc1317>
- Jordan MA, Thrower D, Wilson L (1992) Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. J Cell Sci 102:401–416
- LaFountain JR, Cole RW, Reider CL (2002) Partner telomeres during anaphase in crane fly spermatocytes are connected by an elastic tether that exerts a backward force and resists poleward movement. J Cell Sci 115:1541–1549
- Lorbeer G (1934) Die Zytologie der Lebermoose mit besonderer Berücksichtigung allgemeiner Chromosomenfragen. Jb Wiss Bot 80:567–818
- Metz CW (1938) Chromosome behavior, inheritance and sex determination in *Sciara*. Am Nat 72(743):485–520. <https://doi.org/10.1086/280803>
- Metz CW, Moses MS, Hoppe EN (1926) Chromosome behavior and genetic behavior in *Sciara* (Diptera). Z Indukt Abstamm Vererbungsl 42:237–270
- Mogessie B, Schuh M (2017) Actin protects mammalian eggs against chromosome segregation errors. Science 357(6353):eaal1647. <https://doi.org/10.1126/science.aal1647>
- Nur U (1982) Destruction of specific heterochromatic chromosomes during spermatogenesis in the *Comstockiella* chromosome system (Coccoidea: Homoptera). Chromosoma 85(4):519–530. <https://doi.org/10.1007/BF00327347>
- Oakley HA (1983) Male meiosis in *Mesostoma ehrenbergii ehrenbergii*. Kew Chromosome Conference II Editors PE Brandham, MD Bennett, George Allen and Unwin, London (Boston, Sydney) 195–199
- Oakley HA (1985) Meiosis in *Mesostoma ehrenbergii ehrenbergii* (Turbellaria, Rhabdocoela). III. Univalent chromosome segregation during the first meiotic division in spermatocytes. Chromosoma 91(2):95–100. <https://doi.org/10.1007/BF00294051>
- Osborn M, Weber K (1982) Intermediate filaments: cell-type-specific markers in differentiation and pathology. Cell 31(2 Pt 1):303–306. [https://doi.org/10.1016/0092-8674\(82\)90122-2](https://doi.org/10.1016/0092-8674(82)90122-2)
- Paliulis LV, Forer A (2017) A review of “tethers”: elastic connections between separating partner chromosomes in anaphase. Protoplasma. <https://doi.org/10.1007/s00709-017-1201-1>
- Payne F (1912) The chromosomes of *Gryllotalpa borealis*. Burm Arch Zellforsch 9:141–148
- Pickett-Heaps J, Forer A (2009) Mitosis: spindle evolution and the matrix model. Protoplasma 235(1-4):91–99. <https://doi.org/10.1007/s00709-009-0030-2>
- Pickett-Heaps J, Spurck T (1982) Studies on kinetochore function in mitosis. I. The effects of colchicine and cytochalasin on mitosis in the diatom *Hantzschia amphioxys*. Eur J Cell Biol 28(1):77–82
- Pickett-Heaps J, Forer A, Spurck T (1996) Rethinking anaphase: where “Pac-man” fails and why a role for the spindle matrix is likely. Protoplasma 192(1-2):1–10. <https://doi.org/10.1007/BF01273239>
- Rieder CL, Alexander SP (1990) Kinetochore are transported poleward along a single astral microtubule during chromosome attachment to the spindle in newt lung cells. J Cell Biol 110(1):81–95. <https://doi.org/10.1083/jcb.110.1.81>
- Rieder CL, Salmon ED (1994) Motile kinetochores and polar ejection forces dictate chromosome position on the vertebrate mitotic spindle. J Cell Biol 124(3):223–233. <https://doi.org/10.1083/jcb.124.3.223>
- Schrader F (1921) The chromosomes of *Pseudococcus nipae*. Biol Bull 40(5):259–270. <https://doi.org/10.2307/1536736>
- Schrader F (1923) A study of the chromosomes in three species of *Pseudococcus*. Arch Zellforsch 17:45–62
- Sheykhan R, Baker N, Gomez-Godinez V, Liaw LH, Shah J, Berns MW, Forer A (2013a) The role of actin and myosin in PtK2 spindle length changes induced by laser microbeam irradiations across the spindle. Cytoskeleton 70(5):241–259. <https://doi.org/10.1002/cm.21104>
- Sheykhan R, Shirodkar PV, Forer A (2013b) The role of myosin phosphorylation in anaphase chromosome movement. Eur J Cell Biol 92(4-5):175–186. <https://doi.org/10.1016/j.ejcb.2013.02.002>
- Sheykhan R, Berns MW, Forer A (2017) Elastic tethers between separating anaphase chromosomes coordinate chromosome movements to the two poles. Cytoskeleton. 74(2):91–103. <https://doi.org/10.1002/cm.21347>
- Spurck T, Forer A, Pickett-Heaps J (1997) Ultraviolet microbeam irradiations of epithelial and spermatocyte spindles suggest that forces act on the kinetochore fibre and are not generated by its disassembly. Cell Motil Cytoskeleton 36(2):136–148. [https://doi.org/10.1002/\(SICI\)1097-0169\(1997\)36:2<136::AID-CM4>3.0.CO;2-7](https://doi.org/10.1002/(SICI)1097-0169(1997)36:2<136::AID-CM4>3.0.CO;2-7)
- Stevens NM (1905) Studies in spermatogenesis with especial reference to the “accessory chromosome”. Carnegie Institution of Washington, Washington D.C.
- Stevens NM (1906) Studies in spermatogenesis. II. A comparative study of the heterochromosomes in certain species of Coleoptera, Hemiptera, and Lepidoptera, with especial reference to sex determination. Carnegie Institution of Washington, Washington D.C.
- Vasquez R, Howell B, Yvon AC, Wadsworth P, Cassimeris L (1997) Nanomolar concentrations of nocodazole alter microtubule dynamic instability in vivo and in vitro. Mol Biol Cell 8(6):973–985. <https://doi.org/10.1091/mbc.8.6.973>
- Wang H, Brust-Mascher I, Cheerambathur D, Scholey JM (2010) Coupling between microtubule sliding, plus-end growth and spindle length revealed by kinesin-8 depletion. Cytoskeleton 67(11):715–728. <https://doi.org/10.1002/cm.20482>
- Wilson PJ, Forer A, Wise D (2003) Microtubule distribution during meiosis I in flea-beetle [*Alagoasa (Oedionychus)*] spermatocytes: evidence for direct connections between unpaired sex chromosomes. J Cell Sci 116(7):1235–1247. <https://doi.org/10.1242/jcs.00296>
- Wong R, Forer A (2003) Signalling between chromosomes in crane fly spermatocytes studied using ultraviolet microbeam irradiation. Chromosom Res 11(8):771–786. <https://doi.org/10.1023/B:CHRO.0000005753.97458.20>
- Yamashita YM (2009) The centrosome and asymmetric cell division. Prion 3(2):84–88. <https://doi.org/10.4161/pri.3.2.8821>
- Yang H, Ganguly A, Cabral F (2010) Inhibition of cell migration and cell division correlates with distinct effects of microtubule inhibiting drugs. J Biol Chem 285(42):32242–32250. <https://doi.org/10.1074/jbc.M110.160820>
- Yao C, Rath U, Maiato H, Sharp D, Girton J, Johansen KM, Johansen J (2012) A nuclear-derived proteinaceous matrix embeds the microtubule spindle apparatus during mitosis. Mol Biol Cell 23(18):3532–3541. <https://doi.org/10.1091/mbc.E12-06-0429>

CHAPTER 2

PRECOCIOUS CLEAVAGE FURROWS SIMULTANEOUSLY MOVE AND INGRESS
WHEN KINETOCHORE MICROTUBULES ARE DEPOLYMERIZED IN *Mesostoma
ehrenbergii* SPERMATOCYTES

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Precocious cleavage furrows simultaneously move and ingress when kinetochore microtubules are depolymerized in *Mesostoma ehrenbergii* spermatocytes

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Abstract

A “precocious” cleavage furrow develops and ingresses during early prometaphase in *Mesostoma ehrenbergii* spermatocytes (Forer and Pickett-Heaps Eur J Cell Biol 89:607-618, 2010). In response to chromosome movements which regularly occur during prometaphase and that alter the balance of chromosomes in the two half-spindles, the precocious furrow shifts its position along the cell, moving 2–3 μm towards the half cell with fewer chromosomes (Ferraro-Gideon et al. Cell Biol Int 37:892-898, 2013). This process continues until proper segregation is achieved and the cell enters anaphase with the cleavage furrow again in the middle of the cell. At anaphase, the furrow recommences ingression. Spindle microtubules (MTs) are implicated in various furrow positioning models, and our experiments studied the responses of the precocious furrows to the absence of spindle MTs. We depolymerized spindle MTs during prometaphase using various concentrations of nocodazole (NOC) and colcemid. The expected result is that the furrow should regress and chromosomes remain in the midzone of the cell (Cassimeris et al. J Cell Sci 96:9-15, 1990). Instead, the furrows commenced ingression and all three bivalent chromosomes moved to one pole while the univalent chromosomes, that usually reside at the two poles, either remained at their poles or moved to the opposite pole along with the bivalents, as described elsewhere (Fegaras and Forer 2018). The microtubules were completely depolymerized by the drugs, as indicated by immunofluorescence staining of treated cells (Fegaras and Forer 2018), and in the absence of microtubules, the furrows often ingressed (in 33/61 cells) at a rate similar to normal anaphase ingression ($\sim 1 \mu\text{m}/\text{min}$), while often simultaneously moving toward one pole. Thus, these results indicate that in the absence of anaphase and of spindle microtubules, cleavage furrows resume ingression.

Keywords Meiosis · Cytokinesis · Cleavage furrow · Microtubules · Nocodazole

Introduction

In a typical cell division, anaphase begins before the cleavage furrow forms. In animal cells, the cleavage furrow is a

contractile ring made of actin and myosin II as well as various other regulatory proteins (Eggert et al. 2006). After anaphase begins, the furrow ingresses at the midzone of the cell, eventually cleaving the progenitor cell into two equally sized daughter cells (Barr and Gruneberg 2007). Furrow position is determined before or during anaphase onset, usually during the metaphase-anaphase transition, and is irreversible even if the mitotic apparatus is removed (Rappaport 1981). The three main furrow positioning models are the astral stimulation, astral relaxation, and central spindle stimulation models (Barr and Gruneberg 2007). These models all rely on the presence of microtubules to transmit a signal to the cell cortex, whether it be astral microtubules that produce either a positive or a negative furrow stimulus (Rappaport 1961, 1985; Rappaport and Ebstein 1965) or interzonal (non-kinetochore) microtubules which transmit signaling proteins to the cortex by an unknown mechanism as shown in various

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cell types through addition of drugs that selectively target astral microtubules while conserving interzonal microtubules (von Dassow et al. 2009) or the creation of asterless mutants (Bonaccorsi et al. 1998). Although there are several other theories that we have not mentioned, the two requirements that all these theories share are the presence of microtubules and the commencement of anaphase.

Certain cell types do not require anaphase commencement to have their cleavage furrows form, however. In various diatom species such as *Hantzschia*, *Nitzschia*, *Pinnularia*, *Cymatopleura*, and *Surirella* (Pickett-Heaps et al. 1978, 1979, 1980; Pickett-Heaps 1991), furrows appear at the start of prometaphase. Precocious cleavage furrows are also common in various green algae, such as *Spirogyra* (McIntosh et al. 1995). In these organisms, the precocious furrows partially ingress and arrest until anaphase when they fully ingress and cleave the cell in two. Likewise, spermatocytes from a species of hermaphroditic flatworm *Mesostoma ehrenbergii* have precocious furrows (Forer and Pickett-Heaps 2010). The precocious furrows in *Mesostoma* spermatocytes form in early prometaphase, arrest and slowly ingress somewhat (Ferraro-Gideon et al. 2013; Forer and Pickett-Heaps 2010); they have the same dynamics as regular cleavage furrows, in that anti-actin or anti-myosin drugs cause the furrows to regress (Forer and Pickett-Heaps 2010).

Although the formation of a precocious furrow is not unique to *Mesostoma*, what is unique is that the furrow changes position in the cell in response to chromosome imbalances in the cell (Ferraro-Gideon et al. 2013, 2014). Throughout prometaphase, which can last up to 2 h, univalents often move between the two poles, temporarily resulting in unequal numbers of univalents at the two poles (Oakley 1984). By the start of anaphase, there is one of each kind of univalent at each pole (Oakley 1984). When there are unequal numbers of univalents at the two poles, the furrow shifts 2–3 μm towards the pole with fewer univalents (Ferraro-Gideon et al. 2013, 2014; Forer and Pickett-Heaps 2010). This creates an asymmetrically positioned furrow, with one half cell being larger than the half cell with fewer chromosomes. It is important to note that the asymmetric furrow in *Mesostoma* is not the same as other asymmetrically dividing cells such as *C. elegans* or *Drosophila* embryos in which the spindle is positioned asymmetrically in the cell (Cytrynbaum et al. 2005; Grill et al. 2001; Gonczy 2008). In *Mesostoma*, the spindle remains symmetric throughout division even when the cleavage furrow is not. By anaphase onset there are equal numbers of univalents at the two poles, and a symmetrically placed furrow ingresses to produce two equally sized daughter cells (Forer and Pickett-Heaps 2010; Ferraro-Gideon et al. 2013).

In our experiments, we wanted to test whether microtubules are required for positioning of the cleavage furrow, as required by many models. We chose to target microtubules by adding nocodazole (NOC) to *Mesostoma ehrenbergii*

spermatocytes. NOC has been used to lock the cell cycle in the G2 to M phase as a means to synchronize cell cultures (Poxleitner et al. 2008) and has been used in cancer cell studies (Choi et al. 2011; Mollinedo and Gajate 2003; Jordan and Wilson 2004). NOC targets the dynamic nature of microtubules which in cells constantly switches between phases of growth or shrinking. When microtubules are treated with concentrations of NOC above 1 μM , they enter a third, paused state with arrested growth, as observed in newt lung epithelial cells (Vasquez et al. 1997). In a cell undergoing division, this equates to the kinetochore fiber losing its formerly tight connection to the kinetochore. High concentrations of NOC also cause microtubules to detach from the microtubule organizing center (MTOC), generating microtubule fragments (Yang et al. 2010). Microtubules are a key in furrow positioning models, and they are also considered the primary force producers of chromosome movements. When microtubules are depolymerized during prometaphase or metaphase, it is expected that chromosomes stay in the middle of the cell and arrest at the equator (Cassimeris et al. 1990) and that the cleavage furrow will regress (Alsop and Zhang 2003; Barr and Gruneberg 2007; Glotzer 2004; Murthy and Wadsworth 2008). However, when *Mesostoma* spermatocytes are treated with NOC, in each cell the bivalents (and sometimes the univalents) detach from one pole and move towards the opposite pole (Fegaras and Forer 2018), and the cleavage furrow usually ingresses, as described in the present article.

Our results indicate that after the chromosomes move as a consequence of NOC treatment, the precocious furrow remains ingressed or ingresses further and simultaneously moves towards the half-cell with fewer chromosomes. This movement results either in the furrow reaching the far end of the half-cell and disappearing or the furrow partially cleaving the cell into two grossly unequally sized daughter cells which together look like shmoos (Capp 1948). Thus, furrows can ingress and change position in the apparent absence of spindle microtubules. The mechanism by which it does so remains unknown.

Materials and methods

Living cell preparation

Mesostoma are reared in the lab as described by Hoang et al. (2013). We dissected animals that contain resting eggs. The animals are chosen based on the opaque white color and large size of their testes. We extracted the entire testes using pulled 5, 10, or 15 μL micropipettes (Fisher) and then laid them onto a cover slip into a small drop of 0.2 mg/mL bovine fibrinogen (Calbiochem) diluted in *Mesostoma* Ringers solution (61 mM NaCl, 2.3 mM KCL, 0.5 mM CaCl_2 , and 2.3 mM phosphate buffer, pH 6.8), prepared fresh daily. We used the pulled

needle tip to pierce the testes and break the lobes, after which cells lie flat on the cover slip. Thrombin is added, which causes the fibrinogen to clot and the clot holds the cells in place, as previously described (Forer and Pickett-Heaps 2005; Ferraro-Gideon et al. 2014). We then mounted the coverslip preparations on a perfusion chamber containing *Mesostoma* Ringers solution to submerge the cells and sealed the edges of the coverslip with wax. Live primary spermatocytes were viewed with phase-contrast microscopy using a Nikon 100× oil immersion lens (NA 1.3) and recorded in real-time on a DVD recorder. Cells were periodically streamed with 0.5–1 mL Ringers to exchange their immersion medium before the addition of drug. The cells were then perfused with 2–5 mL of either 5, 10, or 20 μ M NOC (Sigma) or 50 or 100 μ M colcemid (Sigma) in *Mesostoma* Ringers streamed through the perfusion chamber. Both drugs were diluted from a 1000× concentrated stock solution in dimethyl sulfoxide (DMSO); the 0.1% concentration of DMSO has no effect on *Mesostoma* spermatocytes (Forer and Pickett-Heaps 2010). The drugs were usually washed out with 5–10 mL of *Mesostoma* Ringer's solution 10–15 min after initial immersion in the drugs. The cells were followed by video microscopy for 45 to 120 min depending on the experiment.

Cleavage furrow and chromosome movement analysis

We used the free computer program virtual dub (www.virtualdub.org) to time lapse the image sequences into videos at 60 times the original speed. Our in-house program Winimage (Wong and Forer 2003) was used to plot furrow width (the “diameter” of the cell at the position of the furrow) and furrow position along the length of the cell as shown in the diagram in Fig. 7a; the measured furrow width is the distance between black arrowheads in Fig. 7a. Measuring the distance between the two black arrowheads at different times gave data on the rate of furrow ingression or regression, and measuring the distance between one of the black arrowheads and the red “X” (Fig. 7a) gave data on the movement of the furrow along the length of the cell. Furrow movement and ingression graphs were generated in the program SlideWrite 7.0. Adobe Photoshop was used to overlay cell outlines as seen in Figs. 2 and 4 and to create image montages from single video frames. Chromosome velocity as shown in Table 2 was collected in a similar fashion to furrow position, as described in detail in Fegaras and Forer (2018). The position of each bivalent's and/or univalent's kinetochore was plotted in comparison to a fixed point at the cell's edge, near the pole (e.g., the X in Fig. 7a). Distances calculated in WinImage were converted to graphs of distance versus time using the commercial program SlideWrite. Student's *t* test was performed on all data to compare the different times and velocities for furrow and chromosome movement at the different concentrations of drug.

Results

Control cells

Of the several unusual features in *Mesostoma* spermatocytes (Ferraro-Gideon et al. 2013), we concentrate here on the precocious, pre-anaphase cleavage furrow which forms during prometaphase, up to 2 h before anaphase commencement (Forer and Pickett-Heaps 2010). Spermatocytes have 3 bivalent (b) and 4 univalent (u) chromosomes (Fig. 1). Throughout prometaphase, the three bivalents persistently oscillate towards and away from either pole, at speeds roughly 5–6 μ m/min, as first described by Fuge (1987, 1989). The univalents are at the poles throughout prometaphase. By the start of anaphase, they have assorted themselves into one of each kind of univalent at each pole (Fig. 1), which they achieve by moving to opposite poles. When a univalent changes poles, this causes an imbalance in the number of chromosomes at the two poles (Oakley 1984), after which the furrow shifts 2–3 μ m towards the pole with fewer chromosomes (Forer and Pickett-Heaps 2010). Throughout prometaphase, the furrow can either be symmetrically (Fig. 2a, a') or asymmetrically oriented (Fig. 2b, b') depending on the distributions of univalent chromosomes. By the start of anaphase, the univalents have achieved proper distribution and the furrow always is in a symmetrical position. After anaphase, the furrow ingresses, eventually cleaving the cell into two equally sized daughter cells.

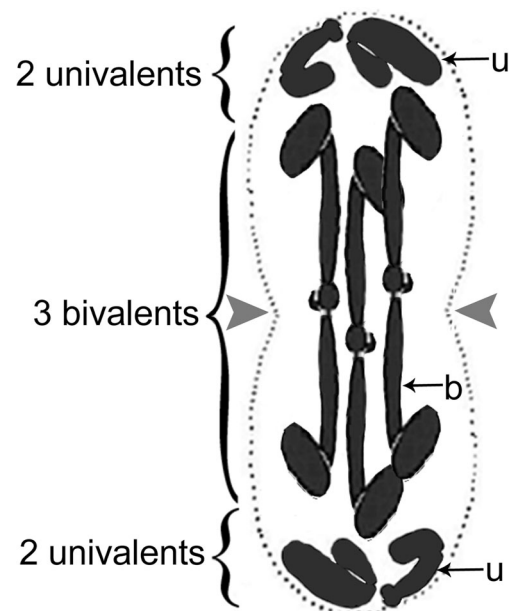


Fig. 1 Illustration of a *Mesostoma* spermatocyte during prometaphase, modified from Husted and Ruebush (1940). There are three bivalents (b) that span across the middle of the cell, and four univalents (u), two at each pole. The position of the precocious furrow is indicated by the arrowheads. In this example, the furrow is symmetrically positioned in the middle of the cell

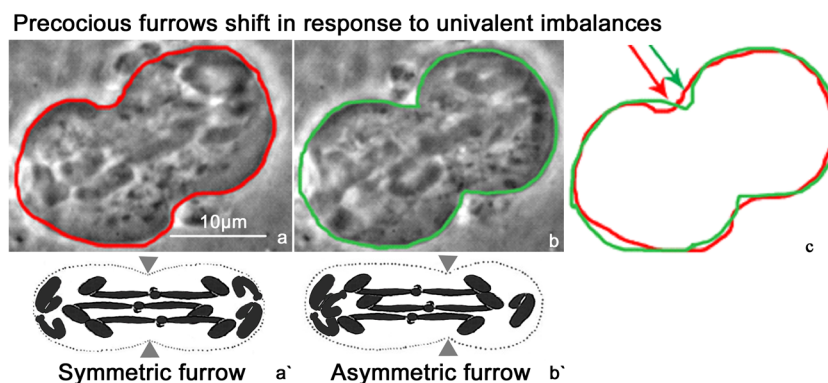


Fig. 2 **a–c** Two frames from a video sequence of one spermatocyte in prometaphase (**a**, **b**). The colored outlines emphasize the cleavage furrow position. **a** Symmetrically positioned cleavage furrow. There are two univalents at each pole. **b** Asymmetric furrow, smaller at the top because there is one univalent at that pole and three at the bottom pole.

c The cell outlines were superposed to show that the cleavage furrow shifted along the length of the cell, moving towards the half-cell with fewer chromosomes. **a'**, **b'** Diagrammatic representations of the univalent distributions in cells with similarly symmetric or asymmetric cleavage furrows

Drug-treated cells

We treated cells with NOC or colcemid to depolymerize the spindle microtubules. The effects on chromosome movement were the same for both anti-microtubule drugs, as were effects on the furrow, but we studied in detail only the effects of NOC on the cleavage furrow.

Chromosome movement

To help understand the effects on the cleavage furrow of removal of microtubules, we first need to describe the effects on the chromosomes. The effects of NOC and colcemid on chromosome movements are described in detail elsewhere (Fegaras and Forer 2018). Briefly, immediately after addition of NOC at all concentrations (5, 10, and 20 μM), the bivalent chromosomes stretch out towards both poles (Fig. 3a–c). They pause there for a short time period, and then detach from only one pole (Fig. 3d). The detached kinetochores quickly move towards the opposite pole and eventually end their movement (Fig. 3e, f). Sometimes, only bivalents detach (Fig. 4i), but sometimes, both the bivalents and univalents detach, both detaching from the same pole (Fig. 4ii). The effects of 50 and 100 μM colcemid are very similar to NOC, verifying that the chromosome response is due to depolymerizing the microtubules. Immunofluorescence observations indicated that the spindle microtubules are almost completely gone when chromosome detachment and movement occur (Fegaras and Forer 2018) and remain absent afterwards as the cleavage furrow ingresses and or moves, which we now describe.

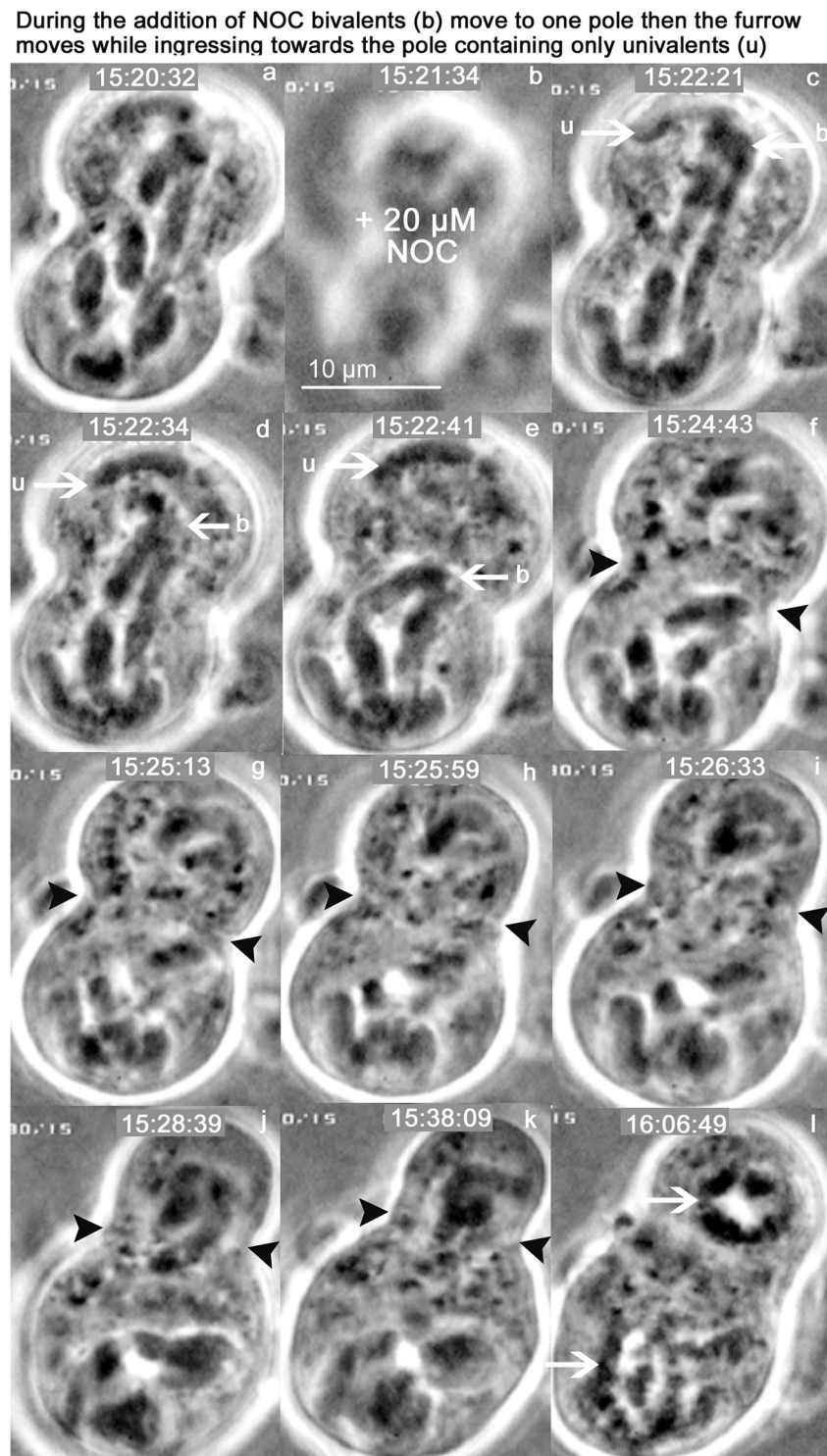
Precocious furrow moves and changes its width

After the kinetochores move to the opposite pole, the cleavage furrow moves and/or changes its width (its ‘diameter’, the distance between the arrowheads in Fig. 7a). The furrow always

moves toward the pole that has fewer chromosomes, but the distances it moves and whether it changes width vary: furrows respond differently in different cells, as illustrated in Fig. 5 and as shown in two supplemental video sequences (S1 and S2). We categorize three different furrow *movements*: (1) partial movement (Fig. 5a), where the furrow moves between 0 and 5 μm , which therefore includes furrows that did not move; (2) halfway movement (Fig. 5c), where the furrow moves between 5 and 10 μm ; and (3) full movement (Fig. 5b), where the furrow moves > 15 μm , off the end of the cell; that is, the furrow contracts to a smaller and smaller diameter as it moves down the cell, and eventually disappears, as in the second image in Fig. 5b. We categorize three different furrow *width* (‘diameter’) *changes*: (1) partial change, where the furrow width increases or decreases slightly if at all, i.e., by less than 2 μm ; (2) ingression (Fig. 5b, c), where the width decreases > 2 μm ; and (3) regression (e.g., Fig. 5a), where the width enlarges > 2 μm . The numbers of cells in each of the combinations that were encountered in this study are shown in Table 1. In 33/61 cells studied, the furrow ingressed in the absence of spindle microtubules, the ingression starting from 0 to 6 min after the chromosomes detached. Full movement occurred in all cells in which both the univalents and bivalents moved to the opposite pole (such as in Fig. 4ii); in these cells, the furrow moved and ingressed until it reached the end of the cell and apparently dissipated since it was no longer seen. Halfway movement of the furrows occurred in cells in which the univalents remained at the otherwise empty poles (such as in Fig. 4i).

In addition to furrow movements and widths, we looked at the timing of width changes and furrow movements—which came first? The most frequent response was that they started at the same time—furrows moved while ingressing. This occurred in 29/61 cells. There were no significant differences between different concentrations of NOC in effects on movement, change in width, or the order of movement vs. width change (Fig. 6), or on the length of time after bivalent detachment that the furrow moved or changed width (Table 2). Thus,

Fig. 3 **a–l** A sequence of images from one cell treated with 20 μ M NOC. After the addition of NOC, chromosomes detach from the top pole and quickly move toward the bottom pole, after which the precocious furrow moves toward the pole with fewer chromosomes (the top pole) while simultaneously ingressing. Arrows point to positions of chromosomes, (b) points to a bivalent, (u) points to a univalent, and black arrowheads point to the furrow. **c–e** The univalents stay at the top pole while the bivalent kinetochores move towards the bottom pole after which the furrow begins to ingress and moves towards the top pole in **f–k**. **l** A reconstituted nucleus at the top pole and one forming at the bottom pole, both of which appear similar to inter-meiotic nuclei in regularly dividing spermatocytes shown in Fegaras and Forer (2018). The furrow still is seen, although it has regressed somewhat from earlier. The cell remained in NOC throughout this sequence



most of the findings were consistent among all three drug concentrations.

The rate of ingression was also consistent among all drug concentrations and is similar to ingression during regular anaphase in cells with intact spindles. As seen in Table 1 and Fig. 6, slightly more than half of cells (33/61) treated with NOC ingressed after chromosome movement to one pole. The rate

of ingression for both drug treated and anaphase cells is approximately 1 μ m/min, range 0.66–1.89 μ m/min (Fig. 7a, b).

While the cells look quite abnormal after treatment with NOC, they are not dead. When the NOC is washed out shortly after the chromosomes detach and move to the one pole, the cells in time look like non-treated cells that progress to meiosis-II: they reconstitute nuclei, the chromatin becomes

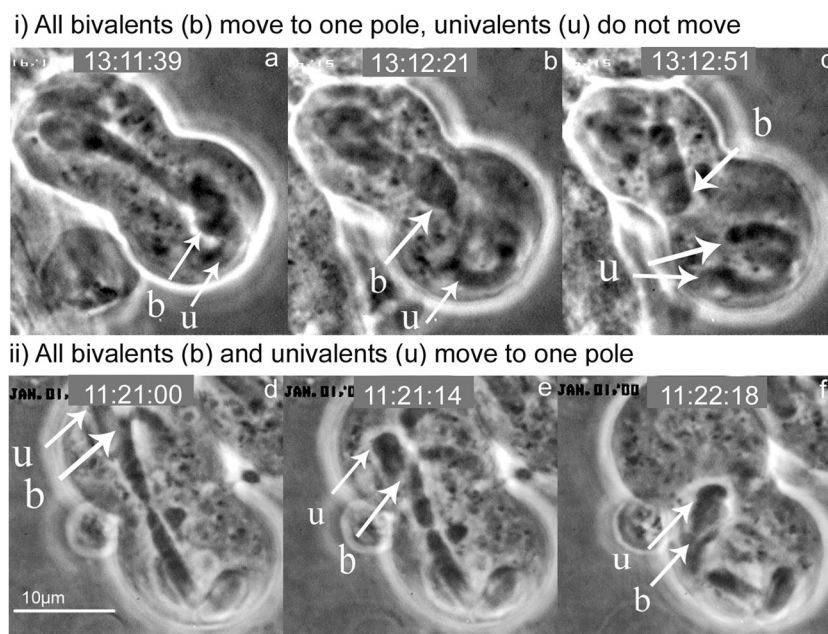


Fig. 4 i–ii Chromosome movements in two different *Mesostoma* spermatoocytes after the addition of NOC. Arrows indicate the positions of a bivalent (b) and univalents (u). **i a–c** Immediately after the addition of 5 μ M NOC the bivalents stretch towards the two poles in **a**, then in **b** the bivalents detach from the bottom pole and move towards the top pole. The univalents do not move with the bivalents, as seen in **c**, and remain at

the bottom pole. **ii d–f** After the addition of 10 μ M NOC, the bivalents stretch towards both poles in **d** then both bivalents and univalents detach from the top pole simultaneously in **e** and begin movement towards the bottom pole. Both the bivalents and univalents complete movement to the bottom pole in **f**. There are no chromosomes left behind at the top pole

Fig. 5 a–c Three different spermatoocytes treated with NOC. The red outlines are seconds after drug addition; the blue outlines are the final furrow positions after the furrow completes its movement and/or width changes. **a** The arrows indicate the positions of the chromosomes. Partial movement, regression of furrow width. Chromosomes do not detach. **b** Full movement, ingression of the furrow. Both bivalents and univalents move into the bottom half cell. **c** Halfway movement, ingression of the furrow. The bivalents move towards the top half cell, while univalents remain behind. The furrow has moved toward the bottom pole, forming a cell that looks like a “shmoo”

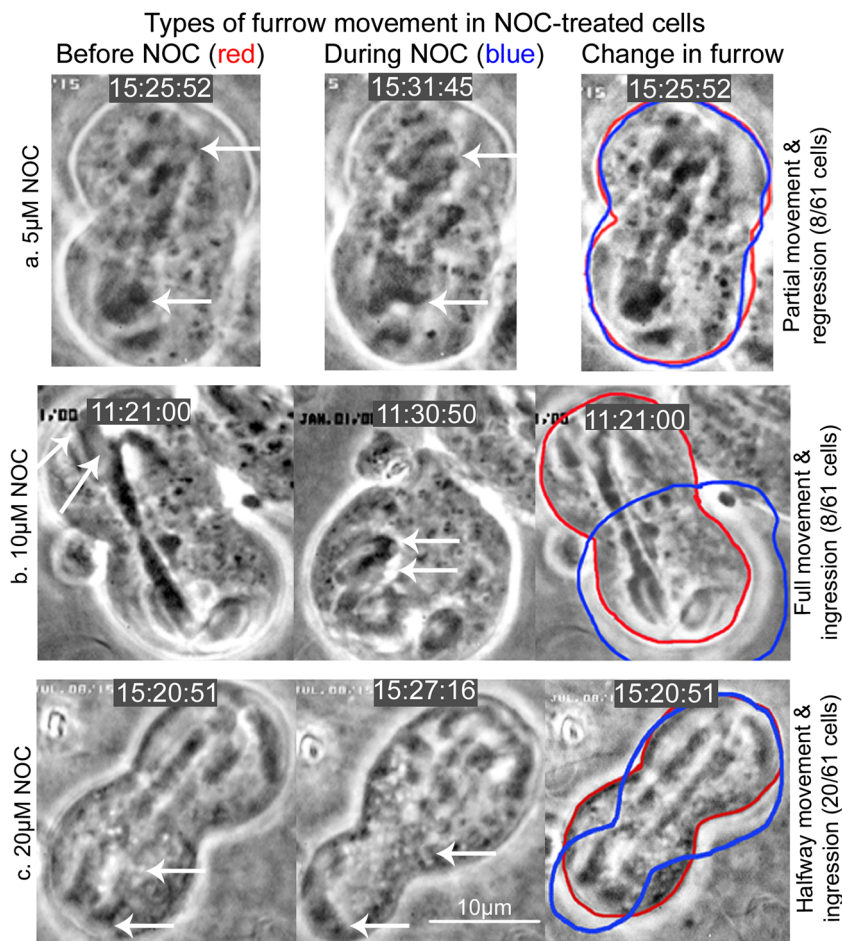


Table 1 The three ways the furrow may move in relation to the three ways the furrow changes width, after the addition of various concentration of NOC. Numbers represent numbers of cells

Conc. of NOC	Partial movement			Full movement			Halfway movement		
	Ingression	Regression	Partial	Ingression	Regression	Partial	Ingression	Regression	Partial
5 μ M	1	2	4	1	0	0	8	0	3
10 μ M	1	2	6	4	0	0	6	0	2
20 μ M	3	4	3	3	0	0	6	0	2
Totals	5	8	13	8	0	0	20	0	7
Totals			26			8			27

arranged around the rim of the nuclei, the nuclei rotate, and then the chromosomes begin to oscillate as if entering second division (Fegaras and Forer 2018). As seen in Fig. 3f–k, the cleavage furrow remained constricted to form unequal sized portions of the cell (a shmoo), and 45 min after the drug was added nuclei reformed (Fig. 3l). In some cells after drug wash-out, the cleavage furrow can even fully ingress and cleave the cell into two unequal portions. In these cells, cleavage occurred between 15 and 45 min after NOC washout (Fig. 8). This was observed in only five cells.

Discussion

The main results of our experiments are as follows: NOC caused all bivalent kinetochores to detach from one pole and the kinetochores moved to the other pole, sometimes accompanied by the univalents. After chromosome movement, the cleavage furrow moved along the length of the cell towards the half-spindle with

fewer chromosomes. In all cells in which both the bivalents and the univalents moved to the other pole and there were no chromosomes in the one half-spindle, the cleavage furrow always moved the entire way to that end of the cell and off the end, as exemplified in Fig. 5b. When univalents remained in the half-spindle, the furrow was not able to travel the entire way but rather it usually moved “halfway”, creating cells that looked like shmoos (Fig. 5c) (Capp 1948). As the furrow moved, it either maintained its width or ingressed further (53/61 cells, Table 1), at rates similar to those that occur in control cells at anaphase (Fig. 7), suggesting that the same acto-myosin mechanisms apply in both circumstances. The continued ingression and movement of the furrow takes place in the absence of microtubules so spindle microtubules determine neither the furrow position nor the continued ingression (or not) of the pre-existing furrow. We do not yet know why the furrow forms in prometaphase, nor why after it appears it does not ingress to cleave the cells until after anaphase, nor why in non-treated cells it moves towards the half-spindle with fewer chromosomes, nor why the furrow ingresses after

Fig. 6 Comparison of furrow movement, ingression, and order of movement vs. ingression in *Mesostoma* spermatocytes treated with various concentrations of NOC. The total cell count is 61 for both furrow movement and width and is 28 for order of movement vs. ingression because only the cells which had both ingression and full or halfway movement were included

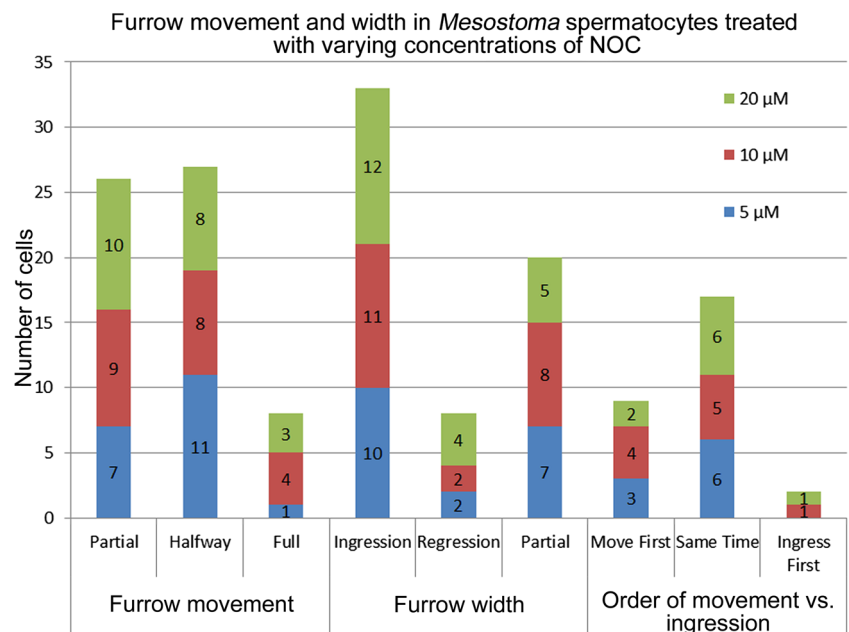


Table 2 Summary of time averages concerning furrow movement and furrow width, as recorded in *Mesostoma* spermatocytes treated with various concentrations of NOC. Times are given in minutes: seconds. Table includes a comparison of the three different types of movement and the three ways the furrow changes width

Conc. of NOC	# of cells	Chromosome velocity ($\mu\text{m}/\text{min}$) $\pm$ SD	Movement		Width			
			How long after chromosome detachment did furrow begin to move? $\pm$ SD	Time furrow spent moving along cell length $\pm$ SD	Movement type (partial; halfway; full) indicated by # of cells	How long after chromosome detachment did furrow begin to change width? $\pm$ SD	Time furrow spent changing width $\pm$ SD	Width change type (ingress; regress; partial) indicated by # of cells
5 μM	19	15.5 $\pm$ 9.07	01:51 $\pm$ 00:47	05:45 $\pm$ 03:39	7; 11; 1	02:04 $\pm$ 00:56	05:32 $\pm$ 03:36	10; 2; 7
10 μM	21	35.1 $\pm$ 24.2	02:17 $\pm$ 01:27	06:41 $\pm$ 02:56	9; 8; 4	02:55 $\pm$ 01:31	06:55 $\pm$ 03:40	12; 2; 7
20 μM	21	57.8 $\pm$ 35.1	01:46 $\pm$ 01:58	04:46 $\pm$ 02:39	10; 8; 3	01:54 $\pm$ 01:55	07:12 $\pm$ 05:41	12; 4; 5

spindle microtubules are depolymerized by NOC, nor why after NOC treatment it moves toward the depleted half-spindle even to the end of the cell.

These furrow behaviors are not explained by prevailing models since the generally accepted ideas are that cleavage furrows form after anaphase and that their positions are determined somehow by direct or indirect interactions with spindle asters or spindle microtubules of some kind (discussed in Forer and Pickett-Heaps 2010). The standard models consider furrows set up by signals arriving at the cortex in late metaphase or early anaphase, and therefore cannot accommodate cleavage furrows forming before then, and none incorporate movement of the furrow along the spindle to accommodate unequal distributions of chromosomes in the spindle. The only data we know of in which furrows moved along the length of the spindle occurred when single centrosomes in metaphase sand dollar zygotes were ablated with a UV laser: the spindles remained in the same positions but the furrows that formed during late anaphase were shifted toward the pole with the ablated centrosome, sometimes shifting so far that the cell formed from the half spindle with the ablated pole excluded both sets of chromosomes (von Dassow et al. 2009). However, the furrows did not shift when single centrosomes were ablated in anaphase, so in those experiments the centrosome influences the furrow position only before the furrow is formed, unlike in our experiments in which pre-formed furrows changed positions.

With no models to rely on, we might speculate that the formation of the precocious furrow and its subsequent behaviors are a consequence of the tensegral nature of the spindle and cell (Ingber 1993, 2003a, b). Ingber (1993) argued from architectural principles that tensional integrity (tensegrity) arises from interconnected tension elements and structural elements (struts), that the cytoskeleton in general is a tensegral network, that the tension and structural components in the cells arise from struts such as microtubules and intermediate filaments, and from tension elements such as the actin filament lattice (and we might add, spindle matrix components such as described in Johansen et al. 2011). The cytoskeleton can act as a mechanochemical and sensory pathway such that mechanical input is translated into local chemical signaling (e.g., Ingber 1991; Liu and Post 2000; White and Frangos 2007; Quinlan et al. 2017). That is, because the cytoskeleton acts as a tensegral network, mechanical perturbations in one part of the structure are transmitted throughout the structure to other regions. As discussed elsewhere (Forer and Pickett-Heaps 2010), the tensegral nature of the spindle and cell might give rise to precocious cleavage furrows seen in *Mesostoma* (Ferraro-Gideon et al. 2013, 2014) and to the shifting of the furrow after ablation of a centrosome (von Dassow et al. 2009). Briefly, in more “normal” cells,

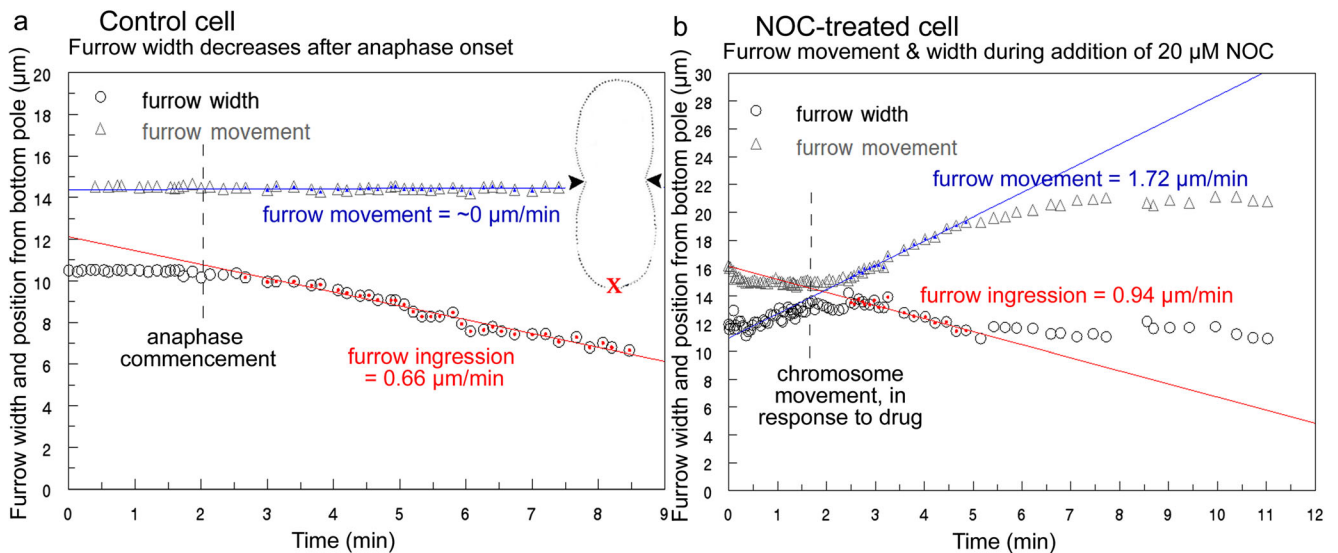


Fig. 7 **a, b** The rate of cleavage furrow ingress in anaphase of control cells is similar to precocious furrow ingress in cells treated with NOC. **a** After anaphase commencement, the furrow ingressed at a rate of $0.66 \mu\text{m}/\text{min}$ and did not change position. The diagram in the upper right corner shows how the furrow positions and widths were determined from the video frames. The positions of each side of the cleavage furrow were plotted (black arrowheads) in comparison to a fixed point at the spindle pole (red X). The distance between

arrowheads represents the furrow width, and the distance between the arrowhead and the X represents the furrow width. **b** Cell treated with $20 \mu\text{M}$ NOC; time zero is at the addition of NOC. After chromosomes detached and moved to one pole, the furrow ingressed at a rate of $0.94 \mu\text{m}/\text{min}$ while simultaneously moving towards the half-cell with fewer chromosomes at a rate of $1.74 \mu\text{m}/\text{min}$. Both the furrow ingress and movement stopped at roughly the same time, about 5 min after perfusion with NOC

a stable tensegral spindle and cytoplasmic structure is achieved after nuclear membrane breakdown. The furrow does not yet ingress because the force exerted by the cell is highest during early stages of cell division, and decreases

in later stages of division, as shown by the deformation of a droplet of ferrofluid (Hiramoto 1975). The furrow appears in anaphase when the structures are disassembling and changing and the tensegral 'weak spots' (e.g., White and

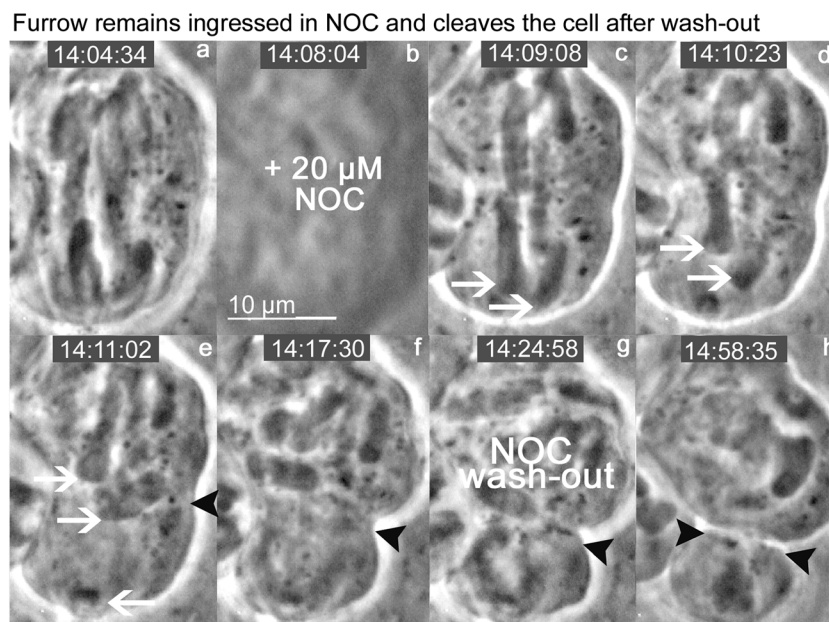


Fig. 8 **a–h** The furrow both moved and ingressed in NOC and completely cleaved the cell after wash-out of NOC. Arrows point to the positions of bivalent kinetochores; the black arrowheads point to the furrow. After addition of $20 \mu\text{M}$ NOC (**b**), the chromosomes stretch out towards the poles (**c**). A few minutes after NOC addition, the bivalents detach from the bottom pole (**d**) and move towards the top pole (**e**). The univalents

remain at the bottom pole. The furrow moves towards the bottom pole while ingressing (**e–g**). After NOC is washed out (**g**), the furrow cleaves the cell into two unequally sized daughter cells (**h**). The top larger cell contains the bivalents and the univalents that were originally present at the top pole, and the smaller bottom cell contains only the univalents

Frangos 2007) are transmitted to the cortex (Ingber 1993). The formation of tensegrally stable structures appears to be prevented in some cases, such as *Mesostoma* spermatocytes, because of the continuous vigorous movements of bivalents throughout prometaphase (Ferraro-Gideon et al. 2013, 2014; Fuge 1987, 1989) and thus precocious furrows appear in these cells during prometaphase. When there is chromosome imbalance due to a univalent excursion (Oakley 1984), the furrows move 2–3 μm in response to altered tensegral “bulk” (such as Fig. 2).

From observing furrow behavior in cells treated with NOC, it appears that the movement or ingression (or not) of the cleavage furrow is related to the ‘bulk’ compressibility properties of the spindle. Whereas in intact spindles, the ‘bulk’ may arise from the spindle itself, after NOC treatment the microtubules are gone and the ‘bulk’ arises from something else, perhaps from proteins which are known to associate with spindle microtubules such as the muscle proteins actin, myosin and titin (Mogessie and Schuh 2017; Fabian et al. 2007; Forer et al. 2003) and the nuclear derived matrix proteins skeletor, megator, chromator, and EAST (Johansen and Johansen 2007; Johansen et al. 2011). After NOC treatment, most of the struts have disappeared, except for microtubules in the centrosomes (Fegaras and Forer 2018) and presumably components of the matrix which have been shown to remain in the cell for some period of time after microtubules are depolymerized (Yao et al. 2012). With little bulk in one half spindle, the furrow moves halfway along the length of the cell in the direction of less bulk to regions with fewer chromosomes and is free to continue to ingress. With zero bulk (when both bivalents and univalents move to the other pole), the furrow even moves off the end of the cell, seeming to compress what is left and pushing it into the half-spindle with all the chromosomes.

In conclusion, precocious cleavage furrows form in prometaphase *Mesostoma* spermatocytes, for reasons unknown. The furrows move up and back along the spindle axis in response to normally occurring imbalances in chromosomes in the two half-spindles. When NOC removes spindle microtubules, the bivalent chromosomes move to one pole, sometimes accompanied by the univalent chromosomes. Shortly thereafter, the otherwise arrested precocious furrows most often resume ingression and simultaneously move along the length of the cell toward the half-spindle with fewer (or no) chromosomes. When univalent chromosomes remain at the one pole, the furrow stops moving and the resultant cell looks like a shmoo, with one small portion attached to a larger portion. When the univalents have moved with the bivalents and the half spindle is empty, the furrow ingresses and moves down the length of the cell until it no longer exists. These very unusual behaviors of the furrow occur in the absence of spindle microtubules and at the present time are not understood.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Alsop GB, Zhang D (2003) Microtubules are the only structural constituent of the spindle apparatus required for induction of cell cleavage. *J Cell Biol* 162:383–390
- Barr FA, Gruneberg U (2007) Cytokinesis: placing and making the final cut. *Cell* 131:847–860
- Bonaccorsi S, Giansanti MG, Gatti M (1998) Spindle self-organization and cytokinesis during male meiosis in asterless mutants of *Drosophila melanogaster*. *J Cell Biol* 142:571–761
- Capp A (1948) The life and times of the Shmoo. Pocket Books, New York
- Cassimeris L, Rieder C, Rupp G, Salmon ED (1990) Stability of microtubule attachment to metaphase kinetochores in PtK1 cells. *J Cell Sci* 96:9–15
- Choi HJ, Fukui M, Zhu BT (2011) Role of cyclin B1/Cdc2 up-regulation in the development of mitotic prometaphase arrest in human breast cancer cells treated with nocodazole. *PLoS One* 6(8):e24312. <https://doi.org/10.1371/journal.pone.0024312>
- Cytrynbaum EN, Sommi P, Brust-Mascher I, Scholey JM, Mogilner A (2005) Early spindle assembly in *Drosophila* embryos: role of a force balance involving cytoskeletal dynamics and nuclear mechanics. *Mol Biol Cell* 16:4967–4981
- Eggert US, Mitchison TJ, Field CM (2006) Animal cytokinesis: from parts list to mechanisms. *Annu Rev Biochem* 75:543–566
- Fabian L, Xia X, Venkitaramani DV, Johansen KM, Johansen J, Andrew DJ, Forer A (2007) Titin in insect spermatocyte spindle fibers associates with microtubules, actin, myosin and the matrix proteins skeletor, megator and chromator. *J Cell Sci* 120:2190–2204
- Fegaras E, Forer A (2018) Chromosomes selectively detach at one pole and quickly move towards the opposite pole when kinetochore microtubules are depolymerized in *Mesostoma ehrenbergii* spermatocytes. *Protoplasma*. <https://doi.org/10.1007/s00709-018-1214-4>
- Ferraro-Gideon J, Hoang C, Forer A (2014) Meiosis-I in *Mesostoma ehrenbergii* spermatocytes includes distance segregation and inter-polar movements of univalent, and vigorous oscillations of bivalents. *Protoplasma* 251:127–143
- Ferraro-Gideon J, Hoang C, Forer A (2013) *Mesostoma ehrenbergii* spermatocytes—a unique and advantageous cell for studying meiosis. *Cell Biol Int* 37(9):892–898
- Forer A, Spurck T, Pickett-Heaps J, Wilson P (2003) Structure of kinetochore fibres in crane-fly spermatocytes after irradiation with an ultraviolet microbeam: neither microtubules nor actin filaments remain in the irradiated region. *Cell Motil Cytoskeleton* 56:173–192
- Forer A, Pickett-Heaps J (2005) Fibrin clots keep non-adhering living cells in place on glass for perfusion or fixation. *Cell Biol Int* 29:721–730
- Forer A, Pickett-Heaps J (2010) Precocious (pre-anaphase) cleavage furrows in *Mesostoma* spermatocytes. *Eur J Cell Biol* 89:607–618
- Fuge H (1989) Rapid kinetochore movements in *Mesostoma ehrenbergii* spermatocytes: action of antagonistic chromosome fibres. *Cell Motil Cytoskeleton* 13:212–220
- Fuge H (1987) Oscillatory movements of bipolar-oriented bivalent kinetochores and spindle forces in male meiosis of *Mesostoma ehrenbergii*. *Eur J Cell Biol* 44:294–298

- Glötzter M (2004) Cleavage furrow positioning. *J Cell Biol* 164(3):347–351
- Gonczy P (2008) Mechanisms of asymmetric cell division: flies and worms pave the way. *Nat Rev* 9:355–366
- Grill SW, Goënczy P, Stelzer EHK (2001) Polarity controls forces governing asymmetric spindle positioning in the *Caenorhabditis elegans* embryo. *Nature* 409:630–633
- Hiramoto Y (1975) Force exerted by the cleavage furrow of sea urchin eggs. *Develop Growth Differ* 17(1):27–38
- Hoang C, Ferraro-Gideon J, Gauthier K, Forer A (2013) Methods for rearing *Mesostoma ehrenbergii* in the laboratory for cell biology experiments, including identification of factors that influence production of different egg types. *Cell Biol Int* 37(10):1089–1105
- Husted L, Ruebush TK (1940) A comparative cytological and morphological study of *Mesostoma ehrenbergii* and *Mesostoma ehrenbergii wardii*. *J Morphol* 67:387–410
- Ingber DE (1991) Integrins as mechanochemical transducers. *Curr Biol* 3: 841–848
- Ingber DE (1993) Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J Cell Sci* 104:613–627
- Ingber DE (2003a) Tensegrity I. Cell structure and hierarchical systems biology. *J Cell Sci* 116:1157–1173
- Ingber DE (2003b) Tensegrity II. How structural networks influence cellular information processing networks. *J Cell Sci* 116:1397–1408
- Johansen KM, Johansen J (2007) Cell and molecular biology of the spindle matrix. *Int Rev Cytol* 263:155–210
- Johansen KM, Forer A, Yao C, Girton J, Johansen J (2011) Do nuclear envelope and intranuclear proteins reorganize during mitosis to form an elastic hydrogel-like spindle matrix? *Chromosom Res* 19:345–365
- Jordan MA, Wilson L (2004) Microtubules as a target for anticancer drugs. *Nat Rev Cancer* 4(4):253–265
- Liu M, Post M (2000) Mechanochemical signal transduction in the fetal lung. *J Appl Physiol* 89:2078–2084
- McIntosh K, Pickett-Heaps JD, Gunning BES (1995) Cytokinesis in *Spirogyra*: integration of cleavage and cell-plate formation. *Int J Plant Sci* 156:1–8
- Mogessie B, Schuh M (2017) Actin protects mammalian eggs against chromosome segregation errors. *Science* 357:eaal 1647
- Mollinedo F, Gajate C (2003) Microtubules, microtubule-interfering agents and apoptosis. *Apoptosis* 8(5):413–450
- Murthy K, Wadsworth P (2008) Dual role for microtubules in regulating cortical contractility during cytokinesis. *J Cell Sci* 121:2350–2359
- Oakley HA (1984) Meiosis in *Mesostoma ehrenbergii ehrenbergii* (Turbellaria, Rhabdozoela). III. Univalent chromosome segregation during the first meiotic division in spermatocytes. *Chromosoma* 91(2):95–100
- Pickett-Heaps JD (1991) Post-mitotic cellular reorganisation in the diatom *Cymatopleura solea*: the role of microtubules and the microtubule Centre. *Cell Motil Cytoskel* 18:279–292
- Pickett-Heaps JD, Tippit DH, Andreozzi A (1978) Cell division in the pennate diatom *Pinnularia*. II Later stages in mitosis. *Biol Cell* 33: 79–84
- Pickett-Heaps JD, Tippit DH, Andreozzi A (1979) Cell division in the pennate diatom *Pinnularia*. V. Observations on living cells. *Biol Cell* 35:295–304
- Pickett-Heaps JD, Tippit DH, Leslie R (1980) Light and electron microscopic observations on cell division in two large pennate diatoms, *Hantzschia* and *Nitzschia*. I. Mitosis in vivo. *Eur J Cell Biol* 21:1–11
- Poxleitner MK, Dawson SC, Cande WZ (2008) Cell cycle synchrony in *Giardia intestinalis* cultures achieved by using nocodazole and aphidicolin. *Eukaryot Cell* 7(4):559–574
- Quinlan RA, Schwarz N, Windoffer R, Richardson C, Hawkins T, Broussard JA, Green KJ, Leube RE (2017) A rim-and-spoke hypothesis to explain the biochemical roles for cytoplasmic intermediate filament networks. *J Cell Sci* 130:3437–3445
- Rappaport R (1985) Repeated furrow formation from a single mitotic apparatus in cylindrical sand dollar eggs. *J Exp Zool* 234:167–171
- Rappaport R (1981) Cytokinesis: cleavage furrow establishment in cylindrical sand dollar eggs. *J Exp Zool* 217:365–375
- Rappaport R, Ebstein RP (1965) Duration of stimulus and latent periods preceding furrow formation in sand dollar eggs. *J Exp Zool* 158: 373–382
- Rappaport R (1961) Experiments concerning the cleavage stimulus in sand dollar eggs. *J Exp Zool* 148:81–89
- Vasquez R, Howell B, Yvon AC, Wadsworth P, Cassimeris L (1997) Nanomolar concentrations of nocodazole alter microtubule dynamic instability in vivo and in vitro. *Mol Biol Cell* 8:973–985
- von Dassow G, Verbrugghe KJC, Miller AL, Sider JR, Bement WM (2009) Action at a distance during cytokinesis. *J Cell Biol* 187(6): 831–845
- White CR, Frangos JA (2007) The shear stress of it all: the cell membrane and mechanochemical transduction. *Phil Trans R Soc B* 362:1459–1467
- Wong R, Forer A (2003) Signalling between chromosomes in crane fly spermatocytes studied using ultraviolet microbeam irradiation. *Chromosom Res* 11:771–786
- Yang H, Ganguly A, Cabral F (2010) Inhibition of cell migration and cell division correlates with distinct effects of microtubule inhibiting drugs. *J Biol Chem* 285(42):32242–32250
- Yao C, Rath U, Maiato H, Sharp D, Girton J, Johansen KM, Johansen J (2012) A nuclear-derived proteinaceous matrix embeds the microtubule spindle apparatus during mitosis. *Mol Biol Cell* 23:3532–3541

CHAPTER 3

ELASTIC ‘TETHERS’ CONNECT SEPARATING ANAPHASE CHROMOSOMES IN A BROAD RANGE OF ANIMAL CELLS

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Research paper

Elastic ‘tethers’ connect separating anaphase chromosomes in a broad range of animal cells



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ABSTRACT

We describe the general occurrence in animal cells of elastic components (“tethers”) that connect individual chromosomes moving to opposite poles during anaphase. Tethers, originally described in crane-fly spermatocytes, exert force on chromosome arms opposite to the direction the anaphase chromosomes move. We show that they exist in a broad range of animal cells. Thus tethers are previously unrecognised components of general mitotic mechanisms that exert force on chromosomes and they need to be accounted for in general models of mitosis in terms of forces on chromosomes and in terms of what their roles might be.

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1. Introduction

This article describes the presence of elastic connections between separating anaphase chromosomes in cells from a broad range of animals, from flatworms to humans, suggesting that such connections are universal. Elastic connections between chromosomes moving to opposite poles represent previously unrecognised forces on anaphase chromosomes, acting in opposite direction to the forces moving chromosomes to the poles. While the composition and function of these connections are unknown, their universal presence has broad implications to how chromosome movements in anaphase are coordinated and controlled.

Elastic physical connections between arms of separating chromosomes were originally demonstrated in anaphase crane-fly spermatocytes by LaFountain et al. (2002), who called them “tethers”. Tethers were identified operationally: fragments cut from ends of arms moved ‘backwards’ to the partner telomeres that were moving to the opposite pole. They moved with speeds considerably greater than the chromosomes while the severed chromosome

continued moving to its original pole. Further experiments by LaFountain et al. (2002) indicated that these ‘backwards’ movements of arm fragments were not due to forces from motors associated with spindle microtubules, and pointed strongly to forces arising from elastic connections between separating telomeres, as follows.

When backward-moving arm fragments were cut into two pieces, the sub-fragment with the telomere kept moving but the other one did not: it stopped. Thus arm-fragment movement is not due to generalised spindle transport forces acting on arms in general but requires the telomere. When the arm-fragment telomere was ablated its movement stopped, and when the fragment itself was not cut but the telomere of the other chromosome (to which the fragment was moving) was ablated, arm-fragment backward movements also stopped. Thus both telomeres need to be intact for backward movement to take place, suggesting that there are elastic connections between separating arms. In meiosis-I, arm fragments from only two of the four arms of each separating half-bivalent pair move backward (LaFountain et al., 2002), which was interpreted to mean that arm fragments connected to their partner move backwards but other arm fragments do not, and that backwards movements of arm fragments require specific connections between partner telomeres. When the backward-moving arm-fragment nears the partner telomere, that intact arm loses length (Sheykhan et al., 2017), it ‘shrinks’, further indicating that

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the connections, the ‘tethers’, are elastic. Thus the forces for movement are applied specifically at the telomere, both telomeres need to be intact, and the forces seem to arise from elastic connections (‘tethers’) that extend between separating telomeres.

Tethers were identified operationally in anaphase meiosis-II cells (LaFountain et al., 2002) as well as meiosis-I cells, so they are not restricted to reduction divisions. The tethers seem to become less elastic as anaphase progresses. Arm fragments produced in early anaphase move fast and reach the telomere of the separating chromosome. Those produced later move across the equator at slower speeds and stop before reaching the partner telomere. Those produced yet later move somewhat toward the partner telomere but not across the equator, or do not move at all. LaFountain et al. (2002) interpreted this behaviour to mean that as anaphase proceeds tethers lose elasticity, as indicated by fragments slowing down, and that eventually tethers are no longer attached to telomeres, as indicated by absence of motion.

In this report we identify and characterise tethers in PtK (marsupial tissue culture) cells, and identify tethers in cells ranging from flatworms to humans, namely from a turbellarian flatworm, two classes of insects, two distinct lineages of arachnids, a marsupial, and humans. Thus, elastic tethers connect separating anaphase chromosomes in animal cells in general, if not universally.

2. Materials and methods

2.1. Cells

Asynchronous or rapidly proliferating PtK2 and U2OS cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 10% bovine calf serum, L-glutamine, and sodium pyruvate at 37 °C and 5% CO₂.

Adult *Pholcus phalangioides* (Fuesslin) (cellar spider) males were collected from a field site near Bucknell University, Lewisburg PA. Adult *Latrodectus mactans* (Fabricius) (black widow spider) males were obtained from Spider Pharm Inc. (Yarnell, AZ). The abdomens of spiders were cut away from the cephalothorax. Testes were removed from the abdomen and placed in a culture chamber as described in Ault et al., 2017. Testes contents were spread under a layer of Kel-F oil (Ohio Valley Specialty Chemical, Marietta, OH).

Juvenile *Acheta domesticus* (Linnaeus) male crickets were purchased from PetCo (Clairemont, San Diego, California). Testes were removed through a small incision cut directly behind the wing buds on the dorsal side of the cricket abdomen. Testes were placed directly into Kel-F oil and testes contents were spread under a layer of Kel-F oil.

Crane flies, *Nephrotoma suturalis* (Loew), were from a laboratory culture (Forer, 1982). Spermatocytes were put into a fibrin clot and immersed in Ringers solution as described in Forer and Pickett-Heaps (2005).

The turbellarian flatworms, *Mesostoma ehrenbergii*, were from laboratory cultures (Hoang et al., 2013). Spermatocytes were removed from animals, placed in fibrin clots, and immersed in Ringers solution as described in Hoang et al. (2013) and Ferraro-Gideon et al. (2014). The highly unusual chromosome movements in these cells are described in detail in Ferraro-Gideon et al. (2014).

2.2. Experimental methods

The viewing, irradiation, and analysis methods are described detail in Forer et al. (2013). In brief, cells were observed using phase-contrast microscopy (using a Zeiss Plan-Neofluar 63X lens, NA 1.4) in a microscope fitted for laser microbeam surgery (Harsono et al., 2012; Shi et al., 2012). Regions of interest were irradiated using a 200-fs pulsed laser (Mai Tai, Newport Co., Irvine, CA, USA)

Table 1

Comparison of PtK2 cell arm fragment and anaphase chromosome velocities.

Arm fragment velocities	1.8 $\mu\text{m}/\text{min} \pm 1.58 \mu\text{m}/\text{min}$ (SD)	N = 50
Anaphase chromosome velocities	0.4 $\mu\text{m}/\text{min} \pm 0.29 \mu\text{m}/\text{min}$ (SD)	N = 37

emitting 740 nm wavelength light. Cells were observed at room temperature, including some of the PtK cells, but for all experiments on U2OS cells and most experiments using PtK cells, the cells were placed on a heated stage maintained at 36.5 °C. Images recorded every 2 or 3 s were cropped, stamped with date and time, and converted into bmp files using *IrfanView* freeware. The bmp files were assembled into avi files using *VirtualDub* freeware. We analysed the results of the experiments by visual observation and by obtaining distance versus time graphs, as described in Forer et al. (2013), using an in-house program, *WinImage* (Wong and Forer, 2003). The graphical data on chromosome arm shortening were only from cuts that caused arms to shorten, except for Figs. 10 and 12, which included all irradiations. Montages were assembled from individual images using Photoshop. The images were adjusted only for image contrast and brightness levels.

3. Results

3.1. Tethers in PtK2 cells

Tethers connect separating anaphase chromosomes in PtK2 cells. When chromosome arms in anaphase cells are cut using a laser microbeam, the resultant arm fragments move away from the arm stub, toward their partner telomeres, either (a) reaching them, or (b) stopping across the equator before reaching them, or (c) stopping before crossing the equator (Fig. 1). The velocities of arm fragments were faster than anaphase chromosomes in the same cells (Table 1), with fragment velocities as high as 6.2 $\mu\text{m}/\text{min}$.

Arm fragment movements are due to elastic connections between telomeres, because cutting the region between the two telomeres causes the arm fragment to stop moving toward the opposite chromosome (Fig. 2). [This was our operational definition of cutting tethers.] When tethers were cut, some arm fragments reversed direction and moved back towards the arm stub (Fig. 3), as reported for arm fragments in crane-fly spermatocytes (Sheykhani et al., 2017), presumably because the initial arm-severing cut was incomplete and elastic chromosome components remained in the cut region.

Tension from tethers stretches anaphase crane-fly spermatocyte chromosome arms backward, toward the separating partner telomeres (LaFountain et al., 2002). Stretched arms shorten when their tethers are cut (Sheykhani et al., 2017). Thus we cut PtK-cell tethers themselves, to test whether their chromosomes were stretched. After cutting their tethers, arms shortened (Fig. 4). In those cells in which we were able to see both kinetochore and telomere sufficiently clearly to measure arm lengths, the arms shortened to about 89% of their initial value (0.89 ± 0.025 sd, N = 31), over a period of up to a minute, independent of the initial length of the chromosome arm (Fig. 5). The stretch appears to be uniform along the entire length of the chromosome arm, because, independent of the initial length of the stub, the arm stubs shortened to about the same extent (0.85 ± 0.06 sd, N = 10) as entire arms (Fig. 6).

Not all arms shortened when we attempted to cut their tethers. This may be because we did not actually cut them; we have no visible marker of tethers, so it is possible that sometimes they were not cut.

Not all arm fragments moved backwards toward the partner telomere. Lack of movement might be because not all arms are connected to their partners with tethers. For example, in some cells only one or two arm fragments moved backwards toward the other

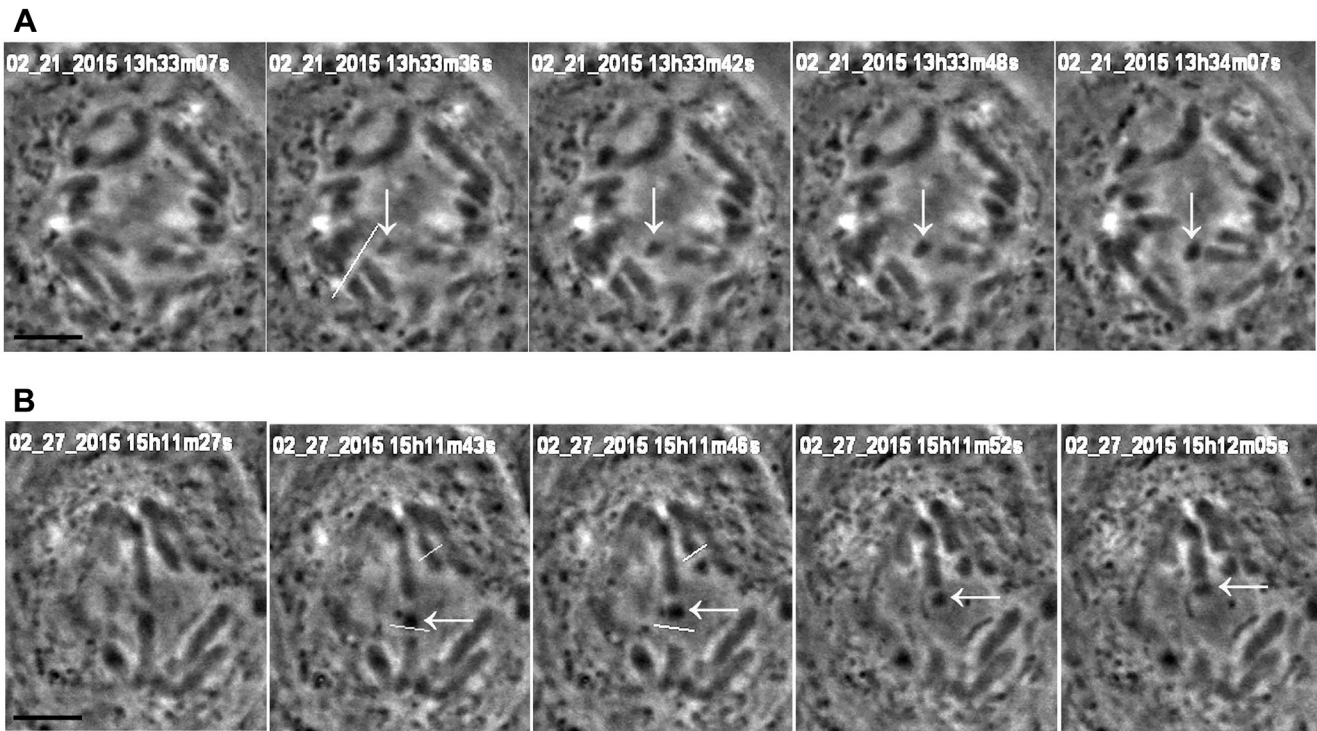


Fig. 1. Arm fragments move across the equator to their partner telomeres in a PtK2 cell. A: the white line in the second image is the laser position immediately after the arms were cut. The arm fragment indicated by the white arrow moved to the partner telomere. The other arm fragments did not. The black line in the first image represents 5 μ m. B: The white lines in the second and third images represent the laser positions just as and just after the arms were cut. The arm fragment indicated by the white arrow moved to the partner telomere and when it did the partner chromosome shortened, and the arm stub shortened. The other arm fragment did not move. The black line in the first image represents 5 μ m.

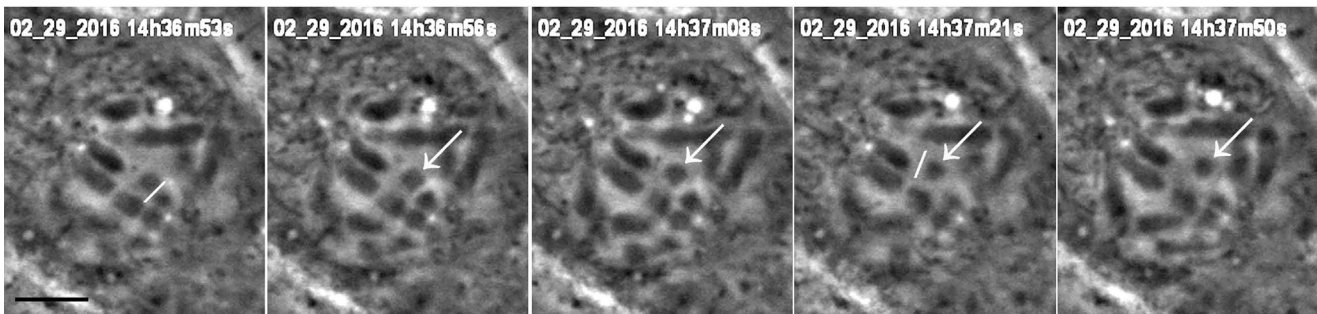


Fig. 2. Cutting the tether stops movement of the arm fragment in a PtK2 cell. The white arrow indicates the arm fragment. The arm fragment moved toward the partner telomere after the arm was cut (white line in the first image) but once the tether was cut (white line in the fourth image) the arm fragment stopped moving and drifted away. The black line in the first image represents 5 μ m.

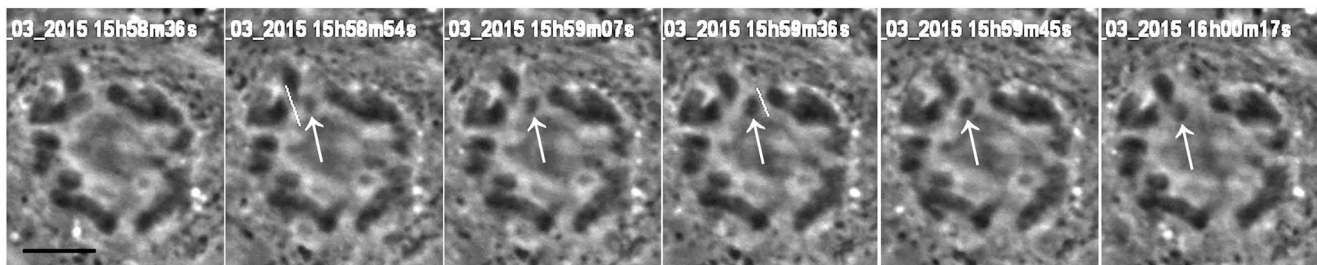


Fig. 3. An arm fragment moved back to the arm stub when its tether was cut in a PtK2 cell. The arm fragment (indicated by a white arrow) moved toward the other telomere after the arm was cut (white line in the second image) and when its tether was cut (white line in the fourth image) the arm fragment moved back to its original arm. The black line in the first image represents 5 μ m.

telomeres while others cut at the same time and same tether length (as measured by inter-telomere distance) did not (Fig. 7, and Supplemental Video S1). It is unclear which arms are connected by

tethers and which not, though it may be that, as in crane-fly spermatocytes, there are tethers between only one of the two arms of each separating chromosome. Another possibility why some arm

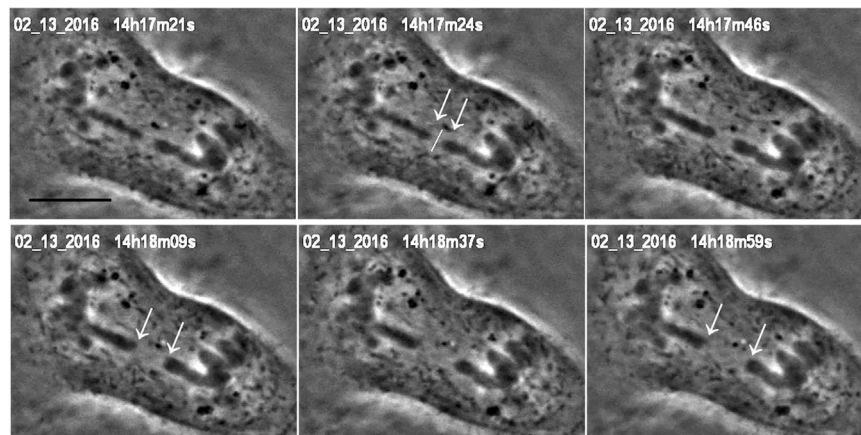


Fig. 4. Arms shrink when their tethers are cut in a PtK2 cell. After their tether was cut (white line in the second image) both chromosome arms shortened. The white arrows indicate the two telomeres. The black line in the first image represents 10 μm .

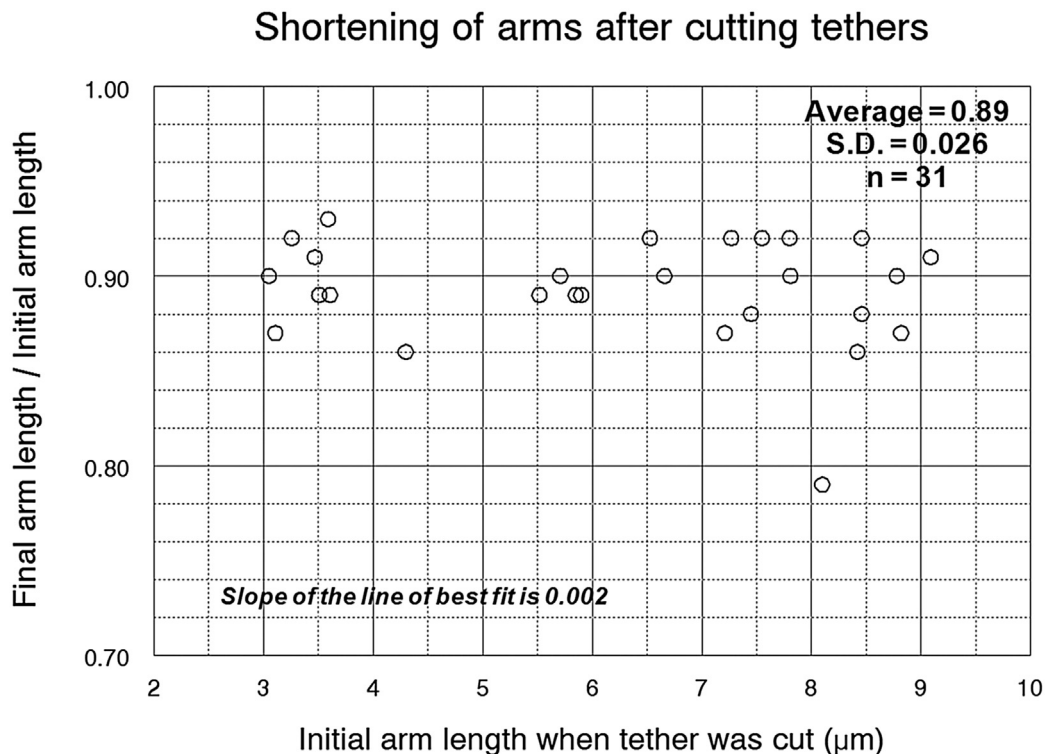


Fig. 5. The percentage shortening of chromosome arms when tethers are cut in PtK2 cells is independent of initial arm length. The ordinate is the final arm length divided by the initial arm length, and the abscissa is the initial arm length when the tether was cut (in μm).

fragments did not move might be that tethers in PtK cells become less elastic as anaphase progresses. To test whether longer tethers are less elastic than shorter ones we compared arm fragment velocities with arm shortening after cutting tethers.

Arm fragment movement varied with tether length. The longer the tethers, (a) the slower were the velocities of arm fragments (Fig. 8), (b) the less likely the arm fragments were to reach the partner telomere or to cross the equator (Fig. 9), and (c) the less likely that arm fragments would move at all (Fig. 10). On the other hand, the *amounts the arms shortened* after cutting tethers did not change with tether length: at all tether lengths the arms shortened on average to 89% of their initial length (Fig. 11). Thus while arm fragment velocities were slower as anaphase progressed, the tension that the arms were under did not change. This suggests

that tethers are present between separating telomeres at all tether lengths, at least to the maximum we studied (9.6 μm), and that their elasticity is reduced as they become longer. As further indication that this is the case, arms shortened after cutting tethers at tether lengths at which fragments did not move: at tether lengths longer than 7 μm , none of 17 arms fragments moved (Fig. 10) yet at those same lengths (>7 μm) arms shortened after 6 out of 8 cuts of tethers (Fig. 12). Thus tethers are present and produce a constant tension on the arms throughout anaphase, but the elasticity of the tethers decreases as the tethers elongate.

We did not detect any change in poleward movement of anaphase chromosomes after their tethers were cut (Fig. 13), so the force from the tethers that stretches chromosome arms is much smaller than the force moving the chromosome to the pole.

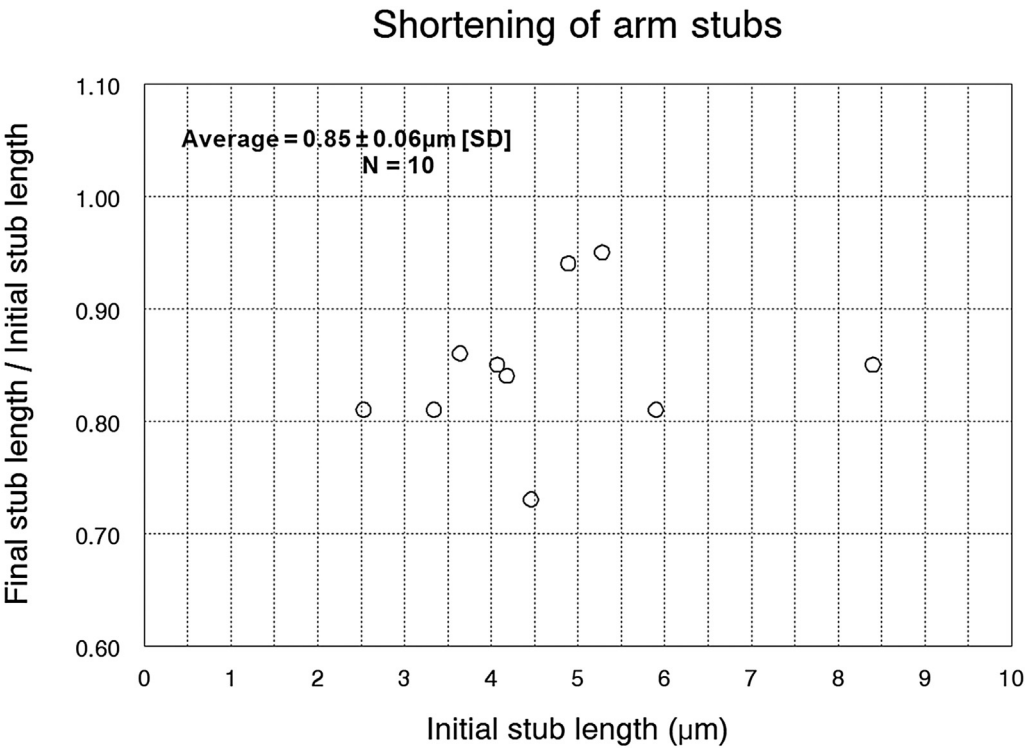


Fig. 6. The percentage shortening of chromosome arm stubs when tethers are cut in PtK2 cells is independent of initial stub length. The ordinate is the final stub length divided by the initial stub length, and the abscissa is the initial stub length (in μm).

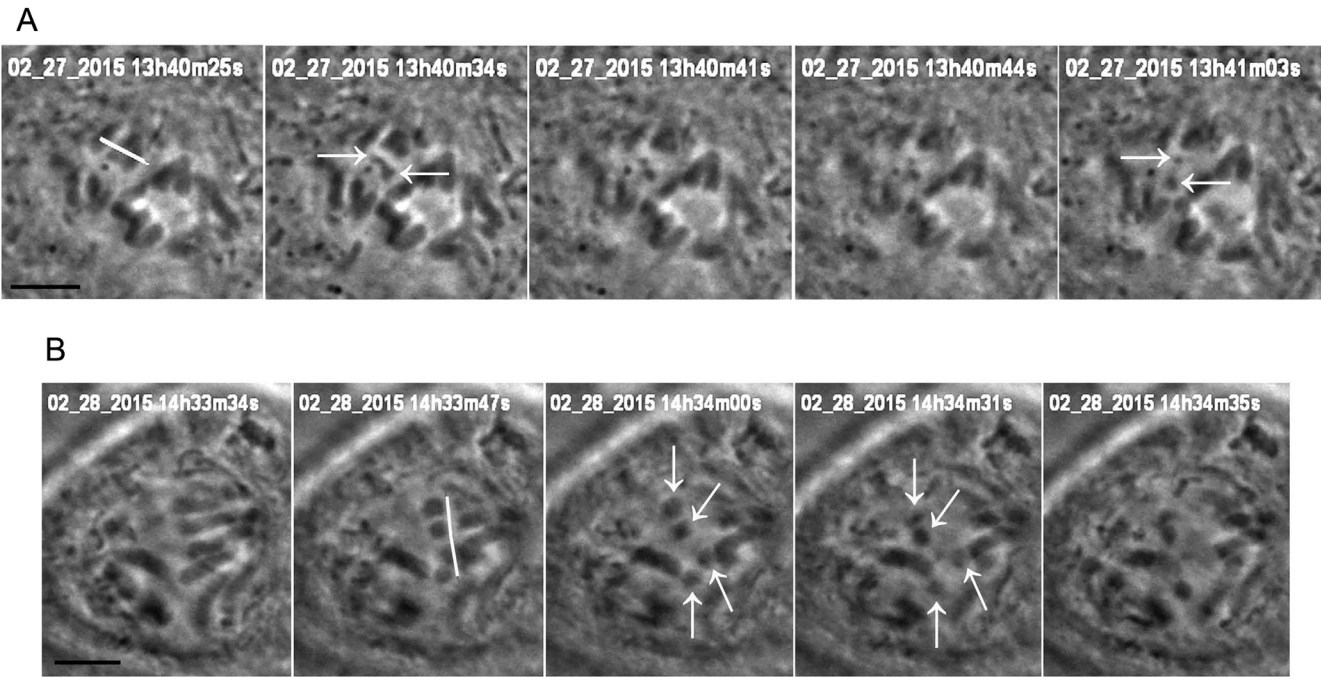


Fig. 7. Not all arm fragments move in PtK2 cells even at the same tether length. A: only one of the two arm fragments (indicated by white arrows) formed when the two arms were cut (white line in the first image) moved toward the opposite telomere. The black line in the first image represents 5 μm. Time-lapsed images of these cuts (and others) in this cell are presented in the Supplemental Video S1. B: one of the four arm fragments (white arrows) formed when the arms were cut (white line in the second image) does not move toward the partner telomere. None of the three that moved reached the other telomeres. The black line in the first image represents 5 μm.

3.2. Tethers in crane-fly spermatocytes

Tether properties described in previous work on crane-fly spermatocytes (LaFountain et al., 2002; Sheykhanian et al., 2017) are the same as those we found in PtK cells. Some issues had not been determined for crane-fly spermatocytes, however, namely, whether the

tethers lose elasticity as they get longer and how much the tethers stretch the chromosome arms to which they are attached. We studied crane-fly spermatocytes to look at these particular issues. In crane-fly spermatocytes, chromosome arms shortened on average to 89% of their initial length, similar to that in PtK cells (Table 2). Arms shortened in late anaphase when their tethers were

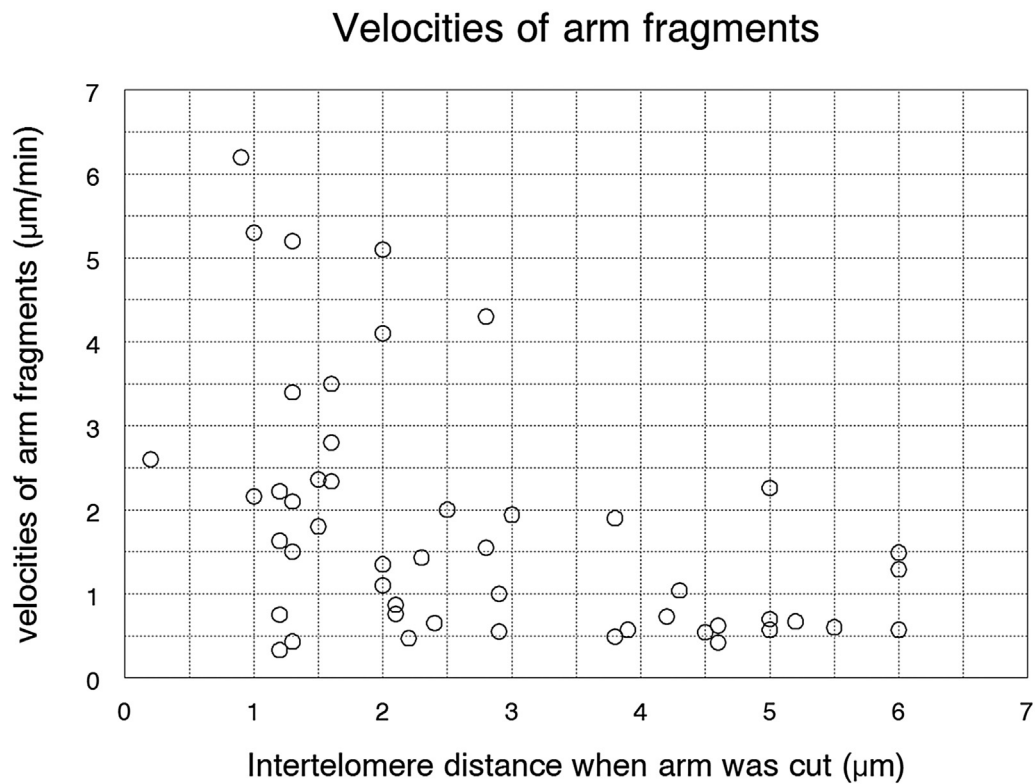


Fig. 8. Arm fragments in PtK2 cells tend to move more slowly when their tethers are longer. The ordinate is the velocities of the arm fragments (in $\mu\text{m}/\text{min}$), and the abscissa is the tether lengths (distance between telomeres) in μm when the arms were cut.

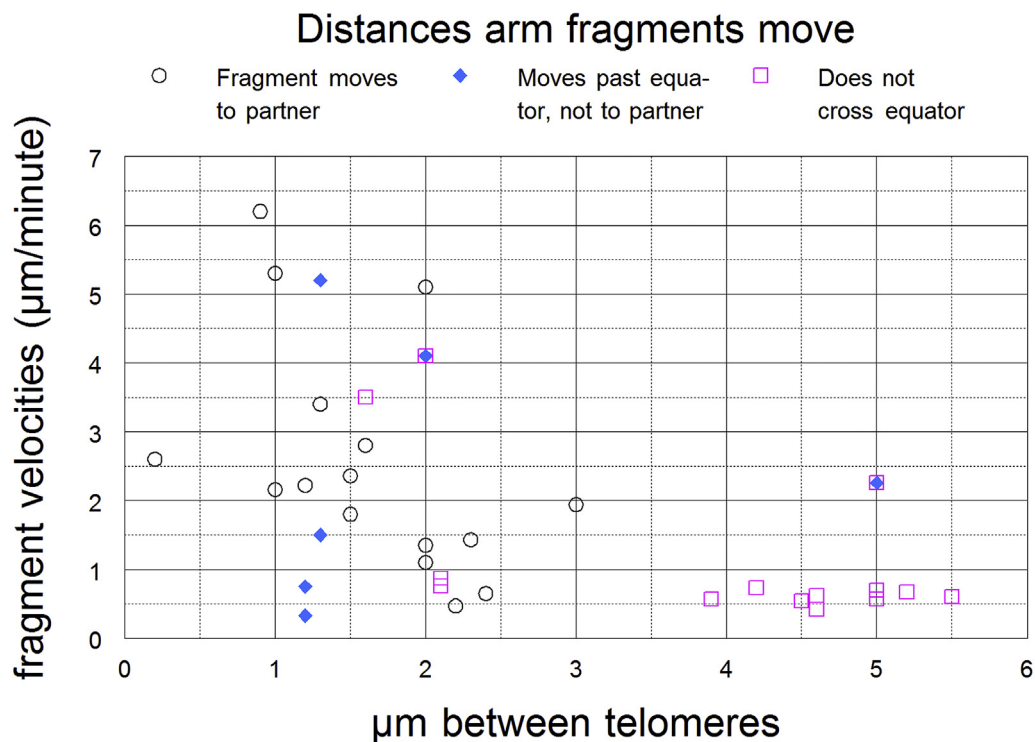


Fig. 9. In PtK2 cells, arm fragments with longer tethers are less likely to move to the opposite telomere, or to move across the equator. The ordinate is the velocities of the arm fragments (in $\mu\text{m}/\text{min}$), the abscissa is the tether lengths (distance between telomeres) in μm when the arms were cut, and the different symbols indicate whether the arm fragment moved to the partner [O], past the equator but not to the partner [◆], or did not cross the equator [□].

cut, up to tether lengths of at least $15 \mu\text{m}$ (the longest we have studied); at these tether lengths arm fragments do not move, or move very slightly (LaFountain et al., 2002). Thus our data sug-

gest that, similar to PtK cells, tethers in crane-fly spermatocytes are present throughout anaphase but lose their elasticity as anaphase progresses.

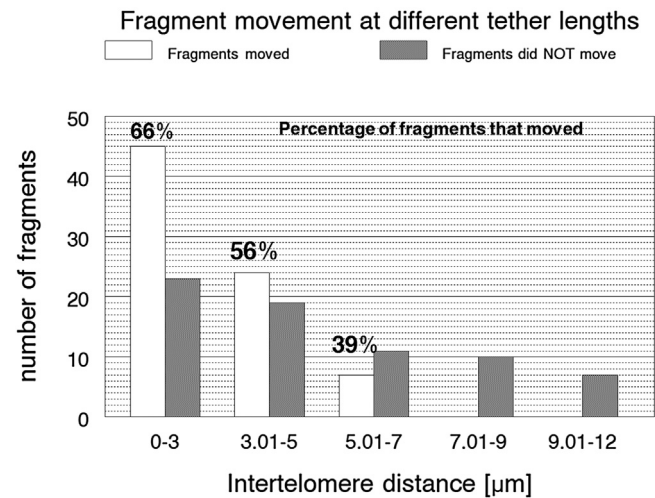


Fig. 10. In PtK2 cells, the longer the tether the less likely that the arm fragment will move across the equator or to the equator. The ordinate is the number of arm fragments and the abscissa is the tether length (inter-telomere distance) in μm. The open bars indicate the arm fragments that moved, the shaded bars the arm fragments that did not move, and the percentages above the open bars indicates the percentages of arm fragments that moved out of the total that were formed in that range of tether lengths.

Table 2
Shortening of chromosome arms after cutting their tethers.

Final length/initial length		
PtK2 cell chromosome arms	0.89 ± 0.025 (SD)	N = 31
PtK2 cell chromosome stubs	0.85 ± 0.06	N = 10
Crane-fly spermatocyte chromosome arms	0.90 ± 0.047	N = 24

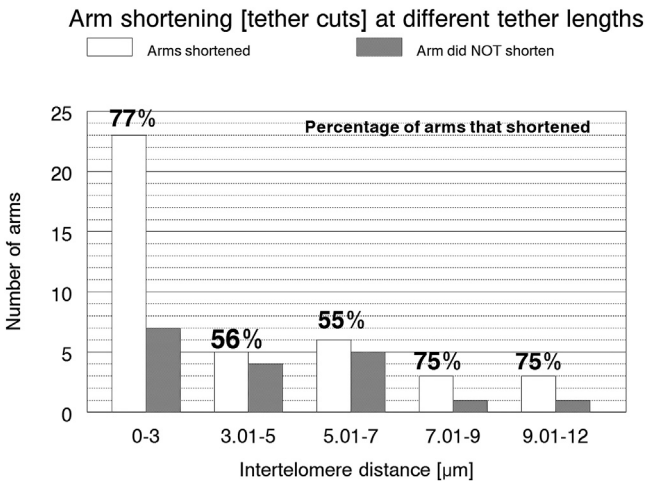


Fig. 12. In PtK2 cells, at all tether lengths chromosome arms shorten when tethers are cut. The ordinate is the number of arms, and the abscissa is the tether length (inter-telomere distance) in μm at the time the tethers were cut. The open bars indicate the arms that shortened, the shaded bars the arms that did not shorten, and the percentages above the open bars indicates the percentages of arms that shortened out of the total that were cut in that range of tether lengths.

3.3. Tethers in other cells

We have identified tethers in other cell types as well, from flatworms to humans. Arm fragments produced in early anaphase moved across the equator to the partner telomeres in spermatocytes from the turbellarian flatworm *Mesostoma*; in spermatocytes from an orthopteran insect, the house cricket *Acheta domesticus*, in both meiosis-I and meiosis-II; in spermatocytes from two arachnids from distinct lineages, cellar spiders (*Pholcus*) and black widow spiders (*Latrodectus*); and in human osteosarcoma cancer cells, U2OS (Fig. 14). We have seen at least 3 examples of rapid arm fragment

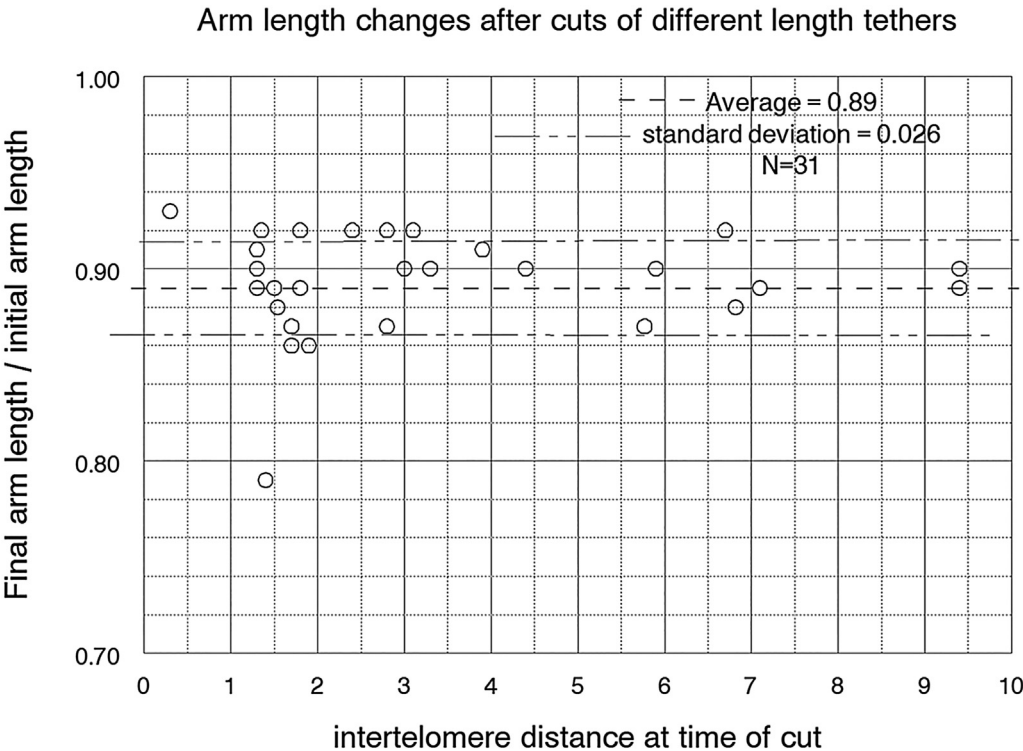


Fig. 11. In PtK2 cells, chromosome arms shorten the by same fractional amount at all tether lengths after their tethers are cut. The ordinate is the final arm length divided by the initial arm length and the abscissa is tether length (inter-telomere distance) in μm at the time the tether was cut.

Table 3
Velocities of arm fragments in different cells.

CELL	Range of velocities	number measured
PtK2 cells	0.5–6.2 $\mu\text{m}/\text{min}$	50
crane-fly (<i>Nephrotoma</i>) primary spermatocytes ^a	1–22 $\mu\text{m}/\text{min}$	37
crane-fly (<i>Nephrotoma</i>) primary spermatocytes ^b	1–18 $\mu\text{m}/\text{min}$	13
Cricket (<i>Acheta</i>) primary spermatocytes	1–11 $\mu\text{m}/\text{min}$	5
Cellar spider (<i>Pholcus</i>) primary spermatocytes	1–7 $\mu\text{m}/\text{min}$	4
Black widow spider (<i>Latrodectus</i>) primary spermatocytes	5–7 $\mu\text{m}/\text{min}$	2
Flatworm (<i>Mesostoma</i>) primary spermatocytes	7–13 $\mu\text{m}/\text{min}$	2
U2OS (human) cells	2–13 $\mu\text{m}/\text{min}$	6

^a Data from LaFountain et al. (2002).

^b Data from Sheykhan et al. (2017).

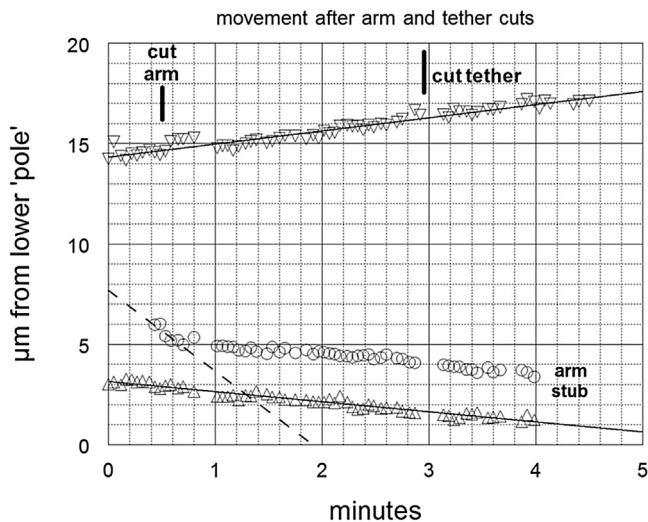


Fig. 13. In PtK2 cells anaphase chromosome movements do not change when arms and/or tethers are cut. The ordinate is the distance (in μm) from a fixed point near the lower 'pole' and the abscissa is time (minutes). The triangles (Δ) and inverted triangles (∇) indicate the positions of the two kinetochores moving to opposite poles. An arm of the chromosome moving to the lower pole was cut at about 0.5 min, and the open circles (O) represent the position of the end of the arm stub; the stub shortens immediately after the cut, and then moves poleward at the same speed as its kinetochore. The tether was cut at about 3 min. Neither kinetochore increased speed when arm or tether were cut.

movements in each of the cells studied. Those whose speeds we have been able to measure are presented in Table 3. In addition, we have verified several other characteristic of tethers in these cells: in particular, in all of the cells not all arm fragments move toward their partner; in flatworm spermatocytes, cricket spermatocytes and human cells tethers stretch arms; and cutting tethers in meiosis-I cricket spermatocytes stops the movements of arm fragments.

4. Discussion

A main conclusion from our experiments is that there are mechanical connections (tethers) between separating anaphase chromosomes in a broad range of cells: flatworm spermatocytes, crane-fly spermatocytes (meiosis-I and -II), cricket spermatocytes (meiosis-I and -II), cellar spider spermatocytes (meiosis-I), black widow spider spermatocytes (meiosis-I), marsupial (PtK2) cells, and human U2OS (osteosarcoma) cells. Detailed characterisation of tethers in PtK2 cells and crane-fly spermatocytes indicates that tethers are elastic, but that they lose elasticity as they become longer: the arm fragments move slower and slower toward the opposite telomere, and eventually do not cross the equator or do not move. Severing tethers releases the connections between separating arms which causes the arms to shorten. Arms shorten at all

tether lengths, whereas arm fragments do not move at longer tether lengths. Thus separating chromosomes are connected by tethers throughout anaphase but the longer the tether the less elastic it is. The force exerted by tethers on separating chromosomes stretches the chromosome arms by around 12% (100/89) regardless of tether length; the stretch seems to be throughout the length of the chromosome arms since the chromosome stubs (that remain after the arm is severed) also contract to the same extent as the unsevered arm, at least in PtK2 cells, in which the arm stubs are long enough to measure length changes. The force exerted by tethers is enough to stretch chromosome arms but is considerably smaller than that exerted on the kinetochore toward the poles since chromosomes do not speed up when tethers are cut (Fig. 13; also LaFountain et al., 2002; Sheykhan et al., 2017).

Because tethers are found in cells from widely separate organisms, from flatworms to humans, it seems likely that they are present in most cell types, and perhaps are even present universally in cells, at least those with localised kinetochores. It seems likely that tethers between partner chromosomes are responsible for the backwards movements observed after cutting individual anaphase spindle fibres in PtK2 cells (Fig. 1C of Elting et al., 2014), which have tethers (this paper), and, by extension, in grasshopper spermatocytes (Chen and Zhang, 2004) and newt fibroblasts (Spurck et al., 1997), in neither of which have tethers been demonstrated. We might also speculate that backwards movements seen after cutting across entire half-spindles in anaphase *Haemaphysalis* cells (Bajer, 1972) are due to tethers, and backwards movements after spindle poles are irradiated in silkworm meiosis-I (Nakanishi and Kato, 1965) are due to tethers.

Our purpose in the experiments presented herein was to test whether tethers were present in a variety of cells, to generalise their presence. We envision that subsequent experiments will deal with the question, what are tethers composed of? We did not pursue this question, and we have no data regarding their composition. But perhaps brief discussion is worthwhile.

It is difficult to get a handle on tethers if one cannot identify them so we would like to be able to see them in living cells, or at least in stained cells. Since cutting between partner telomeres in PtK2 cells caused chromosome arms to shorten 75% of the time (Fig. 12), we expect tethers to extend between separating telomeres in at least 75% of the separating chromosomes, and most likely 100%. In crane-fly spermatocytes tethers were identified operationally for each pair of separating chromosomes, but only for half the arms (La Fountain, 2002b). In trying to identify tethers morphologically we therefore expect to see them between all separating chromosomes, though not necessarily between all separating arms. Connections between separating telomeres in anaphase are not visualised by several usual staining methods (discussed in Sheykhan et al., 2017) and no connections between telomeres were seen in PtK2 cells using standard electron microscopy sectioning methods (Roos, 1973). However, connections between separating telomeres were seen electron microscopically in crane-fly spermatocytes by Fuge

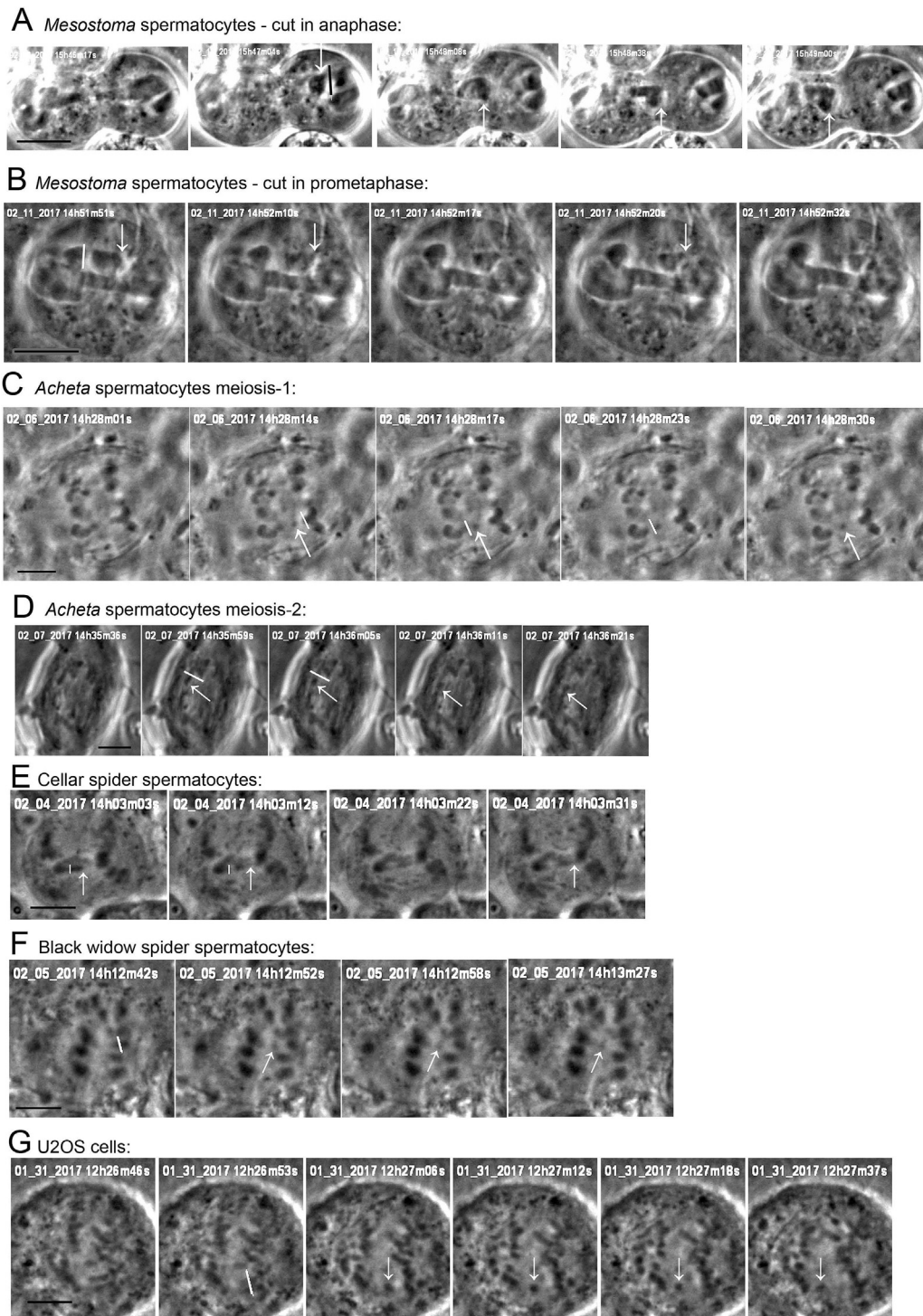


Fig. 14. Arm fragments in different cell types move toward partner telomeres. A: After arms in an anaphase flatworm (*Mesostoma*) spermatocyte were cut (black line in the second image) the arm fragment (indicated by a white arrow) moved across the equator to its partner telomere. B: after an arm was cut in a prometaphase *Mesostoma* spermatocyte, indicated by the white line in the first image, the arm fragment (indicated by a white arrow) moved across the equator to the partner telomere. (Ferraro-Gideon et al., 2014, describe prometaphase in this unusual cell division.) C: after an arm in an anaphase cricket meiosis-I spermatocyte was cut (white line, second image) the resultant arm fragment (indicated by a white arrow) moved toward its partner telomere. When its tether was cut (white line in the third and fourth images) the arm fragment stopped moving and the arm of the partner shortened in length. D: an arm fragment was formed after an arm in an anaphase cricket meiosis-II spermatocyte was cut (white line, second and third images, the second image before the cut and the third after). The arm fragment (indicated by a white arrow) moved backward to the partner telomere. E: after an arm in an anaphase cellular spider spermatocyte was cut (white line, first and second images) the arm fragment (indicated by a white arrow) moved to the partner telomere. F: after an arm in an anaphase black widow spider spermatocyte was cut (white line, first image), the arm fragment (white arrow) moved backwards toward the partner telomere. G: after an arm in an anaphase U2OS cell was cut (white line, second image), the resultant arm fragment (indicated by a white arrow) moved backwards toward its partner telomere. The black lines in the first images in A and B represent 10 μm ; those in C, D, E, F and G represent 5 μm .

(1978), who reported that in fortuitous sections of two crane-fly spermatocytes he identified connections between telomeres in three of the 6 bivalent pairs; in one of the pairs there were connections between two of the 4 pairs of arms, similar to the results from cutting arms in living cells (LaFountain et al., 2002; Sheykhan et al., 2017). Connections between separating chromosomes also were seen electron microscopically in meiosis-I of cockroaches (Krishan and Buck, 1965). Given the difficulty of spotting such elements using the electron microscope (Fuge, 1978), it is tempting to think that these components correspond to tethers, but one does not know how regularly this occurs – i.e., whether all chromosomes are connected this way. Without that correlation one does not know, without further experimentation, whether these images are of tethers. Some bridges also have been seen using light microscopy: Feulgen-positive chromatin bridges that are composed of chromatin and are visible using standard light microscopy after staining for DNA, and ultra-fine DNA bridges that do not stain for DNA but are identified by staining for associated proteins BLM, topoisomerase-III α , or PICH (Chan et al., 2007). Neither of these DNA bridges seem to fit the criteria for tethers, however. Chromatin bridges (sensu Schrader, 1953, pp 45–46) are present only rarely in healthy normal cells, and when present generally retard or stop movements. Ultra-fine (DNA) bridges, studied primarily in human tissue culture cells, also do not fit the criteria if indeed all the bridges are identified by the present staining procedures: in different reports only 20–80% of normal anaphases have 1 or more ultra-fine bridges (Chan et al., 2007; Barefield and Karlseder, 2012; Gemble et al., 2015), yet tethers are present in all cells. Of those anaphase cells that do have ultra-fine bridges, in different reports there are less than 8 bridges per cell (Chan et al., 2007), or 2 or more bridges in only 50% of the cells (Barefield and Karlseder, 2012), or more than 3 bridges per cell in only 10–15% of the cells (Gemble et al., 2015), considerably fewer than the 46 pairs of chromosomes, yet tethers are associated with at least 75% of the chromosomes, if not all. Further, of the bridges studied, most terminate in centromeric DNA or ‘fragile sites’, not telomeres (Chan et al., 2007, 2009; Barefield and Karlseder, 2012; Nielsen et al., 2015). Those extending from telomeres at one or both ends of the bridge were only 15% of the identified ultra-fine bridges (Barefield et al., 2012). Thus, if all the ultrafine DNA bridges are identified by these staining methods, they cannot be components of tethers since not all cells have them and there are far too few per cell that extend between telomeres.

We are aware of only one other description of components extending between separating anaphase chromosomes, those containing the protein titin, described as extending between separating anaphase telomeres in crane-fly spermatocytes (Fabian et al., 2007). Though titin has elastic properties consistent with it being a tether component, we do not know from the published report whether all chromosomes have them, or how often they are seen, so without further description and experimentation one cannot be certain whether they might be components of tethers.

Why do tethers extend between separating anaphase chromosomes? What is their function? One can only speculate at this time, but experiments on crane-fly spermatocytes (Sheykhan et al., 2017) indicate that tethers in crane-fly spermatocytes function to coordinate movements of separating chromosomes; this conclusion derived from studying movements that were linked when tethers were present but that became unlinked when tethers were severed. Thus one function in crane-fly spermatocytes seems to be to modulate movements between partner chromosomes, possibly through the tension that they generate on chromosome arms. Perhaps their evolutionary function is to coordinate chromosome movements in general, to help ensure that chromosomes are directed to their proper poles.

How much force does a tether exert? The force is enough to stretch chromosomes by 12%, but is smaller than the poleward force that acts on the kinetochore. Nonetheless, small as it may be, the forces from tethers oppose the poleward forces at kinetochores, and should be taken into account when considering the balance of forces acting on chromosomes during anaphase. We plan to measure the force that drives arm fragment motion by using an optical trap to stop the movement of arm fragments shortly after they are formed.

In summary, we have shown that tethers extend between the telomeres of separating chromosomes in a broad range of anaphase cells, both meiotic and mitotic. They exert a force that opposes the forces propelling the chromosome to the pole, enough to stretch the chromosome arm by 12%. Tethers are elastic at the start of anaphase, but they lose elasticity as they elongate. Tethers have been found in the several cell types we have studied, the only ones where they have been looked for, as far as we know. From the range of species in which tethers are present, tethers probably are present in most cells, if not universally present during cell division.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejcb.2017.07.001>.

References

- Ault, J.G., Felt, K.D., Nedo, A.O., Doan, R.N., Andreychik, C.M., Paliulis, L.V., 2017. Co-segregation of sex chromosomes in the male black widow spider *Latrodectus mactans* (Araneae, Theridiidae). *Chromosoma*, <http://dx.doi.org/10.1007/s00412-017-0628-7>.
- Bajer, A., 1972. Influence of UV microbeam on spindle fine structure and anaphase chromosome movements. *Chromosomes Today* 3, 63–69.
- Barefield, C., Karlseder, J., 2012. The BLM helicase contributes to telomere maintenance through processing of late-replicating intermediate structures. *Nucleic Acid Res.* 40, 7358–7367.
- Chan, K.-L., North, P.S., Hickson, I.D., 2007. BLM is required for faithful chromosome segregation and its localization defines a class of ultrafine anaphase bridges. *EMBO J.* 26, 3397–3409.
- Chan, K.L., Palmai-Pallag, T., Ying, S., Hickson, I.D., 2009. Replication stress induces sister-chromatid bridging at fragile site loci in mitosis. *Nat. Cell Biol.* 11, 753–761.
- Chen, W., Zhang, D., 2004. Kinetochore fibre dynamics outside the context of the spindle during anaphase. *Nat. Cell Biol.* 6, 227–231.
- Elting, M.W., Hueschen, C.L., Udy, D.B., Dumont, S., 2014. Force on spindle microtubule minus ends moves chromosomes. *J. Cell Biol.* 206, 245–256.
- Fabian, L., Xia, X., Venkitaramani, D.V., Johansen, K.M., Johansen, J., Andrew, D.J., Forer, A., 2007. Titin in insect spermatocyte spindle fibers associates with microtubules, actin, myosin and the matrix proteins skeletor, megator and chromator. *J. Cell Sci.* 120, 2190–2204.
- Ferraro-Gideon, J., Hoang, C., Forer, A., 2014. Meiosis-I in *Mesostoma ehrenbergii* spermatocytes includes distance segregation and inter-polar movements of univalents, and vigorous oscillations of bivalents. *Protoplasma* 251, 127–143.
- Forer, A., Pickett-Heaps, J., 2005. Fibrin clots keep non-adhering living cells in place on glass for perfusion or fixation. *Cell Biol. Int.* 29, 721–730.
- Forer, A., Ferraro-Gideon, J., Berns, M.W., 2013. Distance segregation of sex chromosomes in crane-fly spermatocytes studied using laser microbeam irradiations. *Protoplasma* 250, 1045–1055.
- Forer, A., 1982. Crane fly spermatocytes and spermatids: a system for studying cytoskeletal components. In: Wilson, L. (Ed.), *Methods in Cell Biol.*, vol. 25. Academic Press, New York, pp. 227–252.
- Fuge, H., 1978. Fine structure of anaphase bridges in meiotic chromosomes of the crane fly *Pales*. *Chromosoma* 65, 241–246.
- Gemble, S., Ahuja, A., Buhagiar-Labarchède, G., Onclercq-Delic, R., Dairou, J., Biard, D.S., Lambert, S., Lopes, M., Amor-Guérét, M., 2015. PLoS Genet., <http://dx.doi.org/10.1371/journal.pgen.1005384>.

- Harsono, M.S., Zhu, Q., Shi, L.Z., Duquette, M., Berns, M.W., 2012. Development of a dual joystick-controlled laser trapping and cutting system for optical micromanipulation of chromosomes inside living cells. *J. Biophotonics*, <http://dx.doi.org/10.1002/jbio.201200019>.
- Hoang, C., Ferraro-Gideon, J., Gauthier, K., Forer, A., 2013. Methods for rearing *Mesostoma ehrenbergii* in the laboratory for cell biology experiments, including identification of factors that influence production of different egg types. *Cell Biol. Int.* 37, 1089–1105.
- Krishan, A., Buck, R.C., 1965. Ultrastructure of cell division in insect spermatogenesis. *J. Ultrastruct. Res.* 13, 444–458.
- LaFountain, J.R., Cole, R.W., Reider, C.L., 2002. Partner telomeres during anaphase in crane fly spermatocytes are connected by an elastic tether that exerts a backward force and resists poleward movement. *J. Cell Sci.* 115, 1541–1549.
- Nakanishi, Y.H., Kato, H., 1965. Unusual movement of the daughter chromosome group in telophase cells following the exposure to ultraviolet microbeam irradiation. *Cytologia* 30, 213–221.
- Nielsen, C.F., Huttner, D., Bizard, A.H., Hirano, S., Li, T.-N., Palmai-Pallag, T., Bjerregaard, V.A., Liu, Y., Nigg, E.A., Wang, L.H.-C., Hickson, I.D., 2015. PICH promotes sister chromatid disjunction and co-operates with topoisomerase II in mitosis. *Nat. Commun.* 6, 8962, <http://dx.doi.org/10.1038/ncomms9962>.
- Roos, P., 1973. Light and electron microscopy of rat kangaroo cells in mitosis. I. Formation and breakdown of the mitotic apparatus. *Chromosoma* 40, 43–82.
- Schrader, F., 1953. *Mitosis. The Movements of Chromosomes in Cell Division*, 2nd ed. Columbia University Press, New York.
- Sheykhan, R., Berns, M., Forer, A., 2017. Elastic tethers between separating anaphase chromosomes in crane-fly spermatocytes coordinate chromosome movements to the two poles. *Cytoskeleton* 74, 91–103.
- Shi, L.Z., Zhu, Q., Wu, T., Duquette, M., Gomez, V., Chandsawangbhuwana, C., Harsono, M.S., Hyun, N., Baker, N., Nascimento, J., You, Z., Botvinick, E.B., Berns, M.W., 2012. Integrated optical systems for laser nanosurgery and optical trapping to study cell structure and function. In: Mendez-Vilas, A. (Ed.), *Current Microscopy Contributions to Advances in Science and Technology*, Formatex, Badajoz, Spain. Microscopy Book Series Number 5, pp. 685–695.
- Spurck, T., Forer, A., Pickett-Heaps, J., 1997. Ultraviolet microbeam irradiations of epithelial and spermatocyte spindles suggest that forces act on the kinetochore fibre and are not generated by its disassembly. *Cell Motil. Cytoskel.* 36, 136–148.
- Wong, R., Forer, A., 2003. 'Signalling' between chromosomes in crane-fly spermatocytes studied using ultraviolet microbeam irradiation. *Chromosome Res.* 11, 771–786.

CHAPTER 4

EVIDENCE OF NON-MICROTUBULE SPINDLE FORCES IN *Mesostoma ehrenbergii* SPERMATOCYTES

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Evidence of Non-microtubule Spindle Forces in *Mesostoma ehrenbergii* Spermatocytes

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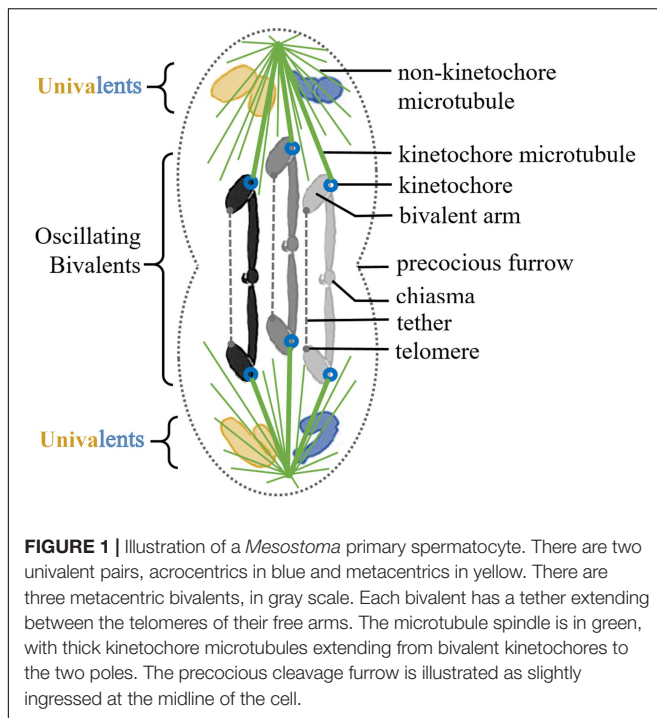
We tested conclusions reached in previous experiments in which *Mesostoma* spermatocyte chromosomes moved rapidly to a pole in the absence of microtubules: after 10 μ M nocodazole (NOC) depolymerized metaphase spindle microtubules, kinetochores from each of the 3 bivalents detached from the same pole and rapidly moved to the other pole, at speeds averaging 37.7 μ m/min. with some as high as 100 μ m/min. We concluded that these very fast movements were due to non-microtubule forces arising from a spindle matrix. However, since the chromosomes stretch out before detaching, there is tension in the chromosomes from the stretch. Thus the movements of detached kinetochores conceivably might be due to recoil from the tension, though we argued against this possibility (Fegaras and Forer, 2018a). In this article we test whether recoil causes the movements. We cut bivalents into 2 pieces, using a femtosecond laser, before addition of NOC. When 1 bivalent was severed, all kinetochores moved to one pole in 12/15 cells; when 2 bivalents were severed, all kinetochores moved to one pole in 4/6 cells; and when all 3 bivalents were severed all kinetochores moved to one pole in 3/9 cells. The bivalent “halves” moved rapidly, with average speeds of 47 μ m/min, velocities that are not significantly different from those in cells without any laser-cut bivalents ($p > 0.05$). Since kinetochores move at the same speeds whether they are part of bivalents or not, NOC-induced chromosome movements are not due to recoil from tension along the full-length bivalent, strongly supporting the idea that non-microtubule forces move chromosomes in *Mesostoma* spermatocytes.

Keywords: meiosis, *Mesostoma*, laser irradiation, microtubules, spindle matrix

INTRODUCTION

The present work studies the mechanism by which chromosomes move rapidly in the absence of microtubules in *Mesostoma* spermatocytes. Before we describe our present experiments we summarize some important features of meiosis-I in *Mesostoma* spermatocytes.

Mesostoma spermatocytes have three bivalent chromosomes and two sets of univalent chromosomes positioned at either pole, as first described in Husted and Ruebush, 1940 (Figure 1). A precocious pre-anaphase cleavage furrow develops and ingresses slightly during early



prometaphase giving the cell its characteristic dumbbell-like shape, then recommences ingression during anaphase at which time it cleaves the cell in the usual manner (Forer and Pickett-Heaps, 2010; Fegaras and Forer, 2018b). The univalent chromosomes are never paired: there are only 3 synaptonemal complexes in pre-division nuclei, corresponding to each of the 3 bivalents (Oakley and Jones, 1982). Throughout prometaphase the three bivalent chromosomes oscillate toward and away from the two poles with excursion distances averaging 4 μm and at speeds averaging 5–6 $\mu\text{m}/\text{min}$ (e.g., Ferraro-Gideon et al., 2014). The chromosomes never form a metaphase plate: oscillations continue until anaphase. At anaphase the bivalent oscillations end abruptly and the segregating chromosomes move toward the two poles at speeds of approximately 1 $\mu\text{m}/\text{min}$ (Fuge, 1987, 1989; Ferraro-Gideon et al., 2013, 2014). For clarity, we must describe several other unusual behaviors in these cells.

Elastic tethers that extend between the separating arms of all anaphase chromosomes in a variety of cells also extend between the arms of separating *Mesostoma* spermatocyte anaphase chromosomes (Forer et al., 2017). Tether elasticity is shown when one cuts an arm: the arm fragment that is produced moves rapidly, telomere toward telomere, to the partner chromosome moving to the other pole. Tethers are elastic early in anaphase, but become inelastic later in anaphase as they elongate (e.g., LaFountain et al., 2002; Forer et al., 2017; Kite and Forer, 2020). In *Mesostoma* spermatocytes each bivalent has one chiasma and 2 free arms that are associated with each kinetochore, and similar elastic tethers extend between the free arms of the bivalents in prometaphase: when one cuts an arm, the arm fragment that is formed moves rapidly, telomere to telomere, to the free arm of the partner half-bivalent (Forer et al., 2017). This does not happen

all the time in *Mesostoma*, but only about half the time. Absence of arm fragment movement indicates that either the tethers are inelastic at that time, or not present.

The anaphase chromosomes in *Mesostoma* spermatocytes may be distributed non-randomly to the daughter cells, i.e., male-derived chromosomes to one pole and female-derived chromosomes to the other. The first line of evidence suggesting this derives from detailed study of univalent chromosome movements. In early prometaphase there often are “faulty” distributions of univalents at the two poles but by anaphase there is one of each kind at each pole (Oakley, 1983, 1985). Univalents move between poles in living cells (Oakley, 1985; Forer and Pickett-Heaps, 2010; Ferraro-Gideon et al., 2014). Proper distribution of univalents seems to arise from their movements between the poles because early in prometaphase the univalents often are distributed wrongly (Oakley, 1983, 1985), such as 3 or 4 at one pole and one or none at the other, or two of one kind at one pole and two of the other kind at the other pole. Since the univalents often change poles more times than necessary to achieve the goal of one of each kind at each pole, in particular, “partner” univalents often switch poles after proper distribution has been achieved (Oakley, 1985), Oakley suggested that there is non-random segregation in these cells in that male-derived chromosomes go to one pole and female-derived chromosomes go to the other (Oakley, 1985): the “gratuitous” univalent excursions are due to sorting out male-derived from female-derived univalents. A second line of evidence that segregation may be non-random is that bipolar oriented bivalents frequently reorient (Ferraro-Gideon et al., 2014; Brady and Paliulis, 2015). One kinetochore releases from one pole, moves to the other pole, and then the other kinetochore moves to the vacated pole (Ferraro-Gideon et al., 2014). That is, the reorientations give rise to segregation to poles different from the original orientation. This may be because the bipolar orientation must segregate male-derived from female-derived chromosomes. A third line of evidence, that we now describe, is from experiments that we follow up on in this article, in which all chromosomes consistently move to one pole after addition of nocodazole (NOC) to depolymerize microtubules.

When microtubules are depolymerized in *Mesostoma* spermatocytes, the bivalents stop mid-oscillation and the sister kinetochores of each half-bivalent move toward their respective poles (Fegaras and Forer, 2018a). This causes the bivalents to stretch, possibly due to non-microtubules associated force production via the spindle matrix and/or actin-myosin. After a few minutes being stretched, all the kinetochores oriented to one pole detach and move rapidly toward the opposite pole, at speeds averaging 38 $\mu\text{m}/\text{min}$ and with instantaneous speeds up to 100 $\mu\text{m}/\text{min}$ (Fegaras and Forer, 2018a,b). One reason that all kinetochores move to the same pole might be because this is related to non-random segregation, because all male partners segregate to one pole and all females to the other.

The movements to the one pole after NOC treatment occurred in the absence of microtubules; Fegaras and Forer (2018a) argued that movements in the absence of microtubules were due to forces arising from the spindle matrix. But because the chromosomes stretch out and elongate by 25–30% of their original length before

detaching, the rapid chromosome movement conceivably may be due to a release in tension along the length of the bivalent. Fegaras and Forer (2018a) argued against this interpretation for various reasons, including that the movements are not linear. We tested this point directly in experiments reported herein. We used a femtosecond laser to cut 1, 2, or 3 bivalents (per cell) into two pieces, two “halves.” After treatment with NOC, “halved” bivalents at one pole moved toward the other pole at the same rapid speeds that non-severed chromosomes move at. Since the movements occur in the absence of microtubules and in the absence of tension along the length of the chromosomes, we suggest that the chromosomes are moved by spindle matrix components such as actin and myosin.

While the focus of our experiments is on the question of what produces force for movement in the absence of microtubules, some of the results extend to the issue of why all kinetochores detach from one pole and move to the other, instead of detaching from poles at random, as they do in other cells (Pickett-Heaps and Spurck, 1982; discussed in Fegaras and Forer, 2018a), and to the issue of how the movements of all the kinetochores are coordinated. Some of our experimental manipulations altered the coordinated movements to one pole and implicated the tethers as being important in that coordination, thereby providing some insight into how the coordinations might occur.

MATERIALS AND METHODS

Living Cell Preparations and Drug Addition

The procedures have been described in detail elsewhere (Fegaras and Forer, 2018a,b). In brief, *Mesostoma ehrenbergii* were reared in lab (Hoang et al., 2013). Testes were extracted from individual animals using pulled 5, 10, or 15 μ L micropipettes (Fisher), and then expelled onto a coverslip into *Mesostoma* Ringers solution (61 mM NaCl, 2.3 mM KCl, 0.5 mM CaCl₂, and 2.3 mM phosphate buffer) that contained 0.2 mg/mL fibrinogen (Calbiochem). Thrombin was added to the fibrinogen to hold the cells in a fibrin clot and the cells in the clot were then immersed in *Mesostoma* Ringers solution in a perfusion chamber (Forer and Pickett-Heaps, 2005). For drug treatment, cells were perfused with 10 μ M nocodazole (NOC, from Sigma) a microtubule depolymerizing agent (Vasquez et al., 1997; Jordan and Wilson, 2004; Poxleitner et al., 2008), in *Mesostoma* Ringers: NOC from a 1000x concentrated stock in dimethyl sulfoxide (DMSO) was diluted with *Mesostoma* Ringer's solution to reach the desired concentration. Cells were viewed with phase-contrast microscopy using a 63x, NA 1.4, Zeiss Plan Apochromatic Lens. We recorded live cells, captured digital images every 2–3 s, and cropped the images and time-stamped them (with data from the recorded file images) using the freeware program IrfanView. Time-lapsed sequences and avi files were made using freeware Virtualdub2, and movement graphs were obtained using our in-house program WinImage (Wong and Forer, 2003), in which the position of each kinetochore was plotted relative to a fixed point at the cell edge. Chromosome movement graphs were generated using the program SlideWrite 7.0. We determined

chromosome speeds from computer generated lines-of-best fit to the points on the movement graphs. Movement graphs of kinetochore positions vs time appeared as sawtooth waves. We considered one complete bivalent oscillation as the distance from peak-to-peak or trough-to-trough of the sawtooth waves. We considered the distance along the *y*-axis between successive trough and peak as the amplitude, representing the distance a kinetochore travels during an oscillation. And we considered the distance along the *x*-axis between successive troughs or peaks as the period, representing the time a bivalent takes to complete an oscillation. Student's *t*-tests were performed when comparing chromosome speeds.

Laser Irradiations

Spermatocytes were observed using phase-contrast microscopy. User-defined positions were irradiated using a 200 fs-pulsed laser (Mai Tai, Newport Co., Irvine, CA, United States) that emitted 740 nm wavelength light. System details can be found in Berns and Greulich, 2007; Harsono et al., 2012; Forer et al., 2017. We recorded digital images every 2 s and performed cuts in three different planes of focus along the *Z*-axis. Bivalents were cut in two different regions: near the chiasma of the bivalent to create bivalent “halves” or near the telomere of a bivalent arm to disable tethers (Figure 2). Laser irradiations of bivalent chromosomes were performed near the middle of the chromosome, near the region of the chiasma; we refer to the resultant pieces as “halves,” or “halved bivalents” because bivalents are not necessarily cut precisely in half. Sometimes cutting a bivalent required more than one series of cuts. During prometaphase oscillations the two kinetochores on one normal bivalent sometimes move in the same direction (i.e., one moves toward its pole and the other moves away from its pole). Sometimes the two kinetochores move in opposite directions (each toward its own pole). We cut bivalents most often when the bivalent kinetochores moved toward opposite poles because chromosomes are more stretched out then and because kinetochore movements tend to briefly pause for a few seconds once they reach a pole, making the “moving target” more stationary (Ferraro-Gideon et al., 2014). We compared oscillations of “halved” bivalents with those of non-cut bivalents by comparing the saw-tooth waves of movement. We considered “half” bivalent oscillations as normal when they have the same oscillation period and velocities as before laser treatment. We considered “half” bivalent oscillations as irregular when they changed 50% or more in period and/or velocity. In cells treated with both NOC and laser cuts, bivalents were cut up to 15 min before NOC was added.

RESULTS

Control Cells and NOC Treated Cells

During prometaphase-I in *Mesostoma* spermatocytes, the three bivalents continuously oscillate toward and away from either pole. When cells are treated with 10 μ M NOC the bivalent chromosomes immediately stop oscillating and each kinetochore moves toward its respective pole as the bivalent stretches out. The chromosomes pause (i.e., remain stretched out) and after a

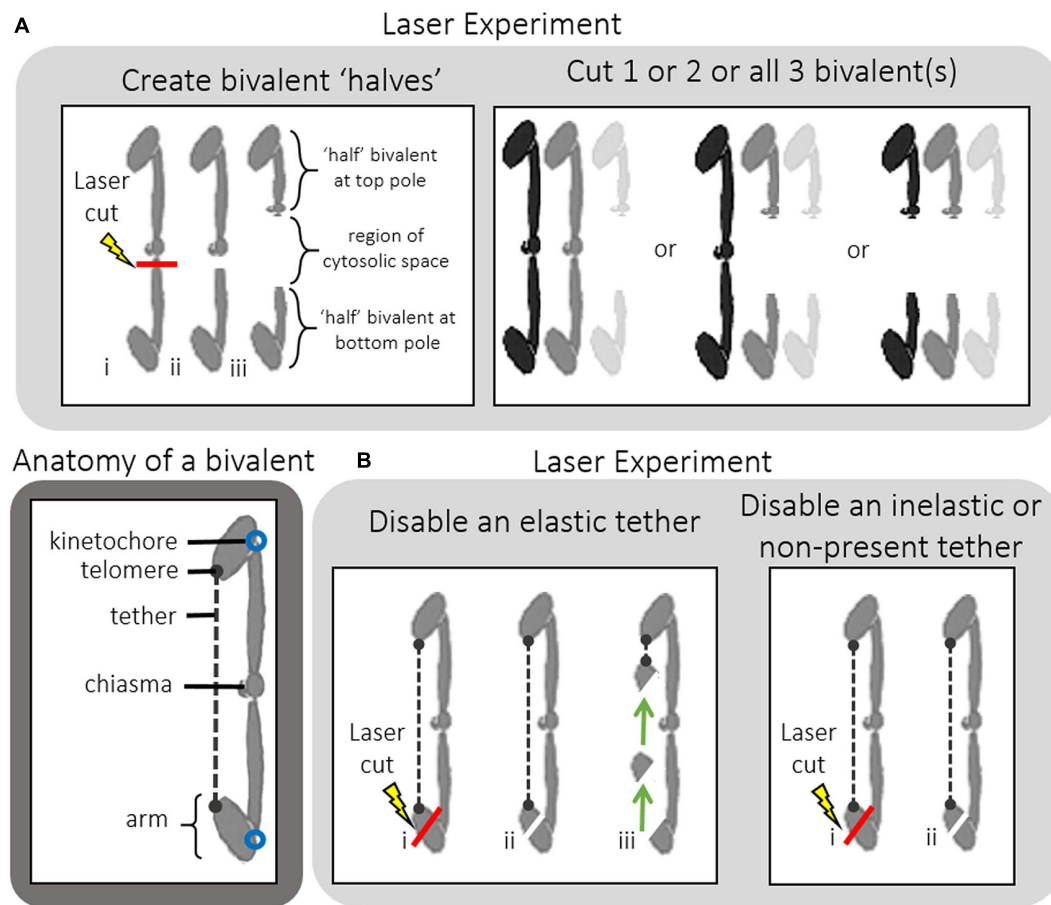


FIGURE 2 | (A, B) Anatomy of a bivalent and a guide to the two types of laser cuts performed on bivalents. **(A)** *Creating bivalent "halves."* Laser irradiations of bivalent chromosomes were performed near the middle of the chromosome, near the chiasma; we refer to the resultant pieces as "halves," or "halved bivalents" because bivalents are not necessarily cut precisely in half. Successful severing of the bivalents was determined by observing a region of cytosolic space between the two bivalent "halves." Either 1, 2, or all 3 bivalents were cut in half within any one given cell, after which NOC was added. **(B)** *Disabling tethers.* Tethers extend between the telomeres on the free arms of each bivalent. In order to functionally disable them a portion of the arm near the tip of one bivalent arm was cut off. If the arm fragment moved toward the opposite arm (as shown by the arrows) this indicated the tether was elastic. If the arm fragment did not move this indicated the tether was inelastic. NOC was then added to these cells.

few minutes all three kinetochores at one pole detach and move toward the opposite pole, after which the cleavage furrow moves toward the vacated half spindle (Fegaras and Forer, 2018a,b).

Bivalent Chromosomes "Halved" With a Laser

Effect of Severing Bivalent(s) Prior to Treatment With NOC

We first describe the behavior of the "halved" bivalents, both as aid to explaining the effects after NOC treatment and because of the relevance of their behavior to the possible non-random segregation in these cells.

We successfully cut bivalents in "half" in 30 cells. The resultant "halved" bivalents either moved to the poles and remained stationary there, or oscillated; the kinetochores of the oscillating halved bivalents moved toward and away from the pole they were associated with when they were part of a bivalent. The uncut

bivalents continued their usual oscillation patterns after bivalents in the same cells were cut. The relative frequency of moving to the pole or continuing oscillating depended on the numbers of bivalents that were cut; the fewer bivalents that were cut per cell the more likely the "halved" bivalents would continue to oscillate (Table 1). In the cells in which more than one bivalent was cut, all "halved" bivalents acted the same: they either all oscillated or all moved directly to the pole. For all oscillating "half" bivalents, the initial oscillations were normal: they had the same velocities and periods as before being cut and the same as the continuing oscillations in not-cut bivalents. After varying numbers of normal oscillations, the oscillations first became irregular, with altered velocities and or periods, and then they ceased, as the "halved" bivalents became stationary at their poles. One such sequence is illustrated in Figure 3: as seen in the graph (Figure 3B, blue circles), the bottom "half" bivalent oscillated normally between 5 and 7 min, irregularly between 7–12 min, and then resided at the pole until NOC was added. The partner "half," on the other

TABLE 1 | Numbers of cells with bivalent “halves” that continue to oscillate or become stationary at their respective poles.

Behavior of bivalent “halves”	Number of bivalents cut			Totals
	1 bivalent	2 bivalents	3 bivalents	
“halves” oscillate	12	4	3	19
“halves” stationary at the poles	3	2	6	11

hand (upper blue circles), oscillated normally from 5 to 14 min, then irregularly from 14 to over 15 min, and then was at the pole until NOC was added (at ~17 min). It is typical that the partner “halves” do not necessarily follow the same pattern: sister kinetochores of the same bivalent (the two “halves”) generally stop oscillating at different times after the bivalent was cut. The one exception is one cell in which all 3 bivalents were cut: each of the “halves” oscillated irregularly for 3 cycles, and all stopped at their poles the same time (**Figure 4**).

Bivalent “halves” that first oscillated and then stopped moving remained at the poles, except in 3 cells in which only 1 bivalent was cut. In each of these cells the single “halves” first stopped at the poles and some time later moved across the equator to the opposite pole, which we refer to as “half” bivalent excursions. In all “half” bivalent excursions the kinetochores led the way. All three “half” bivalent excursions had two phases, initial slower movement and later faster movement. In the cell illustrated in **Figure 5**, for example, the “half” bivalent at the bottom pole finished oscillating (at 8 min on the movement graph, **Figure 5B**), was stationary at its pole for a short time, then began moving toward the opposite pole between 8 and 11 min, and then moved faster between 12 and 13 min (**Figure 5C**). For the 3 cells in which there were “half” bivalent excursions, the initial slower speeds averaged 3.3 $\mu\text{m}/\text{min}$. and the faster 7.6 $\mu\text{m}/\text{min}$.

Effects of Adding NOC to Cells With “Halved” Bivalents

In cells treated only with NOC (i.e., there was no laser cutting), all bivalents stretched, paused, detached from one pole, moved toward the opposite pole with a fast speed, slowed down as they reached the opposite pole, and stopped moving at the opposite pole [described in detail in Fegaras and Forer, 2018a]. In cells treated with NOC after bivalents were severed, at the same time that non-severed bivalents detached and move to the other pole the “halves” at the pole also detached and moved to the opposite poles, moving at the same time that the non-severed chromosomes moved, and at the same speeds as the non-severed chromosome kinetochores (see **Figures 3B, 5B**). The major difference in the behavior sequence after severing chromosomes is that the “halved” bivalents did not stretch out as the non-cut bivalents did, presumably because there was no force in the opposite direction otherwise supplied by the attached partner. As seen in **Figure 3C**, for example, the severed kinetochore (blue circle) is already paused at its respective pole when the not-severed kinetochore (red triangle) is in the stretch phase and both move to the opposite pole at the same time, and with the same speed. That the bivalent and “half”-bivalent movements are at

the same speed suggests that the kinetochores move because of external forces, not those from tension in the stretched bivalent. We looked at these data in more detail to test how closely the speeds match.

Speed of Severed Bivalents Is the Same as That of Non-severed Bivalents

The total average speed of movement of “halved” bivalents in all cells with 1, 2, and 3 severed bivalents was not significantly different than the average speed of kinetochores in control cells (**Figure 6**). Furthermore, the speeds of the “halved” bivalents are very close to the speeds of the non-severed bivalents in the same cells (e.g., **Figures 3B, 5B**). Since “half” bivalents detached and moved to a pole at the same time and speed as non-severed bivalents in the same cell, and moved at the same speed as in control cells, the tension in the stretched chromosome does not contribute much, if anything, to the forces for movement. The forces for movement rather must arise from external non-microtubule forces. Other parameters of the movements of “halved” bivalents were not statistically different to those of non-cut bivalents, namely the time from NOC addition to detachment and the duration of the movements, regardless of whether 1, 2, or 3 bivalents were cut (**Table 2** and **Figure 6**), so the absence of intact bivalents does not affect these parameters either.

We studied three additional issues, all dealing with coordination between chromosomes so that all detach at the same time, all detach from the same pole, and all move to the opposite pole.

After NOC Treatment, do “Half” Bivalents All Move to the Same Pole, as Bivalents do? the Likelihood of Detachment From Solely 1 Pole Decreases as the Number of Severed Chromosomes Increases

After addition of NOC to cells in which no bivalents were severed, the bivalents usually detached from one pole and the detached kinetochores moved to the other pole: they rarely detached from 2 or 0 poles (**Figure 7**). When bivalents detached from 0 poles, the bivalents stayed in the middle of the spindle, and no kinetochores moved toward either pole. When bivalents detached from 2 poles, either both kinetochores of each bivalent detached from both poles and all 3 bivalents were positioned in the middle of the cell, or bivalent kinetochores detached from 1 pole and moved to the other pole but different bivalents in the cell detached from different poles (see the diagram in **Figure 7**). After addition of NOC to cells in which bivalents were cut, the most common response was that “half” bivalents detached from one pole and moved to the other. As more bivalents were cut the likelihood “half” bivalents would detach from 1 pole decreased, and the likelihood they would detach from 2 or 0 poles increased (**Figure 7**). Thus, when one bivalent is severed, the “half” bivalents act as the bivalents do in control cells, but the coordination in detachments and in movements breaks down as more bivalents per cell are cut in half per cell. While this result may suggest that coordination in movements requires physical connection between “halved” bivalents, the

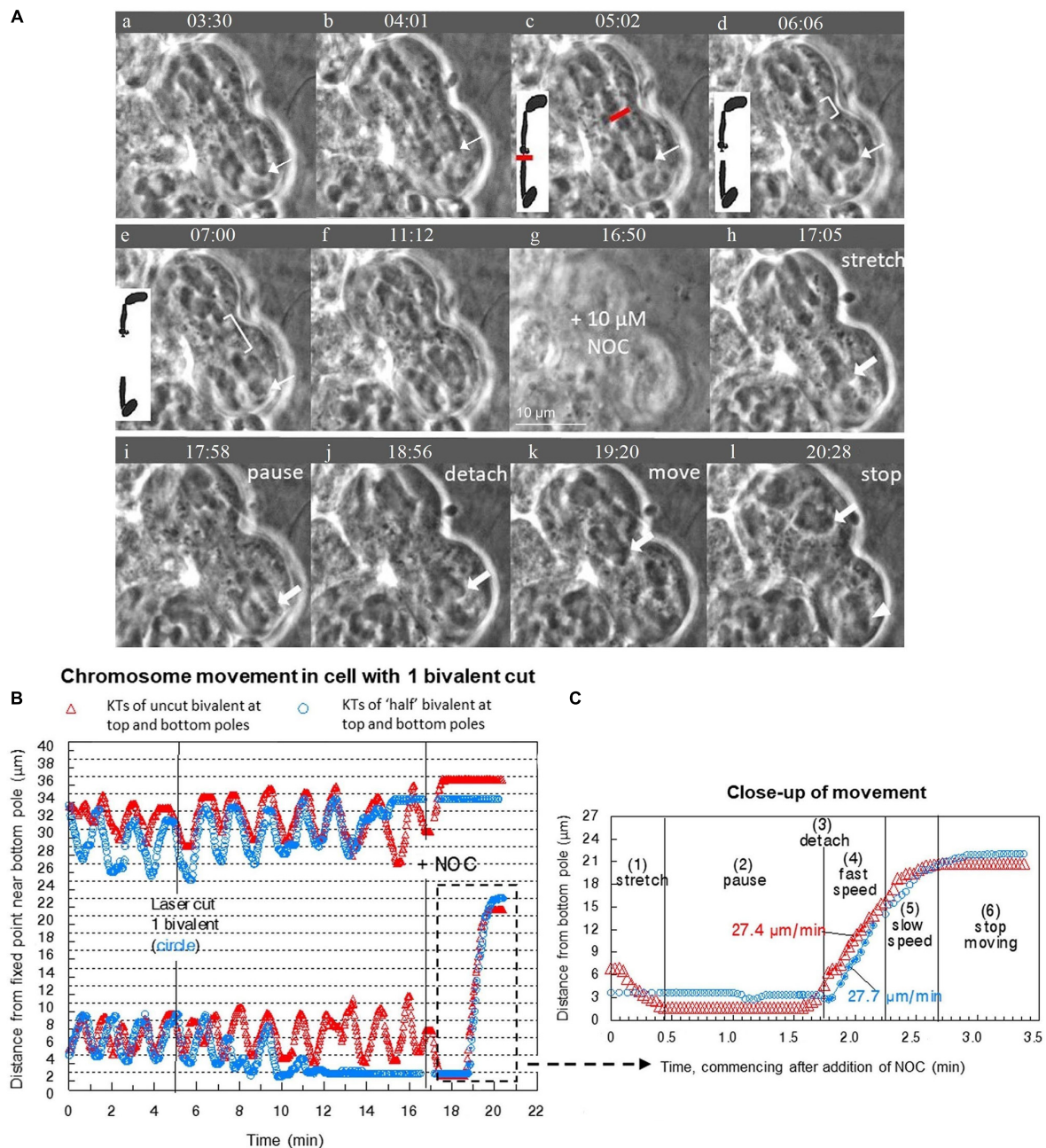


FIGURE 3 | (A–C) After cutting 1 bivalent into two pieces during prometaphase, severed and unsevered bivalents still move after adding NOC to *Mesostoma* spermatocytes. **(A)** Image sequence where one bivalent is cut into 2 pieces and then the “halved” bivalent and the two other bivalents detach from the bottom pole after the addition of NOC. Diagrams are included to clarify the laser cut and separation of “half” bivalents. Between **(a–e)** bivalents oscillate between the two poles, the position of one is indicated by the thin white arrow pointing to the kinetochore at the bottom pole. Two laser cuts were performed in quick succession; the first was not successful. The successful laser cut is shown in **(c)** a clear space is seen between the two pieces, indicated by the white bracket in **(d)** and **(e)**. The gap grows larger in **(e)**. The bottom “halved”-bivalent continued to oscillate, but stopped after several oscillations. The image went out of focus when NOC was added (in **(g)**). NOC caused the bivalents to stretch out, pause, detach from the bottom pole, then move quickly toward the top pole, as described by Fegaras and Forer, 2018a. The movement of kinetochores from the bottom pole is indicated by arrows pointing to kinetochores. The kinetochores (and attached bivalents or “halved” bivalents) stop moving once they near the top pole. The univalents remain behind in the bottom pole, as indicated by the white arrowhead in **(l)**. The cleavage furrow shifted toward the bottom pole. Time, as in all figures, is shown in mins:secs. **(B)** Graph of chromosome movement in the same cell, depicting the two kinetochores of an un-cut bivalent (red) and the two kinetochores of the two “half”-bivalents (blue). **(C)** Close-up of chromosome movement of both the cut and un-cut bivalents in the part of the graph indicated by the hashtag rectangle. Chromosome movement of un-cut bivalents follows the same pattern seen in NOC-only treated cells, which is bivalents stretch, pause, detach, fast speed, slow speed then stop. Bivalents that have been cut in two follow the same pattern, except there is no stretch phase because they are already positioned at their respective poles and there is no countervailing force toward the other pole from the previously-connected partner. The speeds of movement for the two moving kinetochores were determined from the slopes of the lines of best fit.

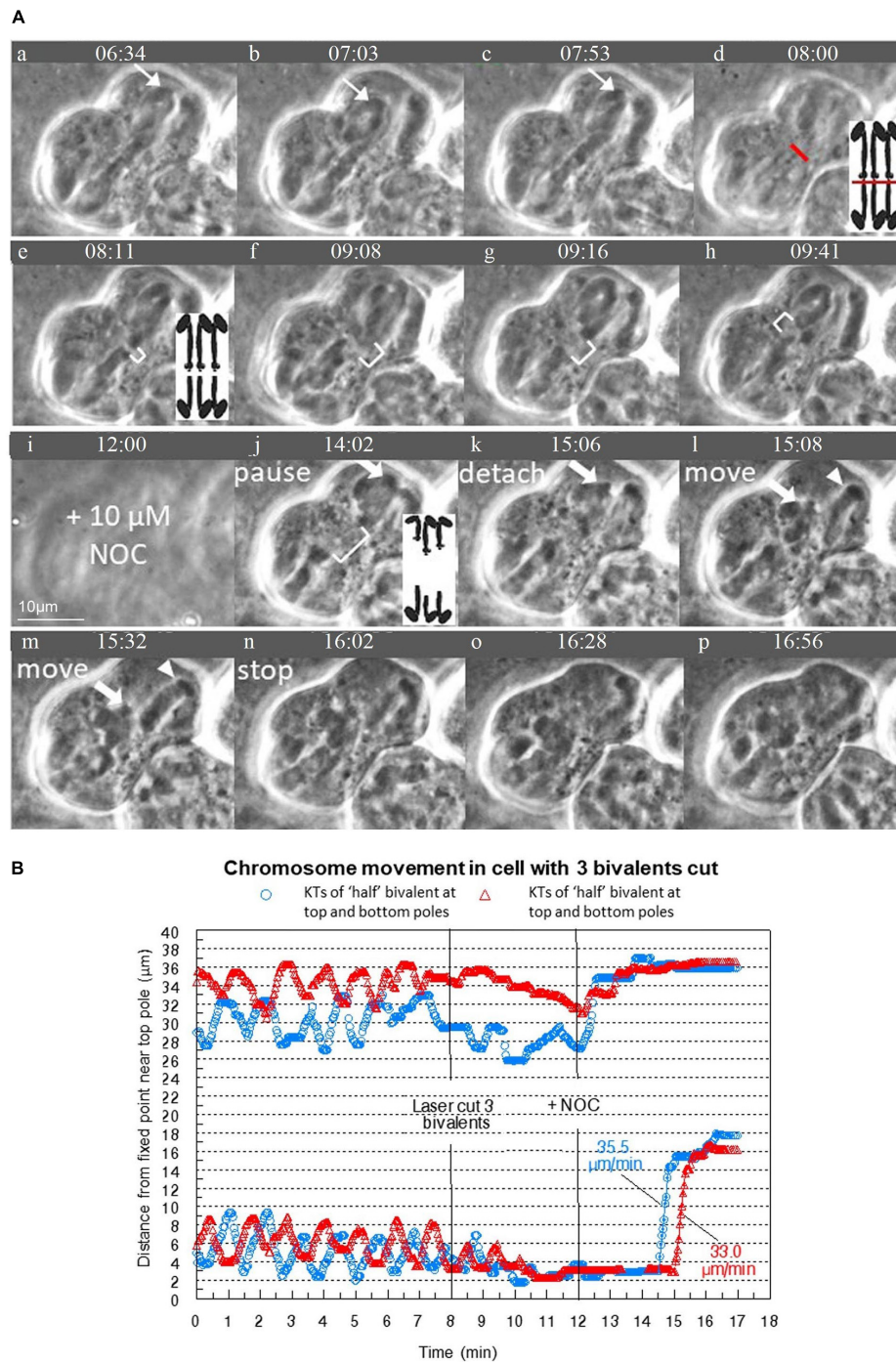


FIGURE 4 | (A,B) Severed chromosomes are able to move after treatment with NOC when three bivalents are severed. **(A)** Image sequence of a cell where all 3 bivalents are severed as indicated by the red line. Diagrams are included to clarify the laser cut and separation of “halved” bivalents. In **(a–c)** the bivalents oscillate between either pole as shown by the thin arrows that point to a kinetochore. **(d)** is out of focus as the laser cut is performed along several places of focus. In **(e–h)** the cytosolic space is visible between the two pieces of the cut bivalents as shown by the white brackets. Throughout this time period, the bivalents oscillate irregularly, eventually stopping at their respective poles. The images of each of the frames is at a slightly different plane of focus to illustrate there is still a clear gap between all 3 of the severed bivalents, as also illustrated in the diagram. NOC was added at **(i)**. After the addition of NOC, the bivalents remain paused at both poles as seen in **(j)** (there is no “stretch” phase). They then detach, move quickly at first, then slowly toward the bottom pole, as shown by the thick white arrow, then stop near the bottom pole. The univalents do not move with the bivalents and remain at the top pole, as indicated by the arrowhead. The cleavage furrow moved toward the top pole. Time is shown in mins:secs. **(B)** Graph of the movement of four “halved” bivalents that were in the same plane of focus. The other two “halves” are in another plane of focus; their movements were not graphed but that the third bivalent was severed and that the “halves” moved to one pole were verified in the recorded image sequence in **(A)**. The speeds of movement for the severed kinetochores were determined from the lines of best fit.

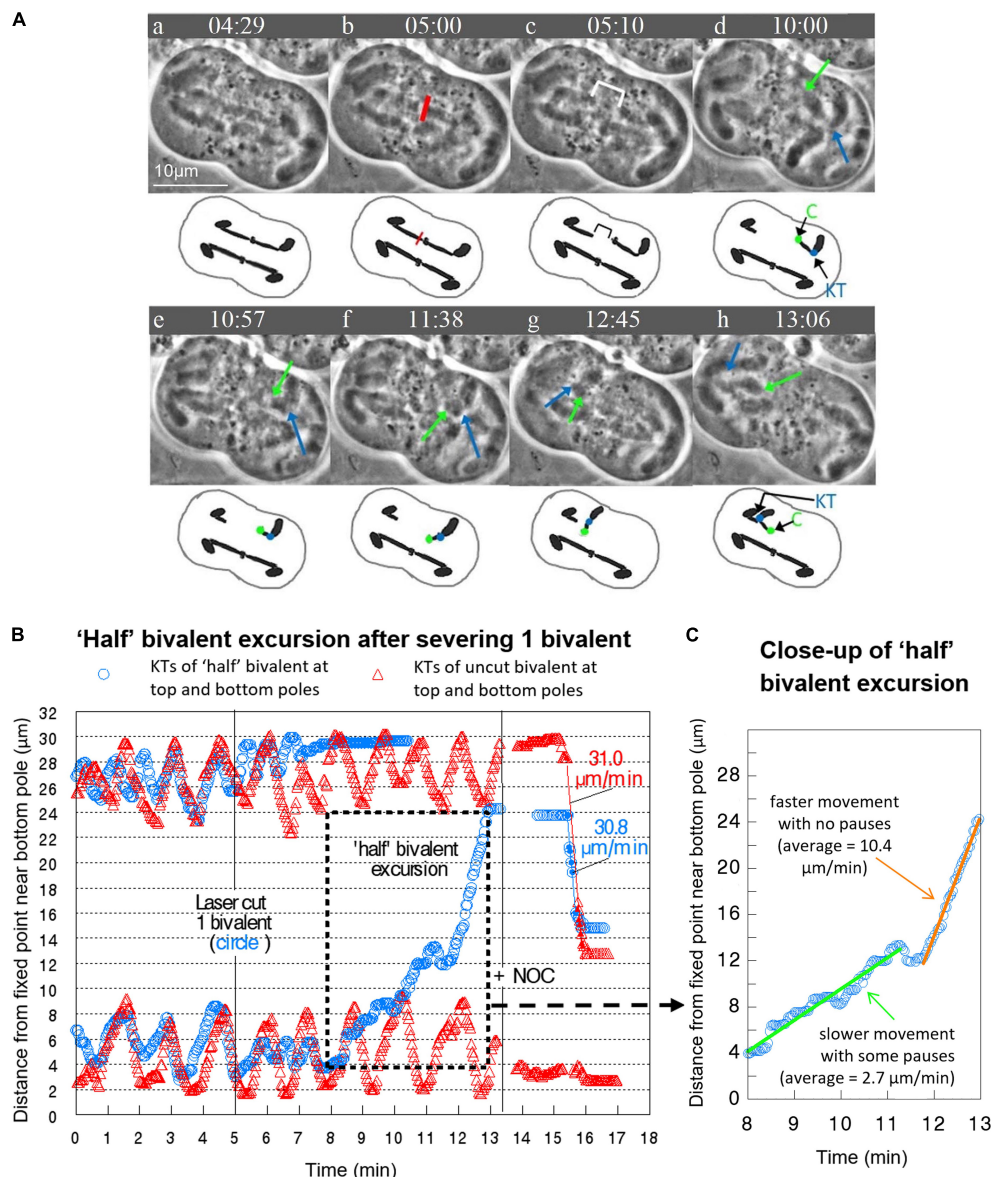


FIGURE 5 | (A–C) Prior to addition of drug, severed “half” bivalents sometimes move to the opposite pole, similar to univalent excursions. **(A)** Video sequence of a cell with accompanying diagrams to clarify movement of the “half” bivalent. The red line indicates the laser irradiation of a single bivalent, with the cytosolic space between the two separated “halves” shown by the white bracket in **(c)**. In **(d)**, the bottom “half” bivalent is oriented with the chiasma (C – green dot and arrow) pointing toward the top pole, and the kinetochore (KT – blue dot and arrow) oriented toward the bottom pole. As the bottom “half” bivalent moves toward the top pole from **e** to **g** it rotates so that by **(h)** the kinetochore orients toward the top pole and the chiasma toward the bottom pole. In **(h)** the “half” bivalent reaches the top pole next to its partner “half” bivalent. For simplicity, we excluded from the diagram the univalent and the 1 bivalent that is not visible. Times are shown in mins:secs. **(B)** Movement graph of the excursion by a “half” bivalent in the same cell. The excursion begins at 8 min and reaches the opposite pole at 13 min. **(C)** Close up of the “half” bivalent excursion. There is a period of slower movement with some pauses (the line of best fit is green) followed by faster movement with no pauses (the line of best fit is orange). The average speeds of movement are taken from the lines of best fit.

physical connection that is required may be in the tethers that connect the free arms of the bivalent. Tethers physically connect the free chromosome arms and it may be that that physical connection is important for co-ordinating movements: some of the deleterious effects of severing bivalents on coordination between the chromosomes may be due to the laser inadvertently cutting tethers.

Tethers May Play a Role in Bivalent Detachment During NOC

Elastic tethers connect the telomeres of all separating anaphase chromosomes in a variety of animal cells: the arm fragment cut from one of the arms moves across the equator to the telomere of the partner chromosome (LaFountain et al., 2002; Forer et al., 2017). As summarized in the Introduction, “tethers”

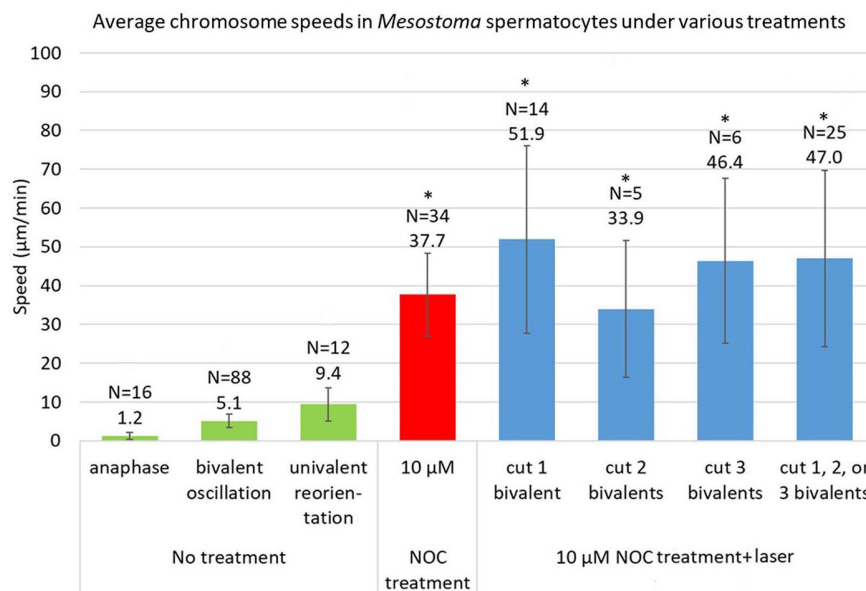


FIGURE 6 | Average speeds of chromosomes in *Mesostoma* spermatocytes with no treatment, NOC-only treatment, and NOC + laser. The data for control cells ("no treatment") is taken from Ferraro-Gideon et al., 2014. The speed of 10 μM NOC-only cells is an average of the fast speeds, and is updated from Fegaras and Forer, 2018a, to include data taken after that article was published. The data for NOC + laser cells include only cells in which bivalents or "halved" bivalents detached from 1 or 2 poles. The average speed of movement for NOC-treated cells vs NOC + laser treated cells are not significantly different. *represents values that are not significantly different, $p > 0.05$, as determined using Student's *t*-test.

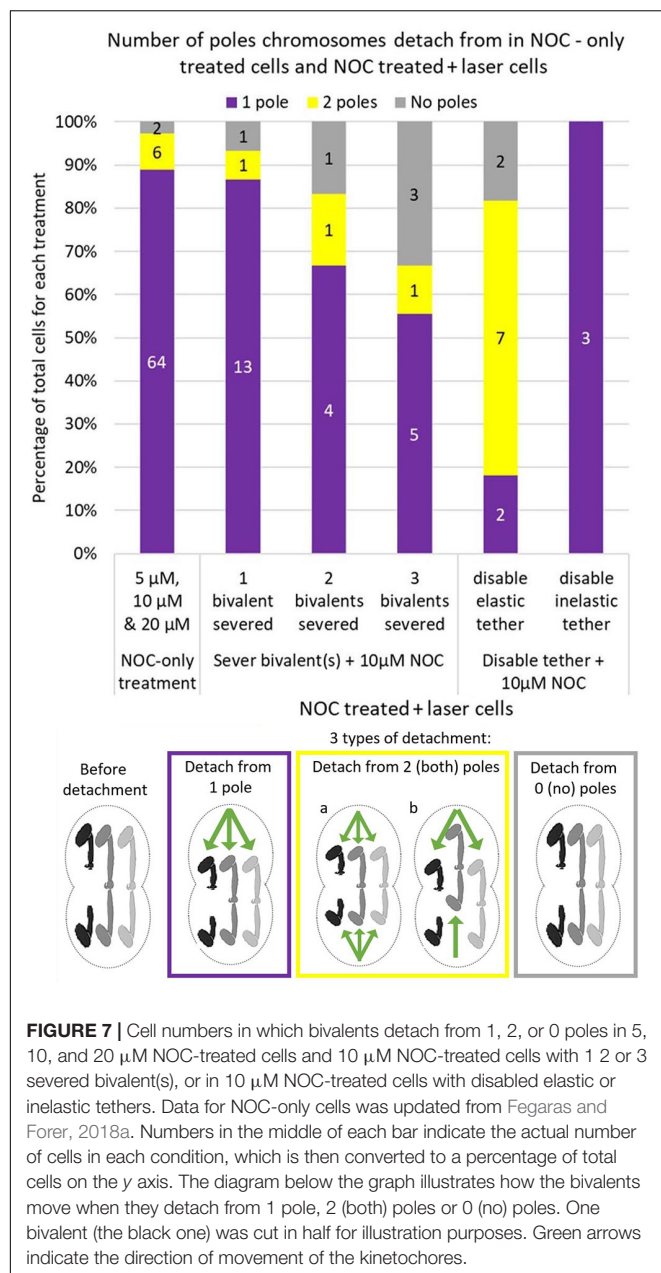
TABLE 2 | Timings and speeds of the chromosome responses for the different treatments.

Treatment <i>n</i> = number of cells	Time from drug addition to detachment (min:sec)	The total duration of kinetochore movement to the opposite pole, fast plus slow movement (min:sec)
10 μM NOC (<i>n</i> = 34)	02:06 ± 00:50 (01:07 03:44)	01:20 ± 00:51 (00:34–02:22)
Cut 1 bivalent + 10 μM NOC (<i>n</i> = 14)	02:04 ± 00:49 (01:01–04:20)	01:00 ± 00:35 (00:27–01:52)
Cut 2 bivalents + 10 μM NOC (<i>n</i> = 5)	01:27 ± 00:51 (00:29–02:29)	00:55 ± 00:19 (00:40–01:17)
Cut 3 bivalents + 10 μM NOC (<i>n</i> = 6)	01:44 ± 00:32 (00:59–02:22)	00:56 ± 00:25 (00:35–01:28)
TOTAL: Cut 1, 2, or 3 bivalent(s) + 10 μM NOC (<i>n</i> = 25)	01:52 ± 00:47 (00:29–04:20)	00:58 ± 00:29 (00:27–01:52)

Cell counts exclude 5 cells which had bivalent detachment from neither pole (see gray values in **Figure 7** for number of cells with detachment from no poles). The values are ± standard deviations; the ranges are given in parentheses; the cell sample size is indicated by the *n* = value. None of the values in any given column are significantly different from others in the same column.

connect separating anaphase chromosomes in a broad range of cells, including *Mesostoma* spermatocytes, and would seem to be present universally in animal cells (Forer et al., 2017). Anaphase "tethers" are elastic in early anaphase but become inelastic as they elongate. Tethers also extend between the telomeres of the free

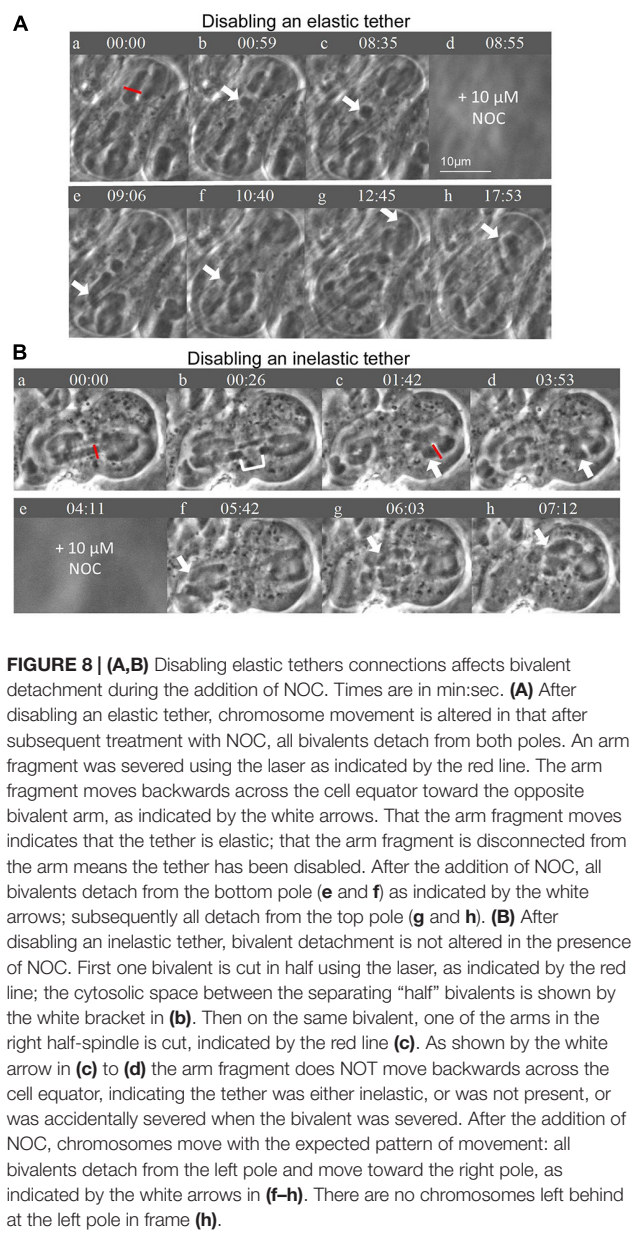
arms of *Mesostoma* spermatocyte bivalents during prometaphase: after severing a portion of the free arm, the arm fragment moves to the telomere of the other free arm (Forer et al., 2017). But not all the time, only in an estimated 50% of all cases (Fegaras-Arch, unpublished). Since tethers produce tension between the arms, we thought that tension from tethers might be involved in determining the detachment of the kinetochores, e.g., in co-ordinating kinetochores so that only one kinetochore detaches, and co-ordinating which pole it detaches from. To test the role of tethers we disabled them directly by cutting a section from the tip of a free arm [**Figure 2B**, Laser experiment]; this disconnects the mechanical connection between the arms and hence to the kinetochore to which it originally was attached. Since tethers cannot be visualized, one needs to cut arms to test if an elastic tether is present (Paliulis and Forer, 2018). If elastic tethers are present, the arm fragment moves to the partner telomere (LaFountain et al., 2002), as illustrated in the cartoon in **Figure 2B**. In the cell shown in **Figure 8A**, one of the free bivalent arms at the top pole was severed and the arm fragment moved backwards across the equator toward the telomere of the opposite arm, nearer the bottom pole. In the cell shown in **Figure 8B**, on the other hand, the arm was severed but the arm fragment did not move backwards, indicating that either a tether was not elastic or it was not present. In our experiments, we severed tethers of one bivalent, added NOC, and asked if bivalent behavior was altered. When we disabled *elastic* tethers and treated those cells with NOC, bivalent detachment was altered: there was increased frequency of cells in which bivalents either detached from both poles or did not detach from either pole (**Figure 7**). In comparison, when we severed *inelastic* tethers, bivalent behavior



was normal: in 3/3 cells all bivalents detached from 1 pole and the detached kinetochores moved to the other (Figure 7). This experiment points to elastic tethers having an important role in co-ordinating the movements of bivalent kinetochores so that they all move to the same pole (after treatment with NOC), that tethers must physically connect and put tension on free arms in order for the coordinated after-NOC movements to occur.

“Half” Bivalent Oscillations Predict Which Pole Kinetochores Will Detach From

It is a puzzle that after treatment with NOC all bivalents detach from one pole at the same time, and all kinetochores move to the opposite pole. None of the features of cell division in



Mesostoma spermatocytes that we have looked at have allowed us to predict which pole the kinetochores will detach from (Fegaras and Forer, 2018a), but detailed analysis of the “half” bivalent movements seem to indicate that there might be differences between the two spindle poles. We compared the behavior of “half” bivalents at the two poles, the pole from which all kinetochores detached after NOC was added vs that at the pole where kinetochores did not detach. The parameters we looked at are listed in Table 3. Two of the parameters indicated which pole that kinetochores would detach from, the “half” bivalent that stopped oscillating first, and the length of time the “half” bivalents oscillated (normal plus irregular oscillations). At the pole from which kinetochores subsequently detached, the “half” bivalent stopped oscillating before the partner “half” bivalent

TABLE 3 | Comparison of “half” bivalents oscillations at the pole that DID detach, vs. DID NOT detach.

Laser cut(s)	“Half” bivalents at pole from which kinetochores subsequently DID detach			“Half” bivalents at pole from which kinetochores subsequently DID NOT detach		
	Average # of normal oscillations	Average # of irregular oscillations	Average duration of normal plus irregular oscillations (min:sec)	Average # of normal oscillations	Average # of irregular oscillations	Average duration of normal plus irregular oscillations (min:sec)
Cut 1 bivalent (<i>n</i> = 12)	2.3 ± 1.9	2.2 ± 1.2	03:35 ± 02:01*	3.0 ± 1.7	2.4 ± 1.0	04:57 ± 03:01*
Cut 2 bivalents (<i>n</i> = 4)	0	2.5	–	2.5	1	–
Cut 3 bivalents (<i>n</i> = 3)	1	3	–	1	2	–

Data in this table is only of the cells tabulated in the “halves’ oscillate” row in **Table 1**, excluding cells with “bivalent ‘halves’ stationary at the poles”. The ± standard deviations are included where applicable. Values with *are significantly different at *p* < 0.05 using Student’s *t*-test. The average duration of normal plus irregular oscillations is statistically greater for “half” bivalents at the pole from which there was no detachment, but there appears to be no significant difference between the numbers of normal oscillations, or of the numbers of irregular oscillations.

did in 12/15 cells (illustrated graphically in **Figure 3B**), and the duration of oscillations was shorter than that of the partner “half” bivalent (averaging 3 min:35 s vs 4 min:57 s).

DISCUSSION

Force to Move Chromosomes

A main conclusion from our experiments is that after removal of spindle microtubules with NOC (Fegaras and Forer, 2018a) “halved” bivalents move with the same rapid speeds as normal bivalents in the same cell. Therefore, rapid kinetochore movement in the absence of spindle microtubules is not due to tension in the stretched bivalents and must be due to some spindle component(s) other than microtubules. What could the force producers be?

In the presence of 10 μM NOC, chromosomes in *Mesostoma* spermatocytes with no severed bivalents stretch and then selectively detach from one pole and move toward the opposite pole at speeds that average 37.7 μm/min. (**Figure 6** and Fegaras and Forer, 2018a). Experiments presented here show that this movement is not due to recoil of stretched chromosomes because when bivalents are cut, the “halved” bivalents still detach from one pole and move toward the opposite pole, and they do so at an average speed of 47.0 μm/min. (**Figure 6**). There does not appear to be a statistically significant difference in the average speeds for cells treated with 10 μM NOC without cutting bivalents vs after cutting 1, 2, or 3 bivalents (*p* > 0.05). Since the “half” bivalents and the non-cut bivalents move at the same (or very similar) speeds, this indicates that similar forces act on the kinetochores of the moving “halved” bivalents and of the full bivalents, and that the forces do not derive from tension in the stretched bivalents. Nor is the force for these kinetochore movements from microtubules, because immunofluorescence staining shows that NOC treatment removes microtubules from the spindle: most spindle microtubules are depolymerized by the NOC. Any that remain are highly fragmented and not attached to the pole, and none are in contact with the moving chromosomes (Fegaras and Forer, 2018a). Since no microtubules are present, the movement is due to some force different from microtubules.

We suggest that what may be moving the chromosomes is actin and myosin, perhaps as components of the spindle matrix (Johansen et al., 2011). In the spindle matrix model, microtubules play a passive role in chromosome movement, acting as governors rather than force producers (Spurck et al., 1997; Johansen and Johansen, 2007; Pickett-Heaps and Forer, 2009; Johansen et al., 2011). What may instead be providing the force for chromosome movement is actin, myosin, or some other spindle matrix proteins (Fabian and Forer, 2007; Fabian et al., 2007; Forer et al., 2015). We can test this hypothesis by using various enhancers and inhibitors that target actin and myosin in NOC-treated cells. If perturbing these proteins alters chromosome movement, that would support our suggestion that actin and myosin play a role in the chromosome movements that occur after NOC treatment.

Coordination Between Chromosomes: Detachment From One Pole

In NOC treated cells all 3 bivalents detach from one pole, almost always the same pole, and all three kinetochores move rapidly to the other pole (**Figure 7**). Halved bivalents act the same way: most detach from one pole (**Figure 7**), always the same pole that the un-cut bivalents detached from (when there were fewer than 3 halved bivalents in the cell). Our data suggest that elastic tethers are important for these coordinations. After severing arms in *Mesostoma* spermatocytes, the arm fragments sometimes move to their partners, and sometimes they do not (also noted by Ferraro-Gideon, 2013), indicating that sometimes there are elastic tethers between the arms, and sometimes tethers either are not present or are inelastic. In our experiments, in cells in which we disabled an elastic tether, bivalents had an increased frequency of detachment from 2 poles after treatment with NOC (**Figures 7, 8A**). Severing an arm that did not have an elastic tether, on the other hand, did not alter chromosome detachment from 1 pole after addition of NOC (**Figures 6, 8B**). We think that tethers produce tension between the two free arms of each bivalent, and that this tension may be involved in co-ordinating chromosome movement to one pole after treatment with NOC. If tethers are involved in this way, this is consistent with the role of tethers found in crane-fly spermatocytes, in which tethers appear to be involved in co-ordinating movements of separating

anaphase chromosomes (Sheykhani et al., 2017; Paliulis and Forer, 2018; Forer and Berns, 2020).

Damage to tethers may be the reason that cutting more bivalents per cell results in reduced coordination: as more bivalents are cut the more likely it is that coordination is altered and that halved bivalents do not all go to the same pole (**Figure 7**). We suggest that this is because the likelihood of accidentally cutting a tether increases as one severs more bivalents because that requires several larger cuts in various focal planes. It is tricky to avoid cutting tethers even when cutting 1 bivalent in “half,” and it is very difficult to avoid cutting tethers when cutting 2 or 3 bivalents, all in several focal planes. Therefore, the decrease in detachment from 1 pole may be due to the fact we accidentally cut elastic tethers while cutting 2 or 3 bivalents in the same cell.

Mesostoma spermatocytes so far are unique in having tethers between chromosome arms prior to anaphase, having been found between the ends of the free arms in prometaphase. In other cells, in early anaphase tethers are elastic; in later anaphase the longer tethers are inelastic (Forer et al., 2017), and in crane-fly spermatocytes, at least, the difference seems to be coordinated with phosphorylation and dephosphorylation events (Kite and Forer, 2020). Tethers in *Mesostoma* may be similar: it is possible that *Mesostoma* tethers alternate between phosphorylation states, or perhaps not all tethers are phosphorylated in any one given cell, and that is why sometimes severed arm tips might not move.

Coordination Between Chromosomes: Oscillation of “Halved” Bivalents as Markers for Which Pole the Kinetochores Will Move to After NOC Treatment

“Halved” bivalents usually continued to oscillate for some period of time after the bivalents were cut in two by the laser. In general, partner “halved” bivalents start by oscillating regularly, then they oscillate irregularly, and then they stop at their respective poles (**Figures 3B, 4B, 5B**), usually at different times: one “halved” bivalent generally stops several oscillations before its partner. When kinetochores subsequently detach from one pole and move to the other, they usually (12/15 cells) move from the pole at which “half” bivalent oscillations stopped first. The detailed timings shown in **Table 3** confirm that the duration of both normal and irregular oscillations was significantly shorter at the pole from which kinetochores DID detach. This suggests that some difference in the spindle forces at that pole plays a role in chromosome detachment from that pole. We don’t know, however, whether the difference arises from something inherent in that particular one of the 2 poles, or rather whether there are inherent differences in the 2 “half” bivalents (e.g., male-derived vs female-derived).

Other Issues

That the “half” bivalents continue to oscillate counters the argument by Fuge about the mechanisms of the regular oscillations of bivalents in prometaphase. Fuge (1987) argued that the normally-occurring bivalent oscillations arise from forces from opposite poles transmitted by tension in the chromosomes: the pulling forces from the spindle fibers were countered by

tension along the chromosome and that this interplay caused the normal oscillations. Our experiments counter this argument since “halved” bivalents still oscillate, at least for a while. Thus, the forces for the continuing oscillations are not from opposite kinetochores but must arise from something inherent in the forces produced by the spindle.

Why do regular oscillations stop after several cycles of normal and then irregular oscillations begin (**Table 3**)? While the lack of tension along the length of the “halved” bivalents may eventually stop the oscillations, there may be another contributing factor. Prometaphase bivalents often go through periods where their oscillations shift in phase; graphically these look similar to the irregular oscillations of a severed “half” bivalent (e.g., **Figures 6, 7** in Ferraro-Gideon et al., 2014; **Figure 7B** in Fegaras and Forer, 2018a). Phase shifts may or may not occur in control cells in any given sequence we film, and they can occur multiple times to any one bivalent at anytime throughout prometaphase. It is possible that whatever mechanisms are involved in causing phase shifts, and that take place during a phase shift, are also at work during the period of dampened oscillations in “halved” bivalents.

“Half” bivalents eventually pause at the poles after oscillating. Some of them then move across the spindle to the opposite pole (**Figures 5A–C**). During “half” bivalent excursions the kinetochore of one “half” bivalent detaches from a pole, swings around toward the opposite pole, and moves toward the opposite pole, in a similar manner to univalent excursions in *Mesostoma* (Ferraro-Gideon et al., 2013, 2014), and to sex chromosomes in other cell types such as grasshopper spermatocytes (Nicklas, 1961; Ault, 1984, 1986) and crane-fly spermatocytes (Bauer et al., 1961). We do not know what controls these movements. Excursions of univalent chromosomes from pole to pole seem to be needed to obtain proper distributions of the two pairs of univalent chromosomes (Oakley, 1985), so *Mesostoma* spermatocytes indeed have mechanisms to aid pole-to-pole movements. Though we have no idea what they are, it is not surprising that they act on “halved” bivalents that are at the poles together with the univalent chromosomes. Since anaphase seems to be inhibited until proper distribution of univalents is obtained at the poles, it may be that “halved” bivalents inhabiting a pole may trigger the movement to the opposite pole through whatever mechanism triggers the univalent chromosomes to move. Since improper distribution of univalents seems to inhibit anaphase onset (Oakley, 1985) it may be that the presence of “halved” bivalents at the poles inhibits anaphase. This is speculation on our part, and further testing is needed.

In Summary

One main conclusion from our experiments is that non-microtubule spindle components can move chromosomes in *Mesostoma* spermatocytes. After microtubules are removed with NOC, all chromosome kinetochores move at rapid speeds (up to 100 $\mu\text{m}/\text{min}$) to the opposite poles. Our experiments have eliminated tension in elongated chromosomes as producing these forces since the same movements occur in “halved” bivalents which do not have bipolar connections. Chromosome

movements still occur when 1, 2, or 3 bivalent(s) are severed into “halved” bivalents, and the movements are at very close to the same rapid speeds as in not-cut bivalents. A second conclusion is that elastic tethers may be required to coordinate kinetochore movements. Cutting more than one bivalent per cell alters the coordination by which all kinetochores release from one pole and move to the opposite pole. Because directly disabling one elastic tether in a cell alters the coordinated movements in that cell, we suggest that the altered coordination in moving to one pole when all bivalents are severed is because of collateral damage to tethers when cutting multiple bivalents.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

REFERENCES

- Ault, J. G. (1984). Unipolar orientation stability of the sex univalent in the grasshopper (*Melanoplus sanguinipes*). *Chromosoma* 89, 201–205. doi: 10.1007/bf00295000
- Ault, J. G. (1986). Stable versus unstable orientations of sex chromosomes in two grasshopper species. *Chromosoma* 93, 298–304. doi: 10.1007/bf00327587
- Bauer, H., Dietz, R., and Robbelen, C. (1961). Die spermatocytenteilungen der tipuliden III. Mitteilung. Das bewegungsverhalten der chromosomen in translokationsheterozygoten von *Tipula oleracea*. *Chromosoma* 12, 116–189. doi: 10.1007/bf00328918
- Berns, M. W., and Greulich, K. O. (2007). *Laser Micromanipulation of Cells and Tissues: Methods in Cell Biology*. San Diego, CA: Academic Press.
- Brady, M., and Paliulis, L. V. (2015). Chromosome interaction over a distance in meiosis. *R. Soc. Open Sci.* 2, 1–10.
- Fabian, L., and Forer, A. (2007). Possible roles of actin and myosin during anaphase chromosome movements in locust spermatocytes. *Protoplasma* 231, 201–213. doi: 10.1007/s00709-007-0262-y
- Fabian, L., Troscianczuk, J., and Forer, A. (2007). Calyculin A, an enhancer of myosin, speeds up anaphase chromosome movement. *BMC Cell Chrom.* 6:1. doi: 10.1186/1475-9268-6-1
- Fegaras, E., and Forer, A. (2018a). Chromosomes selectively detach at one pole and quickly move towards the opposite pole when kinetochore microtubules are depolymerized in *Mesostoma ehrenbergii* spermatocytes. *Protoplasma* 255, 1205–1224. doi: 10.1007/s00709-018-1214-4
- Fegaras, E., and Forer, A. (2018b). Precocious cleavage furrows simultaneously move and ingress when kinetochore microtubules are depolymerized in *Mesostoma ehrenbergii* spermatocytes. *Protoplasma* 255, 1401–1411. doi: 10.1007/s00709-018-1239-8
- Ferraro-Gideon, J. (2013). *Rearing Mesostoma ehrenbergii and Studying Chromosome Movements During Meiosis in Their Spermatocytes*. Ph. D thesis, New York University, Toronto.
- Ferraro-Gideon, J., Hoang, C., and Forer, A. (2013). *Mesostoma ehrenbergii* spermatocytes—a unique and advantageous cell for studying meiosis. *Cell Biol. Int.* 37, 892–898. doi: 10.1002/cbin.10130
- Ferraro-Gideon, J., Hoang, C., and Forer, A. (2014). Meiosis-I in *Mesostoma ehrenbergii* spermatocytes includes distance segregation and inter-polar movements of univalents, and vigorous oscillations of bivalents. *Protoplasma* 251, 127–143. doi: 10.1007/s00709-013-0532-9
- Forer, A., and Berns, M. W. (2020). Elastic tethers between separating anaphase chromosomes regulate the Poleward speeds of the attached chromosomes in crane-fly spermatocytes. *Front. Mol. Biosci.* 7:161. doi: 10.3389/fmolb.2020.00161

AUTHOR CONTRIBUTIONS

EF-A conducted all original experiments, analyzed the data, created the figures and tables, and wrote the manuscript. EF-A, AF, and MB edited manuscript drafts and AF contributed to planning the experiments. The experiments were done in the lab of MB. All authors contributed to the article and approved the submitted version.

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- Forer, A., Duquette, M. L., Paliulis, L. V., Fegaras, E., Ono, M., Preece, D., et al. (2017). Elastic ‘tethers’ connect separating anaphase chromosomes in a broad range of animal cells. *Eur. J. Cell Biol.* 96, 504–514. doi: 10.1016/j.ejcb.2017.07.001
- Forer, A., Johansen, K., and Johansen, J. (2015). Movement of chromosomes with severed kinetochore microtubules. *Protoplasma* 252, 775–781. doi: 10.1007/s00709-014-0752-7
- Forer, A., and Pickett-Heaps, J. (2010). Precocious (pre-anaphase) cleavage furrows in *Mesostoma spermatocytes*. *Eur. J. Cell Biol.* 89, 607–618. doi: 10.1016/j.ejcb.2010.03.001
- Forer, A., and Pickett-Heaps, J. D. (2005). Fibrin clots keep non-adhering living cells in place on glass for perfusion or fixation. *Cell Biol. Int.* 29, 721–730. doi: 10.1016/j.cellbi.2005.04.010
- Fuge, H. (1987). Oscillatory movements of bipolar-oriented bivalent kinetochores and spindle forces in male meiosis of *Mesostoma ehrenbergii*. *Eur. J. Cell Biol.* 44, 294–298.
- Fuge, H. (1989). Rapid kinetochore movements in *Mesostoma ehrenbergii* spermatocytes: action of antagonistic spindle fibres. *Cell Motil. Cytosk.* 13, 212–220. doi: 10.1002/cm.970130308
- Harsono, M. S., Zhu, Q., Shi, L. Z., Duquette, M., and Berns, M. W. (2012). Development of a dual joystick-controlled laser trapping and cutting system for optical micromanipulation of chromosomes inside living cells. *J. Biophoton.* 6, 197–204. doi: 10.1002/jbio.201200019
- Hoang, C., Ferraro-Gideon, J., Gauthier, K., and Forer, A. (2013). Methods for rearing *Mesostoma ehrenbergii* in the laboratory for cell biology experiments, including identification of factors that influence production of different egg types. *Cell Biol. Int.* 37, 1089–1105. doi: 10.1002/cbin.10129
- Husted, L., and Ruebush, T. K. (1940). A comparative cytological and morphological study of *Mesostoma ehrenbergii* and *Mesostoma ehrenbergii wardii*. *J. Morph.* 67, 387–410. doi: 10.1002/jmor.1050670302
- Johansen, K. M., Forer, A., Yao, C., Girtton, J., and Johansen, J. (2011). Do nuclear envelope and intranuclear proteins reorganize during mitosis to form an elastic hydrogel-like spindle matrix? *Chromosome Res.* 19, 345–365. doi: 10.1007/s10577-011-9187-6
- Johansen, K. M., and Johansen, J. (2007). Cell and molecular biology of the spindle matrix. *Int. Rev. Cytol.* 263, 155–210. doi: 10.1016/s0074-7696(07)63004-6
- Jordan, M. A., and Wilson, L. (2004). Microtubules as a target for anticancer drugs. *Nat. Rev. Cancer* 4, 253–265. doi: 10.1038/nrc1317
- Kite, E., and Forer, A. (2020). The role of phosphorylation in the elasticity of the tethers that connect telomere of separating anaphase chromosomes. *Nucleus* 11, 19–31. doi: 10.1080/19491034.2019.1710329
- LaFountain, J. R., Cole, R. W., and Rieder, C. L. (2002). Partner telomeres during anaphase in crane-fly spermatocytes are connected by an elastic tether that

- exerts a backward force and resists poleward motion. *J. Cell Sci.* 115(Pt 7), 1541–1549.
- Nicklas, B. R. (1961). Recurrent pole-to-pole movements of the sex chromosomes during prometaphase I in *Melanoplus differentialis* spermatocytes. *Chromosoma* 12, 97–115. doi: 10.1007/bf00328917
- Oakley, H. A. (1983). “Male meiosis in *Mesostoma ehrenbergii ehrenbergii*,” in *Kew Chromosome Conference II, Proceedings of the Second Chromosome Conference*, eds P. E. Brandham and M. B. Bennett (London: George Allen and Unwin), 195–199.
- Oakley, H. A. (1985). Meiosis in *Mesostoma ehrenbergii ehrenbergii* (Turbellaria, Rhabdocoela). III. Univalent chromosome segregation during the first meiotic division in spermatocytes. *Chromosoma* 91, 95–100. doi: 10.1007/bf00294051
- Oakley, H. A., and Jones, G. H. (1982). Meiosis in *Mesostoma ehrenbergii ehrenbergii* (Turbellaria, Rhabdocoela). I. chromosome pairing, synaptonemal complexes and chiasma localization in spermatogenesis. *Chromosoma* 85, 311–322. doi: 10.1007/bf00330355
- Paliulis, L. V., and Forer, A. (2018). A review of “tethers”: elastic connections between separating partner chromosomes in anaphase. *Protoplasma* 255, 733–740. doi: 10.1007/s00709-017-1201-1
- Pickett-Heaps, J., and Forer, A. (2009). Mitosis: spindle evolution and the matrix model. *Protoplasma* 235, 91–99. doi: 10.1007/s00709-009-0030-2
- Pickett-Heaps, J. D., and Spurck, T. P. (1982). Studies on kinetochore function in mitosis. I. The effects of colchicine and cytochalasin on mitosis in the diatom *Hantzschia amphioxys*. *Eur. J. Cell Biol.* 28, 77–82.
- Poxleitner, M. K., Dawson, S. C., and Cande, W. Z. (2008). Cell cycle synchrony in *Giardia intestinalis* cultures achieved by using nocodazole and aphidicolin. *Eukaryot Cell* 7, 559–574.
- Sheykhan, R., Berns, M. W., and Forer, A. (2017). Elastic tethers between separating anaphase chromosomes coordinate chromosome movements to the two poles. *Cytoskeleton* 74, 91–103. doi: 10.1002/cm.21347
- Spurck, T., Forer, A., and Pickett-Heaps, J. (1997). Ultraviolet microbeam irradiations of epithelial and spermatocyte spindles suggest that forces act on the kinetochore fibre and are not generated by its disassembly. *Cell Motil. Cytosk.* 36, 136–148. doi: 10.1002/(sici)1097-0169(1997)36:2<136::aid-cm4>3.0.co;2-7
- Vasquez, R., Howell, B., Yvon, A. C., Wadsworth, P., and Cassimeris, L. (1997). Nanomolar concentrations of nocodazole alter microtubule dynamic instability in vivo and in vitro. *Mol. Biol. Cell* 8, 973–985. doi: 10.1091/mbc.8.6.973
- Wong, R., and Forer, A. (2003). “Signalling” between chromosomes in crane-fly spermatocytes studies using ultraviolet microbeam irradiation. *Chromosome Res.* 11, 771–786. doi: 10.1023/b:chro.0000005753.97458.20

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 5

TITLE

MYOSIN IS IMPLICATED IN THE FORCE PRODUCTION THAT CAUSES CHROMOSOME MOVEMENT IN *Mesostoma ehrenbergii* SPERMATOCYTES

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5.1 ABSTRACT

The three bivalent chromosomes of *Mesostoma ehrenbergii* primary spermatocytes continuously oscillate towards and away from their respective pole throughout prometaphase I, for up to two hours before anaphase commences, at average speeds of 5-7 $\mu\text{m}/\text{min}$. Drugs that affect myosin affect the oscillations, changing the chromosome oscillation speed, duration, and distance. after treating cells with several drugs that either inhibit or enhance myosin function. The average speeds of bivalent oscillations were slowed down by myosin inhibitors (ML-7, Y-27632, and BDM) were sped up by myosin enhancers (calyculin A and okadaic acid). The same drugs also affected NOC-induced chromosome movement. In previous experiments, after nocodazole (NOC) was added to depolymerize the microtubules, bivalents stopped oscillating, paused briefly at their respective poles, then detached from one pole and in the absence of spindle microtubules quickly moved towards the opposite pole with initial velocities of up to 200 $\mu\text{m}/\text{min}$. or more (Fegaras and Forer 2018ab). Similar results were also seen with the addition of colcemid. In the experiments presented herein, cells were pretreated for approximately 5 minutes with the myosin altering drug of choice, then 10 μM NOC was added. In these cells, the time to chromosome detachment and the speed of NOC-induced movement were significantly altered.

Overall, these results indicate that myosin is involved in the regular bivalent oscillations which occur throughout prometaphase in *Mesostoma* spermatocytes, and in the NOC-induced high-speed chromosome movements that occur in the absence of microtubules.

5.2 INTRODUCTION

The purpose of our experiments is to test the possible role of myosin in *Mesostoma*-spermatocyte chromosome movements by assessing whether myosin inhibitors and/or enhancers affect the regular bivalent oscillations and/or affect the NOC-induced chromosome movement. To test whether myosin is involved in regular oscillatory movement of bivalents in prometaphase *Mesostoma* spermatocytes we added drugs during prometaphase and studied whether they altered chromosome movements. To test whether myosin is involved in rapid movements that occur when microtubules (MTs) are depolymerised by NOC we added the myosin altering drug of choice during prometaphase and shortly thereafter added nocodazole (NOC) and studied whether those movements were altered. As background we briefly describe the unusual persistent bivalent oscillations in *Mesostoma* control cells and the NOC-induced chromosome movements.

Mesostoma ehrenbergii primary spermatocytes have three large bivalent chromosomes which span across the middle of the cell, and two sets of smaller univalent chromosomes positioned at either pole (Husted and Ruebush 1940; Fuge 1987, 1989). By the time cells enter prometaphase I, each bivalent is bipolarly attached with each set of sister chromatids co-oriented towards their respective pole (Fuge 1987; Brady and Paliulis 2015). Throughout prometaphase I, these bivalent chromosomes consistently oscillate towards and away from their respective poles without stopping. There is no traditional metaphase: the bivalents disjoin and move poleward directly from oscillating without settling into a metaphase plate. Bivalents oscillate for up to two hours before anaphase commences and oscillate at average speeds between $\sim 5\text{--}7\text{ }\mu\text{m}/\text{min}$ (Ferraro-Gideon et al., 2013 A and B, 2014). We tested whether myosin might be involved in these relatively fast bivalent oscillations because myosin has been implicated in chromosome movement in other cell types.

In traditional theories of chromosome movement, microtubules and their associated motor proteins are the main producers of force in the spindle, either by depolymerizing tubulin at the kinetochore (PacMan) or at the pole (Flux) (Rieder and Salmon, 1994). However, there are many other non-microtubule proteins found within the meiotic spindle which may contribute to, or even be responsible for, chromosome movement. This list includes proteins such as actin and its motor protein myosin, and the spindle matrix proteins (Pickett-Heaps and Forer, 2009). Actin and myosin have both been visualized in the spindles of various other animal cells (Xu et al., 2013; Silverman-Gavrila and Forer, 2003; reviewed in Forer et al., 2003; Sandquist et al., 2011), and myosin has been implicated in anaphase chromosome movement in a variety of cell types (as reviewed in Sheykhan et al., 2013). The addition of the anti-myosin drug BDM, and anti-actin drugs cytochalasin D, latrunculin B, swinholide A, and jasplakinolide all cause anaphase movements to slow down in crane-fly spermatocyte cells (Silverman-Gavrila and Forer, 2000; Fabian and Forer, 2007; Xie and Forer 2008), whereas the myosin activator Calyculin A (CalA) increases anaphase speeds (Fabian et al., 2007; Kite 2020). Therefore, if myosin also plays a role in chromosome movements in *Mesostoma*, using drugs which target myosin should perturb the bivalent oscillations.

The various myosin targeting drugs we used all have a different mechanism of action. Myosin activity is regulated through several different pathways which alter the phosphorylation of the myosin regulatory light chain (Komatsu et al., 2000; Seller, 1991; Trybus, 1991), on either the threonine 18 or serine 19 site (Ikebe & Hartshorne, 1985; Tan et al., 1992). Phosphorylation occurs via multiple pathways which may include myosin light chain kinase, Rho-associated protein kinase (ROCK), and several others. These different kinases can either monophosphorylate or diphosphorylate the myosin regulatory light chain to activate myosin

(Amano et al., 1996; Cremonesi & Hartshorne, 2008; Sakurada et al., 1998). To deactivate myosin, only a single dephosphorylation pathway is involved. Protein phosphatase 1 (PP1) regulates myosin activity through the dephosphorylation of the myosin regulatory light chain, inhibiting myosin catalytic activity.

We treated cells with the myosin inhibitor ML-7 which inhibits myosin light chain kinase (MLCK) (Saitoh et al., 1987). H-7 was added to some cells as a control for possible side effects of ML-7 because H-7 inhibits MLCK at a lower affinity than ML-7 but also inhibits the same non-MLCK targets as ML-7 at higher affinity (Quick et al., 1992). To impede myosin function in a different way we also treated cells with BDM which inhibits myosin ATPase (Higuchi and Takemori, 1989; Forer and Fabian 2005), and with 200 μ M Y-27632, which works upstream from MLCK by blocking ROCK (Uehata et al., 1997). To enhance myosin function, we treated cells with the myosin enhancer CalA, which is a PP1 inhibitor, but which also affects PP2A (Ishihara et al., 1989). CalA prevents dephosphorylation of myosin and thereby hyperactivates myosin (Ishihara et al., 1989). To determine whether the effect is due to perturbing PP1 or PP2A, we used another myosin enhancer, okadaic acid, a toxin which also targets Ser/Thr phosphatases (Dounay and Forsyth, 2002). It inhibits the activity of both PP1 and PP2A, but unlike CalA, Okadaic acid has a much higher affinity for PP2A. As seen in the results section, these various myosin inhibitors and enhancers had a significant effect on several parameters of the bivalent oscillations, suggesting myosin plays a role in the bivalent oscillations. We then tested whether myosin is involved in the rapid movements that occur when MTs are depolymerised by nocodazole (NOC).

In several recent experiments (Fegaras and Forer, 2018ab; Fegaras-Arch, Forer and Berns 2020), nocodazole (NOC), a known microtubule depolymerizer (Vasquez 1997; Yang et al.,

2010), was added to *Mesostoma ehrenbergii* primary spermatocytes during prometaphase to depolymerise microtubules. Theoretically, if microtubules are quickly depolymerized, the chromosomes should arrest at the metaphase plate and not move (Rieder and Salmon, 1994). However, in these experiments, NOC addition caused all three bivalents to stretch and extend towards the poles, and after a few minutes in 52/59 cells all three chromosomes detached from the same pole and quickly moved towards the opposite pole, with initial velocities of up to 200 $\mu\text{m}/\text{min}$. or more (Fegaras and Forer, 2018a). Hereinafter, this fast bivalent movement to one pole is referred to as “NOC-induced chromosome movement”. Confocal microscopy confirmed that there were no remaining kinetochore-microtubule connections at the detached pole, indicating that the rapid chromosome movement is not due to microtubules (Fegaras and Forer, 2018a). NOC-induced movements also cannot be due to elastic recoil along the length of bivalents because when chromosomes are severed in half by a laser prior to adding NOC to the cell, half-bivalents will still detach and move towards the opposite pole (Fegaras-Arch, Forer and Berns, 2020). We thought that the microtubules don’t produce the force, perhaps myosin is responsible for the quick NOC-induced chromosome movements. To test this in our experiments, NOC was added to cells that were previously treated with the various myosin inhibiting or enhancing drugs, which caused the NOC-induced chromosome movement to be significantly altered, suggesting myosin plays a role in the NOC-induced rapid chromosome movement.

5.3 MATERIALS and METHODS

5.3.1 Living Cell Preparations:

The procedures have been described in detail elsewhere (Fegaras and Forer, 2018 a and b). Briefly, testes were extracted from living *Mesostoma ehrenbergii* using a pulled glass needle (Fisher) and then expelled onto a coverslip into *Mesostoma* Ringers solution (61 mM NaCl, 2.3 mM KCL, 0.5 mM CaCl₂ and 2.3 mM phosphate buffer) that contained 0.2mg/mL fibrinogen (Calbiochem). Immediately after, Thrombin (Sigma) was added to cause formation of a fibrin clot to hold the cells in place (Forer and Pickett-Heaps, 2005, 2010; Ferraro-Gideon et al., 2014). The preparation was then mounted onto a perfusion chamber slide (illustrated in Forer and Pickett-Heaps, 2005), perfused with *Mesostoma* Ringers solution and viewed with phase-contrast microscopy using a Nikon 100x oil immersion lens (NA 1.3). Images of living cells were recorded in real-time on a DVD recorder. *Mesostoma* primary spermatocytes were periodically perfused with additional *Mesostoma* Ringers solution and observed for at least 3 or 4 cycles of bivalent oscillations to ensure the cells were healthy. *Mesostoma* spermatocytes in prometaphase-I were then treated with single drugs or double drugs as outlined below.

5.3.2 Single-drug treatments:

Once a prometaphase-I *Mesostoma* spermatocyte with healthy bivalent oscillations was identified, single-drug procedures were performed at room temperature. The myosin-altering drugs were added individually. Each was perfused over the cell at these final concentrations: 200 μ M Y-27632 [(R)-(+)-trans-N- (4-pyridyl)-4-(laminoethyl)- cyclohexanecarboxamide; LC Laboratories]; 10, 15 or 20 μ M ML-7 [1~(5-iodonaphthalene-l-sulfonyl)-lH-hexahydro-l,4-diazepine hydrochloride; Toronto Research Chemicals]; 1, 2.5, 5 or 10 nM CalA [Calyculin A; LC Laboratories]; 5 or 10 nM Okadaic acid (LC laboratories); 20 mM BDM [2,3-butanedione 2-

monoxime;Sigma]; and 20 μ M H-7 [Sigma]. Blebbistatin, a myosin inhibitor, was not used because it does not work on cells from ‘lower’ animals such as those in insects (Straight et al., 2003). Stock solutions of all drugs, excluding BDM, were dissolved in DMSO and stored at 1000 times the desired concentration at - 40°C. The BDM was made up in *Mesostoma* Ringers solution at the desired concentration. As control, addition of 0.1% DMSO by itself had no effect on bivalent oscillations (Forer and Pickett-Heaps 2010). The bivalent oscillations were observed in the presence of the myosin-altering drug of choice for 5-10 minutes after which the drugs were washed out by perfusion with 5-10mL of *Mesostoma* Ringers solution. In some cases, the cells underwent anaphase during drug addition; there was no wash-out in these cells.

5.3.3 Double-drug treatments:

Once a prometaphase-I *Mesostoma* spermatocyte with healthy bivalent oscillations was identified, the following double drug procedure was performed at room temperature. The first drug perfused over the cell was one of the following myosin altering drugs: 20 mM BDM, 10 μ M ML-7, 200 μ M Y-27632, 1 nM CalA, or 10 μ M H-7 (Sigma) which acted as a ML-7 control. Then, approximately 5 minutes later, 10 μ M NOC (Nocodazole; Sigma) plus the myosin altering drug that was added in the first step, were both perfused over the cell. Addition of the myosin-altering drug of choice to the NOC solution was to ensure that perfusing NOC alone would not wash out the myosin-altering drug. In those cells in which drugs were washed out, 5-10mL of *Mesostoma* Ringers solution was 15-20 minutes after the drugs were initially added.

5.3.4 Data Analysis:

We recorded live cells on DVDs and created time-lapsed images using VirtualDub (www.virtualdub.org). The position of each kinetochore was plotted relative to a fixed point at the cell edge where the centriole is (Ferraro-Gideon et al., 2013 A and B), using our in-house

program WinImage (Wong and Forer, 2003). Chromosome movement graphs were generated in the commercially available program SlideWrite 7.0 and the line of best fit, representing the speeds of chromosomes, were generated by the SlideWrite program. Adobe Photoshop was used to generate montages from a series of single images.

5.3.5 Analyzing bivalent movement:

As each bi-polarly attached bivalent chromosomes oscillate, co-aligned sister kinetochores on each set of sister chromatids (also known as a homologue) move towards and away from their respective poles. One complete oscillation is measured from peak to peak or trough to trough on the movement graph. The duration of one oscillation, also known as the period, is measured as the time between trough to trough or peak to peak. The distance a bivalent travels towards or away from the pole, or the amplitude, is measured as the distance from trough to peak to peak to trough. Refer to Figure 2. When NOC is added to cells, bivalents detach from one pole and quickly move towards the opposite pole. This fast bivalent movement to one pole shall be referred to throughout the text as “NOC-induced chromosome movement”.

5.3.6 Statistical Analysis:

Two-tail Student's t-tests were conducted to determine if the velocities, amplitudes and periods before and after drug and double-drug treatment were significantly different. For some data, the difference in amplitude, period and velocity were calculated as percentages. For this data, the percentages were first converted to proportions. All the proportions were converted into arc-sin values, and then t-tests performed on the arc-sin values (Sokal and Rohlf, 1995).

5.4 RESULTS

5.4.1 Control Cells

Mesostoma spermatocytes have three bivalent chromosomes positioned in the middle of the spindle, and two sets of univalent chromosomes positioned at either pole (Figure 1). A precocious pre-anaphase cleavage furrow is present throughout prometaphase (there is no metaphase plate as such), giving the cell its characteristic dumbbell-like shape (Figure 1). Each bivalent is bipolarly attached with each set of sister chromatids co-oriented towards their respective pole (Figure 1). Throughout prometaphase, the three bivalent chromosomes continuously oscillate towards and away from their respective poles (Fuge 1987, 1989; Ferraro-Gideon et al., 2013 A, 2014). The positions of the sister kinetochores can be tracked and plotted, as seen in Figure 2. In the experiments presented here the average speeds of bivalent oscillations towards the pole are statistically faster than bivalent oscillations away from the pole (7.7 $\mu\text{m}/\text{min}$ vs 6.4 $\mu\text{m}/\text{min}$ respectively) (Table 1). The average amplitudes and periods for prometaphase bivalent oscillations are 3.6 μm and 74 seconds (Table 1). These measurements are similar to those of other researchers (Table 1).

5.4.2 Single myosin-targeting drug treated cells

Various myosin inhibitors and enhancers altered velocities of *Mesostoma* bivalent oscillations towards and away from the pole, amplitudes of oscillations and periods of the oscillations. In general, myosin inhibitors decreased the velocities and amplitudes of oscillations, whereas the activator increased the velocities and amplitudes (Table 2, Figure 3A-E). The effects each drug had on bivalent oscillations will now be discussed in detail.

Effects of ML-7 and Y-27632 on bivalent oscillations

Cells were treated with 3 different concentrations of ML-7 (10, 15 & 20 μ M). There was a noticeable decrease in chromosome velocities towards and away from the pole and in the distance they moved. Bivalent oscillations became very irregular; kinetochores quickly jerked back and forth making the peaks very sharp rather than the typical smooth curve (Figure 4A). Similar results were obtained when primary spermatocytes were treated with 200 μ M Y-27632. Bivalent oscillations became irregular and jerky in comparison to the smoother movement observed directly before drug addition (Figure 4B). ML-7 affected one direction of movement slightly more than the other. The majority of the kinetochore velocities towards the pole decreased by somewhat more than those away from the pole (Table 2). Y-27632 seemed to have the opposite effect as the movement away from the pole was altered more than movement towards the pole. When ML-7 and Y-27632 were washed out from cells recovery was within 1-2 minutes. With most parameters recovery was to almost 100% of the original pre-drug treatment values. Because H7 had no effect, we conclude the effects of ML-7 were due to effects on MLCK.

Effects of cells treated with both ML-7 + Y-27632 simultaneously:

We treated cells with both to see if inhibiting multiple myosin inhibition pathways would have an additive effect to bivalent oscillations, or perhaps cause oscillations to completely stop. When both drugs were added together, the decrease in bivalent oscillations velocity and amplitude was not significantly different than cells treated with ML-7 and Y-27632 separately (Figure 4C). When primary spermatocytes were treated with both kinase inhibitors movements both towards and away from the pole were equally affected (Table 2). Since bivalent oscillations

did not completely stop, this suggests there are redundant pathways, as suggested by Sheykhani et al, (2013).

Effects of BDM on bivalent oscillations

20 mM BDM caused decrease in the velocities of both towards and away from the pole oscillations (Figure 4D, Table 2) and both directions were affected the same. The amplitudes of the oscillations decreased, and the period either increased or decreased. The period change varied greatly between cells. Bivalents within the same cell responded differently, and even the pairs of sister kinetochores on the same bivalent responded differently. For example, as the period of the sister kinetochores oriented towards the top pole increased, the sister kinetochores oriented towards the other pole decreased. Drug effects were observed within 1 minute of drug addition, and recovery was observed within 1-2 minutes after washout of BDM.

Effects of CalA on bivalent oscillations

Cells were treated with 10, 5, 2.5, and 1 nM concentrations of calyculin A. Observable effects of the drug were seen within 2 minutes of drug addition. There was an increase in velocities of the kinetochore movements both towards and away from the pole, and the amplitude also significantly increased (Figure 4E). Calyculin A did not affect one direction more compared to the other and the distribution was fairly even with the majority of velocities increasing by 81-90% (Table 2). All concentrations of CalA treatment caused a decrease in the period. In all concentrations the spindles collapsed after approximately 5 minutes. Okadaic acid was added to several cells to determine whether these results were obtained as a result of inhibiting PP1 or PP2A (Dounay and Forsyth, 2002). Okadaic acid (5 and 10 nM) had no major effects on bivalent oscillations or any other observable parameters, suggesting that the results seen with CalA are a result of inhibiting PP1 activity (results not shown).

5.4.3 NOC only treated cells

Chromosome movement following the addition of the MT depolymerizing drug NOC in *Mesostoma* primary spermatocytes follows a very specific 6-stage pattern of movement, as previously characterized (Fegaras and Forer, 2018a). Briefly, the bivalents stretch out towards their respective poles, pause at the poles for a period of time, then the bivalents detach from only one pole (and in some cases the univalent detaches as well), moving quickly at first and then slowing down as they approach the opposite pole (Figure 5A and B). This pattern of movement was consistent among all concentrations of NOC that was used (5 μ M, 10 μ M and 20 μ M). With increasing concentrations of NOC, the average time to detachment decreased whereas the speed of chromosome movement increased.

5.4.4 Double-drug, myosin targeting drug + NOC

In our experiments, *Mesostoma* primary spermatocytes were first treated with various myosin inhibitors/enhancers for approximately 5 minutes, then the cells were treated with a double-drug mixture of 10 μ M NOC + the original myosin inhibitor or enhancer. For example, Fig 6A-D show a cell treated with ML-7 then ML-7 + NOC. The bivalent chromosomes were oscillating in their usual fashion before addition of ML-7 (Figure 6A). The bivalent oscillations continued after addition of ML-7 in Figure 6B, but eventually slowed down (Fig 6C) over the next 5 minutes until ML-7 + NOC was added. After the addition of NOC in Figure 6D, the bivalents followed the usual pattern of NOC-induced detachment and movement to one pole, but at a significantly slower speed in comparison to the chromosome movement in Figure 5. These speeds will now be discussed in further detail.

Chromosomes in *Mesostoma* cells treated with NOC + ML-7 moved more slowly to the opposite pole as compared to NOC alone (Figure 7). To ensure this response was not due to a

side effect ML-7, we also treated cells with H-7. Chromosomes in these cells detached at a similar speed to cells treated with NOC alone. Both BDM and Y-27632 decreased chromosome detachment speed. In cells treated with NOC + CalA, chromosome movement sped up (Figure 7, Table 3).

Our experiments had some surprising results giving some insight into control of directionally of kinetochore movement. In addition to altering the speeds of movement to the opposite pole, myosin inhibitors also increased the time to detachment and completion of movement, and CalA decreased those times (Table 3). All myosin inhibitors had an effect on the numbers of poles chromosomes detached from. In cells treated with 10 μ M NOC alone, 64/72 cells detached from one pole (Table 3, Figure 8A). However, with myosin inhibitors + NOC bivalents became less likely to detach from only one pole and instead detached from 2 poles or did not detach at all. Treatment with CalA did not appear to have any effect on the likelihood of detachment from 1 pole. These results with myosin inhibitors and enhancers indicate that myosin is involved with chromosome movement in NOC-treated cells, in force production and in whether the kinetochores detach from one pole.

5.5 DISCUSSION

A main conclusion of this work is that myosin is involved in regular oscillations of bivalents during prometaphase and in NOC-induced chromosome movements in the absence of MTs. I used three different drugs that reduced myosin motor activity, ML-7, Y-27632, and BDM. All drugs significantly decreased bivalent oscillation velocities towards and away from the poles and reduced the amplitude of bivalent oscillations (Table 2). I also used one drug that increased myosin activity, CalA. This drug increased bivalent oscillation velocities towards and away from the pole and increased the amplitude of bivalent oscillations (Table 2). These same drugs had similar effects to the NOC-induced movement when added in combination with NOC (Figure 6 and Table 3). Could these changes in bivalent movement be due to non-specific effects of the drugs? The various drugs we used all act on different parts of myosin activation – MLCK, myosin ATPase, ROCK, and PP1 and PP2A (Saitoh et al., 1987; Higuchi and Takemori, 1989; Uehata et al., 1997; Ishihara et al., 1989). All the drugs have different side effects but the common denominator of all is they all effect myosin function in some way, therefore these effects are due to perturbation of myosin.

Further, I used several myosin targeting drugs which acted like controls. I treated various cells with H-7 which acts as a control for ML-7. H-7 effects other (non-MLCK) possible targets of ML-7 (Quick et al., 1992). The bivalent oscillation speeds in cells treated with H-7 had no detectable change and did not differ from controls (Table 2, Figure 7, Table 3), therefore the effect on bivalent oscillation speeds we see with ML-7 must be on MLCK. The same reasoning goes for CalA. I treated various cells with okadaic acid which has less of an effect on PP1 than PP2 (Dounay and Forsyth, 2002). Okadaic acid had no detectable effect on chromosome movement (Table 2), therefore the effect we see with CalA is due to PP1. Myosin has been

visualized in the spindles of various other animal cells (Xu et al., 2013; Silverman-Gavrila and Forer, 2003; reviewed in Forer et al., 2003; Sandquist et al., 2011), and until we know where myosin is localized in *Mesostoma* spindles in relation to actin, microtubules, and other spindle components, we cannot specify in detail how spindle myosin might function. Taken altogether, my results indicate the effects are on myosin function and therefore enforces our conclusion that myosin is involved in force generation for bivalent oscillations and for movement without MTs in NOC-induced movements.

This work also deals with two other main issues. The first is the issue of why the effects of ML-7 + Y-27632 are not additive (Table 2). ML-7 and Y-27632 block two different pathways of myosin phosphorylation, MLCK and ROCK (Saitoh et al., 1987; Uehata et al., 1997). In cells treated with both ML-7 + Y-27632, chromosomes did not move slower as one might expect. Since the chromosome velocities, amplitude and period of cells treated with both ML-7 + Y-27632 is not statistically different than cells treated with either ML-7 or Y-27632 alone (Table 2), this suggests there may be redundant myosin phosphorylation pathways in this cell. This has been previously demonstrated by Sheykhani et al, in crane-fly spermatocytes (2013). In their results, the addition of either ML-7 or Y-27632 caused a decrease in anaphase chromosomes speed. They then challenged this by adding CalA in the presence of either ML-7 or Y-27632. The chromosomes which were moving at a slower speed accelerated once CalA was added to the cell. This result shows that more than one phosphorylation pathway is used to activate myosin within the spindle to contribute to force production. It would be beneficial in future experiments with *Mesostoma ehrenbergii* to test for alternate pathways of myosin function, for example with drugs that target citron kinase and integrin-linked kinase (Davis et al., 2011; Fielding et al., 2008), and add them to cells in parallel with ML-7 and Y-27632 to see if movement can be

completely stopped. Likewise, in future experiments one could also challenge myosin inhibitors by adding CalA to determine if there are more phosphorylation pathways.

The second issue is the effect on sidedness. The anti-myosin drugs alter the poles that chromosomes move to: instead of all going to one pole, chromosomes are more likely to detach from both poles or no poles, as seen in Figure 8. This result is surprising and completely unexpected, although there may be a simple explanation. A completely different experimental treatment also altered the detachment from one pole in NOC-treated cells: disabling bivalent tethers (Fegaras-Arch et al, 2020, Chapter 4). In this experiment, I disabled bivalent tethers by cutting free arms. As more tethers were disabled, the likelihood of detachment from one pole also decreased. The study currently being reported is a radically different treatment but gave same results. Tethers are a relatively newly discovered component in dividing cells, and we know tethers put force on chromosome arms at the telomeres and from there to the kinetochore (Paliulis and Forer 2018). This force might be the common denominator: data presented herein suggests that myosin puts force on kinetochore to move during oscillations and NOC movements, and we know that tethers indirectly put force on kinetochores (via telomeres and arm stretching). My current experiment points to two important findings: (1) myosin produces force on kinetochores, as seen by the effects on oscillation parameters and on NOC-induced chromosome movements, and (2) whether all bivalent kinetochores go to the same pole are not only dependant on forces at/on kinetochores, but also forces that arise from the spindle apparatus, which includes myosin, and from tethers.

In conclusion, the purpose of these experiments was to better understand whether myosin is involved in the force production of chromosome movement in *Mesostoma ehrenbergii* spermatocytes. The activity of myosin was either inhibited or enhanced by adding various

myosin targeting drugs to live cells. Myosin appears to be involved in both regular chromosome movement and NOC-induced chromosomes movement because chromosome speed and various other parameters of chromosome movement was altered. My results also reinforce the finding that there appears to be redundant mechanisms of myosin phosphorylation, and show that the force myosin exerts on kinetochores may play a role in co-ordinating chromosome movement, much like tethers are speculated to (Paliulis and Forer 2018). Future work on visualizing myosin in *Mesostoma* spermatocytes and by performing more double drug experiments with CalA as suggested, may provide further insight into these findings.

5.6 FIGURES AND FIGURE LEGENDS

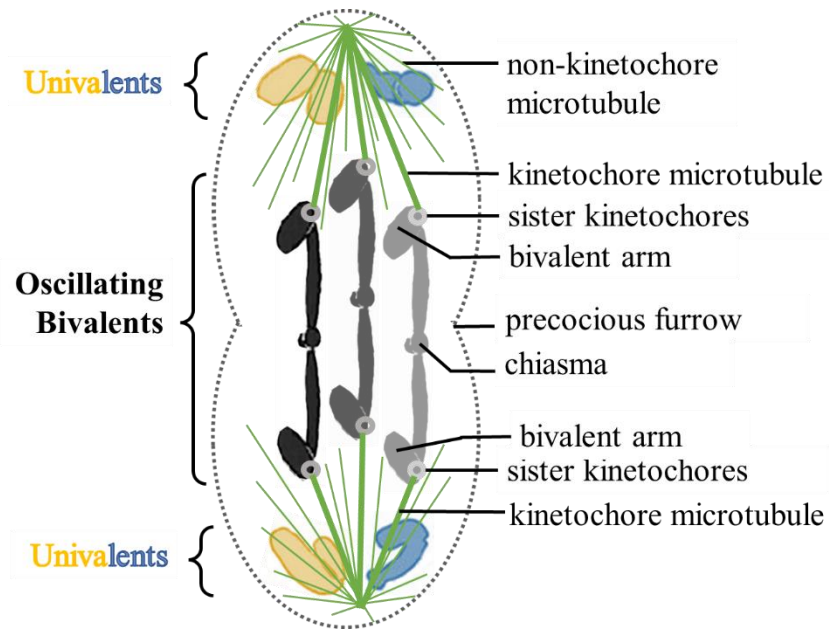


Figure 1. Illustration of a *Mesostoma* primary spermatocyte. There are three metacentric bivalents as shown in various shades of grey, and four univalents, with the two kinds indicated by blue and green. The various components of the cell are labelled. Image modified from Fegaras-Arch, Forer and Berns 2020.

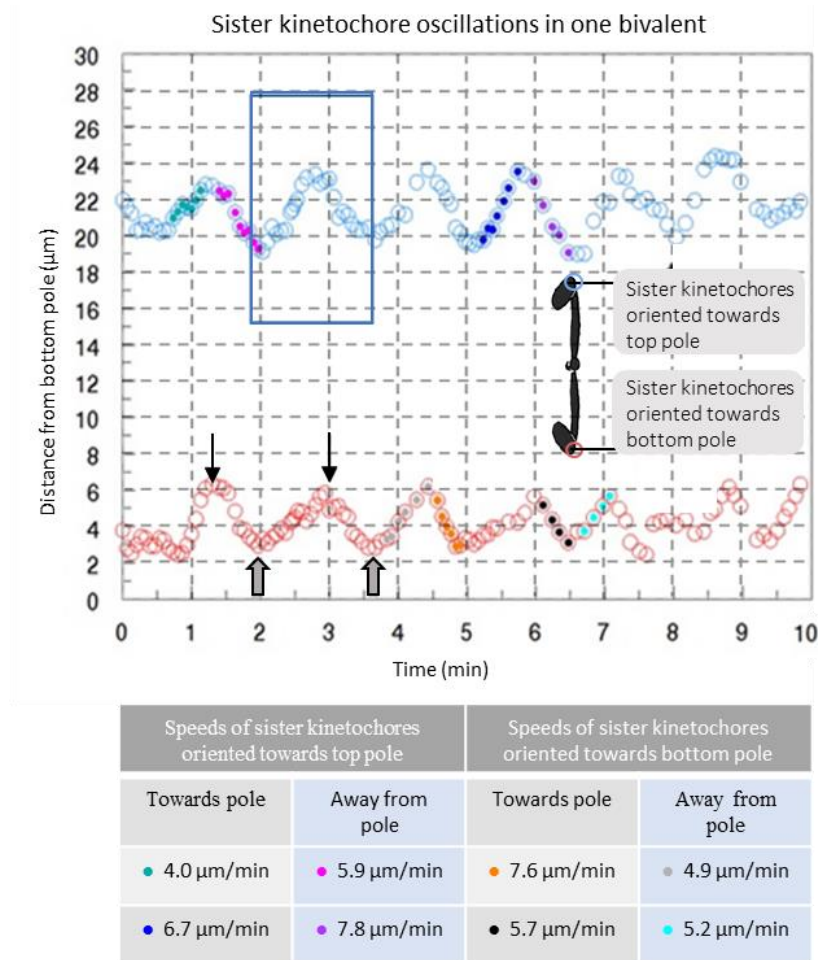


Figure 2. Graphical representation of typical bivalent oscillations during prometaphase, which appear as saw tooth waves. The positions of the top and bottom sister kinetochores of each bivalent are plotted approximately every 2 seconds and are shown as either blue open circle or red open circles. The solid filled circles of various colours located within the blue and red open circles represents the points we used to determine the lines of best fit and the corresponding kinetochore velocities (as indicated in the table below). The blue rectangle indicates one complete oscillation of the sister kinetochores oriented towards the top pole. The kinetochores first move towards the top pole, pause briefly near the top pole, then move away from the top pole. The duration of one oscillation, also known as the period, is measured as the time between trough to trough or peak to peak. The distance a bivalent travels during one oscillation, or the amplitude, is measured as the distance from trough to peak to peak to trough. The black arrows point to peaks and the grey arrows point to troughs.

Table 1. Comparison of various oscillation parameters for control *Mesostoma* spermatocyte bivalent kinetochores during prometaphase I. The data collected in the Fuge studies were taken from a different subspecies of *Mesostoma* and therefore reflect slightly different speeds, as discussed in Oakley (1985). † n= 15 cells; *p ≤ 0.01 for difference towards and away from the pole.

	Bivalent oscillations in control <i>Mesostoma</i> primary spermatocytes		
	Fuge (1987, 1989)	Ferraro-Gideon, Hoang, and Forer (2014)	Data from experiments present here†
Average velocity	8-10 µm/min	To pole: 6.24 µm/min Away from pole: 5.23 µm/min	To pole: 7.66 ± 2 (SD) µm/min* Away from pole: 6.44 ± 1.28 (SD) µm/min*
Average Amplitude	5-7 µm	4 µm	3.58 ± 0.55 (SD) µm
Average Period	1 minute 40 seconds	1 minute 32.5 seconds	1 minute 14 seconds

Table 2. Summary of the effects of myosin inhibitors and enhancing drugs on several parameters of bivalent movement, including the velocity, amplitude and period change. 3 cycles measured while drug present (approximately 5 minutes). No detectable change applies to values that did not change by greater than 5% before vs. during drug treatment. Values are $\pm$ SE, standard error. Statistical significance students t-test values * $p \leq 0.05$, ** $p \leq 0.01$

	Myosin Inhibiting Drugs					Myosin Activating Drugs	
	H-7	ML-7	Y-27632	ML-7 + Y-27632	BDM	CalA	Okadaic acid
Number of cells	6	20	11	6	10	9	7
Number of kinetochores	13	32	23	12	19	12	14
Velocities towards pole	No detectable change	Decrease (by 41%*)	Decrease (by 38% *)	Decrease (by 39%*)	Decrease (by 48%**)	Increase (by 89%**)	No detectable change
Velocities away from pole	No detectable change	Decrease (by 41%*)	Decrease (by 36%*)	Decrease (by 40%*)	Decrease (by 47%*)	Increase (by 85%**)	No detectable change
Amplitude	No detectable change	Decrease (by 46%*)	Decrease (by 40%*)	Decrease (by 41%*)	Decrease (by 62%**)	Increase (by 64%*)	No detectable change
Period	No detectable change	No detectable change	No detectable change	No data available	Decrease (by 52%*) -and- Increase (by 23%*)	Decrease (by 19.5%*)	No detectable change

Figure 3 A-C. Effects of single drugs on bivalent oscillations.

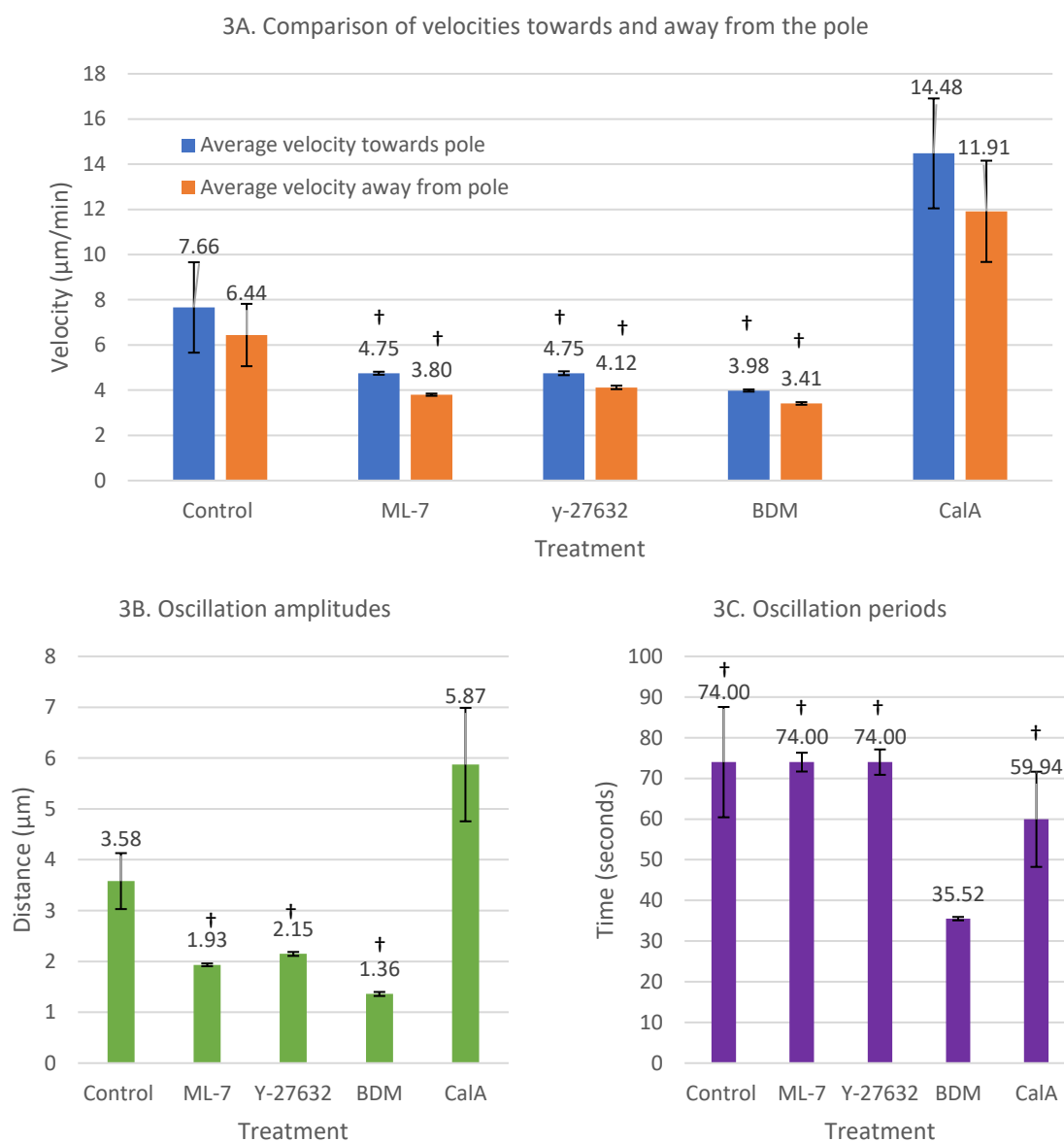
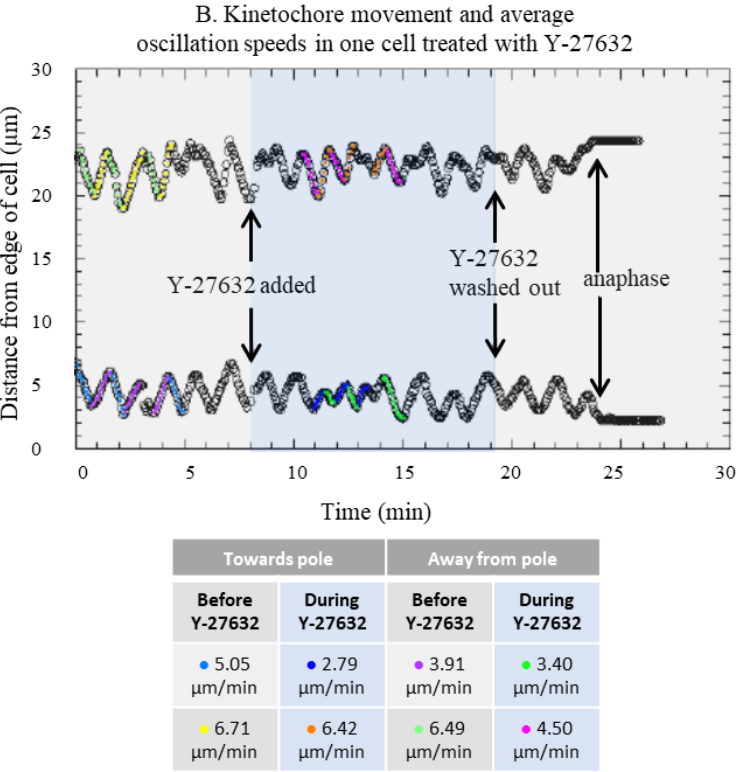
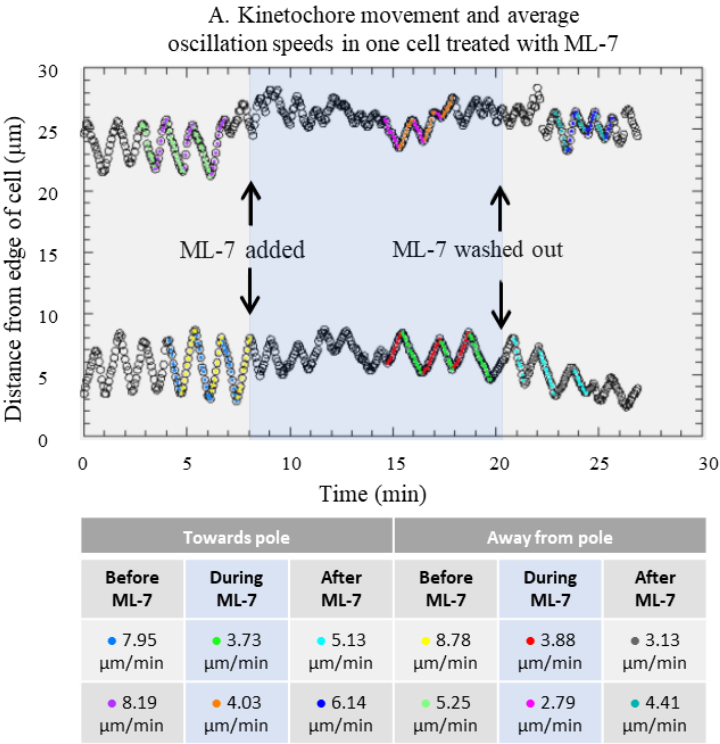
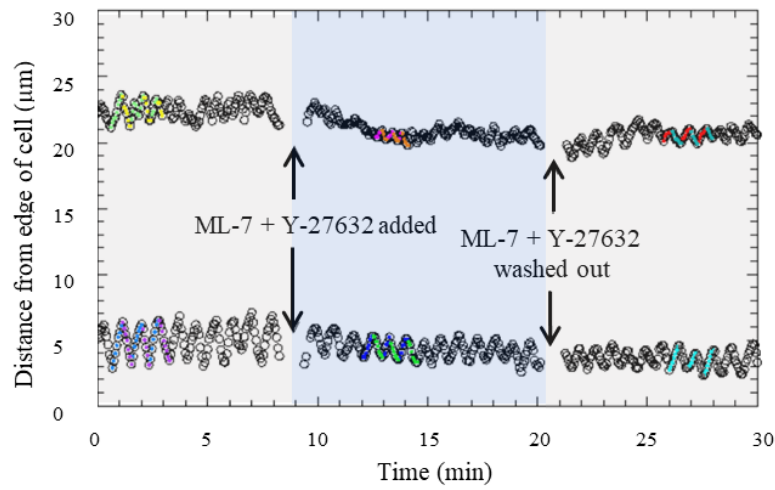


Figure 3 A-C. Graphs showing the average speeds towards and/or away from the pole, the average amplitude and the average period of *Mesostoma* spermatocytes under varying treatments. Error bars indicate $\pm$ standard error. † Represents values that are not significantly different, $p > 0.05$.

Figure 4A-F. Plots of Kinetochore movements.

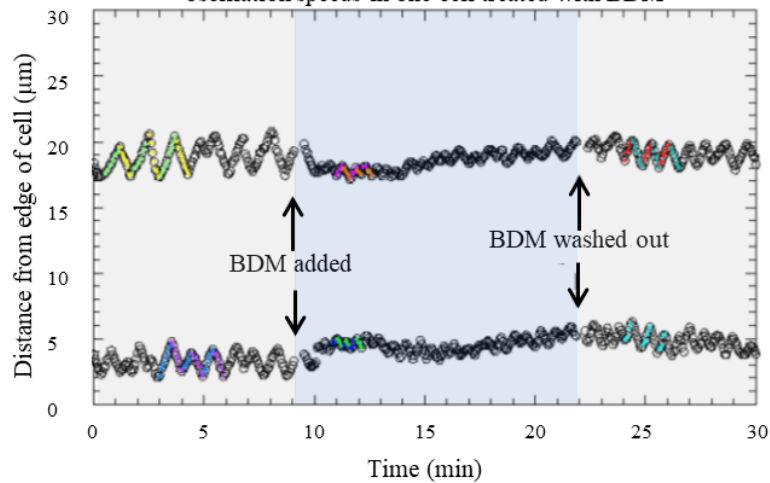


C. Kinetochore movement and average oscillation speeds in one cell treated with ML-7 + Y-27632



Towards pole			Away from pole		
Before ML-7 + Y-27632	During ML-7 + Y-27632	After ML-7 + Y-27632	Before ML-7 + Y-27632	During ML-7 + Y-27632	After ML-7 + Y-27632
● 8.25 $\mu\text{m}/\text{min}$	● 4.08 $\mu\text{m}/\text{min}$	● 5.17 $\mu\text{m}/\text{min}$	● 7.66 $\mu\text{m}/\text{min}$	● 4.66 $\mu\text{m}/\text{min}$	● 4.29 $\mu\text{m}/\text{min}$
● 4.59 $\mu\text{m}/\text{min}$	● 3.46 $\mu\text{m}/\text{min}$	● 2.39 $\mu\text{m}/\text{min}$	● 6.14 $\mu\text{m}/\text{min}$	● 3.12 $\mu\text{m}/\text{min}$	● 2.95 $\mu\text{m}/\text{min}$

D. Kinetochore movement and average oscillation speeds in one cell treated with BDM



Towards pole			Away from pole		
Before BDM	During BDM	After BDM	Before BDM	During BDM	After BDM
● 4.38 $\mu\text{m}/\text{min}$	● 2.52 $\mu\text{m}/\text{min}$	● 5.40 $\mu\text{m}/\text{min}$	● 4.12 $\mu\text{m}/\text{min}$	● 2.41 $\mu\text{m}/\text{min}$	● 4.72 $\mu\text{m}/\text{min}$
● 2.75 $\mu\text{m}/\text{min}$	● 2.24 $\mu\text{m}/\text{min}$	● 4.36 $\mu\text{m}/\text{min}$	● 5.85 $\mu\text{m}/\text{min}$	● 2.25 $\mu\text{m}/\text{min}$	● 5.04 $\mu\text{m}/\text{min}$

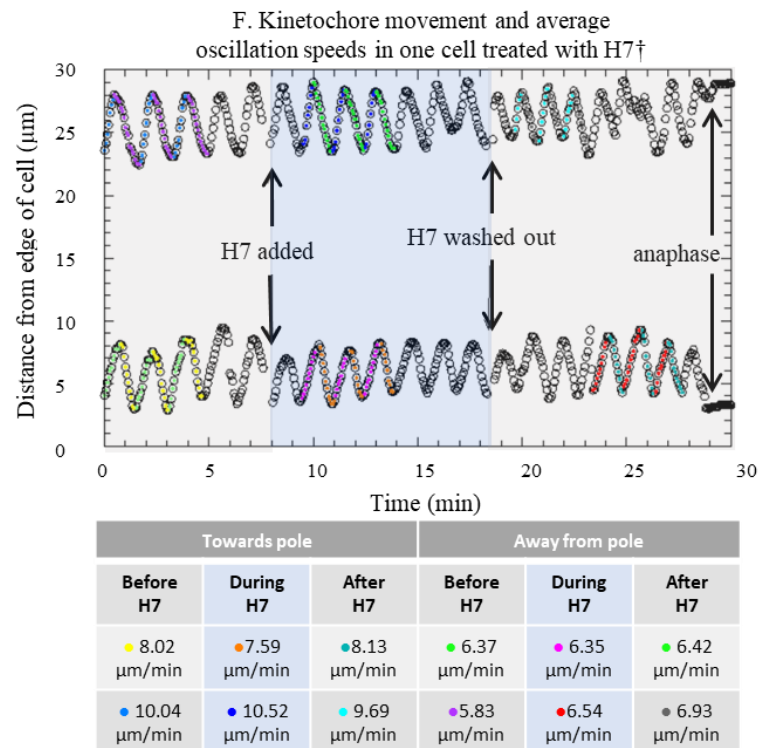
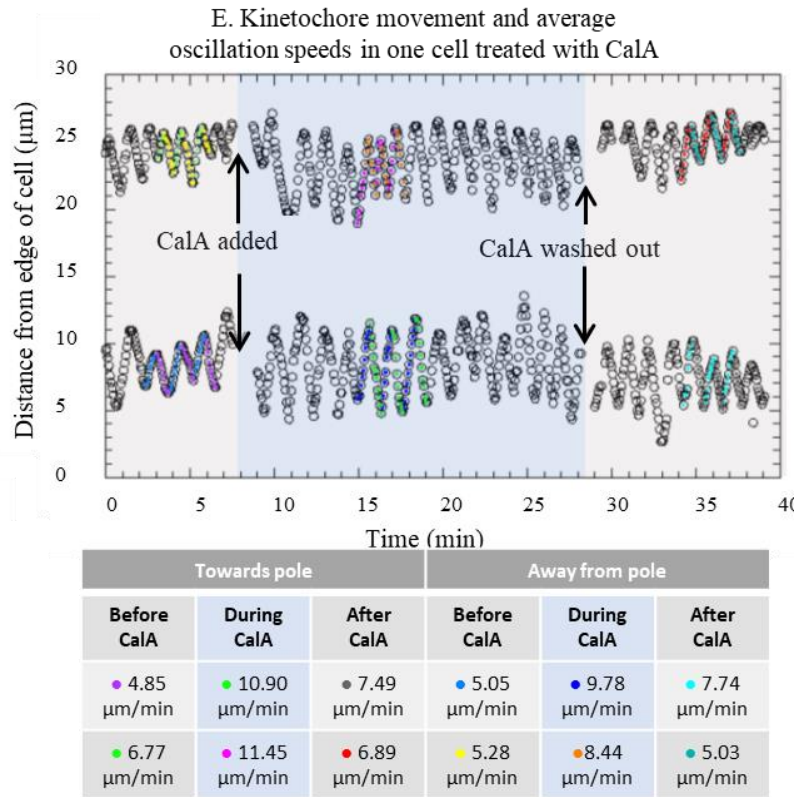
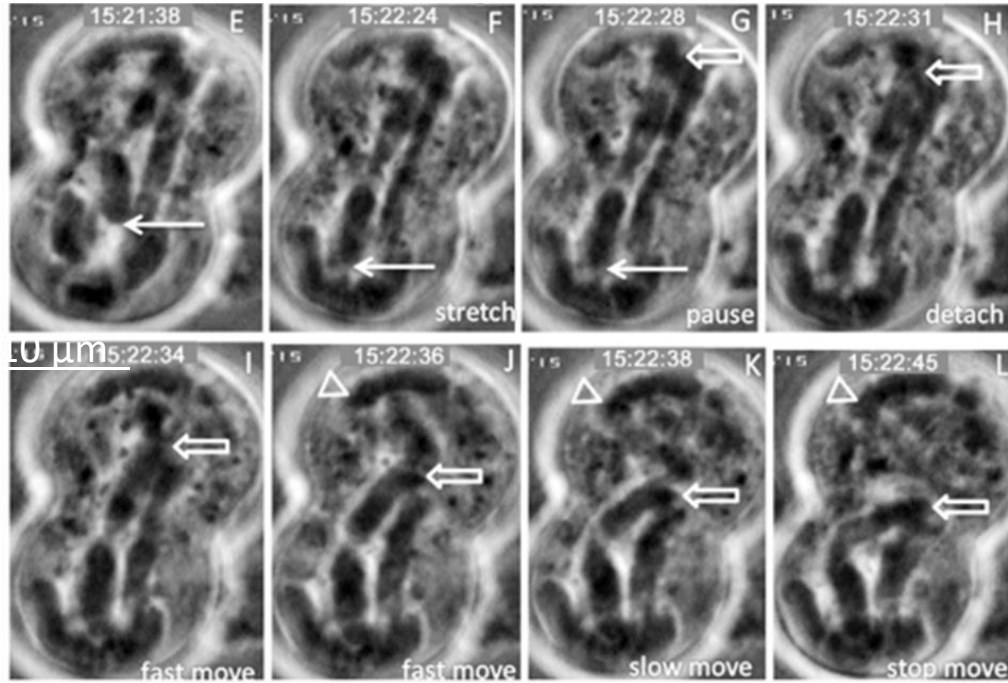


Figure 4 A-F Movement graphs of the top and bottom kinetochore of one bivalent in 6 different *Mesostoma* spermatocytes, treated with the indicated drug: ML-7, Y-27632, ML-7+Y-27632, BDM, CalA or H7. Arrows indicate when the drug was added and again when the drug was washed out with *Mesostoma* Ringers. The blue shaded regions in the graphs and tables indicates values taking during the addition of drug, and the grey shaded regions in the graphs and tables indicates values taking in the presence of *Mesostoma* Ringers with no drug present. In graph B and F. there is also an arrow that indicates when the cell goes through anaphase after drug was washed out. Tables below each graph show the average kinetochore velocities. Kinetochore speeds were taken from the lines of best fit that were measured as solid filled coloured circles in the graphs.

Figure 5 A and B. Figure montage and movement graph of typical NOC induced chromosome movement in *Mesostoma* spermatocytes.

A. Figure montage of a *Mesostoma* spermatocyte after the addition of NOC



B. Pattern of chromosome movement in the presence of 10 μ M NOC

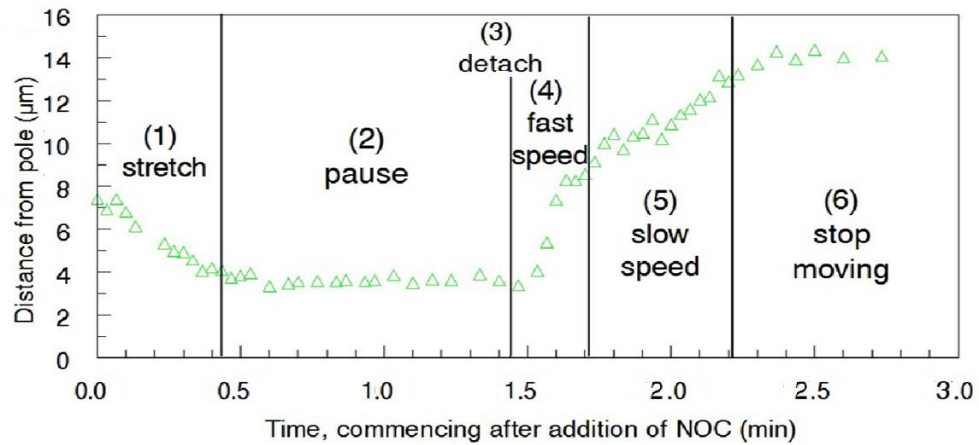


Figure 5 A and B. A. In this figure sequence, bivalent stretching towards the bottom pole is indicated by the thin arrow, and chromosome detachment from the top pole is shown by the thick arrow. The positions of the univalents are shown by the triangles, for this particular cell they remained at the top pole and the bivalents moved towards the bottom pole. Figure modified from Fegaras and Forer 2018a. B. Chromosomes respond to NOC addition in a very specific pattern, where the chromosomes first stretch out towards either pole, pause there for a period of time,

detach at only one pole and move extremely fast towards the opposite pole, slowing down as they reach the pole and then eventually stop.

Figure 6 A-D. Figure montage and movement graph of typical double drug chromosome movement in *Mesostoma* spermatocytes.

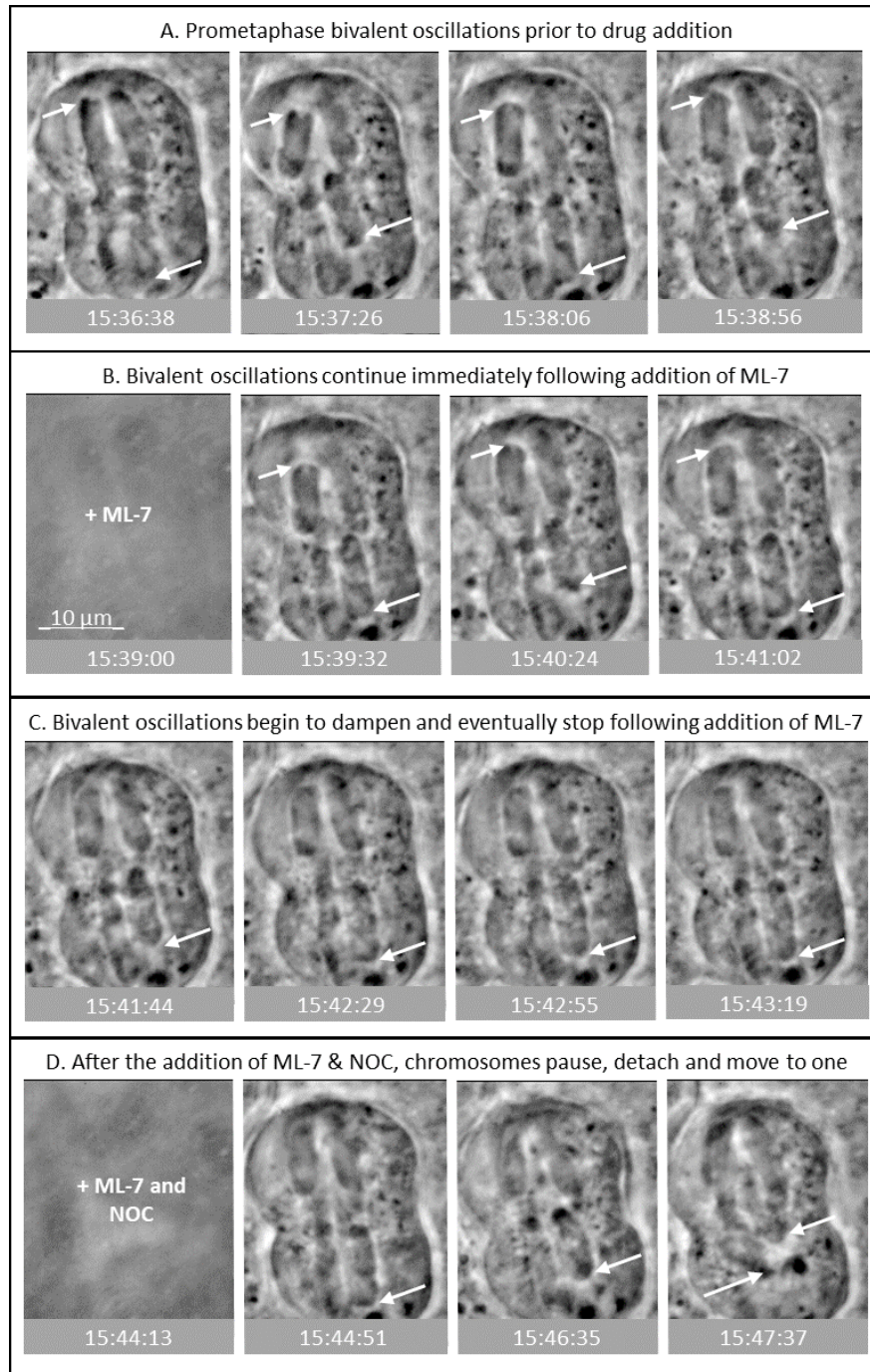


Figure 6 A-D. Image sequence of one cell treated with 10μM ML-7 then 10μM ML-7 + 10 μM NOC. White arrows point to the position of bivalent kinetochores. Time is shown in hours:minutes:seconds. In A, the bivalents at both the top and bottom poles oscillate towards and away from their respective pole in the usual fashion. In B, at 15:39:00, 10μM ML-7 is perfused onto the cell. The bivalents continue to oscillate immediately following drug addition, then the

oscillations begin to dampen in C. The bivalent at the bottom pole does not oscillate as far away from the pole as it did prior to drug addition. In D, at 15:44:13, 10 μ M ML-7 + 10 μ M NOC is perfused onto the cell. Immediately following addition of NOC the bivalents first pause, then detach from the bottom pole and move towards the top pole. Both visible bivalents move towards the top pole.

Figure 7. Bivalent speeds in *Mesostoma* spermatocytes

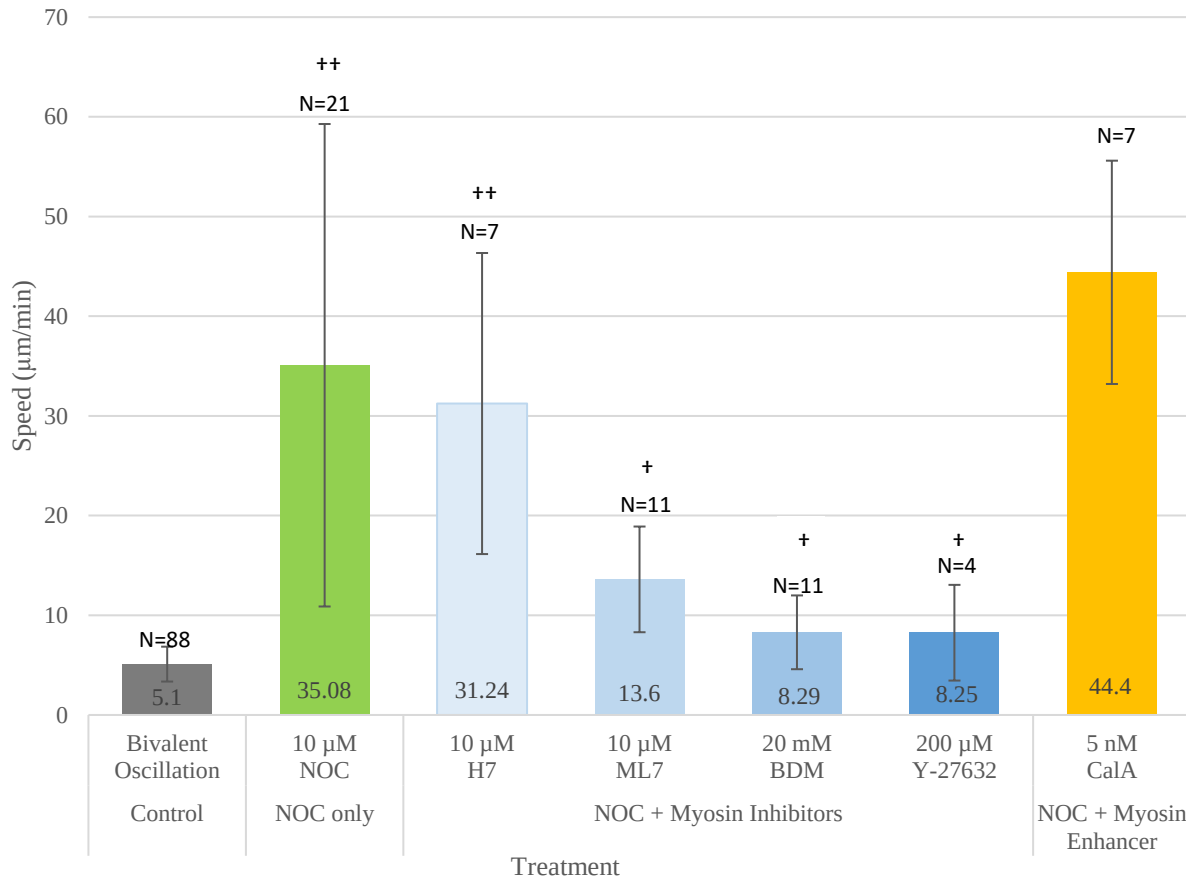


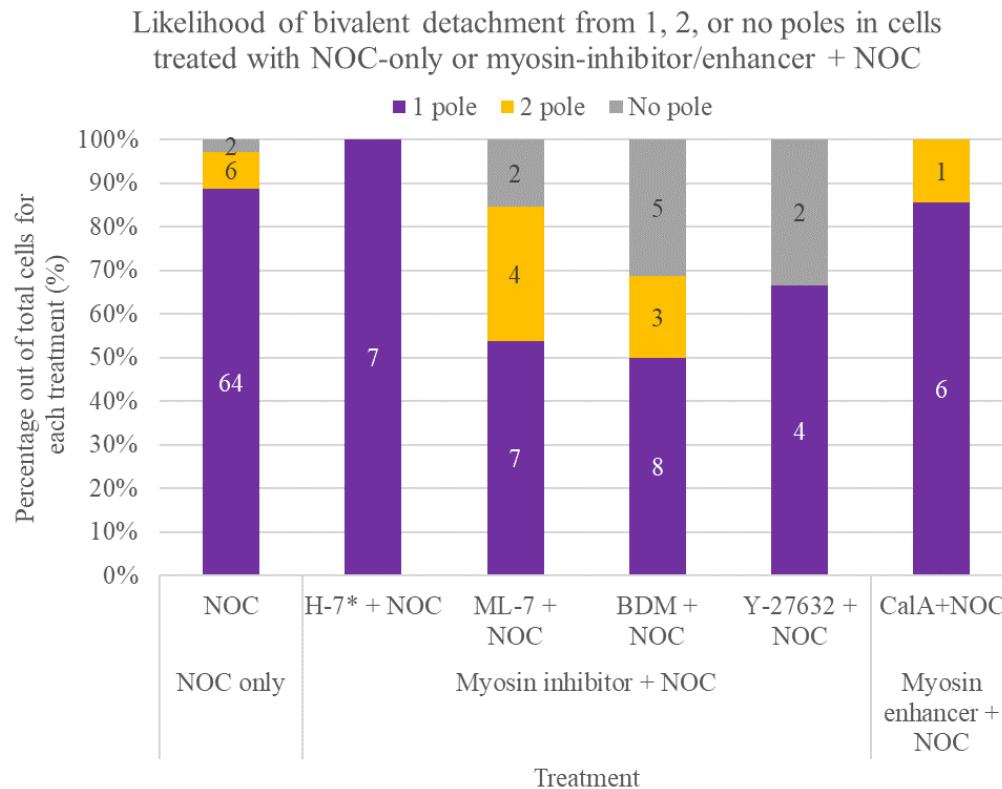
Figure 7. Myosin inhibitors + 10 μM NOC decrease chromosome speeds, and myosin enhancer + 10 μM NOC increase chromosome speeds. Graph is of speeds in cells that had detachment. Cells with no detachment or no bivalent movement after addition of NOC were excluded. The average speeds are included at the base of each bar in the bar graph, and standard deviations are represented by the error bars. N = cell count. Data for 10 μM NOC was taken from Fegaras and Forer 2018a. Data for bivalent oscillations was taken from Ferraro-Gideon et al, 2013 † and †† values are not statistically significant using Students T-test, $p > 0.05$. H7 is a control for ML-7. Cells treated with H7 + NOC are not statistically different from NOC treated cells.

Table 3. Myosin inhibitors + 10 μ M NOC increase time from drug addition to chromosome detachment and time to complete movement. Myosin enhancer + 10 μ M NOC decrease both of the above. Also, depending on the myosin inhibitor, chromosomes will more likely detach from 2 poles or that they will detach from no poles as opposed to only 1 pole. CalA does not appear to alter the number of poles that chromosomes detach from. Data for 10 μ M NOC was taken from Fegaras and Forer 2018a. Times are indicated by minutes:seconds. * Values are statistically significant using Students T-test, $p < 0.05$. † H7 is a control for ML7. Cells treated with H7 + NOC are not statistically different from NOC treated cells.

	Drug (n=bivalent count)	Time from NOC addition to detachment (min:sec)	Bivalents detach from:			Time for completion of movement (min:sec)	Speed of chromosome movement (μ m/min) $\pm$ SD and range
			No poles	1 pole	2 poles		
Control	10 μ M NOC (n=21)	02:06 $\pm$ 00:50*	2	64	6	01:20 $\pm$ 00:51*	35.1 $\pm$ 24.2* (4.3-97.8)
Myosin inhibiting drug treatment	10 μ M H7† + 10 μ M NOC (n=7)	2:45 $\pm$ 00:39	0	7	0	01:37 $\pm$ 00:26	31.24 $\pm$ 15.1 (6.5-56.2)
	10 μ M ML7 + 10 μ M NOC (n=13)	4:32 $\pm$ 01:45*	2	7	4	03:32 $\pm$ 01:52*	13.6 $\pm$ 5.3* (6.6-21.5)
	20mM BDM + 10 μ M NOC (n=16)	5:07 $\pm$ 02:10*	5	8	3	03:56 $\pm$ 02:33*	8.9 $\pm$ 3.7* (2.5-13.4)
	200 μ M Y-27632 + 10 μ M NOC (n=6)	4:37 $\pm$ 02:02*	2	4	0	03:30 $\pm$ 02:01*	8.25 $\pm$ 4.8* (3.1-16.2)
Myosin enhancer	1nM CalA + 10 μ M NOC (n=7)	1:30 $\pm$ 00:36*	0	6	1	0:45 $\pm$ 00:37*	44.4 $\pm$ 11.2* (32.6-66.4)

Figure 8 A and B. Likelihood of detachment from 1 pole decreases when NOC is added in conjunction with various myosin-inhibiting drugs.

A



B

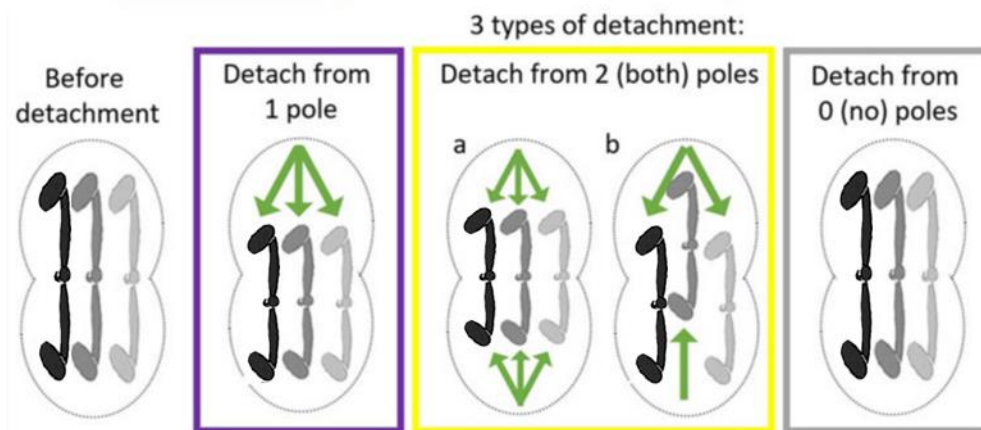


Figure 8 A and B. Likelihood of detachment from 1 pole decreases when NOC is added in conjunction with various myosin-inhibiting drugs. NOC-only data taken from Fegaras-Arch and Forer 2020. Numbers in the middle of each bar indicate the actual number of cells in each condition, which is then converted to a percentage of total cells on the y axis. * H7 is a control for ML7. Diagram is modified from Fegaras-Arch, Berns and Forer 2020. B. Diagram illustrating the 3 types of bivalent detachment. Green arrows at the poles indicate the direction of bivalent movement.

5.7 REFERENCES

- Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem.* 271: 20246
- Brady M, Paliulis L. 2015. Chromosome interaction over a distance in meiosis. *Royal Society Open Science.* 2: 1-10.
- Cremona CR, Hartshorne DJ. 2008. Myosins: A Superfamily of Molecular Motors. 7: 171- 222.
- Davis MI, Hunt JP, Herrgard S, Ciceri P, Wodicka LM, Pallares G, Hocker M, Treiber DK, Zarrinkar PP. 2011. Comprehensive analysis of kinase inhibitor selectivity. *Nat. Biotechnol.* 29: 1046-1051.
- Dounay A. 2002. Okadaic acid: the archetypal serine/threonine protein phosphatase inhibitor. *Current Medicinal Chemistry.* 9: 1939-1980.
- Fabian L, Forer A. 2007. Possible roles of actin and myosin during anaphase chromosome movements in locust spermatocytes. *Protoplasma.* 231:201–213.
- Fegaras E, Forer A. 2018 A. Chromosomes selectively detach at one pole and quickly move towards the opposite pole when kinetochore microtubules are depolymerized in *Mesostoma ehrenbergii* spermatocytes. *Protoplasma.* 255(4):1205-1224
- Fegaras E, Forer A. 2018 B. Precocious cleavage furrows simultaneously move and ingress when kinetochore microtubules are depolymerized in *Mesostoma ehrenbergii* spermatocytes. *Protoplasma.* 255:1401–1411
- Fegaras-Arch E, Berns M, Forer A. 2020. Evidence of non-microtubule spindle forces in *Mesostoma ehrenbergii* spermatocytes. *Front. Mol. Biosci.* 7:557990.
- Ferraro-Gideon J, Hoang C, Forer A. 2014. Meiosis-I in *Mesostoma ehrenbergii* spermatocytes includes distance segregation and inter-polar movements of univalents, and vigorous oscillations of bivalents. *Protoplasma.* 251: 127-143.
- Ferraro-Gideon J, Hoang C, Forer A. 2013. *Mesostoma ehrenbergii* spermatocytes--a unique and advantageous cell for studying meiosis. *Cell Biol Int.* 37(9): 892-898.
- Ferraro-Gideon J, Sheykhan R, Zhu Q, Duquette ML, Berns MW, Forer A. 2013. Measurements of forces produced by the mitotic spindle using optical tweezers. *Mol Biol Cell.* 24: 1375-1386.

- Fielding A, Dobрева I, McDonald P, Foster L, Dedhar S. 2008. Integrin-linked kinase localizes to the centrosome and regulates mitotic spindle organization. *J Cell Biol.* 180: 681-689.
- Forer A, Fabian L. 2005. Does 2,3-butanedione monoxime inhibit nonmuscle myosin? *Protoplasma.* 225: 1-4.
- Forer A, Pickett-Heaps JD. 2010. Precocious (pre-anaphase) cleavage furrows in *Mesostoma* spermatocytes. *Eur J Cell Biol.* 89: 607–618.
- Forer A, Pickett-Heaps JD. 2005. Fibrin clots keep non-adhering living cells in place on glass for perfusion or fixation. *Cell Biol Int.* 29:721–730.
- Forer A, Spurck T, Pickett-Heaps JD, Wilson PJ. 2003. Structure of kinetochore fibres in crane-fly spermatocytes after irradiation with an ultraviolet microbeam: neither microtubules nor actin filaments remain in the irradiated region. *Cell motility and the cytoskeleton.* 56(3), 173–192.
- Fuge H. 1987. Oscillatory movements of bipolar-oriented bivalent kinetochores and spindle forces in male meiosis of *Mesostoma ehrenbergii*. *Eur J Cell Biol.* 44:294- 298.
- Fuge H. 1989. Rapid kinetochore movements in *Mesostoma ehrenbergii* spermatocytes: action of antagonistic chromosome fibre. *Cell Motil Cytoskeleton.* 13:212- 220.
- Gorbsky GJ, Sammak PJ, Borisy GG. 1988. Microtubule dynamics and chromosome motion visualized in living anaphase cells. *J. Cell Biol.* 106: 1185-1192.
- Higuchi H, Takemori S. 1989. Butanedione monoxime suppresses contraction and ATPase activity of rabbit skeletal muscle. *J Biochem.* 105(4):638-43.
- Husted L, Ruebush TK. 1940. A comparative cytological and morphological study of *Mesostoma ehrenbergii ehrenbergii* and *Mesostoma ehrenbergii wardii*. *J Morphol.* 67: 387-410.
- Ikebe M, Hartshome DJ. 1985. Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. *JB iol Chem.* 260(18):10027-31.
- Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto K, Uemura D. 1989. Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. *Biochem Biophys Res Commun.* 159(3):871-7.
- 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.

- Komatsu S, Yano T, Shibata M, Tuft RA, Ikebe M. 2000. Effects of the regulatory light chain phosphorylation of myosin II on mitosis and cytokinesis of mammalian cells. *J Biol Chem.* 275: 34512-20.
- Mitchison TJ, Maddox P, Gaetz J, Groen A, Shirasu M, Desai A, Salmon ED, Kapoor TM. 2005. Roles of polymerization dynamics, opposed motors, and a tensile element in governing the length of *Xenopus* extract meiotic spindles. *Mol Biol Cell.* 16:3064–3076.
- Pickett-Heaps J, Forer A. 2009 Mitosis: spindle evolution and the matrix model. *Protoplasma.* 235(1-4):91–99.
- Quick J, Ware JA, Driedger PE. 1992. The structure and biological activities of the widely used protein kinase inhibitor H-7 differ depending on the commercial source. *Biochem Biophys Res Commun.* 187:657–663.
- Rieder CL, Salmon ED. 1994. Motile kinetochores and polar ejection forces dictate chromosome position on the vertebrate mitotic spindle. *J Cell Biol.* 124(3):223–233.
- Saitoh M, Ishikawa T, Matsushima S, Naka M, Hidaka H. 1987. Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. *J Biol Chem.* 262:7796–7801.
- Sakurada K, Seto M, Sasaki Y. 1998. Dynamics of myosin light chain phosphorylation at Ser19 and Thr18/Ser19 in smooth muscle cells in culture. *Am. J. Physiol.* 274:1563-1572.
- Sandquist JC, Kita AM, Bement WM. 2011. And the dead shall rise: actin and myosin return to the spindle. *Dev Cell.* 21:410–419.
- Sellers JR. 1991. Regulation of cytoplasmic and smooth muscle myosin. *Current opinion in cell biology.* 3(1), 98–104.
- Sheykhan R, Shirodkar PV, Forer A. 2013. The role of myosin phosphorylation in anaphase chromosome movement. *Eur J Cell Biol.* 92(4-5):175–186.
- Silverman-Gavrila RV, Forer A. 2000. Evidence that actin and myosin are involved in the poleward flux of tubulin in metaphase kinetochore microtubules of crane-fly spermatocytes. *J Cell Sci.* 113: 597–609.
- Silverman-Gavrila RV, Forer A. 2003. Myosin localization during meiosis I of crane-fly spermatocytes gives indications about its role in division. *Cell Motil Cytoskeleton.* 55:97–113.
- Sokal RR, Rohlf FJ. 1995. *Biometry: The Principles and Practice of Statistics in Biological Research.* 3rd Edition, W.H. Freeman and Co., New York.

- Straight AF, Cheung A, Limouze J, Chen I, Westwood NJ, Sellers JR, Mitchison TJ. 2003. Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. *Science*. 299(5613): 1743–1747.
- Tan JL, Ravid S, Spudich JA. 1992. Control of nonmuscle myosins by phosphorylation. *Annu Rev Biochem*. 61:721-59.
- Trybus KM. 1991. Regulation of Smooth Muscle Myosin. *Cell Motility and the Cytoskeleton*. 18:81-85.
- Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. 1997. Calcium sensitization of smooth muscle mediated by rho-associated protein kinase in hypertension. *Nature*. 389: 990-994.
- Vasquez R, Howell B, Yvon AC, Wadsworth P, Cassimeris L. 1997. Nanomolar concentrations of nocodazole alter microtubule dynamic instability in vivo and in vitro. *Mol Biol Cell*. 8(6):973–985
- Wong R, Forer A. 2003. “Signalling” between chromosomes in crane-fly spermatocytes studies using ultraviolet microbeam irradiation. *Chromosome Res*. 11:771–786.
- Xie L, Forer A. 2008. Jasplakinolide, an actin stabilizing agent, alters anaphase chromosome movements in crane-fly spermatocytes. *Cell Motility and the Cytoskeleton*. 65(11):876-889.
- Xu C, Liu Z, Zhang L, Zhao C, Yuan S, Zhang F. 2013. Organization of actin cytoskeleton during meiosis I in a wheat thermo-sensitive genic male sterile line. *Protoplasma*. 250:415–422.
- Yang H, Ganguly A, Cabral F. 2010. Inhibition of cell migration and cell division correlates with distinct effects of microtubule inhibiting drugs. *J Biol Chem*. 285(42):32242–32250.

GENERAL DISCUSSION

A. Overview

My thesis deals with better understanding the mechanisms and proteins involved in producing the force that drives chromosome movement during meiosis. I investigated the role of microtubules because they are canonically viewed as the main mechanism of chromosome movement. I developed and characterized a cell system devoid of microtubules by using NOC. Using this novel approach, I was able to show chromosomes can not only move, but can move even faster in the absence of microtubules. I was also able to illustrate that myosin and actin appear to be the main cause of chromosome movement because chromosomes sped up or slowed down when enhancers or inhibitors were added to these microtubule-free cells. These results directly illustrate that microtubules are not the main mechanism of chromosome movement but rather act as governors that limit the speed of chromosome movement, since chromosomes move faster in the absence of microtubules. The importance of my main findings are they refute the microtubule-based models for chromosome movement and instead support the spindle matrix model by suggesting that actin and myosin produce the forces for chromosome movement rather than the canonically accepted microtubules (Picket-Heaps and Forer 2009). However, more work needs to be done to accurately define the mechanisms of chromosome movement.

My thesis also provides insight into other key cell division theories such as non-random segregation, mechanisms of furrow ingression, and tethers. I was also able to show precocious furrow can ingress and move in the absence of microtubules (Fegaras and Forer 2018b). By using the femtosecond laser, I was the first to show the presence of tethers that extend along the free arms of a bivalent, and I showed that tethers may play a role in co-ordinating chromosome

movement because disconnecting a tether significantly decreases the likelihood of detachment from 1 pole.

B. Discussion

In *Mesostoma* spermatocytes microtubules depolymerize in the presence of 10 μM NOC and chromosomes detach from one pole and move towards the opposite pole at an average speed of 35.1 $\mu\text{m}/\text{min}$, which is over 6x faster than in control cells (Fegaras and Forer 2018a). Since similar results were found using the microtubule-depolymerizing drug colcemid, this suggests this result is due to targeting microtubules. Current theories of chromosome movement cannot account for these findings since they all assume microtubules produce the forces to move chromosomes (Gorbsky et al., 1988; Mitchison et al. 2005). Instead of microtubules, what could be providing the force for these chromosome movements? We have argued in Fegaras and Forer (2018a) that the fast movements after NOC are not due to release of chromosome tension, as shown by the way chromosomes curl towards one side while moving (Figure 4B in Fegaras and Forer, 2018a). My more recent experiments have more definitively ruled out this possibility.

Movement is not due to recoil of stretched chromosomes because when 1, 2, or 3 bivalents are cut, the ‘halved’ bivalents are still able to detach from one pole and move toward the opposite pole at about the same speed or faster than non-cut bivalents in NOC treated cells, the average speed of which is 47.0 $\mu\text{m}/\text{min}$ (range 12.8 - 104.3 $\mu\text{m}/\text{min}$). On average, non-severed bivalents in cells which contain bivalent ‘halves’ move faster in comparison to cells in which no bivalents have been severed, but not-severed bivalent(s) and halved bivalent(s) in the same cell move at almost identical speeds. The difference in average speeds for the non-cut bivalents in these two conditions could be an artefact due to other conditions – e.g., different temperatures for the cells in the two populations, or different timing during prometaphase – but if

not, it indicates that for some reason we don't yet understand that kinetochore speeds are faster when there are halved bivalents in the cell than when there are none. Regardless, since the 'halves' and the non-cut bivalents move at the same (or very similar) speeds, this indicates that similar forces act on the kinetochores of the moving halved bivalents and full bivalents.

The force for these kinetochore movements is not from microtubules. NOC treatment removes microtubules from the spindle: as deduced from immunofluorescence staining, most spindle microtubules are depolymerised by the NOC, any that remain are highly fragmented and not attached to the pole, and none are in contact with the moving chromosomes (Fegaras and Forer 2018a). Since 'halved' bivalents move in the absence of spindle microtubules at the same speed as non-severed bivalents, since there is no stretching or recoil possible in the 'halved' bivalents, and since no microtubules are present, the movement is due to some force different from microtubules.

I suggest that what may be moving the chromosomes is actin and myosin, perhaps as components of the spindle matrix. In the spindle matrix model, microtubules play a more passive role in chromosome movement (Johansen and Johansen 2007; 2011, Pickett-Heaps and Forer 2009, Spurck et al 1997) and what may instead be providing the force for chromosome movement is actin, myosin or some other spindle matrix proteins (Forer et al 2015).

I have not tested for the presence of spindle matrix proteins directly since I have no tools to test for their function in *Mesostoma* spermatocytes. But perturbing these proteins in *Drosophila melanogaster* interferes with normal spindle formation and chromosome segregation (Rath et al., 2004; Ding et al., 2009). Data presented in this thesis suggest that some structure underlies the spindle, to which myosin binds, and, given that a matrix exists, it is possible that the matrix provides a substrate for motor proteins to interact with. The focus of my work instead

was focusing on actin and myosin's involvement in chromosome movement. We can test this speculation by using various enhancers and inhibitors that target actin and myosin in NOC-treated cells. If perturbing these proteins alters chromosome movement that would support our suggestion that actin and myosin play a role in the chromosome movements that occur after NOC treatment.

We tested for the role of myosin using various anti-myosin and myosin enhancing drugs. We reasoned that if BDM, ML-7, and Y-27632 all have the same effect on chromosome movement, then the effect must be via myosin and not an alternate effect of the drugs. The speed of bivalent detachment decreased, and the time to detachment increased with all these drugs (Chapter 5, Table 2). H-7, which effects other (non-MLCK) possible targets of ML-7 (Quick et al., 1992), had no effect, further confirming this conclusion. Also, preliminary results with CalA, a myosin enhancer, show it increases chromosome speed (Chapter 5, Table 2). Myosin has been visualized in the spindle of various other animals, (Xu et al., 2013; Silverman-Gavrila and Forer, 2003; reviewed in Forer et al., 2003; Sandquist et al., 2011) and until we know where myosin is localized in *Mesostoma* spindles in relation to actin, microtubules, and other spindle components, we cannot specify in detail how spindle myosin might function; nonetheless, myosin appears to be involved in NOC-induced chromosomes movements.

Figure 1 outlines a general movement model illustrating how chromosomes may move in *Mesostoma* spermatocytes. In this proposed model, the microtubules are cross-linked to actin, and myosin directly interacts with the spindle matrix (Johansen and Johansen 2007). Thus, in this system, myosin acts indirectly on the microtubules and can account for our findings where microtubules impede chromosome movement (Forer et al., 2008). This model is also consistent

with other findings using the laser and UV microbeam (Forer et al., 2008, Sheykhani et al., 2013).

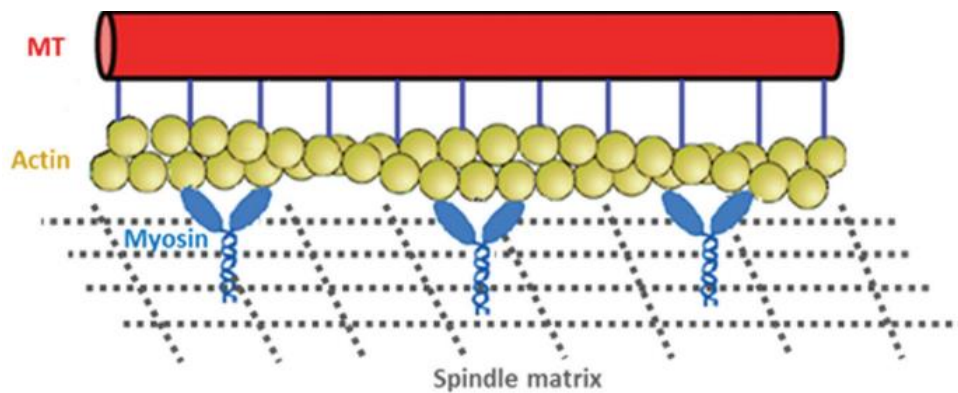


Figure 1. A general model illustrating how chromosomes may move in the spindle matrix model and the interaction between microtubules in red, myosin in blue, and actin in yellow. Figure modified from Sheykhani et al., 2013.

Just as this data questions the involvement of microtubules in chromosome movement, it also questions the models of cell cleavage that requires spindle microtubules to form the cleavage furrow. We also sought to answer the question, why is NOC-induced chromosome movement almost always towards only one pole? This may be because *Mesostoma* spermatocytes are speculated to divide via non-random segregation (Brady and Paliulis, 2015). The main pieces of evidence are univalents re-orient too many times during one cell division to be due to chance and univalents of the same kind switch positions (Oakley 1985), and bivalent reorientations occur wherein kinetochores on attached bivalents switch the poles to which they are attached (Ferraro-Gideon et al., 2014). Non-random segregation in *Mesostoma* spermatocytes may explain why chromosome movement was selectively and consistently to only one pole in 64/72 cells treated with NOC (Fegaras and Forer, 2018a). This sidedness may be an indicator that spindle organization is different at each pole, or that the force distribution in the cell is asymmetric and oriented towards one pole.

The precocious cleavage furrow may also provide an indication of unequal force distribution within the cell. Although the formation of a precocious cleavage furrow is not unique, for example precocious furrows are also present in *C. elegans* spermatocytes (Wang et al 2021), what is unique is that the *Mesostoma* spermatocyte cleavage furrow can move in response to imbalances brought about by chromosome reorientations. Perhaps, the matrix can sense the differences in forces at the two poles when unequal numbers of chromosomes and thereby causing the furrow to move along the cell.

Another possibility is it may be due to tethers, which are a recently discovered universal component in dividing animal cells (Forer et al., 2017). My research was the first to show tethers are present connecting the telomeres of bivalent arms in *Mesostoma* primary spermatocytes during prometaphase and anaphase, of which I convincingly did (Forer et al., 2017). Since tethers are speculated to aid in co-ordinating chromosome movements (Sheykhani et al., 2017; Paliulis and Forer, 2018; Forer and Berns, 2020) we speculated that tethers may play a key role in co-ordinating these NOC-induced chromosome movements to one pole. If they do, then severing tethers would affect the likelihood of detachment from one pole and maybe not be able to detach at all. In my results, when the tether is disabled, not all the chromosomes in the cell move. This tether finding may also provide insight into the results when I cut bivalents. In cells with one bivalent cut in half then treated with 10 μ M NOC, 12 out of 16 cells had bivalent detachment from only one pole. As 2 and 3 bivalents are cut, and the likelihood of detachment from only one pole appears to decrease, Thus, I think that tethers, and the tension they produce between two arms of each bivalent, may be involved in co-ordinating chromosome movement to one pole after treatment with NOC.

Tethers have different states where they can be either inelastic or elastic. Only by disabling an elastic tether is there an effect on chromosome detachment from one pole after NOC addition. Short tethers, in early anaphase, are elastic; longer tethers, in later anaphase, are inelastic (Forer et al., 2017). The difference in elasticity states is related to phosphorylation and dephosphorylation events (Kite and Forer, 2020). The timing of tether phosphorylation / dephosphorylation during prometaphase in *Mesostoma* has not yet been studied, but it is possible *Mesostoma* tethers alternate between phosphorylation states, or perhaps not all tethers are phosphorylated in any one given cell, or perhaps some arms are not connected by tethers [as in other cells, Forer et al., 2017].

After severing arms in *Mesostoma* spermatocytes, the arm fragments sometimes move to their partners, and sometimes do not (also noted by Ferraro-Gideon, 2013 B). This indicates that sometimes there are elastic tethers between the arms, and sometimes tethers either are not present or are inelastic. Our data suggest that only elastic tethers assist chromosomes to coordinate their movements to one pole after NOC treatment, much as tethers coordinate the movements of partner chromosomes in anaphase in crane fly spermatocytes (Sheykhani et al. 2017; Forer and Berns, 2020). In our experiments, after NOC addition bivalents with disabled elastic tethers moved to both poles or to no poles instead of to only 1 pole. Severing an arm without a tether (or with a tether that is inelastic) does not affect the chromosome movements that occur after addition of NOC.

C. Future experiments

There are several experiments that I think would be useful to consider performing on *Mesostoma* spermatocytes. I will briefly explain each proposed experiment and discuss how they might help to answer the two main inquiries of my research, which are: what provides the force for

chromosome movement in the absence of microtubules and why is chromosome movement towards only one pole.

The first experiment I propose is to create fluorescently labelled tubulin to follow spindle changes in live time during metaphase oscillations and anaphase progression. One method would be to use Life-Act labelling of tubulin. However, there are issues with using Life-Act labelling because it is based on taxol binding to microtubules. Previous studies with *Mesostoma* primary spermatocytes showed that when taxol was added at various concentration during prometaphase, it caused bivalent oscillations to slow down and eventually stop (Hoang 2013). LifeAct can also cause several unwanted effects in the cell (Flores et al. 2019). Another alternative might be to use a specialized method of polarising light field microscopy that can generate three-dimensional maps of birefringence, its position and orientation at spatial resolution of about 2 μm (Chandler et al 2017; Tran and Oldenbourg 2018). One might be able to use such a technique to view microtubules in living *Mesostoma* cells.

If one could visualize the spindle microtubule, it would be advantageous to study the spindle breakdown after the addition of NOC and to determine the exact timing when kinetochore microtubules no longer attach to the kinetochores. This would help to confirm that chromosome movement during NOC is only occurring once all microtubules are fully depolymerised in the cell, thus validating my NOC results. It would also be a useful tool to study prometaphase bivalent oscillations and chromosome reorientations such as the univalent excursions and bivalent reorientations, as suggested by Ferraro-Gideon (2013). It might help explain the mechanism behind the persistent bivalent oscillations towards and away from the pole, and to determine whether Flux and/or PacMan are occurring during oscillations. Also, we do not know how univalent excursions occur, do univalents momentarily from a connection with

a microtubule from the opposite pole? The same goes for bivalent reorientations, perhaps bivalents momentarily lose connection at one pole and forms a connection with the opposite pole. If one could visualize the microtubule spindle in real time, this would greatly help to answer these questions.

I faced some challenges and limitations with immunofluorescence staining, and the only spindle protein I was able to successfully visualize was tubulin. I attempted to optimize our *Mesostoma* spermatocyte immunofluorescence staining protocol. I have tried various lysis buffers solutions, I have also added lysis buffer with varying concentrations of glutaraldehyde, glutaraldehyde only followed by lysis buffer, 4% paraformaldehyde, and cold methanol treatment, all for varying lengths of time. I have also tried a citrate acid buffer pre-wash. I have tried 24-hour incubation of antibodies at room temperature, as well as microwave staining using the PELCO BioWave microwave. The microwave method has shown some promising results and is worthwhile to continue investigating. If a successful protocol to view other proteins within this cell can be established, it would be ideal to stain for a broad range of proteins in the *Mesostoma* spermatocyte spindle such as actin, myosin, and titin, as well as various spindle matrix protein candidates such as skeletor, megator, chromator, and EAST. Another option is to use an antigen retrieval machine (Aptum Biologics Inc), which can help enable the antibody to access the target protein within the sample (Vaught et al 2009; Alferez et al 2008; Zhu et al 2004). If these proteins can be visualized, I suggest the follow beneficial experiments.

Several experiments have shown that spindle matrix proteins are localised to spindle fibres of different organisms (reviewed by Johansen and Johansen, 2007; Johansen et al., 2011). Immunostaining work in crane-fly spermatocytes showed that skeletor, megator, and chromator are localised around chromosomes, kinetochore microtubules and non-kinetochore microtubules

(Fabian et al., 2007). It would be beneficial to visualize these protein candidate distribution patterns in the *Mesostoma* spindle during meiosis, to see if they colocalize with the microtubule spindle and to see if they are near or contact the chromosomes and/or kinetochores. This would be an advantageous experiment to perform because it is essential to show that these proteins are near or in contact with chromosomes and/or connect chromosomes to the pole if they are to be convincing candidates for chromosome movement. It would also be useful to see if these structures remain in the absence of microtubules brought about by NOC addition, and for how long.

To address the sidedness issue, one can see whether these proteins are asymmetrically distributed in the cell and are concentrated at one pole or perhaps they are oriented towards one pole. This could provide some insight into the sidedness issue and why the force for chromosome movement seems to be towards only one pole. This may even help one to predetermine which pole the chromosomes will detach from. For example, actin has a particular orientation so maybe that is the orientation the chromosomes move.

In addition to the aforementioned proteins, titin would be useful to visualize because it is suspected to be a major component in tethers. We do not yet know what tethers are comprised of (Paliulis and Forer, 2018), but it is speculated they may be made of the protein titin since it is present in regions where tethers are located in crane-flies (Fabian and Forer, 2007). Being able to visualize tethers may also provide insight into the results when I cut more than 1 bivalent into half-bivalents in a cell. In cells with 1 bivalent cut in half, 12 out of 16 cells had bivalent detachment from only one pole. When 2 and 3 bivalents are cut, the likelihood of detachment from only one pole appears to decrease, suggesting the cells require tension along at least 1 or 2 bivalents to move. However, the likelihood of accidentally cutting a tether increases as I sever

more bivalents because I have to make larger cuts and more cuts on various focal planes. So these preliminary results may be due to the fact I am also accidentally cutting a tether while I attempt to cut 2 or 3 bivalents at the same time in the same cell, and it is the disabled tether that is causing this un-coordinated movement rather than the fact I've cut multiple bivalents. The value of this proposed experiment is one could obtain immunofluorescence staining results to visualize whether the tether has been accidentally severed.

In order to show more definitively that *Mesostoma* divide via non-random segregation, I would like to propose a series of experiments to create heteromorphic bivalents by crossing two different *Mesostoma* sub-species. In our North American sub-species, the *Mesostoma ehrenbergii wardii*, all three bivalents are metacentric in shape and have large free “arms”. In comparison, in the European sub-species the *Mesostoma ehrenbergii ehrenbergii* (Oakley and Jones 1982), two of the three bivalents are acrocentric and so they have very small “arms” (as discussed in Ferraro-Gideon et al 2013 A). Crossing the two species will create heteromorphic bivalents in their offspring, where one bivalent homologue has a large arm and the other homologue has a small arm. If all the small armed homologous chromosomes orient towards one pole this will verify these cells divide non-randomly. We will try to capture some of the European ones while in France and Spain or acquire some overwintering eggs from a colleague in Argentina. This experiment would be a produce a valuable and consistent cell-line one could use to better investigate non-random segregation in these cells.

The last study I suggest is to study second division in secondary *Mesostoma* spermatocytes and add NOC to these cells to see if movement of half-bivalents is random or non-random. In Fegaras and Forer 2018a, I provided key findings and a figure sequence of a secondary spermatocyte, and Ferraro-Gideon (2013 A) commented on their findings from one

cell. We first need to get some more insight into the regular chromosome movements of these cells before we add NOC. A continuation of this study is currently underway by another student in the lab and hopefully these experiments will shed some light on secondary spermatocyte division. It would be advantageous to add NOC to second division spermatocytes to confirm that non-random segregation only occurs in meiosis I.

D. Conclusion

In conclusion, *Mesostoma* are a newly emerging model organism with only a handful of researchers who have experimented on their very unique cell division. My PhD experiments have shown that non-microtubule spindle components can move chromosomes in *Mesostoma* spermatocytes. After microtubules are removed with NOC, all chromosome kinetochores move at rapid speeds (up to 100µm/min) to the opposite poles. Our experiments have eliminated tension in elongated chromosomes as producing these forces since the same movements occur in ‘halved’ bivalents which do not have bipolar connections. Chromosome movements still occur when more than one of the 3 bivalents are cut into two ‘halved’ bivalents, at the same rapid speeds as in not-cut bivalents, but with altered coordination in moving to the same pole. Because directly disabling the tethers alters the coordinated movements, we suggest that the altered coordination in moving to one pole is because of damage to elastic tethers that connect the free arms of these bivalents.

GENERAL REFERENCES

- Ahmad FJ, Hughey J, Wittmann T, Hyman A, Greaser M, Baas, PW. 2000. Motor proteins regulate force interactions between microtubules and microfilaments in the axon. *Nat Cell Biol.* 2: 276-80.
- Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. 2008. *Molecular biology of the cell: Chapter 16. The Cytoskeleton.* 8th Ed. Garland Science Publishing, Inc. New York and London. 965-1052.
- Alferez D, Wilkinson RW, Watkins J, Poulson R, Mandir N, Wedge SR, Pyrah IT, Smith NR, Jackson L, Ryan AJ, Goodlad RA. *Mol Cancer Ther.* 2008 Mar;7(3):590-8. Dual inhibition of VEGFR and EGFR signaling reduces the incidence and size of intestinal adenomas in *Apc(Min/+)* mice.
- Alushin GM, Lander GC, Kellogg EH, Zhang R, Baker D, Nogales E. 2014. High- resolution microtubule structures reveal the structural transitions in alpha beta- tubulin upon GTP hydrolysis. *Cell.* 157: 1117-1129.
- Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rhokinase). *J Biol Chem.* 271: 20246-9.
- Ampe C, Vandekerckhove J. 1999. Actin. *In: Guidebook to the cytoskeletal and motor proteins.* Kreis T. Vale R. (eds). Print. 11-14.
- Andrews FM. 1915. Die Wirkung der Zentrifugalkraft auf Pflanzen. *Jahrb Wiss Bot* 56: 221-253
- Barefield C, Karlseder J. 2012. The BLM helicase contributes to telomere maintenance through processing of late-replicating intermediate structures. *Nucleic acids research*, 40(15), 7358-7367.
- Barr FA, Gruneberg U. 2007. Cytokinesis: placing and making the final cut. *Cell* 131, 847–860.
- Bedini C, Lanfranchi A. 1990. The eyes of *Mesostoma ehrenbergii* (Focke, 1836) (Platyhelminthes, Rhabdocoela). Fine structure and photoreceptor membrane turnover. *Acta Zool.* 71:125-133.
- Behnke O, Forer A. 1976. Evidence for four classes of microtubules in individual cells. *J Cell Sci.* Jun;2(2):169-92.
- Behnke O, Forer A. 1972. Vinblastine as a cause of direct transformation of some microtubules into helical structures. *Experimental cell research*, 73(2), 506–509.

- Behnke O, Forer A, Emmersen J. 1971. Actin in sperm tails and meiotic spindles. *Nature*, 234(5329), 408–410.
- Berns MB. 2020. Laser scissors and tweezers to study chromosomes: a review. *Front. Bioeng. Biotechnol.* doi: 10.3389/fbioe.2020.00721
- Berns MW, Greulich KO. 2007. Laser micromanipulation of cells and tissues. *Methods in Cell Biology*. San Diego (CA): Academic Press.
- Bershadsky AD, Vasiliev JM. 1988. *Cellular Organelles: Cytoskeleton*. Plenum Press. New York and London. Print.
- Bieling P, Laan L, Schek H, Munteanu EL, Sandblad L, Dogterom M, Brunner D, Surrey T. 2007. Reconstitution of a microtubule plus-end tracking system in vitro. *Nature* 450, 1100–1105.
- Blachoin L, Boujemaa-Paterski R, Sykes C, Plastino J. 2014. Actin dynamics, architecture, and mechanics in cell motility. *Physiol Rev.* 94: 235-263.
- Blaustein L, Dumont HJ. 1990. Typhloplanid flatworms (*Mesostoma* and related genera) mechanisms of predation and evidence that they structure aquatic invertebrate communities. *Hydrobiologia* 198:61-77.
- Bonaccorsi S, Giansanti MG, Gatti M. 1998. Spindle self-organization and cytokinesis during male meiosis in asterless mutants of *Drosophila melanogaster*. *J. Cell. Biol.* 142; 571-761.
- Brady M, Paliulis LV. 2015. Chromosome interaction over a distance in meiosis. *R. Soc. Open sci.* 2:150029. <http://dx.doi.org/10.1098/rsos.150029>.
- Bray D. 2001. *Cell movements from molecules to motility*. New York: Garland Publishing.
- Bresslau E. 1903. Die sommer und wintereier der Rhabdocoelen sussen wassers und ihre biologischebedeutung. *Verh Dt Zool Ges.* 126-139.
- Brouhard GJ, 2015. Dynamic instability 30 years later: complexities in microtubule growth and catastrophe. *Mol Biol Cell* 1207-1210.
- Brown SW, Nelson-Rees WA. 1961. Radiation analysis of a Lecanoid genetic system. *Genetics.* 46:983-1007.
- Brown SW, Nur U. 1964. Heterochromatic chromosomes in the Coccids. *Science.* 145:130-136.
- Bugyi B, Kellermayer N. 2019. The discovery of action: “to see what everyone else has seen, and to think what nobody has thought”. *J Muscle Res. Cell. Motil.* 41, 3-9.

- Burgess DR, Chang F. 2005. Site selection for the cleavage furrow at cytokinesis. *Trends in cell biology*, 15(3), 156–162.
- Buster DW, Zhang D, Sharp DJ. 2007. Poleward tubulin flux in spindles: regulation and function in mitotic cells. *Molecular biology of the cell*, 18(8), 3094–3104.
- Camenzind R, Nicklas RB. 1968. The non-random chromosome segregation in spermatocytes of *Gryllotalpa hexadactyla*. *Chromosoma*. 24:324-335.
- Carlson JG. 1952. Microdissection studies of the dividing neuroblast of the grasshopper *Chortophaga viridifasciata* (De Geer). *Chromoosma* 5: 199-220
- Carnoy J. 1885. La cytodierèse chez les Arthropodes. Aug Peeters Libraire, Louvain
- Case TJ, Washin RK. 1979. Flatworm control of mosquito larvae in rice fields. *Science* 206:1412-1414.
- Cassimeris L, Inoue S, Salmon ED. 1988. Microtubule dynamics in the chromosomal spindle fiber: analysis by fluorescence and high-resolution polarization microscopy. *Cell Motil Cytoskeleton*. 10(1-2):185-96.
- Cassimeris L, Spittle C. 2001. Regulation of microtubule-associated proteins. *Int. Rev. Cytol.* 210: 163-226.
- Champion MD, Hawley RS. 2002. Playing for half the deck: the molecular biology of meiosis. *Nat Cell Biol.* 4:50-6.
- Chan KL, North PS, Hickson ID. 2007. BLM is required for faithful chromosome segregation and its localization defines a class of ultrafine anaphase bridges. *The EMBO journal*, 26(14), 3397-3409.
- Chandler T, Mehta S, Shroff H, Oldenbourg R, La Riviere PJ. 2017. Single-fluorophore orientation determination with multiview polarized illumination: modeling and microscope design. *Opt Express*, 25(25):31309-31325.
- Chang P, Coughlin M, Mitchison TJ. 2005. Tankyrase-1 polymerization of poly(ADP-ribose) is required for spindle structure and function. *Nature cell biology*, 7(11), 1133–1139.
- Chang P, Jacobson MK, Mitchison TJ. 2004. Poly(ADP-ribose) is required for spindle assembly and structure. *Nature*, 432(7017), 645–649.
- Cheeseman IM, Desai A. 2008. Molecular architecture of the kinetochore-microtubule interface. *Nature*. 9: 33-46.
- Chretien D, Wade R. 1991. New data on the microtubule surface lattice. *Biol Cell*. 71: 161-174.

- Cimino MC. 1972 A. Egg-production, polyploidization and evolution in a diploid all-female fish of the genus *Poeciliopsis*. *Evolution*. 26(2): 294-306.
- Cimino MC. 1972 B. Meiosis in triploid all-female fish (*Poeciliopsis*, *Poeciliidae*). *Science* 175: 1484-1486.
- Civelekoglu-Scholey G, Sharp DJ, Mogilner A, Scholey JM. 2006. Model of chromosome motility in *Drosophila* embryos: Adaptation of a general mechanism for rapid mitosis. *Biophys J* 90: 3966-3986
- Civelekoglu-Scholey G, He B, Shen M, Wan X, Roscioli E, Bowden B, Cimini D. 2013. Dynamic bonds and polar ejection force distribution explain kinetochore oscillations in PtK1 cells. *The Journal of cell biology*, 201(4), 577–593.
- Cleveland DW. 1999. Tubulin and associated proteins. Introduction. *In: Guidebook to the cytoskeletal and motor proteins*. Kreis T. Vale R. (eds). Print. 189-193.
- Cremona CR, Hartshorne DJ. 2008. Myosins: A Superfamily of Molecular Motors. 7: 171- 222
- Crick FHC. 1953. The packing of α -helices: simple coiled-coils. *Acta Crystallogr*. 6:689–697.
- Cytrynbaum EN, Sommi P, Brust-Mascher I, Scholey JM, Mogilner A. 2005. Early spindle assembly in *Drosophila* embryos: role of a force balance involving cytoskeletal dynamics and nuclear mechanics. *Mol Biol Cell* 16:4967–4981
- Czaban BB, Forer A. 1994. Rhodamine-phalloidin and anti-tubulin antibody staining of spindle fibres that were irradiated with an ultraviolet microbeam. *Protoplasma*. 178:18–27.
- Dawley RM. 1989. Evolution and ecology of unisexual vertebrates: An introduction to unisexual vertebrates. Bulletin 466. New York, New York. 1-18. Print.
- de Forges H, Pilon A, Cantaloube I, Pallandre A, Haghiri-Gosnet AM, Perez F, Poüs C. 2016. Localized Mechanical Stress Promotes Microtubule Rescue. *Curr Biol*. 26 (24): 3399-3406.
- DeRoeck ERM, Artois T, Brendonck L. 2005. Consumptive and non-consumptive effects of turbellarian (*Mesostoma* sp.) predation on anostracans. *Hydrobiologia*. 542:103-111.
- Desai A, Mitchison TJ. 1998. Preparation and characterization of caged fluorescein tubulin. *Methods in enzymology*, 298, 125–132.
- Ding Y, Yao C, Lince-Faria M, Rath U, Cai W, Maiato H, Girton J, Johansen KM, Johansen J. 2009. Chromator is required for proper microtubule spindle formation and mitosis in *Drosophila*. *Developmental biology*, 334(1), 253–263.

- Domenici L, Gremigni V. 1977. Fine structure and functional role of the coverings of the eggs in *Mesostoma ehrenbergii* (Focke). *Zoomorphology* 88:247-257.
- Dutcher SK. 2003. Long-lost relatives reappear: identification of new members of the tubulin superfamily. *Curr Opin Microbiol.* 6(6): 634-640.
- Echeverri CJ, Paschal BM, Vaughan KT, Vallee RB. 1996. Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *The Journal of cell biology*, 132(4), 617–633.
- Eggert US, Mitchison TJ, Field CM. 2006. Animal cytokinesis: from parts list to mechanisms. *Annu. Rev. Biochem.* 75, 543–566.
- Enos AP, Morris NR. 1990. Mutation of a gene that encodes a kinesin-like protein blocks nuclear division in *A. nidulans*. *Cell*, 60(6), 1019–1027.
- Er Rafik M, Doucet J, Briki F. 2004. The Intermediate Filament Architecture as Determined by X-Ray Diffraction Modeling of Hard α -Keratin. *Biophys J.* 86(6): 3893–3904.
- Erickson HP. 2007. Evolution of the cytoskeleton. *BioEssays.* 29: 668-677.
- Erickson HP. 2017. The discovery of the prokaryotic cytoskeleton: 25th anniversary. *Mol Biol Cell.* 28: 357-358.
- Esteban MR, Campos MCC, Perondini ALP Goday C. 1997. Role of microtubules and microtubule organizing centers on meiotic chromosome elimination in *Sciara ocellaris*. *J Cell Sci.* 110: 721-730.
- Even-Ram S, Doyle AD, Conti MA, Matsumoto K, Adelstein RS, Yamada KM. 2007. Myosin IIA regulates cell motility and actomyosin-microtubule crosstalk. *Nature cell biology*, 9(3), 299–309.
- Fabian L, Forer A. 2005. Redundant mechanisms for anaphase chromosome movements: Crane-fly spermatocyte spindles normally use actin filaments but also can function without them. *Protoplasma.* 225:169-184.
- Fabian L, Troszianczuk J, Forer A. 2007. Calyculin A, an enhancer of myosin, speeds up anaphase chromosome movement. *Cell Chromosome* 6:1 doi:10.1186/1475-9268-6-1
- Fabian L, Xia X, Venkitaramani DV, Johansen KM, Johansen J, Andrew DJ, Forer A. 2007. Titin in insect spermatocyte spindle fibers associates with microtubules, actin, myosin and the matrix proteins skeletor, megator and chromator. *J Cell Sci* 120:2190-2204

- Fabian L, Forer A. 2007. Possible roles of actin and myosin during anaphase chromosome movements in locust spermatocytes. *Protoplasma* 231:201–213.
- Ferguson FF, Hayes Jr WJ. 1941. A synopsis of the genus *Mesostoma ehrenbergii* 1835. *J Elisha Mitchell Sc Soc.* 57, 1-37
- Ferraro-Gideon J. 2013. Rearing *Mesostoma ehrenbergii* and studying chromosome movements during meiosis in their spermatocytes. PhD Dissertation, York University.
- Ferraro-Gideon J, Hoang C, Forer A. 2013 A. *Mesostoma ehrenbergii* spermatocytes—A unique and advantageous cell for studying meiosis. *Cell Biology International*, 37(9), 892-898.
- Ferraro-Gideon J, Sheykhan R, Zhu Q, Duquette ML, Berns MW, Forer A. 2013 B. Measurements of forces produced by the mitotic spindle using optical tweezers. *Mol Biol Cell* 24: 1375-1386.
- Ferraro-Gideon J, Hoang C, Forer A. 2014. Meiosis-I in *Mesostoma ehrenbergii* spermatocytes includes distance segregation and inter-polar movements of univalent, and vigorous oscillations of bivalents. *Protoplasma*. 251: 127-143.
- Fiore L, Ioalè P. 1973. Regulation of the production of subitaneous and dormant eggs in the Turbellarian *Mesostoma ehrenbergii* (Focke). *Monit Zool Ital.* 7:203-224
- Flemming W. 1882. *Zellsubstanz, Kern und Zelltheilung*. F. C. W. Vogel, Leipzig.
- Flores LR, Keeling MC, Zhang X. *et al.* 2019. Lifeact-TagGFP2 alters F-actin organization, cellular morphology and biophysical behaviour. *Sci Rep* **9**, 3241.
- Forer A. 1965. Local reduction of spindle fiber birefringence in living *Nephrotoma suturalis* (Loew) spermatocytes induced by ultraviolet microbeam irradiation. *The Journal of cell biology*, 25(1), 95–117.
- Forer A. 1966. Characterization of the mitotic traction system, and evidence that birefringent spindle fibers neither produce nor transmit force for chromosome movement. *Chromosoma* 19: 44-98
- Forer A. 1985. Does actin produce the force that moves a chromosome to the pole during anaphase?. *Canadian journal of biochemistry and cell biology = Revue canadienne de biochimie et biologie cellulaire*, 63(6), 585–598.
- Forer A. 1988. Do anaphase chromosomes chew their way to the pole or are they pulled by actin?. *Journal of cell science*, 91 (Pt 4), 449–453.
- Forer A. 2008. The diversity of cytoplasmic microtubules. *Journal of cell science*, 121(Pt 1), 7–9.

- Forer A, Berns MW. 2020. Elastic Tethers Between Separating Anaphase Chromosomes Regulate the Poleward Speeds of the Attached Chromosomes in Crane-Fly Spermatocytes. *Front Mol Biosci.* 7:161.
- Forer A, Duquette ML, Paliulis LV, Fegaras E, Ono M, Preece D, Berns MW. 2017. Elastic 'tethers' connect separating anaphase chromosomes in a broad range of animal cells. *European journal of cell biology*, 96(6), 504–514.
- Forer A, Goldman RD. 1969. Comparisons of isolated and in vivo mitotic apparatuses. *Nature*, 222(5194), 689–690.
- Forer A, Jackson WT. 1979. Actin in spindles of *Haemaphysalis katherinae* endosperm. I. General results using various glycerination methods. *Journal of cell science*, 37, 323–347.
- Forer A, Johansen KM, Johansen J. 2015. Movement of chromosomes with severed kinetochore microtubules. *Protoplasma* 252, 775–781.
- Forer A, Koch C. 1973. Influence of autosome movements and of sex-chromosome movements on sex-chromosome segregation in crane fly spermatocytes. *Chromosoma*. 40(4):417-42.
- Forer A, Pickett-Heaps JD. 2010. Precocious (pre-anaphase) cleavage furrows in *Mesostoma* spermatocytes. *Eur J Cell Bio* 89: 607-618
- Forer A, Pickett-Heaps JD, Spurck T. 2008. What generates flux of tubulin in kinetochore microtubules?. *Protoplasma*, 232(3-4), 137–141.
- Forer A, Spurck T, Pickett-Heaps JD. 2007. Actin and myosin inhibitors block elongation of kinetochore fibre stubs in metaphase crane-fly spermatocytes. *Protoplasma*, 232(1-2), 79–85.
- Forer A, Spurck T, Pickett-Heaps JD, Wilson PJ. 2003. Structure of kinetochore fibres in crane-fly spermatocytes after irradiation with an ultraviolet microbeam: neither microtubules nor actin filaments remain in the irradiated region. *Cell motility and the cytoskeleton*, 56(3), 173–192.
- Forer A, Wilson PJ. 1994. A model for chromosome movement during mitosis. *Protoplasma*.179:95-105.
- Forer A, Wilson PJ. 1999. Evidence that kinetochore fibre microtubules shorten predominantly at the pole in anaphase flea-beetle spermatocytes. *Chrom. Res.* 8:151-163.
- Fuchs E, Cleveland DW. 1998. A structural scaffolding of intermediate filaments in health and disease. *Science*, 279:514-519.

- Fuge H, Falke D. 1991. Morphological aspects of chromosome spindle fibres in *Mesostoma*: “microtubular fir-tree” structures and microtubule association with kinetochores and chromatin. *Protoplasma*. 160:39-48.
- Fuge H. 1997. Nonrandom chromosome segregation in male meiosis of a Sciarid fly: elimination of paternal chromosomes in first division is mediated by non-kinetochore microtubules. *Cell Motil. Cytoskeleton*. 36: 84-94.
- Fuge H. 1994. Unorthodox male meiosis in *Trichosia pubescence* (Sciaridae). Chromosome elimination involves polar organelle degeneration and monocentric spindles in first and second division. *J Cell Sci*. 107: 299-312.
- Fuge H. 1989. Rapid kinetochore movements in *Mesostoma ehrenbergii* spermatocytes: action of antagonistic chromosome fibre. *Cell Motil Cytoskeleton*. 13:212-220.
- Fuge H. 1987. Oscillatory movements of bipolar-oriented bivalent kinetochores and spindle forces in male meiosis of *Mesostoma ehrenbergii*. *Eur J Cell Biol*. 44:294-298.
- Ganem NJ, Compton DA. 2006. Functional roles of poleward microtubule flux during mitosis. *Cell Cycle*. 5:5, 481-485.
- Gemble S, Ahuja A, Buhagiar-Labarchède G, Onclercq-Delic R, Dairou J, Biard DS, Lambert S, Lopes M, Amor-Guérét M. 2015. Pyrimidine pool disequilibrium induced by a cytidine deaminase deficiency inhibits PARP-1 activity, leading to the under replication of DNA. *PLoS genetics*, 11(7).
- Gerbi SA. 1986. Unusual chromosome movements in sciarid flies. In Hennig W, ed: *Germ Line-Soma Differentiation. Results and Problems of Cell Differentiation*. New York: Springer Verlag. Vol 13. P71-104.
- Gibbons IR. 1963. Studies on the protein components of cilia from tetrahymena pyriformis. *Proceedings of the National Academy of Sciences of the United States of America*, 50(5), 1002–1010.
- Goday C, Esteban MR. 2001. Chromosome elimination in sciarid flies. *BioEssays*. 23:242-250.
- Goldman RD, Chou YH. 1999. Intermediate filaments and associated proteins. *In: Guidebook to the cytoskeletal and motor proteins*. Kreis T. Vale R. (eds). Print. 281-284.
- Gonczy P. 2008. Mechanisms of asymmetric cell division: flies and worms pave the way. *Nat Rev* 9:355–366.

- Gorbsky GJ, Sammak PJ, Borisy GG. 1987. Chromosomes move poleward in anaphase along stationary microtubules that coordinately disassemble from their kinetochore ends. *J Cell Biol.* 104(1):9-18.
- Gorbsky GJ, Sammak PJ, Borisy GG. 1988. Microtubule dynamics and chromosome motion visualized in living anaphase cells. *J Cell Biol.* 106(4): 1185-92.
- Gordon GW, Inoué A. 1979. Unexpected increase in poleward velocities of mitotic chromosomes after UV irradiation of their kinetochore fibers. *J Cell Biol* 83: 376a
- Graff LQ. Turbellaria. II. Rhabdocoelida. 1913. In: *Das Tierreich.* p. 35.
- Granzier HL, Labeit S. 2006. The Giant Muscle Protein Titin is an Adjustable Molecular Spring. *Exercise and Sport Sciences Reviews*, 34, 50-53.
- Green-Marroquin BL, Kupfer H, Virkki N, Wise DA. 2001. Orientation of nonrandomly segregating sex chromosomes in spermatocytes of the flea beetle, *Alagoasa bicolor* L. *Chromosoma.* 110:32-38.
- Grill SW, Goënczy P, Stelzer EHK. 2001. Polarity controls forces governing asymmetric spindle positioning in the *Caenorhabditis elegans* embryo. *Nature* 409:630–633
- Gruen LC, Woods EF. 1983. Structural studies on the microfibrillar proteins of wool. *Biochem. J.* 209:587–595.
- Guo Y, Zheng Y. 2015. Lamins position the nuclear pores and centrosome by modulating dynein. *Mol Biol Cell.* 26: 3379-3389.
- Hagan I, Yanagida M. 1990. Novel potential mitotic motor protein encoded by the fission yeast *cut7+* gene. *Nature*, 347(6293), 563–566.
- Hamdani N, Herwig M, Linke WA. 2017. Tampering with springs: phosphorylation of titin affecting the mechanical function of cardiomyocytes. *Biophysical Reviews*, 9(3), 225-237
- Harsono MS, Zhu Q, Shi LZ, Duquette M, Berns MW. 2013. Development of a dual joystick-controlled laser trapping and cutting system for optical micromanipulation of chromosomes inside living cells. *Journal of biophotonics*, 6(2), 197–204.
- Hartman MA, Spinach JA. 2012. The myosin superfamily at a glance. *J Cell Sci.* 125(7): 1627-1632.
- Heitkamp U. 1977. Zur fortpflanzungsbiologie von *Mesostoma ehrenbergii* (Focke, 1836)(Turbellaria). *Hydrobiologia.* 55:21-31

- Herrmann H, Aebi U. 1998. Intermediate filament assembly: fibrillogenesis is driven by decisive dimer–dimer interactions, *Curr. Opin. Struct. Biol.* 8: 177–185.
- Herrmann H, Aebi U. 2004. Intermediate filaments: molecular structure, assembly mechanism, and integration into functionally distinct intracellular Scaffolds. *Annual review of biochemistry*, 73, 749–789.
- Hildebrandt ER, Hoyt MA. 2000. Mitotic motors in *Saccharomyces cerevisiae*. *Biochimica et biophysica acta*, 1496(1), 99–116.
- Hoang C, Ferraro-Gideon J, Gauthier K, Forer A. 2013. Methods for rearing *Mesostoma ehrenbergii* in the laboratory for cell biology experiments, including identification of factors that influence production of different egg types. *Cell Biol Int.* 37(10):1089-105.
- Honda Y, Tsuchiya K, Sumiyoshi E, Haruta N, Sugimoto A. 2017. Tubulin isotype substitution revealed that isotype combination modulates microtubule dynamics in *C. elegans* embryos. *J Cell Sci.* 130: 1652-1661.
- Howard L. 2001. Mechanics of motor proteins and the cytoskeleton. Ch 7: Structures of cytoskeletal filaments. Sinauer Associates, Inc. Sunderland, Massachusetts. Print. 117-134.
- Howell BJ, McEwen BF, Canman JC, Hoffman DB, Farrar EM, Rieder CL, Salmon ED. 2001. Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. *The Journal of cell biology*, 155(7), 1159–1172.
- Hubbs CL, Hubbs LC. 1932. Apparent parthenogenesis in nature in a form of fish hybrid origin. *Science*. 76, 628-630.
- Hubbs CL, Hubbs LC. 1946. Breeding experiments with the invariable female, strictly matroclinous fish, *Mollienisia formosa*. *Rec Genetics Soc Amer.* 14, 48.
- Hughes-Schrader S. 1969. Distance segregation and compound sex chromosomes in Mantispids (*Neuroptera: Mantispidae*). *Chromosoma* 27: 109-129.
- Husted L, Ruebush TK. 1940. A comparative cytological and morphological study of *Mesostoma ehrenbergii ehrenbergii* and *Mesostoma ehrenbergii wardii*. *J Morphol.* 67:387-410.
- Huxley AF. 1957. Muscle structure and theories of contraction. *Prog. Biophys. Biophys. Chem.* 7:255-318.
- Huxley AF, Simmons RM. 1971. Proposed mechanism of force generation in striated muscle. *Biochim. Biophys. Acta.* 12:387-394.

- Ikebe M, Hartshome DJ. 1985. Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. *J Biol Chem.* 260(18):10027-31.
- Ino MC. 1972. Egg production, polyploidization and evolution in a diploid all female fish of the genus *Poeciliopsis*. *Evolution.* 26, 294-306.
- Inoue S. 1952. The effect of colchicine on the microscopic and submicroscopic structure of the mitotic spindle. *Exp Cell Res Suppl* 2: 305–318
- Johansen J, Johansen KM. 2009. The spindle matrix through the cell cycle in *Drosophila*. *Fly.* 3, 1–8.
- Johansen KM, Johansen J. 2007. Cell and molecular biology of the spindle matrix. *Int Rev Cytol.* 263, 155–206.
- Johansen KM, Forer A, Yao C, Girton J, Johansen J. 2011. Do nuclear envelope and intranuclear proteins reorganize during mitosis to form an elastic, hydrogellike spindle matrix? *Chromosome Res.* 19, 345-365.
- Kalita G, Goswami MM. 2012. Occurrence of *Mesostoma tetragonum* (Muller) (Turbellaria) in the Deepar wetlands of Assam, India. *J Threat Taxa.* 4:2609-2613.
- Kallman KD. 1962. Gynogenesis in the teleost *Mollienisia formosa* (Girard), with a discussion of the detection of parthenogenesis in vertebrates by tissue transplantation. *J Genet.* 58, 7-21.
- Karki S, Holzbaur EL. 1999. Cytoplasmic dynein and dynactin in cell division and intracellular transport. *Current opinion in cell biology*, 11(1), 45–53.
- Karp G. 2003. *Cell and molecular biology: Concepts and experiments*. Danvers: John Wiley and Sons Inc
- Karsenti E, Vernos I. 2001. The mitotic spindle: a self-made machine. *Science* (New York, N.Y.), 294(5542), 543–547.
- Khodjakov A, Kapoor T. 2005. Microtubule flux: what is it good for? *Current biology: CB*, 15(23), R966–R968.
- 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.
- Kolasa J, Fletcher M, Main AJ. 1985. New records for two mosquito predators [Turbellaria: *Mesostoma*] in the northeastern United States. *Entomophaga.* 30:83-85.

- Kolasa J, Mead AP. 1981. A new species of freshwater turbellarian from Africa, predatory on mosquitoes: *Mesostoma zariae* n. sp. (Typhloplanoida). *Hydrobiologia* 84:19-22
- Kolasa J. 1984. Predation on mosquitoes by juveniles of *Mesostoma* spp. (Turbellaria). *Freshwater Invertebrate Biology*. 3:42-47.
- Kolasa J. Schwartz SS. 1988. Two new *Mesostoma* species (Turbellaria, Rhabdocoela) from Australia. *Zool Scr.* 17, 329-335.
- Koshland DE, Mitchison TJ, Kirschner MW. 1988. Polewards chromosome movement driven by microtubule depolymerization in vitro. *Nature*. 331(6156):499-504.
- Kosova B, Panté N, Rollenhagen C, Podtelejnikov A, Mann M, Aebersold U, Hurt E. 2000. Mlp2p, a component of nuclear pore attached intranuclear filaments, associates with nic96p. *The Journal of biological chemistry*, 275(1), 343–350.
- Kubai D, Wise D. 1981. Non-random chromosome segregation in *Neocurtilla* (*Gryllotalpa*) *hexadactyla*: an ultrastructural study. *J. Cell Biol.* 88: 281-293.
- Kubai DF. 1987. Nonrandom chromosome arrangements in germ line nuclei of *Sciara coprophila* males: the basis for nonrandom chromosomes segregation on the meiosis I spindle. *J Cell Biol.* 105, 2433-2446.
- Kühne W. 1864. Untersuchungen über das Protoplasma und die Contractilität. Leipzig: W. Engelmann
- Kupfer H, Wise D. 2000. Behavior of sex chromosomes, autosomes, and the spindle during non-random segregation in a flea beetle. *Genome*. 43: 521-527.
- Kupfer H, Wise D. 2000. The pattern of sex chromosome kinetochore phosphorylation during non-random segregation in a flea beetle. *Biochem. Cell Biol.* 78: 93-98.
- LaFountain JR, Jr Cohan CS, Siegel AJ, LaFountain DJ. 2004. Direct visualization of microtubule flux during metaphase and anaphase in crane-fly spermatocytes. *Molecular biology of the cell*, 15(12), 5724–5732.
- LaFountain JR, Cole RW, Rieder CL. 2002. Partner telomeres during anaphase in crane-fly spermatocytes are connected by an elastic tether that exerts a backward force and resists poleward motion. *J Cell Sci.* 115, 1541-1549.
- Lenart P, Bacher CP, Daigle N, Hand AR, Eils R, Terasaki M, Ellenberg J. 2005. A contractile nuclear actin network drives chromosome congression in oocytes. *Nature*. 436: 812-818.

- Li X, Nicklas RB. 1995. Mitotic forces control a cell-cycle checkpoint. *Nature*. 16(6515): 630-632.
- Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. 2000. *Molecular Cell Biology*. 4th edition. Section 19.6, Intermediate Filaments. W. H. Freeman. New York. Print.
- Lorbeer, G. 1934. Die Zytologie der Lebermoose mit besonderer Berticksichtigung allgemeiner Chromo- somenfragen. I. Jahrb. Wiss. Bot. 80: 567-818
- Lydersen BK, Pettijohn DE. 1980. Human-specific nuclear protein that associates with the polar region of the mitotic apparatus: distribution in a human/hamster hybrid cell. *Cell*, 22(2 Pt 2), 489–499.
- Ma L, Tsai MY, Wang S, Lu B, Chen R, Yates JR III, Zhu X, Zheng Y. 2009. Requirement for Nudel and dynein for assembly of the lamin B spindle matrix. *Nature cell biology*, 11(3), 247–256.
- Maddox PS, Oegema K, Desai A, Cheeseman IM. 2004. Holo"er than thou: Chromosome segregation and kinetochore function in *C. elegans*. *Chromo Res*. 12(6) 641-653.
- Maiato H, Rieder CL, and Khodjakov A. 2004. Kinetochore-driven formation of kinetochore fibers contributes to spindle assembly during animal mitosis. *J Cell Biol*. 167: 831-40.
- Maney T, Ginkel LM, Hunter AW, Wordeman L. 2000. The kinetochore of higher eucaryotes: a molecular view. *International review of cytology*, 194, 67–131.
- Margolis RL, Wilson L. 1978. Opposite end assembly and disassembly of microtubules at steady state in vitro. *Cell*. 13(1):1-8.
- Margolis RL, Wilson L. 1981. Microtubule treadmills--possible molecular machinery. *Nature*. 293(5835), 705–711.
- McIntosh JR, Hepler PK, Van Wie DG. 1969. Model for mitosis. *Nature*. 224, 659-663.
- McIntosh K, Pickett-Heaps JD, Gunning BES. 1995. Cytokinesis in *Spirogyra*: integration of cleavage and cell-plate formation. *Int. J. Plant Sci*. 156, 1–8.
- Mendel GJ. 1865. "Versuche über Pflanzen-Hybriden" [Experiments Concerning Plant Hybrids]" [1866]. In *Verhandlungen des naturforschenden Vereines in Brünn* [Proceedings of the Natural History Society of Brünn] 4: 3–47. Reprinted 1966 in *Fundamenta Genetica*, ed. Jaroslav Kříženecký, 15–56.

- Merdes A, Ramyar K, Vechio JD, Cleveland DW. 1996. A complex of NuMA and cytoplasmic dynein is essential for mitotic spindle assembly. *Cell*, 87(3), 447–458.
- Metz CW, Moses MS, Hoppe EN. 1926. Chromosome behavior and genetic behavior in *Sciara* (Diptera). I. Chromosome behavior in the spermatocyte divisions. *Z. induct Abstamm-u Vererbungs.* 45: 184-201.
- Metz CW. 1938. Chromosome behaviour, inheritance and sex determination in *Sciara*. *Am. Nat.* 72: 485-520.
- Meunier S, Vernos I. 2016. Acentrosomal Microtubule Assembly in Mitosis: The Where, When, and How. *Trends Cell Biol.* 26(2):80-87.
- Miller MK, Granzier H, Ehler E, Gregorio CC. 2004. The sensitive giant: the role of titin-based stretch sensing complexes in the heart. *Trends in cell biology*, 14(3), 119–126.
- Mimori-Kiyosue Y, Tsukita S. 2003. “Search-and-capture” of microtubules through plus-end-binding proteins (+TIPs). *J Biochem.* 134: 321-326.
- Mitchison TJ. 1989. Mitosis: basic concepts. *Current opinion in cell biology*, 1(1), 67–74.
- Mitchison TJ, Maddox P, Gaetz J, Groen A, Shirasu M, Desai A, Salmon ED, Kapoor TM. 2005. Roles of polymerization dynamics, opposed motors, and a tensile element in governing the length of *Xenopus* extract meiotic spindles. *Molec. Biol. Cell.* 16: 3064-76.
- Mitchison T, Kirschner M. 1984. Dynamic Instability of Microtubule Growth. *Nature*, 312, 237-242.
- Mitchison TJ, Maddox P, Gaetz J, Groen A, Shirasu M, Desai A, Salmon ED, Kapoor TM. 2005. Roles of polymerization dynamics, opposed motors, and a tensile element in governing the length of *Xenopus* extract meiotic spindles. *Molecular biology of the cell*, 16(6), 3064–3076.
- Miyamoto DT, Perlman ZE, Burbank KS, Groen AC, Mitchison TJ. 2004. The kinesin Eg5 drives poleward microtubule flux in *Xenopus laevis* egg extract spindles. *The Journal of cell biology*, 167(5), 813–818.
- Mogessie B, Schuh M. 2017. Actin protects mammalian eggs against chromosome segregation errors. *Science*, 357(6353), [eaal1647]. <https://doi.org/10.1126/science.aal1647>
- Montgomery TH. 1899. The spermatogenesis in *Pentatoma* up to the formation of the spermatid. *Zool Jahrb Abt f Anat* 12:1-88

- Moore DP, Orr-Weaver TL. 1998. Chromosome segregation during meiosis: building an unambivalent bivalent. *Curr Top Dev Biol.* 37: 263-299.
- Morgan DO. 2007. *The cell cycle: principles of control*. London: New Science Press Ltd.
- Murata-Hori M, Suizu F, Iwasaki T, Kikuchi A, Hosoya H. 1999. ZIP kinase identified as a novel myosin regulatory light chain kinase in HeLa cells. *FEBS letters*, 451(1), 81–84.
- Nicklas RB, Koch CA. 1969. Chromosome micromanipulation. 3. Spindle fiber tension and the reorientation of mal-oriented chromosomes. *J Cell Biol.* 43(1):40-50.
- Nicklas RB. 1989. The motor for poleward chromosome movement in anaphase is in or near the kinetochore. *J Cell Biol.* 109(5):2245-55.
- Nogales E, Whittaker M, Milligan RA, Downing KH. 1999. High-resolution model of the microtubule. *Cell.* 9: 79–88.
- Nogales E, Wolf SG, Downing KH. 1998. Structure of the $\alpha\beta$ -tubulin dimer by electron crystallography. *Nature.* 391: 199–203.
- Nur U. 1982. Destruction of specific heterochromatic chromosomes during spermatogenesis in the *Comstockiella* chromosome system (*Coccoidea: Homoptera*). *Chromosoma.* 85: 519-530.
- Oakley BR. 2000. An abundance of tubulins. *T Cell Biol.* 10: 537-542.
- Oakley HA, Jones GH. 1982. Meiosis in *Mesostoma ehrenbergii ehrenbergii* (Turbellaria, Rhabdocoela) I. chromosome pairing, synaptonemal complexes and chiasma localization in spermatogenesis. *Chromosoma* 85: 311-322.
- Oakley HA. 1983. Male meiosis in *Mesostoma ehrenbergii ehrenbergii*. *Key Chromosome Conference II*.
- Oakley HA. 1985. Meiosis in *Mesostoma ehrenbergii ehrenbergii* (Turbellaria, Rhabdocoela) III. univalent chromosome segregation during the first meiotic division in spermatocytes. *Chromosoma* 91:95-100.
- Ohkura H. 2015. Meiosis: An Overview of Key Differences from Mitosis. *Cold Spring Harb Perspect Biol* 185-198. Print.
- Page SL, Hawley RA. 2003. Chromosome choreography: The meiotic ballet. *Science.* 301: 785-789.
- Paliulis LV, Nicklas RB. 2000. The reduction of chromosome number in meiosis is determined by properties built into the chromosomes. *J Cell Biol.* 150(6): 1223-1231.

- Paliulis LV, Forer A. 2018. A review of “tethers”: elastic connections between separating partner chromosomes in anaphase. *Protoplasma*. 255:733-740.
- Parry DAD, Strelkov SV, Burkhard P, Aebi U, Herrmann H. 2007. Towards a molecular description of intermediate filament structure and assembly. *Exp. Cell Res.* 313: 2204-2216.
- Pauling L, Corey RB, Branson HR. 1951. The structure of proteins: two hydrogen-bonded helical configurations of the polypeptide chain. *Proc. Natl. Acad. Sci. USA*. 37:205–211.
- Paweletz N. 2001. Walther Flemming: pioneer of mitosis research. *Nat Rev Mol Cell Biol.* 2(1): 72-5.
- Payne F. 1916. A study of the germ cells of *Gryllotalpa borealis* and *Gryllotalpa vulgaris*. *J. Morph.* 28: 287-327.
- Payne F. 1912. The chromosomes of *Gryllotalpa borealis*. *Archiv f. Zellforsch.* 9: 141-148.
- Pfarr CM, Coue M, Grissom PM, Hays TS, Porter ME, McIntosh JR. 1990. Cytoplasmic dynein is localized to kinetochores during mitosis. *Nature*. 345(6272):263-5.
- Pickett-Heaps JD. 1991. Post-mitotic cellular reorganisation in the diatom *Cymatopleura solea*: the role of microtubules and the microtubule centre. *Cell Motil. Cytoskel.* 18, 279–292.
- Pickett-Heaps JD, Forer A. 2009. Mitosis: spindle evolution and the matrix model. *Protoplasma* 235: 91-99.
- Pickett-Heaps JD, Forer A. 2001. Pac-Man does not resolve the enduring problem of anaphase chromosome movement. *Protoplasma* 215:16–20.
- Pickett-Heaps JD, Forer A, Spurck T. 1996. Rethinking anaphase: where “PACMAN” fails and why a role for the spindle matrix is likely. *Protoplasma*. 192, 1–10.
- Pickett-Heaps JD, Spurck TP. 1982. Studies on kinetochore function in mitosis. I. The effects of colchicine and cytochalasin on mitosis in the diatom *Hantzschia amphioxys*. *European journal of cell biology*, 28(1), 77–82.
- Pickett-Heaps J, Spurck T, Tippit D. 1984. Chromosome motion and the spindle matrix. *The Journal of cell biology*, 99(1 Pt 2), 137s–143s.
- Pickett-Heaps JD, Tippit DH. 1980. Light and electron microscopic observations on cell division in two large pinnate diatoms, *Hantzschia* and *Nitzschia*. I. Mitosis in vivo. *Eur J Cell Bio* 21: 1-11.

- Pickett-Heaps JD, Tippit DH, Andreozzi JA. 1979 Cell division in the pinnate diatom *Pinnularia*. V- Observations on live cells. *Biol Cellulaire* 35: 295-304.
- Pickett-Heaps JD, Tippit DH, Andreozzi A. 1978. Cell division in the pennate diatom *Pinnularia*. II. Later stages in mitosis. ***Biol. Cellulaire***. 33, 79–84.
- Pickett-Heaps JD, Tippit DH, Leslie R. 1980. Light and electronmicroscopic observations on cell division in two large pennate diatoms, *Hantzschia* and *Nitzschia*. I. Mitosis in vivo. *Europ. J. Cell Biol.* 21, 1–11.
- Pollard TD. 1990. Actin. *Curr Op Cell Biol.* 2: 33-40.
- Pollard TD, Cooper JA. 1986. Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. *Annu Rev Biochem.* 55: 987–1035.
- Pring M, Weber A, Bubb M. 1992. Profilin-actin complexes directly elongate actin filaments at the barbed end. *Biochemistry* 31: 1827–1836.
- Qi H, Rath U, Ding Y, Ji Y, Blacketer MJ, Girton J, Johansen J, Johansen KM. 2005. EAST interacts with Megator and localizes to the putative spindle matrix during mitosis in *Drosophila*. *J Cell Biochem.* 95(6): 1284-91.
- Qi H, Rath U, Wang D, Xu YZ, Ding Y, Zhang W, Blacketer M, Paddy M, Girton J, Johansen J, Johansen KM. 2004. Megator, an essential coiled-coil protein localizes to the putative spindle matrix during mitosis. *Mol Biol Cell.* 15, 4854– 4865.
- Quick J, Ware JA, Driedger PE. 1992. The structure and biological activities of the widely used protein kinase inhibitor, H7, differ depending on the commercial source. *Biochemical and biophysical research communications*, 187(2), 657–663.
- Rabitsch KP, Petronczki M, Javerzat JP, Genier S, Chwalla B, Schleiffer A, Tanaka TU, Nasmyth K. 2003. Kinetochore recruitment of two nucleolar proteins is required for homolog segregation in meiosis I. *Dev Cell.* 4(4): 535-548.
- Rappaport R. 1961. Experiments concerning the cleavage stimulus in sand dollar eggs. *J exp. Zool.* 148: 81-89.
- Rappaport R. 1981. Cytokinesis: cleavage furrow establishment in cylindrical sand dollar eggs. *J. Exp. Zool.* 217: 365-375.
- Rappaport R. 1985. Repeated furrow formation from a single mitotic apparatus in cylindrical sand dollar eggs. *J Exp Zool.* 234: 167-171.

- Rappaport R, Ebstein RP. 1965. Duration of stimulus and latent periods preceding furrow formation in sand dollar eggs. *J. Exp. Zool.* 158: 373-382.
- Rath U, Rogers GC, Tan D, Gomez-Ferreria MA, Buster DW, Sosa HJ, Sharp DJ. 2009. The *Drosophila* kinesin-13, KLP59D, impacts Pacman- and Flux-based chromosome movement. *Molecular biology of the cell*, 20(22), 4696–4705.
- Rath U, Wang D, Ding Y, Xu YZ, Blacketer MJ, Girton J, Johansen J, Johansen KM. 2004. Chromator, a novel and essential chromodomain protein interacts directly with the spindle matrix protein skeleton in *Drosophila*. *J Cell Biochem.* 93, 1033– 1047.
- Reymann AC, Suarez C, Guerin C, Martiel JL, Staiger CJ, Blanchoin L, Boujemaa-Paterski R. 2011. Turnover of branched actin filament networks by stochastic fragmentation with ADF/cofilin. *Mol Biol Cell.* 22: 2541–2550.
- Rieder CL, Schultz A, Cole R, Sluder G. 1994. Anaphase onset in vertebrate somatic cells in controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J Cell Biol.* 127(5): 1301-1310.
- Rieder CL, Salmon ED. 1994. Motile kinetochores and polar ejection forces dictate chromosome position on the vertebrate mitotic spindle. *J. Cell Biol.* 124, 223–233.
- Rodriguez OC, Schaefer AW, Mandato CA, Forscher P, Bement WM, Waterman-Storror CM. 2003. Conserved microtubule-actin interactions in cell movement and morphogenesis. *Nature.* 5(7): 599-609.
- Rogers GC, Rogers SL, Sharp DJ. 2005. Spindle microtubules in flux. *Journal of cell science*, 118(Pt 6), 1105–1116.
- Rogers GC, Rogers SL, Schwimmer TA, Ems-McClung SC, Walczak CE, Vale R D, Scholey JM, Sharp DJ. 2004. Two mitotic kinesins cooperate to drive sister chromatid separation during anaphase. *Nature*, 427(6972), 364–370.
- Rothschild J. 1956. Fertilization. John Wiley & Sons: New York, New York. Print.
- Sagata N. 1996. Meiotic metaphase arrest in animal oocytes: its mechanisms and biological significance. *Trends Cell Biol.* 6 (1): 22 -28.
- Sandquist JC, Kita AM, Bement WM. 2011. And the dead shall rise: actin and Myosin return to the spindle. *Dev Cell.* 21(3):410–419
- Scholey JM, Rogers GC, Sharp DJ. 2001. Mitosis, microtubules, and the matrix. *The Journal of cell biology*, 154(2), 261–266.

- Schrader F. 1921. The chromosomes of *Pseudococcus nipae*. Biological Bulletin. 40(5): 259-270.
- Schrader F. 1923. A study of the chromosomes in three species of *Pseudococcus*. Archiv. Fur Zellforsch. 17:45-62.
- Schuh M, Ellenberg J. 2008. A new model for asymmetric spindle positioning in mouse oocytes. Current biology : CB, 18(24), 1986–1992.
- Schultz RJ. 1973. Unisexual fish: laboratory synthesis of a “species”. Science. 179: 180-181.
- Schultz RJ. 1966. Hybridization experiments with an all-female fish of the genus *Poeciliopsis*. Biol. Bull. 130: 415-429.
- Schultz RJ. 1967. Gynogenesis and triploidy in the viviparous fish *Poeciliopsis*. Science. 157: 1564-1567
- Schwacha A, Kleckner N. 1995. Identification of double holiday junctions as intermediates in meiotic recombination. Cell. 83: 783-791.
- Sharp DJ, Rogers GC, Scholey JM. 2000. Cytoplasmic dynein is required for poleward chromosome movement during mitosis in *Drosophila* embryos. Nat. Cell Biol. 2,922-930.
- Sheykhani R, Berns M, Forer A. 2017. Elastic tethers between separating anaphase chromosomes in crane-fly spermatocytes coordinate chromosome movements to the two poles. Cytoskeleton 74:91-103
- Sheykhani R, Baker N, Gomez-Godinez V, Liaw L, Shah J, Berns M, Forer A. 2013. The role of actin and myosin in PtK2 spindle length changes induced by laser microbeam irradiations across the spindle. Cytoskeleton. 70:241-259.
- Sillers PJ, Forer A. 1983. Action spectrum for changes in spindle fibre birefringence after ultraviolet microbeam irradiations of single chromosomal spindle fibres in crane-fly spermatocytes. Journal of cell science, 62, 1–25
- Silverman-Gavrila RV, Forer A. 2003. Myosin localization during meiosis I of crane-fly spermatocytes gives indications about its role in division. Cell Motil. Cytoskel. 55, 97–113
- Silverman-Gavrila RV, Forer A. 2001. Effects of anti-myosin drugs on anaphase chromosome movement and cytokinesis in crane-fly spermatocytes. Cell Motil Cytoskeleton. 50, 180-197.

- Silverman-Gavrila RV, Forer A. 2000. Evidence that actin and myosin are involved in the poleward flux of tubulin in metaphase kinetochore microtubules of crane-fly spermatocytes. *J. Cell Sci.* 113, 597–609.
- Silverthorn DU. 2016. "Muscles". *Human Physiology: An Integrated Approach* (7th ed.). San Francisco, CA: Pearson. pp. 377–416.
- Smith E. 2020. 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. Masters dissertation, York University.
- Snyder JA, Armstrong L, Stonington OG, Spurck TP, Pickett-Heaps JD. 1991. UV-microbeam irradiations of the mitotic spindle: spindle forces and structural analysis of lesions. *European journal of cell biology*, 55(1), 122–132.
- Spurck T, Forer A, and Pickett-Heaps J. 1997. Ultraviolet microbeam irradiations of epithelial and spermatocyte spindles suggest that forces act on the kinetochore fibre and are not generated by its disassembly. *Cell Motil Cytoskeleton* 36:136-48.
- Spurck TP, Stonington OG, Snyder JA, Pickett-Heaps JD, Bajer A, Mole-Bajer J. 1990. UV microbeam irradiations of the mitotic spindle. II. Spindle fiber dynamics and force production. *J. Cell Biol.* Ill: 1505-18.
- Stanton RA, Gernert KM, Nettles JH, Aneja R. 2011. Drugs That Target Dynamic Microtubules: A New Molecular Perspective. *Medicinal Research Reviews*, 31(3).
- Steffen W, Linck RW. 1992. Evidence for a non-tubulin spindle matrix and for spindle components immunologically related to tektin filaments. *Journal of cell science*, 101 (Pt 4), 809–822.
- Steuer ER, Wordeman L, Schroer TA, Sheetz MP. 1990. Localization of cytoplasmic dynein to mitotic spindles and kinetochores. *Nature*, 345(6272), 266–268.
- Stevens NM. 1905. Studies in spermatogenesis with especial reference to the “accessory chromosome.” *Carnegie Institution of Washington Publication* 36:1-33
- Stevens NM. 1909. Further studies on the chromosomes of the Coleoptera. *J Exp Zool* 6:101-113
- Strambio de Castillia C, Rout MP. 1999. TAPping into transport. *Nature cell biology*, 1(2), E31–E33.
- Straub FB. 1942. Actin. *Studies Med Inst Szeged* 2: 3–15.

- Suozzi KC, Wu X, Fuchs E. 2012. Spectraplakins: Master orchestrators of cytoskeletal dynamics. *J Cell Biol.* 197(4): 465-475.
- Svitkina TM, Borisy GC. 1999. Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. *J Cell Biol* 145: 1009–1026.
- Szent-Györgyi A. 1941. Towards a new biochemistry?. *Science (New York, N.Y.)*, 93(2426), 609–611.
- Tan JL, Ravid S, Spudich JA. 1992. Control of nonmuscle myosins by phosphorylation. *Annu Rev Biochem.* 61:721-59.
- Tanaka TU, Desai A. 2008. Kinetochore-Microtubule Interactions: The Means to the End. *Current Opinion in Cell Biology.* 20(1), 53–63.
- Tippit DH, Pickett-Heaps JD. 1977. Mitosis in the pennate diatom *Surirella ovalis*. *The Journal of cell biology*, 73(3), 705–727.
- Toth A, et al. 2000. Functional genomics identifies monopolin: a kinetochore protein required for segregation of homologs during meiosis I. *Cell.* 103(7): 1155-1168.
- Tran MT, Oldenbourg R. 2018. Mapping birefringence in three dimensions using polarized light field microscopy: the case of the juvenile clamshell. *J. Microsc.*, 271(3):315-324. <http://dx.doi.org/10.1111/jmi.12721>
- Tranchida MC, Macia A, Brusa F, Micieli MV, Garcia JJ. 2009. Predation potential of three flatworm species (Platyhelminthes: Turbellaria) on mosquitoes (Diptera: Culicidae). *Biological Control.* 49, 270-276.
- Trochine C, Modenutti B, Balseiro E. 2006. Influence of spatial heterogeneity on predation by the flatworm *Mesostoma ehrenbergii* (Focke) on calanoid and cyclopoid copepods. *J Plankton Res.* 28:267-274.
- Trochine C, Modenutti B, Balseiro E. 2005. When prey mating increases predation risk: the relationship between the flatworm *Mesostoma ehrenbergii* and the copepod *Boeckella gracilis*. *Arch Hydrobiol.* 163:555-569.
- Trybus KM. 1991. Regulation of Smooth Muscle Myosin. *Cell Motility and the Cytoskeleton.* 18:81-85.
- Tskhovrebova L, Trinick J. 2003. Titin: properties and family relationships. *Nature Reviews Molecular Cell Biology*, 4, 679-689

- Tuxworth RI, Titus MA. 2000. Unconventional myosins: anchors in the membrane traffic relay. *Traffic* (Copenhagen, Denmark), 1(1), 11–18.
- Tyska MJ, Warshaw DM. 2002. The myosin power stroke. *Cell motility and the cytoskeleton*, 51(1), 1–15.
- Umemoto S, Bengur AR, Sellers JR. 1989. Effect of multiple phosphorylations of smooth muscle and cytoplasmic myosins on movement in an in vitro motility assay. *J Biol Chem*. 264(3): 1431-6.
- Vale RD. 1999. Molecular motors and associated proteins. Introduction. In: *Guidebook to the cytoskeletal and motor proteins*. Kreis T. Vale R. (eds). Print. 367-377.
- van Hemert MJ, Lamers GE, Klein DC, Oosterkamp TH, Steensma HY, van Heusden GP. 2002. The *Saccharomyces cerevisiae* Fin1 protein forms cell cycle-specific filaments between spindle pole bodies. *Proceedings of the National Academy of Sciences of the United States of America*, 99(8), 5390–5393.
- Vaught D, Chen J, Brantley-Sieders DM. 2009. *Molecular Biology of the Cell* Vol. 20, 2572–2581. Regulation of Mammary Gland Branching Morphogenesis by EphA2 Receptor Tyrosine Kinase
- Vemu A, Atherton J, Spector JO, Szyk A, Moores CA, Roll-Mecak A. 2016. Structure and Dynamics of Single-isoform Recombinant Neuronal Human Tubulin. *J Biol Chem*. 291(25):12907-15.
- Verhey KJ, Gaertig J. 2007. The tubulin code. *Cell Cycle* 6, 2152–2160
- Vicente-Manzanares M, Ma X, Adelstein RS, Horwitz AR. 2009. "Non-muscle myosin II takes centre stage in cell adhesion and migration". *Nature Reviews. Molecular Cell Biology*. 10 (11): 778–90.
- Virkki N. 1971. Formation and maintenance of the distance sex bivalent in *Oedionychina* (*Coleoptera, Alticidae*). *Hereditas*. 68:305-312.
- von Dassow G, Verbrugghe KJC, Miller AL, Sider JR, Bement WM. 2009. Action at a distance during cytokinesis. *Journal of Cell Biology*. 187(6): 831-845.
- Walker DL, Wang D, Jin Y, Rath U, Wang Y, Johansen J, Johansen KM. 2000. Skeletor, a novel chromosomal protein that redistributes during mitosis provides evidence for the formation of a spindle matrix. *J Cell Biol*. 151, 1401–1411.

- Wang R, Zhang D, Zheng C, Wu S, Glotzer M, Tse YC. 2021. Cortical recruitment of centralspindlin and RhoA effectors during meiosis I of *Caenorhabditis elegans* primary spermatocyte. *Journal of Cell Science*. 134 doi:10.1242/jcs.238543
- Whalen RG, Butler-Browne GS, Gros F. 1976. Protein synthesis and actin heterogeneity in calf muscle cells in culture. 73(6): 2018-2022.
- Wiche G. 1999. Plectin. *In: Guidebook to the cytoskeletal and motor proteins*. Kreis T. Vale R. (eds). Print. 341-344.
- Wieser S, Pines J. 2015. The biochemistry of mitosis. *Mitosis*. Cold Spring Harbour Laboratory Press. 15-30.
- Wilson EB. 1905. Studies on chromosomes. I. The behavior of the idiochromosomes in Hemiptera. *J Exp Zool* 2:371-405
- Wilson L, Jordan MA. 1994. Pharmacological probes of microtubule function.
- Wilson DP, Sutherland C, Borman MA, Deng JT, Macdonald JA, Walsh MP. 2005. Integrin-linked kinase is responsible for Ca²⁺-independent myosin diphosphorylation and contraction of vascular smooth muscle. *The Biochemical journal*, 392(Pt 3), 641–648.
- Wilson PJ, Forer A, Leggiadro C. 1994. Evidence that kinetochore microtubules in crane-fly spermatocytes disassemble during anaphase primarily at the poleward end. *Journal of cell science*, 107 (Pt 11), 3015–3027.
- Wilson PJ, Forer A, Wise D. 2003. Microtubule distribution during meiosis I in flea-beetle [Alagoasa (*Oedionychus*)] spermatocytes: evidence for direct connections between unpaired sex chromosomes. *Journal of cell science*, 116(Pt 7), 1235–1247.
- Wise D, Sillers PJ, Forer A. 1984. Non-random chromosome segregation in *Neocurtilla hexadactyla* is controlled by chromosomal spindle fibers: an ultraviolet microbeam analysis. *J Cell Sci*. 69: 1-17.
- Woodrum DT, Rich SA, Pollard TD. 1975. Evidence for biased bidirectional polymerization of actin filaments using heavy meromyosin prepared by an improved method. *J Cell Biol*. 67: 231-237.
- Woodworth WM. 1897. Contribution to the morphology of the Turbellaria. II. On some Turbellaria from Illinois. *Bull Mus Comp Zool*. 31:1-16.
- Wühr M, Mitchison TJ, Field CM. 2008. Mitosis: new roles for myosin-X and actin at the spindle. *Current biology : CB*, 18(19), R912–R914.

- Xu D, Yin C, Wang S, Xiao Y. 2013. JAK-STAT in lipid metabolism of adipocytes. *JAK-STAT*, 2(4), e27203.
- Yamashiro S, Totsukawa G, Yamakita Y, Sasaki Y, Madaule P, Ishizaki T, Narumiya S, Matsumura F. 2003. Citron kinase, a Rho-dependent kinase, induces diphosphorylation of regulatory light chain of myosin II. *Mol Biol Cell*. 14(5): 1745-56.
- Yanagida M. 2015. The role of model organisms in the history of mitosis research. *Mitosis*. Cold Spring Harbour Laboratory Press. 1-14. Print.
- Yang Y, Dowling J, Yu QC, Kouklis P, Cleveland DW, Fuchs E. 1996. An essential cytoskeletal linker protein connecting actin microfilaments to intermediate filaments. *Cell*. 86: 655-665.
- Yao C, Rath U, Maiato H, Sharp D, Girton J, Johansen KM, Johansen J. 2012. A nuclear-derived proteinaceous matrix embeds the microtubule spindle apparatus during mitosis. *Molecular biology of the cell*, 23(18), 3532–3541.
- Yeh E, Yang C, Chin E, Maddox P, Salmon ED, Lew DJ, Bloom K. 2000. Dynamic positioning of mitotic spindles in yeast: role of microtubule motors and cortical determinants. *Molecular biology of the cell*, 11(11), 3949–3961.
- Yu I, Garnham CP, Roll-Mecak A. 2015. Writing and reading the tubulin code. *J. Biol. Chem.* 290, 17163–17172 7.
- Zhang D, Nicklas RB. 1999. Micromanipulation of chromosomes and spindles in insect spermatocytes. *Methods Cell Biol*. 61:209-18.
- Zhu L, Tran T, Rukstalis JM, Sun P, Damsz B, Konieczny SF. *Mol Cell Biol*. 2004 Apr;24(7):2673-81. Inhibition of Mist1 homodimer formation induces pancreatic acinar-to-ductal metaplasia.
- Zimowska G, Aris JP, Paddy MR. 1997. A *Drosophila* Tpr protein homolog is localized both in the extrachromosomal channel network and to nuclear pore complexes. *Journal of cell science*, 110 (Pt 8), 927–944.