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The role of the kinetochore in chromosome segregation during Meiosis I

Par
Evelyne Turrin

Programme de biologie moléculaire, Faculté de médecine

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Ce mémoire intitulé

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Présenté par

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Résumé

La ségrégation des chromosomes est un processus complexe permettant la division égale du matériel génétique entre les cellules filles. Contrairement aux cellules somatiques, ce processus est sujet à des erreurs dans les cellules germinales telles que les ovocytes. Lorsque des erreurs surviennent lors de la ségrégation des chromosomes durant la méiose cela peut conduire à une aneuploïdie. L'aneuploïdie est la présence d'un nombre incorrect de chromosomes dans une cellule et est connue pour causer l'infertilité et des arrêts de grossesses chez l'humain. L'incidence de l'aneuploïdie augmente avec l'âge maternel (1).

Le kinétochore est une structure cellulaire impliqué dans la ségrégation des chromosomes. Il est composé de plus de 100 protéines et se situe entre les microtubules et les centromères. Les microtubules se lient aux kinétochores, et ces derniers s'attachent sur les centromères afin de séparer les chromosomes homologues durant la méiose et les chromatides des sœurs pendant la mitose (1–3). Dans les cellules somatiques, cette structure est bien connue (2). Pourtant, moins d'informations sont connues à dans l'ovocyte de mammifère en développement au cours de la méiose I (3,4).

Ce projet vise à étudier le rôle du kinétochore durant la ségrégation des chromosomes dans l'ovocyte de souris en développement. Plus spécifiquement, l'assemblage, le désassemblage, la dynamique et la tension des protéines du kinétochore seront évalués. Ce projet permettra de mieux comprendre le rôle du kinétochore durant la méiose I, ses implications durant la séparation des chromosomes, et éventuellement ses implications dans l'aneuploïdie.

Mots-clés : Kinétochore - Ovocyte - Ségrégation des chromosomes - Méiose I - Aneuploïdie - Microtubules- Rupture de la vésicale germinale- Cellules somatiques - Delta - CDK1-CycB

Abstract

Chromosome segregation is an intricate process in dividing genetic material equally between daughter cells. This process, unlike in somatic cells, is error prone in germ cells like the oocyte. When errors occur during meiosis in segregating chromosomes, aneuploidy results when the cell has an incorrect number of chromosomes. This can result in infertility and birth defects in human reproduction. The incidences of aneuploidy are also seen to increase with increasing maternal age (1).

The kinetochore is a cellular structure at the heart of chromosome segregation. It is composed of more than 100 proteins and is located between the microtubules and the centromeres. The microtubules attach onto the kinetochores, which themselves attach onto the centromeres, in order to pull the homologous chromosomes apart during meiosis and the sister chromatids during mitosis (1–3). Much is known about this multi-protein structure in somatic cells (2). Yet, very little is known about this in the developing mammalian oocyte during Meiosis I (1,3,4).

This project aims to investigate the role of the kinetochore in chromosome segregation in a developing mouse oocyte. More specifically, kinetochore protein assembly, disassembly, dynamics and tension will be assessed. This project will achieve a better understanding of the kinetochore's role in Meiosis I, its implications in chromosome segregation in a developing mouse oocyte, and how it may be involved in aneuploidy.

Keywords: Kinetochore - Oocyte - Chromosome Segregation - Meiosis I - Aneuploidy - Microtubules - Germinal Vesicle Breakdown (GVBD) - Somatic cells - Delta - CDK1-CycB

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List of Abbreviations

APC/C- anaphase promoting complex/cyclosome

AurA- Aurora Kinase A

AurB- Aurora Kinase B

AurC- Aurora Kinase C

BSA- bovine serum albumin

cAMP- cyclic adenosine monophosphate

CCAN- constitutive centromere associated network

CDK1- cyclin dependent kinase 1

CENP- centromeric protein

CLASP- cytoplasmic linker associated proteins

CycB- cyclin B

GVBD- germinal vesicle breakdown

HJURP- Holiday junction recognition protein

IBMX- 3-siobotyl-1-methyl-xanthine

IVF- *in-vitro* fertilization

IVT- *in-vitro* transcription

MAPs- microtubule associated proteins

MPF- M-phase promoting factor

MTOCs- microtubule organizing centres

NEDB- nuclear envelope breakdown

PBS- phosphate buffered saline

PFA- paraformaldehyde

PGS- preimplantation genetic screening

PGT- preimplantation genetic testing

PGT-A- preimplantation genetic testing for aneuploidy

PMSG- pregnant mare serum gonadotropin

PMP- protein metallophosphatases

RE- restriction enzyme

SAC- spindle assembly checkpoint

Dedicated to Mom, Sabrina, Bruno, Simona, and Leah

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Chapter 1- Introduction

Chromosome segregation: Mitosis vs Meiosis

Chromosome segregation is an intricate, complicated, and important cellular process (5). Chromosome segregation, in somatic cells, is the process of dividing the genetic material equally from the parent cell into the resulting two daughter cells (5). In order for this to occur without errors, three things are required: something to identify each chromosome, something to attach the DNA to other cellular players to be able to be moved around, and something to create the force necessary to move around the DNA (2). These cellular players are the centromere, the kinetochore, and the microtubule, respectively (2). When chromosome segregation occurs correctly, chromosomes will move into the middle of the cell along the centre line termed the metaphase plate (6). As this occurs, microtubules emanate from the centrosomes to attach onto the kinetochores (6). These centrosomes have centrioles that help with microtubule polymerization (7). The formation of the spindle stems from the centrioles (1). This is organized spatially to be on either end of the cell and this will allow the microtubules to attach onto the chromosomes (7). In somatic cells, microtubules attach onto the kinetochores upon nuclear envelope breakdown (NEBD) (8). The kinetochores make direct contact with the centromeres, a structure in the middle of the chromosomes in humans (or at the end of chromosomes in mice) that hold the sister chromatids together and a site of recognition for the kinetochore to attach onto (1,6). Cohesin proteins are also responsible to keep the sister chromatids together prior to their separation (8). The cohesin proteins also provide tension to the kinetochores in opposite response to what the kinetochore feels at the microtubules; the microtubules are also generating a pulling force (7). The microtubules emanate from either end of the centrosomes, coming from spindle poles on opposite ends of the cell. This is termed bipolar attachment (6). When proceeding from metaphase to anaphase, the microtubules attached onto the kinetochores will pull apart the sister chromatids to either pole of the cell (1). Chromatids are held together by cohesin and this cleavage occurs when the separase protein is able to cleave cohesin; separase performs its action at anaphase (1). Telophase and cytokinesis then occur and form two identical daughter cells with equal amount of DNA material (9). This process of chromosome segregation is seen to occur in somatic cells (2,9).

The process of meiotic chromosome segregation occurs differently to what is observed in mitotic chromosome segregation. The first difference being sister chromatids are separated during mitosis whereas homologous chromosomes are separated during Meiosis I, and then followed by sister chromatids being co-segregated after the separation of the homologous chromosomes (3,7). The same terminology applies to kinetochores; in mitosis, sister kinetochores are pulled to either side whereas in Meiosis I, homologous kinetochores are pulled to either side of the cell (7,10).

The oocyte is larger compared to the somatic cell; mouse oocytes are roughly 80μm in diameter and human oocytes are 130μm in diameter (7). Oocytes undergo two divisions, Meiosis I and Meiosis II, to produce an oocyte that has half the genetic material than what it began with (1). Prior to entering meiosis, oocytes are arrested in prophase where they grow in size (7,12). Oocytes are ready for meiosis when they have a germinal vesicle (GV); this is when the DNA is compacted into a circular shape in the middle of the cell (7,13). Both Meiosis I and II result in asymmetrical cell division, where a large oocyte is formed and a smaller polar body is also formed that later dies (1,14). In Meiosis I, homologous chromosomes are separated that result in two daughter cells. Following in Meiosis II, sister pairs are separated, resulting in haploid cells that have half the genetic material to the parent cell (1,7). In order for the homologous chromosomes to separate in Meiosis I, microtubules that come from the same pole will attach onto sister kinetochores (3). This differs from somatic cells where microtubules come from opposite poles to pull apart the sister chromatids (2).

There are similarities and differences between cellular structures and proteins between the oocyte and somatic cell. Cohesin proteins that hold the chromatid arms together are present in both somatic and meiotic cells (7,10,15). Somatic cells contain centrosomes with centrioles that will nucleate microtubules. Mammalian oocytes do not contain either of these (7). This means that spindle pole location is not pre-determined, and spindle assembly and microtubule polymerization occur without a centrosome in the oocyte (1,7). Mouse oocytes contain multiple clusters of microtubule-organizing centres (MTOCs) to overcome this challenge (1,7). This is because mouse oocytes contain acentrosomal spindles, and the use of MTOCs are needed for bipolarity to occur and a bipolar spindle to form (8). MTOCs only cluster in mouse oocytes and do not cluster in human oocytes (7).

To recapitulate, the oocyte undergoes an intricate process in Meiosis I and Meiosis II in order to form the oocyte that will later be fertilised to become an egg. In Meiosis I at anaphase I and telophase I, an asymmetrical division occurs, where a larger, secondary oocyte is formed and the primary polar body is formed that later dies (1). NEBD or GVBD occurs where the DNA becomes condensed, the chromosomal ring in the middle becomes no more and the microtubules begin to polymerize (7,12). Afterwards, Meiosis II occurs where a haploid oocyte will result, with the secondary polar body forming and dying in conjugation (1).

Meiosis I is timed in both human oocytes and mouse oocytes (1,3,8). Mice oocytes are a well-established model for studying human oocytes. Both species undergo GVBD, where the nuclear envelope dissolves (1). Once this occurs, then spindle formation and migration occur (1).

The placement of the spindle is important because this ensures asymmetric cell division. Size of the oocytes and timing of oocyte maturation differ in humans to mice. Mouse oocytes are smaller in size (60-70μm in diameter) and human oocytes are larger (100-120μm in diameter) (1,17). In mice, it takes approximately 20 minutes for GBVD to complete, whereas in humans, it takes approximately 1 hour for GVBD to complete. In mice, it takes 3 to 4 hours after GVBD to form the bipolar spindle and microtubules and kinetochores are only stably attached 6 to 8 hours after GVBD (8). In humans, it takes 16 hours for the spindle to form and up to 20 hours for the kinetochore to make a stable attachment (7,18). It is also observed that some cellular structures tend to lose their structure and geometry with increase in female biological age. This increases the chance to observe a phenomenon termed aneuploidy (1,3,4).

Aneuploidy

The process of chromosome segregation should occur with high fidelity, ensuring proper distribution of genomic material. Yet, this process is prone to errors in the oocyte during meiosis (7). The oocyte is a large cell with a large cytoplasm. It undergoes an asymmetric cell division, with an improperly functioning spindle assembly checkpoint (1,14). The purpose of this asymmetry is to have one large egg capable to be fertilized as the end result (1,14). When chromosome segregation errors occur, a phenomenon termed aneuploidy results. Aneuploidy is when the incorrect number of chromosomes are present in the cell. In contrary, an euploid cell is when there is the correct number of chromosomes present in the cell (1). Aneuploidy is

predominately observed in cancer and infertility. In cancer, uncontrolled cell division occurs with improper checkpoints and it is shown that aneuploid cells are found in tumours and aid in their growth (1,19). Aneuploidy is also associated with an increase risk in infertility and birth defects; the risk of aneuploidy increases with increased maternal age (1,21). The mouse is an important model for studying aneuploidy, as 1 to 2 year old mouse oocytes show similar artefacts to that of older women with aneuploidy (4,20,22).

The concept of aneuploidy has been at the centre of debate for a number of years (23). It is also observed for genetic screen tests in determining if an embryo is healthy for implantation into the women, such as in *in-vitro* fertilization (IVF) treatments (24). These genetic screen tests like pre-implantation genetic screening (PGS) are under debate (23,25). Here, embryologists look for euploid embryos to discard those that are aneuploid (25,26). Now, PGS is termed PGT-A (preimplantation genetic testing aneuploidy) (27,28). The euploid embryos are deemed healthy with a high chance for successful pregnancy (23,24). Yet, most embryos are mosaic, having a mix of euploid and aneuploid cells (29). Some experts state these mosaic embryos should not be implanted and others say that mosaic embryos have the potential to result in successful implantation (23,27,28). The human oocytes and embryos discarded from fertility clinics and used for research are deemed unhealthy, but demonstrate potential to develop into healthy embryos and complete Meiosis I faithfully (3,29,30). This debate is ongoing and provides societal context for a scientific problem.

Aneuploidy can result due to poor microtubule attachments onto kinetochores, poor kinetochore attachments, misalignment of chromosomes along the metaphase plate, and loss of cohesin (1,4). There are two main types of oocyte chromosome segregation errors: sister chromatids separating too early and non-disjunction of chromosomes (1,31). The first type of error occurs due to loss of cohesion between the sister chromatids (in Meiosis II) or loss of cohesion between sister homologous chromosomes (in Meiosis I) (1,4). The errors in Meiosis II occur more frequently than the errors in Meiosis I (7,31,32). The second error, chromosomal non-disjunction, occurs when a pair of sister chromatids displace to the wrong cell during cell division due to defects in spindle assembly (1). Both types of errors are observed in mice and in human oocytes (1,31).

Oocytes are highly specialized in their function to undergo fertilization (7). The cellular factors that oocytes possess impact why this cell type is error prone in its cell division (7). In human

oocytes, it is observed that the spindle goes from apolar to bipolar while the microtubules assemble onto it. This bipolarity of the spindle is difficult to maintain and fluctuations of the spindle result in apolar or multipolar attachments, leading to chromosome segregation errors (7,10,15). Human oocytes lack MTOC clusters that are found in mouse oocytes and hence human oocytes are more error prone than mouse oocytes (7,30). These spindle errors are one cause of aneuploidy (7,10).

The gradual loss of cohesin is another cause of aneuploidy. Cohesin holds sister chromatids together and is dismantled by separase at anaphase (1,7,8). It is believed that mammalian oocytes begin with the set number of cohesin proteins prior to oocyte establishment and loss of cohesin occurs with increasing maternal age (1,22,33). This results in chromosome segregation errors (1,22). In older mice, a loss of cohesin has been observed (1,34,35). There was also found an increased rate of chromosome misalignments in older mouse oocytes (4,22). The majority of chromosome misalignments were bioriented and this increases the chance of chromosome segregation errors (4). In older mouse oocytes, there are also defects in spindle and microtubule assembly (1,36,37). Loss of cohesion is not solely responsible for aneuploidy and impacts minimally defective microtubules during Meiosis I (4).

Kinetochore defects and increased separation are also observed with increased maternal age (3). The young mouse kinetochore in the oocyte appears clustered whereas the kinetochore pair in older mice with aneuploidy appears separated (4,34,35). Also, older oocytes require more time to create proper kinetochore-microtubule attachments than younger oocytes (4). This is likely attributed to loss of cohesin proteins in aged oocytes (4,34,35). This loss of cohesion in older oocytes in Meiosis I coincides with the different kinetochore geometry observed (4). The protein Meikin is needed to ensure that sister kinetochores stay together. In older mice, there is less cohesin and Meikin, hence sister kinetochores are separated (3,34,38). Kinetochores from older mouse oocytes make less stable microtubule attachments and fewer robust end-on attachments compared to kinetochores from younger mice (2,4,8). Kinetochores in human oocytes undergoing Meiosis I appear similar to Meikin-deficient mice because these kinetochores appear to be separated due to less cohesin in older human oocytes (3,34,38). This loss contributes to aneuploidy (1).

Kinetochore-microtubule attachment errors occur independently of kinetochore geometry and contribute to aneuploidy (4). It was observed in human oocytes that inter-kinetochore distance increases with age independently of pre-existing fertility problems, and is part of aging. Yet, this may also contribute to aneuploidy (3). The kinetochore separation in human oocytes is greater than that observed in mice and contributes to why human oocytes are more prone to aneuploidy than mouse oocytes (3,7,30). The kinetochore is an integral structure to chromosome segregation and will be discussed in detail.

The kinetochore and its functions

The kinetochore is a cellular structure located between the microtubules and the chromosomes, and is where the microtubules attach onto in order to pull apart the chromosomes during chromosome segregation (2). The kinetochore attaches directly onto the centromeres, that hold the homologous chromosomes together during Meiosis (3,4). The kinetochore has 3 main functions. First, it is where the microtubules attach onto in order to pull the chromosomes apart during chromosome segregation. Second, it is partly responsible for force generation in pulling apart and moving the chromosomes (39). Third, it serves as a signaling hub between the chromosomes and the microtubule to ensure proper chromosome segregation (2,6). More specifically, it helps control the spindle assembly checkpoint (SAC) to ensure proper microtubule-kinetochore attachment (2,6,39).

The kinetochore has been studied in many model organisms including *Saccharomyces cerevisiae*, the yeast, maize, the mouse and in humans (3,39). There are similarities and differences of the kinetochore between these models. The yeast kinetochore only binds to one microtubule at a time, making it an easy model to study (39). During Meiosis I, the kinetochore of the budding yeast and the mouse appear fused together, whereas kinetochores in the human oocyte appear as distinct units and are able to make individual attachments onto microtubule spindles (3,38,40). Reasons why this occurs are less understood, but this contributes to the high incidence of aneuploidy observed in human oocytes (3). The MTOCs in mice cluster together, making the kinetochores that it attaches onto closer together in space and the lack of MTOCs in humans can impact the kinetochore separation (1,3,7,8). Differences between the model organism (mouse) and the one in which treatment is desired (human) should be noted.

The kinetochore and its proteins

The kinetochore is very well studied in somatic cells and limited in oocytes (2,6,8). The kinetochore is a dynamic structure that in vertebrate cells is composed of more than 100 proteins (2). Present at each kinetochore are many copies of each of these proteins (2,39). The kinetochore proteins can be subdivided into 3 protein groups each with a distinct function; the inner kinetochore proteins, the outer kinetochore proteins, and the regulatory proteins that are loosely associated to the kinetochore (Fig.1.1) (2,3). The following groups of proteins are found in the somatic cell and in the oocyte. Homologues of these proteins exist in other species (2,3,8,39,40).

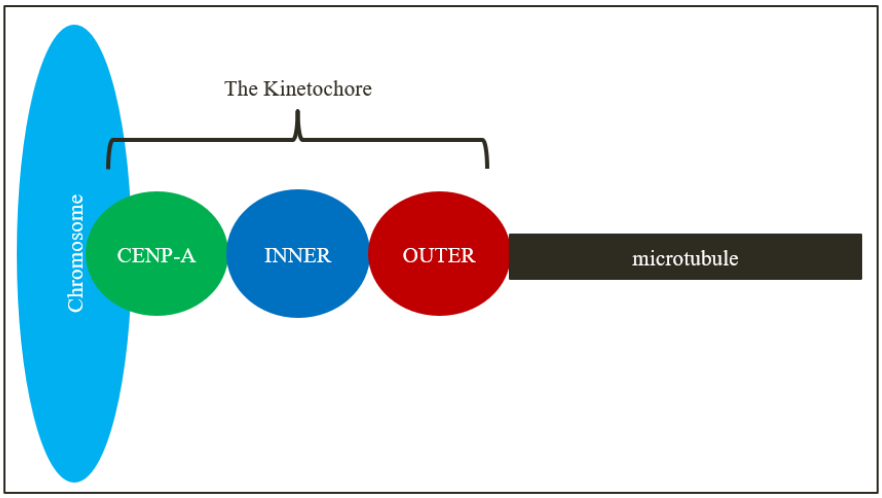

Figure 1.1: Organization of kinetochore proteins in the cell- *CENP-A and other CCAN proteins are the closest to the chromosomes. The inner kinetochore proteins are located between the centromeric proteins and the outer kinetochore. The outer kinetochore makes direct contact to the microtubule (2).*

The kinetochore and its proteins: the inner kinetochore

The inner kinetochore proteins make direct attachments onto the centromeres to connect the DNA and the kinetochore (2). The placement of the inner kinetochore proteins is required for the remainder of the kinetochore to assemble (2). A subgroup of the inner kinetochore proteins is called the constitutive centromere associated network (CCAN). The CCAN is composed of 16 kinetochore proteins that can be divided into 5 complexes and is always associated with the centromeres during the cell cycle (6,41). These protein complexes are CENP-C, CENP-

T/W/S/X, CENP-L/N, CENP-H/I/K/M, and CENP-O/P/Q/R/U (6,48). The first 4 complexes mentioned are necessary or chromosome segregation to take place (6).

CENP-A is centromeric protein A. CENP-A is a histone H3 variant located at the centromeres that is able to form nucleosomes (2,42). In nucleosomes, CENP-A takes the place of H3 at the end of the kinetochore and forms a nucleosome in complex with histone H2A/B and histone H4 (6,42). CENP-A is required for the kinetochore proteins to assemble at the centromeres (2). CENP-A is deposited onto the centromeres via acetylation by histone H4 Lys5 and Lys12 and is then monomethylated by histone H4 Lys20 in order for the inner kinetochore and other CCAN proteins to assemble onto CENP-A (6). The methylation serves as an epigenetic marker for the CCAN to recognize CENP-A in its assembly (6). In this way, CENP-A also functions as an epigenetic marker for the other kinetochore proteins to localize properly (2,6,43,44). CENP-A deposition begins at G1 in vertebrates. CENP-A will only be deposited on active centromeres completed by the histone chaperone HJURP (Holiday junction recognition protein) and the Mis18 complex (2,45,46). Mis18 will signal HJURP to the centromeres, that will then bring CENP-A onto the nucleosomes (2,45). During this time at G1, CENP-C is already present at the centromeres in somatic cells and serves as the receptor for Mis18 so CENP-A can accumulate (2,47). This ensures one location on the centromere for CENP-A to be deposited to provide one location where the kinetochore can attach to (2).

CENP-C is an important inner kinetochore protein that aggregates towards the kinetochore base and is needed to ensure that CENP-A is deposited at the centromeres (2,42,49). CENP-C binds directly to CENP-A and is an important link between the centromere and the inner kinetochore (Fig.1.2) (2,6,50).

CENP-T-W-S-X binds to DNA and is needed for stable attachment between the kinetochore and the centromere (2,50). Similar to CENP-C, this complex is also required to link the centromere to the outer kinetochore, in order for the outer kinetochore to be under the influence of the microtubules (2). CENP-T does not bind to CENP-A like CENP-C, but binds to centromeric DNA (6). CENP-T/W/S/X and CENP-A are responsible and required for the interaction between the kinetochore and the DNA (50). Both CENP-C and CENP-T arms of outer kinetochore assembly are necessary for human kinetochore function (39,51). How and where the inner

kinetochore proteins assemble and organize dictates how the outer kinetochore proteins assemble onto the inner kinetochore platform in space (39).

The kinetochore and its proteins: the outer kinetochore

The outer kinetochore proteins interact directly with the microtubules and are responsible for the connection between the inner kinetochore proteins and the microtubules (Fig.1.2) (2). The outer kinetochore dictates organizing events that occur at the microtubule plus-end and are partly responsible for generating force within the kinetochore. It is also suggested it may play a role in ensuring checkpoint activity by helping control the SAC and in correcting misattachments between the microtubule and the kinetochore (2,39,42).

The outer kinetochore is composed of three independent protein complexes that when combined form the KMN network (2). These protein complexes are the KNL-1 complex (KNL1C), the Mis12 complex (Mis12C), and the Ndc80 complex (Ndc80C) (2). KNL1C is composed of Zwint-1 and KNL1 (6). Mis12C is composed of Mis12, Dsn1, Nsl1, and PMF1 (Nnf1) (6,52). Ndc80C is composed of Spc24, Spc25, Nuf2, and Ndc80 (Hec1) (6). Unlike the CCAN that is constantly present at the centromeres, these outer kinetochore proteins are recruited onto the inner kinetochore proteins during mitosis (6). Ndc80C is the only outer kinetochore complex to form the connection between the inner kinetochore proteins and the microtubules via Nuf2 and Hec1 (2,6,42). The Mis12C binds to both the Ndc80C and KNL1C (6). KNL1C communicates with signaling proteins like Bub1 part of the SAC and phosphatases like protein phosphatase 1 (PP1) (6). More specifically, KNL1C is where Bub1 will bind to (42). Each outer kinetochore protein complex has its own function.

CENP-C and CENP-T are seen to recruit the outer kinetochore proteins onto the CCAN via two different pathways (2,6). In somatic cells, CENP-C recruits Mis12C and KNL1C during interphase (6,53). Ndc80C is the last outer kinetochore protein complex to be recruited and this occurs after NEBD (6). Unlike KNL1C and Mis12C, Ndc80C is not found in the nucleus during interphase (6,41). The CENP-T pathway for outer kinetochore protein recruitment differs highly compared to that of the CENP-C pathway (6). CENP-T will directly bind to Ndc80C without requiring KNL1C or Mis12C (6,54). In the CENP-C pathway, it is essential for KNL1C and Mis12C to bind prior to Ndc80C (6,54,55). This suggests that the CENP-C and CENP-T pathways may be differentially regulated (6). These connections of the outer kinetochore arms to

CENP-C and CENP-T are required for proper interactions of the outer kinetochore to the microtubules (2,50,56,57).

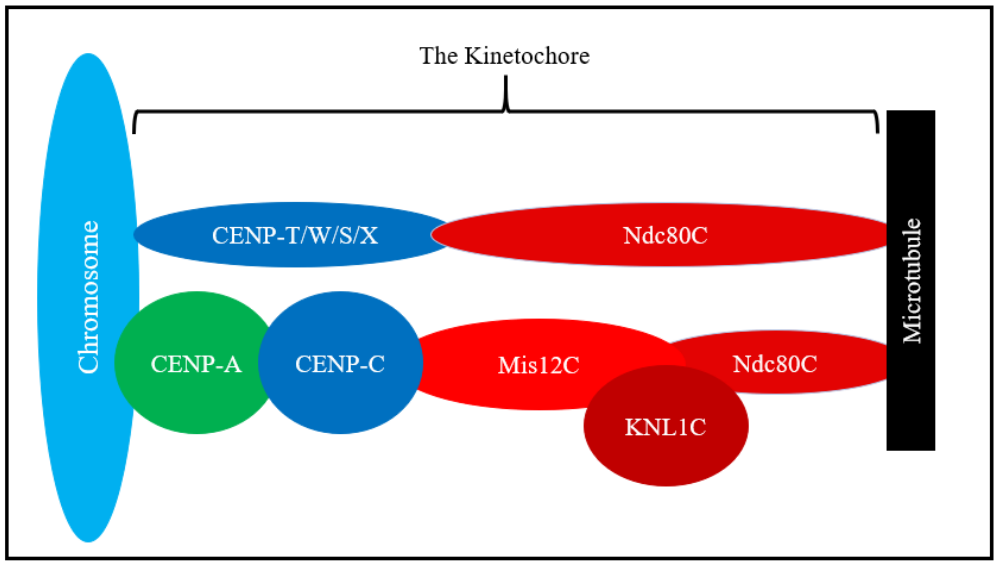

Figure 1.2: Organization of inner and outer kinetochore proteins in the cell- *CENP-A is in green, inner kinetochore proteins (CENP-C and CENP-T/W/S/X) are in blue, and outer kinetochore complexes (Ndc80C, Mis12C and KNL1C are in red). Inner and outer kinetochore proteins make direct attachments to one another, and Ndc80C makes interactions with the microtubules (2).*

The kinetochore and its proteins: the loosely associated proteins

The loosely associated kinetochore proteins are in control of signaling cascades and help in the communication between the kinetochore and the microtubules, the centromere, and the rest of the cell (2,6). Kinetochore assembly and disassembly is a well controlled process in somatic cells and these proteins that include Ska1 (spindle-associated and kinetochore-associated) complex, Bub1, Mad1 and Mad2 in vertebrates play an important role (2,6). Ska1 is needed for Ndc80C to bind to the depolymerizing end of the microtubule when force is being applied (2). Kinases like Aurora B (AurB) and the cyclin-dependent kinase 1 (CDK1) are highly involved in kinetochore assembly and disassembly in somatic cells and oocytes (8,10,41).

Kinetochore assembly

Kinetochore assembly and disassembly is well controlled in somatic cells. At the start of mitosis, the kinetochore remains detached from the microtubules (39). After NEDB, inner kinetochore

proteins rapidly assemble onto the centromeric proteins and within 20 minutes, outer kinetochore proteins assemble onto the inner kinetochore proteins (2). The inner kinetochore always assembles prior to the outer kinetochore in somatic cells of vertebrates, and the outer kinetochore always disassembles prior to the inner kinetochore (2,6,42). After inner kinetochore protein assembly, KNL1C and Mis12C assemble first at S phase, followed by Ndc80C assembly at the end of G2 (41,58). At anaphase, the KMN network comes off from the inner kinetochore platform (41).

During mitosis, kinetochore assembly occurs in a specific order in somatic cells. First, the histone H3 variant CENP-A remains constant during mitosis. Second, inner kinetochore proteins CENP-C and CENP-T raise by 50% within 10 minutes of nuclear envelope break down and decrease 50% within 10 minutes of anaphase (41). Third, the outer kinetochore assembles. Before NEBD, Mis12C is present at centromeres, but reaches its maximum levels during mitosis (41,58). The majority of Ndc80C accumulates after prophase and is incorporated into the nucleus at prophase (41). Disassembly of the outer kinetochore is well timed, beginning 6 minutes prior to anaphase onset and disassembly terminated within 20 minutes post anaphase (41).

In oocytes, kinetochore assembly is less understood and timing differs to that of somatic cells. Signaling cascades have been studied in the oocyte, but kinetochore assembly in the oocyte has been less investigated (3,4,8). It appears that the human kinetochore in meiosis has similar architecture to that of the human kinetochore in somatic cells with the inner and outer kinetochore being distinct parts (3).

The kinetochore and signaling cascades

The kinetochore is under control of many signaling cascades in the cell, the main one being M-phase promoting factor (MPF), that is composed of Cyclin B (CycB)-CDK1 (cyclin-dependent kinase 1) (6,60). CycB-CDK1 also controls when cells begin mitosis, and is the main kinase during M-phase (1,6,61). It is observed in somatic cells that as the level of CDK1 increases, so does the frequency of kinetochore protein assembly; the level of CDK1 activity controls the rate at which the mammalian kinetochore assembles (1,2,41). CDK1 levels increase rapidly in the mitotic cell, allowing for the kinetochore to assemble without delay (1,61). The assembly of CENP-C and CENP-T onto the mammalian centromeres is controlled by phosphorylation by

CDK1 seen at interphase (2). Phosphorylation by CDK1 at CENP-T is required for the interaction between CENP-T and the Ndc80C, in order to have proper contact between the inner and outer kinetochore arms (6). CDK1 does not only phosphorylate kinetochore proteins but phosphorylates other loosely attached proteins in order for them to bind to the kinetochore (6). CDK1 will phosphorylate the spindle-associated and kinetochore-associated (Ska) complex in order for it to bind to the end of Ndc80C to allow Ndc80C to interact with the microtubule (6,62,63). The phosphorylation driven primarily by CDK1 encourages the protein-protein interactions necessary for proper kinetochore protein communication and assembly (2).

CDK1 in oocytes exhibits different patterns of expression than in the somatic cell. In the somatic cell, CDK1 levels stay constant during mitosis and decrease after anaphase. In the oocyte, CDK1 activity increases slowly during Meiosis I and will peak at metaphase 6 hours post GVBD (1,8). This activity coincides with stable kinetochore microtubule interactions (8). Since CDK1 is dependent upon CycB, CycB levels in oocytes follow the same trends as CDK1 in the mouse oocyte (8). Also, CDK1 activity is required for stable end-on attachments between microtubules and kinetochores in oocytes (8). This is why CDK1 activity is gradual in oocytes because it puts off stable attachments for later on in meiosis, avoiding segregation errors (8). The slow accumulation of CDK1 also ensures slow kinetochore attachments and assembly (1). Yet, correctly oriented chromosomes could lack proper attachments to kinetochore-microtubules, since this assembly can occur even as delayed into late Meiosis I (1,64). It is thought that this slow assembly reduces errors in the oocyte by ensuring proper attachments (1,8).

Aurora B kinase (AurB) is also involved in kinetochore protein assembly. AurB aids in the interaction between CENP-C and the outer kinetochore by phosphorylating Dsn1, a protein of the Mis12C and allowing this outer kinetochore complex to localize to CENP-C (6). AurB is more involved with kinetochore protein disassembly by correcting for misattachments between the kinetochore and microtubule (1,2,10,42). AurB encourages turnover of the microtubule at improper attachments between the microtubule-kinetochore interface (1,65). AurB will recognize incorrect attachments and will phosphorylate outer kinetochore complexes Ndc80C and the Dam1 and Ska1 complex (2,58,62,66). This phosphorylation inhibits protein interactions between the kinetochore and microtubule and allows proper kinetochore-microtubule attachments to be made (2,58,62). In somatic cells, AurB is part of the chromosomal passenger

complex (CPC) and is able to act specifically at the inner centromere, only phosphorylating improperly attached kinetochores (2,6,67).

AurB behaves the same in mouse oocytes during Meiosis I as that in somatic cells during mitosis. Upon phosphorylation, it destabilizes the interactions between the kinetochore and the microtubule (8). Oocytes possess another homolog of Aurora kinase called Aurora Kinase C (AurC). In mammalian oocytes, AurC has similar functions to AurB (1). Yet, why two versions of the kinase are required in oocytes remain less understood (1,68). AurA also phosphorylates misattachments during Meiosis I, as seen in mitosis (7,69,70). AurB/C acts differently in meiosis in oocytes than does AurB in mitosis in somatic cells (7). Upon correct attachment in mitosis, AurB phosphorylation decreases whereas even with correct attachment in meiosis, AurB/C phosphorylation levels remain high (7,69). This is because since the kinetochore is being pulled in towards the centromere, it moves closer to the inner centromere where AurB/C is located (7). This is when phosphatase PP2A acts during metaphase to counteract this kinase and to stabilize attachments (7). Attachment of stable kinetochore-microtubule attachments is slow during meiosis. AurB/C activity is high until the spindle achieves bipolarity, and after Aurora activity decrease while CDK1 levels slowly increase (7,64). The main kinases that control kinetochore assembly and disassembly are CDK and the Aurora Kinases (7,8,41). Yet, other kinases are also involved in ensuring correct microtubule-kinetochore attachments, like Bub1, Mps1, and Polo-like Kinase 1 (Plk1) (2).

The kinetochore and its attachments to the microtubule

The kinetochore can make correct attachments to the microtubule via lateral attachments when the kinetochore binds to the microtubule from the side or end-on attachments when the kinetochore binds to the plus end of the microtubule (2). End-on attachments are stronger and more stable than lateral attachments (2,42). Ndc80C creates the kinetochore-microtubule attachments that often begin as lateral and then switch to the stable end-on attachment before proceeding with mitosis (39,71,72,73).

Kinetochore-microtubule attachments are also seen to contribute to aneuploidy. Young mice oocytes have more proper polar attachments than oocytes from aged mice, with older mice showing fewer end-on attachments (4,35,74). This demonstrates that timing of proper

attachments is malfunctioning in older oocytes and contributes to misattachments seen in aneuploidy (4).

The kinetochore can make different attachments to the microtubules depending where the oocyte is in its maturation. In Meiosis I, sister kinetochores make connections to the same pole and are conserved as a pair to divide the homologous chromosomes to its respective side, termed amphitelic attachments (1,75). In Meiosis II and in mitosis, sister kinetochores attach to microtubules coming from spindle poles on opposite ends of the cell to divide sister chromatids (1). Yet, correct kinetochore-microtubule attachments do not always occur and incorrect attachments can result. Some of these incorrect attachments include merotelic attachments, syntelic attachments, monotelic attachments, and no attachment (2,3,39). Merotelic attachments occur when one kinetochore makes connections to microtubules coming from both poles of the spindle poles (2,39). In somatic cells, merotelic attachments are not detected by the SAC and lead to chromosome segregation errors (4). When this occurs, a pull can be felt by the chromosome coming from both poles during chromosome segregation at anaphase. Hence, this improper attachment can lead to improper segregation and chromosome lag (1). Merotelic attachments also occur when the kinetochore-microtubule attaches to a spindle that fails to undergo bipolarization (7,10,15). Unlike in mitosis where kinetochore microtubule attachments are corrected prior to metaphase, kinetochore-microtubule attachments in meiosis can have merotelic attachments into metaphase and these need to be corrected (7,64,74,76). Merotelic and lateral attachments increase in older mouse and human oocytes that have less cohesin and cohesion (3,4,34,35). These misattachments can also occur due to a change in kinetochore geometry in older oocytes (4,77).

Merotelic attachments are not recognized by the SAC (spindle assembly checkpoint), but are corrected by tension sensors at the kinetochore (4,7,77). As AurB/C levels decrease, the increase in tension is sensed and merotelic attachments switch to end-on stable attachments (7). This is thought to occur upon the dephosphorylation of the substrates after AurB/C activity is completed (7,64).

The second misattachment, syntelic attachments (monopolar attachments) occur when both sister kinetochores make connections to one spindle pole (2,39,50). The third misattachment, monotelic attachments, occur when only one of the kinetochores in a sister pair make an

attachment (3). This is observed when an attachment occurs prematurely or when improper error-correction occurs (3,64). The fourth misattachment, unattached occurs when one or both sister kinetochores do not connect to the microtubule (2,50). In somatic cells, a correct bi-oriented attachment needs to occur between the sister kinetochores and the microtubules (2). The kinases ensure correct kinetochore-microtubule attachments for the cell to progress to anaphase; when errors are detected, the cell cycle is halted until correct attachments are made (2). This occurs with oocytes where in Meiosis I, amphitelic attachment is achieved in order to be able to properly segregate chromosomes to either end during anaphase (1).

In human oocytes during Meiosis I, the kinetochore can have different attachments to the microtubule. Sister kinetochore pairs can have one kinetochore make a connection to a microtubule or sister kinetochore pairs make connections to microtubules at the same time (3). The later attachment ensures that each kinetochore makes connections to a separate spindle (3). The homologous chromosomes can make attachments to spindles in Meiosis I in two ways, so the kinetochore pair in the human oocyte can act independently of each other (3). Hence, in human oocytes, there are 4 places where the kinetochore can attach onto independent of each other; making bi-orientation more difficult to achieve (3,64). This contributes to how error prone Meiosis I is (3).

The kinetochore and its control factors

Two control factors of M-phase progression include the SAC and the anaphase promoting complex/cyclosome (APC/C), which are both involved in Meiosis I (78). Anaphase is delayed upon SAC activation when incorrect kinetochore-microtubule attachments are sensed by the SAC at the kinetochore-microtubule interface (2,7,79). Correct attachments must occur before proceeding with the cell cycle to ensure correct chromosome segregation will take place (2,8). The proteins of the SAC include Bub1, BubR1/Mad3, Bub3, Mad1, Mad2, and Mps1. The SAC is a group of proteins that are part of the regulatory proteins that help the kinetochore to communicate with the rest of the cell (2,79). These SAC proteins aggregate to the kinetochore when improper attachments occur (1,80). This SAC signal is activated upon Bub1 phosphorylation and this will allow Bub1 to bind to Mad1 (6). The SAC also operates by the use of Mad2 in somatic cells that binds to kinetochores when there are misattachments between the kinetochore and the microtubule (2,81,82). Mad2 will form the mitotic checkpoint complex

(MCC) by binding with Mad3 and BubR (2,83). MCC then binds to inactive Cdc20 and this will prevent anaphase. Cdc20 will activate the APC once bound to it, once correct attachments occur and the cell will continue to anaphase (2,81,82). This is one way that the kinetochore behaves as a signaling hub for communication with the cell in ensuring proper chromosome segregation (2).

In oocytes, the SAC also functions during Meiosis I, though at low intensity. It is found to be inactivated in late MI, even when misaligned chromosomes and misattachments between the kinetochore and microtubule are present (8,84,85). The SAC in oocytes cannot sense minor misattachments or misaligned chromosomes and allows errors to persist in mice and human oocytes by not inhibiting the APC/C (1,84,85). This is thought to occur due the oocyte's large size. (1).

The way kinetochore assembly and signal transduction occurs in the oocyte during meiosis differs greatly to that in the somatic cells during mitosis (8). Sister kinetochores in meiosis need to attach to one pole only to ensure that homologous kinetochores will be pulled to either end of the cell; this separation of homologous kinetochores is needed for separation of homologous chromosomes (7,86). It is also observed that the stable attachment between kinetochores and microtubules is different between mitosis and Meiosis I. In mitosis, stable kinetochore and microtubule attachments occur after NEBD when the kinetochores are bioriented (7,10). In meiosis, the stability increases steadily well into metaphase, when the kinetochores are switching from apolar to bipolar (7,8,84,85). This activity coincides with CDK1 levels in the cell (7,8,41). As well, though the SAC is well functioning in somatic cells, this error correction method presents itself with less fidelity in meiotic cells (1,8). Therefore, kinetochore assembly and signaling transduction is well known in the somatic cell, but needs to be investigated in oocytes (8).

Kinetochore disassembly

Phosphatases are used to disassemble the kinetochore and continue the cell cycle (2). Yet, how the kinetochore disassembles is less understood than how the kinetochore assembles in somatic cells (6). The kinetochore only begins to disassemble upon high levels of the APC/C at anaphase onset (41). It is known that the CycB-CDK1 levels are also seen to dictate the rate at which the kinetochore disassembles. CycB-CDK1 activity decreases upon the degradation of CycB (6). It is

observed that a combination of decreasing CDK1 levels and the action of protein phosphatases are required for the outer kinetochore to begin in its disassembly (41). This cues the end of mitosis and kinetochore disassembly. These phosphatases include the protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) (6). PP1 opposes the activity of AurB by dephosphorylating the substrates of AurB, like Dsn1 of the Mis12C (6,87). This occurs at anaphase when AurB moves from the inner centromere to along the spindle (6). This is how the CENP-C-Mis12C interaction between the inner and outer kinetochore terminates. The PP2A phosphatase will dephosphorylate the substrates phosphorylated by CDK1, specifically the CENP-T-Ndc80C interaction between the other inner and outer kinetochore arm (6). This dephosphorylation is essential in order for outer kinetochore protein disassembly to occur (41). Ndc80C is the outer most component of the outer kinetochore in contact with the microtubule, and in order to obliterate this interaction, dephosphorylation must occur (41). Outer kinetochore protein disassembly occurs independently of activity at the microtubule, and is governed by phosphorylation and dephosphorylation patterns (41). In somatic cells, CDK1 levels are seen to peak right after NEBD, and will stay constant until after anaphase where they drop (6,41).

In oocytes, the role of kinetochore disassembly is less known, but CDK1 levels appear to control kinetochore assembly. CDK1 levels in the oocyte accumulate slowly after prophase and peak at metaphase(1,8). Also, Protein Kinase C (PKC) in the oocyte plays a role in controlling anaphase onset, and is involved in kinetochore disassembly (60). Yet, kinetochore disassembly and its other protein regulators in the oocyte are much less understood (1,4,8)

The kinetochore and distance: the concept of delta

The kinetochore is a flexible structure that is dynamic and compliant, with the distance between kinetochore proteins changing over time (6,42,88). This distance that separates kinetochore proteins is termed delta (42). It is observed in somatic cells that the inner kinetochore proteins undergo the most displacement whereas the outer kinetochore proteins appear noncompliant (42). This was deduced by using anticancer drugs like taxol that inhibits microtubule polymerization prematurely activating the SAC (42,82,89). When cells were treated with taxol at metaphase when the kinetochore was under the most tension from the microtubules, this allowed the kinetochore to be viewed under no tension (42,88). The kinetochore proteins were observed to approach one another, whereas under partial influence from the microtubule they remain

separated (42). This demonstrates that the kinetochore is a compliant and dynamic structure (42). Kinetochore compliance in oocytes remains to be investigated (3,4)

Measurements of delta in somatic cells describe the stiffness or compliance of the kinetochore proteins. It was observed in somatic cells that kinetochore proteins change their delta throughout mitosis, with emphasis during metaphase when maximum tension is observed and chromosomes oscillate at the metaphase plate (42,88). It was found in somatic cells that only CENP-A and CENP-C are compliant proteins during metaphase oscillations of the chromosomes, whereas all other kinetochore proteins remain noncompliant (42). Oscillations occur when chromosomes change rapidly between poleward and antipoleward movement due to microtubule influence. Due to the oscillations, either the kinetochore can be noncompliant and stiff or the kinetochore can be compliant and move along with the oscillations (42,90,91).

The Ndc80C was found to be a stiff complex in somatic cells. Under treatment of taxol, Ndc80C remained noncompliant, unlike the inner kinetochore proteins (42). The Ndc80C remained fixed at metaphase even when under high tension from the microtubules in relation to the inner kinetochore (42). This result was surprising since Ndc80C helps to transduce the force derived from the microtubules (42,92). Nocodazole is another drug used to obliterate the microtubules, similar to Taxol. This occurs by nocodazole binding to β-tubulin and preventing polymerization (42,93). This drug was used depolymerize microtubules to view Ndc80C under no tension and it was observed that the Ndc80C proteins became closer together (42). This suggests that the Ndc80C may be flexible from within, but as a whole the structure does not move (42).

In somatic cells, CENP-C was the inner kinetochore protein observed to be the most compliant. The remainder of the CCAN was found to be noncompliant during metaphase oscillations with Ndc80C, and including complexes KNL1C and Mis12C (42). Under taxol treatment, it was observed that CCAN proteins such as CENP-A, CENP-C, and CENP-T all moved towards the Ndc80C, rather than towards the centromere (42). Components of the KNL1C and the Mis12C were also seen to move during Taxol treatment (42). A possibility for this occurrence is the existence of two arms to the kinetochore; CENP-C attaching to Mis12C, KNL1C and Ndc80C, and CENP-T-W-S-X attaching to Ndc80C (2,42). The CENP-C arm with the KMN network is compliant and more flexible whereas the CENP-T arm with Ndc80C remains stiff and noncompliant (42). However, in taxol treated cells, the Ndc80C arms moves towards the

centromere, compared to the KMN network arm which remains in place. This insinuates an uncoupling of the kinetochore arms under this treatment and that the kinetochore arms may act independently of each other. Overall, it was found that the link between the centromeres and the CCAN was compliant, whereas the link between the CCAN and to the outer kinetochore is noncompliant in somatic cells (42).

The kinetochore delta measurements in somatic cells was completed, but has yet to be done in oocytes. The oocyte has different CDK1 level activity than in somatic cells and is possible that delta will differ in the oocyte compared to the somatic cell (1,8). One preliminary study observed the distances between the meiotic human sister kinetochores increase with age (3,34,64,94). Also, the intra-kinetochore distance from the inner to the outer kinetochore increases with age in relation to cohesion loss (3,34). The kinetochore distance is greater in human oocytes compared to mouse oocytes in relation to aneuploidy (3). Yet, the distances surrounding the meiotic kinetochore is understudied and worth investigation (3).

The kinetochore and force

The term force has multiple applications in different fields. It is a vector, where it has both speed (magnitude) and a direction. In physics terms, F=ma, where ‘m’ is mass in kilograms and ‘a’ is acceleration in m/s^2. A force upon an object can create or destroy, make bonds or break bonds, and can create or release tension (50,95). The kinetochore, and not solely the microtubules, is partly responsible in generating the force to pull apart the chromosomes during chromosome segregation in somatic cells (50,88). The kinetochore is under both passive and active force within itself; the active forces create the force and the passive forces are observed at points of friction (88). The kinetochore is able to slide over itself and is able to polymerize and depolymerize (50,88).

Force at the kinetochore has two main functions. The first is to signal correct attachments of the kinetochores to the microtubules and the second is to pull apart the chromosomes for chromosome segregation to occur (50). The kinetochore transduces the force that is derived from the microtubules, in order to correctly orient and pull apart the paired sister chromatids in mitosis (50,88). The kinetochore is under different amounts of force during cell division due to the varying length of microtubules during the cell cycle (50). The greatest amount of force and

tension observed at the kinetochore is during metaphase when opposite spindle poles make connections to the sister chromatids to align the chromosomes in a bioriented manner (42,50,88,96). Two main movements can occur at the kinetochore. Poleward movement predominately occurs at anaphase when the kinetochore is being pulled toward the opposite pulls of the cell (50,88). Antipoleward movement occurs when the kinetochore is being moved away from the cell membrane extremities and towards the centre of the cell (50,88). Both of these movements affect the intra-kinetochore and interkinetochore distances (50). Oscillating between these two movements occur when the chromosomes are lined at the metaphase plate (88). During these oscillations, there is also force coming from the chromosomes as they are getting ready to separate their sister chromatids (50,96). It was observed that during poleward movement, the distances between the kinetochore proteins decrease and the kinetochore compresses within itself, due to active forces at the kinetochore (88). When the microtubule is depolymerizing, active forces are produced at the kinetochore and this creates a pull of the chromosomes towards the poles (88). The opposite is observed upon polymerization of the microtubule, and friction is created at the kinetochore due to passive forces as a result. This passivity is generated by antipoleward movement, and the sliding motion of the kinetochores going over the microtubules (88,97,98). Mechanical compliance of the kinetochore allows the kinetochore to respond physically to a force acting upon it, either via compression or stretching (88).

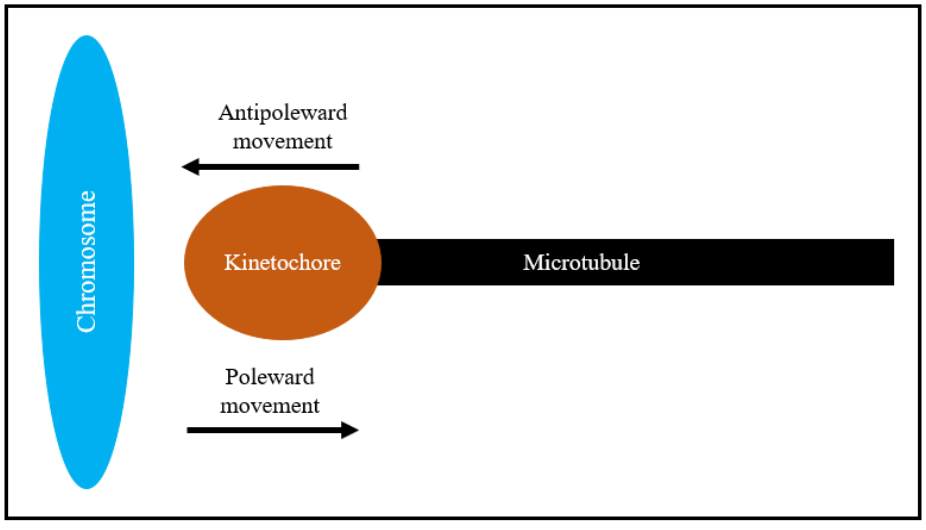

Figure 1.3: Movement of the kinetochore- *The kinetochore can move in an antipoleward fashion towards the chromosome, or in a poleward fashion towards the microtubules (50).*

Microtubules are responsible for pulling apart the chromosomes via force derived from their polymerization and depolymerization. The kinetochore is also another force transducer; it is responsible for generating the force from the microtubules onto the chromosomes to pull them apart. (39,88). This interaction between the kinetochore and the microtubule is dynamic throughout the cell cycle. In somatic cells, beginning at prometaphase, microtubules bring kinetochores to the metaphase plate where the centromere-kinetochore-microtubule attachment is made. Following this at anaphase, the kinetochores still attached to the centromeres pull apart the homologous chromosomes to either end of the cell with the force generated by the microtubule (2). The kinetochore begins its attachment to the microtubule laterally, but converts to the more robust end-on attachment when pulling the chromosomes apart during chromosome segregation. (2,4,8). Upon making end-on attachments, the force to pull apart the chromosomes can come almost all from the microtubules depolymerizing. This allows the kinetochore to maintain the connection between the centromere and the microtubule and not generate all the force (2,99).

The force required to pull apart the chromosome derive mainly from the microtubules upon their depolymerization. When microtubules depolymerize, this process is also called catastrophe and it can generate a significant force up to 65pN (50,100). The kinetochore must find a way to use this force with control and decrease the rate of the microtubule; by forming precise attachments and modulating the tension (50,101–103). Also, the kinetochore is able to withstand a high amount of force and remains stable under high tension (50). The kinetochore can withstand up to 700pN of force, when the typical force to break a bond is 100pN, demonstrating its robustness (50,104).

The kinetochore is able to diffuse the force from the microtubules to its proteins in the arms of the outer-inner kinetochore, allowing the proteins to experience the force at the same time (50). Ndc80C is the outer kinetochore protein complex that attaches to the microtubule; 10 to 20 Ndc80C proteins can attach to one end of a microtubule, making for a robust interaction and stable way to diffuse the force (50,105–107). In humans, 20 microtubules can be attached to one kinetochore via its plus end, demonstrating the stability of the kinetochore (39,42). This occurs as the microtubules undergo polymerization and depolymerization (39).

Several motor proteins generate force at the microtubule-kinetochore interface including the kinetochore motors dynein and kinesin (50). The spindle poles and kinetochore can also generate a force; not solely the microtubules (50,88,108,109). When incorrect attachments of the

kinetochore-microtubule arise, the forces from the kinetochore can aide in moving the chromosomes by regulating tension and help control the microtubule in its movements (50). The kinetochore also uses its force and the force from the microtubule to correctly guide the chromosomes spatially (2). In humans, the kinetochore works with the microtubules in order to move the chromosomes over a distance of 5μm while experiencing forces of 100pN (39,103,110).

Proteins termed MAPs (microtubule-associated proteins) act at the kinetochore to help promote polymerization and depolymerization of the microtubules (39). These proteins are the cytoplasmic linker associated proteins (Clasp1 and Clasp2) and the kinesin-like proteins (Kif2c and Kif18a), respectively (2,111,112). These proteins aid the kinetochore by helping control microtubule dynamics, hence allowing the kinetochore to use its force and the force coming from the microtubules to correctly guide the chromosomes in their placement and segregation (2). More specifically, Clasp proteins encourage polymerization and inhibit depolymerization of the microtubule (42,112). Other motor proteins include the CENP-E motor protein and the CENP-F coiled- coil protein (39,42). CENP-E is a type of kinesin protein dependent upon Bub1 and is partly responsible for ensuring correct kinetochore-microtubule attachments. Bub1 is needed for CENP-E to localize to kinetochores (42,113–115). CENP-F, like CENP-E also requires Bub1 to localize to the kinetochore (42,58,113). Many of the motor proteins are redundant in their function. It is thought that since the kinetochore is robust with many attachments, using these redundant proteins helps to ensure the kinetochore functions properly (39).

The kinetochore is able to sense when misattachments occur at the microtubule-kinetochore interface. This is due to tension sensed at the kinetochore; improper attachments produce less tension at the kinetochore than do proper attachments (50,88). The kinetochore can sense the distance between its pairs (interkinetochore distance) and within a kinetochore, the inner and outer kinetochore distance (intrakinetochore distance) (50,116). The kinetochore assembly is a timely process and the distance between kinetochore proteins in a single kinetochore can be measured over time. Therefore, the distance between inner kinetochore components and outer kinetochore components is coupled and when misattachments occur, these distances become uncoupled (50,88,116). This is a way for the kinetochore to verify its assembly (50). The distances between the kinetochore proteins are dependent upon force from within the kinetochore

and from the microtubule (50,88). This is well observed in somatic cells, and more needs to be investigated in oocytes (7,50,88).

AurB is an important kinase able to correct for misattachments between the kinetochore and the microtubule by sensing tension between these two cellular components (50,67,117). When AurB senses a misattachment, or lack of tension, its substrates are under a high amount of phosphorylation (50,51,117). This phosphorylation weakens the affinity of the outer kinetochore to the microtubule and keeps the kinetochore unattached to the microtubule, awaiting a proper attachment (39,50).

When correct biorientation is achieved, the substrates of AurB become dephosphorylated. This is when distance and spatialization have an impact (50,67,117). AurB is located at the inner centromere region whereas the majority of its substrates are located at the outer kinetochore (42,50). When proper attachment and tension is achieved, the outer kinetochore becomes further away from the inner kinetochore/centromere (50). This distance prevents AurB from phosphorylating its substrates; hence tension and force also play a role in ensuring correct kinetochore-microtubule attachments (50). When the activity of AurB is decreased, the tension is stabilized and so are the attachments between the kinetochore and the microtubule (8). In monopolar attachments, there is less tension between the kinetochore and microtubule attachments than observed in proper bipolar attachments (15,39,65). When this loss of tension occurs due to improper attachments, other error correction mechanisms ensue. How this lack of tension activates AurB is less understood, but is a way in correcting improper attachments (39).

In oocytes, the same was observed where AurB plays a role in tension and ensuring kinetochore-microtubule attachments during MI (8). Yet, AurB/C is less sensitive in its activation and inactivation in meiosis than in mitosis. Phosphorylation of AurB stops in mitosis as tension persists and the substrates of the outer kinetochore go further away from AurB (7). In oocytes, it phosphorylation by AurB/C onto kinetochore substrates still persists, even when the kinetochore becomes stretched (1,64). This is an inherent problem. AurB/C prevents improper kinetochore-microtubule attachments and encourages this binding turnover (1,65). In oocytes, even when maximum tension of the chromosome and kinetochore persist, microtubule-kinetochore turnover could also persist if phosphorylation by AurB/C keeps continuing (1,64). If turnover keeps occurring, then reattachments can occur, leading to mis-attachments when wanting to attach

again, and hence segregation error. This is another reason why the oocyte is prone to chromosome segregation (1).

Chapter 2- The Rationale and Hypothesis

The Rationale

The kinetochore, its assembly, and its signaling cascades have been extensively studied in the somatic cell (2). Yet, much less is known about the kinetochore in the developing mammalian oocyte in regards to its assembly, proteins, and signaling cascades (1,7,18). There are many differences between the oocyte and the somatic cell in regards to kinetochore assembly and dynamics. First, CDK1 levels are different in the mammalian oocyte compared to that in somatic cells (1,8). Second, the oocyte has another homolog of Aurora kinase than somatic cells (1,39). Third, the oocyte posses an error prone chromosome segregation and is subject to aneuploidy more than the somatic cell (1,3,4). The kinetochore is at the centre of chromosome segregation and may be involved in aneuploidy (3,4). This project aims to investigate the kinetochore in developing mammalian oocytes during Meiosis I, and achieve a better understanding of this structure and its potential role in aneuploidy. The kinetochore appears to be flexible and compliant in somatic cells and oocyte findings will be compared to known somatic data (42).

The Hypothesis

In regards to the oocyte kinetochore, it is hypothesized that oocyte kinetochore protein assembly will undergo different timing and kinetics compared to what is observed in the somatic cell. It is also hypothesized that the kinetochore's flexibility, compliance, and delta will differ in the mammalian oocyte during Meiosis I than what is observed in somatic cells during mitosis. This project is divided into two aims that focus on: 1) kinetochore protein assembly and 2) kinetochore protein dynamics. The first aim of this project hopes to look at the kinetochore protein assembly and observe where along Meiosis I this compares and contrasts with actions in mitosis. The second aim of this project will observe kinetochore compliance in the mouse oocyte and compare that to the somatic cell.

Chapter 3- Materials and Methods

Methods

The aims of this project were completed via 3 major sets of experiments: First, viewing kinetochore assembly in fixed oocytes via staining with antibodies. Second, viewing kinetochore assembly in live oocytes via microinjection with mRNAs that will be translated to proteins that are fluorescently tagged. Third, fixing oocytes and staining with antibodies after microinjection to measure delta of kinetochore proteins.

Dissection

Prior to dissection, 5 collection dishes *(Culture Dish; Thomas Scientific #9380D40)* must be prepared. 2 dishes with 5mL each of IBMX:M2 media 1:1000, *(M2 medium; Sigma-Aldrich® #M7167* and IBMX (3-isobotyl-1-methyl-xanthine) *(IBMX; Sigma-Aldrich ® # I5879)* and placed on the heat pad set to 37°C. 1 dish with 20µL drops of IBMX:M2 in a flower pattern, covered with mineral oil to be placed on the heat pad *(Mineral Oil (light oil/neat); Sigma-Aldrich® #M8410*). 1 dish with 20µL drops of IBMX:M16 (1:1000) in a flower pattern *(M16 medium; Sigma-Aldrich® #M7292*) and 1 dish with 20µL of M16 medium to be placed in the incubator, set to 37°C with 5% CO_2. IBMX is a drug that is a non-selective phosphodiesterase inhibitor. It is able to halt meiosis by keeping cAMP concentrations high in the oocyte, keeping the oocyte under meiotic arrest (118)**.**

Young CD-1 female mice aged 3 to 4 months *(Charles-River #022CD1)* were injected with 0.2mL pregnant mare serum gonadotropin (PMSG) *(PMSG Genway Biotech; GWB-2AE30A),* two days prior to sacrifice (4). Injections of PMSG were administered via syringe with a 27gauge needle and injected subcutaneously *(1mL syringe; BD Biosciences #309659* and *Precision Guide Needle 27G; BD Biosciences #305109)*. Injections were performed by holding the back and neck of the mouse with one hand, flipping the mouse so that the stomach is facing up, and injecting in the lower abdomen close to the legs. PMSG is a gonadotropin and causes the ovaries to undergo superovulation; this promotes folliculargenesis and increased oocyte formation. Injections were given Day 1 in the afternoon (between 16:00-18:00) (119–121).

On Day 3 in the morning (07:00-10:00), CD-1 female mice were sacrificed for their ovaries. Sacrifices and dissections were performed in the animal facility. Mice were placed in a biological safety hood within a chamber for which to manipulate the mice**.** The ventilation box was connected to two tubes; one supplied oxygen and the other isoflurane. The mice began in the box with only oxygen supplied to them, and slowly, the isoflurane concentration was increased to 3-4%. This anesthetized the mice. They were only removed from the box once a reflex test was administered to confirm anesthesia, and sacrificed by neck dislocation. Post dislocation, a reflex text is administered, and then dissection to extract the ovaries can commence.

The mouse was flipped onto its back and ethanol was sprayed on its stomach to wet the hair and prevent it from contaminating the ovaries. The skin was cut at the pelvic line and opened up to expose the peritoneal cavity**.** This lining was cut to expose the stomach, intestines, ovaries and fallopian tubes. The intestinal organs were pushed up towards the upper chest to expose the fallopian tubes and ovaries. The fallopian tubes were pulled up to locate the ovaries. The fallopian tube was cut at the base of the ovaries to isolate both ovaries from the mouse. The ovaries were placed in a 1.5mL Eppendorf tube (*Sigma-Aldrich® #Z336769*) filled with IBMX:M2 (1:1000).

The ovaries in IBMX:M2 medium were dissected under the microscope without a heat pad. In between manipulations, the culture dishes were placed back on the heating pad (if containing M2) or back in the incubator (if containing M16). Under a dissecting microscope, the ovaries were placed in Dish #1 with 5mL IBMX:M2. The fat was stripped away from the ovaries via tweezer manipulation (*Ideal-Tek, 5.SA*). The fat was left in Dish #1 and the ovaries were placed in Dish #2 containing 5mL IBMX:M2. Tweezers in one hand held the ovary in place and in the other hand was a syringe with a needle 27gauge needle. The syringe with the needle was used to scratch away the ovary to dissect it and release the oocytes from within it.

Oocytes covered in a surrounding layer of granulosa cells must be transferred from Dish #2 to Dish #3 containing the IBMX:M2 drops. This was completed via mouthpipetting. With a Bunsen burner, the tip of a glass pipette was molded into just about the size of a diameter of a mouse oocyte. This was confirmed under the microscope. Once this was completed, a P1000 filtered tip was added to it, which was then added to the rubber mouth pipette (*Aspirator Tube; Sigma-Aldrich® #A5177-5EA*). The plastic holder of the aspirator was placed against the lips where an air flow controlled the rate at which and how many oocytes were taken in. Prior to taking

oocytes, a small amount of media was aspirated before aspirating the oocytes. Oocytes were aspirated from Dish #2, and then released into Dish #3. Only those that are surrounded by granulosa cells were selected for via pipetting. Once all the oocytes were transferred into a drop in Dish #3, they were washed away of the granulosa cells that surround them. The oocytes were moved to each drop to wash away the granulosa cells in each drop. This was done by moving quickly the oocyte in and out of the glass pipette tip by inhaling and exhaling quickly. Granulosa cells were slowly lost away from the oocytes in each drop and were devoid of surrounding granulosa cells at the end. Oocytes were transferred from Dish #3 with IBMX:M2 drops to Dish #4 (in the incubator) with IBMX:M16 drops.

Dish #4 was taken from the incubator and placed on the microscope. A small amount of IBMX:M16 media was aspirated into the glass pipette tip first. Oocytes were transferred from Dish #3 of IBMX:M2 to Dish #4 of IBMX:M16, were washed from in every drop of IBMX:M16 to equilibrate, and then placed in the incubator to recover from the manipulation. Either, the oocytes were fixed and stained with antibodies or microinjected with mRNAs that will be translated to proteins that are fluorescently tagged in live experiments.

Fixation and antibody staining

Oocytes were fixed at different time points of Meiosis I. Some oocytes remained in Dish #3 IBMX:M16 to be fixed at Hour 0 of Meiosis I. The remaining oocytes were transferred and washed from Dish #3 IBMX:M16 to Dish #4 M16, and placed back in the incubator to allow continuation of Meiosis I. Every 2 hours until the 10 hour time point, equal number of oocyte groups were removed from the incubator and fixed. Oocytes at Hour 0 were at the GV stage, up until oocytes at the Hour 10 stage with some extruding polar bodies. Prior to fixation, a 96 well plate (*Mikrotest plate; Sarstedt company #D-51588*) was prepared as follows.

Oocytes were moved from left to right, and the solutions were placed in the 96 well from left to right (Fig. 2.1). Every solution was covered with 1 to 3 drops of mineral oil except the Triton drop. The first drop contained 20µL of 2% PFA:PBS *(Paraformaldehyde; Sigma-Aldrich® #P6148 and Phosphate Buffered Saline Dulbecco 'A' Tablets; OXOID™ #BR0014).* The next 3 drops contained 20µL of 1% BSA:PBS *(Bovine Serum Albumin; Sigma-Aldrich® #A7906).* The next drop contained 10µL 0.25% Triton X *(Triton X-100; Sigma-Aldrich® #T9284)* (only placed at the time of oocyte transfer). The following 3 drops contained 20µL of 1% BSA:PBS. The

ninth drop contained 20µL of 3% BSA:PBS. After preparation of the 96 well plate, oocytes were fixed every 2 hours following IBMX wash up until Hour 10. Aluminum foil *(Wrapex; 45cm x 100cm)* covered the 96 well plate between manipulations. The oocytes were placed in 2% PFA:PBS for 20 minutes at room temperature for fixation. Next, they were moved in the next 3 drops of 1% BSA:PBS for 5 minutes each to wash at room temperature. Then, the oocytes were in the Triton X drop for 10 minutes at room temperature. The oocytes were washed again for 3 drops of 1% BSA:PBS for 5 minutes each at room temperature. Next, the oocytes are placed in 3% BSA:PBS overnight at 4°C to block.

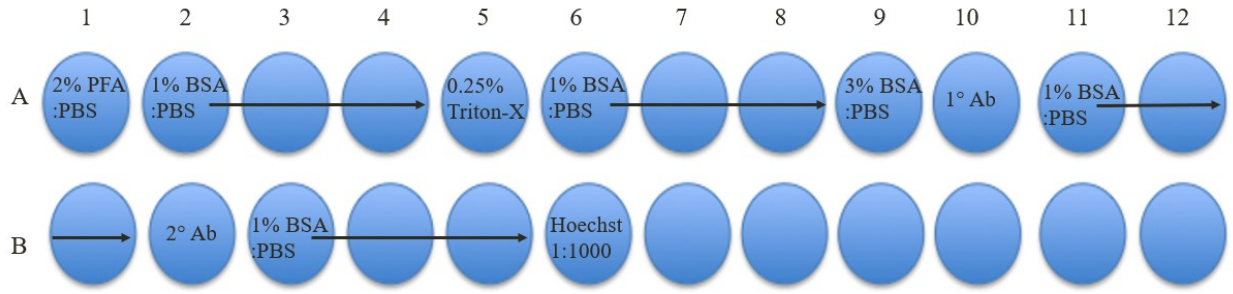

Figure 3.1: Fixation protocol for oocytes- *The 96 well plate is prepared with solutions as shown, and oocytes are mouthpipetted from left to right in row A, and continue on with antibody staining into row B. Antibodies are specific to experiment.*

The following day, 5 to 10µL of the primary antibody was placed in well 10 and covered with mineral oil. The oocytes were stained with the primary antibody for 1 hour at 37°C and wrapped in aluminum foil to minimize light exposure. After, 3 washes of 5 minutes each at room temperature were performed in 1% BSA:PBS. Then, the oocytes were placed in a well containing 5 to10µL of the secondary antibody covered with oil. They were then placed at 37°C for 1 hour covered in aluminum foil. Prior to imaging, the oocytes were dyed with Hoescht *(Hoechst 33342; Invitrogen™ #H1399)* at a 1:1000 dilution in 1% BSA:PBS to stain the chromosomes. After antibody staining, the oocytes were imaged the same day on the confocal microscope to optimize the stain from the antibodies.

The specific antibodies were used as followed and all diluted in 1% BSA:PBS. There were three rounds of staining for three separate groups of oocytes, one for each part of the kinetochore. The first group of oocytes were only stained with CENP-A at 1:200 *(Centromeric Protein A; New*

England BioLabs (NEB) #2048S), the centromeric part of the kinetochore. The second group was stained only with CENP-C at 1:250 dilution *(Covance Costum Antisera #PA5758; gift from Ben Black),* the inner kinetochore protein. The third group was stained only with Hec1 at 1:500 dilution *(Hec1; gift from the Bazurek lab),* the outer kinetochore protein. All the kinetochore protein antibodies were of rabbit isotype, hence staining for them at the same time would cause unspecific signaling and was avoided. The secondary antibody used for each of the kinetochore proteins was the same anti-rabbit antibody at 1:1000 dilution *(Goat anti-rabbit IgG (H+L) cross-absorbed secondary antibody, Alexa Fluor 488; Invitrogen™ # = A-11008; source = Invitrogen™).* Yet, each group of oocytes, following primary and secondary staining for the kinetochore protein, were also stained with primary antibody for β-tubulin at 1:1000 dilution *(β-tubulin; Sigma-Aldrich® #T4026)* and corresponding secondary antibody *(Goat anti-mouse IgG (H+L) cross-absorbed secondary antibody, Alexa Fluor 633; Invitrogen™ catalogue # = A-21050).* Finally, prior to imaging, the oocytes were dyed with Hoechst.

Protocols for antibody use of CENP-A and CENP-C differ slightly. For CENP-A, oocytes must be incubated for 2 hours at 30°C in a λ-phosphatase treatment that was covered with mineral oil. This treatment was composed of 1μL λ-phosphatase, 5μL PMP (Protein MetalloPhosphatases) buffer, 5μL $MnCl^2$ *(all from NEB in a kit)* and 39μL of ultra pure water *(CulGenex™ #CG490).* The oocytes were then stained with the CENP-A antibody for 1 hour at 37°C. For the CENP-C antibody, 2μL of 0.1% of sodium azide was added to an 8μL aliquot of antisera. Following fixation, oocytes were imaged as described in the confocal microscopy protocol further below.

Microinjection

For a live experiment, oocytes were microinjected with mRNAs that are translated to proteins that are fluorescently tagged. The microinjection rig must be assembled prior microinjecting the oocytes. The microinjection rig was comprised of a Leica microscope, a holding pipette that was attached to an oil rig, a needle with a wire inside that was connected to a current provider, a pneumatic picopump to provide a current, a wire attached to a rig *(from Kanetec)* that went in the solution to close the current, and a platform to place the oocytes. The holding pipette and the needle were both attached to separate pivot systems that can be moved with handles *(from Narishige)*.

To finalize the microinjection set up, the bottom of a culture dish *(Thomas Scientific #9380D40)* had a square drawn on it with a marker. This was to indicate where to place the IBMX:M2 medium. Approximately 120µL of the medium was placed in an even layer, with oil placed on top to submerge the large drop underneath. This was placed carefully on the platform under the microscope. Next, the holding pipette was placed in its holder and carefully placed in the drop. Verification by the microscope was necessary to ensure the holding pipette was placed in the middle of the drop and not touching the bottom of the dish. To make the holding pipette, a glass capillary *(Borosilicate glass; Sutter Instruments #B100-75-10)* was placed in a horizontal puller *(Flaming/Brown micropipette puller Sutter Instruments)* that made two needles, separating the capillary in half. One of the needles was then placed in a microscope holder *(Micro-forge MF-900 Microscope: Narishige)* to shape it. It was cut, bent and shaped via a heated glass bulb, to the measurements necessary to hold an oocyte in place. Next, the holding pipette was placed on the microinjection rig. The oocytes were then washed from the IBMX:M16 media to the IBMX:M2 media in drops on a culture dish. Once washed, the oocytes were transferred from the washing drops to the dish on the microinjection rig platform. The needle in the holder was assembled last. To make the needle, a glass capillary *(Havard apparatus #GC1505-15)* was placed in a vertical puller *(Sutter Instrument),* that was then pulled in half to make two needles. The top needle was carefully taken, and inside the needle was pipetted 1µL of the mRNA. The needle was then placed on the microinjection rig with the wire inside of it. The wire provided the current to the needle to inject the oocytes with the probes. The needle was then lowered into the IBMX:M2 media. One at a time, an oocyte was taken by the holding pipette and injected swiftly and carefully with the needle. Once all the oocytes had been injected, they were washed from the IBMX:M2 media and back into the IBMX:M16 media. The oocytes were then placed again into the incubator to recover from their manipulation and to allow time to observe expression of the proteins. Approximately 2 hours later, verification was observed under the fluorescent microscope. After microinjection, the oocytes were imaged live on the microscope (see "confocal microscopy" for protocol).

For these experiments, 3 probes were used: CENP-B-eGFP *(Gift from Michael Lampson; Addgene plasmid # 107264),* SPC24-mCHERRY *(Gift from Keith T. Jones lab),* and MajSat-TALE-mClover *(Addgene plasmid #47878).* All three plasmids are resistant to Ampicillin. CENP-B-eGFP has a T7 promoter and its restriction enzyme (RE) for linearization is NdeI

(NEBL #RO111S). SPC24-mCHERRY has a T3 promoter and its RE for linearization is SfiI *(NEBL #R0123S)*. MajSat-TALE-mClover has a T7 promoter and its RE for linearization is NotI *(NEBL #R0184S)*. Either CENP-B and Spc24 were coinjected (1:2 ratio CENP-B:Spc24) or TALE and Spc24 were coinjected (1:1 ratio) for live experiments. The protocol below describes how the probes were made.

Making mRNAs that will be translated to proteins that are fluorescently tagged

The mRNAs come from a plasmid that contains the genes of interest next to the gene for the fluorescent protein of choice, i.e. H2B:RFP plasmid DNA. In short, the plasmid DNA was linearized, purified, and underwent an in vitro transcription (IVT) reaction to get the resulting mRNA that be translated to a protein that is fluorescently. After IVT reaction, the mRNA also underwent poly(A) tailing and purification to increase stability.

Making mRNAs: transformation and inoculation procedure

Agar plates that contain ampicillin were used. The plasmids provided here were received on a piece of paper. DH5α cells were used for the transformation. Two Eppendorf tubes of 50µL of DH5α cells were prepared; one as a control where 5µl of water was added and one where the 5µl of plasmid was added. After setting it on ice for 30 minutes, then 90 seconds heatshock at 42℃ in a thermocycler, and then 10 minutes on ice again, each of these samples had 500µL of LB Broth *(Lennox L Broth Base; Invitrogen ™ #12780-052)* added to it. The sample was then incubated for 45 minutes at 37℃ and then centrifuged for 5 minutes at 4000rpm. The supernatant was discarded and each sample was plated onto a separate agar dish containing ampicillin. The culture dish was placed on a nonshaking incubator overnight.

The next day, inoculation was performed. The agar plates were put in the fridge to prevent colonies from spreading. Around a Bunsen burner, 3 Eppendorf tubes each for a separate colony were filled with 6.5mL LB broth, 6.5µL ampicillin, and one colony each from the plasmid plate. These samples were left in a shaking incubator overnight.

Making mRNAs: DNA mini prep

The mini prep protocol was followed as outlined by the QIAprep Spin MiniPrepKit *(Qiagen #27106)*. The end product was the plasmid in a liquid solution and must be linearized.

From the previous step, the sample from the shaking incubator overnight must be spun down and the supernatant must be discarded. With the remaining pellet, 250µl Buffer P1 *(Resuspension Buffer; Qiagen #19051)* of the miniprep was added and vortexed briefly. Then, 250µL of Buffer P2 *(Lysis Buffer; Qiagen #1014940)* was added, the HCl neutralizes the solution and turns it blue; mixed by inversion. Then, 350µL Buffer N3 *(Neutralization Buffer; Qiagen #1014796)* was added, also mixed by inversion. The sample was centrifuged for 10 minutes at maximum, the supernatant was placed in a separate tube, and the step was repeated. The column was now centrifuged for 1 minute at maximum with the flowthrough at the end discarded. 500µl of Buffer PB *(Binding Buffer; Qiagen #105089)* was placed on the column, centrifuged for 1minute at maximum, and flow through was discarded. This step was repeated with 750µl of Buffer PE *(Wash Buffer; Qiagen #101520).* The empty column was then centrifuged for 1minute (plasmid is in the filter). Then, 30µl of Buffer EB was placed *(Elution Buffer; Qiagen #19086),* sit still for a minute and then spun down. It was then again centrifuged for 1 minute at maximum, the liquid was put back again on the column, sit still for a minute, and then centrifuged one last time. The sample was then linearized next.

Making mRNAs: linearize DNA

Per plasmid, a 50µL reaction mix was made containing the following: 7.5µL plasmid, 5µL CutSmart buffer at 10x conc *(NEBL #B7S045),* 36.5µL ultra pure water, and 1µL restriction enzyme specific to the plasmid being used. This was mixed via pipetting the solution carefully or flicking the Eppendorf tube. It was placed in the thermocycler at 37℃ overnight. To inactivate the enzyme and perform extraction of the DNA, the temperature and time must be verified per enzyme (i.e. it is 65℃ for 20 minutes for CENP-B and TALE, and no heat inactivation for Spc24). Linearization was confirmed via gel.

Making mRNAs: extraction via DNA mini prep

The extraction via DNA miniprep was performed as followed with the QIAprep Spin MiniPrepKit *(Qiagen #27106)*. The liquid from the previous step was put in a new spin column, centrifuged for 1 minute for maximum and the flowthrough was discarded. The column was first washed with 500µl of Buffer PB, centrifuged at maximum for 1minute, and flow through was discarded. Next, the column was washed once more with 750µl with Buffer PE, centrifuged for 1 minute at maximum and flowthrough is discarded. The column is centrifuged 1 minute more, and

then placed in a clean tube. The DNA was then eluted with 50µl of Buffer EB (10mM TrisCl, pH8.5), sat for 1 minute and centrifuged 1 minute at maximum. The DNA was then extracted, run on a gel to confirm, and IVT reaction was performed.

Making mRNAs- IVT *(in-vitro* transcription) reaction

The following reaction must be performed to transcribe the DNA to RNA. The enzyme used was specific to each promoter site used per plasmid. For example, Spc24 used a T3 promoter, and TALE and CENP-B used a T7 promoter. The T3 promoter kit was used *(Invitrogen™ MEGAscript™ T3 Transcription Kit #AM1338)* and so was the T7 promoter kit used *(Invitrogen™ MEGAscript™ T7 Transcription Kit #AM1334).*

For each plasmid, 20µl of reaction was made containing: 2µL of 10x Reaction (RXT) buffer, 10µL of 2x DNTPS, 2µL of template (the sample from before), 4µL of ultra pure water, and 2µL of the enzyme that were provided in the kit except for the template. The mixture was spun down and flicked to mix and put in a thermocycler overnight set at 37℃. The next day, 1µL of TURBO DNAse was added to remove extra template, flicked to mix, and put at thermocycler at 37℃ for 20 minutes. A gel was run to confirm.

Making mRNAs: Poly(A) tailing reaction

A Poly(A) tailing kit *(Invitrogen™ #AM1350)* was used for the following reaction. After IVT reaction from the previous step, the following was added: 36µL of ultra pure water, 20µL of 5x *E*-PAP (*E.coli* Poly(A) Polymerase I) buffer, 10µL of MnCl2 and 10µL of ATP and 4µL of *E*-PAP enzyme. It was flicked to mix and put in a thermocycler for 80 minutes at 37℃ for the three samples.

Making mRNAs- RNA cleanup

RNeasy kit *(RNeasy Minikit; Qiagen #74104)* was used for the RNA cleanup, provided by Invitrogen. From the PolyATail reaction, the mixture was transferred to another Eppendorf tube. 350µl of the Buffer RLT *(Lysis Buffer; Qiagen #1015750)* was added to it and 250µl of 99% ethanol; this was centrifuged for 30 seconds at 8000rpm and the flowthrough was discarded. After, 500µL of the Buffer RPE *(Wash buffer; Qiagen #1017974)* was added to this, again, centrifuged for 30 seconds at 8000g and flow through was discarded; this step was repeated

except centrifuged for 2 minutes at 8000g. The column was then transferred to an empty Eppendorf and centrifuged for 1 minute at maximum. Then, the column was moved again and 30μL of ultra pure water was added, spun down, and centrifuged for 1 minute at 8000g. The eluate was put back onto column and recentrifuged for 1 minute at maximum speed. The eluate was collected and the RNA was collected. The sample was aliquoted and stored in the -80℃.

Preparing oocytes to measure delta

For the delta procedure, oocytes were injected solely with CENP-B. They were then placed in the incubator to recover and fixed every two hours as described above. At the 8 hour time point prior to fixation, half of the oocytes were placed in 100μM solution of nocodazole *(Sigma-Aldrich® #487928)* in M16 media for 20 minutes in the incubator and then fixed. Nocodazole is an anticancer agent and depolymerizes the microtubules by binding to β-tubulin and preventing microtubule polymerization (122,123). Afterwards, the oocytes were either stained with CENP-C or Hec1 as described above.

Confocal microscopy

The SP8 Laser-scan-head type, (fitted with a hybrid HyD detector) confocal microscope from Leica was used for all experiments.

For live imaging, the settings were as follows: all lasers were turned on, as either Spc24-mCHERRY was coinjected with MajSat-TALE-mClover or CENP-B-eGFP. As well, the cells were stained with Hoechst prior to putting on the microscope. The scan parameters were as follows: acquisition mode at xyzt, format at1024x1024, speed at 400Hx, bidirectional X at on, zoom factor and z-stack varied on a 20x objective. Z-stack images would be taken every 10 minutes. For line average, line accumulation, frame average, and frame accumulation were all set to 1. The sequential scan was set to 'between frames'. For Sequence 1, CENP-B or TALE was used, with HyD1 laser on and laser 488 was used with range between 498-540 with gain at 100 at 1% laser power. Also on Sequence 1, Brightfield PMT trans was on with gain at 250. For Sequence 2, Spc24 was used, with PMT lasers and PMT trans off, and HyD1 laser on and laser 520 was used with range between 555-750 with gain at 100 at 1% laser power. For Hoechst, laser 405 was used with range between 415-485 with gain at 100 at 1% laser power. The live experiments were left running over night for a duration of 16 hours to be able to view Meiosis I.

For fixed imaging, Z-stack 3D images were obtained with the following settings with all lasers turned on. The secondary antibodies used for the kinetochore antibodies (Hec-1, CENP-A, and CENP-C), were all based in rabbit and in order to save materials, were imaged separately to not cause cross signaling. For CENP-C/CENP-A/Hec1 was imaged on laser 488, microtubules were imaged on laser 520, DNA was stained with Hoechst and imaged on laser 405. Microscope settings were as follows: format at 1024x1024, speed at 400Hx, bidirectional X was on, zoom factor and z-stack varied. Line average, line accumulation, frame average and frame accumulation were all set to 1. 63x objective with oil was used. Since fixed imaging was taking place for samples at 0,2,4,6,8,10 hours, the sample with the brightest intensity had to be found first to set the maximum laser power intensity and gain. Afterwards, the laser power and gain would stay fixed for the duration of the experiment when imaging the different time groups. For example, for Hec1, that was at 2 hours, CENP-A was constant throughout relatively, and CENP-C was at 8 hours. The sequential scan was set to 'between frames'. First, CENP-C settings were as follows: Sequence 1 for CENP-C with HyD1 laser 488 at 0.3% laser power and gain at 100 (PMT Trans on and gain at 300), Sequence 2 for tubulin with HyD1 laser 568 at 0.7% laser power and gain at 120, Sequence 3 for DNA with HyD1 laser 405 at 1% laser power and gain at 120. Second, CENP-A settings were as follows: Sequence 1 for CENP-A with HyD1 laser 488 at 1.4% laser power and gain at 60 (PMT trans on and gain at 350), Sequence 2 for tubulin with HyD1 laser 568 at 1.2% laser power and gain at 100, and Sequence 3 for DNA with HyD1 laser 405 at 1.5% laser power and gain at 100. Third, Hec1 settings were as follows: Sequence 1 for Hec1 with HyD1 laser 488 at 1.2% laser power and gain at 40 (PMT trans on and gain at 350), Sequence 2 for tubulin with HyD1 laser 568 at 1.5% laser power and gain at 50, and Sequence 3 for DNA with HyD1 laser 405 at 1.8% laser power and gain at 70. This allowed to produce the fixed images at high resolution (3).

For imaging delta, the microscope settings were as follows: format at 1024x1024, speed at 400Hx, bidirectional X at on, zoom factor and z-stack varied. The sequential scanner was set to 'between frames'. The lasers were all on and set for: Sequence 1 for DNA stained with Hoechst at HyD1 laser 405, Sequence 2 for CENP-B at HyD1 laser 488, Sequence 3 for CENP-C or Hec-1 at HyD1 laser 520 (stained with AlexaFluor®568), and Sequence 4 for β-tubulin microtubules at HyD1 laser 647 (stained with AlexaFluor®633). This was done at 63x objective and line average, line accumulation, frame average and frame accumulation were all set to 1. It was

essential that the microtubules were on the same plane as the chromosomes when passing through the z-stack to ensure when measuring the distance between the kinetochores that it on the same plane as the chromosomes.

ImageJ and FIJI image analysis

How protein intensity is measured

All analysis was performed by using Image-J. Protein intensity was measured for every fixed time point and for every hour for live imaging. For live imaging, 5 kinetochore pairs were measured at every hour from six oocytes, having a total of n= 30 kinetochores per time point. For fixed imaging, 14 kinetochore pairs were measured at every time point with a varying number of oocytes (having the n number change per kinetochore protein per time point). See 'Results' below for details.

The protocol for measuring the protein intensity for live and fixed data was the same. The middle z-stack was resolved where the chromosomes were found parallel to the plane of the oocyte. This is where the most kinetochores were resolved. At this z-stack, protein intensity was measured for the kinetochore proteins. A line was drawn through the kinetochore protein and with pressing 'control M' on the keyboard, the maximum intensity was recorded. This allowed to observe maximum fluorescence emitted from the probes. At every time point, background noise intensity from within the cell was also sampled. The background intensity was subtracted from the maximum intensity to reveal the true intensity without background signal. At every time point, the intensity was plotted by taking the average of the maximum intensities. Significance was measured with an unpaired T test at 99% confidence.

How delta is measured

Delta was measured differently compared to measuring signal intensity (Fig. 2.2). The middle Z stack was found where the microtubules and chromosomes were on the same plane. A kinetochore pair was selected that was attached to either end of the same chromosome. A line was drawn through the kinetochore pairs along the line of the chromosome. This line measured protein intensity along distance; the distance corresponded to the length of the line drawn out. This line was drawn once and by selecting for each protein, a graph was able to be generated. This graph measured protein intensity along a function of distance. Each graph had two peaks at

either extremity with noise in the middle close to the x-axis. The two peaks corresponded to peak intensity from the kinetochore proteins at either end of the chromosome, with the middle noise corresponding to the chromosome in the middle. There were two ends: end A and end B corresponding to Delta 1 and Delta 2. The distance along the x-axis corresponding to the peak intensity of the proteins must be found. These distances were subtracted from each other at either end of the chromosome, that would result in the distance that is Delta 1 and Delta 2. Significance was measured with an unpaired T test with 99% confidence.

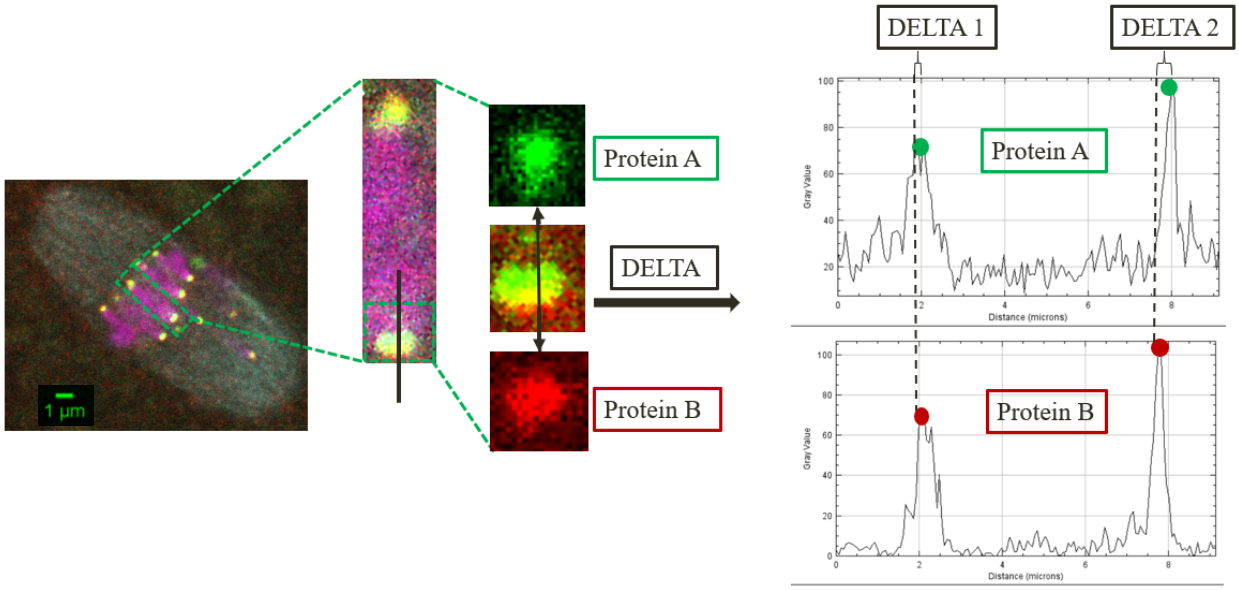

Figure 3.2: How to measure delta in ImageJ software- *From left to right, imaged was an oocyte at 8 hours. Microtubules (cyan) must be on the same plane as the chromosomes (magenta). One chromosome with kinetochore pairs were selected for, and a line was drawn across the kinetochore pairs to measure protein intensity as a function of distance. Graphs were generated, with distance in nm on the x-axis and protein intensity on the y-axis. The distance corresponding to the peak intensity was found for each protein. These distances were then subtracted from each other to get the difference corresponding to the distance that separates the proteins.*

Chapter 4- Results

Results Aim 1: Outer kinetochore protein assembly differs in mammalian oocyte to that in somatic cells

Fixed assay demonstrates differing Hec1 protein assembly in mammalian oocyte than that in somatic cell

How is kinetochore protein assembly different in the mammalian oocyte compared to that in the somatic cell? For the fixed assay, three kinetochore proteins were sampled, each from one different part of the kinetochore. CENP-A, the histone H3 variant, was sampled as the centromere protein. CENP-C, was sampled as the inner kinetochore protein part of the CCAN. And Hec-1 was sampled from the Ndc80C as part of the outer kinetochore (2,6).

In the figures below, the 3 sets of fixed oocytes are observed: the first for CENP-A (Fig. 4.1), the second for CENP-C (Fig. 4.2), and the third for Hec1 (Fig. 4.3). All the kinetochore proteins are observed in green, DNA was stained with Hoechst and is viewed in red, and the microtubules are viewed in blue.

From these images, kinetochore protein intensity for the centromeric, inner and outer kinetochore protein was measured every 2 hours post IBMX treatment. This was achieved by using ImageJ and FIJI. Every green dot represents an individual kinetochore, and signal intensity was measured. The final value taken was the signal intensity having subtracting the background noise from it (the dark parts of the oocyte). The signal intensity was measured for 14 kinetochores per oocyte per time point. The average of these values can be observed on Fig 4.4. Per time point and for each kinetochore antibody, the sample size of the number of oocytes varied. In the images below, N(o) represents the total number of oocytes sampled per time point and N(k) represents the total number of kinetochores sampled per time point. This graph of protein intensity also equates to how the kinetochore proteins are assembling.

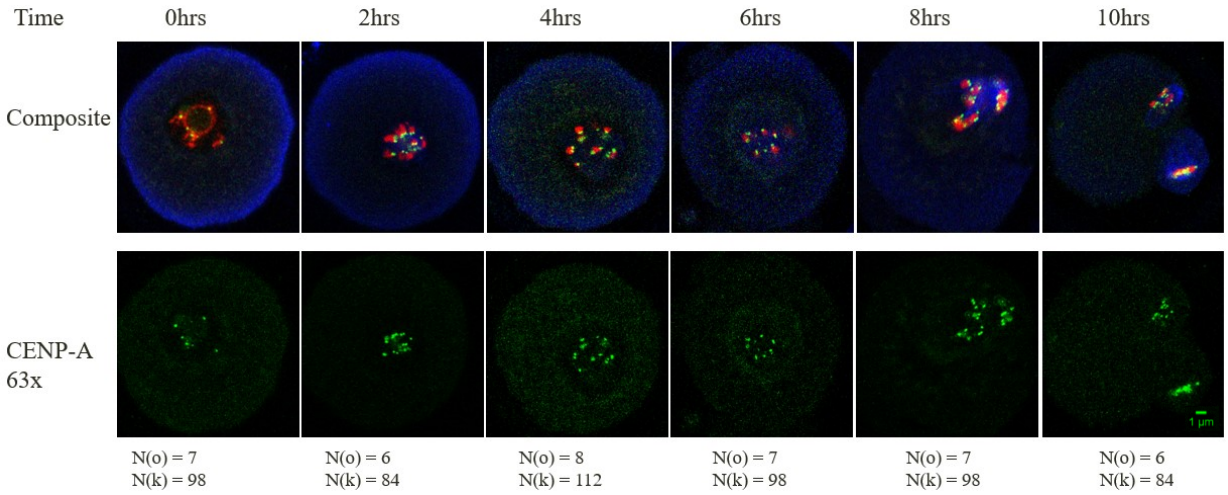

Figure 4.1 CENP-A protein assembly- *Protein assembly of CENP-A was imaged for 10 hours post IBMX release at 63x objective. Three structures were observed: CENP-A in green, microtubules in blue, and DNA in red. N(o) was number of oocytes and N(k) is number of kinetochores for each time point.*

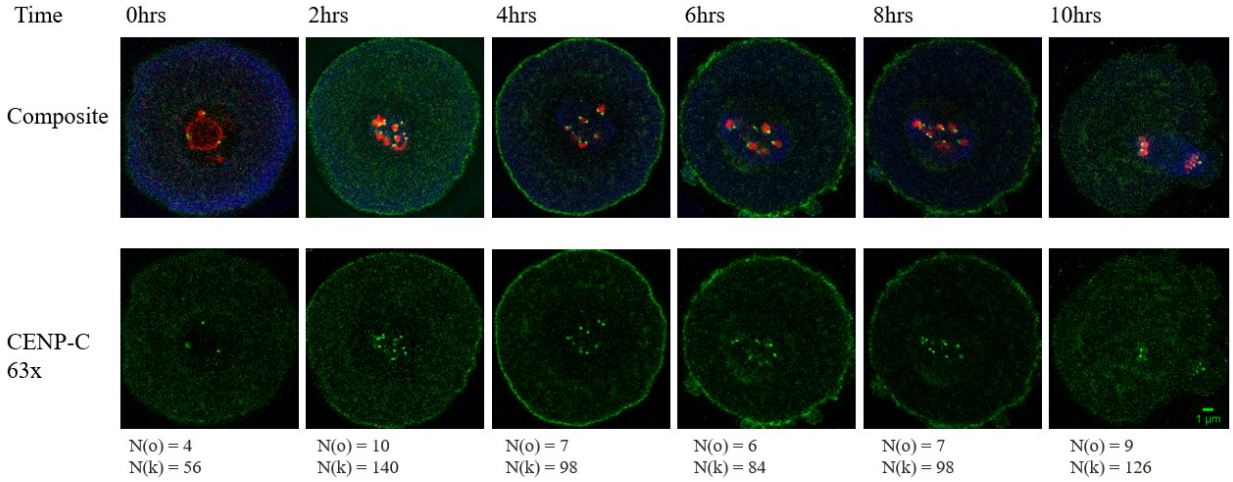

Figure 4.2 CENP-C protein assembly: *Protein assembly of CENP-C was imaged for 10 hours post IBMX release at 63x objective. Three structures were observed: CENP-C in green, microtubules in blue, and DNA in red. N(o) was number of oocytes and N(k) is number of kinetochores for each time point.*

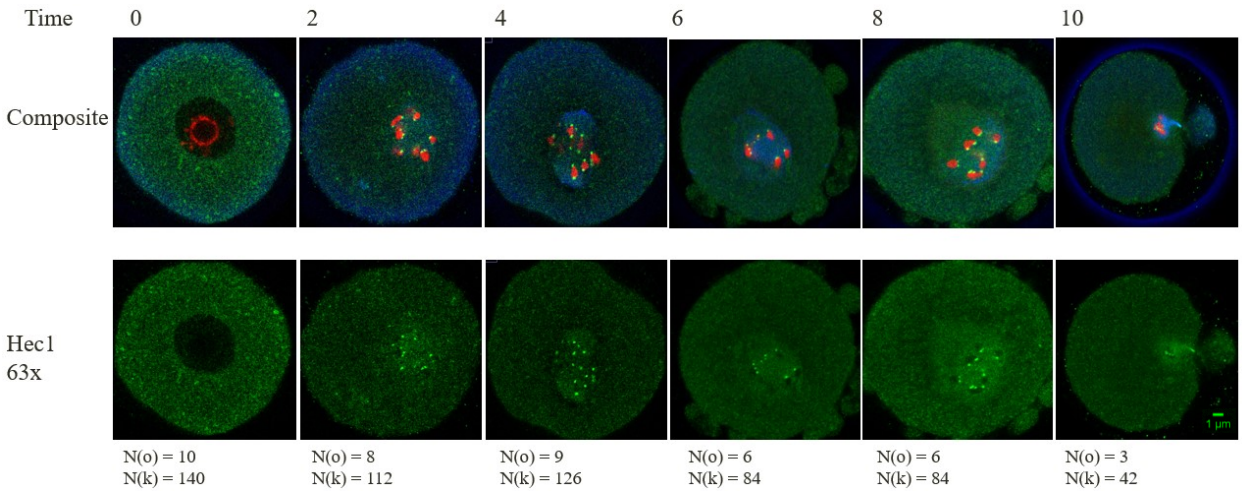

Figure 4.3 Hec1 protein assembly*: Protein assembly of Hec1 was imaged for 10 hours post IBMX release at 63x objective. In the image above, three structures were observed: Hec1 in green, microtubules in blue, and DNA in red. N(o) was number of oocytes and N(k) is number of kinetochores for each time point.*

First, CENP-A levels stayed relatively constant throughout meiosis unsurprisingly (Fig. 4.4). This is also observed in mitosis in somatic cells (2). CENP-A experienced minimum signal intensity at the 4 hour time point and its maximum signal intensity at the 8 hour time point. In this assay, CDK1-CycB levels are gradual and peak 6 to 8 hours post GVBD around metaphase (1,8). Even with changing levels of CDK1, CENP-A levels stayed relatively constant. There was slight increase at the 8 hour time point, but this would follow CDK1 level trends (1,8). Since CENP-A is a histone H3 variant and is present through the whole process of mitosis and meiosis at the centromeres, hence it is unsurprising that its levels for this protein are constant in mitosis and meiosis (2). Hence, in regards to CENP-A, it appears to already be assembled in the oocyte prior to GVBD and protein levels stay relatively constant throughout meiosis in the Meiosis I oocyte.

The second protein to observe was CENP-C, the inner kinetochore protein. It was slightly present prior to GVBD at the 0 hour time point (Fig. 4.4). After GVBD occurred, CENP-C protein levels began to gradually increase, reaching maximum signal intensity at the 6 hour time point and then decreasing. This is unsurprising considering CDK1 levels in the oocyte follow this trend, and CDK1 dictates kinetochore assembly in the somatic and meiotic cell (41). Yet, it was surprising how greatly CENP-C protein assembly trends in the meiotic cell differ to that in

the somatic cell. In the somatic cell, CENP-C stays relatively constant throughout mitosis (2,41). During Meiosis I, CENP-C gradually increased until metaphase at around the 6 hour time point and then decreased. A sharp decrease was observed from the 8 hour time point to the 10 hour time point. This occurred at the time when the oocyte moved from anaphase I (8 hours) to its first polar body extrusion (10 hours) (1). These findings demonstrate that CENP-C protein assembly is gradual and slow in the oocyte undergoing Meiosis I (2,41).

The third and last kinetochore protein to observe assembly trends was Hec1, the outer kinetochore protein (Fig 4.4). Based on this assay, Hec1 was not present at the kinetochore prior to GVBD. Yet, it was observed to reach maximum signal intensity at the 2 hour time point right after the cell undergoes GVBD. After it reached its maximum levels of protein assembly, Hec1 decreased rapidly at the 4 hour time point. After this initial decrease, Hec1 continued to decrease until it stayed relatively constant at the 8 hour time point and plateaued here. This was very surprising, as this trend did not follow to what was previously observed in somatic cells (2,41). In somatic cells, it is well established that the inner kinetochore assembly is achieved rapidly after NEBD prior to outer kinetochore assembly onto the centromere at prophase (2,41). Here, in this assay for the oocyte, the opposite appeared to happen. Hec1 of Ndc80C assembled rapidly after GVBD, decreased and plateaued right after, and assembled prior to the inner kinetochore CENP-C in the oocyte. For reasons why this occurs is unknown and should be explored. It is also possible that this method for measuring signal intensities is not appropriate and further methods for measuring signal intensities should be explored as well.

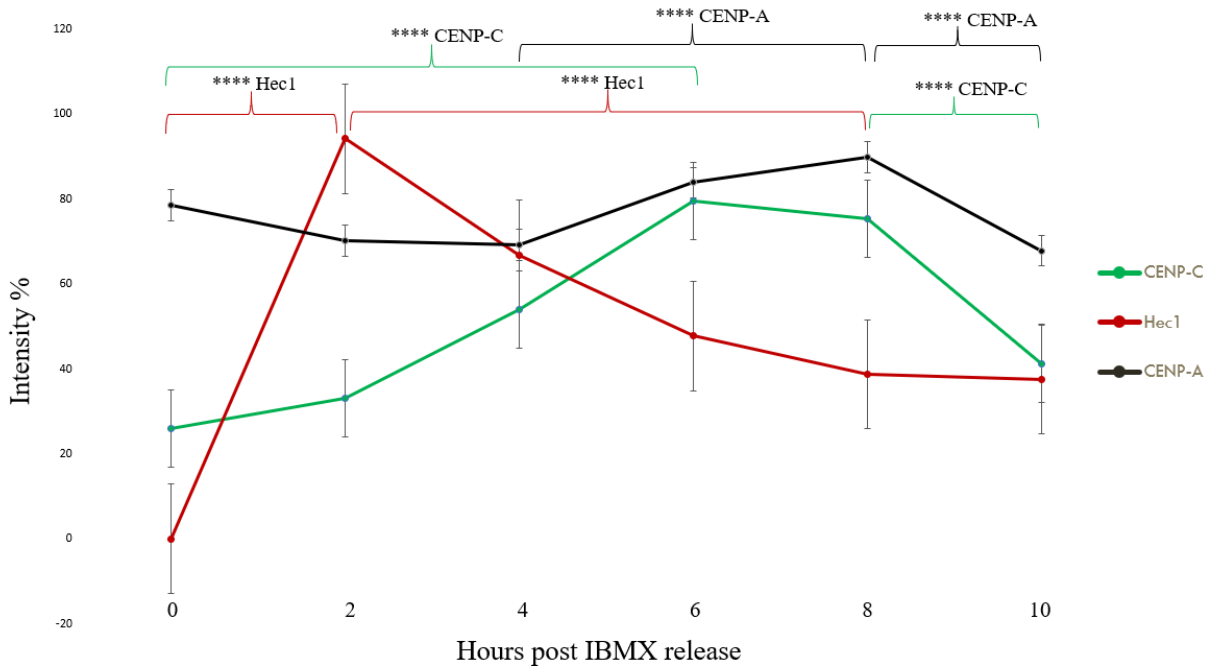

Figure 4.4: Kinetochore protein assembly from fixed assay: intensity vs time- *Average value signal intensity (after staining for CENP-A, CENP-C and Hec1) as a percentage was plotted as a function of hours post IBMX release of the oocytes. CENP-A was plotted in black, CENP-C was plotted in green, and Hec1 was plotted in red. Significance was calculated with an unpaired T test; **** corresponds to p<0.0001 (see Supplemental Information).*

Live assay demonstrates similar kinetochore protein assembly in the mammalian oocyte to that found in fixed assay

Do live assays demonstrate the same pattern of kinetochore protein assembly in the mammalian oocyte as they do in the fixed assay? Each part of the kinetochore was also sampled for the live experiments. Yet, different proteins were sampled compared to those from the fixed data set because these mRNAs (that will be translated to proteins that are fluorescently tagged) were already present in-house. For the centromeric protein, TALE the major satellite protein was measured. For the inner kinetochore protein, CENP-B was measured and lastly, for the outer kinetochore protein, Spc24 of the Ndc80C was measured. Oocytes were microinjected with mRNA that will be translated to proteins that are fluorescently tagged. Either the oocytes were co-injected with CENP-B-eGFP in conjugation with Spc24-mCHERRY (1:2) or with MajSat-TALE-mClover in conjugation with Spc24-mCHERRY (1:2). The oocytes were imaged live overnight on the SP8 confocal microscope to observe meiotic development of Meiosis I.

Protein intensity was measured using ImageJ and FIJI. For every time point and for every, 5 kinetochore proteins were measured with 6 oocytes at each time point. This allowed for a total of 30 kinetochore proteins to be measured for TALE, CENP-B and Spc24 at every point. Three different proteins were used for each part of the kinetochore to see if what was observed from fixed cells was a coincidence or a trend.

From the live experiments, this is what was observed (Fig. 4.5). First, TALE stayed relatively constant throughout; this is unsurprising. Similar to CENP-A, it is always found at the centromeres. The same was also observed in somatic cells (2,41). TALE is present prior to GVBD at maximum signal intensity and its minimum signal intensity is observed at the 6 hour time point. These values indicate that TALE stayed constant throughout meiosis.

Second, CENP-B for the inner kinetochore protein was viewed in its assembly (Fig. 4.5). It was observed that CENP-B is not present prior to GVBD in this live assay, unlike its fixed counterpart CENP-C which is slightly present prior to GVBD. Reasons could be that these are two different proteins, or that the protocols for live and fixed experiments are different. Yet, CENP-B appeared in similar to trends to that of CENP-C. After GVBD, CENP-B accumulated gradually over time, reaching its maximum signal intensity at the 9 hour time point. Two other maximum signal intensities to note are at the 6 hour time point and at the 8 hour time point. CENP-B appears to accumulate gradually and reach maximum levels around metaphase. It is unsurprising that two inner kinetochore proteins CENP-C and CENP-B behaved similarly in the oocyte, and follow CDK1 trends in the oocyte (2,41).

Third, the assembly trends that Spc24 followed were very surprising (Fig. 4.5). Yet, they were very similar to what was observed for Hec1 in the fixed experiments, in that the outer kinetochore protein achieved maximum signal intensity prior to the inner kinetochore proteins. For Spc24, it was not present prior to GVBD. Yet, after GVBD, it increased rapidly, reached its maximum before 5 hours, and then decreased and plateaued. Spc24 had two peaks of maximum protein assembly, one at the 2 hour time point and it reached its maximum at the 4 hour time point. The lowest value it reached was at the 10 hour time point upon which some polar body formation takes place (1). This outer kinetochore assembly in the oocyte is surprising for a number of reasons. First, in somatic cells, the inner kinetochore assembles prior to the outer kinetochore (2,6). Here in these assays sampling the oocyte, it appears as if the outer kinetochore

assembles prior to the inner kinetochore, in both fixed and live experiments. Why this occurs, it is uncertain, but possible reasons will be discussed in detail later.

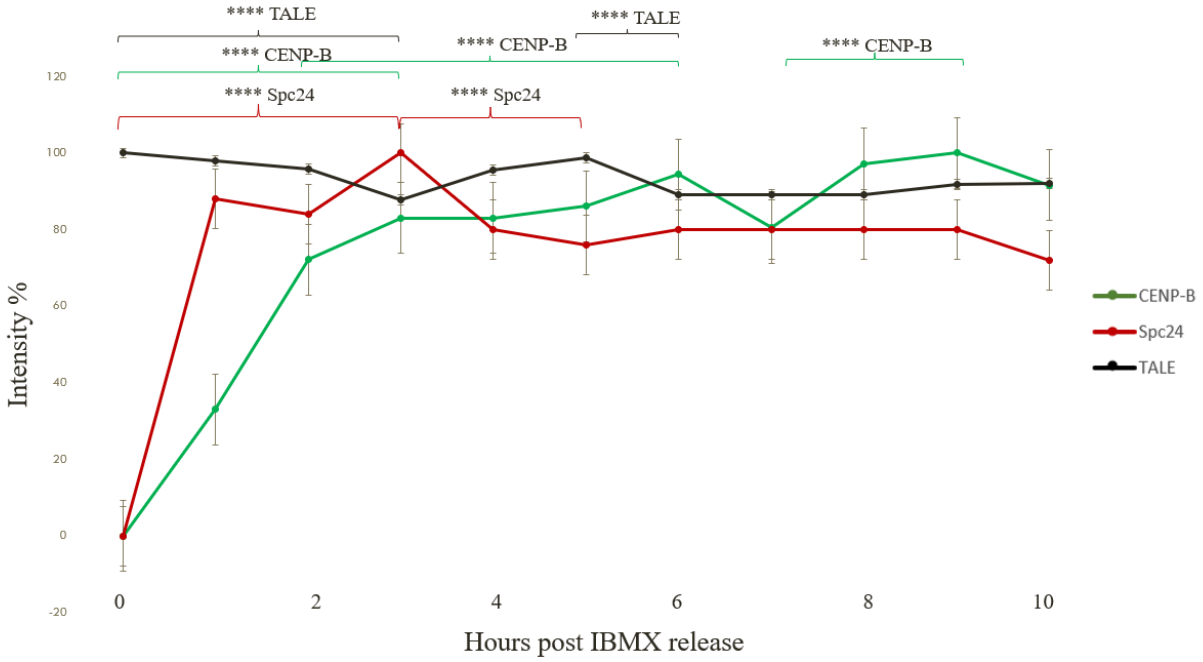

Figure 4.5: Kinetochore protein assembly from live data: protein intensity vs time- *Protein intensity as a percentage was plotted as a function of hours post IBMX release of the oocytes. TALE was plotted in black, CENP-B was plotted in green, and Spc24 was plotted in red. Significance was calculated with an unpaired T test; **** corresponds to p<0.0001 (see Supplemental Information).*

Results aim 2: The kinetochore in the mammalian oocyte is a compliant structure

Fixed assay demonstrates potential Hec1 kinetochore protein compliance in the mammalian oocyte

Is the kinetochore a compliant structure in the mammalian oocyte? In somatic cells, it was discovered that the kinetochore is a compliant, flexible, stretchy structure with some proteins more compliant than others (42,88). This was begun to be investigated for oocytes in this project. Distance (delta) was measured between two inner kinetochore proteins (CENP-B and CENP-C) and between an inner and an outer kinetochore protein (CENP-B and Hec1) every two hours

during Meiosis I development. To observe the protein distances under no tension from the microtubules, nocodazole treatment was used at 8 hours post IBMX release at around the time of metaphase (Fig 4.6) (93,122,123).

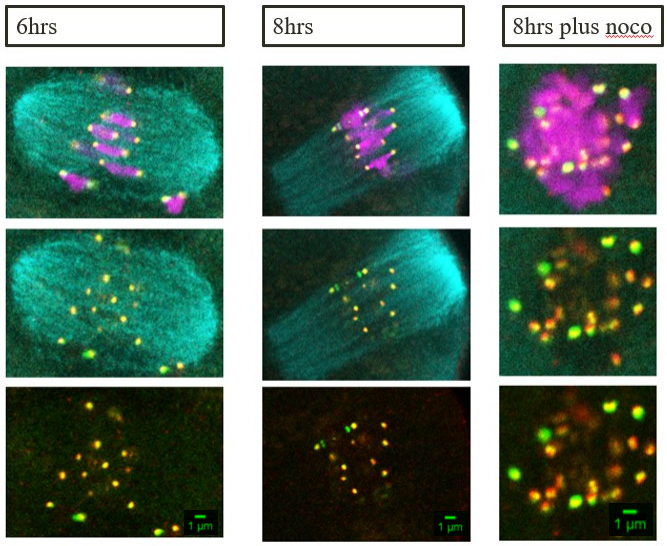

Figure 4.6: Delta assay- *Oocytes at 6 hours, 8 hours, and 8 hours with nocodazole treatment post IBMX release were viewed. The following were viewed: microtubules in cyan, DNA in magenta, CENP-C in red and CENP-B in green. Any variation of yellow to orange corresponds to an overlap of CENP-C and CENP-B. Delta was measured for oocytes that had the microtubules on the same plan as the DNA.*

Some key observations can be made from the graph (Fig. 4.7). The x-axis is time in hour post IBMX release, and the y-axis is the distance from CENP-B. In somatic cells, it is found that the inner kinetochore proteins are clustered and close in space together, and further away from the outer kinetochore proteins (42). It is surprising to see that in the oocyte, the inner kinetochore protein CENP-C is closer to Hec1 than it is to CENP-B. It stays like this up until the 6 hour time point post IBMX. This is when CENP-C is the furthest away from CENP-B with a 121.858nm separation. Unsurprisingly, CENP-C becomes closer to CENP-B under nocodazole treatment at 8 hours (at 64.099nm) than it was at the 2 hour time point. This makes sense as there is no tension from the microtubules. Yet, there is still a separation between these two proteins; they appear to not overlap, even under nocodazole treatment. Possible reasons for this will be discussed later.

For Hec-1, it was unsurprising to see how far away it is from CENP-B. At the 2 hour time point, Hec1 is 95.248 nm away from CENP-B, and this distance increases to 119.947nm at the 4 hour time point, 130.485nm at the 6 hour time point, and finally to 154.025nm at the 8 hour time point (Fig. 4.7). This dramatic stretch of Hec1 is surprising for a number of reasons. In the somatic cell, it is observed that mostly the inner kinetochore proteins are compliant. One of the proteins that is noncompliant in the somatic cell is the Ndc80C (42). In the oocyte, the opposite seems to appear. CENP-C, the inner kinetochore protein, undergoes a stretch, but only minimal at approximately 40nm (between the 2 and 8 hour time point). Hec1 appears to move the most away from its other kinetochore parts; moving approximately 60nm between the 2 and 8 hour time point. This demonstrates that the linkage between Hec1 and CENP-B may be compliant and that the Ndc80C may move in the oocyte. It was surprising to see the sharp increase in movement of Hec1 from the 6 to 8 hour time point. Possible reasons for this will be discussed later. It was observed under nocodazole treatment that Hec1 did not collapse onto CENP-C and they were separated by approximately 14nm. Also, Hec1 approached CENP-B by approximately 76nm under nocodazole treatment. This demonstrates that Hec1 is a very compliant protein in the oocyte.

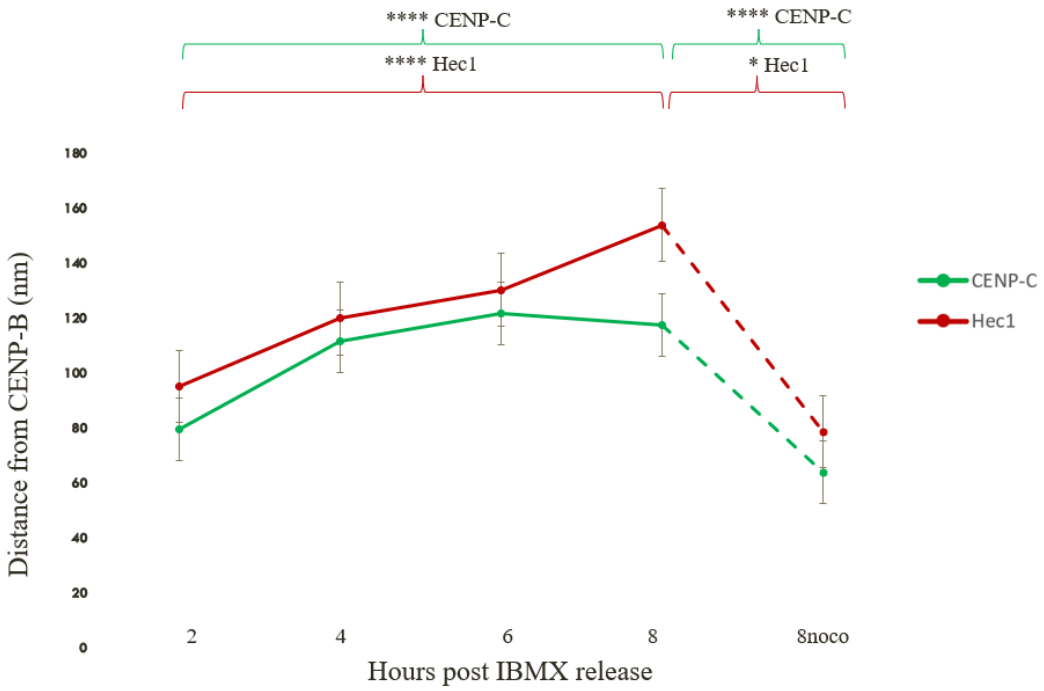

Figure 4.7: Measuring delta in reference to CENP-B: distance vs time- *Hec1 (red) and CENP-C (green) were measured their distance in reference to CENP-B (set as 0 on the y-axis). Distance was plotted as nanometers along a function of time post IBMX release.*

It is surprising to see that CENP-C moved gradually away from CENP-B, and that Hec1 showed the greatest change in movement away from CENP-B during Meiosis I (Fig. 4.8). This is the opposite to what was observed in somatic cells (42). In somatic cells, it is believed that the outer kinetochore proteins stay fixed and help transduce the force coming from the microtubules to the inner kinetochore proteins (39,42). The reasons why the opposite is occurring in oocytes is less understood.

At the 2 hour time post GVBD, there are few proper kinetochore-microtubule attachments present at the oocyte (1). CENP-C and Hec1 at the 2 hour time point were found to be further away from CENP-B than they were under nocodazole treatment. As well, CENP-C and Hec1 did not collapse onto each other during nocodazole treatment with complete obliteration of the microtubules, but rather maintained a distance apart. This suggests that the kinetochore may be under control by its own force. If this is true, this behaviour of the kinetochore is also observed in somatic cells (88). It was found in somatic cells that kinetochores are a site of active and passive force generation within themselves; it is possible that kinetochores in oocytes also have the ability to do this (42,88). Yet, these results are preliminary and further experiments, including negative controls and measuring for chromatic aberrations, will have to be performed to reach a more conclusive interpretation.

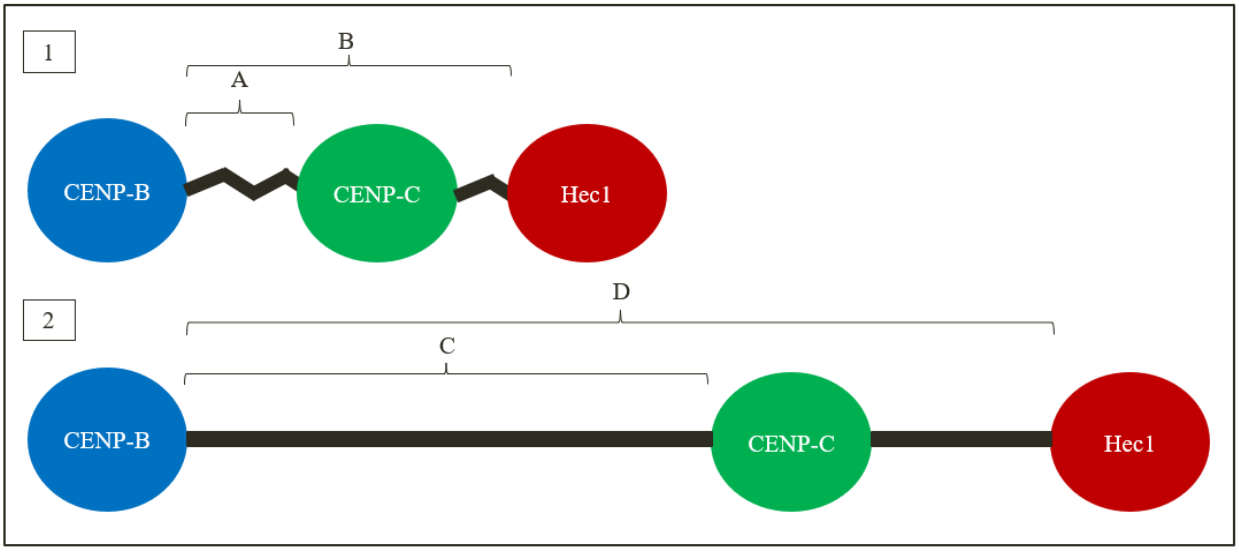

Figure 4.8: Stretch in the oocyte kinetochore- *Situation 1 describes the kinetochore in the oocyte under nocodazole treatment, at basal stretch. 65nm (A) separates CENP-C from CENP-B and 78nm (B) separates Hec1 from CENP-B. Situation 2 describes the kinetochore in the oocyte at maximum stretch at 8 hours post IBMX release. 117nm (C) separates CENP-C from CENP-B and 154nm (D) separates Hec1 from CENP-B*

Chapter 5- Discussion

Aim 1: protein assembly discussion

Two sets of experiments were performed to view kinetochore protein assembly in the developing mouse oocyte. In the fixed assay, CENP-A (centromeric protein), CENP-C (inner kinetochore protein), and Hec1 (outer kinetochore protein) were sampled. In the live assay, TALE (major satellite protein), CENP-B (inner kinetochore protein), and Spc24 (outer kinetochore protein), were sampled (2). For each set of centromeric, inner, and outer kinetochore proteins, the same trends were observed in both the fixed and live experiments.

In somatic cells, CDK1 phosphorylation levels are seen to control the rate at which the kinetochore assembles. These CDK1 levels increase and stay relatively constant after NEBD, and then decrease after anaphase (1,2,41). In the oocyte, different trends are observed; CDK1 levels accumulate gradually after GVBD, peak at metaphase, and then decrease (8). What was observed from the protein assembly and disassembly data can be discussed in relation to signaling cascades in the oocytes.

First, centromeric proteins CENP-A and TALE stayed relatively constant during Meiosis I. This is unsurprising considering the nature of the protein; they are constitutively present at the centromere (2). Though the oocyte is a highly specialized cell compared to the somatic cell, in terms of considering the nature of this protein, it is unsurprising that CENP-A stays constant throughout Meiosis I.

Second, inner kinetochore proteins CENP-B and CENP-C followed surprising results. It was observed in this assay that CDK1 levels are different in the oocyte than in the somatic cell, and this allowed us to hypothesize that the kinetochore protein assembly in the oocyte might differ compared to that in the somatic cell (8,41). It was surprising to see in these results how gradual protein accumulation occurred with the inner kinetochore protein. CENP-C was slightly present prior to GVBD whereas CENP-B was not present prior to GVBD. Afterwards, these proteins accumulated gradually, only to peak at around metaphase, and then decrease. Possible reasons for why this occurs is that kinetochore assembly could be slow in the oocyte to ensure for correct assembly. The inner kinetochore assembles onto the centromeric proteins. It is also well known that the oocyte is extremely error prone (1). Hence, it is possible that this slow level of

kinetochore protein assembly is occurring to minimize the errors that might occur. Slow attachment of the kinetochore would allow slow attachment of the microtubules, hence allowing to maximize end-on attachments. Though microtubule attachments made on the oocyte kinetochore are often lateral attachments, end-on attachments are the most robust and desirable (1,4,8). As well, if proper attachments between the microtubule and kinetochore are made the first time, this prevents correction mechanisms from occurring and allowing the cell to save energy (4,8). This might be one way that the oocyte tries to avoid kinetochore-microtubule attachment error by slowing down kinetochore protein assembly.

Third, outer kinetochore proteins Hec1 and Spc24 were observed to have the most surprising results. In somatic cells, the outer kinetochore is observed to accumulate and assemble last. More specifically, somatic kinetochore proteins first accumulate a centromeric base, then the inner kinetochore, and then the outer kinetochore (2,6). In the oocyte, the same thing does not appear to follow in the assays performed. The outer kinetochore proteins were not present prior to GVBD, but accumulated fast right after GVBD. They reached their maximum, decreased and then plateaued by the time metaphase and anaphase ensued. This is surprising for a number of reasons. In the oocyte, it appears from these preliminary results that the outer kinetochore might assemble prior to the inner kinetochore. The outer kinetochore is the link to the microtubules to the rest of the kinetochore (2). It could be that the outer kinetochore assembles first to help signal to the microtubules for where onto the kinetochore to attach for chromosome segregation to take place. Since chromosome segregation is highly error prone in the oocyte and kinetochore-microtubule misattachments occur, it could be that the outer kinetochore assembles first and early on in Meiosis I so that the signal has enough time to reach the microtubules (1,4,7). The oocyte is a large cell and it could be also that more time is needed for the outer kinetochore to signal to the microtubule, and then in response, the microtubule will know where to attach later on in meiosis (1). Hence, this reasons why the outer kinetochore proteins are present in high quantities early on after GVBD. The more outer kinetochore proteins accumulated, the stronger the signal to the microtubules is sent out. This would ensure proper kinetochore-microtubule attachments if the microtubule knows where to locate to based from the signals coming from the kinetochore (4,7).

It is possible that the differing kinetochore assembly in the oocyte could also be explained because the oocyte is an extremely error prone cell, so the cell must have mechanisms in place for it to minimize these errors (1,7). Syntelic, monotelic, merotelic and unattached kinetochore-microtubule attachment errors can result (1). It could be that the outer kinetochore needs to assemble prior to the inner kinetochore proteins, in order for the inner kinetochore proteins to know where to assemble. It is possible that the inner kinetochore proteins are weak in response to incoming signaling cascades, and hence rely on both the centromeric proteins and the outer kinetochore proteins to signal to the inner kinetochore proteins to know where to assemble.

The patterns observed here for outer kinetochore protein assembly to be entirely different to that of the inner kinetochore protein assembly insinuates that the outer kinetochore proteins in oocytes may not follow CDK1 level phosphorylation trends that the inner kinetochore proteins do (2,8). The outer kinetochore could be subjected to another kinase or a different signalling cascade. This should be investigated and to better understand the rate at which kinetochore protein assembly occurs during Meiosis I and for a better understanding of chromosome segregation in the mouse oocyte.

It was observed that the outer kinetochore proteins begin to disassemble prior to metaphase completion; hence, it is likely that some microtubules make attachments to the inner kinetochore. In the oocyte, it was observed in these assays that the inner kinetochore is present until anaphase and then begins to disassemble. It is surprising to see here that the outer kinetochore begins to disassemble prior to metaphase, and remains low in quantity for metaphase and anaphase; this is not observed in somatic cells (2,6,41). Since these outer kinetochore proteins begin to be disassemble, it could be possible that either the microtubule will lose connection to the kinetochore entirely, or decide to bind to the inner kinetochore. It would be more favourable for the former to occur. This is because if the microtubules make a connection to the inner kinetochore, when it is meant to connect to the outer kinetochore, this could result in a chromosome segregation action that was not meant to occur (2). This is when chromosome segregation errors take place, aneuploidy, infertility and birth defects arise (1,3,4). It would be interesting to investigate if the microtubule can bind to the inner kinetochore proteins as a correction mechanism, and how faithful this type of chromosome segregation would be if this attachment were to occur.

Unfortunately, it is very possible that this result could be an artefact. This is because only the middle Z confocal slice was measured as opposed to measuring all outer kinetochore proteins in all Z confocal slices. As well, it would be curious to observe how measuring signal intensity via taking the area under the curve would be similar or different to that of taking maximum signal intensity. This will have to be taken into consideration in future experiments of this type.

Aim 2: delta discussion

The second aim of the project was to look at kinetochore delta, and observe if this varied during Meiosis I in the developing oocyte. The distance was measured between CENP-B and CENP-C, two inner kinetochore proteins, and between CENP-B and Hec1, an inner and an outer kinetochore protein (2).

CENP-C, an inner kinetochore protein, was found to be closer to Hec1, an outer kinetochore protein, than CENP-B another inner kinetochore protein (2). This is very surprising because in somatic cells, all the CCAN proteins are found to be close together, and further away from the outer kinetochore proteins (42). A reason why the opposite is the case in oocytes is because the outer kinetochore might play a signaling role in recruiting the inner kinetochore to the oocyte. In the kinetochore protein assembly experiments, it was observed that the outer kinetochore assembles prior to the inner kinetochore in oocytes for these assays. This could be because the inner kinetochore relies on signaling from the outer kinetochore in order to know where to localize and where to be recruited. Observing that CENP-C is closer to Hec1 than CENP-B, also adds supporting evidence to this theory. For instance, at the 2 hour time point, CENP-C was 79.683nm away from CENP-B, where it was only about 15nm away from Hec1 at this time. If the outer kinetochore is involved in recruiting the inner kinetochore, it would be likely that the inner kinetochore in the oocyte would be closer to the outer kinetochore than the centromeric nucleosomes, like CENP-A (or like CENP-B where it is also buried in the centromere) (2,42). This is one possible reason.

Second surprising result was observing how the link between Hec1 and CENP-B appeared to be compliant. In the oocyte, Hec1 was seen to move much more than CENP-C. For example, CENP-C was seen to move a maximum of approximately 40nm, whereas Hec1 moved approximately 60nm from the 2 hour time point to the 8 hour time point. As well, Hec1 had a

significant increase of 24nm stretch between the 6 and 8 hour time point alone. This was surprising because in the somatic cell, Hec1 and the Ndc80C are noncompliant and it is the inner kinetochore that is more compliant (42). It was observed here that the outer kinetochore is recruited prior to the inner kinetochore in the oocyte, and in total, the outer kinetochore moves 20nm more away from the centromeric protein CENP-B than does CENP-C. Possible reasons for this is that the outer kinetochore is under greater influence from the microtubules than the inner kinetochore. Yet, around this time at 6 to 8 hours, Hec1 levels start to decrease and the outer kinetochore begins to disassemble. Some possibilities could occur that cause the outer kinetochore to be compliant at this time. The first is that there is a force coming from the microtubules that pull the outer kinetochore away from the rest of the kinetochore. The second is that there could be a force within the kinetochore, pushing away the outer kinetochore from the inner kinetochore, so that the outer kinetochore can disassemble away from and not interfere with the centromeric proteins and inner kinetochore at around metaphase (50). This is because this is occurring at the same time chromosome segregation is taking place (41). It is possible that at the time of chromosome segregation when the outer kinetochore is beginning to disassemble and plateau in protein levels, that microtubules would either lose the connection to the outer kinetochore or make a connection with the inner kinetochore. From this set of experiments, it appears that the microtubules could only bind to the outer kinetochore, and when disassembling, the microtubules would lose the connection to the inner kinetochore. This is because at the time of metaphase and anaphase, the outer kinetochore is 36nm away from the inner kinetochore at 8 hours post IBMX washout. It is likely that the microtubule would lose connection with the kinetochore, rather than compensate and stretch 36nm towards the inner kinetochore very suddenly. Experiments would have to be performed to confirm this. Also, the outer kinetochore being far away from the inner kinetochore at this time would prevent incorrect attachments with the inner kinetochore. As the outer kinetochore would be the first thing that the microtubules see. The distance apart of the kinetochore proteins could be a way of preventing misattachments, and trying to lower errors in an already error prone cell.

It was observed here that unlike in somatic cells, Hec1 appears to be compliant to the link with CENP-B in these assays (42). It could be that there is partial force coming from the microtubules providing this extra stretch. As well, the outer kinetochore moving means it is the most subject to the microtubule forces, and this is logical since it is the closest to the

microtubules. Also, if the link between Hec1 and CENP-B is compliant, making Hec1 a flexible protein; this helps support ways the oocyte corrects for kinetochore-microtubule attachment errors. Hence, it will take less energy to move around in space to correct for kinetochore-microtubule misattachments if the protein (Hec1) is flexible, rather than if it were noncompliant. From these experiments, it appears that the inner kinetochore is focused with making correct and slow attachments, whereas the outer kinetochore is focused on transducing force to the inner kinetochore and being flexible.

Third surprising result was from the nocodazole data. At 2 hours, Hec1 and CENP-C are 95.248nm and 79.683nm away from CENP-B respectively. At the 8 hour time point under nocodazole treatment, Hec1 and CENP-C are now 78.87nm and 64.099nm away from CENP-B respectively. Nocodazole obliterates the spindle and so there is no tension from the microtubule pulling at the kinetochore (93,123). At the 2 hour time point, Hec1 and CENP-C are approximately 15nm away from each other, and under nocodazole treatment, Hec1 and CENP-C are approximately 14nm apart. The proteins were not seen to collapse on each other during the nocodazole treatment, and remained a distance apart. This indicates that there is internal tension and force keeping the kinetochore proteins apart, even under no influence from the microtubule. Also, it is logical that the distance between Hec1 and CENP-C are the same at the 2 hour time point and under nocodazole treatment, as there is little force coming from the microtubule onto the kinetochore at the 2 hour point. Yet, at the 2 hour time point, CENP-C is 14nm further away from CENP-B than under nocodazole treatment. Also, at the 2 hour time point, Hec1 is 17nm further away from CENP-B than under nocodazole treatment. This suggests that there is some pull from the kinetochore pulling the inner and outer kinetochore proteins away from the centromere, but not enough to disrupt their natural separation. In the somatic kinetochore CENP-C was seen to move 16nm closer to Spc24/25, also part of the Ndc80C (42). Here in the oocyte, Hec1 from the Ndc80C was sampled and the distance between this complex and CENP-C did not change between the 2 hour time point and under nocodazole treatment. This demonstrates that compared to the somatic cell, in the oocyte the inner kinetochore is less compliant and the outer kinetochore is more compliant (42). Also, like in the somatic kinetochore, it appears there is internal tension and forces acting from within the kinetochore in the oocyte (88).

Fourth, the kinetochore undergoes a large stretch in the oocyte compared to that in the somatic cell. In the somatic cell, CENP-A and Hec1 are observed to have a 107nm distance between them at metaphase, whereas in the oocyte, CENP-B and Hec1 have a 154.025nm distance apart (42). CENP-B, like CENP-A, is also associated with the centromeres (2,42)**.** The oocyte kinetochore is observed to have more stretch than the somatic kinetochore. This is unsurprising considering how large the oocyte is, with a diameter of the mouse oocyte being roughly 70μm (1). This assay is observed to have a kinetochore that is more compliant in the oocyte than in the somatic cell (42,88). The mechanisms in the oocyte are present to minimize chromosome segregation errors. Future experiments would be to measure the forces from within the oocyte kinetochore (intra-kinetochore forces) during Meiosis I, and observe how the force observed at the kinetochore coincides with the delta measured here.

Overall, from these results, the most surprising findings were that the outer kinetochore in the oocyte assembles prior to the inner kinetochore. It appears that the oocyte kinetochore assembles from the outside in, rather than from the inside out like in somatic cells (2,6). This could be because the inner kinetochore relies on signaling from the outer kinetochore in order to know where to go for kinetochore assembly. Also, the outer kinetochore is more compliant than the inner kinetochore and is further away from the inner kinetochore than was observed in the somatic cell (42). It is likely the kinetochore behaves like this in the oocyte to minimize chromosome segregation errors and misattachments between the kinetochore and the microtubule. This assembly could be one way of ensuring proper attachment and hence proper segregation to an imperfect process (3,4). Yet, these are only preliminary results, and further experiments would have to be performed to validate this data. For example, using different labeling techniques would be a way compare results.

Similar to the first set of results, it is very possible that the delta results could be an artefact. This is because no negative control was performed to observe if the results were true or if they were chromatic aberrations. Also, only the middle Z confocal slice was measured as opposed to measuring all outer kinetochore proteins in all Z confocal slices. Finally, standard confocal microscopy was used to remain consistent with results obtained from the standard confocal microscope observed in aim 1. Yet, if repeated again, this experiment should be performed with

super-resolution microscopy to obtain more accurate results. These factors will have to be taken into account for future delta experiments.

Chapter 6- In Summary

The kinetochore is a dynamic and integrate structure in the developing mouse oocyte during Meiosis I. It was hypothesized that the kinetochore in the oocyte would be different to that than in the somatic cell, and that was observed here. This Masters gave further insight into the kinetochore assembly, disassembly and kinetics that were otherwise less studied before in the mammalian oocyte during Meiosis I (3,4). This is of much importance because the kinetochore is the central component during chromosome segregation in both the somatic and germ cell (1,2). Yet, these results are preliminary and as described above, further experiments would have to be performed to reach a proper conclusion.

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Supplemental Information

Aim 1: Significance for fixed results (unpaired T test)

CENP-A Time	P value	P value Significance	Significant?
0 vs 2 hrs	P = 0.095	DNE	No
0 vs 8 hrs	P = 0.0288	*	Yes
2 vs 4 hrs	P = 0.7629	DNE	No
2 vs 8 hrs	P < 0.0001	****	Yes
4 vs 6 hrs	P = 0.0007	***	Yes
4 vs 8 hrs	P < 0.0001	****	Yes
6 vs 8 hrs	P = 0.2326	DNE	No
8 vs 10 hrs	P < 0.0001	****	Yes

CENP-C Time	P value	P value Signifance	Significant?
0 vs 2 hrs	P = 0.001	*	Yes
0 vs 6 hrs	P < 0.0001	****	Yes
2 vs 4 hrs	P < 0.0001	****	Yes
2 vs 6 hrs	P < 0.0001	****	Yes
4 vs 6 hrs	P < 0.0001	****	Yes
6 vs 8 hrs	P = 0.3323	DNE	No
6 vs 10 hrs	P < 0.0001	****	Yes
8 vs 10 hrs	P < 0.0001	****	Yes

Hec1 Time	P value	P value Significance	Significant?
0 vs 2 hrs	P < 0.0001	****	Yes
2 vs 4 hrs	P < 0.0001	****	Yes
2 vs 6 hrs	P < 0.0001	****	Yes
2 vs 8 hrs	P < 0.0001	****	Yes
2 vs 10 hrs	P < 0.0001	****	Yes
4 vs 6 hrs	P < 0.0001	****	Yes
6 vs 8 hrs	P = 0.004	***	Yes
6 vs 10 hrs	P = 0.0001	***	Yes
8 vs 10 hrs	P = 0.642	DNE	No

Aim 1: Significance for live results (unpaired T test)

TALE Time	P value	P value Significance	Significant?
0 vs 1 hrs	P = 0.06	DNE	No
0 vs 2 hrs	P < 0.0001	****	Yes
0 vs 3 hrs	P < 0.0001	****	Yes
0 vs 6 hrs	P < 0.0001	****	Yes
2 vs 3 hrs	P < 0.0001	****	Yes
3 vs 4 hrs	P < 0.0001	****	Yes
3 vs 5 hrs	P < 0.0001	****	Yes
5 vs 6 hrs	P < 0.0001	****	Yes

5 vs 10 hrs	P < 0.0001	****	Yes
6 vs 10 hrs	P = 0.0008	***	Yes

CENP-B Time	**P value**	**P value Significance**	**Significant?**
0 vs 1 hrs	P < 0.0001	****	Yes
0 vs 2 hrs	P < 0.0001	****	Yes
0 vs 3 hrs	P < 0.0001	****	Yes
0 vs 9 hrs	P < 0.0001	****	Yes
1 vs 9 hrs	P < 0.0001	****	Yes
2 vs 6 hrs	P < 0.0001	****	Yes
6 vs 9 hrs	P = 0.0196	*	Yes
7 vs 9 hrs	P < 0.0001	****	Yes
9 vs 10 hrs	P = 0.0005	***	Yes

Spc24 Time	**P value**	**P value Significance**	**Significant?**
0 vs 3 hrs	P < 0.0001	****	Yes
1 vs 3 hrs	P < 0.0001	****	Yes
2 vs 3 hrs	P < 0.0001	****	Yes
3 vs 4 hrs	P < 0.0001	****	Yes
3 vs 5 hrs	P < 0.0001	****	Yes
3 vs 8 hrs	P < 0.0001	****	Yes

3 vs 10 hrs	P < 0.0001	****	Yes

Aim 2: Significance for delta results (unpaired T test)

CENP-C Time	**P value**	**P value Significance**	**Significant?**
2 vs 4 hrs	P = 0.0001	***	Yes
2 vs 8 hrs	P < 0.0001	****	Yes
4 vs 8 hrs	P = 0.5439	DNE	No
4 vs 6 hrs	P = 0.2471	DNE	No
6 vs 8 hrs	P = 0.6347	DNE	No
2 vs 8noco	P = 0.0221	*	Yes
4 vs 8noco	P < 0.0001	****	Yes
6 vs 8noco	P < 0.0001	****	Yes
8 vs 8noco	P < 0.0001	****	Yes

Hec1 Time	**P value**	**P value Significance**	**Significant?**
2 vs 4 hrs	P = 0.0073	**	Yes
2 vs 6 hrs	P < 0.0001	****	Yes
2 vs 8 hrs	P < 0.0001	****	No
4 vs 6 hrs	P = 0.1787	DNE	No
6 vs 8 hrs	P = 0.0136	*	Yes
2 vs 8noco	P = 0.0496	*	Yes
8 vs 8noco	P = 0.0136	*	Yes