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Meiotic Chromosome Segregation in *C. elegans*:

Discovering a new look for CENP-A

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Joost W. Monen

Committee in charge:

Professor Karen Oegema, Chair
Professor Don W. Cleveland
Professor Arshad Desai
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2008

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2008

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Publications

Green RA, Audhya A, Pozniakovsky A, Dammermann A, Pemble H, **Monen J**, Portier N, Hyman A, Desai A, K. Oegema. "Expression and Imaging of Fluorescent Proteins in the *C. elegans* Gonad and Early Embryo." In K. Sullivan (Ed.), Methods in Cell Biology: *Fluorescent Proteins* 2008 (85):179-218.

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ABSTRACT OF THE DISSERTATION

Meiotic Chromosome Segregation in *C. elegans*: Discovering a new look for CENP-A

by

Joost W. Monen

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2008

Professor Karen Oegema, Chair

In this dissertation, I use the nematode, *Caenorhabditis elegans*, to understand mechanisms involved in chromosome segregation during meiosis, by exploring the role that known mitotic kinetochore proteins play during meiotic segregation. During mitosis, the histone-H3 variant CENP-A is known to be the nucleosomal subunit at the centromere that is responsible for

directing kinetochore assembly. In cells lacking CENP-A, chromosomes are unable to segregate leading to aneuploidy and cell-death. Interestingly, I demonstrate that during meiosis CeCENP-A no longer directs outer kinetochore assembly. Furthermore, in embryos depleted of CeCENP-A, meiotic chromosome segregation appears completely normal, whereas subsequent mitotic divisions completely fail. Outer kinetochore components localize to a cup-like structure, which likely is involved in aligning chromosomes at the metaphase plate. I speculate that this new mechanism for chromosome segregation in meiosis may be a requirement to facilitate proper segregation of recombined chromosome pairs.

During these meiotic studies, I discovered a striking CeCENP-A cleavage event, which I spend the remainder of this dissertation describing, and postulate that this cleavage event may be involved in maintenance of the centromere during mitotic divisions. Centromere specification is thought to be propagated by an epigenetic mark produced by CENP-A. The mechanism for how CENP-A achieves this mark is unknown. In a variety of studies presented here, I show that CeCENP-A is a substrate for Separase-mediated cleavage and discuss its possible implications on maintaining the epigenetic mark. This cleavage event is best demonstrated during meiosis in embryos expressing N-terminally tagged GFP::CeCENP-A. During mitosis, I show that Separase is unable to cleave centromeric CeCENP-A under wild-type conditions. However, in the absence of the kinetochore, centromeric CeCENP-A is

cleaved, indicating that the kinetochore protects CeCENP-A while non-centromeric CeCENP-A is susceptible to cleavage. Worms expressing an uncleavable mutant form of CeCENP-A show an increase in embryonic lethality, and worms solely expressing a pre-cleaved form of CeCENP-A completely lose CeCENP-A localization and function, resulting in complete embryonic lethality. These data suggest that CeCENP-A cleavage may be a way to inactivate CeCENP-A loading. I propose that cleavage of improperly loaded CeCENP-A onto chromosome arms may be a mechanism used by the cell to ensure that the epigenetic mark for CeCENP-A loading remains strictly at the centromere.

Chapter 1: Introduction

1.1 Cell Division and Chromosome Segregation

Cell division is a vital process in the survival of all living organisms, culminating in two exact (daughter) cells stemming from an original (mother) cell. The events that transpire during the process of cell division have been shown to be highly conserved: from single-celled organisms, such as yeast, all the way up to complex vertebrates, such as humans (Mitchison and Salmon, 2001; Scholey et al., 2003). This important biological phenomenon has been studied for over a century, when it was first noted that chromosomes align in the center of the cell at metaphase and segregate to opposite poles to form two cells, each with its own set of chromosomes (Flemming, 1882). It was these initial studies that coined the term “mitosis” for this process, derived from the Greek word meaning thread. We now know the importance of properly segregating chromosomes in order to transmit an exact copy of genetic information. There are dire consequences that can result when failures occur in this process, including cell death and promotion of cancer (Rajagopalan and Lengauer, 2004).

Since the discovery of mitosis, many advances have been made in understanding the components and mechanisms that facilitate the proper segregation of chromosomes. In particular, two major components ensuring chromosomal segregation are the mitotic spindle and the kinetochore (Maiato

et al., 2004). The cytoskeletal protein tubulin can dynamically assemble and disassemble into microtubule polymers, and does so during mitosis to form the bipolar mitotic spindle that (along with motor proteins) provides the necessary force to segregate chromosomes to opposite poles (Walczak and Heald, 2008). The proteinaceous structure responsible for facilitating the connection between spindle microtubules and chromosomes is called the kinetochore (Cleveland et al., 2003; Maiato et al., 2004).

1.2 The Kinetochore

Composed of more than 70 known proteins, the kinetochore provides an attachment site for special microtubule bundles, termed k(inetochore)-fibers, and is composed of several layers that were first described using electron microscopy (Brinkley and Stubblefield, 1966; Rieder, 1982). The tri-laminar structure is composed of an outer-plate, a linker layer, and an inner-plate. The outer-plate of the kinetochore is composed of proteins involved in the dynamic interactions between the k-fibers and the kinetochore itself, and is best visualized in cells treated with microtubule poisons. The more stable inner-plate of the kinetochore is composed of a chromatin structure, termed the centromere, which contains nucleosomes incorporating the specialized histone-variant CENP-A (Earnshaw et al., 1986; Palmer et al., 1991), as well as other structural components making up the base of the kinetochore (Figure 1.1a).

The controlled movements of chromosomes during mitosis are initiated by kinetochores following breakdown of the nuclear envelope. The objective of the cell is to ensure that the genetic material is perfectly divided, and does so by bi-orienting all of the chromosomes at the metaphase plate, with sister kinetochores facing opposite poles. During progression from prometaphase to metaphase, the kinetochore monitors the attachment state and activates signaling pathways to avoid anaphase onset when incorrect kinetochore attachments are present, preventing loss of genomic information. At metaphase, the kinetochore machinery involved in ensuring proper alignment sends a signal to the cell initiating anaphase onset. One of the major mechanisms associated with anaphase onset is the cleavage of cohesin between sister chromatids, which releases the tension between k-fibers stemming from opposite poles, thus allowing for sister chromatids to seperate from one another. Much of the business end for the specific tasks that the kinetochore must accomplish are attributed to the microtubule-interacting outer-plate proteins; however, none of these mechanisms could function without the inner-plate base of the kinetochore, specifically the highly conserved centromeric protein CENP-A.

1.3 CENP-A as the base of the kinetochore

Originally identified from anti-centromere-antibodies in Raynaud's disease patients, CENP-A has since been shown to be the histone-H3 variant

present in nucleosomes at the centromere of the chromosome (Earnshaw et al., 1986; Palmer et al., 1991). Chromosomes are composed of chromatin that is organized and wrapped tightly around a core octamer of proteins forming the nucleosome, and become condensed-compact structures during mitosis (Figure 1.1a). The nucleosome is composed of 2 sets of Histone-H2A, Histone-H2B, Histone-H3, and Histone-H4, throughout most of the chromosome—known as the chromosome arms. However, at the centromere, Histone-H3 is replaced by CENP-A (Figure 1.1a).

It is at the centromere region of chromosomes where the kinetochore is built and is entirely dependent on the presence of CENP-A. It has been clearly shown in various model systems (including yeast, flies, worms, and vertebrates) that in the absence of a functional copy of CENP-A by way of knock-out, null-mutation, or knock-down the kinetochore fails to assemble. Of course, with the failure to assemble a kinetochore, chromosomes fail to align and segregate properly in mitosis resulting in aneuploidy and cell death (Blower and Karpen, 2001; Howman et al., 2000; Moore and Roth, 2001; Stoler et al., 1995). It is relatively unknown how CENP-A nucleosomes are loaded and propagate. The propagation of the centromere onto a replicated chromosome has been an elusive question in the field, and the best model to date for how this is achieved involves CENP-A acting as an epigenetic mark (Black and Bassett, 2008).

1.4 Centromere Specification: CENP-A as the Epigenetic Mark

The centromere was first defined as the site of primary constriction of a chromosome as viewed by light microscopy. Over a century later, we now define the centromere as the chromosomal domain that directs the formation of the kinetochore. But the question remains, how does the cell specify where the centromere is established on the chromosome? It was originally postulated that the DNA sequence defined the centromere. However, only in the budding yeast is there a known 125 base pair region required for $Cse4^{CENP\text{-}A}$ loading (Tanaka et al., 1999). In higher organisms, centromeres have repeating DNA sequences known as α-satellite DNA that are AT rich, but it has been shown that these α-satellite regions are not essential for centromere propagation (Cleveland et al., 2003; Sullivan and Willard, 1998). The best evidence for this can be seen in human patients with neo-centromeres. These patients have chromosomes whose centromeres have been moved to a completely different locus on the chromosome, and yet no DNA translocations have taken place, indicating that there is a sequence independent mechanism that defines the centromere (Amor et al., 2004a; Amor and Choo, 2002; Ventura et al., 2004). The prevailing hypothesis is that CENP-A is responsible for making an epigenetic mark that determines the location of the centromere from one chromosome to the next. The mechanism of action for how CENP-A behaves to serve as the epigenetic mark is an ongoing question that is experimentally very difficult to answer, and is of great

interest in the field of mitosis. A more straightforward question that has recently become clearer, is understanding how CENP-A gets loaded into chromatin. Proteins in multiple organisms have been discovered that are required for the centromeric localization of CENP-A. Depletions for both KNL-2 and Mis-18 proteins cause CENP-A to mislocalize in vivo in worms as well as in human tissue culture (Fujita et al., 2007; Maddox et al., 2007). Interestingly, in human tissue culture, these proteins are transiently localized to centromeres during telophase and early G1, coincident with CENP-A loading (Jansen et al., 2007). Because there is no direct biochemical interactions observed between these proteins and CENP-A, the mechanism for how they affect CENP-A loading remains unclear.

1.5 CENP-A Conservation in Eukaryotes

Of all homologous proteins seen from species to species, perhaps histones are some of the most highly conserved; and CENP-A is no exception. Like all histones, CENP-A has a histone-fold domain and an N-terminal tail. The histone fold domain is the region of CENP-A that is bound in nucleosomes and has been shown in both yeast and human cells to contain the key CENP-A targeting domain (CATD), consisting of the α2-helix and preceding loop (L1). By incorporating the CATD into histone-H3, recruitment of normal levels of kinetochore proteins, centromere-generated mitotic checkpoint signaling, chromosome segregation, and viability can be rescued in CENP-A depleted

cells (Black et al., 2007). While this provided great insight into understanding the regions within CENP-A important for centromere targeting, due to technical limitations for depleting endogenous CENP-A in these model systems and because we know that cells are capable of dividing with only 10% endogenous CENP-A levels, it cannot be ruled out that there is a role played by other regions of CENP-A including the N-terminal tail.

The N-terminal tail is quite divergent from species to species, and ranges from around 20 to more than 150 amino acids in current model organisms. It has been hypothesized that there is no major role for the N-terminal tail. However, the best experiments to date addressing this question, indicate that yeast carrying *cse4*$^{CENP\text{-}A}$ mutant genes lacking a portion of the N-terminal tail, are unable to properly divide, and are thus lethal (Chen et al., 2000). Human tissue culture experiments argue the opposite, suggesting mitosis occurs normally in cells expressing truncated forms of CENP-A as well as histone-H3/CENP-A chimeras lacking the CENP-A tail (Black et al., 2007). The major caveat with these experiments is that siRNA in human tissue culture is known to be highly variable, and even a small amount of endogenous CENP-A remaining could be enough to propagate subsequent cell divisions.

1.6 Other Important Kinetochore Proteins

Defining kinetochore composition proved difficult due to low abundance of constituent proteins and because these proteins are largely essential for cell viability. Advances in biochemistry, genetics, proteomics, and RNA-interference (RNAi) have been instrumental in identifying kinetochore components in various model systems. Since the disocovery of CENP-A in 1985, dozens of other kinetochore proteins have been identified. The structural component CENP-C was identified along with CENP-A, and has been shown to directly interact with CENP-A, helping to form the base of the kinetochore (Earnshaw et al., 1986; Earnshaw and Rothfield, 1985). Other kinetochore components were identified soon after in the budding yeast, *S. cerevisiae*, using genetic approaches and taking advantage of the specific centromeric DNA region known only in *S. cerevisiae* (McAinsh et al., 2003; Westermann et al., 2007). Screens done in yeast identified conserved spindle checkpoint components including the Mad (Mitotic Arrest Deficient) proteins, Mad1-3, and the Bub (Budding Uninhibited by Benzimidazole) proteins, Bub1 and Bub3; which all play a role in cell signaling during mitosis, termed the Spindle Assembly Checkpoint (Hoyt et al., 1991). Over the past decade, mass-spectrometry-based approaches have greatly accelerated progress towards defining the composition of the kinetochore (Cheeseman et al., 2004; De Wulf et al., 2003; Foltz et al., 2006; Obuse et al., 2004; Okada et al., 2006; Sauer et al., 2005; Wigge et al., 1998; Wigge and Kilmartin, 2001). These

studies have also helped to group these novel proteins in sub-complexes with distinct roles at the kinetochore. Among these sub-complexes is the KNL-1/Mis-12 complex/NDC-80 complex (KMN) network, which constitutes the core microtubule-binding site of the kinetochore and is essential for kinetochore-microtubule interactions in-vivo (Cheeseman et al., 2006). Although we are making many advances to understanding the composition and many roles of the kinetochore, most of the progress has been made on chromosome segregation during mitosis, and relatively little is known about kinetochore function during meiosis.

1.7 Meiosis vs. Mitosis

Meiosis is the specialized cell division that produces gametes, also known as sex cells, and is instrumental in maintaining genetic diversity in an individual species. Defects in meiotic divisions have been known to cause genetic diseases in humans such as Down Syndrome. Unlike mitosis, meiosis encompasses two rounds of chromosome segregation following one round of chromosome replication. In meiosis I, homologous chromosome pairs segregate from one another in what is termed a reductional division because the genetic information is cut in half. In the following meiosis II division, sister chromatids separate from one another in an equational division, similar to mitosis. These subsequent divisions result in producing haploid (1n) gametes from a diploid (2n) cell. Fusion of two gametes forms a new diploid cell; which,

in the case of sexual reproduction, contains maternal and paternal genetic information. This newly formed diploid cell becomes the early embryo for multi-cellular organisms.

Because there are obvious similarities between meiosis and mitosis, and because of the technical difficulty in studying meiotic divisions, many of the principles for chromosome segregation in mitosis have been assumed to be conserved in meiosis. Recent advancements have made it easier to study meiosis, and it is becoming increasingly apparent that there are significant differences between meiosis and mitosis. Two such differences include monopolar kinetochore attachment and protection of centromeric cohesion during meiosis I.

In order for aligned homologous pairs in meioisis I to be properly segregated, it was proposed that sister kinetochores must be mono-oriented to the same spindle pole and not bi-oriented as is known to occur in mitosis. It was recently discovered in yeast that a novel protein, Monopolin, is responsible for orienting sister kinetochores in this monopolar orientation (Tóth et al., 2000). Other proteins have since been identified to form the Monopolin complex, which has been shown to be conserved in other model systems (Petronczki et al., 2006; Yokobayashi and Watanabe, 2005). Another adaptation discovered in chromosome segregation of homologous pairs is the protection of centromeric cohesion. At metaphase for both meiosis and mitosis, spindle checkpoint proteins signal to the cell to initiate anaphase. A

major downstream target for the spindle checkpoint is the cysteine protease Separase, which is activated at anaphase onset by degradation of its chaperone protein Securin (Ciosk et al., 1998; Uhlmann et al., 1999; Zou et al., 1999). Active Separase quickly cleaves cohesin between sister chromatids, posing a potential problem in meiotic divisions. If cohesin were completely removed after the first meiotic division, then there would be no cohesion left between sister chromatids to allow for proper segregation in meiosis II. It was discovered, 15 years ago in *Drosophila* and later in yeast and other systems, that Shugoshin protects cohesin at centromeres during meiosis I, thus allowing sister chromatids to remain connected in the subsequent meiosis II division (Kerrebrock et al., 1992; Kerrebrock et al., 1995; Kitajima et al., 2004).

1.8 Holocentric Chromosomes

There are currently two different chromosomal architectures known to exist in eukaryotic organisms: monocentric and holocentric. Monocentric chromosomes are the more common form seen in a range of organisms from yeast to vertebrates, in which chromosomes have a localized centromeric region. The kinetochore is built on these centromeric regions and can be visualized by immunofluorescence as small punctate dots. Holocentric chromosomes differ in that their centromere extends across the entire chromosome and the mitotic kinetochore can be visualized as long strips

along the entire length of the chromosome (Figure 1.1b). Holocentric chromosomes have arisen phylogenetically in unrelated species, from lower plants, hemipteran insects, and nematodes, such as the well-characterized model organism, *Caenorhabditis elegans*. Despite their obvious differences in chromosomal architecture, it has been shown that the proteins, which make up the centromere and kinetochore, and the mechanism by which these proteins facilitate chromosome segregation is highly conserved in mitosis. In fact, many advances in the field have been the result of studying these processes in the *C. elegans* early embryo (Cheeseman et al., 2004; Desai et al., 2003; Moore et al., 1999; Moore and Roth, 2001; Oegema et al., 2001). In trying to understand the mechanism for chromosome segregation of holocentric chromosomes, an interesting problem arose relating to segregation of chromosomes during meiosis.

1.9 Meiosis in Organisms with Holocentric Chromosomes

It is easy to understand how the mechanism for mitotic segregation can be conserved between monocentric and holocentric chromosomes. In meiosis however, it was thought that monocentricity is necessary in order to properly segregate homologous pairs. The reason that this was believed to be true is because chiasmata that are formed, as a result of recombination, are what keep homologous pairs from segregating prematurely. In monocentric chromosomes suppression of recombination at the centromere avoids

problems in aligning and segregating homologs. Because we know that in mitosis, the centromere for holocentric chromosomes spans the entire length of the chromosome, it is hard to imagine how during meiotic divisions holocentric chromosomes are able to segregate properly based on the models for monocentric chromosome meiosis (Figure 1.1b). One suggestion from early EM studies is that the kinetochore is telocentric and restricted to the ends of chromosomes during meiosis I in holocentric organisms (Albertson and Thomson, 1993; Comings and Okada, 1972). In order to address this fundamental question, we use the nematode, *C. elegans*.

1.10 *Caenorhabditis elegans*

The use of worms in laboratory research dates back to the late 1800s, but studies performed by Sydney Brenner in the late 60s and early 70s established a particular nematode, *Caenorhabditis elegans*, as a model system to study development and behavior (Brenner, 1974). Brenner focused on investigating development using forward genetic analysis, and along with his colleagues John Sulston and Robert Horvitz received the Nobel Prize for Medicine for their contributions in development and apoptosis (Hengartner and Horvitz, 1994; Horvitz et al., 1994; Sulston et al., 1983). *C. elegans* offer numerous advantages—they are small (1 mm), transparent, and hermaphroditic—and have become a favorite model organism for numerous applications, including studies in development, neurobiology, and aging.

Recently there has been a dramatic expansion in the use of *C. elegans* as a model system to study cell biology with the advent of RNA-mediated interference (RNAi).

1.11 RNA-mediated interference (RNAi)

The major problem with the forward genetic screens, such as those developed by Brenner and colleagues, is that they are slow and laborious and often miss important genes due to their small size or subtle loss of function phenotype, as well as being limited in their capacity to identify essential genes. The advent of RNAi, a type of reverse genetics in which a gene is first selected and its depletion phenotype is then determined, revolutionized analysis of essential processes during cell division. The initial discovery underlying RNAi was first stumbled upon using *C. elegans* during a control experiment designed to show the use of antisense RNA in reducing gene expression. Surprisingly, both sense and antisense RNAs were effective in this capacity (Guo and Kemphues, 1995). Andrew Fire and Craig Mello later explored this puzzling result in depth, showing that injection of double stranded RNA (dsRNA) into *C. elegans* was indeed the cause of gene silencing (Fire et al., 1998). RNAi technology is now used in a broad range of systems but due to the nature of the *C. elegans* gonad, is very effective in depleting targeted proteins in early embryonic development.

1.12 Using *C. elegans* to Study Cell Division

The structure of the *C. elegans* gonad makes the worm particularly amenable to protein depletion using RNAi. Introduction of dsRNA or siRNA leads to the rapid degradation of its target mRNA transcript. However, most systems are limited by the, often slow, turnover of pre-existing protein. In contrast, protein present within the *C. elegans* syncytial gonad following dsRNA delivery is removed by the continual extrusion of maternal cytoplasm into oocytes. Because *C. elegans* extrude 1 embryo every 15 to 20 minutes; after 48 hour incubations, worms treated with dsRNA have typically less than 3% of targeted protein remaining. The reproducibility and penetrance of depletions makes worms ideal for studying the roles of specific proteins during mitosis and meiosis. In particular, this system has helped to identify numerous genes involved in these processes.

1.13 The Kinetochore is Conserved in *C. elegans*

Because kinetochores play an essential role during every cell division, it is difficult to generate null-mutants and knock-outs in most metazoans due to its lethal consequences. Using high-resolution microscopy coupled with RNAi, major screens were conducted discovering numerous novel proteins involved in cytokinesis, polarity, chromosome segregation, as well as other essential processes (Zipperlen et al., 2001). Among those proteins discovered were the kinetochore null (KNL) proteins, KNL-1, KNL-2, and KNL-3. Both KNL-1 and

KNL-3 have since been identified in humans and other model systems, and are both integral in forming the KMN netork, whose role in mitosis is to provide the core attachment site of the kinetochore to microtubules (Cheeseman et al., 2006; Cheeseman et al., 2004). KNL-2 is another conserved protein, which has recently been shown to be required for CENP-A deposition at the centromere (Fujita et al., 2007; Maddox et al., 2007). Aside from these discoveries, localization studies in worms have helped to describe the kinetochore hierarchy that has since been shown to be conserved in other organisms. At the top of this hierarchy in worms, as well as in other systems, is the centromeric protein CENP-A. However, by homology, *C. elegans* appear to have 2 CENP-A homologs.

1.14 Two CENP-A Homologs in *C. elegans*: HCP-3 and CPAR-1

By sequence homology, two CENP-A homologs exist in *C. elegans*, HCP-3 and CPAR-1 (see Fig. 2.1a). They share sequence homology of nearly 80% identity. The initial characterizations in worm CENP-A have focused on HCP-3 and dismiss CPAR-1 as merely a recent duplication; however, neither of them have been individually characterized since RNAi studies result in depletion of both proteins due to their identity, even at the nucleotide level (see Chapter 2).

1.15 Meiosis in *C. elegans*

C. elegans has also proven to be a very useful model organism for studying meiotic events. A dynamic reorganization of chromosome structures occurs during meiosis, ranging from recombining chromosomes in pachytene of prophase, to compact bivalents seen during chromosome segregation. These processes can be easily visualized in worms because of the architecture of the gonad, composed of hundreds of nuclei at different meiotic stages. Oocytes become arrested at the end of prophase I in the gonad, and it is not until around the time of fertilization that the major sperm protein (MSP) triggers spindle formation and the embryo continues to divide in meiosis (Burke and Ward, 1983; Miller et al., 2001). It is this newly fertilized embryo and the divisions following it that is the focus of this dissertation.

As discussed above, meiotic chromosomal segregation of holocentric chromosomes poses a unique set of challenges. It is unclear how a kinetochore can form and function on recombined homologous chromosomes and still remain holocentric. What we do know is that there is only one recombination event per homologous chromosome pair, which is believed to be important in establishing the proper morphology of the bivalent (Hillers and Villeneuve, 2003). It has also been shown that the Aurora-B-Kinase, AIR-2, is important in establishing the axis of cohesion loss during meiosis by phosphorylating Rec-8, indicating that there may be a mechanism in worms which is independent of Shugoshin and may not involve protection of the

cohesin at the centromere (Kaitna et al., 2002; Rogers et al., 2002). Electron microscopy analysis has attempted to elucidate where the kinetochore might be, but it is unclear based on the EM whether a kinetochore structure is even present. Microtubules seem to embed into chromosomes in meiotic embryos, but it is unclear if this is facilitated by a kinetochore structure (Albertson and Thomson, 1993; Comings and Okada, 1972; Pimpinelli and Goday, 1989). And so the question still remains, how do chromosomes segregate during meiosis in organisms with holocentric chromosomes? Do the bivalents become monocentric? Or do chromosomes rely on another mechanism for chromosome segregation? What role does the kinetochore play in meiotic segregation and where does it localize (Figure 1b)? It is these questions that are the focus of my thesis along with other questions stemming from the discoveries I have made along the way.

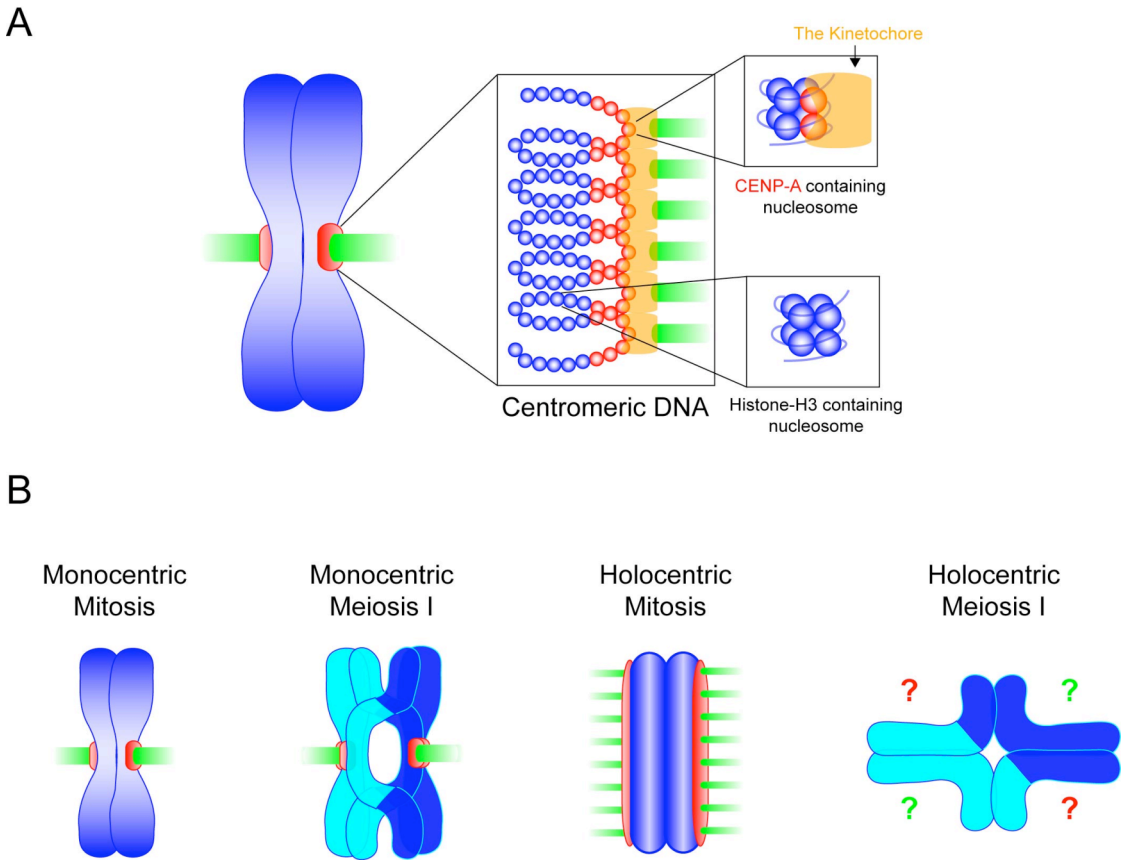

Figure 1.1. Schematics of mitotic and meiotic chromosomes in monocentric and holocentric organisms.
A) A mitotic chromosome highlighting the centromeric region. Histone-containing nucleosomes incorporate CENP-A dimers at the centromere replacing Histone-H3. CENP-A directs kinetochore assembly, and thus facilitates microtubule-to-chromosome attachments during mitosis. B) Four different types of chromosomes segregated in mitosis and meiosis in organisms with monocentric and holocentric chromosomes. Kinetochore assembly and microtubule attachments are well characterized in mitosis, and are believed to be understood during meiosis of monocentric chromosomes. It is unclear how organisms with holocentric chromosomes segregate their chromosomes during meiosis.

Chapter 2: Differential Role of CENP-A in the Segregation of Holocentric *C. elegans* Chromosomes During Meiosis and Mitosis

2.1 Summary

Two distinct chromosome architectures are prevalent among eukaryotes: monocentric, in which localized centromeres restrict kinetochore assembly to a single chromosomal site, and holocentric, in which diffuse kinetochores form along the entire chromosome length. During mitosis, both chromosome types use specialized chromatin, containing the histone H3 variant CENP-A (Amor et al., 2004b; Henikoff et al., 2004; Mellone, 2003), to direct kinetochore assembly (Blower and Karpen, 2001; Buchwitz et al., 1999; Howman et al., 2000; Oegema et al., 2001). For the segregation of recombined homologous chromosomes during meiosis (Hauf and Watanabe, 2004; Petronczki et al., 2003), monocentricity is thought to be critical to limit spindle-based forces to one side of a crossover and prevent recombined chromatids from being simultaneously pulled towards both spindle poles. The mechanisms that allow holocentric chromosomes to avert this fate remain uncharacterized. Here we show that dramatically different mechanisms segregate holocentric chromosomes during meiosis and mitosis in the nematode *Caenorhabditis elegans*. Immediately prior to oocyte meiotic

segregation, outer kinetochore proteins are recruited to cup-like structures on the chromosome surface via a mechanism independent of CENP-A. In striking contrast to mitosis, both oocyte meiotic divisions proceed normally following depletion of either CENP-A or the closely associated centromeric protein CENP-C. These findings highlight a dramatic difference between the segregation of holocentric chromosomes during meiosis and mitosis and demonstrate the potential to uncouple assembly of outer kinetochore proteins from CENP-A chromatin.

2.2 Introduction

Holocentric chromosome architecture is common in widely divergent eukaryotic lineages including nematodes, lower plants and insects (Maddox et al., 2004; Pimpinelli and Goday, 1989). During mitosis, holocentric species assemble diffuse kinetochores along the poleward face of each sister chromatid. The ultrastructure, molecular composition, and assembly of these diffuse kinetochores is very similar to that of kinetochores in monocentric organisms (Buchwitz et al., 1999; Cheeseman et al., 2004; Howe et al., 2001; Moore and Roth, 2001; Oegema et al., 2001). In particular, both holocentric and monocentric organisms assemble mitotic kinetochores on centromeric chromatin in which histone H3 is replaced by the variant CENP-A (Blower and Karpen, 2001; Buchwitz et al., 1999; Howman et al., 2000; Oegema et al., 2001).

In monocentrics, segregation of recombined homologs during meiosis I is thought to be achieved via mechanisms similar to those acting during mitosis, with two important adaptations: selective protection of cohesion between centromeres of sister chromatids, and co-orientation of kinetochores on sister chromatids to face the same spindle pole (Hauf and Watanabe, 2004; Petronczki et al., 2003). The success of these adaptations relies on the fact that centromeres are restricted to a chromosomal region on one side of the crossover, which prevents recombined chromatids from being simultaneously pulled towards both spindle poles. Electron microscopy of holocentric chromosomes has indicated that during mitosis, microtubule attachments along the chromosome length end in a structure that resembles the trilaminar kinetochores of monocentric chromosomes (Albertson and Thomson, 1982; Howe et al., 2001; O'Toole et al., 2003). In contrast, during meiosis a plate-like structure is absent and microtubules appear to directly embed into chromatin (Albertson and Thomson, 1993; Comings and Okada, 1972).

To compare the kinetochores that form on holocentric chromosomes during meiosis to their mitotic counterparts, we examined the localization of kinetochore proteins during oocyte meiosis in the nematode *C. elegans*. During mitosis, both inner (chromatin-proximal) and outer (microtubule-binding interface proximal) kinetochore components localize along the poleward faces of sister chromatids (Cheeseman et al., 2004; Desai et al., 2003; Howe et al., 2001; Moore and Roth, 2001; Oegema et al., 2001). We focused on

CeCENP-A, CeCENP-C and KNL-1 because they represent different levels of mitotic kinetochore structure: CeCENP-A is an inner kinetochore protein that replaces histone H3 in centromeric nucleosomes and directly interacts with DNA, KNL-1 is a component of the outer kinetochore (Desai et al., 2003), and CeCENP-C is an inner kinetochore protein proposed to bridge the two (Cheeseman et al., 2004; Desai et al., 2003). In addition, all three are widely conserved proteins, essential to form mitotic kinetochores that can make microtubule attachments.

2.3 Results

2.3.1 HCP-3 and CPAR-1: The two *C. elegans* CENP-A homologs

C. elegans has two highly homologous genes encoding CENP-A related proteins, *hcp-3* (Buchwitz et al., 1999) and *F54C8.2*, which we have named *cpar-1* for CENP-A Related (Figure 2.1a). *C. briggsae* has only one gene with homology to CENP-A (Stein et al., 2003) suggesting that the second *C. elegans* gene is derived from a recent duplication event. Quantitative western blotting revealed that an antibody raised to the N-terminal tail of HCP-3 and used for all of the analysis presented here, recognizes the tails of both proteins with equal affinity (Figure 2.1a). Western blots of extracts prepared from wild-type worms reveal a prominent band at the expected molecular weight for HCP-3 (Figure 2.1b). In contrast, no band is observed at the expected molecular weight of CPAR-1, indicating that it is expressed at <5%

of the levels of HCP-3, if at all (Figure 2.1b). This result does not exclude the possibility that CPAR-1 is expressed during a narrow developmental time window, such as during late meiotic prophase that is the subject of this study. Therefore, in all of the RNAi experiments described below we used a dsRNA homologous to the *hcp-3* tail that depletes both HCP-3 and an exogenously expressed GFP::CPAR-1 fusion (Suppl. Fig. 2.1a; Figure 2.3a; Suppl. Fig. 2.5b). We also used two different mixtures of dsRNAs homologous to both *hcp-3* and *cpar-1* (Suppl. Fig 2.5b), and in no case were the results different. Since our antibody does not discriminate between HCP-3 and CPAR-1 and a dsRNA generated against the *hcp-3* tail is capable of depleting both proteins, we refer to the two proteins collectively as “CeCENP-A”.

2.3.2 Kinetochore components are recruited to meiotic chromosomes at the diplotene stage of meiosis

In *C. elegans,* nuclei generated in the mitotic zone at the distal end of the syncitial gonad gradually progress through meiotic prophase towards the proximal end of the gonad where they are cellularized to form oocytes (Figure 2.1c) (Hubbard and Greenstein, 2000). The two rounds of meiotic chromosome segregation that generate the haploid oocyte nucleus and two polar bodies, occur after the oocytes are fertilized during passage through the spermatheca. To examine the localization of CeCENP-A, CeCENP-C and KNL-1 during meiotic prophase, we performed immunofluorescence on fixed

gonads. Surprisingly, CeCENP-A, which is present on chromatin throughout the cell cycle during mitotic divisions (Buchwitz et al., 1999; Oegema et al., 2001), was not detected on chromosomes in early meiotic prophase. CeCENP-A was first detected in the late pachytene/diplotene region of the gonad (Figure 2.1d; Suppl. Fig. 2.2), when the synaptonemal complex, which assembles between the paired homologous chromosomes, is in the process of being disassembled (Macqueen et al., 2002; Nabeshima et al., 2005). CeCENP-C and KNL-1, neither of which is present on chromatin throughout the cell cycle in mitotic embryos (Desai et al., 2003; Moore and Roth, 2001; Oegema et al., 2001), were also first detected on chromosomes in late pachytene/diplotene nuclei located at the turn of the gonad arm, although slightly later than CeCENP-A (Figure 2.1d; Suppl. Fig. 2.2). During late meiotic prophase, all three kinetochore proteins were essentially coincident with chromosomal DNA, similar to their localization during mitotic prophase (Desai et al., 2003; Moore and Roth, 2001).

Since CeCENP-A is presumably present and required for mitotic divisions at the distal tip of the gonad (Figure 2.1c), the absence of CeCENP-A from pachytene nuclei and its loading in late pachytene/diplotene is surprising. We have not yet been able to generate a stable strain expressing GFP::HCP-3 to analyze this dynamic behavior. However, we have generated a stable strain expressing GFP::CPAR-1 using the *pie-1* promoter and 3' UTR to drive expression (Suppl. Fig. 2.1). Imaging of living GFP::CPAR-1 worms indicated

a lack of significant localization in pachytene nuclei and loading in late pachytene/diplotene/diakinesis, in agreement with the immunofluorescence analysis (Suppl. Fig. 2.1a). These observations lead us to speculate that CeCENP-A is removed from chromatin during early meiotic prophase, potentially to facilitate recombination.

2.3.3 CeCENP-A localizes to chromatin, whereas outer kinetochore components form a cup-like structure on meiotic chromosomes

During segregation of the bivalent chromosomes on the meiosis I spindle, the localization of CeCENP-A and CeCENP-C remained coincident with chromosomal DNA (Figure 2.2a,b). In contrast, KNL-1 was concentrated on the surface of the chromosomes, where it formed two cup-like structures that enclosed the two halves of each bivalent (Figure 2.2a-d). This localization of KNL-1 is similar to that reported previously for the kinetochore proteins $Nuf2^{HIM-10}$ and HCP-1 (Howe et al., 2001; Moore et al., 1999). The change in the localization of KNL-1 from throughout the chromatin to cup-like structures occurred in oocytes just prior to fertilization (see Figure 2.4c below). The localizations of CeCENP-C and KNL-1 were similar during both meiotic divisions (Figure 2.2a,b). In contrast, using two independently generated antibodies and two different fixation conditions, CeCENP-A levels were dramatically reduced on chromosomes during meiosis II compared to meiosis I (Figure 2.2a,b), suggesting that CeCENP-A is selectively removed from

chromatin between the two meiotic divisions. Live imaging of meiotic embryos expressing GFP::CPAR-1 supported this conclusion. GFP::CPAR-1 localized to bivalent chromosomes during late prophase/prometaphase of meiosis I but was dramatically removed from chromosomes during metaphase-anaphase of the first meiotic division (Suppl. Fig. 2.1b). Analysis of prometaphase, anaphase and telophase stages during both meiotic divisions indicated that KNL-1 delocalized from chromosomes following meiosis I and re-localized during meiosis II (Suppl. Fig. 2.3). In contrast, CeCENP-C persisted on chromosomes between meiosis I and meiosis II and disappeared from chromosomes only late in meiosis II (Suppl. Fig. 2.3).

In addition to its chromosomal staining, KNL-1 was present in linear elements that accumulated within the meiosis I spindle and near the embryo cortex (Figure 2.2a-c; Suppl. Fig. 2.4a). These elements, which are absent during meiosis II, are not fixation artifacts as they are also observed in living embryos expressing GFP fusions of kinetochore proteins that interact with KNL-1 (*not shown*). A previous study analyzing the localization of $Nuf2^{HIM-10}$ during oocyte meiosis also described the presence of linear elements in the meiosis I spindle region (Howe et al., 2001). These linear elements were not present during meiotic divisions in male worms, where KNL-1 localized to the chromosome surface, suggesting that they are specific to the acentrosomal oocyte meiosis I spindle and cortex (Suppl. Fig. 2.4b).

NDC-80, Nuf2^{HIM-10}, KNL-3 and MIS-12, four widely conserved kinetochore components that directly interact with KNL-1 (Cheeseman et al., 2004; Desai et al., 2003), exhibited identical localization to KNL-1 throughout both oocyte meiotic divisions (*not shown*). In addition, BUB-1, a widely conserved outer kinetochore protein kinase that is not directly bound to KNL-1 (Cheeseman et al., 2004), also co-localized with KNL-1 on the chromosome surface and to linear spindle and cortical elements in meiosis I. However, BUB-1 also localized between the two halves of the bivalent during meiosis I and between sister chromatids during meiosis II (Figure 2.2a, b). Localization of BUB-1 to this region, which defines the plane where cohesion will be lost during anaphase of each meiotic division (Rogers et al., 2002), is consistent with prior work implicating this kinase in regulation of chromatid cohesion (Bernard et al., 2001; Kitajima et al., 2004). Thus, in contrast to their similar localization during mitosis (Cheeseman et al., 2004; Desai et al., 2003; Hauf and Watanabe, 2004; Howe et al., 2001; Moore and Roth, 2001; Oegema et al., 2001), kinetochore components exhibit three distinct localization patterns during holocentric meiosis (Figure 2.2e): (1) the inner kinetochore components CeCENP-A and CeCENP-C are present throughout the chromatin, (2) outer kinetochore components, including KNL-1 and four KNL-1 associated proteins, concentrate in two cup-like structures that enclose the ends of the chromosomes facing the spindle poles, (3) BUB-1 localizes to the cup-like structures and also to the central plane between the homologs/sisters

where cohesion will be dissolved. The chromosomal passenger proteins, which target to the region between sister kinetochores prior to anaphase during mitosis in *C. elegans* (Oegema et al., 2001), localize prominently to the plane of cohesion loss during *C. elegans* oocyte meiosis (Oegema et al., 2005; Rogers et al., 2002). To determine if AIR-2, the Aurora-B related kinase subunit of the *C. elegans* passenger complex, excludes KNL-1 and other outer kinetochore components from this region of meiotic chromosomes, we analyzed embryos depleted of AIR-2. We did not observe localization of KNL-1 at the mid-bivalent, indicating that AIR-2 does not exclude KNL-1 from this region (Suppl. Fig. 2.4c). This result suggests that the localization of BUB-1 to the plane of cohesion loss and to cup-like structures reflects a potential dual role for this kinase in chromosome segregation.

2.3.4 Loading of outer kinetochore components require CeCENP-A during meotic prophase

During mitosis, CeCENP-A, CeCENP-C and KNL-1 form a linear assembly pathway (Desai et al., 2003). CeCENP-A containing chromatin directs the recruitment of CeCENP-C, which in turn physically interacts with and directs the recruitment of a 10-protein complex containing KNL-1 that is critical to form the microtubule-binding interface (Cheeseman et al., 2004; Desai et al., 2003). To determine if CeCENP-A directs the chromosomal targeting of kinetochore components during meiosis, we used RNAi to analyze

the consequences of its depletion. In depleted worms, CeCENP-A immunoreactivity was absent in gonads (Figure 2.3b; Suppl. Fig. 2.2). Quantitative western blotting of total worm extracts confirmed that HCP-3 was > 97% depleted (Figure 2.3a). Since we cannot detect any CPAR-1 on western blots of worm extracts (Figure 2.1a), we examined worms expressing GFP::CPAR-1 from an integrated transgene and found that all GFP fluorescence was absent after RNAi, confirming that the dsRNAs used are capable of depleting both proteins (Suppl. Fig. 2.1). Chromosome segregation failed during the first mitotic division of all embryos produced by the injected worms (see Figure 2.5a), as expected following CeCENP-A depletion. Despite the dramatic reduction of CeCENP-A levels, the number of bivalents in developing oocytes was the same as wild-type (WT: 6.0 ± 0.2; n= 58 oocytes; CeCENP-A depleted, 6.0 ± 0.2; n= 64 oocytes) and chromosome morphology appeared normal. Consistent with the recruitment hierarchy established for mitotic kinetochores, both CeCENP-C and KNL-1 failed to localize to chromosomes during diplotene/diakinesis in CeCENP-A depleted worms (Figure 2.3b; n=12 gonads). As depletion of CeCENP-A does not affect CeCENP-C or KNL-1 protein levels (Desai et al., 2003), this result suggests that CeCENP-A is required to form their chromosomal binding site during late meiotic prophase, similar to its role in mitosis.

2.3.5 Contrary to mitosis: Outer kinetochore components are independent of CeCENP-A for loading during meiotic segregation

In fertilized CeCENP-A depleted oocytes undergoing meiosis I and II, little to no CeCENP-C was detected on chromosomes (Figure 2.4a,b), consistent with the penetrant depletion of CeCENP-A. Remarkably, KNL-1 localized normally to the cuplike structures on the surface of the chromosomes during both divisions (Figure 2.4a,b; n=20 meiosis I and 10 meiosis II embryos). An identical result was obtained in worms injected with a mixture of dsRNAs homologous to *hcp-3* and *cpar-1* (Suppl. Fig. 2.4d). In wild-type, KNL-1 undergoes a transition, from localizing throughout the chromatin to concentrating on the chromosome surface and forming linear aggregates in the oocyte immediately preceding the spermatheca (Figure 2.4c). In CeCENP-A depleted worms, the chromosome-wide localization of KNL-1 in distal regions of the gonad was largely absent (Figure 2.3b), but KNL-1 nevertheless became concentrated on the surface of chromosomes and formed cytoplasmic elements in the oocytes immediately preceding the spermatheca (Figure 2.4c). These results suggest that although KNL-1 requires CeCENP-A to localize to chromosomes during late meiotic prophase and during mitosis, its recruitment to the chromosome surface just prior to the assembly of the first meiotic spindle is independent of CeCENP-A (Figure 2.4c). This transition occurs coincident with NEBD, as assessed by co-staining with a monoclonal antibody (mAb414) that recognizes nuclear pores

(Figure 2.4d). Like KNL-1, BUB-1 also targeted normally to chromosomes during meiosis I and II in CeCENP-A depleted embryos (Figure 2.4a,b).

2.3.6 Meiotic chromosome segregation occurs normally in CeCENP-A depleted embryos

In fixed CeCENP-A depleted embryos undergoing meiosis I or II, spindle structure and chromosome segregation did not appear to be significantly perturbed. In contrast, mitotic embryos from the same parent worms exhibited severe defects. To directly analyze segregation, we imaged both meiotic divisions and the first mitosis in embryos co-expressing GFP-histone H2B and GFP̃g-tubulin. *Ex utero* filming made it possible to routinely perform such analysis. In agreement with the analysis in fixed embryos, live imaging indicated that depletion of CeCENP-A did not significantly affect chromosome alignment and segregation during either meiotic division (Figure 2.5a; Suppl. Videos 1-4). Polar body resorption was observed, likely arising from manipulation of fragile embryos prior to eggshell formation, but the frequency of this was not significantly different from similarly imaged control embryos (Suppl. Fig. 2.5a). CeCENP-A was not detectable on chromosomes during meiotic divisions in wild-type males (Suppl. Fig. 2.4b), further suggesting that meiotic segregation does not require CeCENP-A in *C. elegans*. Strikingly, the same CeCENP-A depleted embryos that successfully executed two rounds of apparently normal oocyte meiotic segregation

completely failed to segregate chromosomes during mitosis (Figure 2.5a; Suppl. Videos 2-4; Suppl. Fig. 2.5a). This observation argues against the observed meiotic segregation being due to insufficient depletion of CeCENP-A. Analysis of a single chromosomal locus, marked by integration of an array of Lac operators (Fukushige et al., 1999), confirmed that homologs and sister chromatids were segregating from each other in CeCENP-A depleted embryos (Figure 2.5b). The timing of the meiotic divisions, and the interval between metaphase of meiosis II and metaphase of the first mitotic division were also unaffected by depletion of CeCENP-A (Figure 2.5c). During mitosis, depletion of CeCENP-C results in a severe chromosome segregation defect that is very similar to that seen after depletion of CeCENP-A (Oegema et al., 2001). CeCENP-C depleted embryos also exhibited apparently normal meiotic chromosome segregation followed by severe mitotic segregation defects (Suppl. Fig. 2.5a; Suppl. Video 5).

2.4 Discussion

The results described here surprisingly indicate that CeCENP-A and CeCENP-C, two essential constituents of the specialized chromatin that determines where kinetochores form during mitosis, are dispensable for chromosome segregation during meiosis in *C. elegans*. Remarkably, during the first embryonic mitosis, which occurs in the same cytoplasm only ~20 minutes after anaphase of the second meiotic division, assembly of

kinetochores and chromosome segregation is absolutely dependent on CeCENP-A and CeCENP-C (Desai et al., 2003; Moore and Roth, 2001; Oegema et al., 2001).

Homologous chromosome pairs in *C. elegans* undergo a single crossover event whose position dictates subsequent structural remodeling of the bivalent to define the axes of cohesion loss during each meiotic division (Chan et al., 2004; Hillers and Villeneuve, 2003; Nabeshima et al., 2005). We speculate that the targeting of outer kinetochore components is uncoupled from CeCENP-A/C-containing chromatin during meiosis to direct formation of spindle attachment sites after the axes of cohesion loss during each meiotic division are defined. Consequently, the position of the crossover, rather than CeCENP-A/C chromatin, dictates where outer kinetochore proteins target. Thus, in *C. elegans*, the restriction of crossovers to only one per homologous pair (Hillers and Villeneuve, 2003), crossover position-dependent remodeling of the bivalent in diakinesis (Chan et al., 2004; Nabeshima et al., 2005), and uncoupling of the targeting of outer kinetochore proteins from CeCENP-A/C-chromatin (this study) coordinately act to ensure chromosome segregation during meiosis.

2.5 Methods

2.5.1 dsRNAs, Antibodies & RNA Interference

The oligonuleotide sequences used for the production of dsRNAs

against *hcp-3*, *cpar-1* and *hcp-4* (which encodes CeCENP-C) are listed in Suppl Table 2. The antibody against CeCENP-A was generated and affinity-purified against amino acids 2 –183 of HCP-3. Other antibodies were described previously (Desai et al., 2003; Oegema et al., 2001). L4 hermaphrodites injected with dsRNA were incubated at 20°C for 45-48 hours. Western blots of dsRNA injected worms were performed as described (Desai et al., 2003).

2.5.2 Immunofluorescence

dsRNA-injected and control worms were processed in parallel. 6-8 worms were dissected on poly-lysine coated glass slides in 2.3 µL of 5% sucrose, 100 mM NaCl. A 10x10 mm coverslip was placed on top, the slides were frozen in liquid nitrogen, coverslips were flicked off using a razor blade, and slides were placed in –20°C methanol for 30 minutes. Slides were then processed for immunofluorescence using 1 µg/ml directly labeled antibodies against a-tubulin, CeCENP-A, CeCENP-C, KNL-1, or CeBUB-1 (Desai et al., 2003; Oegema et al., 2001). Four-color 3D image stacks were acquired at 0.2 µm Z steps using a 100x objective, deconvolved and projected for presentation. Gonads were dissected out of hermaphrodites by cutting off the tip of the tail with a scalpel. Whole gonad views were stitched together from individual, overlapping images. Exposure times and image processing were kept constant between RNAi worms and wild-type controls.

2.5.3 Live Imaging

L-15 blastomere culture medium (Edgar, 1995) was used for filming dissected meiotic embryos. Worms were mounted in 8 µl medium on 24x60 mm coverslips taped onto a metal holder and dissected on the coverslip. To prevent compression, vaseline was used as a spacer and an 18x18 mm coverslip placed on top. All live imaging was conducted at 20°C using a Deltavision deconvolution microscope. Chromosome segregation was monitored in embryos from a strain expressing GFP-histone and GFP-g-tubulin. At 30s intervals, 5 sections were acquired at 2 µm Z steps using a 100x objective with 1.5X additional magnification and 2x2 binning. Exposures were 400 ms using a 10% transmission neutral density filter. Z-series were projected to generate timelapse movies.

2.5.4 Monitoring Lac operator array segregation

Strain JM93 (Fukushige et al., 1999) was used for the Lac operator array segregation assay. Lac repressor was purified from *E. coli* as described (Laiken et al., 1972). After fixing in methanol and rehydrating in PBS, samples were incubated with purified Lac repressor in PBST, 5 mM $MgCl_2$, 0.1 mM DTT, and 50 µg/mL of BSA for 90 minutes. The operator array bound Lac repressors were then crosslinked in PBST, 5 mM $MgCl_2$, and 3% formaldehyde. Subsequent processing was similar to the immunofluorescence protocol except that rat anti-tubulin antibodies and

mouse anti-LacI primary antibodies were used followed by FITC conjugated anti-rat and Texas Red conjugated anti-mouse secondary antibodies.

2.6 Acknowledgments

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A.D. and K.O. made the preliminary observations leading to this study. P.M. helped generate and characterize the GFP::CPAR-1 strain. F.H. analyzed relative expression of CENP-A-like proteins. J.M. performed all of the other experiments and J.M., K.O. and A.D. jointly prepared the manuscript.

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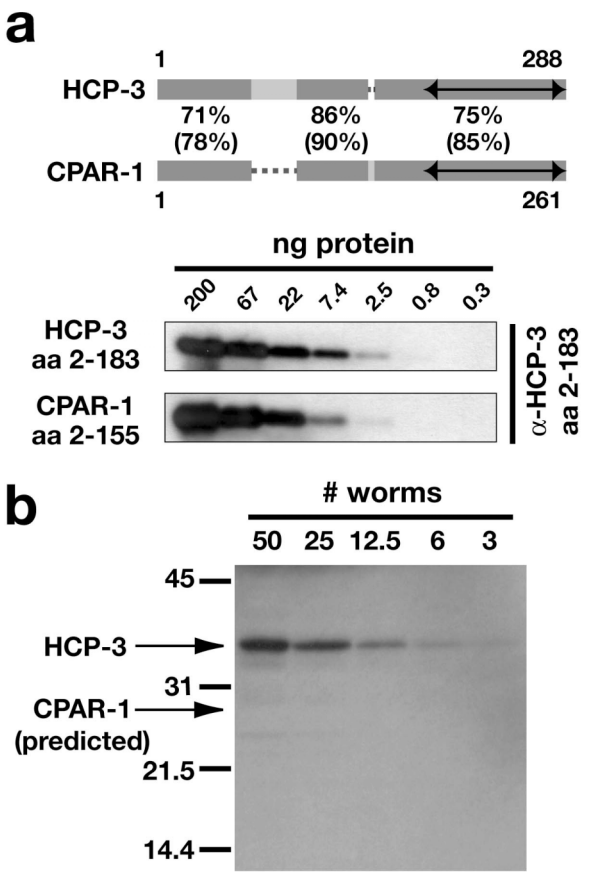

Figure 2.1. The kinetochore components CeCENP-A, CeCENP-C and KNL-1 are recruited to meiotic chromosomes during diplotene
(**a**) Schematic comparing HCP-3 and CPAR-1, the two CENP-A related proteins in the *C. elegans* genome. The extent of amino acid identity and similarity (*in brackets*) are indicated; dashed lines/light gray boxes represent gaps in alignment due to deletions/insertions; the double arrows in the C-termini indicate the histone H3 homology region. A quantitative western blot of the N-terminal tails of HCP-3 and CPAR-1, expressed in bacteria as 6xHis fusion proteins and purified by nickel affinity chromatography followed by electroelution. The blot was probed with an antibody raised against aa 2-183 of HCP-3. (**b**) A western blot of adult hermaphrodite worms probed with the antibody described in (a). The number of worms boiled in sample buffer and loaded are indicated above each lane.

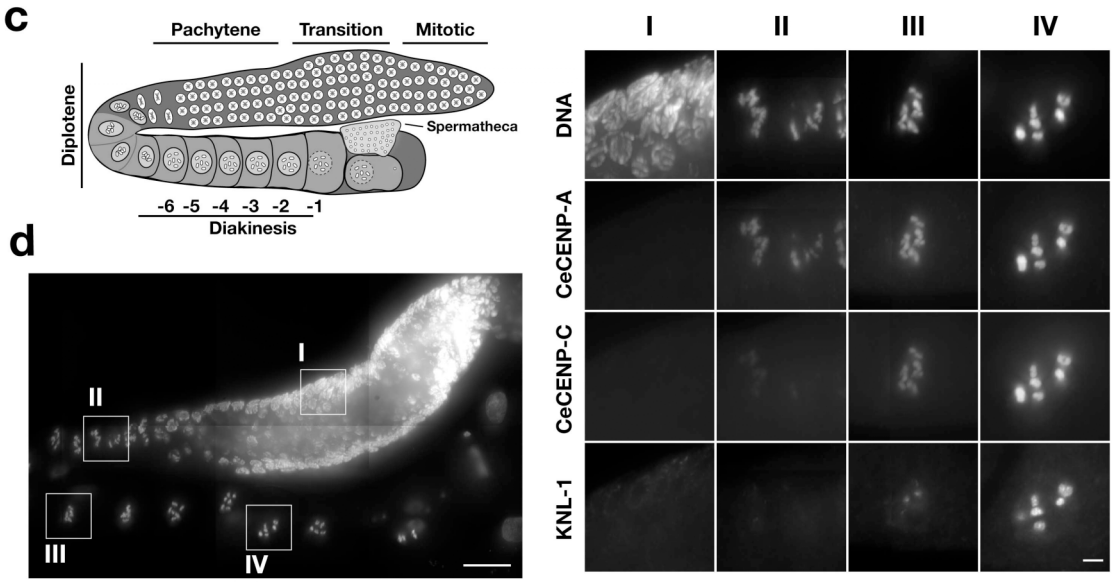

Figure 2.1 (cont.). The kinetochore components CeCENP-A, CeCENP-C and KNL-1 are recruited to meiotic chromosomes during diplotene
(**c**) Schematic of oogenic gonad in *C. elegans*. Nuclei exit the mitotic zone at the distal tip of the gonad and progress through distinct stages of meiotic prophase to generate oocytes containing 6 bivalent chromosomes. Meiotic segregation and embryonic divisions occur following fertilization during passage through the spermatheca. (**d**) Dissected and fixed gonads were co-stained with Hoechst (to label DNA), and antibodies to CeCENP-A, CeCENP-C and KNL-1. The top panel shows DNA staining in the full gonad. Boxes I-IV correspond to the locations of the higher magnification panels below, which show pachytene, diplotene, diplotene/diakinesis transition, and diakinesis, respectively (Scale bars = 25 μm for top panel & 3 μm for boxes; see Suppl. Fig. 2 for full gonad views).

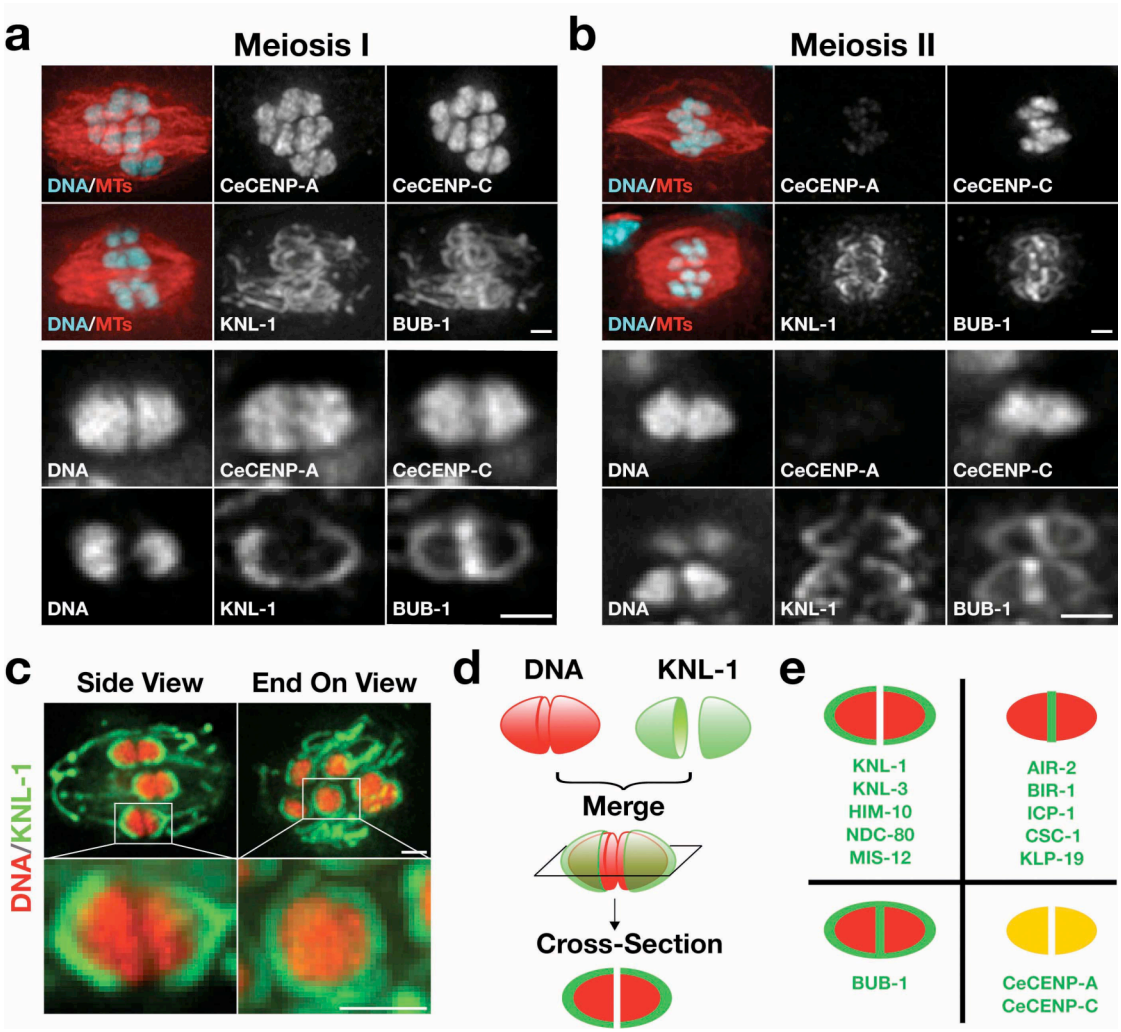

Figure 2.2. Conserved chromatin-proximal and outer kinetochore components exhibit distinct localization patterns during meiosis
(a) & (b) Localization of CeCENP-A, CeCENP-C, KNL-1 and BUB-1 during metaphase of meiosis I (**a**) and meiosis II (**b**). Overlay of DNA (*cyan*) and microtubules (*red*) is shown to the left. Lower panels are higher magnification views of individual bivalents (meiosis I) or sister chromatids (meiosis II). (**c**) Side and end-on views of merged images of a meiosis I spindle stained for KNL-1 (*green*) and DNA (*red*). (**d**) Schematic illustrating the localization of KNL-1 to two cup-like structures that enclose the ends of the chromosomes. (**e**) Summary of the localization patterns observed for components of mitotic kinetochores during meiosis. The localization of the chromosomal passenger complex and the chromokinesin KLP-19 to the central plane where cohesion will be dissolved during each division is also depicted (reviewed in ref. 28 which includes citations for all of the primary literature on these proteins). Scale bars = 1 μm.

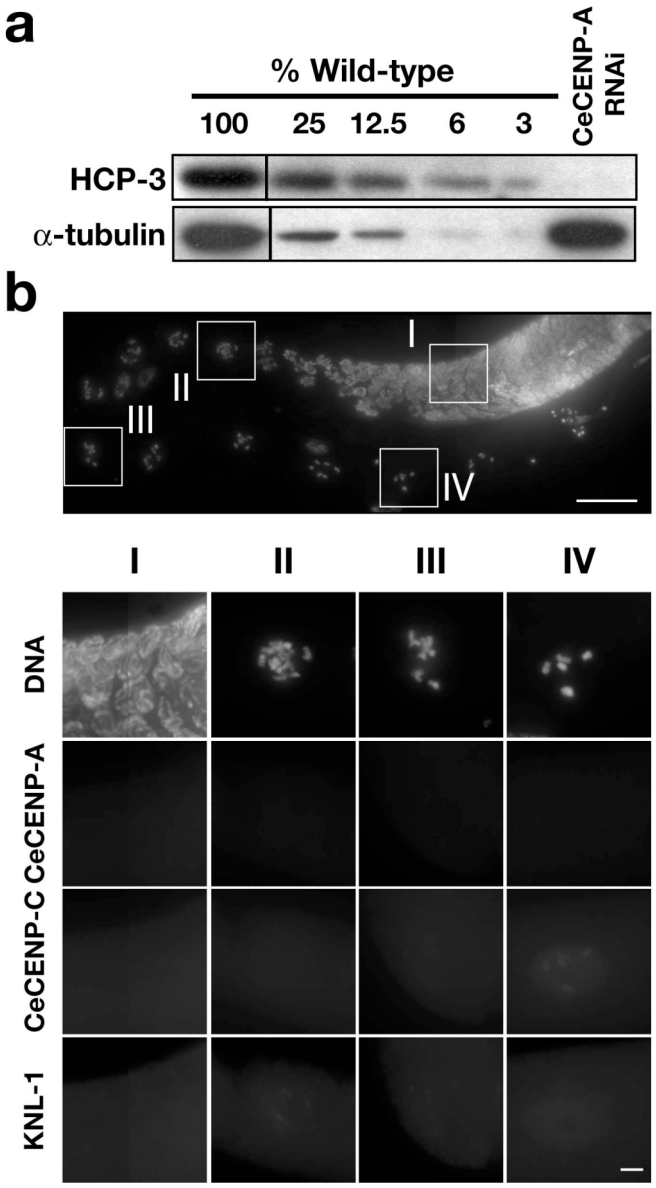

Figure 2.3. The targeting of CeCENP-C and KNL-1 to chromosomes during meiotic prophase requires CeCENP-A

(**a**) Western blot comparing wild-type worms to CeCENP-A RNAi worms indicates that HCP-3 is >97% depleted. (**b**) Gonad arm from CeCENP-A RNAi worm fixed and stained as described in Fig. 1d. Scale bar=3 μm (for full gonad views see Suppl. Fig. 2).

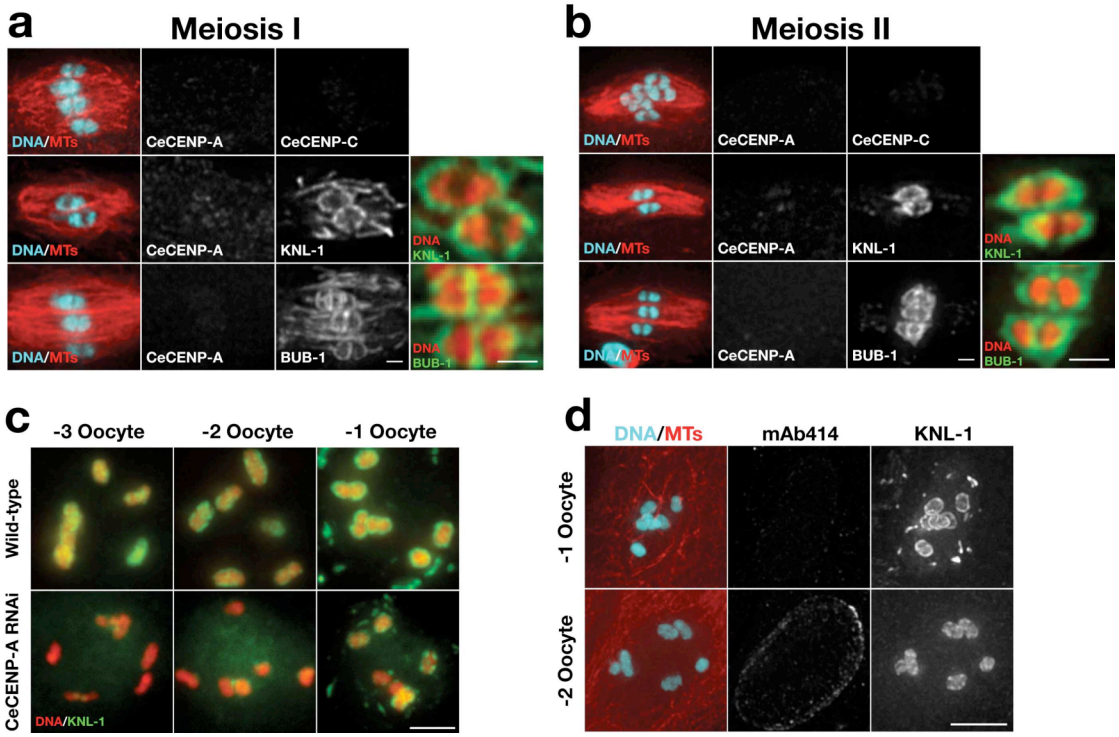

Figure 2.4. Outer kinetochore proteins still localize to the chromosome surface during meiosis in CeCENP-A depleted embryos
(**a**) & (**b**) CeCENP-A depleted embryos fixed in metaphase of meiosis I/II were stained for DNA (*cyan*), microtubules (*red*) and CeCENP-A/CeCENP-C or KNL-1/BUB-1. Higher magnification merged images of DNA/KNL-1 and DNA/BUB-1 are shown on the right (Scale bars = 1 μm). (**c**) The transition in the localization of outer kinetochore proteins to the chromosome surface occurs just prior to fertilization in wild-type (*top panels*). In CeCENP-A depleted worms (*lower panels*), KNL-1 is not detectable on chromosomes in the –3 oocyte and is weakly detected in the –2 oocyte. In the –1 oocyte immediately preceding the spermatheca, KNL-1 starts becoming enriched on the chromosome surface (Scale bar = 3 μm) (**d**) The transition of KNL-1 from localizing throughout the chromatin to localizing on the chromosome surface occurs coincident with nuclear envelope breakdown, assayed by staining with mAb414, which recognizes nuclear pore components (Scale bar = 5 μm).

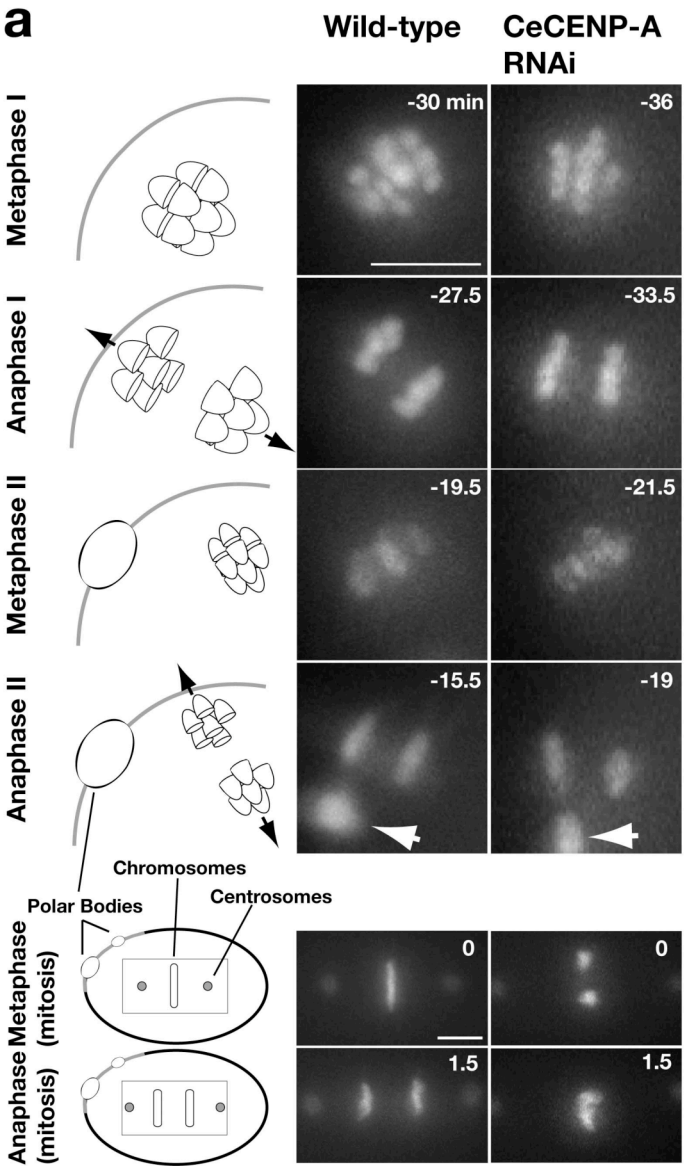

Figure 2.5. Embryos depleted of CeCENP-A or CeCENP-C exhibit normal meiotic segregation followed by a severe mitotic segregation defect
(**a**) The left column shows a schematic of chromosome segregation between metaphase of meiosis I and anaphase of the first mitotic division. Corresponding stills from movies of wild-type and CeCENP-A depleted embryos are shown in the adjacent columns; white arrows indicate polar bodies. In the CeCENP-A depleted embryo, normal meiotic segregation is followed by failure of mitotic segregation (see also Suppl. Table 1 & Suppl. Videos 1-4). A similar result is observed in CeCENP-C depleted embryos (Suppl. Table 1 & Suppl. Video 5). Scale bar = 5 μm.

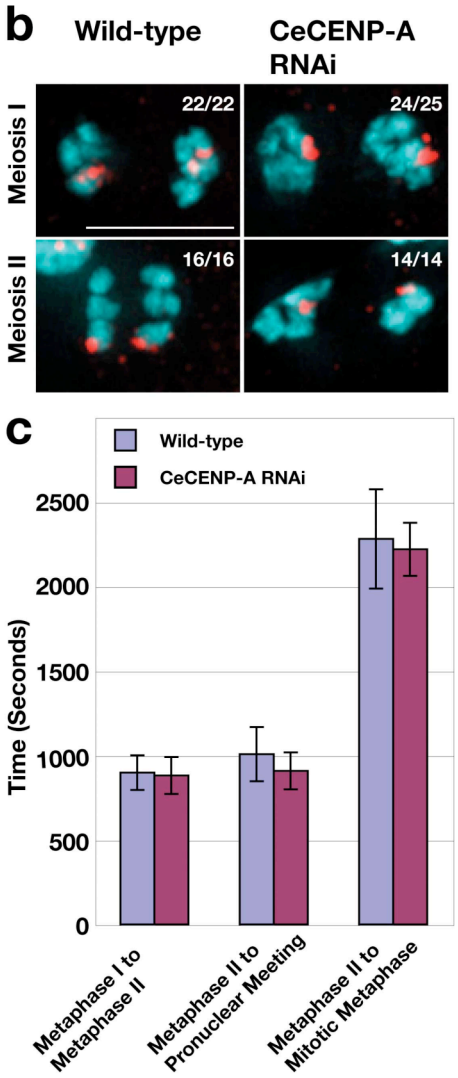

Figure 2.5 (cont.). Embryos depleted of CeCENP-A or CeCENP-C exhibit normal meiotic segregation followed by a severe mitotic segregation defect

(**b**) Reductional and equational segregation occur normally in CeCENP-A depleted embryos. Wild-type and CeCENP-A depleted embryos carrying an integrated Lac operator array were fixed and stained with Lac repressor (*red*) and Hoechst (*cyan*) to monitor the segregation of a specific locus. Anaphase images of meiosis I and II are shown. The numbers in each box correspond to embryos showing proper segregation/total embryos scored. Embryos were co-stained for CeCENP-A to ensure that depletion was effective (*not shown*). Scale bar = 5 μm. (**c**) Timing of meiotic divisions and first embryonic mitosis is not affected by depletion of CeCENP-A. Mean ± SD of 8 wild-type and 11 CeCENP-A(RNAi) embryos is plotted.

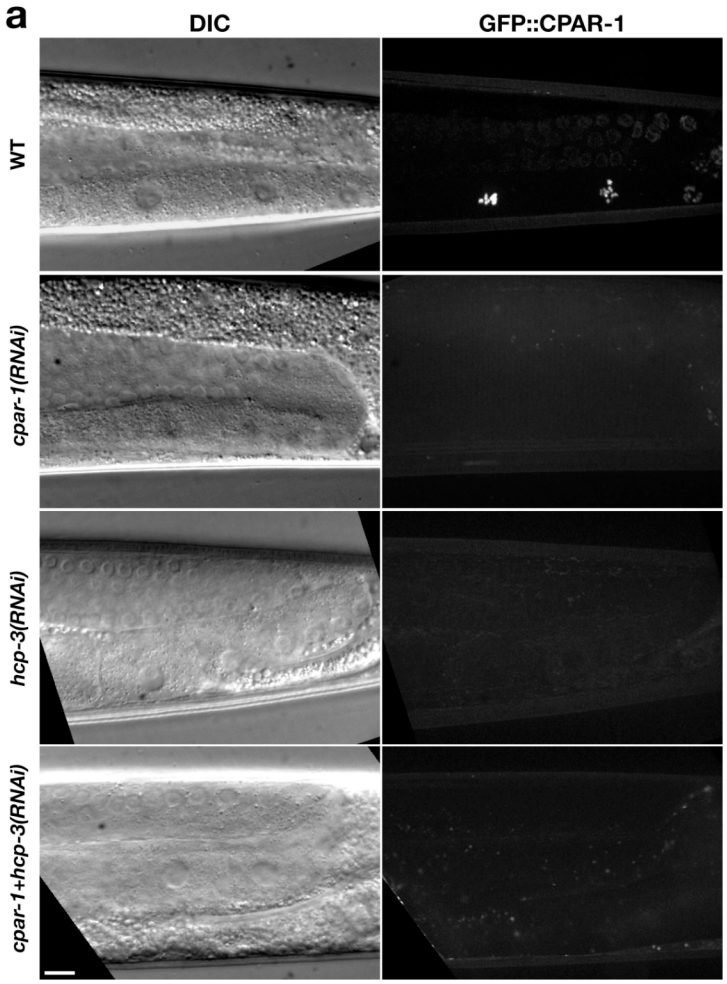

Figure S2.1. Generation and characterization of a strain stably expressing GFP::CPAR-1 in the germline under control of the *pie-1* promoter.

(**a**) Gonads were imaged in anesthetized living worms using a spinning disk confocal microscope. Twenty-one confocal fluorescence z-sections at 1 µm intervals and a single DIC image at the mid point of the z-stack were acquired using a 60X, 1.4NA objective. Maximum intensity projections of the fluorescence z-stacks are shown adjacent to the DIC images. A control worm, a worm injected with a dsRNA homologous to the *cpar-1* tail, a worm injected with a dsRNA homologous to the *hcp-3* tail, and a worm injected with a mixture of *cpar-1+hcp-3* tail dsRNAs are shown. In the control worm (*top panel*), GFP:CPAR-1 is not detectable in nuclei in the pachytene region of the gonad. GFP::CPAR-1 becomes visible in nuclei at the late pachytene/diplotene region and increases in intensity as the meiotic nuclei are cellularized to form oocytes. No GFP:CPAR-1 is detected in any of the dsRNA-injected gonads (Scale bar = 10 µm).

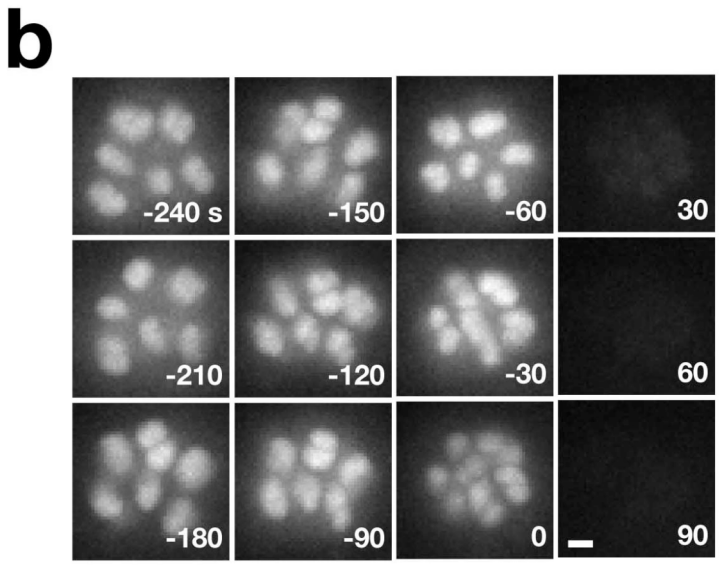

Figure S2.1 (cont.). Generation and characterization of a strain stably expressing GFP::CPAR-1 in the germline under control of the *pie-1* promoter.

(**b**) Selected stills from a timelapse sequence of a recently fertilized embryo expressing GFP::CPAR-1. Images of the region of the embryo containing the meiosis I spindle are shown. Images were acquired every 30s. Times are with respect to anaphase of meiosis I. GFP:CPAR-1 localizes prominently to the aligning chromosomes during the first meiotic division, but is rapidly removed from the condensed chromosomes around the metaphase-anaphase transition of meiosis I. No GFP::CPAR-1 was detected on chromosomes during meiosis II (Scale bar = 1 μm).

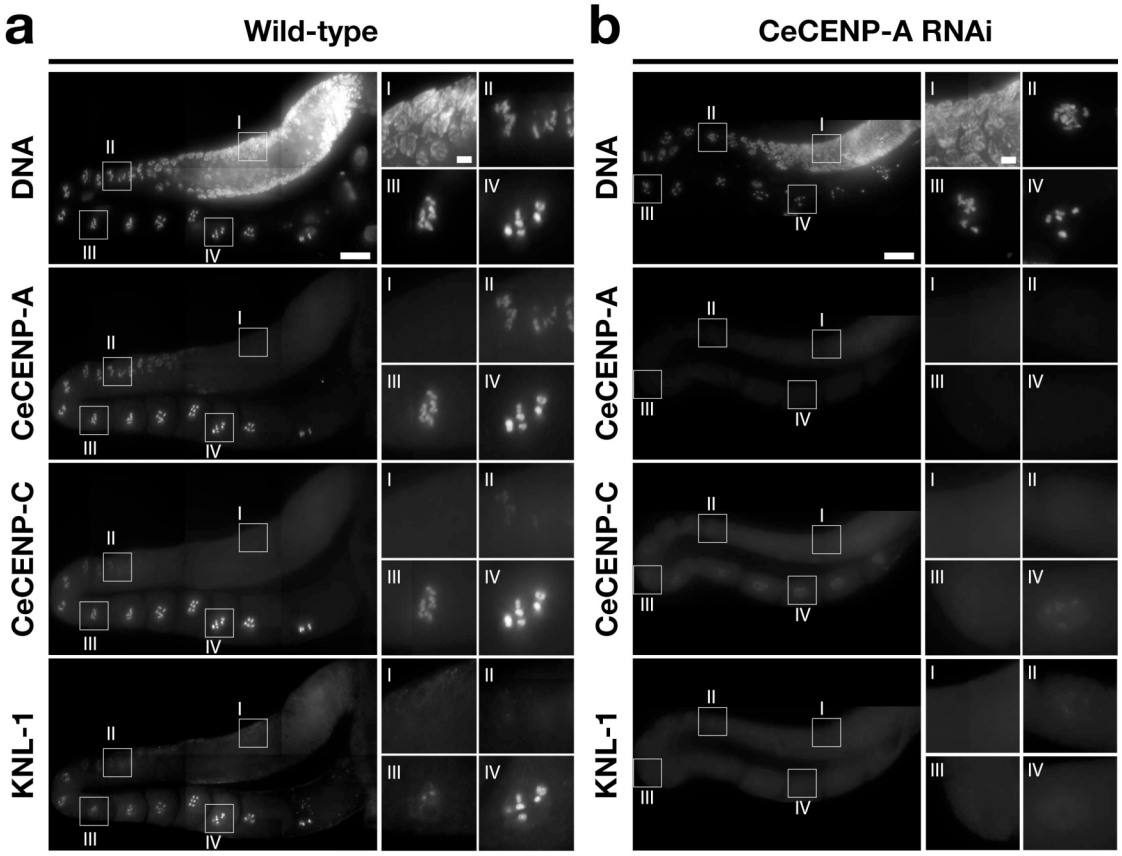

Figure S2.2. Full views of wild-type control and CeCENP-A depleted gonads.

Full views of (**a**) wild-type control and (**b**) CeCENP-A depleted gonads stained for DNA, CeCENP-A, CeCENP-C, and KNL-1. The four boxes correspond to the pachytene, diplotene, diplotene/diakinesis transition, and diakinesis phases of prophase of meiosis I, respectively. CeCENP-A is first detected on chromosomes as nuclei exit the pachytene region of the gonad, followed by CeCENP-C at a slightly later stage and finally KNL-1 around the early diakinesis stage. In the absence of CeCENP-A, both CeCENP-C and KNL-1 fail to localize throughout the region of the gonad depicted here. Condensed versions of the images presented here are shown in Fig. 1 and Fig. 3. (Scale bars = 25 μm for full gonad views and 3 μm for boxes).

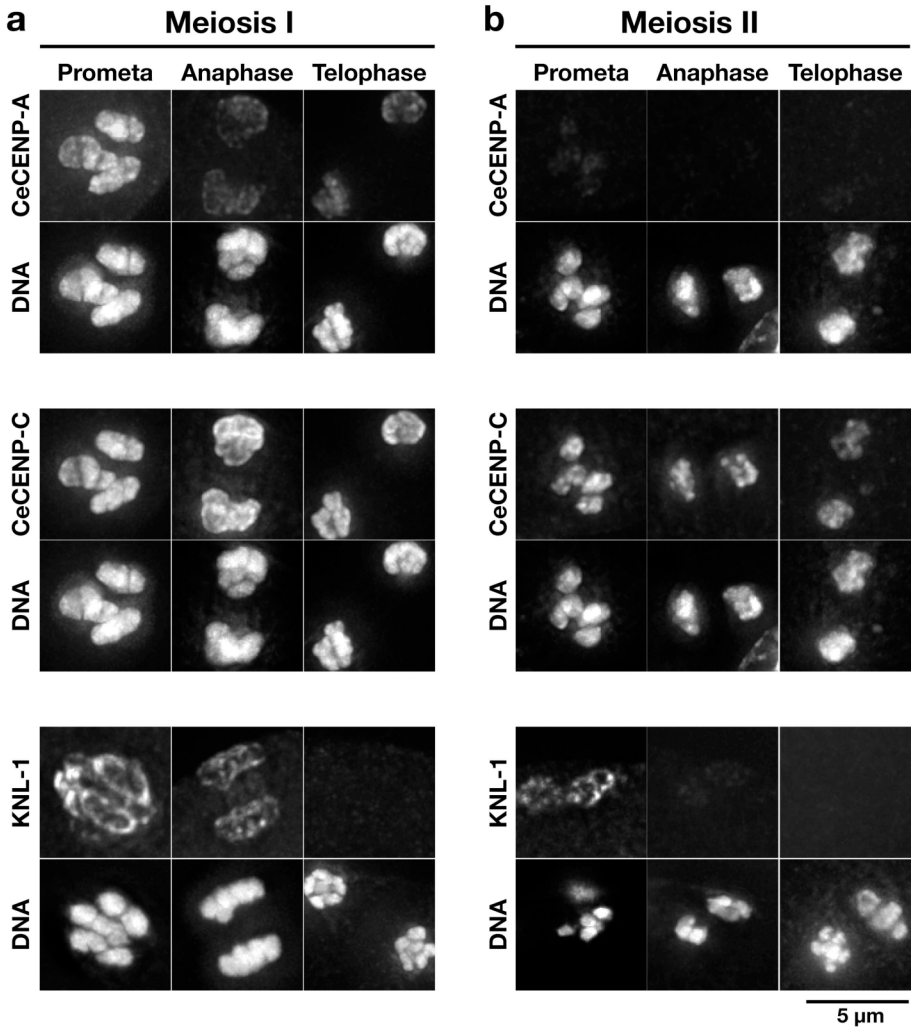

Figure S2.3. Meiotic staining is strikingly different between inner and outer kinetochore proteins.

Fertilized embryos undergoing (a) meiosis I and (b) meiosis II were fixed and stained for CeCENP-A, CeCENP-C, and KNL-1. Images of DNA and each protein during prometaphase, anaphase and telophase stages of both meiotic divisions are shown. (**a**) During meiosis I, CeCENP-A localizes to chromosomes at prometaphase but is largely absent by anaphase/telophase. CeCENP-C remains on chromatin throughout anaphase and telophase during meiosis I. KNL-1 is present in cup like structures as well as in strands throughout the spindle at prometaphase. KNL-1 staining is reduced in anaphase and absent in telophase. (**b**) In meiosis II, CeCENP-A staining is dramatically reduced at all stages compared to prometaphase of meiosis I. CeCENP-C localizes to chromatin in prometaphase, anaphase, and telophase in a manner similar to meiosis I. KNL-1 is present in cup-like structures during prometaphase and metaphase, but is absent after anaphase onset (Scale bar = 5 µm).

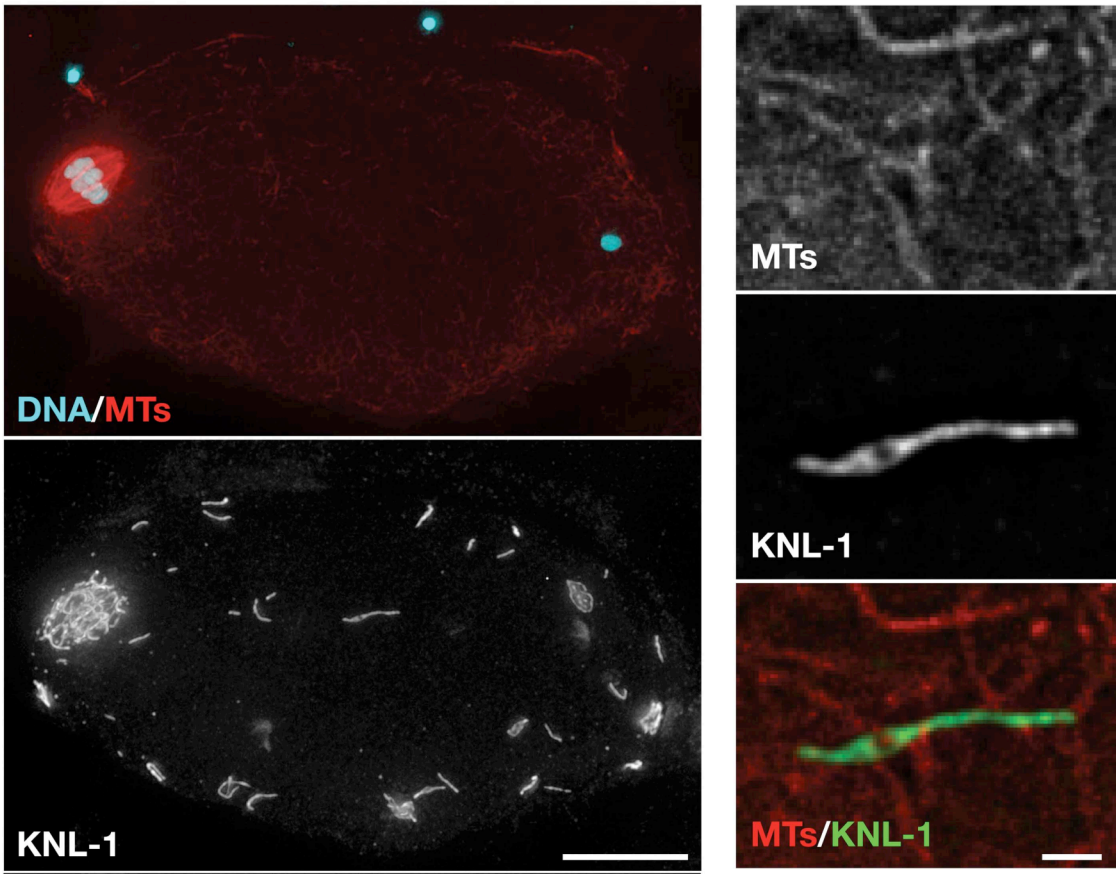

Figure S2.4. Characterization of outer kinetochore proteins during meiosis reveals molecular properties.
KNL-1 linear elements in the spindle and on the cell cortex during oocyte meiosis I (***a***), kinetochore protein localization during male meiosis (***b***), effect of Aurora B (AIR-2) kinase inhibition on kinetochore protein localization (***c***), and consequences of injecting *hcp-3+cpar-1* full length dsRNAs on KNL-1 localization (***d***). (**a**) Projected image of an embryo fixed during metaphase of meiosis I and stained for DNA (*cyan*), microtubules (*red*) and KNL-1. KNL-1 localizes to strands throughout the spindle as well as on the cortical surfaces of the embryo. Other outer kinetochore proteins, such as NDC-80, Nuf2^{HIM-10} and BUB-1, co-localize to these same spindle and cortical elements (*not shown*). (Scale bar = 10 μm). A higher magnification single section shown below indicates that the KNL-1 strand is not coincident with a bundle of cortical microtubules (Scale bar = 1 μm).

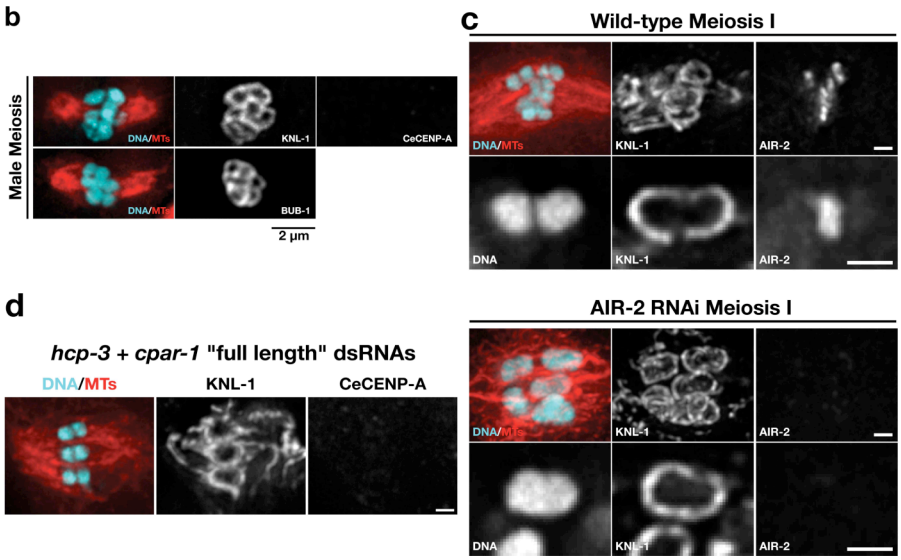

Figure S2.4 (cont.). Characterization of outer kinetochore proteins during meiosis reveals molecular properties.
(**b**) Dissected wild-type male gonads were fixed and stained for DNA (*cyan*), microtubules (*red*) and the kinetochore proteins CeCENP-A, KNL-1, and BUB-1. No chromosomal staining for CeCENP-A was detected (n=99 spermatocytes), whereas KNL-1 (n=59) and BUB-1 (n=39) were always detected localized around the chromosomes. BUB-1 was clearly detected on the axis of cohesion loss in 20% of spermatocytes; this low percentage is likely due to our inability to distinguish this chromosomal axis from the perichromosomal region in these images. There were no KNL-1 or BUB-1 linear elements in any of the spindles or the cortical regions of the analyzed spermatocytes (Scale bar = 2 µm). (**c**) Wild-type and *air-2(RNAi)* embryos undergoing meiosis I segregation were stained for DNA (*cyan*), microtubules (*red*), KNL-1, and AIR-2. Images in the lower row of each panel show a single chromosome at higher magnification. In wild-type embryos, AIR-2 localizes in a reciprocal manner to KNL-1 and is highly enriched in the plane of cohesion loss. After depletion of AIR-2, KNL-1 localization is similar to that in wild-type, except that it appears to encircle the entire bivalent without the gap in the region that normally contains AIR-2. This gap region is also reduced in the DNA images, suggesting a potential difference in the structure of the mid-bivalent region between wild-type and *air-2(RNAi)* embryos. BUB-1 like strong mid-bivalent localization was never observed (n=31 embryos). In a subset of chromosomes in 3 embryos, KNL-1 was weakly detected at a location consistent with the mid-bivalent; however the high density of chromosomes and the linear KNL-1 elements in meiosis I may have contributed to this impression (Scale bar = 1 µm). (**d**) Meiosis I spindle in an embryo from a worm injected with *cpar-1+hcp-3* full length dsRNAs, homologous to the genomic regions of both genes. The embryo was fixed and stained for DNA (*cyan*), microtubules (*red*), KNL-1, and CeCENP-A. (Scale bar = 1 µm).

a

Injected dsRNA(s)	dsRNA Conc. (mg/mL)	Total # of Movies	Meiosis I Defect	Meiosis II Defect	Mitotic "Kinetochore Null" Defect	Polar Body Resorption	Representative Movie
None	N/A	24	0/20	0/19	0/10	5/24	Suppl_Video1
HCP-3 Tail	1.24	23	0/16	0/19	7/7	5/23	Suppl_Video2
HCP-3 Tail + CPAR-1 Tail	2.0 each	12	0/11	0/11	9/9	2/12	Suppl_Video3
HCP-3 Full Length + CPAR-1 Full Length	1.94 each	14	0/11	0/10	10/10	4/14	Suppl_Video4
CeCENP-C	1.36	18	0/16	0/16	10/10	2/17	Suppl_Video5

b

dsRNA	Template used for dsRNA Production	Oligonucleotides used for dsRNA Production
HCP-3 Tail	cDNA	AATTAACCCTCACTAAAGGgccgatgacaccccaattat TAATACGACTCACTATAGGgttccttccggctctcatc
HCP-3 Full Length	Genomic DNA	AATTAACCCTCACTAAAGGgtttcgaccaaaaatgcttcc TAATACGACTCACTATAGGgatgtcgtctgcgtatttccc
CPAR-1 Tail	Genomic DNA	AATTAACCCTCACTAAAGGgttccttccggctatcatcc TAATACGACTCACTATAGGgccgatgacggaccaattat
CPAR-1 Full Length	Genomic DNA	TAATACGACTCACTATAGGtttaatcagagatttggaaggca TAATACGACTCACTATAGGtttcaaagtgcgaaaatttgatt
CeCENP-C	Genomic DNA	AATTAACCCTCACTAAAGGggaaatgtacggagcgaaaa TAATACGACTCACTATAGGacattgttggtgggtccaat

Figure S2.5. Summary of live imaging analysis and information on dsRNAs used for RNA interference..

(**a**) Summary of the live imaging analysis of meiotic and mitotic chromosome segregation for all of the different conditions tested. The interval filmed varied between different embryos, which is why only a subset of the total movies for each condition are in the meiosis I, meiosis II, and mitosis columns. (**b**) Oligonucleotide sequences and templates used to generate dsRNAs for RNA interference . The T3 and T7 promoters on the oligonucleotides are signified by capital letters.

Chapter 3: Separase-mediated CENP-A cleavage provides a fidelity mechanism in the inheritance of centromeric chromatin.

3.1 Summary

Centromere specification is believed to be propogated by an epigenetic mark produced by the centromeric, histone-H3 variant CENP-A (Cleveland et al., 2003; Karpen and Allshire, 1997). The mechanism for how CENP-A achieves this epigenetic mark remains to be described. In this study we discover that CeCENP-A is a substrate for Separase-mediated cleavage in the nematode, *C. elegans*, and discuss its implications on maintaining the epigenetic mark. During mitosis, Separase is unable to cleave centromeric CeCENP-A under wild-type conditions. However, in the absence of the kinetochore, centromeric CeCENP-A is cleaved, indicating that the kinetochore protects CeCENP-A whereas non-centromeric CeCENP-A is susceptible to cleavage. Because in meiosis outer-kinetochore assembly is independent of CeCENP-A (Monen et al., 2005), the cleavage event is very noticeable on chromatin during meiotic segregation in embryos expressing N-terminally tagged GFP::CeCENP-A. Worms solely expressing an uncleavable mutant form of CeCENP-A show an increase in embryonic lethality, and worms solely expressing a pre-cleaved form of CeCENP-A completely lose

CeCENP-A localization and function, resulting in complete embryonic lethality, suggesting that CeCENP-A cleavage inactivates CeCENP-A. We propose that cleavage of improperly loaded CeCENP-A onto chromosome arms is a mechanism used by the cell to ensure that the epigenetic mark for CeCENP-A loading remains strictly at the centromere.

3.2 Introduction

The centromere is a locus of specialized chromatin, which is essential for accurate chromosome segregation during mitosis. The centromere facilitates this by acting as the anchor for kinetochore establishment. The kinetochore complex is thus able to bind to microtubules and establish the chromosome to microtubule interaction necessary for segregation to take place. The highly conserved centromeric protein, CENP-A, has been shown to be essential for establishing the centromere and acts as the base of the kinetochore (Earnshaw et al., 1986; Earnshaw and Rothfield, 1985; Palmer et al., 1991). CENP-A is a histone-H3 variant and replaces histone-H3 in the nucleosomes of centromeric chromatin (Palmer et al., 1987). Studies performed in various model systems—including but not limited to yeast, worms, flies, mouse, and human cells—have clearly demonstrated that knockouts and knockdowns of CENP-A result in complete mitotic failure leading to aneuploidy and cell death (Blower and Karpen, 2001; Howman et al., 2000; Moore and Roth, 2001; Stoler et al., 1995).

A major question in the field is trying to understand how the centromere is propagated from one chromosome to the next after replication. In other words, how does CENP-A know when and where to load on a newly synthesized chromosome? Initial models suggested that DNA sequence played a role in determining where CENP-A would load. While this is true in the ~125 base pair centromere of budding yeast, higher eukaryotes do not appear to have a DNA sequence requirement for establishment of the centromere (Cleveland et al., 2003; Tanaka et al., 1999). Although adenine-thymine rich α-satellite DNA repeats are prevalent in centromeres of higher eukaryotes, they have been shown to not be essential for centromere propagation (Depinet et al., 1997; du Sart et al., 1997; Earnshaw and Rothfield, 1985; Warburton et al., 1997). The currently accepted model suggests that an epigenetic mark is established which directs CENP-A where to go (Amor et al., 2004a; Ventura et al., 2004). The best candidate for this epigenetic mark is, in fact, CENP-A (Cleveland et al., 2003; Karpen and Allshire, 1997). The best evidence to support this model is that there is no apparent centromeric DNA conservation from species to species, and that new centromeres (neo-centromeres) can be established de novo in chromsomomes that have lost their traditional centromere, without DNA translocations (Amor et al., 2004a; Amor and Choo, 2002; Ventura et al., 2004). The only obvious change in these patients carrying neo-centromeres is that CENP-A is propagated from one neo-centromere to the next, implicating

CENP-A as the epigenetic mark. The question still remains however, if CENP-A is indeed the epigenetic mark, how does it act as the mark, and what is the mechanism for how CENP-A is propagated?

A better understood role for CENP-A is its obvious importance during mitosis and cell division. Acting as the base of the kinetochore, we know that chromosomes fail to segregate when CENP-A is absent from cells. Aside from the role of the kinetochore, other structures and processes must work in unison with the forces generated by spindle microtubules, to facilitate the accurate segregation of chromosomes. One such process, is the removal of cohesin between sister chromatids to initiate anaphase onset. We know that a major player in this reaction is the cysteine-protease Separase (Ciosk et al., 1998). It has been shown that Separase is activated, via spindle checkpoint signaling, by the destruction of its chaperone Securin, and cleaves the cohesin subunit SCC-1 allowing for sister chromatid separation (Uhlmann et al., 1999; Zou et al., 1999). Separase has also been implicated in a number of other essential processes including cytokinesis and centrosome duplication (Hauf et al., 2001; Nigg, 2007). Despite this, only one other Separase substrate, the yeast protein Slk19 (which shows no conservation, and displays only a subtle role in spindle stability), has been identified (Sullivan et al., 2001). While we know that Separase is playing important roles in numerous processes, and because it is involved in so many processes; it is hard to understand the

mechanism of action and to assess exactly what specific role Separase plays in these processes without identifying its substrates.

In this study, we have identified CeCENP-A (HCP-3$^{CeCENP-A}$ and CPAR-1$^{CeCENP-A}$), in the nematode *Caenorhabditis elegans*, as a substrate for Separase-mediated cleavage and show evidence suggesting that this cleavage event provides a fidelity mechanism in the replication of centromeric chromatin. This cleavage event was first demonstrated at anaphase I onset in meiosis, and was originally mistaken as a complete removal of CeCENP-A from the nucleosome because immunofluorescence data, coupled with N-terminal GFP::CPAR-1$^{CeCENP-A}$ fusions could not differentiate between removal of the histone versus cleavage of the histone tail. This earlier report also demonstrated that chromosomes are capable of segregating in meiosis while failing in the following mitotic division, suggesting that meiotic chromosome segregation relied on a CeCENP-A independent mechanism to achieve proper segregation. Contrary to mitosis, outer kinetochore protein localization is CeCENP-A independent (Monen et al., 2005). For the remainder of this study I will refer to the two *C. elegans* homologs as HCP-3$^{CeCENP-A}$ and CPAR-1$^{CeCENP-A}$ when attempting to differentiate between the two, but will use CeCENP-A to describe properties they share in common or that cannot be clearly differentiated.

3.3 Results

3.3.1 CeCENP-A is cleaved at anaphase I onset during meiosis

The first worm strain developed in our lab with an integrated transgenic copy of GFP tagged CeCENP-A was the OD82 strain, which expresses an N-terminal GFP fusion to CPAR-1$^{CeCENP-A}$ (Figure 3.1a). Strikingly, this strain displays GFP fluorescence that is extremely bright and localizes to chromosomes in the oocytes of the gonad, but GFP signal is practically unnoticeable in mitotic embryos. The disappearance of GFP signal occurs dramatically at anaphase I onset and does not reappear in meiosis II or the subsequent mitotic divisions, as can be seen in embryos expressing mCherry::H2B and GFP::CPAR-1$^{CeCENP-A}$ (Figure 3.1b, Suppl. Mov. 1). It was not until the development of a second worm strain; OD145, which expresses a CPAR-1$^{CeCENP-A}$::GFP C-terminal tag; that we realized the disappearance of GFP signal seen in OD82 worms might be due to a cleavage event of the N-terminal tail rather than a total removal of CPAR-1$^{CeCENP-A}$ from the nucleosome. In OD145 worms we no longer see the striking removal of GFP signal at anaphase I onset, but rather CPAR-1$^{CeCENP-A}$::GFP remains localized to chromosomes for the remainder of meiosis and into mitosis (Figure 3.1a and 3.1c, Suppl. Mov. 2, see also Suppl. Fig 3.1a-c).

In order to better evaluate if this phenomenon was CPAR-1$^{CeCENP-A}$ specific or if this occured for CeCENP-A in general, we examined HCP-

$3^{CeCENP\text{-}A}$ in the context of meiosis. Despite extensive efforts to make a transgenic worm strain expressing an N-terminal GFP::HCP-$3^{CeCENP\text{-}A}$, we were unsuccessful, likely because HCP-$3^{CeCENP\text{-}A}$ plays an essential role in viability whereas CPAR-$1^{CeCENP\text{-}A}$ does not, and we suspect the N-terminus is essential for proper CeCENP-A function (see figure 3.3f and 3.7d below). A different approach was necessary to address this question, and so we used immunofluorescence to visualize HCP-$3^{CeCENP\text{-}A}$ in meiosis using antibodies targeted to different regions on HCP-$3^{CeCENP\text{-}A}$. The antibodies α-HCP-$3^{CeCENP\text{-}A(150)}$ and α-HCP-$3^{CeCENP\text{-}A(163)}$ were targeted to amino acids 68 to 82 and 105 to 183 of HCP-$3^{CeCENP\text{-}A}$ respectively. Comparing meiotic staining between these two antibodies, α-HCP-$3^{CeCENP\text{-}A(150)}$ signal drops significantly from metaphase I to anaphase I, whereas α-HCP-$3^{CeCENP\text{-}A(163)}$ signal shows no loss in signal intensity between the two stages (Figure 3.1d-e). To control for any deviations in staining from embryo to embryo, the ratio α-HCP-$3^{CeCENP\text{-}A(150)}$: α-HCP-$3^{CeCENP\text{-}A(163)}$ for relative signal intensity was determined for every embryo scored and this ratio was then compared between metaphase I embryos and anaphase I embryos (figure 3.1f). The ratio decreases significantly from metaphase I to anaphase I indicating that HCP-$3^{CeCENP\text{-}A}$ also is cleaved at anaphase I onset. Evidence involving filming of meiosis in embryos expressing GFP::HCP$3^{CeCENP\text{-}A}$ by way of mRNA gonad injections shows that GFP signal (though weakly expressed) is lost at anaphase I onset, further supporting the immunofluorescence data above (Suppl. Fig. 3.2a). It

seems pretty clear that the N-terminal tail of CeCENP-A is removed during meiosis, but in order to understand the significance of this removal, we needed to find out what other factors were involved in this process.

3.3.2 Separase is implicated in CeCENP-A histone-tail removal

In order to uncover proteins involved in the removal of the histone-tail of CeCENP-A, we used RNAi of candidate genes in the OD82 worm strain that displays bright GFP fluorescence in meiotic oocytes but not in mitotic embryos. By depleting these worms of targeted proteins, we were able to easily assess for candidate genes by the presence of GFP signal in mitotic embryos (figure 3.2a). Depletions of meiotic specific proteins, such as MBK-2 (Pellettieri et al., 2003), and proteins involved in degradation, such as CUL-2 (Sonneville and Gönczy, 2004) and CUL-3 (Pintard et al., 2003), displayed no affect (data not shown). However, RNAi of SEP-1^{Separase} in OD82 worms, displayed GFP signal on chromosomes in both oocytes and embryos (Siomos et al., 2001) (Figure 3.2b-c, Suppl. Mov. 3-4). Furthermore, live *in vivo* meiotic analysis shows that embryos attempting to segregate during meiosis I in SEP-1^{Separase} depletions retain GFP signal throughout meiosis and into mitosis (Suppl. Mov. 5). These data suggest that Separase is playing a role in the removal of the histone-tail of CeCENP-A. Because Separase is a cysteine-protease and its best-characterized function is cleavage of SCC-1 at

anaphase onset, it is plausible to suspect that CeCENP-A is a Separase substrate and that Separase is directly involved in cleaving CeCENP-A.

3.3.3 CPAR-1$^{CeCENP-A}$ and HCP-3$^{CeCENP-A}$ share homology but have many different molecular properties.

Before getting to the heart of the question for Separase-mediated cleavage of CeCENP-A, it is important to characterize the CeCENP-A homologs more thoroughly to get a better feel for the individual roles of each. To get a better idea for the specific localization for HCP-3$^{CeCENP-A}$ and CPAR-1$^{CeCENP-A}$, we used the worm strains OD101 and OD145, which express HCP-3$^{CeCENP-A}$::GFP (internally integrated GFP between AAs 173 and 174) and CPAR-1$^{CeCENP-A}$::GFP respectively (Suppl. Fig. 3.1b). Both GFP fusions are distal to the suspected site of Separase cleavage, so we can monitor the location of the histone core for each of the two CeCENP-As. Whole worms were anesthetized and z-stack images were taken of their gonads and uterus containing mitotic embryos (Suppl. Fig. 3.1a). OD56 (mCherry::H2B) worms were also imaged for comparison and to view the morphology of the chromosomes in each of the different stages (Suppl. Fig. 3.1c). HCP-3$^{CeCENP-A}$::GFP signal is lost by the transition zone and pachytene stage of meiosis in the gonad, and reappears on chromosomes later in meiosis and throughout the remainder of meiotic segregation. During mitosis HCP-3$^{CeCENP-A}$::GFP localizes at the kinetochore. CPAR-1$^{CeCENP-A}$::GFP displays a similar pattern

throughout the gonad in meiosis. In striking contrast, CPAR-1$^{CeCENP\text{-}A}$::GFP faintly localizes on chromosomes in mitosis, and in later embryos appears absent entirely (Suppl. Fig. 3.1c). Quantifying GFP signal during these stages indicates that HCP-3$^{CeCENP\text{-}A}$::GFP is brightest in mitosis, whereas CPAR-1$^{CeCENP\text{-}A}$::GFP is brightest during meiosis (Suppl. Fig. 3.1d). These data suggest that perhaps HCP-3$^{CeCENP\text{-}A}$ may be the real CENP-A ortholog in *C. elegans* and that, despite high sequence homology, CPAR-1$^{CeCENP\text{-}A}$::GFP might play a less vital role. To be sure of this, a more in depth characterization between the two CeCENP-As was performed.

There are four major conserved characteristics of CENP-A aside from sequence homology, which define CENP-A orthologs between species. These include the role for CENP-A as the building block for kinetochore assembly, CENP-A loading being dependent on certain known factors, CENP-A localizing at the kinetochore, and lastly CENP-A's essential role during mitotic chromosome segregation. We test all of these known CENP-A characteristics to compare and contrast HCP-3$^{CeCENP\text{-}A}$ and CPAR-1$^{CeCENP\text{-}A}$.

We use immunolocalization of the downstream kinetochore component CeCENP-C (Moore and Roth, 2001; Oegema et al., 2001) to assess the role for HCP-3$^{CeCENP\text{-}A}$ and CPAR-1$^{CeCENP\text{-}A}$ as the building block for kinetochore assembly. By specifically depleting either HCP-3$^{CeCENP\text{-}A}$ or CPAR-1$^{CeCENP\text{-}A}$ via RNAi, we analyze the ability of CeCENP-C to properly localize to the kinetochore using an α-CeCENP-C antibody. To ensure that depletions were

thorough and specific, OD101 and OD145 worms were used in these studies and stained using α-GFP (data not shown). During meiosis, specific depletions of HCP-3$^{CeCENP-A}$ does not affect CENP-C localization, while specific depletions of CPAR-1$^{CeCENP-A}$ prevents CENP-C from localizing to chromosomes, indicating that CPAR-1$^{CeCENP-A}$ and not HCP-3$^{CeCENP-A}$ is required for CeCENP-C targeting during meiosis (Figure 3.3a). While this comes as a bit of a surprise, the only conclusion that can be drawn from this is that CPAR-1$^{CeCENP-A}$ has the capacity to direct CeCENP-C loading in the context of meiosis, but because it has been established in *C. elegans* that outer kinetochore assembly is CeCENP-A independent during meiosis, this may not be a relevant assessment to identify the CENP-A ortholog. A better assessment involves the same analysis during mitosis. Contrary to what we see during meiosis, CENP-C localization is dependent on HCP-3$^{CeCENP-A}$ and not CPAR-1$^{CeCENP-A}$ (Figure 3.3b). This mitotic analysis provides more evidence that HCP-3$^{CeCENP-A}$ is the true CENP-A ortholog.

CENP-A loading is dependent on several proteins, one of which is the conserved myb-domain protein KNL-2 (Fujita et al., 2007; Maddox et al., 2007). To test if HCP-3$^{CeCENP-A}$ and CPAR-1$^{CeCENP-A}$ rely on KNL-2 for loading, we assess immunofluorescence in embryos depleted of KNL-2. To test for CPAR-1$^{CeCENP-A}$ loading we use the OD145 strain (see Figure 3.1a above and Suppl. Fig 3.1a) and use the α-GFP antibody to specifically detect the presence of CPAR-1$^{CeCENP-A}$. In both meiosis and mitosis CPAR-1 appears to

load independently of KNL-2 (Figure 3.3c). The abnormal chromosome morphology in the meiotic KNL-2 RNAi images are characteristically seen, but are the focus of a different study. To analyze HCP-3$^{CeCENP-A}$ loading, OD101 worms (see Suppl. Fig. 3.1a) were used to specifically detect HCP-3$^{CeCENP-A}$::GFP using α-GFP staining. Unlike CPAR-1$^{CeCENP-A}$, HCP-3$^{CeCENP-A}$ does not localize during meiosis or mitosis in KNL-2 depleted embryos (Maddox et al., 2007). These data provide further evidence suggesting that HCP-3$^{CeCENP-A}$ is the *C. elegans* CENP-A ortholog.

Images for CPAR-1$^{CeCENP-A}$ staining (seen in Figure 3.3c) suggest that it may not specifically localize to the kinetochore, but rather throughout chromatin. To better evaluate where both HCP-3$^{CeCENP-A}$ and CPAR-1$^{CeCENP-A}$ localize during mitosis, line-scans of high-resolution mitotic chromosome images were analyzed in OD145 worms comparing DNA (Hoechst), HCP-3$^{CeCENP-A}$ (α-HCP-3$^{CeCENP-A(150)}$) and CPAR-1$^{CeCENP-A}$ (α-GFP). CPAR-1$^{CeCENP-A}$ co-localizes to DNA staining and the line-scan comparisons were indistinguishable from one another (Figure 3.3d). HCP-3$^{CeCENP-A}$, on the other hand, localizes in pair-wise stripes signified by two peaks on the line-scan, characteristic of kinetochore staining (Figure 3.3d).

Thus far we have tested many of the properties known to be characteristic for CENP-A, aside from CENP-A's most important property; its essential role in cell division. To test CENP-A's role in chromosome segregation and viability we specifically deplete N2 worms with either HCP-

3$^{CeCENP\text{-}A}$ or CPAR-1$^{CeCENP\text{-}A}$ and assess the F1 progeny. Worms depleted of HCP-3$^{CeCENP\text{-}A}$ displayed 100% embryonic lethality, whereas CPAR-1$^{CeCENP\text{-}A}$ depleted worms showed no distinguishable difference in embryonic lethality compared to uninjected control worms (Figure 3.3e). Live analysis of embryos expressing GFP::H2B depleted of CPAR-1$^{CeCENP\text{-}A}$ exhibited normal chromosome segregation, whereas embryos depleted of HCP-3$^{CeCENP\text{-}A}$ displayed the kinetochore null (KNL) phenotype characteristic of a CeCENP-A depletion (data not shown). This observation supports the data above that suggests CPAR-1$^{CeCENP\text{-}A}$ does not play a vital role in cell division whereas HCP-3$^{CeCENP\text{-}A}$ is essential for proper kinetochore assembly and function.

Because we now believe that the *C. elegans* CENP-A ortholog is indeed HCP-3$^{CeCENP\text{-}A}$, and not CPAR-1$^{CeCENP\text{-}A}$, we focus our attention to better understanding the role and mechanism of Separase-mediated cleavage on HCP-3$^{CeCENP\text{-}A}$. Having said that, we will continue using CPAR-1$^{CeCENP\text{-}A}$ as a tool to better understand certain CeCENP-A characteristics that they share in common as it pertains to CeCENP-A cleavage.

3.3.4 Separase-mediated cleavage is perturbed in CeCENP-A mutants

To clearly demonstrate the CeCENP-A cleavage event, western blot analysis of embryo extracts and whole worm extracts were performed under wild-type, HCP-3$^{CeCENP\text{-}A}$, and SEP-1^{Separase} conditions (data not shown). Using this approach, we have not been able to find evidence for a cleaved

product. This is likely because the only antibodies that work by blot target regions along the histone-tail of CeCENP-A, and if upon cleavage the N-terminal tail is quickly degraded, as is known to occur for SCC-1 after Separase-mediated cleavage (Uhlmann et al., 1999), then we would not be able to detect cleavage by western blot given the limitations in the system. A different approach was required to address whether Separase was directly cleaving CeCENP-A.

Our new approach to test if Separase cleaves CeCENP-A takes advantage of what is known about Separase substrate recognition. An elegant study performed in budding yeast, very nicely demonstrates that an ExxR motif on both Scc1 and Slk19 is a required element for substrate recognition (Sullivan, 2003). The most stringent mutations involved replacing glutamate with leucine and arginine with glutamine resulting in an LxxQ motif, completely preventing cleavage by Separase. While Separase is quite divergent from organism to organism, this consensus cleavage sequence is believed to be conserved. We analyzed the sequences for HCP-3$^{CeCENP-A}$ and CPAR-1$^{CeCENP-A}$, and there were two ExxR motifs, only one of which located on the N-terminal tail (Figure 3.4a). We thus generated worm strains expressing CeCENP-A mutated at the tail ExxR motif: strain OD180 (CPAR-1$^{CeCENP-A(E68L_R71Q)}$) and strain OD246 (HCP-3$^{CeCENP-A(E101L_R104Q)}$) (Figure 3.4b). These mutations will be referred to as CPAR-1** and HCP-3** for the remainder of this report. Because the cleavage event is best demonstrated by

the dramatic removal of GFP in OD82 (GFP::CPAR-1$^{CeCENP-A}$) worms at anaphase onset, we filmed OD180 (GFP::CPAR-1**) embryos and discovered that GFP was not removed and remained on chromosomes during meiosis II and into mitosis, indicating that the mutation was sufficient to perturb cleavage (Figure 3.4c, Suppl. Mov. 6). Due to the difficulty in making HCP-3$^{CeCENP-A}$ transgenic strains we were only able to make an HCP-3** strain (OD246) with the GFP distal to the cleavage site after amino acid 173 (Figure 3.4b). To assess if cleavage was perturbed in OD246 worms, we depleted endogenous CeCENP-A (both HCP-3$^{CeCENP-A}$ and CPAR-1$^{CeCENP-A}$) and compared α-HCP-3$^{CeCENP-A(150)}$ staining in meiosis between OD136 (HCP-3$^{CeCENP-A}$::GFP) and OD246 embryos. Both transgenes in OD136 and OD246 were re-encoded to have synonymous HCP-3$^{CeCENP-A}$ codons that are resistant to RNAi for endogenous HCP-3$^{CeCENP-A}$. α-HCP-3$^{CeCENP-A(150)}$ levels decrease significantly from metaphase I to anaphase I in OD136 embryo but not for OD246 embryos, indicating that cleavage is also perturbed in HCP-3** mutants (Figure 3.4d-e). These data provide strong evidence suggesting that Separase directly cleaves CeCENP-A. Now that we have established the capacity for Separase to cleave CeCENP-A, we need to analyze whether this cleavage event is also occurring in mitosis.

3.3.5 Centromeric CeCENP-A can be cleaved during mitosis in the absence of the kinetochore

Because HCP-3$^{CeCENP-A}$ plays an essential role during mitosis and CPAR-1$^{CeCENP-A}$ does not (see Figure 3.3e), and because full-length CPAR-1$^{CeCENP-A}$ does not appear to be present on mitotic chromosomes under wild-type conditions (See Figure 3.1b), we focus on HCP-3$^{CeCENP-A}$ as it pertains to Separase-mediated cleavage during mitosis. Using the assay described above for assessing cleavage of HCP-3$^{CeCENP-A}$ in meiosis (see figure 3.1d-f), we analyze immunofluorescence of mitotic embryos and compare staining between α-HCP-3$^{CeCENP-A(150)}$ (antigen proximal of cleavage site) and α-HCP-3$^{CeCENP-A(163)}$ (antigen distal of cleavage site) to assess if cleavage occurs at centromeres during anaphase onset of mitosis. Analysis of wild-type embryos displays no detectable difference for α-HCP-3$^{CeCENP-A(150)}$/α-HCP-3$^{CeCENP-A(163)}$ between metaphase and anaphase, indicating that centromeric bound HCP-3$^{CeCENP-A}$ does not get cleaved at anaphase onset (Figure 3.5a,c).

One major difference between CeCENP-A's role in mitosis versus meiosis, is that in mitosis the kinetochore is dependent on and co-localizes to CeCENP-A (Monen et al., 2005; Moore and Roth, 2001). We speculate that the built kinetochore prevents CeCENP-A substrate accessibility, and is thus protected from Separase-mediated cleavage by the kinetochore. To test this hypothesis, we compare α-HCP-3$^{CeCENP-A(150)}$/α-HCP-3$^{CeCENP-A(163)}$ for mitotic embryos depleted of CeCENP-C, preventing kinetochore assembly. Embryos

were staged based on chromosome and spindle morphology because in CeCENP-C RNAi embryos chromosomes fail to segregate. α-HCP-3$^{CeCENP-A(150)}$ staining was highly diminished between metaphase and anaphase embryos whereas α-HCP-3$^{CeCENP-A(163)}$ staining remained the same (Figure 3.5b). In contrast with wild-type embryos, the α-HCP-3$^{CeCENP-A(150)}$/α-HCP-3$^{CeCENP-A(163)}$ ratio was significantly less in anaphase cells than metaphase cells (Figure 3.5c). These data suggest that the kinetochore may serve as protection for CeCENP-A, preventing access for Separase-mediated cleavage (Figure 3.5d). But what function does cleavage of CeCENP-A have on chromosome segregation?

3.3.6 CeCENP-A cleavage is important for viability

To test the role for Separase-mediated CENP-A cleavage we used the strains OD136 and OD246, which have RNAi resistant transgenes expressing wild-type and uncleavable forms of HCP-3$^{CeCENP-A}$ respectively (Figure 3.6a). Under uninjected control conditions, both OD136 and OD246 embryos load their respective integrants onto centromeres and chromosomes segregate properly in the first mitotic division (Figure 3.6b, Suppl. Mov. 7-8). Surprisingly, when endogenous HCP-3$^{CeCENP-A}$ is depleted, both OD136 and OD246 embryos appear to load their respective HCP-3$^{CeCENP-A}$ integrants and segregate chromosomes properly in the first mitotic division (Figure 3.6c). It is important to note that expression levels for the different integrants in OD136

and OD246 are atleast 20 fold less than endogenous HCP-3 levels, as can be seen by their absence on blots of embryo extracts for these worms (Figure 3.6d). However, the levels of expression between these worms are comparable with one another as viewed by GFP signal quantification at metaphase (Figure 3.6e). Since a phenotype for worms expressing HCP-3** was not directly obvious with our live imaging assays, we needed a new assay to uncover a potential role.

A more informative yet less detailed assay tested whether the integrated transgenes in OD136 and OD246 could rescue viability in HCP-$3^{CeCENP\text{-}A}$ depletions. The major caveat to these experiments is that both strains are obligate heterozygotes, meaning that the transgenes for both strains likely integrated into an essential gene, so approximately ¼ of the F1 progeny are embryonic lethal. When comparing the embryonic lethality in uninjected controls between OD136 and OD246 worms, we see that OD136 lethality is around 14 +/- 8% whereas OD246 lethality is just over 27 +/- 15%, with a p-value of 0.06 (Figure 3.6f). We suspect that this discrepancy between the two strains might be due to a slight dominant-lethal affect caused by the HCP-3** mutant. To test this idea, we depleted both strains of their respective integrants using GFP RNAi. We found that the strains treated with GFP dsRNA were now virtually identical at 15+/- 6% for OD 136 and 18+/- 13% for OD246 with a p-value of 0.64 (Figure 3.6f). We now had a means to compare the two strains because our assay could control for a slight dominant affect by

knocking-down the integrants, and the 2 strains were thus identical to each other when testing for embryonic lethality. The last experiment in this series involved depleting endogenous HCP-3$^{CeCENP-A}$ to test how well the integrated transgenes could rescue viability. Under these conditions, OD136 worms displayed an embryonic lethality of 14 +/- 5%, whereas OD246 worms displayed lethality at 36 +/- 12% (Figure 3.6f). With a p-value of 0.001, the two strains were significantly different, indicating that the HCP-3** mutant protein was perturbing embryonic viability. We now know that preventing CeCENP-A cleavage can have detrimental affects on viability, but in order to understand how this occurs, we need use other means to address this question. Currently, our current chromosome segregation assays are limited to analyzing phenotypes that arise during very early embryonic development, making it very difficult to assess a milder chromosomal segregation defect that could occur in later embryos. If our hypothesis is true, and the Separase-mediated cleavage event plays a role in centromere specificity, it is entirely possible (especially at the low-level transgene expression of the mutant) that the lethality we are seeing is due to events occurring in much later embryos. As a result, despite our initial efforts in characterizing a phenotype for the uncleavable HCP-3** mutant strain, we do not see an obvious defect. New assays need to be developed in order to thoroughly investigate this phenomenon. In the interim, perhaps we can gain insight into the mechanism using a different approach and understand the function of the cleaved HCP-

$3^{CeCENP\text{-}A}$ product by introducing a "pre-cleaved" HCP-$3^{CeCENP\text{-}A}$ mutant transgene.

3.3.7 A pre-cleaved HCP-$3^{CeCENP\text{-}A}$ mutant is unable to localize and function in the absence of full-length HCP-$3^{CeCENP\text{-}A}$

To test the functionality of the CeCENP-A cleavage product, we introduced a transgene expressing a "pre-cleaved" mutant copy of HCP-$3^{CeCENP\text{-}A}$. This worm strain, OD214, carries an N-terminal GFP tag fused to HCP-$3^{105\text{-}288}$, which incorporates the entire sequence of the HCP-$3^{CeCENP\text{-}A}$ cleavage product (Figure 3.7a). In uninjected control worms, GFP::HCP-$3^{105\text{-}288}$ localizes to the centromere (Figure 3.7b). When depleted of endogenous HCP-$3^{CeCENP\text{-}A}$, GFP::HCP-$3^{105\text{-}288}$ is no longer capable of localizing to the centromere, resulting in a KNL phenotype (Figure 3.7b). Expression levels, for both uninjected and RNAi conditions, are the same (Figure 3.7c), so the result we are seeing is not a matter of decreased expression. Testing viability for OD214 worms, we assess the F1 progeny under various conditions (similar to experiment 3.6f above) and score embryonic lethality, using N2 worms as a control. We see 0 +/- 0.1% embryonic lethality for uninjected and GFP RNAi conditions in both strains, and 100% embryonic lethality under endogenous HCP-$3^{CeCENP\text{-}A}$ RNAi conditions (Figure 3.7d). We conclude that the N-terminal tail is important for proper localization and function of HCP-$3^{CeCENP\text{-}A}$,

and thus Separase-mediated cleavage could be a way of inactivating HCP-3$^{CeCENP-A}$.

3.4 Discussion

3.4.1 A model for maintaining the epigenetic mark by way of Separase-mediated CENP-A cleavage

Prior analysis for centromere specification suggested that CENP-A is the epigenetic mark maintaining the centromere from one mitotic division to the next. However, the mechanism for how CENP-A acts as this epigenetic mark has remained unclear. Based on a combination of the RNAi and mutant analysis in this study, we propose that the epigenetic mark is imprinted on the N-terminal histone-tail of CeCENP-A, and that Separase-mediated CeCENP-A cleavage maintains centromere specificity by clearing the mark from non-centromeric CeCENP-A.

In our model, HCP-3$^{CeCENP-A}$ is primarily loaded onto centromeric DNA as a result from the HCP-3$^{CeCENP-A}$ mark from the last division, but like all biological mechanisms, errors do arise. On occasion, HCP-3$^{CeCENP-A}$ is incorrectly loaded onto non-centromeric regions of DNA. During mitosis, the kinetochore is assembled onto centromeric HCP-3$^{CeCENP-A}$, while the minimal amount of non-centromeric HCP-3$^{CeCENP-A}$ is not concentrated enough to direct kinetochore assembly elsewhere. At anaphase onset, Separase cleaves non-centromeric HCP-3$^{CeCENP-A}$, but centromeric HCP-3$^{CeCENP-A}$ is protected by the

presence of the kinetochore. The epigenetic mark has thus been lost on the cleaved HCP-3$^{CeCENP-A}$; and during the subsequent cell cycle, only on the centromere, where full-length HCP-3$^{CeCENP-A}$ resides, will new HCP-3$^{CeCENP-A}$ be loaded (Figure 3.8). And so the cycle continues, with the epigenetic mark for centromere specification protected by the kinetochore, while Separase acts as the cleanup crew, fixing the mistakes by removing the mark from chromosome arms, thus maintaining the integrity of the centromere.

We speculate at the reasons for why we do not see a more dramatic affect on viability and chromosome segregation in our HCP-3** mutant worm strain, OD246. If our model is correct, then an uncleavable form of HCP-3$^{CeCENP-A}$ would likely display a dominant-lethal affect. The major caveat for the system is that the transgenes incorporated express at much lower levels than endogenous HCP-3$^{CeCENP-A}$, as can be seen in figure 3.6d. One can imagine that at such low levels, the presence of HCP-3** is not enough to cause a severe affect on viability and chromosome segregation. Even in endogenous HCP-3$^{CeCENP-A}$ depletions, the low levels of expression for the mutant HCP-3** prevent major problems because most of the free HCP-3** available loads to centromeric DNA and there is just not enough to load elsewhere and cause problems. The fact that these *PIE-1* driven transgenes express at low levels may have been a blessing in disguise. If our model is correct and we got robust expression of our HCP-3** uncleavable mutant

gene, it is possible that it would result in total embryonic lethality and we would never have generated a worm strain.

Although we discovered the phenomenon for CeCENP-A cleavage in meiosis, we do not discuss the possible implications this cleavage may have in the context of meiosis because we believe there may not be a functional role at this stage. However, it was instrumental in this study because it allowed us to characterize properties that may have been overlooked in our mitotic analyses. We were fortunate that outer kinetochore assembly is CeCENP-A independent during meiosis, otherwise we may never have observed this cleavage phenomonon.

3.5 Methods

3.5.1 RNAi and antibody production

RNAi was performed by injection of dsRNA against the target gene indicated as described previously (Desai et al., 2003). Embryos were analyzed from injected adults that were incubated at 20^{o} C for 48 hours after injection. Endogenous HCP-3$^{CeCENP-A}$ dsRNA was designed using standard methods and oligonulecotides AATTAACCCTCACTAAAGGgccgatgacacccc aattat and TAATACGACTCACTATAGGccgtgggagtaatcgacaag were used to create the template DNA. The oligonucleotides used to create CPAR-1$^{CeCENP-A}$ dsRNA were AATTAACCCTCACTAAAGGttgaggaaattgccgagaag and TAATACGACTCACTATAGGccgtgggagtaatcgacaag. All other dsRNA used in

this study have been previously described (Desai et al., 2003; Maddox et al., 2007). α-HCP-3$^{CeCENP\text{-}A(150)}$ was generated and affinity purified against amino acids 68-82 of HCP-3$^{CeCENP\text{-}A}$, and α-HCP-3$^{CeCENP\text{-}A(163)}$ was generated against amino acids 2-183, but purified against amino acids 105-183 of HCP-3$^{CeCENP\text{-}A}$ to specifically target the uncleaved portion of HCP-3$^{CeCENP\text{-}A}$. α-GFP antibodies were generated and purified against the entire GFP sequence. All other antibodies used have been previously described (Desai et al., 2003; Oegema et al., 2001). All antibodies used for immunofluorescence in this study were directly labeled with Cy-2, Cy-3, or Cy-5. Immunofluorescence and western blot procedures have all been previously described (Monen et al., 2005).

3.5.2 Live imaging and GFP fusions

All live images were acquired using a spinning disk confocal microscope (CSU10; McBain Instruments) mounted on an inverted microscope (TE2000e; Nikon), or a DeltaVision-modified inverted microscope (IX70; Olympus). All worm strains created and used in this study were generated via bombardment in a DP38 (*Δunc119*) background. Filming of embryos was performed at 20^{o} C. All GFP fusions used have been described throughout the manuscript.

3.5.3 Antibody and GFP signal quantification

Signal intensity quantification used to determine the presence of cleavage in HCP-3$^{CeCENP\text{-}A}$, was performed by comparing the ratios for α-HCP-3$^{CeCENP\text{-}A(150)}$: α-HCP-3$^{CeCENP\text{-}A(163)}$ between individual embryos. These antibodies were directly labeled with Cy-3 and Cy-5. Average projections of 40 z-plane stacks were analyzed using the Softworx software (Applied Precision) "data inspector" tool to determine the average pixel intensity for a 100 x 100 pixel region encompassing the GFP signal on chromosomes at a given stage. Background levels were determined by calculating the average pixel intensity of a 100 x 100 pixel region, lacking chromosome GFP-signal in the embryo. This background level was subtracted from the average pixel intensity of the signal. A ratio was determined for each embryo by dividing the Cy-3:α-HCP-3$^{CeCENP\text{-}A(150)}$ signal over the Cy-5:α-HCP-3$^{CeCENP\text{-}A(163)}$ signal, and each embryo was also designated to be at prometaphase/metaphase or anaphase/telophase. These ratios were grouped together and averaged to compare metaphase versus anaphase for all perturbations described previously in this study. The only difference between determining GFP signal intensity and the antibody signal intensities described above, was that MetaMorph software was used in place of Softworx software, and the images were of live embryos as opposed to fixed embryos.

3.5.4 Line-scan quantification

Line-scans were performed using Softworx software to compare localization of proteins on individual chromosomes using immunofluorescence. Chromosomes in prometaphase or metaphase were aligned vertically and 20 pixel thick lines quantified antibody intensity levels along a 40 pixel span.

3.5.5 Viability assay

To test viability under various conditions, we analyzed the F1 progeny for the worm in question. After L4 worms were injected with dsRNA, they were allowed to recover for 3 to 5 hours. The worms were then singled out onto individual plates and were allowed to lay eggs for 48 hours. After this 48 hour period, worms were removed and embryos and viable progeny were scored. The plates were monitored over the next few days to determine how many of the embryos laid were embryonic lethal.

3.6 Acknowledgements

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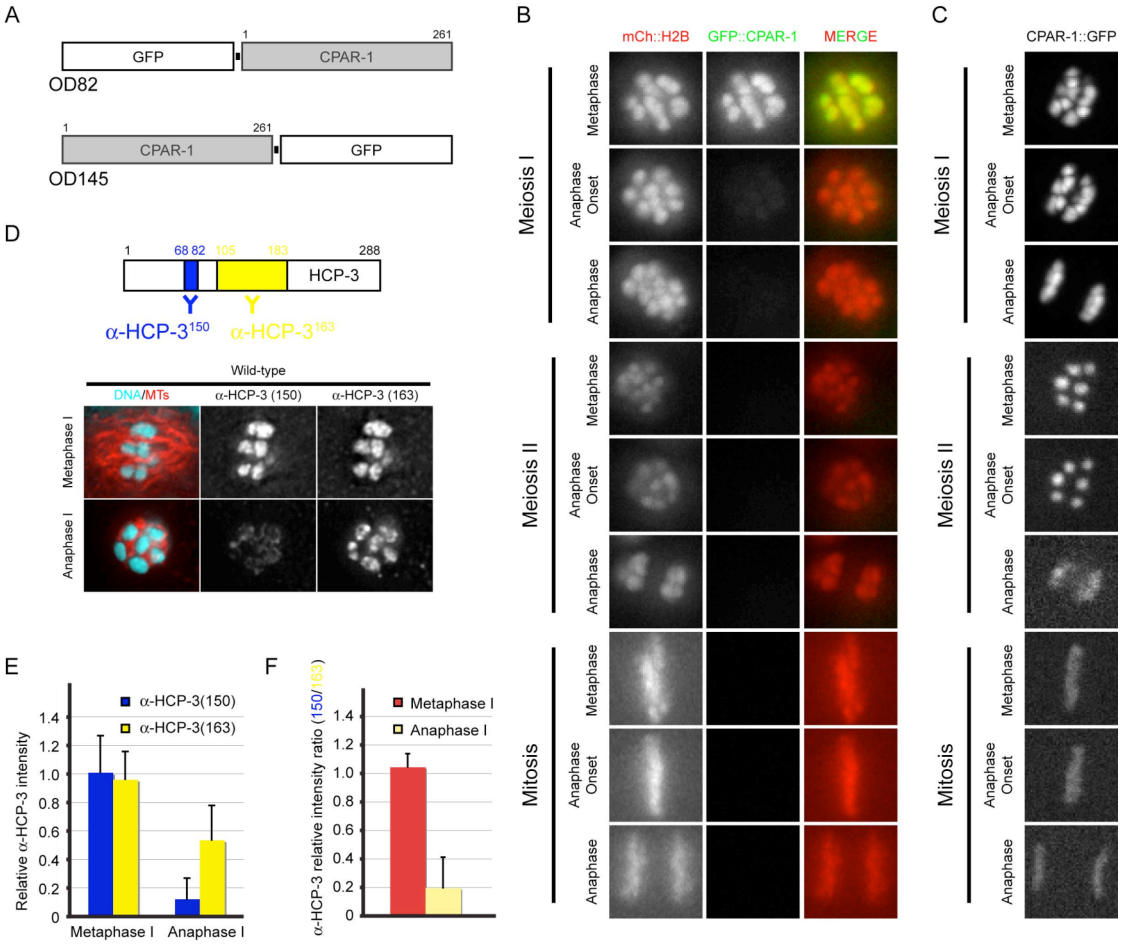

Figure 3.1. CeCENP-A is cleaved at anaphase I onset during meiosis.
A) Transgenic worm strains were made expressing N-terminal (OD82) and C-terminal (OD145) GFP fusions for CPAR-1$^{CeCENP-A}$. B) OD82 worm embryos crossed with mCherry:H2B worms show a rapid removal of GFP signal at anaphase I onset. The GFP signal remains absent throughout meiosis II and mitosis, while chromosome can be monitored by mCh::H2B. C) OD145 do not exhibit the removal seen in OD82 worms and CPAR-1::GFP remains associated to chromosomes into mitosis. D & E) Immunofluorescence shows that α-HCP-3$^{CeCENP-A(150)}$ (proximal to cleavage site) levels significantly decrease between metaphase I and anaphase I; whereas, α-HCP-3$^{CeCENP-A(163)}$ (distal to cleavage site) levels remain similar between metaphase I and anaphase I indicating that HCP-3$^{CeCENP-A}$ is cleaved at anaphase I onset. F) To control for staining variability from embryo to embryo, the ratios for α-HCP-3$^{CeCENP-A(150)}$/α-HCP-3$^{CeCENP-A(163)}$ for each embryo were quantified and then compared between metaphase I and anaphase I. The bar-graph indicates that the ratio significantly drops from metaphase I to anaphase I.

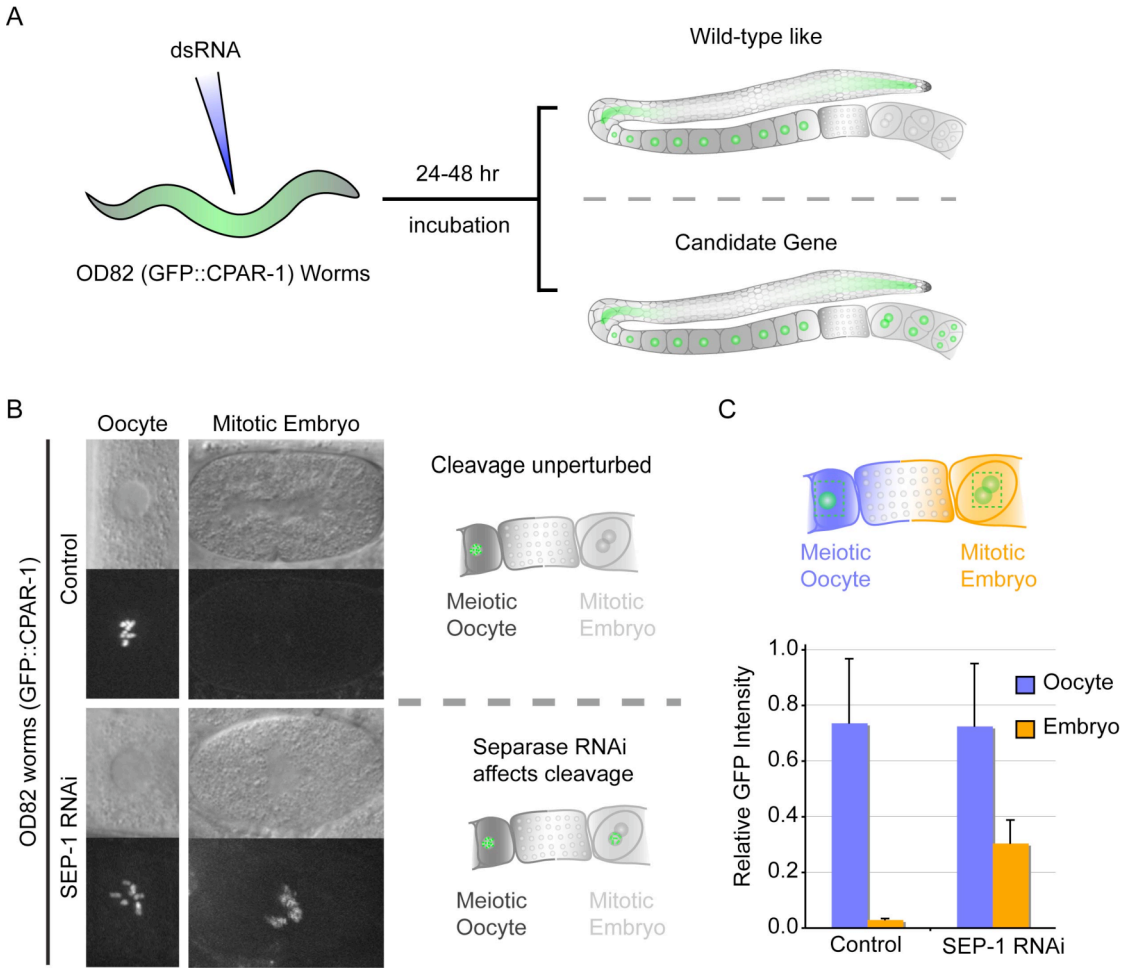

Figure 3.2. Separase is implicated in the cleavage of CeCENP-A.
A) The assay used to test potenitial candidate genes implicated in CeCENP-A removal took advantage of the OD82 strain, which expresses GFP brightly in oocytes but not in embryos. Candidate genes were depleted using standard RNAi injection protocols, and the gonads were then analyzed for GFP expression on mitotic chromosomes. B) Wild-type OD82 worms display oocyte fluorescence but lack embryo fluorescence, wherease Separase RNAi OD82 worms are not capable of GFP removal at anaphase I onset as can be seen by the presence of GFP signal in mitotic embryos depleted of Separase. C) The bar-graph indicates that control embryo GFP fluorescence is only 4% that of control oocyte GFP fluorescence (n=6); whereas in Separase RNAi worms, GFP fluorescence in embryos is 42% that of oocytes.

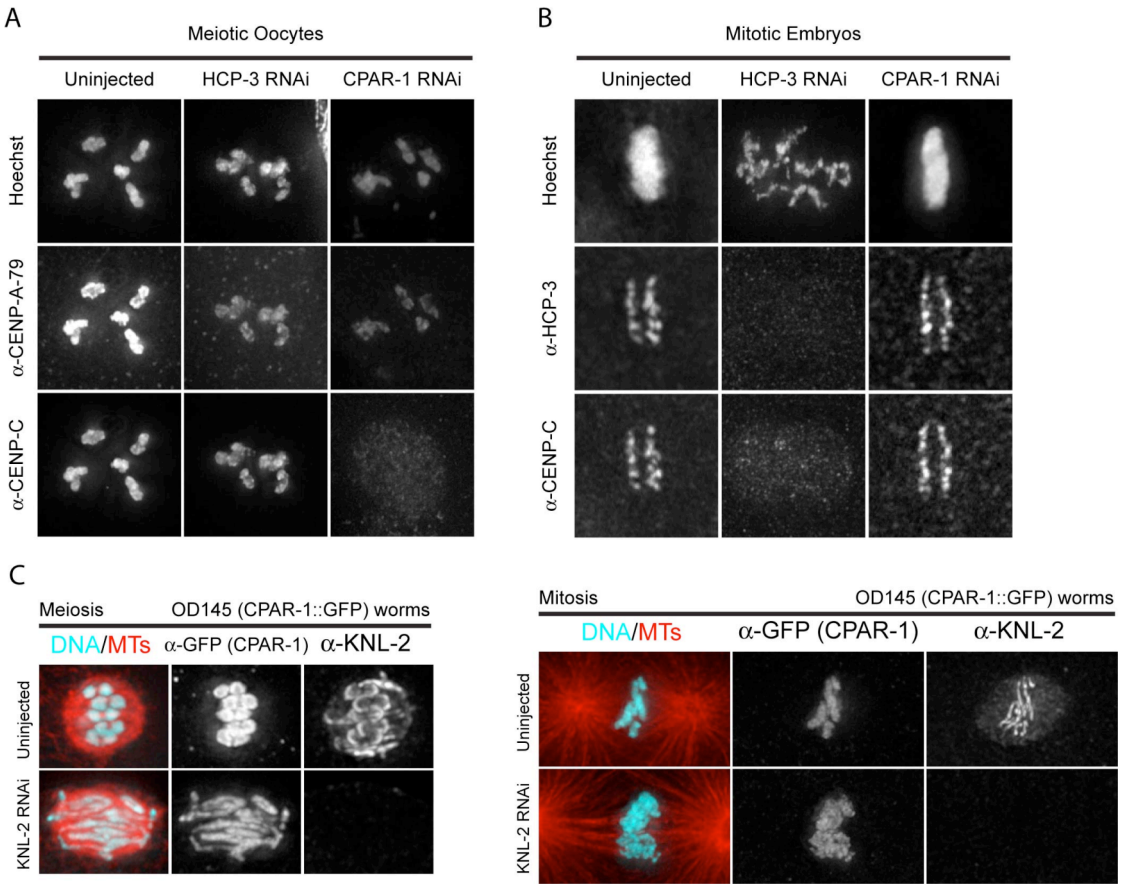

Figure 3.3. Analysis of differing molecular characteristics between HCP-3$^{CeCENP-A}$ and CPAR-1$^{CeCENP-A}$.

A) CENP-C localization is dependent on CPAR-1$^{CeCENP-A}$ and not HCP-3$^{CeCENP-A}$ during meiosis. In uninjected control worms, immunofluorescence suggests that CENP-C localizes to bivalents in late prophase I of meiosis. CENP-C localization is unaffected by depletion of HCP-3$^{CeCENP-A}$ during meiosis. In CPAR-1$^{CeCENP-A}$ depleted worms however, CENP-C is unable to localize to chromosomes. B) Contrary to meiosis, mitotic localization of CENP-C is dependent on HCP-3$^{CeCENP-A}$ and not CPAR-1$^{CeCENP-A}$. During mitosis CENP-C is present at the kintochore in uninjected control worms and is unable to localize properly in HCP-3 depleted embryos. In embryos depleted of CPAR-1$^{CeCENP-A}$, CENP-C is able to localize to kinetochores. C) Contrary to HCP-3$^{CeCENP-A}$, CPAR-1$^{CeCENP-A}$ is capable of localizing to chromosomes in both meiosis and mitosis in the absence of KNL-2. OD145 (CPAR-1$^{CeCENP-A}$::GFP) worms were used to test for CPAR-1$^{CeCENP-A}$ localization in both wild-type and KNL-2 RNAi conditions.

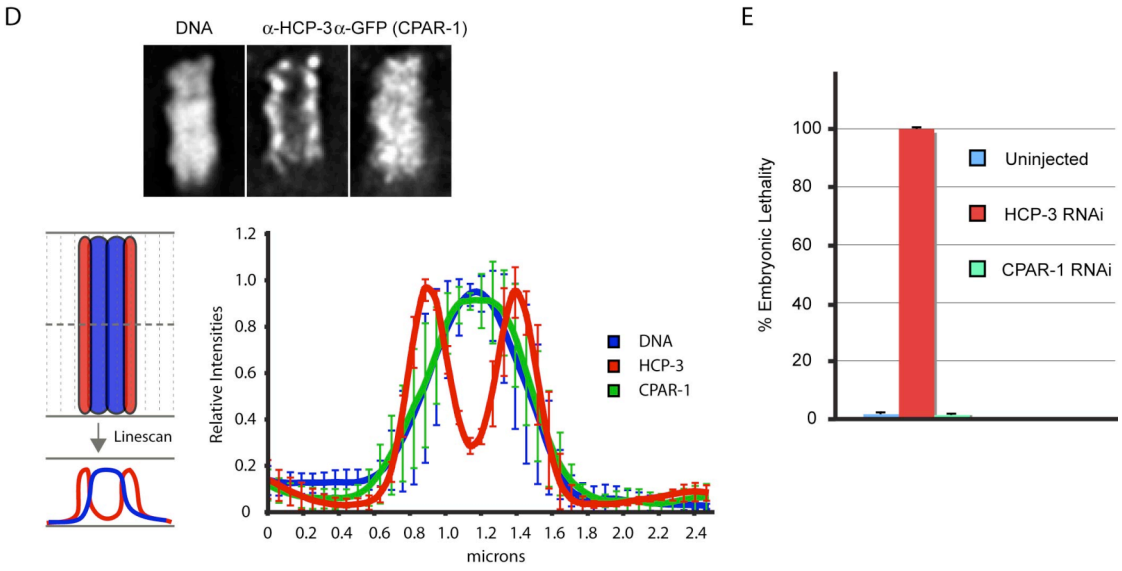

Figure 3.3 (cont.). Analysis of differing molecular characteristics between HCP-3$^{CeCENP-A}$ and CPAR-1$^{CeCENP-A}$.
D) Immunofluorescence at metaphase of mitosis in CPAR-1$^{CeCENP-A}$::GFP (OD145) worms indicates that HCP-3 localizes to the kinetochore and CPAR-1 localizes throughout chromatin. Line-scans of mitotic chromosomes (n=8) were performed to see the antibody intensity profiles for DNA (Hoechst), HCP-3$^{CeCENP-A}$ (α-HCP-3$^{CeCENP-A(150)}$), and CPAR-1$^{CeCENP-A}$ (α-GFP). The graph indicates that HCP-3$^{CeCENP-A}$ has a kinetochore profile, showing two peaks around the 1 peak DNA profile. The CPAR-1$^{CeCENP-A}$ line-scan profile is indistinguishable from that of DNA. E) F1 progeny were assessed for embryonic lethality in uninjected, HCP-3$^{CeCENP-A}$ RNAi, and CPAR-1$^{CeCENP-A}$ RNAi N2 worms. CPAR-1$^{CeCENP-A}$ RNAi progeny were indistinguishable from uninjected control worms, whereas HCP-3$^{CeCENP-A}$ RNAi F1 progeny exhibited 100% embryonic lethality.

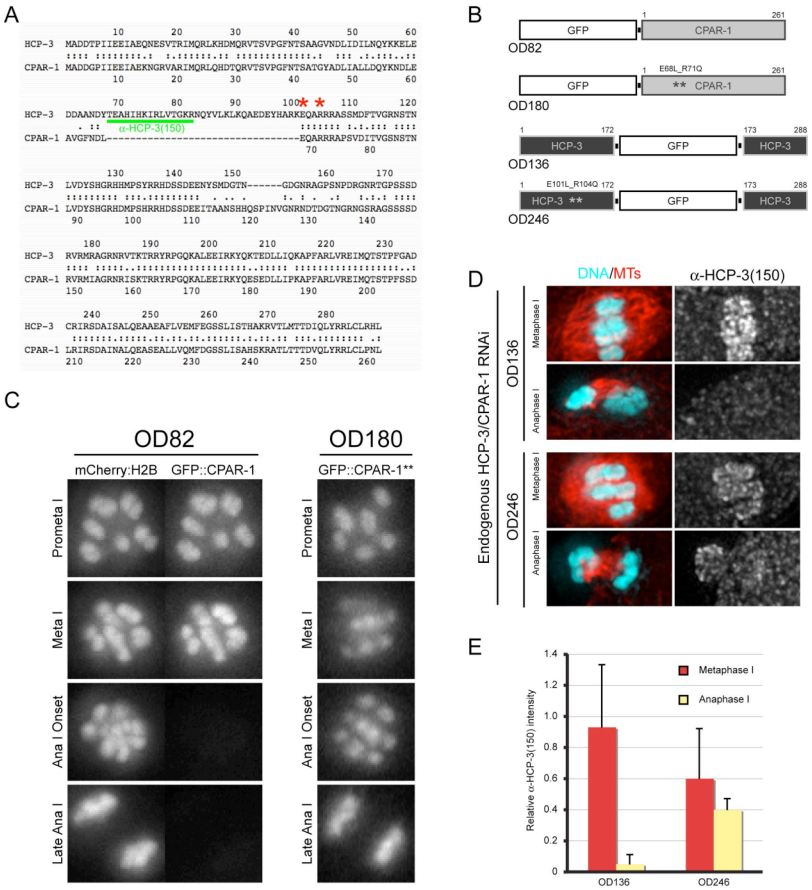

Figure 3.4. CeCENP-A mutatations in the loose consensus sequence for Separase-mediated cleavage perturb CeCENP-A cleavage.

A) A pairwise sequence homology analysis for HCP-3$^{CeCENP-A}$ and CPAR-1$^{CeCENP-A}$ indicates the ExxR conserved Separase cleavage site for both CENP-A homologs. Also indicated is the peptide region for which the α-HCP-3$^{CeCENP-A(150)}$ antibody was raised against. B) Worms analyzed expressed various GFP fusions containing wild-type or (E_L)xx(R_Q) CENP-A mutants. All of these constructs were re-engineered to make them RNAi resistant. C) Cleavage of CPAR-1 does not occur in OD180 worms at anaphase I onset as seen by expression of GFP::CPAR-1**. D) OD136(HCP-3$^{CeCENP-A}$::GFP) and OD246(HCP-3**::GFP) worms depleted of endogenous CeCENP-A were stained for α- HCP-3$^{CeCENP-A(150)}$ showing that staining between metaphase I and anaphase I significantly decreased in OD136 worms but not for OD246 worms. E) Quantification for the experiment shown in panel D indicates that OD136 worms expressing a wild-type RNAi resistant HCP-3$^{CeCENP-A}$ shows a significant decrease in α-HCP-3^{150} levels (p=0.007) between metaphase I and anaphase I, whereas OD246 worms expressing a mutant RNAi resistant HCP-3** shows no statistical difference in α- HCP-3$^{CeCENP-A(150)}$ levels between metaphase I and anaphase I (p=0.49).

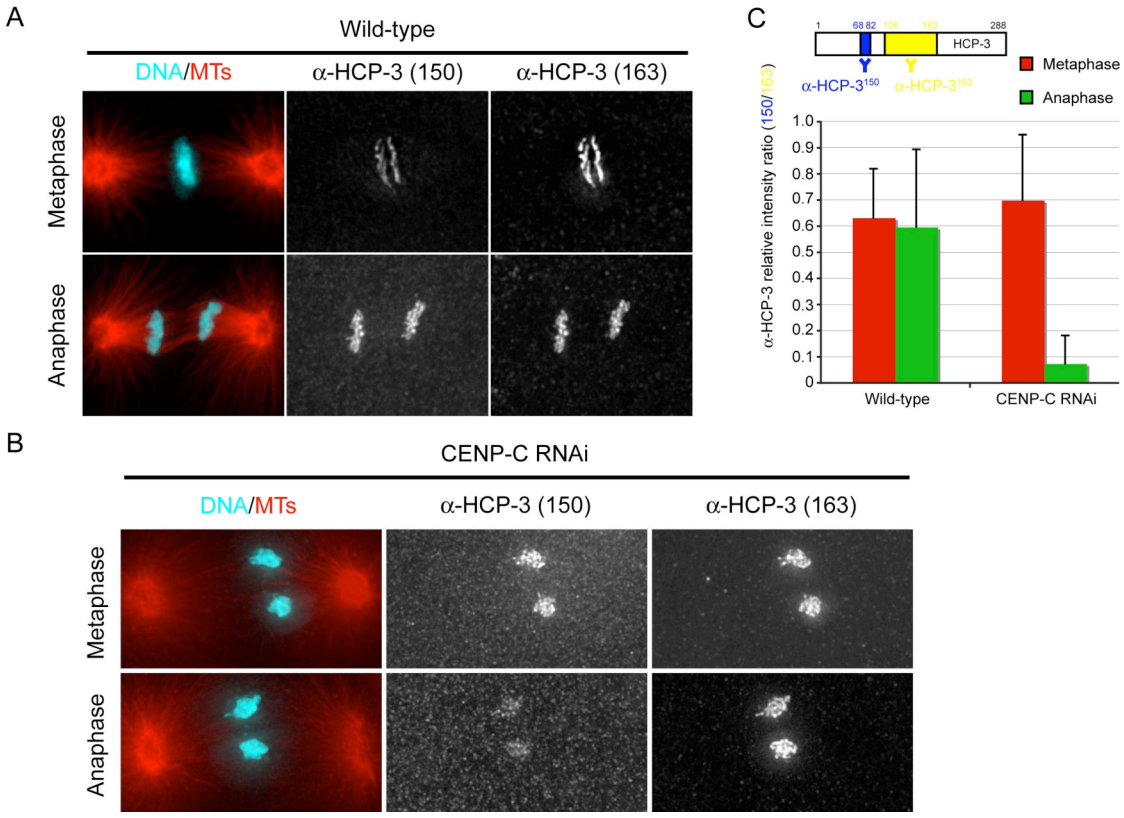

Figure 3.5. Centromeric CeCENP-A can be cleaved during mitosis in the absence of the kinetochore.
A) Both α-HCP-3$^{CeCENP\text{-}A(150)}$ and α-HCP-3$^{CeCENP\text{-}A(163)}$ are present in metaphase and anaphase during mitosis. B) In CeCENP-C RNAi embryos α-HCP-3$^{CeCENP\text{-}A(150)}$ levels diminish between metaphase and anaphase, whereas α-HCP-3$^{CeCENP\text{-}A(163)}$ levels remain constant. C) The HCP-3$^{CeCENP\text{-}A}$ relative antibody intensity levels were quantified and their ratios (α-HCP-3$^{CeCENP\text{-}A(150)}$/α-HCP-3$^{CeCENP\text{-}A(163)}$) were compared between metaphase and anaphase for both wild-type and CENP-C RNAi. There was no ratio difference in wild-type metaphase vs. anaphase; however, there was a significant ratio difference between metaphase and anaphase in CeCENP-C depleted embryos (p=0.002) indicating that HCP-3$^{CeCENP\text{-}A}$ cleavage can occur in embryos incapable of building a kinetochore.

D

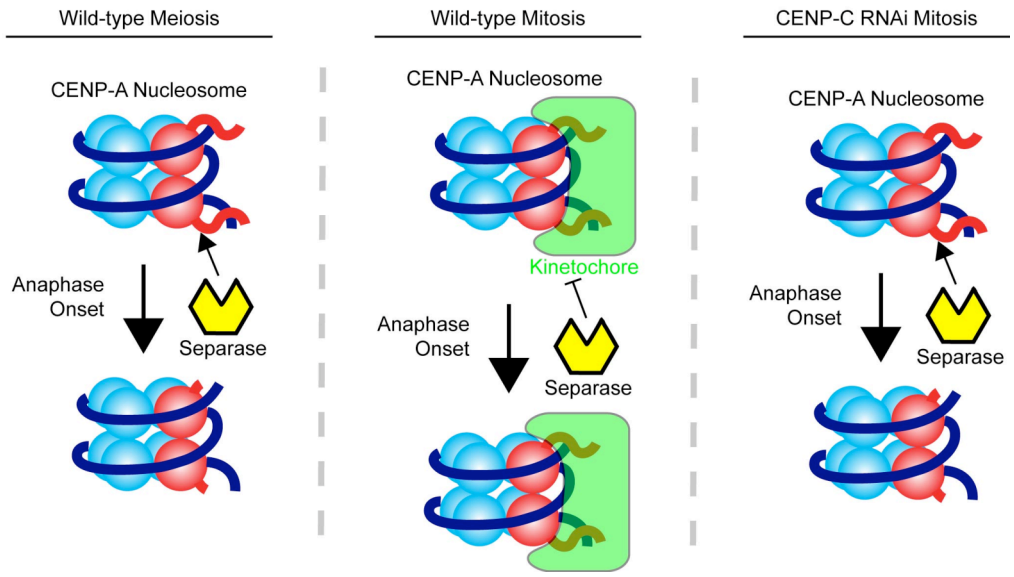

Figure 3.5 (cont.). Centromeric CeCENP-A can be cleaved during mitosis in the absence of the kinetochore.
D) A model for Separase-mediated CENP-A cleavage suggests that Separase is capable of cleaving CENP-A at anaphase onset if Separase has access to the cleavage site. In meiosis site accessibility is not a problem because the outer kinetochore is not built on CENP-A chromatin. The kinetochore prevents accessibility to the CENP-A-Separase cleavage site during mitosis, but in embryos lacking a built kinetochore Separase is capable of cleaving CENP-A.

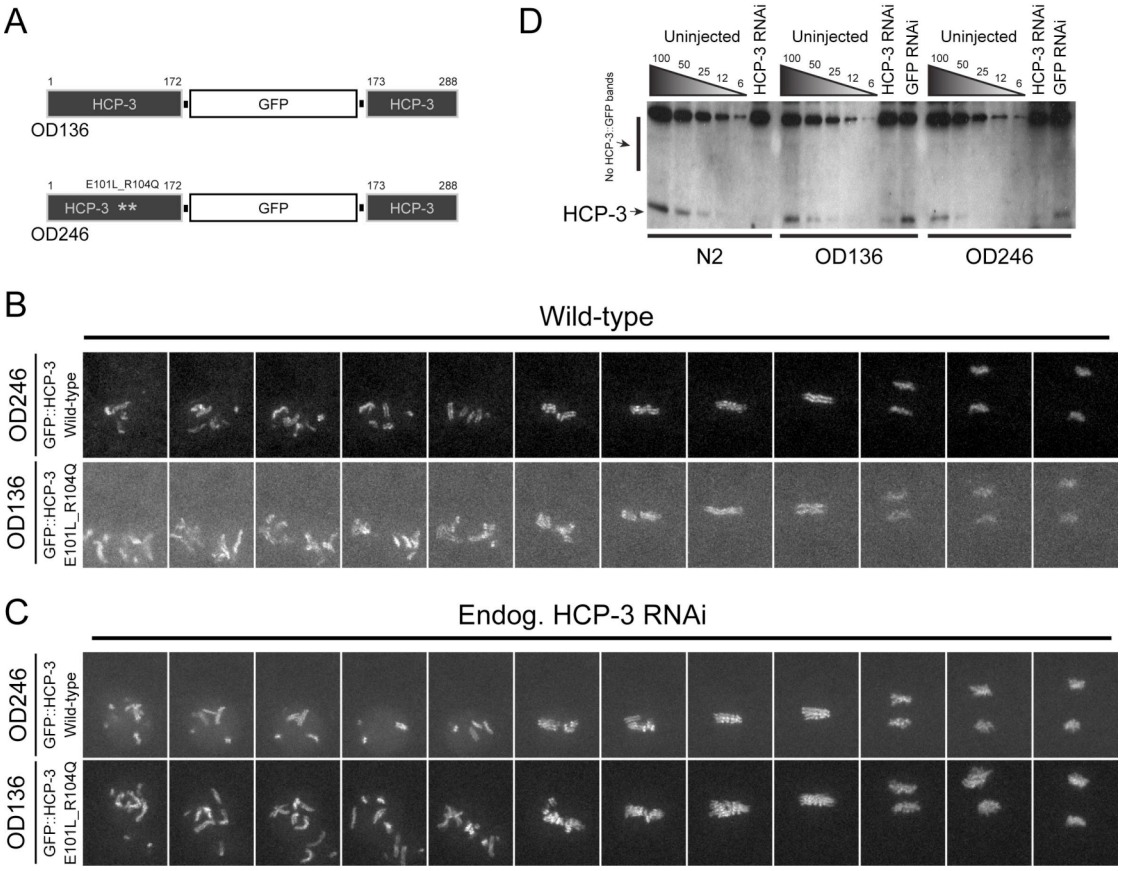

Figure 3.6. CeCENP-A cleavage is important for viability.
A) To test the role for CeCENP-A cleavage, transgenic worm strains were made expressing wild-type HCP-3$^{CeCENP-A}$::GFP (OD136) and uncleavable HCP-3**::GFP (OD246). B) In the presence of endogenous HCP-3$^{CeCENP-A}$; both wild-type and uncleavable HCP-3$^{CeCENP-A}$ integrants localize to the kinetochore. C) In endogenous HCP-3 depletions; both integrants in OD136 and OD246 are still capable of localizing to the kinetochore and appear to restore kinetochore function in the first mitotic division. D) Western blots for α-HCP-3$^{CeCENP-A}$ in N2, OD136, and OD246 worm extracts indicate that transgene expression is undetectable, whereas endogenous HCP-3$^{CeCENP-A}$ can be detected in all strains. Endogenous HCP-3 depletions result in endogenous HCP-3$^{CeCENP-A}$ levels at about 12% wild-type levels. Levels of depletions in GFP RNAi could not be determined due to low-level transgene expression.

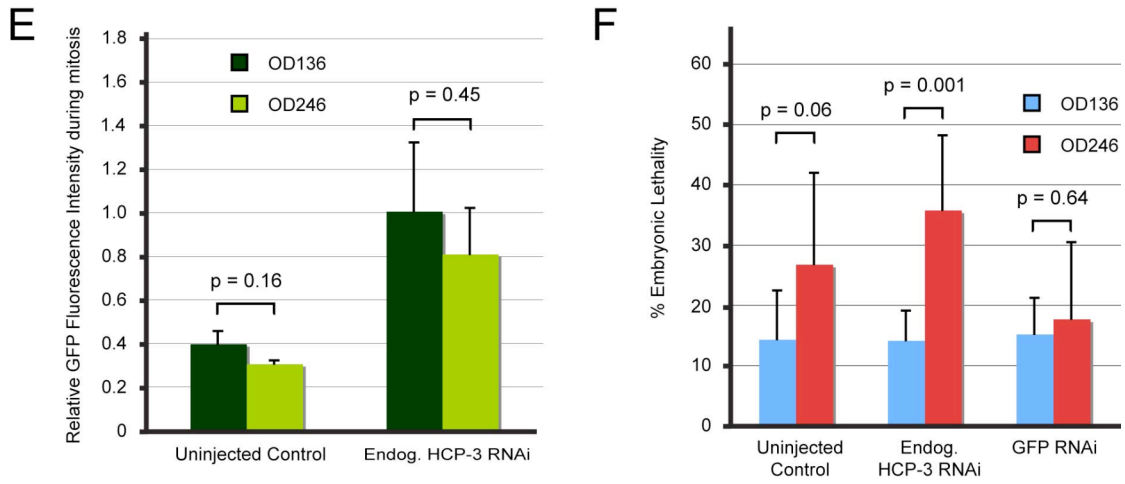

Figure 3.6 (cont.). CeCENP-A cleavage is important for viability.
E) A slight increase in GFP signal at the metaphase plate is detected in OD136 and OD246 in embryos depleted of endogenous HCP-3$^{CeCENP-A}$ vs. uninjected controls. Levels of GFP signal are comparable between both strains. F) Worms were depleted of endogenous HCP-3$^{CeCENP-A}$ to assess the ability of their respective transgenes to rescue embryonic lethality. Embryonic lethality was comparable between OD136 and OD246 worms in the presence of both endogenous and transgenic HCP-3$^{CeCENP-A}$ (uninjected) and in worms only expressing endogenous HCP-3$^{CeCENP-A}$ (GFP RNAi). OD136 and OD246 worms expressing solely their integrated HCP-3$^{CeCENP-A}$ transgenes (endogenous HCP-3 RNAi) were significantly different (p=0.001), with OD246 having a higher incidence of embryonic lethality.

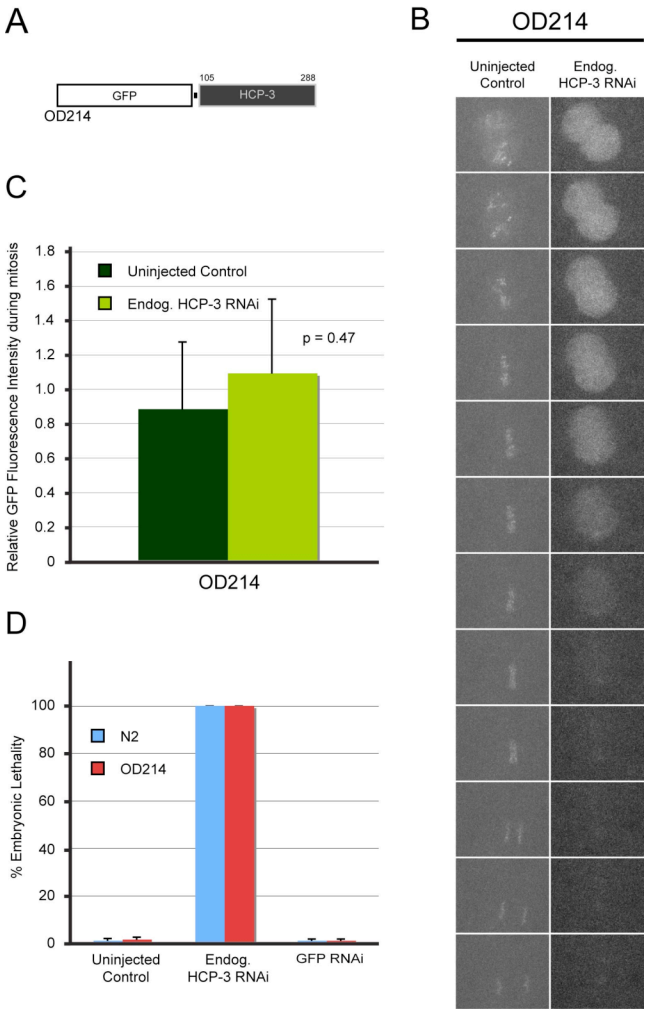

Figure 3.7. Cleaved CeCENP-A products are incapable of proper localization and function at the centromere.
A) To test the function for CeCENP-A cleavage products, transgenic worm strains were made expressing truncated GFP::HCP-3$^{CeCENP\text{-}A(105\text{-}288)}$ (OD214) believed to incorporate the entire CeCENP-A cleavage product (AAs 105-288). B) In the presence of endogenous HCP-3$^{CeCENP\text{-}A}$, GFP::HCP-3$^{CeCENP\text{-}A(105\text{-}288)}$ localizes to the kinetochore. In endogenous HCP-3 depletions, GFP::HCP-3$^{CeCENP\text{-}A(105\text{-}288)}$ is no longer capable of localizing to the kinetochore, and embryos exhibit a KNL phenotype. C) GFP levels, as monitored prior to nuclear envelope breakdown, were equivalent between uninjected and HCP-3$^{CeCENP\text{-}A}$ RNAi embryos in OD214 worms. D) F1 progeny were assessed for embryonic lethality in uninjected, HCP-3$^{CeCENP\text{-}A}$ RNAi, and GFP RNAi for both N2 and OD214 worms. In both strains, GFP RNAi progeny were indistinguishable from uninjected control worms, whereas HCP-3$^{CeCENP\text{-}A}$ RNAi F1 progeny exhibited 100% embryonic lethality.

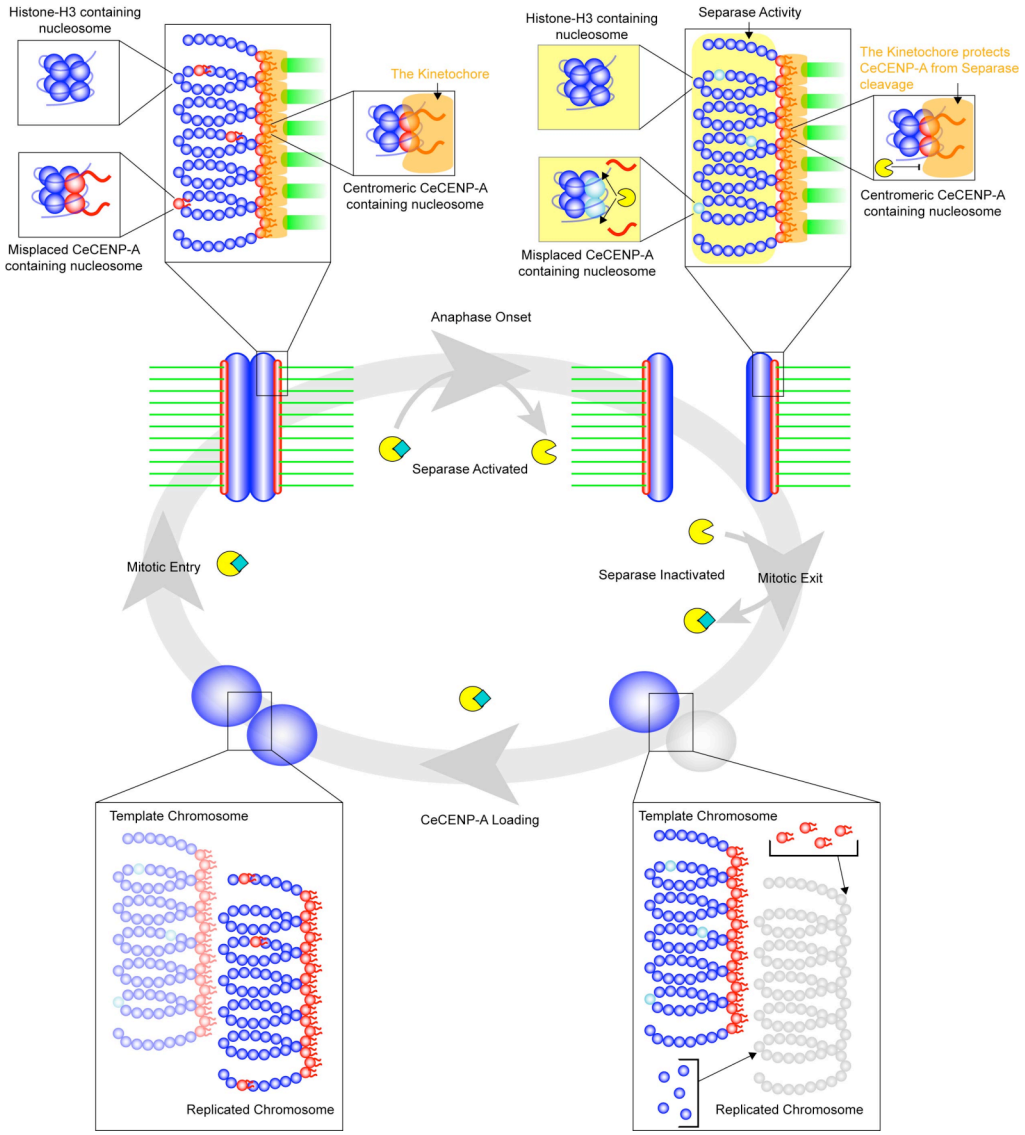

Figure 3.8. Model for the role of Separase-mediated CeCENP-A cleavage on maintaining centromere specificity.
CeCENP-A is loaded onto centromeric DNA and is directed by the CeCENP-A mark from the last division. Incorrect CeCENP-A loading may occur at a low frequency onto non-centromeric regions of DNA. During mitosis, the kinetochore is assembled onto centromeric CeCENP-A while the minimal amount of non-centromeric CeCENP-A is not concentrated enough to direct kinetochore assembly elsewhere. At anaphase onset, Separase cleaves non-centromeric CeCENP-A, but centromeric CeCENP-A is protected by the presence of the kinetochore. The epigenetic mark has thus been lost on the cleaved HCP-3$^{CeCENP-A}$; and during the subsequent cell cycle, only on the centromere, where full-length CeCENP-A resides, will new CeCENP-A be loaded.

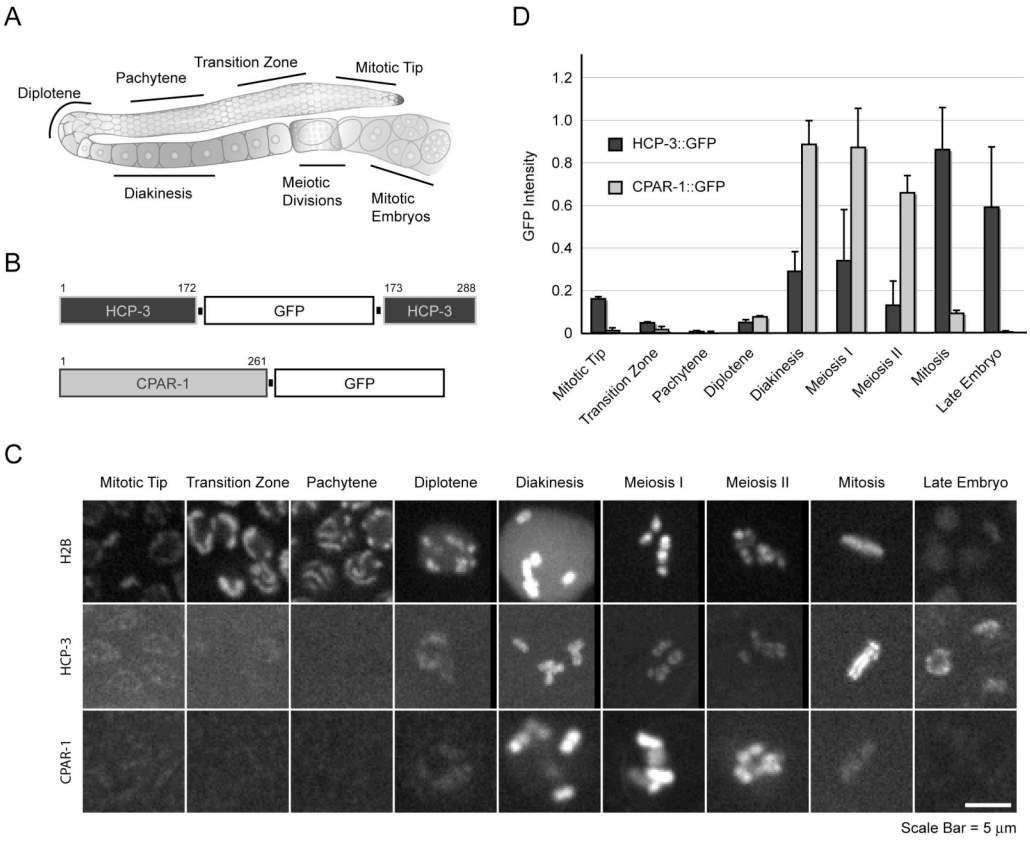

Figure S3.1. The *C. elegans* CENP-A homologs, HCP-3 and CPAR-1, have differing localization profiles throughout meiosis and mitosis.
A) A schematic of the *C. elegans* gonad highlighting various regions where different stages of meiosis and mitosis occur. B) Gonad localization analysis was done using OD101 (HCP-3::GFP) and OD145 (CPAR-1::GFP) worms. The internal GFP in OD101 worms is just proximal to the histone-fold domain of HCP-3, whereas the OD145 worms express a C-terminal GFP tagged CPAR-1. Both transgenes are driven via the *PIE-1* promoter. C) Images at various stages in the gonad and embryos expressing either mCherry::H2B (OD56), HCP-3::GFP (OD101), or CPAR-1::GFP (OD145) display the localization profile for each protein. Neither HCP-3 nor CPAR-1 appear to be present during the transition stage or pachytene stage of meiosis. Both HCP-3 and CPAR-1 begin to localize to chromatin at the diplotene stage of meiosis I and remain throughout meiosis. During the first mitotic division, HCP-3 is kinetochore localized whereas CPAR-1 remains chromatin bound and co-localizes with H2B. D) The bar-graph indicates the relative GFP intensities for both HCP-3 and CPAR-1. HCP-3 appears to be most abundant during mitosis but is still present in meiosis, whereas CPAR-1 localization is most intense during meiosis and becomes exceedingly dimmer as mitotic embryos progress, until CPAR-1::GFP becomes undetectable at late mitotic stages.

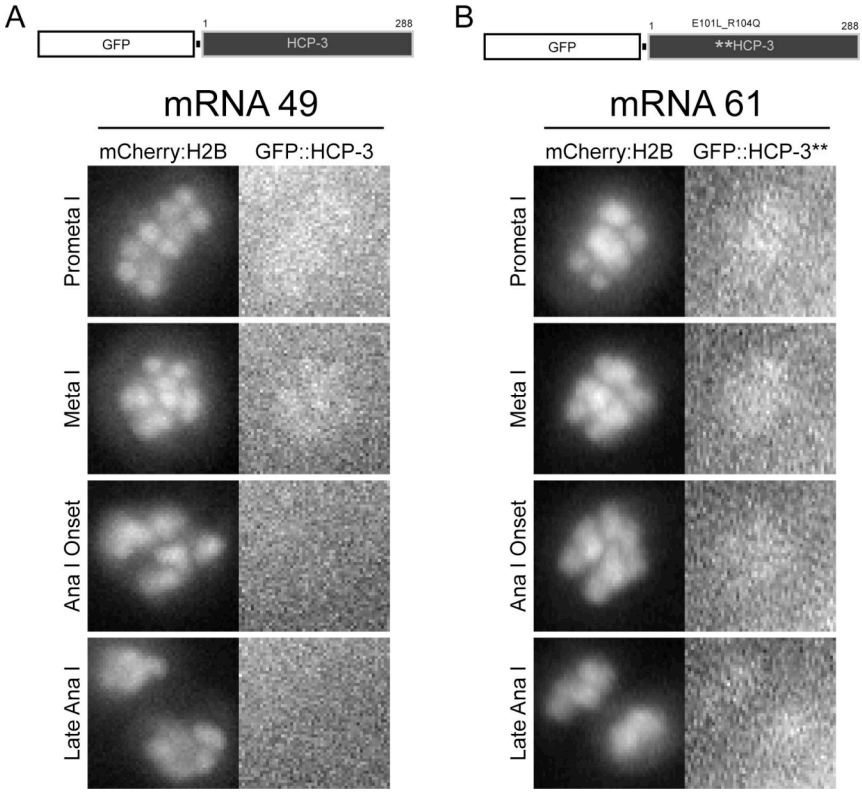

Figure S3.2. N-terminal GFP::HCP-3$^{CeCENP-A}$ and GFP::HCP-3 provide live evidence for CeCENP-A cleavage event.**

A) Worms injected in their gonads with mRNA49 encoding a GFP::HCP-3$^{CeCENP-A}$ protein produce embryos that display the characteristic removal of GFP as seen in GFP::CPAR-1$^{CeCENP-A}$ strains. B) In similar experiments as seen in panel A, substituting mRNA61, which encodes an uncleavable GFP::HCP-3**, results in GFP signal that remains on chromosomes after anaphase I onset.

Chapter 4: Conclusions and Future Directions

In this chapter, I will discuss other studies, which were a bit too preliminary to discuss earlier, and how they relate to my thesis work and to possible advancements in the field. I will also suggest future experiments that would be useful to pursue, as well as exploring alternative models and the ideas surrounding them.

4.1 Chromosome segregation during meiosis

In this dissertation, my research stems from the fundamental question for how chromosomes achieve proper segregation. While much is still unknown in the realm of mitosis, virtually all of the properties known to segregate chromosomes during meiosis are based off of mitotic studies. In order to gain insight into meiotic segregation, I use *C. elegans* as a model system because it is one of the only model systems with the proper tools to study this process in depth. With the study in worms, also comes with it the conundrum that arises given what we know about meiosis and mitosis, and applying those principles to the proper segregation of holocentric chromosomes in meiosis. It was this line of thinking which spurred the questions for my original research. We suggest that the mechanism for outer kinetchore assembly being independent of CeCENP-A may be a way for holocentric chromosomes to avoid the complications that one might expect in

recombined homologous pairs with microtubule attachments throughout. This also implies that somehow the outer kinetochore is able to achieve some form of monocentricity in this new mechanism that I have implicated in meiosis. To explore this further, it is necessary to understand what role the cup-like structure plays in meiosis, as well as understanding how the spindle microtubules act on the chromosomes to generate the necessary forces required to segregate chromosomes.

4.1.1 Exploring the cup-like structure

A natural transition from my earlier studies would have been to understand what role the cup-like structure is playing in meiosis. Because I chose to explore the cleavage event of CeCENP-A and what that all entailed, I was unable to fully understand how and if the cup-like structure was integral to meiotic segregation. Having said that, my preliminary experiments suggest that the cup-like structure is important, but the exact role is not so clear. During my earlier studies I had performed KNL-1 depletions and monitored their segregation. It appeared that chromosomes were mis-segregating as could be visualized using my live-assay. It was obvious however, that these depletions did not produce a KNL-like meiotic phenotype, but rather resulted in one or two mis-segregated chromosomes. Because of the in-depth characterization necessary to understand what was going on, addressing this question has become a new project in the lab and is now the focus of Dr.

Julien Dumont's post-doctoral work. He has since verified some of my previous work and has continued on to discover that double depletions of KNL-1 and the chromokinesin KLP-19, result in a very severe segregation phenotype. It is suspected that the cup-like structure is playing a role in aligning the chromosomes to the metaphase plate, and that chromokinesin is aiding in segregating chromosomes during anaphase. I suspect that there is even more involved in the mechanisms that segregate meiotic chromosomes, and will explain these ideas below. But first, I will discuss what I believe may be the key in forming the cup-like structure.

4.1.2 KNL-2 may be the key to CeCENP-A independent kinetochore assembly during meiosis

One major question still remains, how do outer kinetochore components of the cup-like structure localize independently of CeCENP-A? In other words, what protein(s) is responsible for targeting the cup-like structure to the chromosomes? The best way to address this question is to initially use RNAi coupled with immunofluorescence to determine which of the cup-like components are internal, or upstream of the kinetochore assembly hierarchy. Once this has been established, the best approach would involve immuno-precipitation of said cup-like component in meiotic extracts, followed up by mass spectrometry. The mass-spec data could then be scoured for possible candidates, and these candidates could be tested using our traditional RNAi

and immuno-staining approaches. The major caveat with these experiments is in getting large quantities of meiotic worm extract. Certain temperature sensitive mutants, will arrest embryos in metaphase I of meiosis, but this would still require a large optimization effort.

Based on the data I have assembled over the years, I suspect that KNL-2 may be the key protein involved in preventing the cup-like structure from associating with CeCENP-A. Because KNL-2 carries a Myb-domain DNA binding motif, it would be a very good candidate. We also know that in mitosis, KNL-2 and CeCENP-A are very closely related at the top of the kinetochore assembly pathway (Maddox et al., 2007). During meiosis, KNL-2 is localized in the cup-like structure and appears to be internal to KNL-1 and KNL-3. In KNL-2 depleted embryos, chromosomes are stretched apart during meiotic prometaphase (See Figure 3.3c). However, KNL-1 and KNL-3 still localize to chromatin, but due to the chromosome morphology phenotype it is unclear if they are in cups or all over chromatin. Perhaps in KNL-2 depletions, outer kinetchore components are no longer prevented from associating with CeCENP-A, resulting in microtubule attachments everywhere, and thus leading to chromosomes being ripped to shreds. My idea, is that KNL-2 loads onto the cup-like structure encasing chromosomes in meiosis, thus limiting localization of outer components to associate with just KNL-2 and not CeCENP-A. By preventing outer components from associating with CeCENP-A, microtubule connections are restricted to the cup-like structure and cannot

occur throughout chromosomes. This model would also support our theory that suggests an uncoupling of outer kinetochore components from CeCENP-A is required to properly segregate holocentric chromosomes. While these studies implicating KNL-2 are preliminary, KNL-2 is clearly playing a role in chromosome morphology maintenance and it would be interesting to characterize these studies further.

4.1.3 Does CENP-A play a role during meiosis of monocentric chromosomes?

My research implicates a CeCENP-A independent mechanism for chromosome segregation during meiosis in *C. elegans*. Of course the question still remains if this is strictly a mechanism necessary for segregation of holocentric chromosomes, as we suggest may be the case, or if this is a conserved mechanism even in species with monocentric chromosomes. Unfortunately, addressing the same questions in other species is extremely difficult, and to date these questions remain unanswered. Based on immunostaining data in other systems, CENP-A homologs appear to co-localize with outer kinetochore proteins in distinct punctate regions. This suggests that the mechanism that I describe in this study may not apply to monocentric chromosomes. That being said, I do believe there is much to learn about meiotic segregation, in particular during spindle assembly and function, by studying meiosis in *C. elegans*.

4.1.4 Alternative models for segregating chromosomes in meiosis

Traditional models for chromosome segregation primarily describe a mechanism, which facilitates segregation using a pulling force generated by the spindle microtubules. The basis for these models relies heavily on studies done in model systems where the bipolar spindle has attachments to the cytoskeleton and/or is centrosomally driven. None of these two major components is present during meiotic segregation in *C. elegans*. In fact, most known ooycte meiotic spindles are acentrosomal and do not appear to have attachments to the cortex of the embryo. What I have noticed in my studies is that the spindle microtubules are absent from the poles, and the spindle is entirely made up of mid-zone microtubules during anaphase. This, coupled with the fact that the cup-like structure disappears just after anaphase onset, leads me to believe that chromosomes are not pulled by microtubules, but rather pushed apart by the mid-zone microtubules. This would likely be driven by some sort of mid-zone microtubule motor such as the kinesin ZEN-4. Another protein, which I believe would play a pre-dominant role in this new mechanism, is the microtubule binding protein CLS-2 (Clasp-2). This is based on CLS-2 depletions that I have performed which result in complete failure to assemble the meiotic spindle and thus result in chromosome segregation failure. Immunofluorescence data also suggests that CLS-2 localizes at the mid-zone and is present alongside microtubules during anaphase. In order to get a better understanding for how this spindle segregates chromosomes, I

believe the best strategy would be to couple RNAi of known microtubule motors with live imaging assays looking at spindle dynamics during meiosis. This could give us an indication as to what motors are involved in the process. The fact that meiotic segregation in other systems is also acentrosomal, make studying this process even more appealing. Because of the difficulties associated with studying meiosis in most model organisms, *C. elegans* would be ideal to address questions regarding formation and function of acentrosomal spindles.

4.2 CENP-A cleavage

The discovery that CeCENP-A gets cleaved by Separase was the basis for chapter 3 of this dissertation. I took a very unusual approach, in that I made an interesting observation, I discovered its mechanism of action, and I then pursued the implications for this observation. In so doing, I have made a lot of progress in understanding the molecular properties during this process, but characterizing the phenotype associated with this cleavage still remains a bit elusive. In this section I discuss some of the key experiments necessary to understanding the function for CeCENP-A cleavage as well as discussing an alternative role for CeCENP-A cleavage.

4.2.1 In-depth characterization in uncleavable CeCENP-A mutants

I spent much of my later graduate years trying to understand the function for Separase-mediated CeCENP-A cleavage. Coupled with the fact

that there are 2 CENP-A homologs in *C. elegans*, I spent a lot of time characterizing properties and building reagents to get at the question for what the role of cleavage truly is. We had some pre-conceived notions of what this cleavage may mean, which spurred the adamant pursuit of these studies. Because CENP-A is thought to be the epigenetic mark for centromere specificity, we believed that Separase-mediated cleavage could be involved in generating this mark. How exactly this would work, we were unsure of, but with the right tools, we believed we could get at this question. Now that I have finally generated an uncleavable mutant, we are still left wondering. There are too many caveats with this strain to make any real finite conclusions, and so we are left with more experiments to get at the key question.

The first caveat with the OD246 strain is that it does not express an uncleavable CeCENP-A mutant at endogenous levels (See figure 3.6d). If our initial model is correct, the phenotype would be subtle, just as we are seeing. By making a strain with the endogenous promoter, we would hopefully see an increase in lethality, and get a better feel for the potential phenotype.

There are a few other assays, which I believe could help elucidate whether cleavage is playing a role in centromere specificity. The first would just involve taking very high-resolution images in embryos for both OD136 (wild-type CeCENP-A) and OD246 (uncleavable CeCENP-A). We may be able to detect a higher ratio of GFP signal at non-centromeric regions in OD246 embryos, indicating that the uncleavable CeCENP-A shows less

centromere specificity than wild-type. Also by depleting endogenous CeCENP-A and analyzing the embryos for both strains more thoroughly, we may see a higher incidence of chromosome segregation defects. These two sets of experiments could help to establish our current model.

Current studies in our lab are using ChIP-CHIP technology in order to get a feel for what regions are CeCENP-A bound on *C. elegans* chromosomes. There seems to be a pattern in which genes that are turned off during embryogenesis appear to be bound to CeCENP-A nucleosomes. This makes sense, because one could imagine that any DNA tightly bound within nucleosomes would have a difficult time transcribing. It would be interesting to see if ChIP-CHIP studies performed on the uncleavable CeCENP-A expressing worms showed a different pattern. Even a slight higher incidence for CeCENP-A binding to embryonically transcribed zones would support our idea. If the increase in embryonic lethality that we are seeing in OD246 worms is a result of random silencing of essential genes, it would be highly intriguing, yet it would be very difficult to characterize because of the variability in phenotypes we could see.

In order to better understand the function of the CeCENP-A cleavage, a more in-depth analysis of worms expressing the pre-cleaved CeCENP-A would have to be performed. The first step would be to generate a strain with the GFP at the same location as the other strains to ensure that the N-terminal GFP in OD214 worms is not causing the affect we are seeing and it is truly an

affect caused by the pre-cleaved CeCENP-A. Once this has been verified, it would be interesting to test if the pre-cleaved CeCENP-A is unable to function properly as a result of not being capable to localize properly, or if it has lost all CeCENP-A function. What I mean by this, is if we can get the pre-cleaved CeCENP-A properly localized, and then get rid of full-length CeCENP-A, is the pre-cleaved CeCENP-A enough to rescue embryonic viability. To test this, we could cross the OD214 into a Δ*rde-1* background, which would be RNAi resistant. These worms could then be injected with CeCENP-A dsRNA and mated with wild-type worms. The embryos would initially express both full-length and pre-cleaved CeCENP-A, so CeCENP-A loading and function would be normal. In later embryos, RNAi would kick in and full-length CeCENP-A would be depleted leaving only pre-cleaved CENP-A to propagate the centromere. This experiment would tell us more about the exact characteristics associated with a Separase cleaved CeCENP-A product. This could tell us definitively if the cleaved product is capable of propagating the centromere.

4.2.2 Alternative models for CENP-A cleavage

In chapter 3 I propose the model that the epigenetic mark is imprinted on the N-terminal histone-tail of CeCENP-A, and that Separase-mediated CeCENP-A cleavage maintains centromere specificity by clearing the mark from non-centromeric CeCENP-A. Our hypothesis is partially based on the

data in meiosis indicating that non-kinetochore bound CeCENP-A gets cleaved, and so we are making the assumption that non-centromere, chromatin-bound CeCENP-A is cleaved in mitosis as well, despite not having visual evidence for this in wild-type embryos. While we like this model because of the implications it would have, it is entirely possible that Separase is cleaving free CeCENP-A in the cell versus chormosome arm-bound CeCENP-A. Perhaps by cleaving free CeCENP-A at anaphase onset, it prevents CeCENP-A from loading prematurely, when and where it is not supposed to. Perhaps loading of nucleosomes at chromosome arms occurs after exit out of mitosis, and loading of CeCENP-A must be prevented at this stage—hence cleavage of CeCENP-A as a mechanism to prevent its premature loading. Later in the cell-cycle, newly formed CeCENP-A or distant pools of uncleaved CeCENP-A are able to load when it is required. You could also imagine that with this model, Separase acts as a gate-keeper, by only cleaving CeCENP-A in the area of nucleosome loading, thus preventing CeCENP-A loading.

4.2.3 Is CENP-A cleavage conserved?

Probably the most interesting question to ask would be, is Separase-mediated CENP-A cleavage conserved? This is a question I have asked myself on numerous occasions, but have not thoroughly addressed. Despite the fact that the CENP-A histone-tails are highly divergent, it is entirely

possible the mechanism is conserved. Initially I looked at the N-terminal tails for various species to see if they had an ExxR motif present in their sequences. Many species did have this motif, including humans, *drosophila*, and yeast. I was discouraged when I mutated these sites in yeast and found that there was no lethality phenotype (data not shown). That being said, we know that yeast are quite divergent in that their centromeres only have one $Cse4^{CENP\text{-}A}$ nucleosome per kinetochore and centromere propagation is known to rely on specific DNA sequences, meaning that their centromere specificity does not rely on $Cse4^{CENP\text{-}A}$ as the epigenetic mark as it does in other species. However, because we believe the mechanism is likely involved in maintenance of the centromere over multiple divisions, these initial assays would not be the appropriate assays to assess its role in the fidelity of the cell. Better assays would involve analyzing rates of chromosome loss. This analysis would include sectoring assays and dot-strain assays to determine if Separase cleavage of CENP-A is important for the fidelity of chromosomes over multiple divisions. I also think it would be interesting to explore the possibility in human tissue culture. A quick way to assess this would be to blot tissue culture cells for a metaphase arrest and release experiment. If CENP-A were cleaved, it might be apparent by two bands on the blot just after release. Other experiments could include looking at N-terminally tagged CENP-A in CENP-C depleted cells, to test if the kinetochore is protecting centromeric CENP-A, similar to what we see in worms.

4.3 Concluding Remarks

I believe that in characterizing the kinetochore during meiosis in *C. elegans*, I have made a significant contribution to the field of chromosome segregation. It can no longer be assumed that the principles, which mediate chromosome segregation during mitosis, must also apply to meiosis. It was generally assumed that in meiosis a kinetochore was established by CENP-A, and thus facilitated chromosome-to-microtubule attachments. My work clearly demonstrates that this is not the case for meiotic segregation in *C. elegans*. Whether this is also true in other model systems remains to be seen, but I suspect that as we learn more, it will become apparent that there are numerous properties that meiotic and mitotic chromosome segregation do not share in common.

While it remains elusive as to the exact nature for the role of Separase-mediated CeCENP-A cleavage, there is no doubt that this discovery is very interesting and quite novel. If our model is correct, and this process does play a role in maintaining centromere specificity, then this discovery will make a major impact in the field. With a few key experiments, I believe it will be possible to get a better idea as to the nature of this CeCENP-A cleavage event.

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