THE ROLE OF MEIOSIS-SPECIFIC COHESINS IN ACCURATE CHROMOSOME SEGREGATION

By

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Kim S. McKim, Ph.D.

And approved by

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ABSTRACT OF THE DISSERTATION

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Dissertation Director:

Dr. Kim McKim

Haploid gametes are formed by a specialized cell division called meiosis. During meiosis there is one round of DNA replication, but two rounds of DNA segregation which leaves the daughter cells with half the amount of DNA as the parents. This special division is highly regulated to ensure that exactly one copy of each chromosome is included in each gamete. Once replicated the sister chromatids are held together by cohesion, and the homologous pairs are held together by the synaptonemal complex (SC). Without either of these two complexes, the chromosomes do not segregate properly at meiosis I. Mitotic cohesin is comprised of four subunits SMC1/SMC3/SCC1/SCC3, which form a ring. However, in many organisms there are meiosis specific cohesin subunits that have been discovered, such as Rec8 that substitutes for SCC1. This dissertation aims to gain insight into the meiosis specific complexes in *Drosophila*. There is evidence for two meiosis specific cohesin complexes functioning in *Drosophila*. One of the complexes is comprised of SMC1/SMC3/SOLO/SUNN. This complex has a minor role in SC assembly, but, is required for sister centromere cohesion. The other complex demonstrated is
SMC1/SMC3/C(2)M/SA and is primarily responsible for SC assembly, is not involved in sister centromere cohesion and, is highly dynamic. To confirm this complex forms cohesin rings, we have made point mutations within the regions of C(2)M which are thought to mediate interactions with SMC1 and SMC3. The data collected from these mutations, to date, reveals that N-terminal amino acid residues may not be important. We are working to learn if there are alternate roles for C(2)M outside of interacting with the SMCs or if the interface between the SMCs and C(2)M is different than proposed.
ACKNOWLEDGEMENTS

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Next, I would like to thank Kim McKim for taking me in his lab and mentoring me. This has been a truly unique experience and I am grateful for all of the techniques that I was able to learn while in the lab. I am especially grateful to be able to have access to the confocal microscope where I can take such beautiful images and call them work.

Thank you to all of the lab members past and present that have helped me especially Sarah Radford or “Sarah I have a question!”, as we like to call her. She is always eager to talk science and give advice or fix a problem. Also, thank you to my lab-best-friend, Arunika Das. When I entered this lab you not only showed me the ropes but you befriended me—only to become lab best-friends!

Thank you to my boyfriend Edward Enners for supporting me throughout my time in the lab and thesis writing especially! You are always there for me when I need you and are my crutch to lean on—and you make some pretty mean vegan food too!!
DEDICATION

This thesis is dedicated to my brother and sister, Jacob and Avery. Watching the two of you grow over the years has brought me so much joy. I am so proud of the people that you have become. I love you now and always.
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I. Preface

II. Abstract

III. Introduction

IV. Results

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SA is required for meiotic crossing over and chromosome segregation as is C(2)M
SA and C(3)G dynamics are similar to that of SMC1
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CHAPTER 1: INTRODUCTION

Aneuploidy and maternal age

Aneuploidy, the improper number of chromosomes in a cell, is the result of the chromosomes disjoining improperly during anaphase. There are many causes of missegregation of chromosomes including absence of chiasmata, and loss of cohesion. Premature separation of homologous chromosomes causes nondisjunction (SAKAKIBARA et al. 2015). One cause of the premature release of homologous chromosomes is the loss of cohesion. Since human oocytes are all produced during fetal development, these cells must last the reproductive life of the female. As the female ages, so does her oocytes. The oocyte goes through two major arrests in its life cycle, the first occurs in prophase I. In humans, the oocyte is released from prophase I, after it has been ovulated, arrests again in metaphase II, and waits to be fertilized. In the event of fertilization, meiosis resumes with anaphase II. It has been demonstrated that one case of the maternal age effect is due to a premature loss of cohesion and, therefore, the homologous chromosomes are not segregated properly (HODGES et al. 2005; CHIANG et al. 2010; LISTER et al. 2010). Although Drosophila females can produce new oocytes throughout their life, their oocytes have two arrests: one at prophase I and another post metaphase I. Once the oocyte passes through the oviduct it resumes meiosis again (VON STETINA AND ORR-WEAVER 2011). When Drosophila oocytes are aged, the age effect is also observed (JEFFREYS et al. 2003).

The hypothesis that loss of cohesion (sometimes caused by cohesion exhaustion) is a cause for nondisjunction is supported by the fact that cohesins, in Drosophila, need to be rejuvenated (WENG et al. 2014). It has been shown that if cohesin subunits are not
able to be replaced with new proteins, the level of nondisjunction increases. This indicates that the cohesins are lost and new ones need to be reloaded in their place. It has been shown that SUNN plays an important role in sister centromere cohesion and, therefore, the SOLO/SUNN complex is thought to play an important role in keeping the sisters together before anaphase and may be complex affected by age (KRISHNAN et al. 2014; GYURICZA et al. 2016).

Chromosomal abnormalities caused by too few or too many chromosomes are the leading cause of birth defects, and they can also cause infertility. There are a few chromosomes that when present in three copies, instead of the normal two, are still viable, but leads to genetic abnormalities. Down’s syndrome is caused by a trisomy on the 21st chromosome, Edward’s syndrome by a trisomy of the 18th chromosome and Turner’s syndrome is a monosomy of the X chromosome. Although not technically a trisomy, Kleinfelter’s syndrome is caused by an aneuploidy of the sex chromosomes resulting in a male with the genotype XXY. All of these syndromes are quite rare and only about .2-.3% of newborns are trisomic (HASSOLD AND JACOBS 1984). Trisomies are so rare because three copies of any of the other chromosomes are not compatible with life and will be spontaneously aborted during development of the fetus. Most of the instances of trisomies are the result of an aneuploid egg being fertilized by a euploid sperm. Recent studies have shown that the chance of having a pregnancy resulting from an aneuploid egg increases with the age of the mother from about 2% when the mother is in her 20’s, to 35%, when the mother is in her 40’s (HUNT AND HASSOLD 2010). This phenomenon, known as the maternal age effect, is not well understood, however studies are pointing the finger at cohesins as the culprit (CHIANG et al. 2010; LISTER et al.). One of the reasons that these proteins are being implicated in contributing to the
maternal age effect is because they are required for proper chromosome segregation. Also, they are loaded during pre-meiotic S-phase, which occurs in humans during fetal development when all of a female’s eggs are produced. The same cohesin proteins are thought to stay associated with the chromosomes until the sister chromatids segregate in anaphase, which in humans, can be decades later. Because of this long life span of the proteins it allows for them to get old, break down, and possibly not do their job as well as when they were young, leading to missegregation of the chromosomes. One wrinkle in this hypothesis is the rejuvenation studies performed by Sharon Bickel (WENG et al. 2014). These studies show that certain cohesins may be reloaded on to the chromosomes to maintain proper segregation at anaphase. I will provide evidence in this dissertation supporting both of these hypothesis.

**Meiosis**

Meiosis is a specialized division by which haploid gametes are produced from diploid cells by way of one round of DNA replication followed by two rounds of DNA division. Sister chromatids are held together by a proteinaceous ring called cohesion and the homologs are held together by a proteinaceous ladder like structure called the synaptonemal complex. It is important that these two structures form properly because without them aneuploidy occurs. One role of the cohesion complex in mitosis and meiosis is to hold the chromosomes together so that each one can attach to a different spindle pole during metaphase. This attachment ensures that the chromosomes will be pulled into opposite daughter cells. During meiosis I, homologs separate, but sister chromatids remain together. This is unique to meiosis, and therefore, must require mechanisms unique to the meiotic divisions to ensure that the chromosomes segregate
properly into the gametes. The release of homologs, while sisters remain attached, is regulated by the release of cohesion found on varying parts of the chromosome. During meiosis I, when homologs segregate, cohesion is released on the chromosome arms distal to crossovers. However, during meiosis II the sisters segregate by releasing the cohesion at the centromeres that is holding them together. One hypothesis is that the differential release of these two populations of cohesion is possible because they are different cohesin rings consisting of different subunits at the separate locations. Alternatively, the differential release of the two cohesion populations could be because the two complexes have different protection mechanisms that are removed at different times allowing the rings to be released at separate times.

**Synaptonemal complex and the chromosome axis**

The synaptonemal complex (SC) is a proteinaceous structure that is comprised of many proteins and three main elements. The three main elements of the SC are the lateral elements, which are found at the chromosome axis, transverse filaments, and pillar proteins (Figure 1). In mice the lateral elements include SYCP2 and SYCP3 and the transverse filament is SYCP1. In yeast the lateral elements include Red1 and the transverse filament is Zip1. In *Drosophila* the transverse element is C(3)G and the lateral elements are currently unknown however, this is not surprising because SYCP2, SYCP3, and Red1 are not conserved. Although the lateral elements of the SC in *Drosophila* have not been identified, there are many candidates that may perform that function. For instance, C(2)M and ORD localize to the chromosome axis, where the lateral element lies, and are both required for SC formation (*Balicky et al.* 2002; *Manheim and McKim* 2003). Cohesion proteins such as SMC1, SMC3 and SA also localize to the chromosomes axis and are also required for assembly of the SC (*Tanneti et al.* 2011). In
Drosophila, as in other organisms such as yeast, the mechanism of SC assembly is unknown.

Another family of proteins found at the chromosome axis are the HORMAD proteins. These are a group of proteins that all contain a HORMA domain (Muniyappa et al. 2014). This domain was originally named because it was first discovered in Hop1, Rev7 and Mad2. In mice there are two HORMA domain proteins, HORMAD1 and HORMAD2 and they too localize to the chromosome axis (Fukuda et al. 2010). Interestingly, the HORMA domain proteins are widely conserved from yeast to humans, however, Drosophila does not encode any. This makes us question what proteins are at the chromosome axis that allow for assembly of the SC.

The function of the SC is to hold the homologous pairs together and is required for almost all of the crossover events. Once double strand breaks are resolved as crossovers, and cohesion is in place holding sister chromatids together distal to the crossover, the SC is no longer needed to tether homologs and can be dissolved. The SC is formed in a few distinct steps that initiate during the stage zygotene in prophase of meiosis I. In Drosophila, the first step of SC assembly is building the SC at the centromere. The next step is to build a few foci of SC on each of the chromosome arms; these first two steps are referred to as early and mid-zygotene, respectively. During late zygotene more foci of SC form within the euchromatin, whereas pachytene is characterized by the formation of the SC all over the DNA (Tanneti et al. 2011).

Disruption of the chromosome axis also disrupts SC formation. For instance, when C(2)M and ORD, both proteins found at the chromosome axis, are removed SC assembly is affected (Tanneti et al. 2011). ORD is responsible for the first two stages of SC initiation, and in the absence of ORD the foci at the centromeres are absent. C(2)M,
however, is responsible for the remainder of the SC. Therefore, in the absence of C(2)M, just the centromere SC and a few SC foci on the chromosome arms remain. This indicates that all SC assembly is dependent upon C(2)M or ORD. C(2)M and ORD are both proteins that have been implicated in accurate chromosome segregation (BICKEL et al. 2002; MANHEIM AND MCKIM 2003; WEBBER et al. 2004). ORD in particular has a role in sister chromatid cohesion (BALICKY et al. 2002; BICKEL et al. 2002). In addition, eliminating the cohesion protein SMC3 abolishes all SC assembly. This leads us to the hypothesis that C(2)M and ORD are involved in meiosis-specific cohesin complexes and that there are just two of them in Drosophila. These cohesin proteins are also found at the chromosome axis and are required for SC assembly, possibly making them part of the lateral element of the SC.
Figure 1: Schematic drawing of the synaptonemal complex and cohesin. A) A schematic drawing of the SC showing two looped sister chromatids on each side of the SC. It also can be seen from this schematic that axial elements and cohesins are both at the chromosome axis. B) A schematic drawing of the mitotic cohesin complex comprised of four subunits. Scc1 is Rad21 in *Drosophila* and Scc2 is SA in *Drosophila*. 
Mitotic cohesion

Cohesion is mediated by a proteinaceous ring that consists of four cohesin subunits. Each cohesin ring has two SMCs, one Kleisin, and one stromalin subunit (LOSADA et al. 1998) (Figure 1). The SMC proteins are coiled coil proteins that fold back upon themselves. The N- and C- termini of these proteins encode one half of an ATP hydrolysis domain and once the protein is folded, the domain is fully functional (RANKIN 2015). The other end of the SMC proteins is where the two SMCs dimerize and is known as the "hinge". The hinge is where the DNA enters the ring from whereas the DNA exits through a gate formed by the SMC and Kleisin subunit (CHAN et al. 2012). The Kleisin subunit functions to interact with both SMC proteins and close the tripartite ring (GLIGORIS et al. 2014). The stromalin subunit binds outside of the ring to the Kleisin subunit.

Previous studies have looked at the interface of the Kleisin and the SMC proteins in detail (GLIGORIS et al. 2014). Crystal structures of these proteins were used to identify the residues of the Kleisins that are responsible for mediating the interaction with the SMC proteins (SCHLEIFFER et al. 2003; GLIGORIS et al. 2014). In addition, protein alignments were created showing that these residues are conserved across many organisms, including Drosophila, and that they are present in cohesins both in mitosis and meiosis (SCHLEIFFER et al. 2003). The region between the two SMC binding regions is larger and less studied. This region is where the Stromalin subunit, and possibly others, dock onto the Kleisin. The sequence of the interfacing residues, however, is not well conserved. Also, within this region is a sequence of 10 amino acids termed the ROCC (region of chromatid cohesion), which is also conserved among organisms and is not required for the establishment of cohesion but rather, the maintenance of cohesion.
(ENG et al. 2014). This region is proposed to be a docking point for regulators of cohesion Eco1 and PDS5. It is known that the Drosophila mitotic Kleisin, Rad21, contains the ROCC sequence, however, after looking for this sequence in the Drosophila meiosis specific Kleisins, the sequence cannot be found. This suggests that the cohesins in meiosis have a different method of regulation than their mitotic counterparts.

Sister chromatids are held together by cohesin which is loaded during S-phase of DNA replication (PETERS AND NISHIYAMA 2012). During mitotic growth, the same cohesin rings will remain associated with the DNA until the metaphase- to- anaphase transition. Cohesion loading is mediated by a number of positive regulators of cohesion which aid in closing the ring. Two of these proteins are SCC2 and SCC4, known in Drosophila as Nipped-B and Mau-2, respectively (CIOSK et al. 2000). Another type of cohesion regulator antagonizes the negative regulators and maintains the cohesion ring closed. One of these is Eco1. In addition, there are also negative regulators that mediate the removal of cohesins from the DNA.

Cohesin can be removed from DNA in two ways. One method of cohesin removal is by proteolytic cleavage of the Kleisin subunit by the protease Separase. Separase cuts at specific cleavage sequences found within the Kleisin. This is a highly regulated process that only occurs at the onset of anaphase. Cohesion removal, however, can occur throughout the cell cycle through an alternative pathway (SUMARA et al. 2002). This alternative pathway of cohesin removal, the “prophase pathway”, occurs mostly in prophase and requires WAPL to open the ring. PDS5 and WAPL, two negative regulators, have been shown to make a complex which, in order to act on the ring, interacts with the SA subunit of the cohesion ring (HARTMAN et al. 2000; PANIZZA et al. 2000). This is a non-proteolytic pathway of cohesion removal and opens the ring
between the Kleisin and SMC3 interaction (Chan et al. 2012; Buheitel and Stemmann 2013; Murayama and Uhlmann 2015). This pathway of cohesion removal is regulated by acetylation of the SMC3 subunit by Eco1, an acetyltransferase. In the presence of the acetylation, WAPL will not open the ring. However, in the absence of the acetyl group on SMC3 the cohesion ring is able to be released (Chan et al. 2012).

**Meiotic cohesion**

Meiosis specific cohesin complexes have been discovered in most organisms including *C. elegans, Drosophila, mouse, and yeast*. Each species contain one or more meiosis specific cohesin subunits (Figure 2). Compared to the mitotic subunits, the meiotic ones are not as well studied. However, it has begun to be uncovered in multiple organisms that meiosis specific cohesion complexes play an important role in accurate chromosome segregation. In mice, the meiosis specific stromalin, STAG3, is responsible for nearly all of the SC assembly and sister chromatid cohesion, whereas the mitotic Stromalins (SA1 and SA2) have a minor role in SC assembly (Fukuda et al. 2014; Winters et al. 2014).

Meiosis specific Kleisins are particularly interesting because all eukaryotic organisms have at least one. The meiosis specific Kleisins are paralogs to the mitotic version, Rad21 and are often referred to as Rec8. The first Rec8 was identified in *S. pombe* over twenty-five years ago yet we are still unsure of their meiosis specific function (Ponticelli and Smith 1989). Since then, Rec8 orthologs have been found in many organisms including *S. cerevisiae*. Without this protein, the sister chromatids are completely unable to pair and the SC formation is defective (Klein et al. 1999). In mice, there have been two meiosis specific Kleisins demonstrated, Rec8 and Rad21L. The absence of Rec8 results in lack of synapsis, sister chromatid cohesion and, ultimately,
sterility in both male and female mice (Bannister et al. 2004; Xu et al. 2005). Rad21L is the other meiosis specific kleisin found in mice. It has a similar phenotype to that of Rec8 deletion in that oocyte development arrests early. Previous experiments show that Rad21L and Rec8 localize to the chromosomes in an alternating foci pattern, indicating that they have different functions (Ishiguro et al. 2011; Lee and Hirano 2011). Interestingly, in the absence of both Rad21L and Rec8, no axial or lateral elements formed, indicating that these two proteins are required for all SC formation (LLano et al. 2012). Rad21L, however, is less well studied. It is known that it loads onto chromosomes during S-phase and dissociates quickly. There is, however, Rad21L remaining at the centromere after dissociation. Because it is found at the chromosome axis, it is possible that Rad21L plays a role in pairing the homologous chromosomes and SC assembly (Gutiérrez-Caballero et al. 2011; Ishiguro et al. 2011; Lee and Hirano 2011).

In Drosophila the mitotic cohesion complex consists of SMC1, SMC3, Rad21 and Stromalin. In meiosis, however, this complex does not appear to function. Previous studies indicate that the mitotic cohesion complex has a redundant role in germline mitosis along with a meiotic complex containing SOLO/SUNN (Gyuricza et al. 2016). Because of similar sequence patterns SOLO is a diverged kleisin protein. It localizes primarily to the centromeres with weaker signal on the chromosome arms (Yan and Mckee 2013). SUNN has a similar localization pattern to SOLO, and has homology to the Stromalin family. In Drosophila meiosis there are also two cohesion complexes. One of these complexes is SMC1/SMC3/SOLO/SUNN which also functions in germline mitosis. This complex is required for cohesion in meiosis and is also required in male meiosis (Krishnan et al. 2014; Gyuricza et al. 2016). The other complex is SMC1/SMC3/C(2)M/SA. This complex is similar to the mitotic complex with the exception
of the Kleisin subunit. In this complex C(2)M is the Kleisin that replaces Rad21. C(2)M is required for SC assembly and for crossing over, however, unlike most cohesin complexes, this complex does not appear to have a role in cohesion (MANHEIM AND McKIM 2003; GYURICZA et al. 2016).
**Figure 2:** Cohesin subunits across multiple organisms. Meiosis specific subunits are in blue.

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**Crossing over**

In *Drosophila*, once the SC is assembled, several programmed double strand breaks (DSBs) are formed (MEHROTRA AND MCKIM 2006). The double strand breaks can be repaired as crossovers or non-crossovers. Crossovers mature into chiasmata that ensure the disjunction of homologous chromosomes at meiosis I. Crossovers are initiated by a DSB. In *Drosophila* DSB are initiated by a Spo-11 homolog, Mei-W68 (MCKIM AND HAYASHI-HAGIHARA 1998). In order for DSBs to be repaired as crossover rather than non-crossover a family of proteins, known as exchange proteins, is required. The exchange class consists of MEI-9, ERCC1, MUS312 and HDM (JOYCE AND MCKIM 2009). It has been shown that crossovers are required for accurate chromosome segregation (MCKIM *et al.* 2002). The crossover, in addition to cohesion, will function to hold the homologous chromosomes together once the SC has dissociated.

**The *Drosophila* ovary system**

The oocytes in a *Drosophila* ovary are arranged in a temporal order with the earliest stages of development at the anterior end of the ovariole and the latest stage oocytes at the posterior end. As the oocytes develop they move down the developing chain. Meiotic prophase occurs within the germarium, which contains cysts of 16 cells arranged in developmental order. In region 1, four incomplete mitotic divisions generate 16-cell cysts. In region 2A two of the 16 cells are pro-oocytes and enter zygotene and reach pachytene. Zygote is divided into three stages: early zygote with SC only at the centromeres, then mid zygote with an additional 6-8 euchromatic SC foci, then late zygote with many foci of SC and finally pachytene with threads of SC (TANNETI *et al.* 2011). Double strand breaks are induced in region 2a and are
repaired by region 3 (Mehrotra et al. 2007). Therefore, repair and crossover formation happen between region 2a and 3. By the end of the gerarium, region 3, there is only one pachytene oocyte in each cyst because the other pro-oocyte has reverted to nurse cell status like the other 14 cells.

After the gerarium is the vitellarium which contains 14 stages. Region 3 of the gerarium is stage 1 of the vitellarium. Within the vitellarium the oocyte maintains a long prophase arrest that is equivalent to the prophase arrest in humans which is implicated in the exhaustion of cohesion and the maternal age effect. During stage 14 spindle assembly occurs, the egg activated when it is laid, and fertilization can happen.
Figure 3: Drawing of the *Drosophila* ovaries
The *Drosophila* ovary is comprised of developing chains of oocytes called ovarioles. The left ovary is depicted how it would be within the female. The right ovary has two ovarioles separated from the rest to show the detail. Figure adapted from King et al. (King 1970).
Using the Gal4/UAS system to control expression of transgenics

In order to control expression of various elements used in experiments in this thesis, such as, RNAi or tagged transgenic proteins, we used the Gal4/UAS system. The Gal4/UAS system comes from yeast. UAS is an activating sequence found upstream of genes and, when bound by \textit{GAL4}, promotes transcription. All RNAi and tagged proteins used were under control of the UAS promoter, which is not endogenous to flies, and therefore will not be activated under normal conditions (RORTH 1998). In the \textit{Drosophila} system, the \textit{Gal4} gene is put under control of a \textit{Drosophila} promoter that expresses in the tissue and at the time that is desired of the tagged protein. This system allows the tagged protein to only be expressed where and when the \textit{Gal4} is expressed. In this thesis lines with \textit{Gal4} under control of various promoter were utilized. There are many germline-specific \textit{Gal4} promoters that are used that express at different times of the germline (Figure 4). \textit{P\{GAL4::VP16-nos.UTR\}CG6325MVD1} (referred to as MVD1) is the promoter used when high level expression throughout the germline is desired (RORTH 1998). \textit{P\{GAL4-nos.NGT\}A} (referred to as NGT) is used when more restricted expression throughout zygotene and early pachytene is desired (RORTH 1998). \textit{P\{bam:Gal4-VP16\}} expresses in the mitotic germline divisions and has a short window of expression (MATHIEU et al. 2013). \textit{P\{arm- GAL4\}} expresses in mid/late zygotene (SANSON et al. 1996). The \textit{P\{mato4-GAL-VP16\}} allows expression after the germarium, in the vitellarium, and lasts throughout the germline and into embryogenesis (SUGIMURA AND LILLY 2006; RADFORD et al. 2012).
Figure 4: Schematic drawing of expression patterns of Gal4 lines used. Shown here is a schematic drawing of the germarium and a stage 2 oocyte. The anterior end is to the left. The stages of the oocytes are marked. Above the germarium shows when MVD1, NGT, bam, arm, and matα promoters are expressed.
This dissertation aims to determine the composition of meiosis specific cohesin complexes in *Drosophila* females and their function. *Drosophila* will be used for these experiments because the processes in meiosis are conserved across organisms and it appears from preliminary evidence that the presence of two meiosis specific complexes is conserved. These two meiosis specific complexes in *Drosophila* are in contrast to the potential of upwards of 15 complexes in mammalian meiosis (JESSBERGER 2011).

Although there are a potential of many more complexes in mammals it is likely that there are just two important ones, as in *Drosophila* (LLANO et al. 2012). This is of interest because although meiosis specific complexes have been implicated in many organisms, the function of multiple organisms is unknown. In this dissertation I provide evidence for two complexes, one contain SMC1/SMC3/SOLO/SUNN and the other with SMC1/SMC3/C(2)M/SA. The SOLO SUNN complex is found at the centromere and at distinct foci of euchromatin initiation sites, is involved in sister chromatid cohesion, and new subunits are not incorporated in to the chromosome axis throughout prophase. The C(2)M/SA complex, however, localizes to the chromosome arms, is involved in SC assembly, is not involved in sister chromatid cohesion, and is continually incorporated into the chromosome axis throughout prophase. This data provides evidence that the centromere cohesin proteins are static (SOLO/SUNN) and may be involved in the maternal age effect and it also provides evidence that other cohesins (C(2)M/SA) can be re-loaded during meiosis and are likely not contributing to the maternal age effect. In this dissertation I focus on the role of C(2)M, a meiosis specific Kleisin, functioning outside of the cohesin ring in SC assembly, a novel function for Kleisins.
CHAPTER 2: DYNAMIC AND STABLE COHESINS REGULATE SYNAPTONEMAL COMPLEX ASSEMBLY AND CHROMOSOME SEGREGATION

I. Preface

This chapter was published, as presented here, in Current Biology, July 2016. My contributions to the project and paper were revising and editing the paper and all of the experiments with the exception of Figure 2.
Figure 5: Graphical abstract of chapter 2. Rad21/SA and SOLO/SUNN are present in the complexes in germline mitosis. In meiosis the complexes switch to C(2)M/SA and SOLO/SUNN. This figure also represents that the C(2)M subunit is continually reincorporated into the chromosome axis throughout prophase.
III. Abstract

Assembly of the synaptonemal complex (SC) in *Drosophila* depends on two independent pathways defined by the chromosome axis proteins C(2)M and ORD. Because C(2)M encodes a Kleisin-like protein and ORD is required for sister chromatid cohesion, we tested the hypothesis that these two SC assembly pathways depend on two cohesin complexes. Through single and double mutant analysis to study the mitotic cohesion proteins Stromalin (SA) and Nipped-B (SCC2) in meiosis, we provide evidence that there are at least two meiosis-specific cohesin complexes. One complex depends on C(2)M, SA and Nipped-B. Despite the presence of mitotic cohesins SA and Nipped-B, this pathway has only a minor role in meiotic sister centromere cohesion and is primarily required for homolog interactions. C(2)M is continuously incorporated into pachytene chromosomes even though SC assembly is complete. In contrast, the second complex, which depends on meiosis-specific proteins SOLO, SUNN and ORD is required for sister chromatid cohesion, localizes to the centromeres, and is not incorporated during prophase. Our results show that the two cohesin complexes have unique functions and are regulated differently. Multiple cohesin complexes may provide the diversity of activities required by the meiotic cell. For example, a dynamic complex may allow the chromosomes to regulate meiotic recombination, and a stable complex required may be required for sister-chromatid cohesion.

IV. Introduction

Meiosis I begins with the pairing of homologous chromosomes that are held together by the synaptonemal complex (SC) (Bhalla and Dernburg 2008; Lake and Hawley 2012). The SC may regulate meiotic recombination through interactions with the
chromosome axis, which interacts directly with the chromatin (Mehrotra and McKim 2006; Fowler et al. 2013; Zickler and Kleckner 2015). A crucial component of the axis is cohesin-related proteins. Mitotic cohesin is made up of four subunits: SMC1, SMC3, Stromalin (SA) and the Kleisin Rad21. However, multiple meiosis-specific cohesin complexes have been described in a variety of organisms (Jessberger 2011; Rankin 2015). The function and properties of these meiosis-specific cohesin complexes, however, are poorly understood. Our mutant analysis in Drosophila has shown that SC initiation depends on two independent pathways defined by two sets of cohesin-related genes (Tanneti et al. 2011). One pathway depends on C(2)M, a Kleisin family protein (Manheim and McKim 2003) that physically interacts with the cohesin SMC3 (Heidmann et al. 2004). The second pathway depends on ORD, a cohesion protein that is not conserved (Webber et al. 2004). SC assembly is absent in a c(2)M ord double mutant or oocytes lacking SMC1 and SMC3 (Tanneti et al. 2011), suggesting that cohesin complexes dependent on either C(2)M or ORD are required for all pathways of SC assembly.

Besides SMC1 and SMC3, the Drosophila cohesin subunits that associate with C(2)M are not known. Recent studies have suggested Stromalin has a role in maintaining the SC and cohesion late in meiotic prophase, but its role in SC assembly is not known (Weng et al. 2014). We have examined the function of all known Drosophila cohesin subunits in SC assembly. Our data are consistent with a model that SA and the SCC2 homolog Nipped-B function in a pathway with C(2)M, while ORD functions in a distinct meiosis-specific cohesin pathway with two other proteins, SOLO and SUNN (Yan and McKee 2013; Krishnan et al. 2014)
These two groups of proteins differ not only in their function, but also their loading properties. We have discovered that C(2)M is exchanged during prophase, with subunits being added to and dissociating from the chromosomes throughout pachytene. In contrast, SOLO and SUNN at the centromeres are probably loaded only during premeiotic S-phase. The dissociation of cohesin complexes from meiotic chromosomes, and the failure to replace them, may be a contributing factor to the maternal age effect (CHIANG et al. 2010; TACHIBANA-KONWALSKI et al. 2010; JESSBERGER 2012). Our results modify this model by demonstrating that each cohesin complex is regulated differently.

V. Results
Stromalin is required for SC assembly

To test the hypothesis that C(2)M is part of a cohesin complex required for SC assembly, we investigated the role of other cohesin proteins in SC assembly. In addition to SMC1 and SMC3, the Drosophila mitotic cohesin complex includes the Kleisin, Rad21 (SCC1), encoded by the verthandi (vtd) gene, Stromalin (SCC3), encoded by the SA gene and the cohesin loader Nipped-B (SCC2). All of these proteins are essential; therefore, to generate oocytes lacking each protein, shRNAs were expressed using P{GAL4::VP16-nos.UTR}CG6325MVD1 (herein referred to as MVD1) for germline-specific RNAi (RORTH 1998) (Figure 21). The effect on SC assembly was assayed by examining transverse filament protein C(3)G localization in the germarium of the ovary (PAGE AND HAWLEY 2001). Within a wild-type germarium, zygotene and early pachytene are in region 2a, pachytene then progresses as the oocytes move into region 2b and region 3. Knockdown of vtd (Rad21) did not have any effects on SC assembly (Figure 22), consistent with another report using a different method (URBAN et al. 2014). In contrast, in SA or Nipped-B RNAi oocytes, SC assembly was incomplete (Figure 6B,C).
C(3)G was observed at the centromere, as shown by colocalization with the centromere histone H3 CID, and at several sites in the euchromatin, but there was an absence of C(3)G threads, showing that SC assembly in oocytes lacking SA or Nipped-B did not progress beyond zygotene.

C(2)M localization was absent in SA or Nipped-B RNAi oocytes (Figure 7B, Figure 22), suggesting that the SC assembly defects could be related to a lack of C(2)M. In fact, the SC assembly defect in SA or Nipped-B RNAi was similar to the phenotype we previously observed with c(2)M mutants, where there were about 6-8 C(3)G patches per oocyte (Tanneti et al. 2011) (Figure 6B, C, E, Figure 23). Among all SA RNAi oocytes in the germarium, there was an average of 5.8 euchromatic SC patches per oocyte. However, between region 2a and 3 of the germarium, there was a significant decrease in the number of C(3)G patches in SA and Nipped-B RNAi oocytes (Figure 6B, C, Figure 7G, H, Figure 23). The average number of C(3)G patches in SA RNAi oocytes decreased from 10.5 (n=257) in region 2a to 2.4 (n=16) in region 3, and 43% had only one C(3)G patch that was always at the centromeres. These results suggest that SC assembled along the arms in the absence of SA or Nipped-B is unstable. In c(2)M mutants, the number of C(3)G patches did not decrease (Tanneti et al. 2011) (Figure 6E, Figure 23). These results show that the phenotype of SA and Nipped-B RNAi oocytes is similar to c(2)M mutants, but with some minor quantitative differences.

The SC phenotype of SA RNAi was compared to a null mutant of SA (Figure 21B). The SA86 germline clone null mutant oocytes had patches of C(3)G in region 2a (avg. = 5.6, n=27) that was similar to the RNAi of SA. This decreased in region 3 to an average of 1.6 patches, which always included the centromeres (n=11)(Figure 6D). The results show that the RNAi closely resembles that of the SA null mutant phenotype.
Figure 6: Stromalin and Nipped-B are required for synapsis. SC assembly in A) wild-type, B) $SA$ RNAi, C) $Nipped-B$ RNAi, D) $SA^{86}$ germline clone and E) $c(2)M$ mutant oocytes. Meiotic prophase begins in region 2a, with zygotene and early pachytene oocytes, with later pachytene stages in regions 2b and 3. Shown are representative oocytes in region 2a and region 3, with C(3)G (green) and centromere histone CID (red) and DNA (blue). An arrow indicates synapsis at the centromeres as shown by colocalization of C(3)G and CID. The scale bars = 5µm. See also Figure 21 and Figure 23.
SMC1 on the chromosome arms but not the centromeres depends on SA

To determine if SA and Nipped-B are required for loading of cohesins, we examined SMC1 localization. In wild type pachytene cells with complete SC formation, SMC1 was found at the centromeres and along the chromosome arms in threads (Figure 7D). In c(2)M mutants, where SC assembly was incomplete, SMC1 was observed at the centromere, and not in a thread-like pattern along the chromosome arms (Figure 7E). Conversely, in ord mutants, SMC1 was observed on the chromosome arms (Figure 7F). These observations are similar to a previous study by Khetani and Bickel (KHETANI AND BICKEL 2007). In the c(2)M ord double mutant, in which all C(3)G is absent, SMC1 was also absent (Figure 22F). In SA or Nipped-B RNAi oocytes, SMC1 localization to the arms was almost absent (Figure 7G,H, Figure 22), which was similar to c(2)M mutants and not ord. Based on C(3)G localization at the centromeres (Figure 6), it is likely that the SMC1 remaining in SA or Nipped-B RNAi oocytes is primarily at the centromeres. These results show that SMC1 at the centromere is dependent on ORD while SMC1 on the arms is dependent on C(2)M and SA. SMC1 localization was not affected by vtd RNAi (Figure 22H).
Figure 7: Cohesin localization in cohesin mutants and RNAi knockdowns. C(2)M (red) localization in (A) wild-type and (B) S4 RNAi oocytes with C(3)G in green and DNA in blue. C-H) SMC1 (red) localization and SC assembly with C(3)G (green). The regions of brightest C(3)G and SMC1 are the centromeres (Figure 1) (Khetani and Bickel 2007) (Tanneti et al. 2011) (Takeo et al. 2011). Zygotene (C) in wild-type is observed in some germaria in the most anterior (earliest) region 2a oocytes. Most of the germarium, region 2a, 2b and 3, contains pachytene (A,D) oocytes. The c(2)M (E) and ord (F) mutant oocytes and S4 RNAi (G) oocytes are from the region 2a-2b where pachytene is expected in wild-type. The S4 RNAi oocyte in H is from region 3. In all images, the scale bar = 5 μm. See also Figure 22.
SA functions in the same pathway as C(2)M but not ORD or SUNN

To determine if the SC observed in SA or Nipped-B knockdown oocytes depends on either ORD or C(2)M, we expressed *SA* or *Nipped-B* shRNA in *ord* or *c(2)M* mutants. Oocytes mutant for *c(2)M* and with RNAi to either *SA* or *Nipped-B* had patches of C(3)G at the centromere and in the euchromatin that was similar to the *c(2)M* single mutant (Figure 8, compare B- D to G and I). Like *SA* RNAi, however, the number of C(3)G patches in *SA* RNAi *c(2)M* oocytes significantly decreased between region 2a and region 3 (Figure 8H, Figure 23). A similar but not significant decrease was observed in *Nipped-B* RNAi *c(2)M* (Figure 8J, Figure 23). The absence of a more severe phenotype in these experiments suggests that SA, Nipped-B and C(2)M function in the same pathway to promote sites of SC initiation in late zygotene and the completion of synapsis.

When *SA* shRNA was expressed in the germline of an *ord* mutant, the result was a surprisingly more severe phenotype: the ovaries were rudimentary in size and lacked development of 16 cell cysts with oocytes. The same result was observed when *vtd* shRNA was expressed in the germline of *ord* mutants. These small ovaries were examined for VASA and Spectrin expression, which mark germline and somatic cells, respectively (SANTOS AND LEHMANN 2004). The small ovaries had no VASA expressing cells, showing they lacked germ cells (Figure 8L, M). These results suggest that ORD has a function that is independent of other cohesin subunits such as *vtd* and *SA*. They also show that ORD has a redundant function in the germline mitotic divisions, as has been suggested previously (MIYAZAKI AND ORR-WEAVER 1992).

We repeated these experiments with *sunn* mutants, which has a meiotic phenotype similar to *ord* (KRISHNAN et al. 2014). The *c(2)M; sunn* double mutant had no
evidence of SC assembly, similar to the *c(2)M ord* results (Figure 8F). However, when *SA* shRNA was expressed in a *sunn* mutant, we observed the same absent germline phenotype as *SA* RNAi *ord* mutant oocytes (Figure 8N). Thus, like ORD, SUNN has a role in germline mitosis that is redundant with SA. The synergism between the two groups of genes is consistent with a two cohesin pathway model in the germline. In germline mitosis, SUNN/SOLO/ORD and the canonical SA/RAD21/Nipped-B cohesin pathways function redundantly in cohesion. In meiosis, this changes to SUNN/SOLO/ORD and SA/C(2)M/Nipped-B that are the active cohesin pathways and they are not redundant (Figure 8).
Figure 8: SA and C(2)M function in the same SC assembly pathway while ORD and SUNN function in germline mitosis.

A) SC assembly in wild-type region 2a oocyte. B-E) SC assembly in representative region 2a of single mutant or RNAi oocytes. F-J) SC assembly in c(2)M; sunn double mutant or a c(2)M mutant also expressing RNAi for SA or Nipped-B. Each image shows a representative oocyte from region 2a (F,G,I) or region 3 (H,J). SC protein C(3)G is in green and centromere protein CID is in red. K-N) Lower magnification images showing ovary development in wild-type or females lacking two cohesins. Females are ord or sunn mutants also expressing RNAi for SA or vtd. The germline cells are shown by VASA (red) while the somatic follicle cells are shown with Spectrin (green). The absence of VASA expressing cells shows a failure of germline mitosis. In all images, the DNA is in blue and the scale bars = 5µm.

O) Model for the two meiotic cohesin complexes that function in SC assembly and crossing over. See also Figure 23.
SA depletion does not result in sister centromere defects at meiosis I

Mutations in *ord* or *sunn* have cohesion defects in female meiosis (KRISHNAN et al. 2014) (YAN AND McKee 2013). To determine if SA is required for cohesion in meiosis, we used two cytological assays in SA RNAi oocytes. First, we looked for defects in sister centromere cohesion at metaphase I in stage 14 oocytes using the centromere marker CENP-C. In wild-type, eight CENP-C foci are expected, one for each chromosome, and we observed an average of 7.1 (Figure 9A,E). In *sunn* mutant oocytes, which is required for sister chromatid cohesion (KRISHNAN et al. 2014), there was a significantly elevated number of CENP-C foci relative to wild-type or SA-knockdown oocytes (avg. = 11.1, Figure 9D, E). In SA RNAi oocytes, the number of CENP-C foci was not significantly greater than wild-type, suggesting that SA is not required for sister centromere cohesion (avg = 7.2, Figure 9B, E). Similarly, the number of CENP-C foci was not significantly increased in S4 germline clone oocytes (avg. = 6.9, Figure 9C, E). These results suggest that SUNN, but not SA, is required for sister centromere cohesion at meiosis I.

Second, centromere clustering was examined during pachytene of meiosis. During pachytene, all 8 centromeres are usually found in one or two clusters. We and others previously demonstrated that *ord, sunn* and *solo*, but not *c(2)M*, mutants, have defects in centromere clustering (TAKEO et al. 2011; TANNETI et al. 2011; YAN AND McKee 2013; KRISHNAN et al. 2014). Similarly, *SMC3* RNAi and *c(2)M ord* oocytes had clustering defects, with a significant number of oocytes with more than 2 CID foci (Figure 9F). While there may be a slight increase in the number of CID foci in SA or *Nipped-B* RNAi oocytes compared to wild-type or *c(2)M*, they were significantly less (p < 0.001) than *SMC3* RNAi or *ord c(2)M* (Figure 9F).
Figure 9: Oocytes lacking SA maintain sister-centromere cohesion. Mature stage 14 oocytes, which is where metaphase I occurs, from (A) wild type, (B) SA RNAi, (C) SA germline clone and (D) a sunn mutant are shown with tubulin in green and CENP-C in red to label the centromeres and the DNA in blue. In some cases, precocious anaphase was observed, which is when the karyosome has separated into two groups of chromosomes that appear to be moving towards the poles. Precocious anaphase was elevated in SA RNAi (60%, n=33), SA germline clones (15%, n=27) and the sunn mutant (33%, n=30). This phenotype can be caused by a failure in arm cohesion or reduced crossing (McKIM et al. 1993; BICKEL et al. 2002), and because all these mutants or RNAi are expected to reduce crossing over, this phenotype is not necessarily indicative of a cohesion defect. The scale bars are 5 μm. (E) A dot plot showing the number of CENP-C foci in each stage 14 oocyte. Wild-type, and the two SA genotypes are significantly different than sunn, as shown by a Mann-Whitney test. The horizontal
and vertical lines show the mean and 95% confidence limits. The number of CENP-C foci expected for wild-type is eight, although less could be observed due to overlap of signals. (F) A dot plot showing the number of CID foci in each pachytene oocyte. The SMC3 RNAi and the c(2)M ord double mutant are significantly different than all the other genotypes (p<0.001) by a Mann-Whitney test. The horizontal and vertical lines show the mean and 95% confidence limits.
**C(2)M is integrated into the SC throughout pachytene**

To determine if the two cohesin pathways are under different temporal regulation, we expressed cohesin subunits with a pulse of heat shock and determined when they could be incorporated into the meiotic chromosomes.  *C(2)M* fused to an HA epitope tag (*UASp-c(2)M*<sup>3XHA</sup>) was expressed under the control of a heat shock promoter.  If *C(2)M* can only be incorporated at a specific time in meiosis, and because oocytes are arranged in temporal order, then localization of heat-shock induced *C(2)M* would be observed in a restricted region of the germarium (e.g. region 2a).  If *C(2)M* can be incorporated at multiple stages of meiosis, then localization of heat-shock induced *C(2)M* would be observed at many or all regions of the germarium (e.g. regions 2a, 2b and 3).

With fixation one hour after heat shock, no *C(2)M*-HA was detected (Figure 24). With fixation at 6 hours after heat shock, however, *C(2)M*-HA localization was detected as threads in almost 100% of region 2a, 2b and 3 oocytes, consistent with loading occurring at all stages of meiotic pachytene (Figure 10A, Figure 25A). Since six hours is not enough time for a cyst to change position within the germarium, most of the oocytes were in pachytene at the time of heat shock and incorporated *C(2)M*-HA. At 24 hours after heat shock, some early region 2a oocytes did not incorporate *C(2)M*-HA (Figure 10B, Figure 25B). These oocytes could have been premeiotic at the time of heat shock. Consistent with this conclusion, the number of oocytes that did not incorporate *C(2)M*-HA increased as the time between heat shock and fixation was increased (Figure 24). These results were used to estimate the duration of each stage in the germarium.
Heat shock induced expression of C(2)M was repeated in a c(2)M mutant background to determine if cohesins could be loaded and the SC assembled late. In the absence of heat shock, the typical patchy SC phenotype of c(2)M mutants was observed (Figure 10C, Figure 25C). Following heat shock, full length SC (with C(2)M and C(3)G in threads) was observed in region 2a, 2b and 3 oocytes (Figure 10D, Figure 25D). This suggests that, even if the SC is not assembled early in prophase due to the absence of C(2)M, assembly can resume if C(2)M becomes available later. The newly assembled SC in region 3 of the germarium appeared diffuse and disorganized, suggesting that late SC assembly was abnormal (Figure 10D).

To determine if heat shock induced c(2)M is functional, nondisjunction was measured. Because oocytes are laid in the order they develop, the effect of heat shock at successive stages of oogenesis could be measured. Heat shock induced expression partially reduced the nondisjunction frequency in the eggs laid 7-10 days after heat shock (Figure 11, Table 5). These oocytes were in the germarium, likely region 2a, at the time of heat shock (BHAGAT et al. 2004). These results can be explained by proposing that C(2)M must be loaded during a narrow window of time during early prophase to properly organize the SC and support crossing over. Rescue of the nondisjunction phenotype was not complete, suggesting that a single pulse of C(2)M is not sufficient, or there is a narrow window within the 7-10 day time point where C(2)M is required.

To directly test the effect of adding C(2)M late, we used the armadillo (arm) promoter to express C(2)M (RORTH 1998). In females carrying the combination of arm-GAL4 and UASp-c(2)M3XHA, C(2)M-HA protein expression was delayed in region 2a (Figure 10E, Figure 25). While C(3)G and C(2)M normally appear simultaneously, with
arm-, C(2)M appeared later than the first C(3)G. Furthermore, arm-GAL4 controlled expression of the c(2)M transgene did not rescue the nondisjunction phenotype of a c(2)M mutant, in contrast to MVD1 controlled expression (Table 5) (MANHEIM AND MCKIM 2003). Indeed, when C(2)M was expressed using arm-GAL4; UASp-c(2)M^{3XHA} in c(2)M mutant females, there was a delay in the appearance of SC threads, with more zygotene nuclei in region 2a than typical for wild type (Figure 10F, Figure 25F). These results suggest the timing of C(2)M expression and SC assembly is critical; the SC must assemble early in region 2a in order to promote crossing over.
Figure 10: Meiotic cohesin protein C(2)M is incorporated throughout pachytene
(A-D) C2M-HA was induced with a 1 hour heat shock and then fixed 6 or 24 hours later.
In all images, the DNA is in blue, C(3)G is in green, C(2)M-HA is in red and the scale bar
= 5 μm. Interpreting these experiments takes advantage of the germarium
organization, which contains approximately seven 16-cell cysts, each containing an oocyte. The cysts are arranged in temporal order, where the more posterior oocytes (e.g. region 3) have been in pachytene longer than more anterior ones (e.g. region 2a) and it takes about 12-24 hours for a cyst to move down one position (Figure 24). All oocytes carried the UASp-c(2)M\textsuperscript{30264} transgene either in a wild-type (A,B) or in a c(2)M mutant (C,D) background. C(2)M-HA was induced with a 1 hour heat shock and then fixed 6 (A) or 24 (B,D) hours later, or without heat shock (C). (E,F) Oocytes from the indicated region with C(2)M-HA induced by arm-GAL\textsuperscript{4} in either wild type (E) or a c(2)M mutant (F) background. See also Figure 24 and Figure 25.
Figure 11: Rescue of nondisjunction defect by heat shock-induced expression of C(2)M
A) Schematic showing the timeline of oocyte development (SPRADLING 1993; JIA et al. 2016). B) Nondisjunction in c(2)M; UASp-c(2)M^{3XHA}/hs-Gal4 females measured following 1 hour of heat shock and the number of days indicated within each bar. Significant reductions in nondisjunction were observed in the 6-9 (p=0.003) day period of the 3-day broods and the 7-8 (p=0.003) and 9-10 (p=0.02) periods of the 2-day broods. Nondisjunction was calculated and statistical significance determined as described (ZENG et al. 2010). The data is in Table 5. See also Figure 25.
C(2)M is also removed from the SC throughout pachytene

When C(2)M was expressed using arm-GAL4, C(2)M was present in region 2b and absent in some (9/17, Figure 10F, Figure 25E) but present in other (8/17, Figure 10) region 3 oocytes. The loss of C(2)M in region 3 oocytes corresponds to the known decrease in arm-GAL4 expression in region 2b and 3 (ROTH 1998). Thus, these results show that there was complete turnover of SC-associated C(2)M between region 2b and region 3 oocytes, which is approximately 12-24 hours. Another promoter that expresses for a limited time is bam-GAL4:VP16, which peaks in 8-cell cysts of region 1 but usually extends in early region 2a (MATHIEU et al. 2013). When combined with the UASp-c(2)M3XHA transgene, C(2)M-HA expression was observed early in region 2a but absent in all region 2b or 3 cysts (n=7 germaria) (Figure 25). These experiments show that C(2)M is unloaded from chromosomes during pachytene.

Centromere cohesins SUNN and SOLO are not integrated during pachytene

The heat shock protocol was repeated with UASp transgenes encoding Venus-tagged SUNN (KRISHNAN et al. 2014). When UASp-sunn.Venus was expressed with MVD1, foci of SUNN at the centromeres were observed at all stages (Figure 12A, n=3, Figure 26A). In contrast to C(2)M, however, SUNN was not detectable in most pachytene oocytes 24 hours after heat shock induced expression of UASp-sunn.Venus (Figure 26B, n=10). In four of the 10 germaria, however, SUNN foci were visible in early region 2a (Figure 12B). The absence of foci in most pachytene oocytes, including all oocytes in late region 2a, region 2b and 3, suggests that centromeric SUNN is not loaded during pachytene. Similar results were found with SOLO (YAN AND MCKEE 2013) (Figure 26C,D). Furthermore, when we dissected and fixed ovaries 5 days following
heat shock to allow premeiotic cells to enter meiosis, region 2b centromeric SUNN was observed in 5 out of 9 germaria (Figure 12C, D). These results show that SUNN at the centromeres can only be loaded during a narrow window of meiosis, which is at or earlier than when foci were observed (region 2a) at the 24 hour time point. In germaria where no SUNN was observed, it is likely there were no oocytes that could load SUNN at the time of heat shock.

If these observations reflect the loading properties of cohesin complexes, then SMC1 or SMC3 should follow the patterns of both C(2)M and SUNN. Using a UASp-\textit{SMC1}^{3XHA} transgene, SMC1 that colocalizes with C(3)G at the centromeres and along the chromosome arms was observed throughout pachytene when expression was controlled by \textit{MVD1} (Figure 12E, Figure 26E). When SMC1 expression was induced with heat shock and fixed 24 hours later, both foci and threads were observed, although at different times (Figure 12E, Figure 26F,G). In 8/16 germaria there was thread-like SMC1 in regions 2b and 3, and all of these oocytes lacked SMC1 centromere foci (Figure 12F). The thread-like localization of SMC1 in some region 2b and 3 oocytes indicates that SMC1 is loaded during pachytene. It is unclear why the thread-like SMC1 is not observed in all germaria. Because the SMC1 signal in immunofluorescence experiments is weaker than C(2)M, it is possible there is a detection problem. Alternatively, SMC1 may not be loaded onto the arms during early pachytene. In 12/16 germaria, there was SMC1 foci in region 2a that colocalized with CID (Figure 12F). This restricted appearance is similar to heat-shock induced SUNN and may reflect loading that only occurs early, such as during meiotic S-phase. These region 2a cells with bright heat shock induced SMC1 foci always lacked threads, showing that the centromeric and euchromatic SMC1 proteins are loaded at different times in meiosis.
Figure 12: Centromeric cohesin protein SUNN is not incorporated during meiotic pachytene
(A-D) Oocytes are shown in merged image with SUNN-Venus in red, C(3)G in green and DNA in blue. CID is shown only in a single channel image. SUNN-Venus was expressed constitutively with using MVD1 (A) or was induced with a 1 hour heat shock and then fixed 24 (B) or 5 days later (C,D). The whole germarium in panel D shows a band of SUNN-Venus foci in region 2b (indicated by a bracket). (E) SMC1-HA (red) induced with MVD1 or expressed with 1 hour of heat shock and fixed 24 hours later. C(3)G is in green. (F) SMC1-HA (red) induced with MVD1 or expressed with 1 hour of heat shock and fixed 24 hours later. CID marks the centromeres in green (arrows). In all images, the DNA is in blue and the scale bar = 5 μm. Whole germarium images of each genotype is in Figure 26.
VI. Discussion

The two complexes are directed towards either sister or homolog interactions

Based on similar mutant phenotypes and double mutant analysis, we propose that SC assembly in *Drosophila* depends on two meiotic cohesin complexes. The first includes C(2)M, SA and Nipped-B. The most important function of C(2)M/SA/Nipped-B is SC assembly, which is demonstrated by the more significant SC assembly defects observed in c(2)M mutants and SA or Nipped-B knockdowns compared to sunn, solo or ord mutants. Our cytological results suggest that, like c(2)M (MANHEIM AND MCKIM 2003), SA has only a minor role in meiotic sister-centromere cohesion. Correlating with this difference is that C(2)M, SA or Nipped-B are required for the accumulation of SMC proteins on the chromosome arms but not the centromeres (Khetani AND Bickel 2007). Furthermore, Nipped-B, like C(2)M, localizes to the chromosome arms but not the centromeres (Gause et al. 2008). These observations indicate a significant change in cohesin regulation. While SA and Nipped-B are required for sister chromatid cohesion in mitotic cells, they have a new partner, C(2)M, for a non-cohesion function in meiosis. There are minor differences in the c(2)M and SA phenotypes, which has also been observed with solo and sunn (Krishnan et al. 2014), suggesting there could be additional minor complexes. SA and Nipped-B could be required to maintain sister chromatid cohesion on the chromosome arms in late prophase, a function that C(2)M likely does not have (Weng et al. 2014).

The second proposed meiotic cohesin complex includes SOLO, SUNN and ORD, which are also highly diverged, making homology assignments difficult. Based on sequence features, SUNN may be a SA homolog (Krishnan et al. 2014) while SOLO has
been shown to interact with SMC1 and to have sequence motifs similar to the SMC1 interaction domains of Kleisins (Yan et al. 2010; Yan and McKee 2013). The role of ORD in this context is unclear. It is possible that ORD is a positive regulator like Nipped-B. Genetic evidence shows that ord, sunn and solo are required for sister chromatid cohesion, which correlates with SMC1/3 and SC accumulation at the centromeres (Bickel et al. 2002; Yan and McKee 2013; Krishnan et al. 2014). In addition, there are elevated levels of sister chromatid exchange and abnormal SC structure in ord and solo mutants (Webber et al. 2004) (Yan and McKee 2013).

Surprisingly, we found an important role for the meiotic cohesins SUNN and ORD in mitotic germline cells, which is consistent with prior observations that ORD localizes to centromeric foci in premeiotic cells (Webber et al. 2004) and ord mutants have defects in mitotically dividing germline cells (Miyazaki and Orr-Weaver 1992). Since Rec8 in C. elegans is also observed in premeiotic cells (Pasierbek et al. 2001) (Pasierbek et al. 2001), it may be a conserved feature of meiotic cohesins required for sister chromatid cohesion that they accumulate and function in premeiotic mitotic germline cells.

Differential regulation and dynamics of meiotic cohesins

A striking difference between C(2)M/SA and SOLO/SUNN is that C(2)M incorporation is continuous during pachytene even after the SC is fully assembled. This may result in a dynamic SC, which has been observed in budding yeast (Voelkel-Meiman et al. 2012). Paradoxically, we have also found that C(2)M must load during a narrow window of early prophase in order to support crossover formation. Cohesins have been shown to be loaded during prophase in a number of systems (Rankin 2015) including
In contrast, centromeric SOLO/SUNN can only be loaded prior to meiotic prophase. Sister-chromatid cohesion in mitotic cells is established during S-phase (Peters and Nishiyama 2012) and in mammals, Rec8 cohesin cannot be replenished and dissociates with age (Chiang et al. 2010; Tachibana-Konwalski et al. 2010; Burkhardt et al.). Whereas in mitotic cells the dynamic and stable cohesin complexes involve the same four core subunits, in meiosis, there may be separate cohesin complexes that differ in their regulation and capacity to be replaced or replenished. These observations complement Weng et al. (Weng et al. 2014) who showed there was cohesin replenishment during meiotic prophase, although after pachytene and possibly not at the centromeres. Interestingly, mouse Nipbl (Nipped-B) (Kuleszewicz et al. 2013) and the meiosis-specific SMC1β (Biswas et al. 2013) show pronounced accumulation starting at leptotene, indicating that, as in Drosophila, some mouse cohesins are loaded during pachytene while other cohesins are stable.

**Evolutionary conservation and function of multiple cohesins complexes**

Meiosis-specific cohesin complexes appear to be a highly conserved feature of meiosis (Kitajima et al. 2003; Fukuda et al. 2014; Hopkins et al. 2014; Winters et al. 2014; Rankin 2015). While there is some variation in the constituents of each cohesin complex, our results suggesting two major pathways contributing to SC assembly help explain the results with coh-3/coh-4 and rec-8 in C. elegans (Severson et al. 2009) or rad21l and rec8 in mouse (Llano et al. 2012). Only the double mutants in each case eliminate all SC. The role of the respective Kleisins could also be conserved. Like C(2)M, Rad21L has been proposed to be primarily responsible for inter-homolog chromosome interactions (Lee and Hirano; Ishiguro et al. 2014). Multiple cohesin
complexes may be required because some cohesins need to be loaded at a specific time (S-phase for cohesion) while others need to be exchanged during pachytene. A dynamic cohesin complex may be important to provide plasticity to the meiotic chromosomes and allow them to respond to DSBs and regulate crossover formation, crossover interference and chromosome segregation. Alternatively, different cohesin complexes may accumulate at different locations. If meiotic cohesins, directly or indirectly, interact with SC proteins, they may have a strong influence on the pattern of SC assembly and influence the frequency and distribution of DSBs and crossovers.

VI. Materials and Methods

Drosophila strain and genetics

*Drosophila* stocks and crosses were maintained on standard medium at 25°C. The following mutant alleles were used unless otherwise noted: *ord*5 and *ord*10 (BICKEL et al. 1997), *c*(2)M*0810*, *c*(2)M*P2115*. Germline RNAi was performed using the following stocks from the Transgenic RNAi Project (TRiP) at Harvard Medical School (MANHEIM AND MCKIM 2003): *Nipped-B* (HMS00401 and GL00574), *Stromalin* (HMS00272 and GL00534), *vtd* (GL00522) and *SMC3* (GL00518). These transgenic lines express short hairpin microRNAs under the control of the GAL4/UAS system. They were crossed to *P{w^{mc}=tubP-GAL4}LL7* for ubiquitous expression (LEE AND LUO 1999). For germline expression, the following GAL4 lines were used: *P{w^{mc}= GAL4::VP16-nos.UTR}CG6325^{D1}*(RORTH 1998), *P{w^{mc}, GAL4-Hsp70, PB}89-2-1*(BRAND AND PERRIMON 1993), *P{w*, bam::Gal4-VP16}*(MATHIEU et al. 2013) and *P{w^{mW.hs}= GAL4-arm.S}4a P{w^{mW.hs}= GAL4-arm.S}4b* (SANSON et al. 1996). For the two genes with two hairpins, *SA* and *Nipped-B*, similar phenotypes were observed with both, although the
Valium 22 based lines (GL#) were used because they are optimized for germline expression (NI et al. 2011).

**P-element excision and Stromalin germline clones**

To create a mutation in the *SA* coding region, we mobilized the *P{EPgy2}EY20282* transposable element. The transposable element is 114 bp upstream of the *SA* coding region. Since we expected *SA* to be an essential gene, excisions were screened for a deletion by crossing to *Df(2L)Exel7029/Cyo*, a deficiency of the region, and testing for lethality. DNA for PCR was prepared from balanced adult flies. PCR was used to determine if any portion of the *P*-element remained by using a primer annealing to the repeat sequences flanking the *P*-element and either a reverse primer downstream of the *P*-element or a forward primer upstream. The excisions were further characterized by PCR using primers within the *SA* gene. For this PCR, embryos homozygous for the excision chromosomes were selected against a GFP-tagged second chromosome balancer (CASSO et al. 2000). DNA from homozygous embryos was prepared the same as adult flies. It was determined that excision *SA*86 has deleted more than 96 bp but less than 813 bp of the coding region.

A recombinant chromosome was made using the mutant *SA*86 allele and *al dp b pr P(neoFRT)40A* to create *SA*86 *pr P(neoFRT)40A*. This recombinant was used to generate germline mutant clones using the FRT/FLP system (CHOU AND PERRIMON 1996). Homozygous mutant cells in the germlines of *SA*86 *pr P(neoFRT)40A / P(Ubi-mRFP.nls)2L P(neoFRT)40A* females that were expressing FLPase were identified by the lack of the RFP signal.
**qRT-PCR to measure the level of mRNA knockdown**

Using *P{tubP-GAL4}LL7* to induce ubiquitous expression of shRNAs from the Transgenic RNAi Project (TRiP) collection, *vtd*, *SA*, and *Nipped-B* caused lethality, indicating the targeted proteins were being knocked down. Quantitative RT-PCR was used to measure the level of mRNA knockdown using shRNA expressed with *P{w^mC=GAL4::VP16-nos.UTR}CG6325^{Mwj}*. RNA was prepared from stage 14 oocytes using TRIzol® Reagent (Life Technologies). cDNA was made from the harvested RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan® Assays were used to measure the level of expression of the gene of interest. Assays used were *vtd Dm03419424_g1*, *Stromalin Dm01842873_g1*, *Nipped-B Dm2587436_g1*, *SMC3 Dm01813788_g1* and *Rpl140 Dm02134593_g1* for a control. A StepOnePlus™System (Applied Biosystems) was used to run the qRT-PCR reaction and analyze the results.

**Cytology and immunofluorescence of germaria**

For immunolocalization experiments, females were aged at 25° temperature for about 16 hours with yeast paste and ovaries were dissected and fixed using the “Buffer A” protocol (McKim *et al.* 2009). The ovaries from 10-15 flies were dissected in 1x Robb’s media in one well of a two well plate and moved to the second clean well containing fresh media. A tungsten needle was used to remove the ovariolar sheath and to tease the ovaries apart. After no more than 20 minutes, the separated ovaries were moved to the cap of a graduated 1.5 ml Eppendorf tube containing 500 μl of Buffer A fixative solution for 10 min at room temperature. Following several washes, the primary antibodies were diluted into a volume of 300 μl. Primary antibodies were mouse anti-
C(3)G used at 1:500 (Page and Hawley 2001), rabbit anti-C(3)G used at 1:500 (Anderson et al. 2005), rabbit anti-C(2)M used at 1:400 (Manheim and McKim 2003), rabbit anti-CID used at 1:100 (Henikoff et al. 2000), chicken anti-CID used at 1:100 (Blower and Karpen 2001), a combination of two mouse anti-ORB antibodies (4H8 and 6H4) used at 1:100 (Lantz et al. 1994), rat anti-SMC1 used at 1:250 (Malmanche et al. 2007), rat anti-HA used at 1:15 (Roche), mouse anti-Spectrin used at 1:50 (DSHB), rat anti-VASA used at 1:300 (DSHB), and a rabbit anti-GFP antibody used at 1:400 (Life Technologies). The secondary antibodies were conjugated to either Cy3, Cy5 (Jackson Labs) or Alexa fluor 488 (Invitrogen). Chromosomes were stained with Hoechst 33342 at 1:10,000 (10mg/ml solution) for seven minutes at room temperature. Images were collected using a Leica TCS SP5 or SP8 confocal microscope with a 63X, N.A. 1.4 lens. In most cases, whole germaria were imaged by collecting optical sections through the entire tissue. These data are presented as maximum projections of a confocal image stack encompassing an entire oocyte nucleus. The analysis of the images, however, was performed by examining one section at a time. All the data comes from at least two independent experiments.

For immunolocalization experiments on females with underdeveloped ovaries, females were aged and yeasted the same way as larger ovaries. After dissecting the ovaries from 10-15 flies, a tungsten needle was used to remove the ovariolar sheath. After no more than 20 minutes, the 1x Robb’s solution was removed from the well and 150 μl of Buffer A fixative solution was added to the well with the ovaries for 10 min at room temperature. After several washes in the well of the plate ovaries were incubated with primary antibodies diluted in 150 μl in a humidified chamber overnight at 4°C.
same primary and secondary antibodies and chromosome stain were used at the same concentrations as with larger ovaries.

**Cytology and immunofluorescence of late stage oocytes**

Late-stage oocytes were prepared using formaldehyde/heptane fixation (RADFORD et al. 2015). Approximately 100–300 mated females were fattened on yeast with males for 3–5 days then briefly pulsed in a blender to disrupt abdomens. Late-stage oocytes were separated from bulk fly tissues and then fixed in a 5% formaldehyde solution. After 2.5 minutes an equal amount of heptane was added and the oocytes were vortexed. Chorion and vitelline membranes were removed by rolling between the frosted part of a glass slide and a coverslip. Rolled oocytes were extracted in PBS/1% Triton X-100 for 1.5–2 hr and blocked in PBS/0.1% Tween 20/0.5% BSA for 1 hr, and then antibodies were added. Primary antibodies used were mouse anti-α-tubulin conjugated to FITC used at 1:50 (clone DM1A, Sigma), rabbit anti-CENP-C used at 1:5000 (HEEGER et al. 2005). Secondary antibody used was goat anti-rabbit used at 1:250 conjugated to Cy3 (Jackson Immunoresearch). DNA was labeled with Hoechst 33342 (1:1000, Invitrogen). Images were collected on a Leica TCS SP5, or SP8 confocal microscope with a 63X, N.A. 1.4 lens. Images are shown as maximum projections of complete image stacks. Image analysis was performed with Imaris image analysis software (Bitplane).

**Pulse expression of transgene lines using a heat shock promoter**

For reincorporation studies, tagged transgenes UASp-c(2)M3XHA (MANHEIM AND MCKIM 2003), UASp-solo.Venus (YAN AND MCKEE 2013), and UASp-sunn.Venus (KRISHNAN et al. 2014) and UASp-SMC13XHA (see below) were expressed using P(w^mc GAL4-Hsp70, PB)89-2-1. Female flies containing both P(w^mc GAL4-Hsp70, PB)89-2-1 and the
transgene were heat-shocked at 37°C for one hour. After heat shock females were aged at room temperature for 24 hours with yeast. SUNN.Venus expressing flies were aged for 5 days and they were given yeast paste 24 hours before dissection. Ovaries were dissected, fixed and prepared using the “Buffer A” protocol as stated above.

The frequency of \(X\) chromosome nondisjunction (INDJEIAN et al.) was determined by crossing the females to \(y \ w/\mathcal{B}^Y\) males and calculated as \(2(X\text{-ND progeny})/\text{total progeny}\), where total progeny = \([2(X\text{-ND progeny}) + (\text{regular progeny})]\). For experiments to measure \(X\)-chromosome nondisjunction following heat shock, the females were transferred to new food (or “brooded“) every 2 or 3 days.

**Construction of SMC1 transgene**

Full length coding sequence of SMC1 subcloned into pENTR-D/TOPO (Invitrogen) was obtained from Oren Schuldiner (SCHULDINER et al. 2008). An expression vector encoding full-length SMC1 fused to a C-terminal 3×HA tag under control of the \(UASp\) promoter was created by a Clonase LR reaction with the pPWH vector (DGRC). Germline transformations were performed by Model Systems Genomics (Durham, NC) to generate transgene lines.
CHAPTER 3: MEIOSIS SPECIFIC KLEISIN,C(2)M, FUNCTIONS WITH OTHER COHESIN SUBUNITS, BUT POSSIBLY NOT IN TRADITIONAL RING STRUCTURE

I. Preface

This chapter will be published after a few more experiments are conducted. My contributions to this project and paper were writing the paper and all of the experiments.

II. Abstract

Meiosis is the process by which sexually reproducing diploid organisms make haploid gametes. During this process sister chromatids are held together by the cohesion complex and, homologous pairs, by the synaptonemal complex (SC). Both of these complexes are important for accurate segregation, as they ensure that the chromosomes can be attached to the proper spindle pole in metaphase. If the chromosomes are not attached to opposite spindle poles they will not segregate properly. Meiosis-specific cohesin complexes have been demonstrated in many organisms. These complexes are comprised of some meiosis specific subunits mixed with subunits that are also found in mitosis, making these complexes as a whole, unique ones. Although meiosis specific complexes have been identified in many organisms, the function of them is still not well understood. In Drosophila, there are two meiosis specific complexes, one that contains SMC1/SMC3/SUNN/SOLO and the other complex contains SMC1/SMC3/C(2)M/SA. It has been shown that the SUNN/SOLO complex is involved in keeping sister centromeres together, but the C(2)M/SA complex is not. The C(2)M/SA complex is responsible for a majority of the synaptonemal complex (SC) assembly. We have explored the structure of the C(2)M complex and how this complex
promotes SC assembly. The experiments presented here provide evidence that C(2)M can induce the assembly of SC in a germline mitotic cell.

III. Introduction

During metaphase of meiosis I, sister chromatids are held together by the cohesin complex of proteins and the homologous pairs are held together with the synaptonemal complex (SC). In *Drosophila*, meiotic recombination starts with formation of double strand breaks (DSBs). DSBs can be repaired as crossovers or non-crossovers. If the DSB is repaired through the crossover pathway and the sister chromatids are held with cohesion, a chiasma will be formed. Once chiasmata are formed, the SC dissociates and the homologs remain tethered. This allows for the homologous pairs to remain attached so they can line up at metaphase I and segregate at anaphase I. Without either the cohesin complexes or the SC, chromosomes will not segregate accurately and aneuploidy results.

Mitotic cohesin complexes are well studied, however, there is still much to learn about meiotic cohesin complexes. Meiotic cohesin complexes contain some different subunits than mitotic cohesion complexes and have been identified in many organisms including *C. elegans*, *Drosophila*, yeast, and mouse. Most organisms have meiosis-specific subunits, and all of these have a meiosis-specific Kleisin. In *C. elegans*, the meiotic-specific Kleisin proteins are named Rec8, Coh-3 and Coh-4, in *Drosophila* the meiotic Kleisins are SOLO and C(2)M, in fission yeast it is Rec8, and in mouse there are Rec8 and Rad21L. These Kleisins all have a role in accurate chromosome segregation during oocyte formation. The function of the Kleisin is to interact with both of the SMC proteins and to close the ring, enabling strands of DNA to be closed within. The Kleisin has three domains, two head domains that interact with the SMC proteins, and a linker
domain that connects the two. The linker domain is also the region of the protein that interacts with the Stromalin subunit and, therefore, we refer to it as the Stromalin binding domain (SABD).

We previously explored the function of the *Drosophila* meiosis-specific cohesin complexes ([Gyuricza et al. 2016](#)). The C(2)M/SA complex is not involved in keeping sister chromatid centromeres together, however, the complex is involved in assembly of the majority of the SC. Because the complex is not involved in the canonical role of cohesin complexes (sister chromatid cohesion), we are interested in testing if the complex has the same structure as the mitotic complex. Previous studies have identified residues that are responsible for mediating the interaction between the Kleisin and SMC proteins (Figure 13). Based on sequence alignments, C(2)M residue F89 should mediate the interaction with SCM3, while F524 and L528 should mediate the interaction with SMC1. In *S. cerevisiae*, the homologous amino acids are Y82, F528 and L532 respectively ([Haering et al. 2004](#); [Gligoris et al. 2014](#)). In order to test if the interactions between C(2)M and the SMC proteins are important for the function of the complex, we mutated each of these three residues to alanine.

These experiments have provided new insight into the function of the meiosis-specific complex C(2)M/SA and the reason meiosis-specific complexes are present and conserved among so many organisms. While our evidence is consistent with this complex forming a ring, as typical cohesion rings do, we will test if a closed ring is required or if a “broken” ring structure is sufficient to do the job of the meiosis-specific cohesin. Finally, these experiments will shed light on the mechanism by which C(2)M is able to assemble the SC.
Figure 13: 3D Models of the interaction between the SMCs and Rad21.
A). The interaction between SMC3 and Rad21 in *Saccharomyces cerevisiae*. Blue is SMC3, cyan is Rad21, and in magenta is Y82, the amino acid homologous to the *Drosophila* amino acid mutated in these studies. B). A blow up to show the interaction between SMC3 and Rad21. C). The interaction between SMC1 and Rad21. Blue is SMC1, cyan is Rad21 and in magenta are F528, L532, the two amino acids homologous to the *Drosophila* amino acids mutated in these studies.
**IV. Results**

**SA localization is dependent upon C(2)M and Nipped-B but not VTD**

In order to determine the localization of SA, a MYC tagged transgene under control of *UASp* was created. We expected that SA would localize to the chromosome axis as this is where many other cohesins localize (Gause et al. 2008). In a wild type background, when expressed with MVD1, a germline-specific promoter, SA-MYC was detected in a more diffuse pattern than was expected. SA-MYC was also detected along the chromosome axis in the oocytes, and in many more cells than just the oocyte (Figure 14A). The more diffuse staining of SA-MYC could be due to excess transgene expression in the nucleus, or SA-MYC localizing to the chromatin and not just at the chromosome axis. However, when SA-MYC was expressed with *P(GAL4-nos.NGT)*A (referred to here as NGT), a promoter known to not express as highly as MVD1 in the germarium, the diffuse staining persisted (not shown). This suggests the observed expression pattern is not due to overexpression. When SA-MYC was expressed with MVD1 in a c(2)M mutant background, the SA-MYC localization was abolished, even in the regions, such as the diffuse chromatin, where C(2)M is not detected (Figure 14B). This indicates that C(2)M may be localizing to locations we are not able to detect or that C(2)M is able to affect SA without a direct interaction. Either way, it is clear that SA-MYC is dependent upon C(2)M. This could indicate that the C2M/SA complex has roles outside of the chromosome axis. It also could mean that when expressed with MVD1 or NGT, SA is too highly expressed and there are large amounts of it filling up the nucleus and in the absence of C2M, SA is not stable and degraded.

Since SA is dependent upon the meiosis-specific Kleisin, C(2)M, we tested if it is also dependent upon VTD. VTD is the Kleisin subunit SA interacts with in mitosis and is
proposed to interact with in germline mitosis (GYURICZA et al. 2016). vtd RNAi however, has no SC assembly defect and therefore it may not to interact with SA during the meiotic divisions (GYURICZA et al. 2016). To test this hypothesis, we expressed the SA-MYC transgene in a vtd shRNAi background. We observed that in the presence of vtd RNAi SA is localized to the chromosome axis indicating that VTD does not play a role in SA localization (Figure 14C).

Nipped B is a regulator of the mitotic cohesin complex SMC1/SMC3/VTD/SA. This complex shares many of its subunits with the meiotic SMC1/SMC3/C(2)M/SA. It also appears to share its regulator, Nipped-B. Nipped-B knockdown has a similar cytological phenotype to both SA knockdown and C(2)M mutants (GYURICZA et al. 2016). In order to test if SA localization was dependent upon Nipped-B, we co-expressed Nipped-B shRNA and SA-MYC. In the presence of the Nipped-B shRNA, SA-MYC is not able to localize to the chromosomes (Figure 14D). However, in some cells, particularly very early region 2a and region 3, there is some SA in the cytoplasm (Figure 14D). This result is not as severe as the abolition of SA localization in a c(2)M null mutant. This discrepancy is possibly due to the fact that in the Nipped-B shRNA the mRNA message is only depleted by 70% (GYURICZA et al. 2016). Absent SA localization in late region 2a and 2b is consistent with those regions having the highest amount of expression of the shRNA with the MVD1 promoter (Figure 14D).
Figure 14: SA-MYC localization is dependent upon C(2)M and Nipped-B but not VTD. C(3)G is in green, MYC is in red, and blue is the DNA. A) SA-MYC in a WT background. B) SA-MYC in a c(2)M mutant background. C) and D) are in vtd and Nipped-B RNAi backgrounds respectively. SA localizes to the chromosome axis in WT A) and vtd RNAi C), but is unable to localize in c(2)M B) and Nipped-B RNAi D). E) Proposed meiotic cohesin complexes. Scale bars are 5µm.
SA is required for meiotic crossing over and chromosome segregation as is C(2)M

Synapsis defects such as those observed in S4 knockdowns should result in reductions in crossing over and a corresponding increase in nondisjunction (Lake and Hawley 2012), (Manheim and McKim 2003). To knock down cohesin proteins during prophase, but allow for cohesin expression during embryogenesis so that we can genetically examine chromosome segregation, we used a transgenic line, P{GAL4-nos.NGT}A (referred to here as NGT), that expresses GAL4 in the germarium but not in later stages (Parikh and Radford, per. Comm.). This pattern of expression should cause protein deletion in early prophase oocytes but allow protein loading into the oocyte for embryonic development. Indeed, high levels of X-chromosome nondisjunction were observed when NGT was used to express SMC3 or S4 shRNA in the germarium (Table 1, Table 2), consistent with a defect in SC assembly and crossover formation.

To measure crossing over, S4 and SMC3 shRNA were expressed in females heterozygous for a marked X-chromosome (y w cv m f • y+/ y w) which allows us to detect crossovers by looking for these visible markers (Table 1). All of these visible marker are recessive and therefore are only visible when the fly is homozygous. When y is mutated a yellow body results, when w is mutated white eyes result, mutated cv yields wings lacking cross veins, mutated m leaves the flies with smaller wings, and mutated f results in the bristles being frayed. The centromere is represented by the • symbol. In these females, one of the X-chromosome centromeres was marked with the y+ marker, which allows sister chromatid nondisjunction events to be detected. Only if the sister chromatids do not segregate properly will we see a female fly that is y+. As expected from the nondisjunction data, the knockdowns of S4 and SMC3 had severe
effects on crossing over (Table 1). It is expected that knockdown of cohesins have an
effect on sister chromatid segregation because it is known that in the absence of
cohesion there is a higher instance of non-disjunction. Surprisingly, however, no
nondisjunction of sister chromatids was detected. This same result was obtained using
a different method to detect sister chromatid nondisjunction (not shown). Overall, when
cohesion proteins are knocked down, there is a higher instance of inaccurate
chromosome segregation and a decrease in crossing over, consistent with the published
SA cytology results. Our explanation for the lack of sister chromatid nondisjunction in
SMC3 knockdown oocytes is there may be a different threshold for cohesion and SC
assembly functions. It is possible that a smaller amount of cohesin complexes are
sufficient for cohesion than SC assembly function. Alternatively, SMC3 protein required
for cohesion may be loaded in late prophase where NGT does not express.
Table 1: Crossing over and sister chromatid segregation in *SMC3*, *SA* and *c(2)M* RNAi females

X-chromosome genotype was either *yw cv m f* • *y+ w* or *yw FM7*

*y♀* indicates sister chromatid non-disjunction

<table>
<thead>
<tr>
<th>Genotype</th>
<th><em>cv m</em> (m.u.)</th>
<th><em>mf</em> (m.u.)</th>
<th>Total males</th>
<th><em>y♀</em> or B+♀</th>
<th>NDJ %</th>
<th>Total progeny</th>
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<tr>
<td><em>ORD bam</em></td>
<td>23.6</td>
<td>18.8</td>
<td>560</td>
<td>2.8</td>
<td>1076</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>21</td>
<td>19</td>
<td>719</td>
<td>0.4</td>
<td>1614</td>
<td></td>
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<tr>
<td><em>SMC3 NGT</em></td>
<td>1.2</td>
<td>1.6</td>
<td>428</td>
<td>1</td>
<td>35.7</td>
<td>1198</td>
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<tr>
<td><em>SMC3 bam</em></td>
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<td>4.0</td>
<td>201</td>
<td>26.2</td>
<td>478</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>17.8</td>
<td>17.6</td>
<td>653</td>
<td>0</td>
<td>1464</td>
<td></td>
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<tr>
<td><em>SA NGT</em></td>
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<td>31.4</td>
<td>1434</td>
<td></td>
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<td><em>c(2)M MVD1</em></td>
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<td>25.7</td>
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<td><em>c(2)M NGT</em></td>
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<td>11.0</td>
<td>190</td>
<td>20.1</td>
<td>624</td>
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Table 2: Nondisjunction with different GAL4 lines with various shRNA lines

w1118 is used as the wild type control. Knocking down each of the cohesin subunits
results in a increased level of non-disjunction. Genotype of the female yw/+;
shRNA/Gal4. Genotype of the male is y Hw w/B^Y.

<table>
<thead>
<tr>
<th>RNAi Genotype</th>
<th>Oocyte genotype</th>
<th>Nondisjunction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XXY ♀</td>
<td>XO ♂</td>
</tr>
<tr>
<td><strong>w1118</strong></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td><strong>SMC3 NGT</strong></td>
<td>109</td>
<td>122</td>
</tr>
<tr>
<td><strong>SA NGT</strong></td>
<td>159</td>
<td>153</td>
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<tr>
<td><strong>ord NGT</strong></td>
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<td>2</td>
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<tr>
<td><strong>ord MVD1</strong></td>
<td>69</td>
<td>71</td>
</tr>
<tr>
<td><strong>c(2)M NGT</strong></td>
<td>95</td>
<td>99</td>
</tr>
<tr>
<td><strong>c(2)M MVD1</strong></td>
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<td>38</td>
</tr>
<tr>
<td><strong>c(2)M arm</strong></td>
<td>42</td>
<td>81</td>
</tr>
<tr>
<td><strong>vtd NGT</strong></td>
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<td>1</td>
</tr>
</tbody>
</table>
**SA and C(3)G dynamics are similar to that of SMC1**

The germarium is the first stage of the *Drosophila* ovary. It is arranged in a temporal manner with the earliest stages being most anterior and the later stages being more posterior. This arrangement of the ovaries allows us to be able to see many time points of oocyte development in one image. We have previously published that C(2)M-HA is able to be incorporated into the chromosome axis throughout prophase, in contrast to SMC1 which incorporates at distinct times in prophase (Gyuricza et al. 2016). In order to determine if the dynamics of SA and C(3)G are similar to SMC1 or C(2)M, we assayed for incorporation after a one hour pulse expression of the transgenic protein with a heatshock promoter (Gyuricza et al. 2016). The oocytes were fixed at various time points after the one hour heatshock. Unless otherwise noted the oocytes were fixed 24 hours after heatshock.

As previously published, SMC1 and C(2)M have different incorporation patterns when studied with this assay. SMC1 was incorporated in threads during late pachytene and in foci associated with the centromere in zygotene or early pachytene. C(2)M on the other hand is incorporated in large amounts in oocytes and nurse cells throughout the germarium. Both SA-MYC and C(3)G-Flag are incorporated in threads during late pachytene but not during earlier stages (Figure 15). This is similar to SMC1 and indicates that there may be more than one population of C(2)M, one that is associated with the other cohesins and is less dynamic and another whose function is unknown and is highly dynamic. The SA-MYC ovary was fixed at three different time points after heatshock, 3 hours, 6 hours, and 24 hours. Incorporation of the SA-MYC was only seen in the 24 hour time point. This indicates that SA dynamics are different than that of C(2)M, which we were able to see after both the 3 and 6 hour time points.
Figure 15: SA and C(3)G have similar dynamics, different from SMC1. Green shows the C(3)G, red is the transgene tag. In panel A it is MYC and in B Flag. Blue is the DNA. Shown in each panel is a selected oocyte from an early stage (2A) and a later stage (3) as well as a whole germarium. This shows that both SA and C(3)G are not incorporated early but are late in the germarium. Panel C shows the proposed meiotic cohesin complexes. Scale bars are 5µm.
**Mutational analysis of C(2)M**

In order to characterize how C(2)M promotes SC assembly and how it interacts with the SMC proteins, a mutational analysis was conducted. A previously published wild type C(2)M transgene tagged with HA on its N-terminus, HA-C(2)M, was used as a control (Manheim and McKim 2003). In both wild type and a c(2)M mutant background, the HA-C(2)M is able to localize properly to the chromosome axis and rescue the SC assembly phenotype of the c(2)M mutant (Figure 17A). Nondisjunction in progeny arising from females with this transgene expressed with MVD1 in both a wild type and mutant background and both had wild type levels of nondisjunction (Table 3). This data indicates that this transgene does not have a dominant phenotype when expressed in a wild type background and is able to rescue the mutant phenotype.

Another C(2)M transgene, that has a HA tag on the C-terminus, was expressed with MVD1 in both the wild type and mutant background. In the wild type background, C(2)M-HA was able to localize to the chromosome axis normally however, in the mutant background, in the absence of endogenous C(2)M, C(2)M-HA was not able to localize and rescue the mutant phenotype (Figure 17C). Nondisjunction was also measured and C(2)M-HA expressed in a wildtype background showed normal levels of non-disjunction, whereas C(2)M-HA expressed in the mutant background showed a significantly higher level of non-disjunction (Table 4). C(2)M-HA is not able to interact with the chromosome axis, in the absence of wild type C(2)M, suggesting that the C-terminus of C(2)M is required for this interaction. Because C(2)M-HA is able to localize in the presence of endogenous C(2)M but not when endogenous C(2)M is lacking, we hypothesize that the wild-type C(2)M protein is able to mediate interactions between the chromosome axis and other C(2)M subunits such as C(2)M-HA.
C(2)M has three domains, the N-terminus, Stromalin Binding Domain, and the C-terminus. Shown here are the locations of the three point mutations that are explored here.

Figure 16: Schematic of domains within C(2)M

C(2)M has three domains, the N-terminus, Stromalin Binding Domain, and the C-terminus. Shown here are the locations of the three point mutations that are explored here.
We also made point mutations in the HA-C(2)M transgene (Figure 16). These mutants were designed to abolish the interaction between C(2)M and the SMC proteins. The three point mutants are F89A which should disrupt interactions with SMC3 and F524A and L528A which should disrupt interaction with SMC1 (SCHLEIFFER et al. 2003). When each of these transgenes was expressed in a wild type background, they were able to localize in threads along the chromosome axis, just as wild type C(2)M does (Figure 17A). Nondisjunction levels of HA-C(2)M- F89A and L528A in a wild type background are also similar to wild type indicating that these transgenes are not having a dominant negative phenotype (Table 4). The HA-C(2)M- F89A transgene was also expressed in a c(2)M null mutant background to determine if the mutated transgene could rescue the null phenotype. The HA-C(2)M-F89A localized to the chromosome axis in the absence of endogenous C(2)M and rescued the patchy C(3)G phenotype, as determined by the presence of C(3)G threads (Figure 17C). The transgene also rescued the c(2)M mutant phenotype of increased levels of nondisjunction (Table 4). This indicates that the F89 site is not critical for the C(2)M SC assembly function. This could be because this is not the residue that is responsible for mediating the interaction between C(2)M and SMC3, or because C(2)M does not need to interact with SMC3 to promote SC assembly. Yeast two-hybrid experiments will be performed to determine if F89A abolishes the interaction between C(2)M and SMC3. Co-immunoprecipitations will also be performed, however, because of the ring structure, preventing the C(2)M – SMC3 interaction is not expected to affect a co-IP result. Instead, these experiments could be performed with expression of a N-terminal domain fragment (see below) with F89A. Likewise, these experiments will be performed with the remaining mutant transgenes F524A and L528A.
Figure 17: C(2)M transgenes incorporate into the chromosome axis. C(3)G is in green, the indicated C(2)M transgene is in red and DNA is in blue. A) Full length C(2)M tagged on both the C- and N-terminal ends as well as the three point mutants (F89A, F524A and L528A) in a Wildtype background. B) N-terminal portion of C(2)M in a wild type background. C) Full length C(2)M tagged on the C-terminal end and F89A transgene expressed in a mutant C(2)M background. Scale bars are 5µm.
Table 3: Nondisjunction of C(2)M transgenes in WT background
Genotype of the male is y Hw W/By

<table>
<thead>
<tr>
<th>Female Genotype</th>
<th>Oocyte genotype</th>
<th>Nondisjunction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XXY ♀</td>
<td>XO ♂</td>
</tr>
<tr>
<td>HA-c(2)M/ MVD1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>c(2)M-HA/ MVD1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>HA-c(2)M F89A/MVD1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HA-c(2)M L528A/MVD1</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>HA-c(2)M NTERM/MVD1</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 4: Nondisjunction of C(2)M transgene in mutant c(2)M background
Genotype of the male is y Hw W/B^y

<table>
<thead>
<tr>
<th>Female Genotype</th>
<th>Oocyte genotype</th>
<th>Nondisjunction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XXY ♀</td>
<td>XO ♂</td>
</tr>
<tr>
<td>c(2)M/c(2)M; HA-c(2)M/MVD</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>c(2)M/c(2)M; c(2)M-HA/MVD</td>
<td>86</td>
<td>68</td>
</tr>
<tr>
<td>c(2)M/c(2)M; HA-c(2)M F89A/MVD</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>c(2)M/c(2)M; HA-c(2)M NTERM/MVD1</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>
**C(2)M domains aid in determining differential roles of the protein**

In hopes of separating the different functions of C(2)M, each of its three domains were cloned separately. The N-terminal fragment of C(2)M tagged with HA was expressed in a wild-type background with MVD1 and based on colocalization with CID, was only detected at the centromeres (Figure 17B). This is interesting because wild type C(2)M does not localize to the centromere. The centromere is where the SMC1/SMC3/SOLO/SUNN complex localizes and the C(2)M complex is normally excluded. It could be that the other domains of C(2)M (either the C-terminal domain or the Stromalin binding domain) are required to localize C(2)M to its proper location and in its absence C(2)M localizes to centromeres. The other two domains will be tested in the same manner. If it becomes clear that either the C-terminal domain or the Stromalin binding domain are required for proper localization, then we would expect that domain to be able to localize properly in the absence of the rest of the protein.

**Inducing germline mitosis to form SC**

C(2)M expression is seen in a wild type germarium in region 2a (MANHEIM AND MCKIM 2003). The region 1 cells, cells that are undergoing mitotic divisions, have cohesion complexes that contain SMC1/SMC3/SOLO/SUNN and SMC1/SMC3/Rad21/SA. However, when a C(2)M-HA transgene is expressed using MVD1, C(2)M is incorporated throughout the germarium along the chromosome axis including oocytes in region 1. It is also observed that C(2)M is overexpressed under control of MVD1, threads of C(2)M (and in turn C(3)G) form in more cells per cyst than normal. These results indicate that the presence of C(2)M may be sufficient to initiate SC assembly because all other cohesins are present in region 1 of the germarium.
In order to test if C(2)M is able to initiate the switch from germline mitosis to meiosis and initiate SC assembly, we used a shRNAi against *bag of marbles (Bam)*. It has previously been published that in the absence of *bag of marbles* (Bamba, 2002 #2368) gametogenesis does not occur, but instead, in place of cells that have entered meiosis is a vast amount of mitotically dividing germline cells (McKearin and Spradling, 1990). This results in a bag of germline stem cells instead of a Germaine with developing oocytes. There is no C(3)G staining, indicating that the cells are not entering meiosis (Figure 18B). The large amount of cells in mitotic germline divisions was used to test the hypothesis that C(2)M is able to initiate the meiotic divisions and assembly of the SC. When the C(2)M-HA transgene was expressed with MVD1 in a *bam* shRNAi background, most of the mitotic cells in the germline started to assemble SC, indicating that C(2)M is sufficient to initiate SC assembly in mitotic cells (Figure 18C). This may point to C(2)M being a factor that can trigger the onset of SC assembly.

It is of use for us to have a large amount of synchronously staged oocytes to perform biochemical assays on such as co-immunoprecipitations. In order to see if we were able to induce a large amount of cells in the germarium to enter meiosis at the same time, we induced expression of a *bam* transgene with heatshock in a *bam* mutant background. The heatshock was a one hour pulse and then the germarium were fixed at 1, 2 and 5 day time points. The *bam* mutant normally has very small patches of C(3)G staining and the germarium are much larger than normal with an abnormally large amount of cells per cyst. The one day time point looked much the same. In the two day time point there was a large amount of C(3)G, that almost formed threads but still looked abnormal compared to wild type threads of C(3)G. The five day time point...
reverted back to the mutant phenotype indicating that the wave of heat shocked
oocytes had progressed to the vitellarium (Figure 18D).
Figure 18: Expression of C(2)M in meiotic germline induced SC assembly. C(3)G is in green and marks the SC. HA is in red and DNA is in blue. A shows C(2)M-HA expression in a wildtype background using the MVD1 promoter, which begins expression in the pre-meiotic divisions. A’ is a blow up of the pre-meiotic region, region 1, and shows SC assembly in this region. B shows the bam RNAi also expressed with MVD1. In this panel there are no cells forming much SC and the gerarium is much larger than normal. B’ shows a blow up of the earliest region in the bam RNAi. C shows C(2)M-HA expressed in a bam RNAi background. SC is able to assemble in this case as well. Scale bars are 5µm.
V. Discussion
Two populations of C(2)M: one associated with cohesins, the other not

*Drosophila* SC assembly is dependent upon two pathways made up of two groups of cohesin proteins, SMC1/SMC3/C(2)M/SA and SMC1/SMC3/SOLO/SUNN (TANNE ET AL. 2011; GYURICZA ET AL. 2016). Whether or not these pathways are actually dependent upon these proteins forming a ring like structure like the rings used for cohesion is not well understood. One meiosis-specific Kleisin in mice, and possible C(2)M ortholog, Rad21L, does interact with the other cohesin proteins to form a ring (LEE, 2011 #2522). From my experiments it is, however, clear that the localization of SA is dependent upon C(2)M and Nipped-B but not VTD, the Kleisin subunit in *Drosophila* mitosis. This can be explained because VTD is not functioning in meiosis, but rather in mitosis. The fact that the localization of SA is dependent on C(2)M and Nipped-B supports that they are all functioning together to promote SC assembly. Similarly, the fact that SA and C(2)M have similar levels of nondisjunction and crossing over levels, and neither are required for sister chromatid cohesion, also supports that they are working together in the same pathway.

The only difference that has been found between SA and C(2)M subunits are the dynamics. I found that the dynamics of SA and C(3)G are more similar to the dynamics of SMC1 rather than that of C(2)M. This could be because there are two populations of C(2)M. One that is associated with the rest of the cohesin proteins such as SMC1 and SA and is less dynamic. The other population of C(2)M is not associated with other cohesins and is highly dynamic. This is similar to how meiosis-specific Kleisins function in *C. elegans*. Coh-3/Coh-4 is thought to have both a population that associates with the cohesins and does cohesion, as well as a population that does not interact with the
cohesins and is involved in SC assembly (Severson, 2014 #3098). Another possibility is that C(2)M is always associated with the cohesin ring, but its dynamics differ because the C(2)M subunit can be exchanged while the rest of the ring remains intact and associated with the chromosomes. This raises the question: If there is a population of C(2)M not functioning with cohesins, what proteins does it work with and what does it do?

**Mutational analysis of C(2)M may lead to a novel role for the Kleisin**

In order to determine if the population of C(2)M functioning with the other cohesins is forming a traditional ring structure, I made mutants to abolish the amino acid residues thought to mediate this interaction. However, even in the absence of any one of these residues, C(2)M was still able to localize in a wild type background. More experiments will be done to determine if the mutants can localize in the absence of endogenous C(2)M. It is likely, from the preliminary data collected, that at least one of these mutants will be able to localize in the mutant background and rescue nondisjunction levels. This indicates that either these residues are not important for C(2)M to interact with the SMC proteins or the interaction between C(2)M and the SMCs is not required for SC assembly. It may be that the mutated sites are not important because the ring does not need to be closed on both sides to function. Also, it appears that there are many conserved amino acid residues in the region of the proposed interaction site, it is possible that another amino acid is responsible for mediating the interaction. Given that the the two C-terminal domains have not been tested for rescue, it is also possible that interactions between C(2)M and SMC1 are more important than the interactions with SMC3.
Transgenes have been made to express each of the three C(2)M domains, the N-terminus, the central SA binding region, and the C-terminus. By looking at the localization as well as nondisjunction in wild type and mutant backgrounds, we may be able to determine if the different domains have different functions. From these experiments we may be able to determine the difference in function of the two populations of C(2)M. The dynamics of each of the domains of C(2)M will be determined using the heat shock experiment explained earlier. By determining if the dynamics of each domain are similar to the full length C(2)M or SMC1, we can tell which pathway it is functioning with. We also have created swap constructs where the middle Stromalin binding domain of C(2)M is switched with that of Rad21 and the vice versa. These constructs will let us know what part of C(2)M is required for its meiotic function and makes it different from Rad21.

**Is C(2)M the key to entering meiosis?**

There are two lines of evidence suggesting that C(2)M may be the key to SC assembly. The first one is that C(2)M is not expressed in germline mitosis and is only seen after meiosis begins (Manheim, 2003 #1836). Secondly, when C(2)M is overexpressed in germline mitosis, the cells begin to form patches of C(3)G, something that does not normally happen until the cell has entered zygotene of meiotic prophase I. From the experiments presented here it is evident that C(2)M can induce the assembly of SC in a germline mitotic cell. This was also observed in *bam* mutants and *bam* shRNA backgrounds which are arrested in germline mitosis and are continually dividing (Chen and McKearin 2003). These results suggest that C(2)M expression is sufficient to induce SC assembly. These results are also of practical interest for making large quantities of oocytes that are synchronously staged. Normally early stages oocytes are a small
percentage of the germline, however inducing them all to enter meiosis at the same time potentially can synchronize them. This makes almost all of the cells begin to form SC and can be used for material in biochemical studies.
VI. Materials and methods

Drosophila strains and genetics

Both Drosophila stocks and crosses were kept on standard medium at 25°C. The following mutant alleles were used unless otherwise noted: ord6 and ord10 (BICKEL et al. 1997), c(2)Mp0810, c(2)Mf2115 (MANHEIM and MCKIM 2003). Germline RNAi was performed using the following stocks from the Transgenic RNAi Project (TRiP) at Harvard Medical School (Ni et al. 2011): Nipped-B (GL00574), Stromalin (GL00534), vtd (GL00522) and SMC3 (GL00518). These transgeneic lines express shRNA against the desired RNA and are under control of the GAL4/UAS system. They were crossed to P{w+mc=tubP-GAL4}LL7 for ubiquitous expression to be sure that the transgeneic was lethal because in all of these cases the shRNA is against an essential gene (LEE AND LUO 1999). For germline expression, the following GAL4 lines were used: P{w+mc=GAL4::VP16-nos.UTR}CG6325 (RORTH 1998), P{w+mc,GAL4-Hsp70,PB}89-2-1 (BRAND AND PERRIMON 1993), P{w+, bam:Gal4-VP16} (MATHIEU et al. 2013) and P{w+mW.hs=GAL4-arm.S}4a P{w+mW.hs=GAL4-arm.S}4b (SANSON et al. 1996).

Cytology and Immunofluorescence

For immunolocalization experiments, females were aged at 25° temperature for about 16 hours with yeast paste and ovaries were dissected and fixed using the “Buffer A” protocol (MCKIM et al. 2009). The ovaries from 10 flies were dissected in 1x Robb’s media. A needle was used to remove the ovariolar sheath. After no more than 20 minutes, the separated ovaries were moved to a 1.5 ml Eppendorf tube containing 500 μl of Buffer A fixative solution for 10 min at room temperature. Following several washes, the primary antibodies were diluted into a volume of 300 μl. Primary antibodies were mouse anti-C(3)G used at 1:500 (PAGE AND HAWLEY 2001), rabbit anti-C(3)G used
at 1:500 (Anderson et al. 2005), rabbit anti-C(2)M used at 1:400 (Manheim and McKim 2003), rabbit anti-CID used at 1:100 (Henikoff et al. 2000), a combination of two mouse anti-ORB antibodies (4H8 and 6H4) used at 1:100 (Lantz et al. 1994), rat anti-SMC1 used at 1:250 (Lantz et al. 1994), rat anti-HA used at 1:15 (Roche), mouse anti-Spectrin used at 1:50 (DSHB), rat anti-VASA used at 1:300 (DSHB), rabbit anti-GFP antibody used at 1:400 (Life Technologies) mouse anti-MYC 9E10 used at 1:50 (Roche), and a mouse anti-FLAG used a 1:500 (Thermofisher) The secondary antibodies were conjugated to either Cy3, Cy5 (Jackson Labs) or Alexa fluor 488 (Invitrogen). Chromosomes were stained with Hoechst 33342 at 1:10,000 (10mg/ml solution) for seven minutes at room temperature. Images were collected using a Leica TCS SP5 or SP8 confocal microscope with a 63X, N.A. 1.4 lens. Whole germaria were imaged by collecting optical sections through the entire tissue. These data are presented as maximum projections of a confocal image stack encompassing an entire oocyte nucleus. The analysis of the images, however, was performed by examining one section at a time. All the data comes from at least two independent experiments.

**Pulse expression of transgene lines using a heat shock promoter**

For reincorporation studies, tagged transgenes UASp-\textit{SA}\textsuperscript{MYC}, UASp-\textit{solo}.\textit{Venus} (Yan and McKee 2013), and UASp-\textit{sunn}.\textit{Venus} (Krishnan et al. 2014) and UASp-SMC1\textsuperscript{3XHA} (see below) were expressed using \textit{P\{w\textsuperscript{+mc} GAL4-Hsp70, PB\}}\textit{89-2-1}. Female flies containing both \textit{P\{w\textsuperscript{+mc} GAL4-Hsp70, PB\}}\textit{89-2-1} and the transgene were heat-shocked at 37°C for one hour. After heat shock females were aged at room temperature for 24 hours with yeast. Ovaries were dissected, fixed and prepared using the "Buffer A" protocol as stated above.
**Measuring Nondisjunction**

The frequency of \(X\) chromosome nondisjunction (INDJEIAN et al.) was determined by crossing the females to \(y \ w/ B^S Y\) males and calculated as \(2(X\text{-ND progeny})/\text{total progeny}\), where total progeny = [2(\(X\text{-ND progeny}\) + (regular progeny))].

Construction of SA-MYC transgene

**Construction of SA-MYC transgene**

Full length cDNA of SA was obtained from the *Drosophila* Gene Collection (LD34181). The C-Terminal half was amplified by PCR with the following primers.

5’ GATCC AACATCATCGAGAGC3’

5’GCCTCGAGGCGCCCCTGTAATCAGGATC3’

These primers introduce a Xho1 site after the coding region and abolish the stop codon. It was blunt cloned into PST BLUE. Using the restriction sites EcoRI Xho1 the C-terminal fragment was sub cloned into pENTR4 (Invitrogen) The N-terminal half was excised from the cDNA clone using the restriction sites BglI and HindIII and also ligated into the same pENTR4 construct. An expression vector encoding full-length SA fused to a C-terminal MYC tag under control of the *UASp* promoter was created by a Clonase LR reaction with the pPWM vector (DGRC). Germline transformations were performed by Model Systems Genomics (Durham, NC) to generate transgene lines.
Site Directed Mutagenesis

In order to introduce point mutants into the coding region of C(2)M the Change-IT™ Multiple Mutation Site Directed Mutagenesis Kit (Affymetrix) was used. The following primers were used in the reaction:

F89A:
5'-tgtttttttgagttgccgatattgccagatgccaagtgatctac-3'
5'-gtgatccacttggcatctggcaatccgcaactccccaaaacca-3'

F524A
5'-gcatcaaggcttcgtaagccgcctccattttgtttgtatc-3'
5'-caagaataaccaatggagggcggctaacgcttgatgc-3'

L528A
5'-taaaatggtctgaacaagataggccaaatggagaaaggctaacgcttg-3'
5'-cttgcagctcttccatcattgctatctgtctcgagaccatgta-3'

Gibson Assembly to clone fragments of C(2)M

In order to clone each of the domains individually and to make the swaps with portions of Rad21 and C(2)M into pENTR4 Gibson Assembly (New England Biolabs) was used. Full length C(2)M was used as the template.

The following primers were used in the reactions:

C(2)M N-Term+ HA
5'gccactttgtacaaaaagcaggctcacATGGGACCAATTCAGTC3'
5’aagaaagctgggtctagatatctcgagtgcCTCTGATTTGTTGACCAC3’

**C(2)M SA Binding**

5’gccaaactttgtcaaaaaaaagcaggctccacgGACACGAAGGTATTACTTGAC3’

5’aagaaagctgggtctagatatctcgagtgcGATATCTCGAGTGCGGCC3’

**C(2)M C-Term**

5’gccaaactttgtcaaaaaaagcaggctccacGAGAATTATCATAATGAAATGAGC3’

5’aagaaagctgggtctagatatctcgagtgcGATATCTCGAGTGCGGCC3’

**CRC Swap**

C2M N-Term Fragment

5’gccaaactttgtcaaaaaaagcaggctccacATGGGAACCAATTTCAGTC3’

5’tcgaaaagccatctttACATTGGTCAAGTAATACCTTC3’

Rad 21 SA Binding

5’ttacttgaccaatgtAAGATGGCTTTTGCACCAG3’

5’gagactcatcattactCTCTTTTAAAAACACGTTC

C2M C-Term

5’gatttaaaagagaGTAATGATGAGTCTTTAAGCGTATTGGGCATTAATCCGAAAATCACGG3’

5’aagaaagctgggtctagatatctcgagtgcCGAGCTCGGCCCCCA3’

**RCR Swap**

Rad 21 N -Term
5'tacaaaaaagcaggctccacATGTCTATGAGCACATTATTTTG3’
5'tggtacgtgtaATCTTGACAAAGGCTTC3’

C2M SA Binding

5'tgtcaagattACACGTACCAACTTGGATTAC3’
5’ggtgggcggcGCTGTAGGTATCATCATGATTATC3’

Rad21 C-Term

5’tacctacagcGCCGCCACCTTTTTTATTG3’
5’ggtctagatatctcagtcTTTTTTTTTTTTTTTTTTGAGTTGTACTTATGTC3’
CHAPTER 4: CONCLUSION

Presented here is a collection of data supporting the hypothesis that there are two pathways for SC assembly in Drosophila female meiosis and they are dependent upon two cohesin complexes. I have characterized the protein subunits that are involved in each of the pathways. The first pathway contains SMC1, SMC3, SOLO, and SUNN and the second pathways depends on SMC1, SMC3, C(2)M and SA. Although we have no biochemical data yet, we refer to these as cohesin complexes because each of the pathways contains one of each of the four subunits required in a cohesin complex. Interestingly, one of the two complexes functioning in meiosis is only one subunit (C(2)M) different from the complexes functioning in germline mitosis. The two complexes in germline mitosis are, first, SMC1, SMC3, Rad21, and SA and second, SMC1, SMC3, SOLO, and SUNN (Figure 19) The complexes in germline mitosis and meiosis are almost identical with the exception of Rad21 switching to C(2)M in meiosis.

The transition from germline mitosis to meiosis requires Rad21 to be swapped for C(2)M in an unknown way (Figure 19). There are a few possible mechanisms for the switch from Rad21 to C(2)M, one being that C(2)M has a greater affinity for the SMCs and when C(2)M is present it will be present in more complexes. The other possibility is that there is a protein modification that allows C(2)M to interact with the rest of the subunits, or a protein modification that makes it so Rad21 no longer can interact with the SMCs. C(2)M also is the only protein needed for meiosis that is not present in the germline mitotic cells. Thus, the germline mitotic cells appear poised for meiosis. Germline mitotic cells are expressing many of the proteins required for meiosis, such as ORD, SUNN, SOLO and the SC transverse element C(3)G (CHRISTOPHOROU et al. 2013;
GYURICZA et al. 2016). One of the key differences between the mitotic and meiotic germline is the expression of C(2)M. This indicates that C(2)M could be a key to the initiation of meiosis. In fact, when C(2)M was expressed in germline mitotic cells, they began to assemble SC, a process unique to meiosis. The presence of C(2)M on the chromosomes appears to trigger SC assembly. This could be through a direct interaction with SC subunits or by modifying chromatin structure, similar to how cohesins can modify chromatin structure in mitotic cells and affect transcription. We also cannot rule out the interesting possibility that, in addition to direct affects on chromosome structure, the effects of C(2)M on the chromatin could promote transcription of meiosis genes like c(3)G.
Figure 19: Schematic of the cohesin subunits functioning in germline mitosis and meiosis. In germline mitosis two cohesin complexes are present: SMC1/SMC3/SOLO/SUNN and SMC1/SMC3/Rad21/SA. These complexes only differ in one subunit from the complexes in meiosis, which are SMC1/SMC3/SOLO/SUNN and SMC1/SMC3/C(2)M/SA. During the transition from germline mitosis to meiosis Rad21 needs to be changed from C(2)M.
Meiosis-specific cohesin are found in many organisms, however their function and why there is a need for multiple complexes is not well understood. I was able to tease apart some key differences between these two complexes. First, it appears that the SOLO/SUNN complex is involved in sister centromere cohesion but the C(2)M/SA complex is not. This is interesting because all mitotic cohesins are used for cohesion, providing a new role for cohesin proteins in meiosis. Second, the C(2)M/SA complex is responsible for most of the SC assembly whereas the SOLO/SUNN complex plays a minor role. The SOLO/SUNN complex is required for SC at a small number of sites, the centromeres and a few euchromatic loci. Lastly, I determined that the C(2)M/SA complex is incorporated into the chromosome axis throughout prophase, whereas the SOLO/SUNN complex is at the centromere and is more static. Determining that the complex responsible for sister centromere cohesion is not replaced throughout prophase supports the maternal age effect being caused by the loss of cohesion (HUNT AND HASSOLD 2010; LISTER et al. 2010). This is due to the fact that in human females, meiotic cohesion is established during S-phase while they are a fetus and the cohesion is not removed until the egg is ovulated. The long arrest that the oocytes go through and the inability to exchange the cohesion proteins leads to cohesin exhaustion. Because this complex is not able to be switched out for newer complexes, it is susceptible to deterioration and the effects of aging. The other complex, C(2)M/SA appears to be reincorporated quickly and likely does not contribute to the maternal age effect.

These experiments support the idea that cohesin exhaustion is contributing to the maternal age effect, and that other cohesins are able to be reloaded throughout meiosis, two competing ideas. The SOLO/SUNN complex is a static complex that is only
loaded during S-phase and is responsible for cohesion of the sister chromatids. This is the complex that has the potential to get old and degraded and contribute to the maternal age effect. The other complex C(2)M/SA, on the other hand, is able to be reloaded throughout prophase and, therefore, has a less likely chance of exhaustion. This is evidence to support that cohesin are able to reload and remain youthful throughout meiosis. Although one complex can reload, the static complex may play a role in the maternal age effect.
Figure 20: Proteinalignments of the N- and C-Termini of many Kleisins

A) The N-terminus of Kleisins across many organisms. This is the portion predicted to interact with SMC3. B) C-terminus of Kleisins across many organisms. This is the portion predicted to interact with SMC1.
In humans, there could also be a static (Rec8) and dynamic complex (Rad21L), as in flies. However, the static complexes are the ones that will contribute to the maternal age effect as those subunits are going to age throughout meiosis. Although C(2)M is able to incorporate throughout prophase, the reason for this is not known. In fact, many questions still remain about the C(2)M/SA complex: Is the only function to assemble SC? How does it assemble SC? Is the complex actually functioning as a ring? Is the ring closed? The differences between these two complexes may be because they are able to organize chromatin differently or that SA/C(2)M can interact with SC but SUNN/SOLO cannot. We also do not understand the mechanisms for another important difference between the complexes, SA/C(2)M localize to different chromosome locations than SUNN/SOLO. We speculate that this is dependent on the SA binding domain or the C-terminal domain.

In order to address these questions I delved deeper into the C(2)M/SA complex by making a series of mutations in C(2)M to tease apart the functions of C(2)M. The first set of mutations that were made were point mutations in the residues proposed to mediate interactions with the SMC proteins in order to determine if C(2)M needs to make those connections to function properly (Figure 20). My results show that the connection between C(2)M and SMC3 is either not important, or the residue that we mutated is in fact not mediating the interaction. This could be because a closed ring is not important for this complex to function. Because the F89A point mutation was able to rescue a c(2)M mutant future plans include mutating other, more conserved, amino acid residues in the region of the SMC3 binding domain. These residues include L81, G84, I97 and D99.
The second set of transgenics constructed contains each of the three domains of C(2)M separately. This will help is to determine if there are multiple functions of C(2)M and which portion of C(2)M is able to confer them. C(2)M may be able to assemble SC by directly interacting with C(3)G, a transverse filament protein of the SC. By performing co-immunoprecipitation experiments with each of the three domain transgenes I will be able to determine which portion of C(2)M makes that interaction and what each of their localization pattern is. Because the N-terminus localizes to the centromere, an abnormal location for C(2)M, the other domain must regulate the localization sites of C(2)M.

The experiments found in this dissertation bring new light into the function of meiosis-specific cohesins. I have assigned distinct functions to the complexes and have begun to shed light on how the meiosis-specific cohesin complexes are able to confer different functions than mitotic cohesin complexes. The different functions of meiotic complexes is necessary for multiple functions of meiosis that are very different from or do not exist in mitosis. During meiosis I, cohesion between sister centromeres must remain tethered while cohesion on the arms is released in order for homologs to separate. This differential release calls for differential functions of meiosis-specific cohesins. The role of cohesins in SC assembly and crossover formation requires a different complex entirely, that may interact with and regulate the chromatin differently and at different sites than the other cohesin complex.
APPENDIX 1: SUPPLEMENTARY FIGURES

Figure 21: RNAi and mutations in cohesin genes.
A) qRT-PCR analysis of RNAi knockdown in oocytes when shRNA was expressed with \( P\{w^{111C}=GAL4::VP16-nos.UTR\}CG6325^{KD1} \). Expression of cohesin gene mRNA in oocytes when the indicated hairpin is expressed relative to wild-type controls, using \( Rpll140 \) as an internal control for variations in total RNA amounts. B) Molecular map of the \( SA \) region, showing the location of the original P-element and deletion in the \( SA^{86} \) allele. The \( SA^{86} \) allele, which is a deletion of more than 96 bp but less than 813 bp of the coding region, was chosen for analysis of the mutant phenotype because it deleted the beginning of the \( SA \) coding region without extending into either of the flanking genes (\( Gas41 \) and \( CG13775 \)).
Figure 22: Cohesin localization in *Nipped-B* and *vtd* RNAi oocytes.

A) *vtd* (Rad21) RNAi oocyte is shown with C(3)G in green and CID in red. B-D) C(2)M (red) localization in (B) wild-type, (C) *Nipped-B* and (D) *vtd* RNAi oocytes. E-H) SMC1 localization in (E) wild-type, (F) *c(2)M ord* double mutant, (G) *Nipped-B* RNAi and (H) *vtd* RNAi oocytes. In B-H, the oocyte is identified by the cytoplasmic protein ORB in green. In all images the DNA is in blue and the scale bars = 5 µm.
Figure 23: SC assembly defects in cohesin RNAi oocytes. The number of C(3)G foci plotted as a function of germinarium stage. Error bars are standard error of the mean. The reduction in foci numbers between regions 2a and 3 is significant in SA RNAi (avg. = 10.5 and 2.4), c(2)M; SA RNAi (avg. = 11.9 and 3.1) and Nipped-B RNAi (avg. = 6.5 and 2.5) (p < 0.001) by a Mann-Whitney test but not significant in c(2)M and c(2)M; Nipped-B RNAi.
Figure 24: Time course analysis of C(2)M dynamics.
Whole mounted germaria are shown with the earliest stages (anterior) towards the top. Each cyst moves down the germarium in an anterior-to-posterior direction. Each image represents a projection of an image stack taken at the indicated time point following heat shock. ORB staining (green) is a cytoplasmic marker which accumulates in the cytoplasm of the oocytes. C(2)M tagged with HA (red) is visible at 24 hours after heat shock. At 48, 72 and 96 hours after heat shock, there are progressively more cysts lacking C(2)M-HA staining. This is because these cysts entered meiotic prophase after the heat shock induced C(2)M-HA production. Based on how many of these cysts there are, we estimated the relative age of each cyst. At 48, 72 and 96 hours, there were an average of 4, 5 and 6 cysts without C(2)M-HA staining, respectively. This suggests that it takes 24 hours for a cyst to move down one position in the germarium. Given there is approximately one zygotene cysts per germarium, the duration of zygotene is probably less than 12 hours.
Figure 25: C(2)M is loaded and unloaded throughout prophase.
Whole mounted germaria are shown with the earliest stages (anterior) towards the top. Each cyst moves down the germarium in an anterior-to-posterior direction. Zygotene and early pachytene is in region 2A, mid-pachytene is in region 2b and late pachytene begins in region 3. C2M-HA induction from the UASp-c(2)M^{3XHA} transgene with (A-D) a 1 hour heat shock and then fixed 6 or 24 hours later, or (E,F) arm-GAL4 or (G) bam-GAL4. The arrows indicate oocytes that are lacking induced C(2)M. In a wild-type background (B,E,G), these oocytes have C(3)G. In a c(2)M mutant background (F), these oocytes also lack C(3)G. The brackets (C,F) indicate a large number of zygotene oocytes in region 2a. In all images, the DNA is in blue, C(3)G is in green, C(2)M-HA is in red and the scale bar = 5 μm. Below the merged images is the HA channel.
Figure 26: Lack of dynamics with SUNN and SOLO.

(A-D) Whole germarium with SUNN-Venus or SOLO-Venus in red and C(3)G in green. Venus fusion proteins were expressed in all germ lines cells with *MVD1* (A,C) or were induced with a 1 hour heat shock and then fixed 24 hours later (B,D). The example in D is a germarium lacking centromere SOLO, although 6/22 had centromere foci in early region 2a. Higher magnification images of region 2a oocytes from C and D show examples of oocytes with (C') and without (D') SOLO foci. (E-G) Whole germarium with SMC1-HA in red and C(3)G in green. SMC1 was expressed in all germ lines cells with *MVD1* (E) or induced with a 1 hour heat shock and then fixed 24 hours later (F,G). A germarium is shown with threads of SMC1 in regions 2b and 3 (F) or with SMC1 foci in region 2a. DNA is in blue and the scale bar = 5 μm.
Table 5: Rescue of c(2)M mutants using *UASp-c(2)M^{EXHA}* transgenic lines regulated by arm-GAL4 or *hs-GAL4* related to Figure 6.

<table>
<thead>
<tr>
<th>GAL4 line</th>
<th>% X ND a</th>
<th>Total Progeny</th>
</tr>
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<td><em>hsGAL4 – no heat shock</em> b</td>
<td>13.5</td>
<td>1761</td>
</tr>
<tr>
<td>No GAL4 c</td>
<td>20.2</td>
<td>218</td>
</tr>
<tr>
<td><em>GAL4::VP16-nos</em></td>
<td>0.6</td>
<td>1698</td>
</tr>
<tr>
<td><em>GAL4-arm</em></td>
<td>15.7</td>
<td>1173</td>
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<table>
<thead>
<tr>
<th>hsGAL4</th>
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<th>2-day</th>
<th>3-day</th>
<th>2-day</th>
</tr>
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<tbody>
<tr>
<td>Brood A b</td>
<td>13.8</td>
<td>17.9</td>
<td>2399</td>
<td>1821</td>
</tr>
<tr>
<td>B</td>
<td>14.4</td>
<td>18.4</td>
<td>3789</td>
<td>1974</td>
</tr>
<tr>
<td>C</td>
<td>11.2</td>
<td>19.9</td>
<td>3914</td>
<td>920</td>
</tr>
<tr>
<td>D</td>
<td>15.0</td>
<td>12.7</td>
<td>3678</td>
<td>844</td>
</tr>
<tr>
<td>E</td>
<td>15.1</td>
<td>12.3</td>
<td>3133</td>
<td>701</td>
</tr>
<tr>
<td>F</td>
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<tr>
<td>G</td>
<td></td>
<td>17.6</td>
<td></td>
<td>1005</td>
</tr>
</tbody>
</table>

a ND = nondisjunction

b The genotype of the experiment females was c(2)M; *UASp-c(2)M^{EXHA}*/ hsGAL4.

c The genotype of the control females was c(2)M; *UASp-c(2)M^{EXHA}*/+. The frequency of nondisjunction is higher than in the no heat shock control, possibly because of low level expression from the *hs-GAL4* in the absence of heat shock.
APPENDIX 2: ZIP3 HOMOLOG AS A MARKER FOR CROSSOVERS

Meiotic recombination is one requirement of accurate chromosome segregation. In *Drosophila* females the process of recombination occurs after the chromosomes are fully synapsed and double-strand breaks (DSBs) are formed. DSBs can be repaired in two ways, non-crossover or crossovers. Zip3 is a protein conserved among many organisms that is known to function in crossover formation (Macqueen and Roeder 2009), (Henderson and Keeney 2004; Bhalla et al. 2008). *Drosophila* has three Zip3 homologs: *CG2709*, also known as *vilya*, *CG31053 (Zip3 (3))* and *CG12200 Zip3(X)*. However, at the time of these experiments only two homologs were recognized, therefore, the experiments in this appendix are characterizing two of the homologs. Previously in the McKim lab these two genes were briefly studied and reagents made. The reagents previously reported in the master’s thesis of Sanese White-Brown were utilized in these experiments.

One reason we were particularly interested in this family of proteins is because it has homology to proteins found to localize to sites of crossovers. With the absence of a reliable crossover marker in *Drosophila*, these proteins were of interest. First, it was determined that these proteins are functioning in the pathway for crossover formation. In the absence of just the Zip3 homolog on the X chromosome crossover levels remain normal, however in the absence of both of the homologs crossovers are reduced (Table 6). With this promising data large quantities of the protein were expressed, isolated, and purified (Figure 27). Once the protein was concentrated and dialyzed into PBS much of the protein precipitated out of solution. The concentrated protein was then run on an SDS PAGE again to estimate the concentration. The first round of purification yielded
approximately 300µg and the second 600µg. The protein was sent to Covance to be injected into rabbits in hopes of creating a suitable antibody for immunofluorescence. The antibody was tested on a wildtype background and on a mutant Zip3(3) background (Figure 28). From these experiments it seems that the antibody does not recognize the Zip3 protein.
Table 6: Crossing over in Zip3(3); and Zip3(X) RNAi females

<table>
<thead>
<tr>
<th>Genotype</th>
<th>al dp (m.u.)</th>
<th>dp b (m.u.)</th>
<th>b pr (m.u.)</th>
<th>pr cn (m.u.)</th>
<th>Total males</th>
</tr>
</thead>
<tbody>
<tr>
<td>zip3(3);Zip3(X)RNAi</td>
<td>0</td>
<td>2</td>
<td>.6</td>
<td>0</td>
<td>146</td>
</tr>
<tr>
<td>+</td>
<td>6</td>
<td>29</td>
<td>2</td>
<td>0</td>
<td>308</td>
</tr>
<tr>
<td>Zip3(X) RNAi</td>
<td>3</td>
<td>19</td>
<td>5</td>
<td>.2</td>
<td>1390</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
<td>24</td>
<td>8</td>
<td>2</td>
<td>928</td>
</tr>
</tbody>
</table>
Figure 27: SDS PAGE of various stages of Zip3(3) purification. Panel A is one of 16 gels that are the Zip3(3) protein run on a gel after purification by 6X-HIS beads. Panel B shows a small portion of the protein after electro-elution and dialysis into PBS. After the dialysis step much of the protein was lost as it formed crystals while dialyzing into PBS.
Figure 28: Testing the Zip3(3) antibody in both wild type and Zip3(3)- mutant background.

Green is C(3)G a marker for SC, red is Zip3(3) and blue is the DNA. Animal #136 and #137 were two rabbits that were injected with the Zip3(3) antigen. The Zip3(3) antibody does not appear to be recognizing anything specifically. We would expect to see it at the chromosome axis, possibly in foci found at sites of crossovers.
APPENDIX 3: THE REGULATOR OF COHESION SAN DOES NOT HAVE AN EFFECT ON SC ASSEMBLY

In order to test if the mitotic regulator of cohesion SAN (Separation Anxiety) has a role in meiosis and on which complex it acts we used a shRNA against the SAN transcript generated by the Transgenic RNAi project at Harvard University (TRiP) GL00592. When the shRNAi was expressed in the germline with MVD1 nondisjunction was measured and the cytological phenotype of C(3)G was looked at. When the shRNA was expressed with MVD1, a promoter that expresses throughout the germline at high levels, the flies were sterile. This is likely due to the fact that SAN is required for the mitotic divisions during embryogenesis. Because of this the shRNA was expressed with Bam GAL4 a promoter that is restricted to the earliest stages of meiosis. The nondisjunction levels are comparable to wildtype and the C(3)G appears to also be forming normally (Figure 29 Table 7). This indicates that SAN is not functioning in Drosophila meiosis. If SAN was functioning in meiosis it would be expected to see a defect in the SC as SAN is supposed to be required for the maintenance of cohesion by acetylating SMC3. SAN is an acetyltransferase and is thought to antagonize WAPL a deacetylase. The acetyl group on SMC3 is protection from cohesion removal.

In addition we also looked at WAPL (Wings Apart-Like) a negative regulator of cohesion in mitosis to see if it was functioning in meiosis and on which complex. We obtained a shRNA from TRiP (GL00576) and determined that it was not sufficiently knocking down the gene because after expression with a ubiquitous promoter (tubulin). Therefore, we obtained a mutant allele from the Bloomington Stock Center and made a recombinant chromosome with an FRT site. This was used to make homozygous mutant oocytes in the germline using the germline clone site specific recombination technique.
In this experiment homozygous mutant clones will not be GFP but instead contain two mutant \textit{WAPL} alleles. It was seen that the C(3)G is able to assemble and form properly in the WAPL mutant and DSBs are repaired normally as seen by the Y-H2AV signal not persisting later than region 2b (Figure 30). These results, however, does not mean that WAPL is not functioning in meiosis. As a negative regulator of cohesion it is expected that WAPL will not have a role in the assembly of cohesion onto the DNA. There still may be a defect in the removal of cohesin that cannot be detected in this assay. Other members of the lab will look into the role of WAPL in the removal of cohesins in late stage oocytes.
Table 7: Nondisjunction in *SAN* RNAi

<table>
<thead>
<tr>
<th>RNAi Genotype</th>
<th>Oocyte genotype</th>
<th>Nondisjunction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XXY ♀</td>
<td>XO ♂</td>
</tr>
<tr>
<td><em>w1118</em></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td><em>SAN Bam</em></td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

* The control data shown here is the same presented in Chapter 3. These experiments were all done together.
Figure 29: \textit{SAV} RNAi has no effect on SC assembly

\textit{C(3)G} marks the SC and is shown in green. CID is in red and DNA is in blue. In both wild type and \textit{SAV} RNAi the SC progresses to pachytene, threads of SC all over the chromosome axis.
Figure 30: WAPL Germline clones have no effect on SC assembly. Green marks the C(3)G, red is Y-H2AV, and blue is DNA. A is showing a germarium with a clone in region 3. The region 3 oocyte was blown up in A’. A” shows just the Y-H2Av channel. In this experiment clones were identified by the lack of GFP signal. B shows the GFP signal from the same germarium. There is a lack of GFP signal around the region 3 oocyte indicating that it is a WAPL mutant clone.
APPENDIX 4: THE ROLE OF COHESION REGULATOR NIPPED-B IN MEIOSIS

In addition to the Nipped-B data that is included in chapter 2 presented here is more data to further characterize the phenotype of the shRNA. When the shRNA was expressed with MVD1 or Matα the flies were sterile. This is potentially due to the fact that Nipped-B is required for mitotic divisions and the MVD1 promoter expresses highly during embryogenesis, not necessarily due to a meiotic phenotype. For this reason we also expressed the shRNA with two other promoters that express in the germline. The first driver is Bam, a promoter that is restricted to early stages of meiosis. The second promoter NGT is similar to MVD1 (throughout the germline) but expresses at lower levels. When expressed with Bam the non-disjunction levels were normal, however, when expressed with NGT the non-disjunction levels were increased to 14% (Table 8). In order to test if the sterility with the shRNA expressed with Matα is due to the chromosomes not segregating properly or a because of a defect in embryogenesis Fluorescent in-situ hybridization (FISH) was used. Probes were used to recognize the second and X chromosome in stage 14 (late stage) oocytes. At this stage the homologs should be oriented to opposite poles unless there is an orientation defect. An orientation defect at this stage would mean that the chromosomes were going to segregate improperly and result in aneuploidy. When we used FISH to look at the chromosomes in the absence of Nipped-B the chromosomes were bi-oriented (n=16), as in wild type (Figure 31).
Table 8: Nondisjunction in *Nipped-B* RNAi

<table>
<thead>
<tr>
<th>RNAi Genotype</th>
<th>Oocyte genotype</th>
<th>Nondisjunction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XXY ♀</td>
<td>XO ♂</td>
</tr>
<tr>
<td><em>w1118</em></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td><em>NB Bam</em></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><em>NB NGT</em></td>
<td>62</td>
<td>70</td>
</tr>
</tbody>
</table>

* The control data shown here is the same presented in Chapter 3. These experiments were all done together.
A. Chromosome probes: X 2n

Normal Segregation  Nondisjunction

B.

WT Mata>Nipped-B RNAi

Figure 31: Fluorescent in-Situ hybridization in Nipped-B RNAi. Green is tubulin which marks the spindle, red marks the second chromosomes, white marks the X chromosomes and the DNA is in blue. A is a schematic drawing of normal segregation versus chromosomes that are going to non-disjoin. B shows representative oocytes from the FISH experiment. In all (n=16) of the Nipped-B RNAi oocytes the chromosomes were bi-oriented.
APPENDIX 5: USING THE CHROMOSOME SPREAD PROTOCOL TO ALLOW FOR BETTER ANTIBODY PENETRATION

In order to see antibody staining that was not visible with whole mounted ovaries we used a chromosome spread protocol obtained from the lab of Sharon Bickel. Dr. Bickel adapted this protocol from Peters et al (PETERS et al. 1997)

The following protocol includes the changes I have made to suit our needs:

1. Make 1% paraformaldehyde fix.
   - 22.5ml water in falcon tube
   - 0.25g paraformaldehyde
   - 50µl of 1N NaOH
   Put it in a 50-60˚ water bath to dissolve it.
   When dissolved, cool to room temp and add1000µl of 50 mM Boric Acid to pH.
   After pHing, add 350ul of 10% Triton-X100.

2. Make 1mL of 100mM sucrose (200µl of 500mM sucrose and 800µl dH₂O).

3. Make 5 ml hypo-extraction buffer:
   Make fresh each time immediately before use and pH should be at 8.2-8.4.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>(final concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (600mM) pH 8.2</td>
<td>250µl (30mM)</td>
</tr>
<tr>
<td>Sucrose (500mM)</td>
<td>500µl (50mM)</td>
</tr>
<tr>
<td>Trisodium Citrate Dihydrate (170mM)</td>
<td>500µl (17mM)</td>
</tr>
<tr>
<td>EDTA (250mM)</td>
<td>100µl (5mM)</td>
</tr>
<tr>
<td>DTT (1.0M - thaw right before use)</td>
<td>2.5µl (0.5mM)</td>
</tr>
<tr>
<td>Pefabloc (200mM - thaw right before use)</td>
<td>12.5µl (0.1mM)</td>
</tr>
<tr>
<td>Sterile mQ-H₂O</td>
<td>q.s. to 5 ml (3635 µl)</td>
</tr>
</tbody>
</table>
4. Dissect out ovaries from approximately 7 females in 1xPBS for no longer than 5-10 minutes with two forceps. (Later steps are easier if one uses fairly young ovaries in which the number of st8 and older oocytes are reduced. Fatten virgins with yeast one night only.) Remove PBS and rinse once in hypo-extraction buffer, making sure that ovaries sink in the buffer. Fill dish with ~500µl hypo-buffer, make sure that all are immersed and incubate 20'-30'.

5. Put a drop of hypo-buffer in a shallow 2-well dish and transfer 14 ovaries to drop. Using forceps and a micro-knife cut off transparent tip of an ovary removing all oocytes with yolk.

6. Put 40µl of 100mM sucrose in the other well of the dish and transfer the ovary tip to the sucrose with forceps. Repeat with the other 13 ovaries putting all in the same 40µl of 100mM sucrose.

7. Using forceps and a micro-knife, mince all the ovary tips so that no intact ovarioles remain. (Ultimate goal is to have single egg chambers.) Pipette up and down several times (10-20 times) using a p10 with a p2 tip that has been BSA coated immediately beforehand.

8. Dip a slide in the paraformaldehyde fix for ~15 sec. and while holding the slide horizontal, dab off the excess fix from the bottom of the slide with a kimwipe. Hold the slide at an angle and place 10µl of the minced ovary mixture (in multiple drops) onto the middle of the long side of the slide. Tilt and rotate the surface of the slide to allow the tissue to move around and to spread out the cells.

9. Allow slides to dry very slowly overnight in a humidified chamber, lid slightly open.

10. Next morning, open up the humidified chamber to allow final drying. When slides are dry, (~2 hours or more), place them in a 0.4% solution of Kodak Photo-Flo 200 in water in a coplin jar (200µl into ~50ml of water) for 2 minutes. Remove the slides and dab the edges dry on a paper towel. Allow them to air dry in a dry coplin jar for about 1 hour.

11. Best if antibodies are applied within the next two days.

   If necessary, store dry slides at -20 to -30°C.

12. Make a well around the sample on the slide with rubber cement. It may be helpful to etch the glass first as well.

13. Block and Primary Antibodies
   A. Rehydrate in PBS 15’ by placing 100uL of PBS on top of the cells on the slide.
B. Incubate in 200ul block (5% NGS/2% BSA/0.1% triton X) 1 hour on slide in humidified chamber, under parafilm cover. (Overnight under parafilm works too.)
C. Rinse 3x in 2% BSA/0.1% triton X in PBS
D. Dilute primary antibodies in 2% BSA/0.1% triton X in PBS in humidified chamber, 100ul on slide under parafilm. Incubate overnight at 4°C under parafilm.

14. Secondary Antibody
A. Rinse 3x in 0.1% triton X in PBS and wash 3X10’ on slide.
B. Dilute secondary in 2% BSA/0.1% triton X in PBS.
C. 100ul on slide under parafilm and incubate for 1 hour in chamber at room temperature.
D. Rinse 3x in 0.1% triton X in PBS and wash 3X10’ on slide.

15. Mounting
A. Rinse 3x in PBS, add 400ul Dapi at 1µg/ml in PBS on slide for 10’.
B. Rinse in PBS on slide.
C. Dab off excess liquid with kimwipe.
D. Drop 7.5ul of Slowfade mounting media onto tissue on slide and lower coverslip onto slide.
E. Store sealed slides at 4ºC
Figure 32: Cytology using the chromosome spread protocol
A). A W⁻ oocyte prepared using the chromosome spread protocol stained with C(3)G. B).
A SUNN:Venus oocyte expressed with MVD1 stained with C(3)G in green and Venus in Red.B’). The Venus channel separate.
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