

**CHARACTERIZATION OF A HYPOMORPHIC ALLELE OF SPC25, SPINDLE POLE
BODY COMPONENT 25, DURING MITOSIS AND MALE MEIOSIS IN DROSOPHILA
MELANOGASTER**

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Degree
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DEDICATION

I dedicate this dissertation to one person, my mother *Paula Goe Adams*. She passed away May 1, 2023, before I could complete this degree. My mother lived a life of compassion and grace, in which she devoted her heart and soul to her children. The daily encouragement and unconditional love she gave me during some of the hardest times of my education journey will never be forgotten. In my last conversation with her she reminded me of how far I had come, how hard I had worked for my higher education, and how proud she was of me. I am forever grateful for a mother like her who taught me to be independent, strong, and dedicated, but also to enjoy and cherish life. She is truly missed, and I remember her encouragement every day. Without the time I was blessed with my mother's grace, I don't know that I would have been able to complete this huge accomplishment.

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ABSTRACT

Accuracy of chromosome segregation during division is vital for maintaining genomic integrity and essential for cellular function and viability. Failed chromosome segregation leads to aneuploidy, which has different consequences for mitotic and meiotic cells. Aneuploid products of mitosis often have unregulated gene expression which can cause cellular over-proliferation or apoptosis. Chromosome nondisjunction (NDJ) events in meiosis generate aneuploid sperm or eggs. If incorporated during fertilization, their presence can lead to a range of consequences from spontaneous abortion to genetic conditions such as Down Syndrome or other aneuploid-associated syndromes. In this study, we identify a unique allele of *spc25*, *spindle pole body component 25*, which generates two mutant alternatively spliced *spc25* transcripts, encoding two mutant Spc25 proteins. One protein contains an amino acid substitution, D71E (aspartate 71 glutamic acid), and a four amino-acid motif insertion, which we refer to as Spc25^{ER}. The other protein lacks its carboxy-terminal (CT) domain. Spc25, Spc24, Nuf2, and Ndc80 proteins form the Ndc80 complex, which establishes end-on kinetochore-microtubule (KT-MT) attachments. Stabilized end-on KT-MT attachments are required for proper biorientation and segregation of chromosomes. We show that the D71E substitution in Spc25^{ER} causes chromosome NDJ events in both meiosis I and II. Analysis of Nuf2 localization in mutants that express Spc25^{ER} shows premature loss of Nuf2 during anaphase. We propose that Spc25^{ER} incorporation into the Ndc80 complex leads to its instability when spindle forces are exerted onto kinetochores for the poleward movement of chromosomes. Our results also support previous work that shows that the CT region of Spc25 is critical for its localization to the kinetochore region.

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CHAPTER ONE

INTRODUCTION

1.1 Two Types of Cell Division: Mitosis vs. Meiosis

1.1.1 Mitosis produces cells with equal amount of genomic material

Mitosis is an evolutionarily conserved process which is a part of the cell cycle responsible for cellular division. In somatic cells of multicellular organisms, mitosis is essential for the development, growth, homeostatic maintenance of organs, and repairment of damaged cells. Additionally, mitosis is vital for asexually reproducing organisms to reproduce. Mitosis is characterized by six consecutive stages: prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis which are carefully carried out to generate two new daughter cells, each containing the same amount of genetic material as their mother cell (Yanagida et al, 2014).

Prior to the start of mitosis, the S phase of the cell cycle is responsible for replicating DNA, producing two copies of every chromosome. This process ensures that each daughter cell inherits all genetic information. During prophase, the genome is transcriptionally active, leading to an increase in cell material and volume. In prometaphase, chromosomes undergo condensation which leads to a compacted structure to facilitate their transmission when division occurs. Such compaction also prevents the damage or loss of any genetic material during segregation of sister chromatids. During this stage, a proteinaceous structure called the kinetochore (KT) forms at the centromeric region of the chromosome which provides physical attachment of chromatids to microtubules of the spindle (McIntosh, 2016).

Kinetochore – microtubule (KT-MT) attachments facilitate alignment of the chromosomes independently at the equator of the spindle during metaphase. Crosstalk between

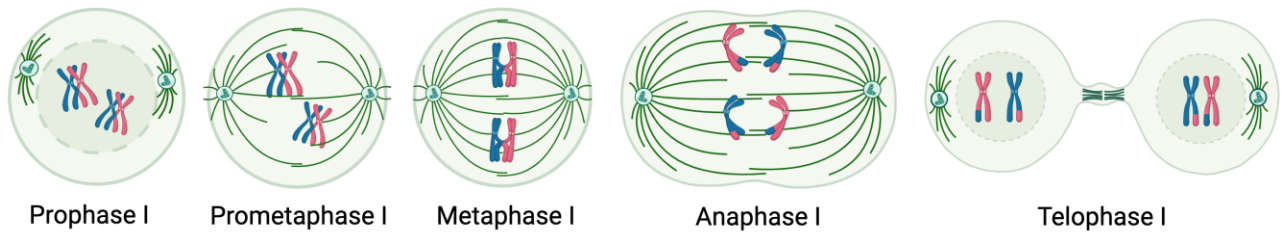
the KT and cell signaling regulators ultimately leads to satisfaction of signal assembly checkpoints to permit chromosome segregation during anaphase (Lara-Gonzalez et al., 2021). The anaphase promoting complex (APC) activates the protease, Separase. It selectively targets cohesin rings which encircle duplicated sister chromatids. This release of cohesion leads to the poleward movement of chromatids towards spindle poles for which they are attached to, with the centromeric region leading and chromatid arms following behind (Uhlmann et al., 2001). At the completion of chromosome segregation, regeneration of nuclear envelopes in each daughter cell encloses the chromosomes, which is followed by the process of cytokinesis to complete the physical separation of the two new cells.

1.1.2 Meiosis produces up to four cells each with half the amount of genomic material

Meiosis is a specialized cell division which occurs only within the germline cells of sexually reproducing organisms. This type of cell division gives rise to sperm or eggs in males or females, respectively, that each with their haploid nature will form a zygote upon fertilization. During meiosis, two consecutive rounds of cell division, termed meiosis I and meiosis II, occur without an intervening DNA replication phase. Like mitosis, the stages of prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis occur in both meiosis I and meiosis II, shown in **Figure 1.1**.

Meiosis I includes unique features which do not occur in mitosis or meiosis II. In most eukaryotic organisms, pairing of homologous chromosomes permits the process of recombination during which exchange of genetic information occurs and is the mechanism of genetic variation within species. As a result of meiotic recombination, paired homologs become physically linked by chiasmata, which are remnants of crossing over. Paired homologs align on

Meiosis I



Meiosis II

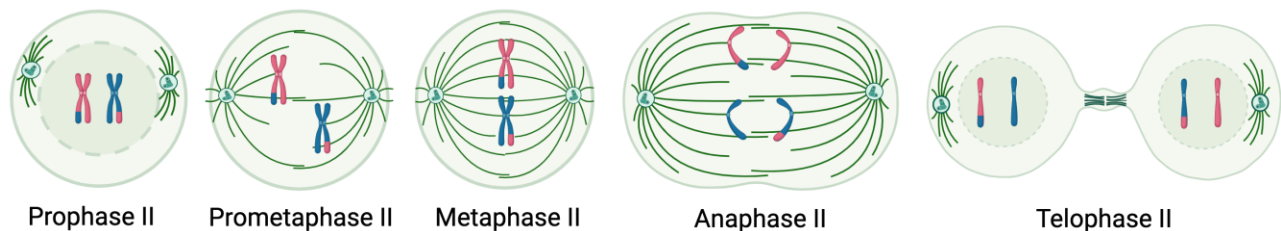


Figure 1.1 Graphical summary of the major stages of meiosis.

(Top panel) After S phase of the cell cycle, meiosis begins in prophase I. In most eukaryotes, pairing of homologous chromosomes allows crossing over to occur, shown in the top panel with two blue and pink chromosomes are paired and have cross-over events between non-sister chromatids. In prometaphase I, paired homologs are captured by microtubules of the meiotic spindle. Biorientation of homologs occurs during metaphase I and permits homolog segregation in anaphase I. Anaphase I is referred to as a reductional division, as it reduces the ploidy number from $2n$ to n . Telophase I nuclei are shown, containing one copy of each chromosome, still with duplicated sister chromatids. **(Bottom panel)** Meiosis II begins directly after telophase I, without an additional DNA replication phase. Meiosis II is like mitosis with respect to the following stages: in prometaphase and metaphase, paired sister chromatids are independently captured by microtubules and aligned on the metaphase plate. Sister chromatids achieve biorientation via individual sister kinetochores attached to opposite poles. Sister chromatid separation occurs during anaphase II, resulting in telophase II nuclei containing only one chromatid per chromosome. Created in <https://BioRender.com>.

the metaphase I plate, which sets the stage for homolog segregation during anaphase I. Anaphase I is referred to as a reductional division, generating the haploid nature of this special cell type (Hughes et al., 2018).

In meiosis II, like mitosis, duplicated chromatids independently align on the metaphase plate and separate during anaphase. Products of meiosis differ between males and females. At completion of male meiosis and sperm individualization, an overall process referred to as *spermatogenesis*, four gametes are produced (Griswold et al., 2016). On the other hand, completion of female meiosis and egg generation, an overall process referred to as *oogenesis*, only leads to the formation of a single oocyte (Bilinski et al., 2017).

1.2 Consequences of Inaccurate Chromosome Segregation

Cells implement a highly sophisticated system to coordinate the alignment and disjunction of chromosomes during cell division. However, errors in chromosome segregation can occur. Unequal segregation of chromatids, a process referred to *chromosome nondisjunction (NDJ)*, may occur if cells lack proper regulation or functioning of gene products required for proper chromosome segregation. Abnormalities in spindle morphology, chromosome structure or dysregulation of signaling networks controlling cell division are just some examples of the many causes of chromosome NDJ events (Gisselsson, 2008). This section explains the types of chromosome NDJ that can occur during mitosis and meiosis and the impact of each type of error.

1.2.1 Mitotic chromosome nondisjunction events generates aneuploid somatic cells

Proper gene dosage regulates production of cellular components, which is vital for maintaining the homeostasis of cells and tissues. The consequence of a chromosome NDJ event generates aneuploid cells, which either that lack or acquire additional chromosomes, and creates imbalance

in gene dosage (Stingele et al., 2012). As mitosis is responsible for generating most of the major cell types within organisms, mitotic chromosome NDJ events can negatively impact cellular functioning and severely affect the health and survival of an organism. Segregation of chromosomes during oogenesis or embryogenesis is more error prone when compared to somatic cells. Chromosome NDJ events that occur during early divisions of an embryo create embryonic mosaicism, where the embryo contains a mixture of normal and aneuploid cells. If chromosome mis-segregation occurs during development, there can be a dramatic increase the number of aneuploid cells within the embryo, which can lead to embryonic lethality or developmental abnormalities and disease (Horakova et al., 2024).

Aneuploidy often cause major changes in the gene expression profile or cause cells to undergo apoptosis, or cell death. The imbalance in gene dosage affects regulation of mRNA levels and protein levels, which can have a profound effect on cellular signaling and genome stability (Potapova et al., 2017). Mitotic NDJ events and aneuploidy have been implicated in tumorigenesis and the formation of cancer. Genomic instability, unregulated gene expression or dysregulated cell cycles are common occurrences associated with tumor formation and metastasis of tumor cells (Upender et al., 2004).

1.2.2 Meiotic chromosome nondisjunction events generates aneuploid gametes

Meiotic chromosome nondisjunction (NDJ) during either meiosis I or meiosis II ultimately leads to the generation of aneuploid gametes. Aneuploid sperm or eggs are problematic if they are engaged in fertilization. Embryos generated with aneuploid sperm or eggs have disrupted development due to the unbalanced gene dosage, which ultimately severely affects offspring viability (Potapova et al., 2017). In the most severe cases, meiotic NDJ events account for 50%

of spontaneous abortions occurring in the first trimester of pregnancy (Hyde et al., 2015).

Aneuploidy for some human chromosomes does not result in lethality but negatively affects the development and overall physical health of individuals. Children born with trisomy for chromosomes 13 and 18 have severe developmental abnormalities which result in a short life expectancy, with only 9.7% and 12.3% living up to five years, respectively. Trisomy for chromosome 21, referred to as Down syndrome, has been shown to have the longest lifespan, but individuals have neurological underdevelopment resulting in mental retardation and learning disabilities along with other health conditions (Krivega et al., 2022).

Meiotic chromosome NDJ of sex chromosomes can cause other types of disorders like Turner Syndrome (TS) and Klinefelter Syndrome (KS). These individuals either lack (XO) or have an additional sex chromosome (XXY), respectively. TS affects approximately 50 out of 100,000 women, and they have array of disorders such as infertility, hypergonadotrophic hypogonadism, neurocognitive problems, congenital heart malformations and other metabolic abnormalities. Healthy women inherit two X chromosomes, and if both X chromosomes were transcriptionally active women would have double gene expression of X linked genes. To control gene dosage for X-linked genes, the epigenetic regulation process of X chromosome inactivation randomly inactivates transcription of one of the X chromosomes in every cell in females. Since individuals with TS only have one X chromosome (XO individuals) but no Y chromosome to encode for the male sex, they are biologically female. In these TS females, X-linked genes are susceptible to major dysregulation due to the active X-inactivation process. XO individuals' genomes have been shown to be hypomethylated when compared to XX healthy individuals, and some reports have even shown that RNA expression of autosomal genes are drastically different

in XO individuals (Gravholt et al., 2019). Another example of sex chromosome aneuploidy occurs in individuals with Klinefelter Syndrome (KS), which contain an extra sex chromosome with the karyotype XXY. They suffer from similar disorders such as fertility issues and cognitive impairment along with being at a greater risk to develop type 2 diabetes, cardiovascular disease and cancer (Gravholt et al., 2018).

The relationship between the changes in gene expression and the complex phenotypes observed in either TS or KS patients is still not well understood. However, understanding their genetic origin and possible disrupted mechanisms by which they occur is also important. These syndromes are caused meiosis I or meiosis II NDJ events during gametogenesis of either parent.

Meiosis I NDJ occurs when the paired homologous chromosomes do not separate and are both transmitted to the same pole. After completion of meiosis II, two classes of aneuploid gametes are produced in which two lack the chromosome ($n - 1$) and the other two contain the additional chromosome. Consequentially, the impact of homolog NDJ events is more severe than meiosis II NDJ events as all gametes produced from meiosis I NDJ are all aneuploid (**Figure 1.2**). Homolog NDJ events can be caused by several instances such as disrupted or lack of meiotic recombination, incorrect microtubule – kinetochore attachments, or instability in the meiotic spindle (Charalambous et al., 2023); (Wasielak-Politowska et al., 2022).

Failure for sister chromatids to separate during anaphase II of meiosis also results in the generation of aneuploid gametes. In the case of meiosis II NDJ, half of the gametes will be euploid and the other half will be monosomic or trisomic (**Figure 1.2**). Sister chromatid NDJ events can also occur by perturbations in meiotic spindle attachments or dysregulated timing of chromosome segregation. Premature removal or lack of removal of cohesin rings, which encircle

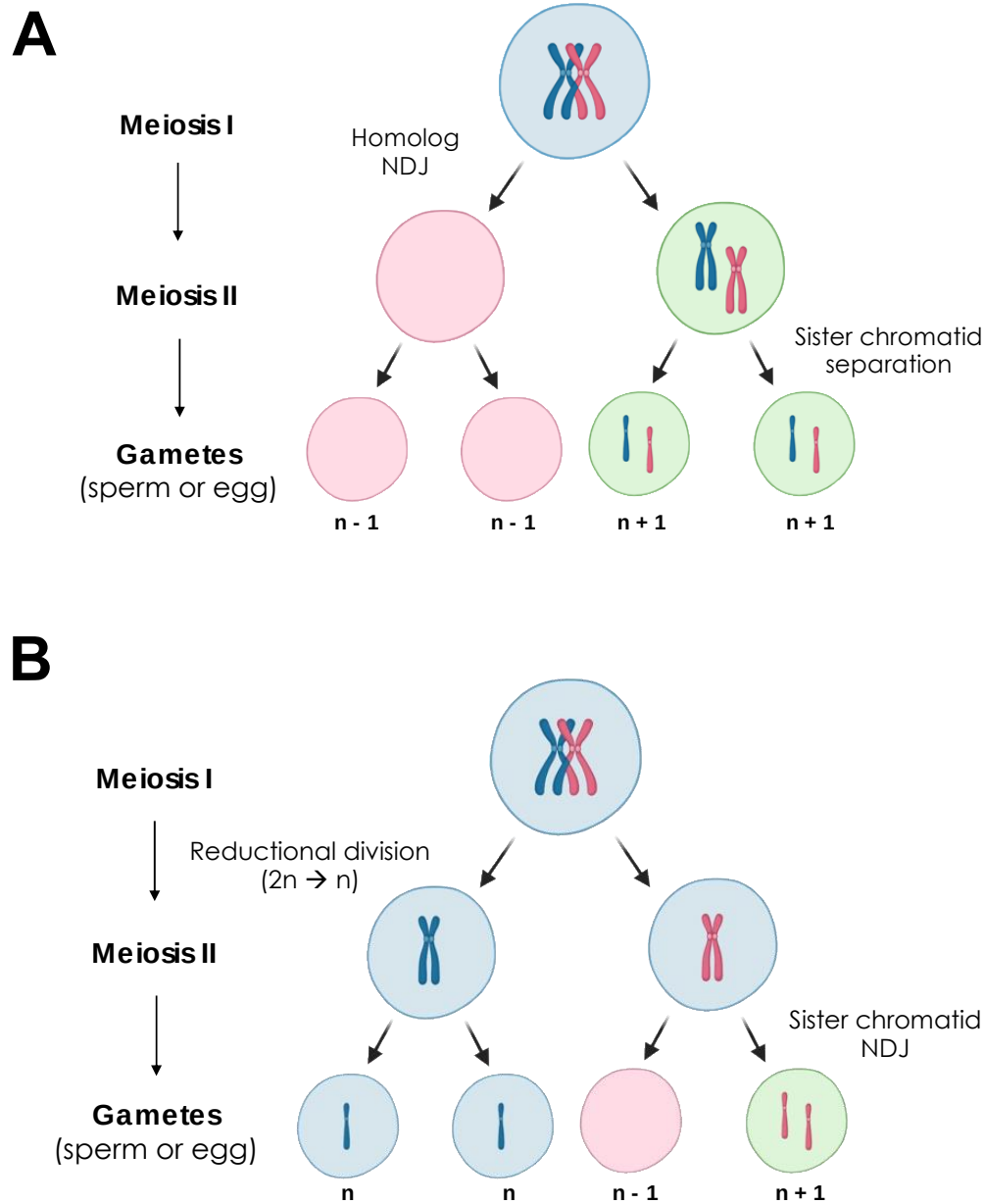


Figure 1.2 Nondisjunction (NDJ) of homologous chromosomes and sister chromatids in meiosis. **A** Meiosis I NDJ occurs when homologs do not segregate properly, resulting in secondary meocytes containing either two (green cell) or no copies (pink cell) of a specific chromosome. At the end of meiosis II, all gametes are aneuploid. **B** Meiosis II NDJ occurs when sister chromatids do not properly separate during anaphase II, resulting in the production of aneuploid gametes that contain two (green cell) or no (pink cell) copies of a specific chromosome. Created in <https://BioRender.com>

duplicated chromatids, can either be a major mechanism by which sperm or eggs can become aneuploid (Ioannou et al., 2015).

In relationship to human fertility, events of meiosis NDJ occur more often during oogenesis when compared to spermatogenesis. Women 20 - 30 years of age on average have eggs in which 20% are aneuploid whereas in males only 1-2% of sperm generated will be aneuploid. With increase in age of women, the number of eggs becoming aneuploid also increases, which is directly implicated in women over 35 having an increased risk of bearing children with chromosomal abnormalities. Many factors have been investigated that may contribute to the increase in number of aneuploid eggs with age, ranging from how the process of oogenesis in mammals occurs to exposure to environmental factors causing DNA or cellular damage (Charalambous et al., 2023).

At the molecular level, chromosome NDJ in either mitosis or meiosis occurs when mechanisms essential in segregating chromosomes are disrupted. The next section will highlight and detail the major cellular components that mediate proper chromosome segregation.

1.3 Mechanisms to Ensure Accurate Chromosome Segregation

1.3.1 Chromosomal protein complexes directly influence chromosome structure

Two major groups of chromosomal protein complexes which are well conserved in eukaryotic organisms play essential roles in facilitating proper chromosome segregation during both mitosis and meiosis. Condensins and cohesins which are formed by multi-component protein complexes, each execute unique functions, shown in **Figure 1.3**. This section will discuss the composition, function and importance of each complex.

Condensins play an active role in spatially organizing the genome in a form conducive of

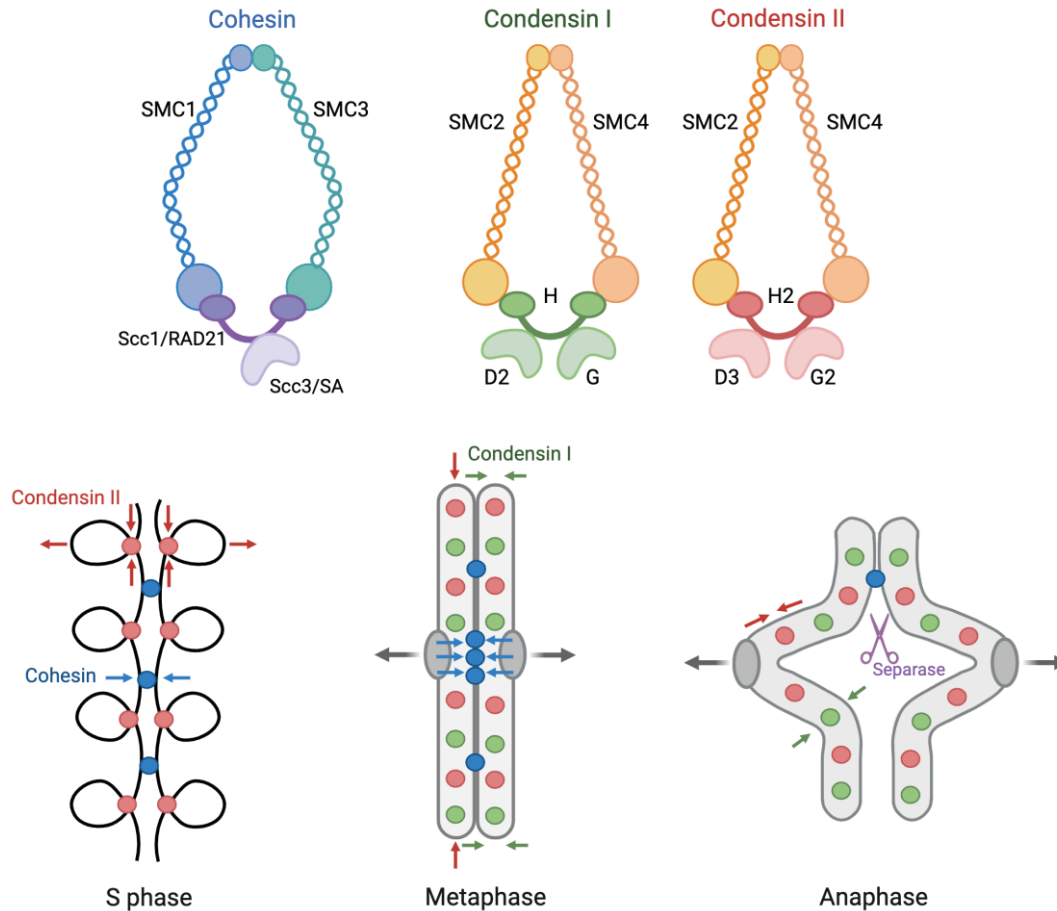


Figure 1.3 Structure of cohesin and condensin rings and their roles during the cell cycle. (Top panel) Cohesin and Condensins form ring-like structures, both containing SMC (Structural Maintenance of Chromosomes) proteins and non-SMC components such as a kleisin subunit to close the ring and additional subunits which stabilize the ring. Cohesin rings in mitosis are composed of SMC1, SMC3, a Scc1/RAD21 homolog, and a Stromalin homolog, Scc3/SA. Two types of Condensins, Condensin II and Condensin I both contain the core SMC proteins SMC2 and SMC4 but differ in their kleisin and regulatory subunits. Condensin II contains the CAP-H (H) kleisin subunit and CAP-D2 (D2) and CAP-G (G) subunits. Condensin I contains the CAP-H2 (H2) kleisin subunit and CAP-D3 (D3) and CAP-G2 (G2) subunits. (Bottom panel) Throughout the cell cycle, cohesins and condensins play important roles in chromosome structure and organization to ensure proper chromosome segregation during cellular division. During S phase in which DNA replication occurs, cohesins are loaded onto duplicated sister chromatids to bound them together until anaphase onset. Condensin II complexes facilitate early compaction of individual chromatids, keeping each sister chromatid individualized while cohesins are linking sisters together. During both prometaphase and metaphase, cohesins are mostly localized to the centromeric region of sister chromatids, and the Condensin I complex is largely responsible for the lateral compaction of a single chromatid. Evidence does support that Condensin II also helps in axial shortening of the chromatid. During anaphase, cohesins are subject to cleavage by Separase activity (purple scissors) and maintenance of both condensin complexes ensures chromosomes remain condensed for their transmission to opposite poles. Figure adapted from Hirano, 2015; Created in <https://BioRender.com>

cellular needs. In eukaryotes, two types of condensin complexes have been identified, condensin I and condensin II. Within these two complexes, they share the same core SMC (structural maintenance of chromosomes) proteins, SMC2 and SMC4, which form a V-shaped heterodimer. Condensin I and II complexes differ in the three proteins which form a bridge-like structure, referred to as the kleisin subunit, which binds the two ATPase domains of SMC2 and SMC4. CAP-H and CAP-H2 are the kleisin subunits for condensin I and condensin II, respectively. Additional association of two HEAT protein subunits, CAP-D2/CAP-G and CAP-D3/CAP-G2 to condensin I and condensin II, respectively, completes the condensin holocomplex. Within this holocomplex, the binding of ATP and ATP hydrolysis by the two ATPase domains of SMC2 and SMC4 permits the condensin complex to entrap a DNA strand. The active entrapment of DNA strands is how condensins perform their activity in changing chromatid structure (Hirano, 2016).

Understanding the adaption of two different condensin complexes in eukaryotic cells has come from studies of their localization and requirement within different organisms throughout the cell cycle. In general, studies have shown that condensin II is in the nucleus prior to DNA replication and active during interphase (Bauer et al., 2012); (Ono et. al, 2013). Work in *Drosophila* has shown that condensin II functions to oppose homolog pairing in somatic cells in which gene expression regulation can occur by the process of transvection (Hirano, 2015). Additionally, chromosome capture and 3-dimensional (3D) genome structure studies in vertebrates demonstrate that condensin II activity promotes loop extrusion, a model in which portions of the DNA are physically positioned to regulate gene expression. Also, condensin II has been shown to have a role in early compaction of chromatids after DNA replication while cohesins are loaded onto duplicated sisters. Condensin I localizes to DNA only after nuclear

envelope breakdown during prometaphase and promotes the lateral compaction of individual sister chromatids, giving rise to the shape of metaphasic chromosomes (Hirano, 2015). The highly compacted nature of chromatids is a crucial requirement to allow easy transmission of chromatids without causing DNA damage or chromosomal loss during chromosome segregation (Kim et al., 2013).

Cohesins form protein complexes very similar to the above mentioned condensin complexes, as shown in **Figure 1.3**. Like condensins, cohesins use SMC proteins, SMC1 and SMC3 in the case of the cohesin complex, to form a V-shaped heterodimer. Both SMC1 and SMC3 consist of an ATPase domain at the distal ends of the arms of the V-shaped structure, and they dimerize at their opposite ends forming the hinge domain. A kleisin subunit, homologs of SCC1/RAD21, binds the two ATPase domains of the SMC1/SMC3 heterodimer connecting the three proteins to form a ring complex. A fourth member of the cohesin complex, homologs of the Stromalin (SA/STAG) family, also contains HEAT domains and physically interacts with the kleisin which stabilizes the fully formed complex. The most recognized function of cohesin rings is their ability to encircle duplicated chromatids after DNA replication and provide cohesion of chromatids until their separation during chromosome segregation. Multiple cohesin rings coat the arms and centromeric region of sister chromatids during mitosis (Nasmyth et al., 2009).

Removal of cohesins occurs by two different pathways. The pool of cohesins decorating chromosome arms are unloaded by the protein WAPL in what is referred to as the prophase pathway. Studies have shown that cohesin ring removal during prophase is required for sister chromatid decatenation, which eliminates DNA entanglements that if not removed negatively affects chromosome segregation (Buheitel et al., 2013). The other removal process is a carefully

regulated pathway dependent on the activity status of the cysteine protease Separase. Prior to anaphase onset, Separase is maintained in an inactive state by interactions with its inhibitor Securin. Activation of Separase occurs upon activation of the anaphase promoting complex (APC) which targets key proteins, Securin being one, to the proteasome. Active Separase targets the kleisin subunit of centromeric cohesin rings by recognizing D/EXXR amino acid motifs within the alpha kleisin subunit, leading to its release from chromosomes (Holt et al., 2008). This is the key point at which chromosomes submit to spindle their transmission to opposite poles.

1.3.2 Kinetochores mediate connections between chromosomes and the spindle

During cell division, chromosomes are aligned to the metaphase plate and subject to bipolar forces emitted by spindle microtubules. The kinetochore (KT) plays a center role in mediating these movements as they physically connect chromosomes to microtubules (MTs) of the spindle. Proteins within the KT are major communicators with spindle assembly checkpoint signaling and become a focal point during chromosome transmission as they lead chromosomes to move to their respective poles. As KT's have several responsibilities, they can be considered to be one of the most important structures to ensure accurate segregation.

The KT consists of a large network of multiple protein complexes that forms at the centromeric region of the chromosome. Assembly of KT's at the centromere of chromosome is dictated by the presence of centromere specific proteins. CENP-A (CID in *Drosophila melanogaster*), is a histone variant incorporated at centromeric sequences (Blower et al., 2001). CENP-A interacts with another centromeric protein CENP-C which provides the localization site for the core KT complexes to assemble (Schittenhelm et al., 2007); Przewloka et al., 2007). The complexity of the network of centromeric proteins highly differs between mammalian and

Drosophila centromeres (**Figure 1.4**). At the mammalian centromere, up to 16 centromeric proteins compose a CCAN (constitutive centromere-associated network) which provide a large platform for kinetochore assembly (Musacchio et al., 2017). The fly centromere lacks this CCAN network, following a much simpler centromere where CENP-C directly binds components of the core kinetochore (Przewloka et al., 2011).

The Mis12 complex, a KNL-1/Spc105 homolog, and the Ndc80 complex, referred to as the KMN network, all constitute the core KT. The Mis12 complex resides at the inner most region of the KT and consists of four proteins Mis12, Nnf1, Nsl1, and Dsn1 in most eukaryotes. In *Drosophila*, the Dsn1 subunit has not yet been identified (Schittenhelm et al., 2007); (Przewloka et al., 2007). Uniquely, in *D. melanogaster* there appears to have been a gene duplication event as two paralogs of *Nnf1* are encoded, *Nnf1a* and *Nnf1b*. The functions of the two *Nnf1* paralogs are overlapping but differ in their spatiotemporal localization patterns in a cell type and developmental manner (Blattner et al., 2017).

In human cells, the Mis12 complex constitutively associates with the centromere throughout the cell cycle, though at lower levels during interphase and higher levels during prophase. In flies only the Mis12 subunit associates with the centromere constitutively throughout the cell cycle, though still following a similar pattern of lower levels before the start of mitosis and then increased localization during prophase (Venkei et al., 2011). As this points out that localization timing of the Mis12 complex components in mammals and flies may differ; in both organisms however, the presence of the Mis12 complex is required for localization of the core kinetochore protein Spc105 and the Ndc80 complex. Within the complex, the Nnf1 subunit directly binds CENP-C, residing close to the centromeric region (Przewloka et al., 2011).

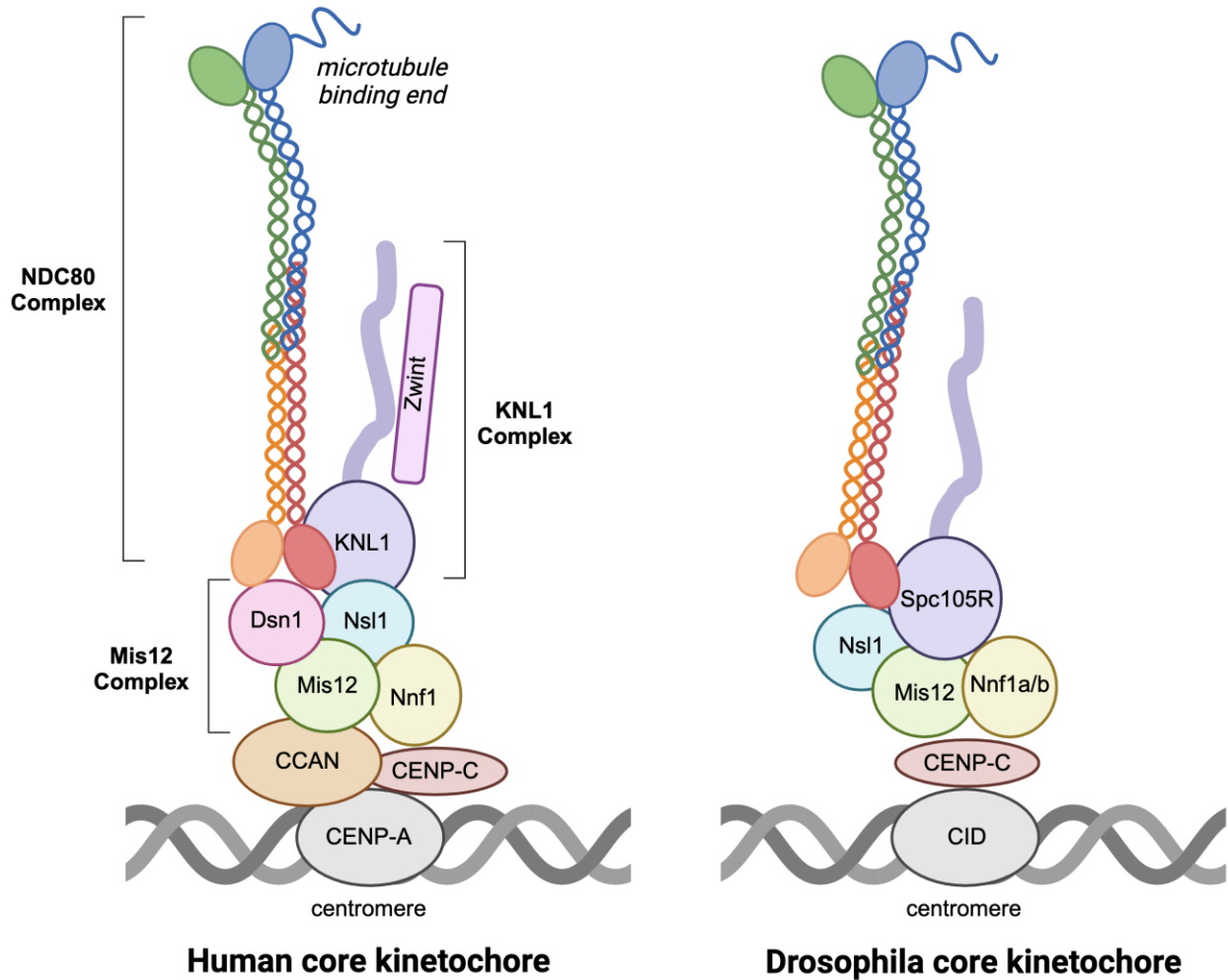


Figure 1.4 Representation of similarities and differences between human and Drosophila core kinetochore composition.

Shown on the left, the human centromere contains not only CENP-C but also a network of centromeric proteins referred to the CCAN (Constitutive Centromere-Associated Network). In addition to this, there are two key differences within the KMN (KNL1/Spc105, Mis12/Ndc80) core kinetochore network. In humans, the Mis12 complex contains Dsn1 and KNL1 has an additional member, Zwint. Both Dsn1 and Zwint have not been identified within the Drosophila core kinetochore. Figure adapted from Singleton, 2016 and created in <https://BioRender.com>

On the other side of the Mis12 complex, Nsl1 subunit directly interacts with subunits of the Ndc80 complex and Spc105, pointing towards the outer KT region (Feijao et al., 2013).

In *S. pombe*, *C. elegans*, *D. melanogaster*, and humans Spc105 localization is required for kinetochore assembly. Spc105/KNL1 homologs interact with both core KT complexes, Mis12 and Ndc80, while additionally interacting with spindle assembly checkpoint (SAC) proteins, regulators of chromosome segregation, and microtubules of the spindle. In human cells, the Spc105 homolog Blinkin has been shown to interact with members of the SAC such as Bub1 and BubR1 (Kiyomitsu, 2007). In fission yeast, Spc105 homolog, Spc7, immunoprecipitates with microtubule binding proteins, and the Spc105 homolog in *C. elegans* directly interacts with microtubules (Kerres et al., 2004); (Cheeseman et al., 2006).

Spc105 homologs are large proteins with signature [S/G]ILK and RRSVF motifs found within their amino-terminus (NT) region, MELT repeats within their middle region, and a coiled-coil domain at their carboxy-terminus (CT). The CT region interacts with members of the Mis12 complex Mis12 and Nsl1 and Spc25 of the Ndc80 complex (Venkei et al., 2012). Thus, the CT of Spc105 is essential in its localization to the kinetochore region (Schittenhelm et al., 2009). The NT region of Spc105 contains its microtubule binding domain along with Protein Phosphatase 1 (PP1) binding sites. In *D. melanogaster* mitotic cells, binding of PP1 to Spc105 leads to dephosphorylation of its internal MELT repeats, which in turn reduces the affinity of SAC protein Bub3 binding to Spc105. This removal of Bub3 is a part of the SAC satisfaction process to permit chromosome segregation (Audett et al., 2022).

Establishment of KT-MT attachments is a dynamic process (**Figure 1.5**). Microtubules can be captured by the kinetochore through lateral attachments mediated primarily by

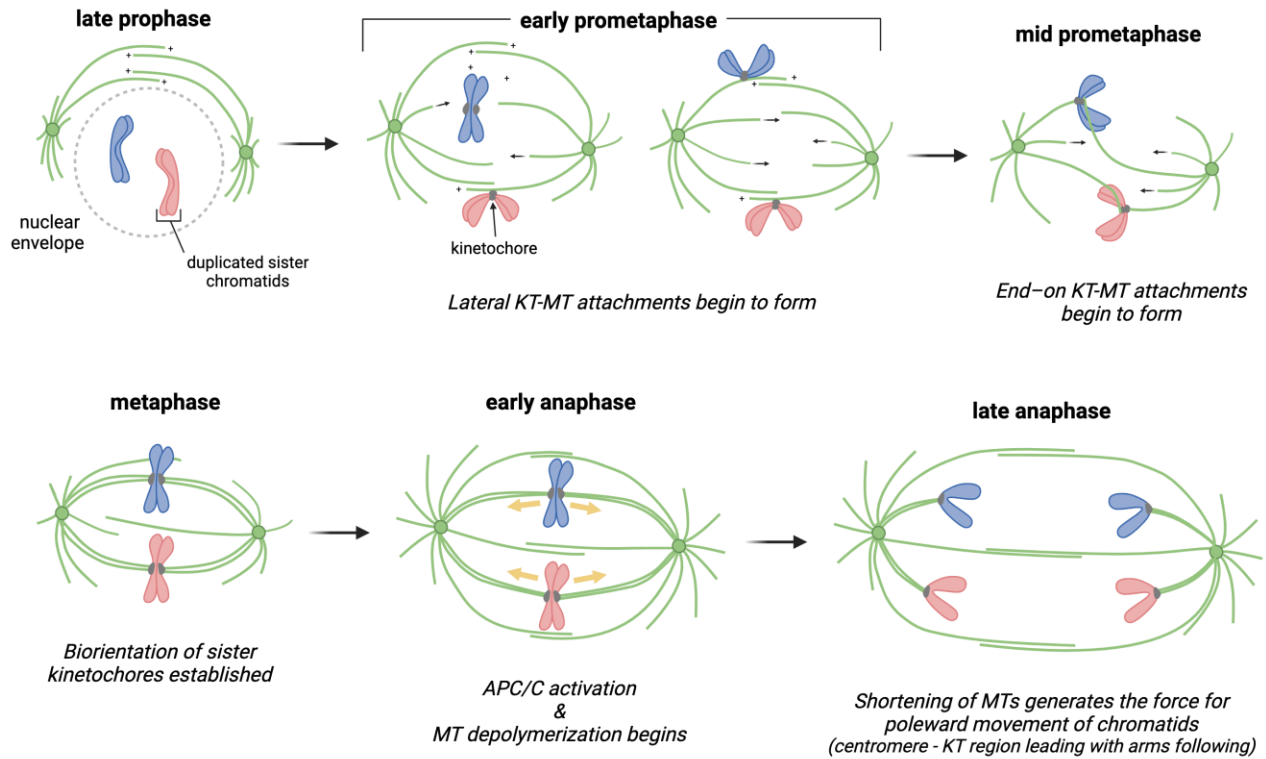


Figure 1.5 Establishing kinetochore-microtubule (KT-MT) attachments and chromosomal movements during mitosis. The dynamic nature of microtubules (green lines) and KT-MT attachments established from prometaphase to metaphase are responsible for chromosomal congression and ultimately chromosome segregation during anaphase. Created in <https://BioRender.com>

KNL1/Spc105 homologs. Lateral KT-MT attachment involves the kinetochore attaching to the side of the microtubule which then can move chromosomes along the microtubule by dynein motor protein activity. Hence, such initial KT-MT docking plays major role in chromosomal movement and congression to the metaphase plate (Tanaka, 2010). However, for chromosomes to be properly transmitted to opposite spindle poles, KT-MT attachments must adopt stable, end-on connections. End-on KT-MT attachments referred to the perpendicular configuration between the KT and MT, with the MTs directly embedded into the KT region (Bunning et al., 2023).

As shown in **Figure 1.6**, The Ndc80 complex, composed of four proteins Ndc80, Nuf2, Spc24 and Spc25, functions to establish end-on KT-MT attachments (Tanaka, 2010). Spc25 and Spc24 form a heterodimer and interact via their CT regions with both the Mis12 complex subunit Nsl1 and Spc105. Localization of Spc25 has been shown to be dependent on localization of both the Mis12 complex and Spc105 (Przewloka et al., 2007); (Schittenhelm et al., 2007); (Musacchio et al., 2017). Once the Spc25/Spc24 dimer localizes, Ndc80 and Nuf2 as a formed heterodimer localize to the KT and interact with Spc25/Spc24 to form a heterotetramer. NT coiled coil domains of both Spc25 and Spc24 and the CT coiled coil domains of both Ndc80 and Nuf2 form the heterotetramerization conjunction of all four components (Valverde et al., 2016). This fully forms the Ndc80 complex, which takes on a resemblance of a dumbbell. As the Spc25/Spc24 dimer interacts with the inner KT, the Ndc80/Nuf2 resides in the outer KT (Feijao et al., 2013); (Musacchio et al., 2017). Dimerization of the CT regions of Ndc80 and Nuf2 forms a dual calponin homolog (CH) domain, a common fold found in other microtubule and actin binding proteins. Most homologs of Ndc80 also contain an unstructured “tail” domain at the NT which can be phosphorylated to regulate direct MT binding and an internal “loop” region to facilitate

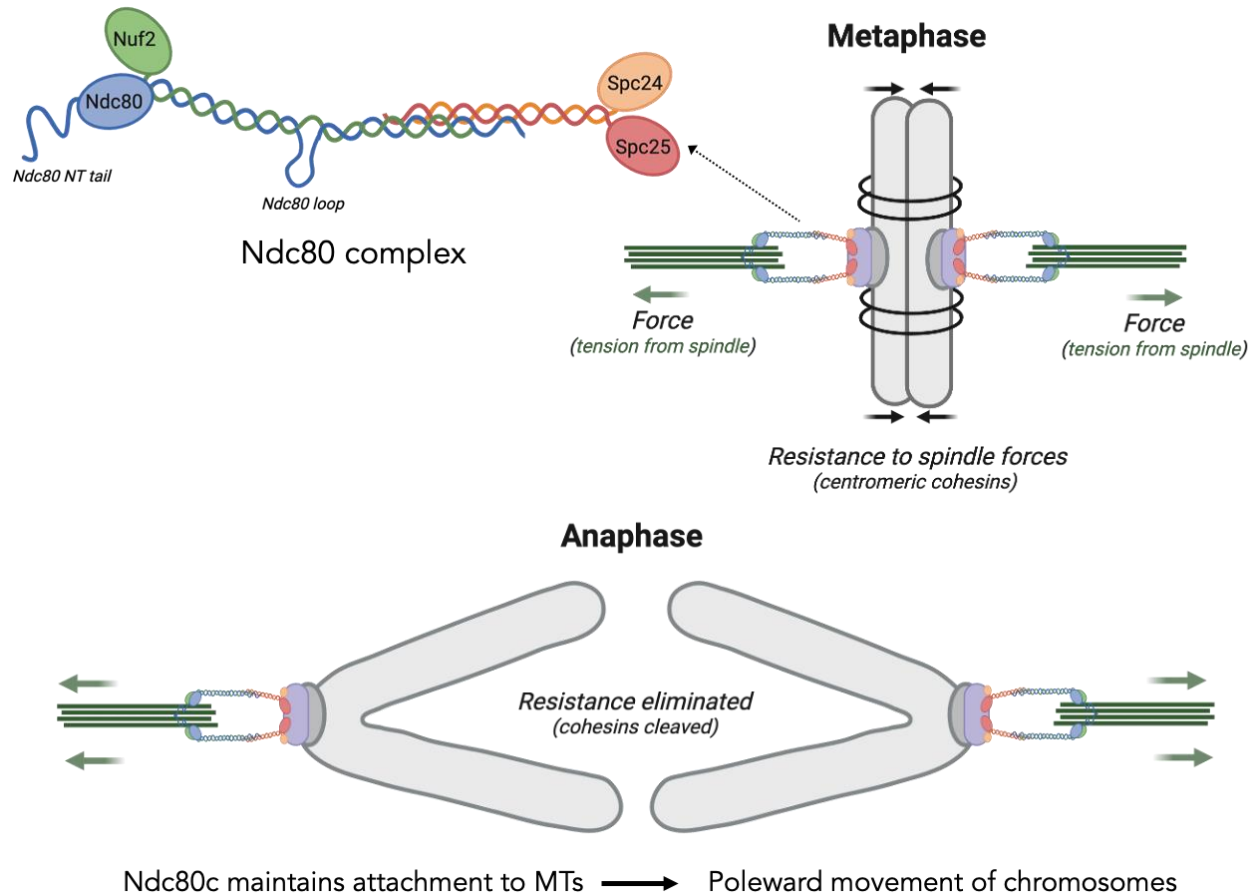


Figure 1.6 Structure and function of the Ndc80 kinetochore complex.

The Ndc80 complex, composed of Spc25, Spc24, Nuf2, and Ndc80, localizes to the KT region and establishes end-on KT-MT attachments. The complex is required for sister KT biorientation, which then generates tension across the KT and centromere region of paired chromatids. During anaphase, release of centromeric cohesin complexes from chromatids leads to the poleward movement of chromosomes. Attachment to MTs during chromosome transmission to opposite poles is mediated by the Ndc80 complex. Figure was created in <https://BioRender.com>

flexibility of the complex during MT binding (Tooley et al., 2011).

Once formed, the core KT recruits loading of microtubule regulatory proteins and members of the spindle assembly checkpoint (SAC) signaling, all of which monitor KT-MT attachments. For accurate segregation of chromatids in mitosis and meiosis II, sister kinetochores must biorient towards the opposite poles. Correct, or amphitelic, KT-MT attachments are formed when sister centromeres and kinetochores achieve a back-to-back orientation, each bound to microtubules emanating from opposite spindle poles. In meiosis I, proper biorientation and segregation of homologs requires sister KTs to mono-orient to the same pole. A pair sister KTs in meiosis I adopt the side-by-side configuration to allow for homolog biorientation (**Figure 1.7A**).

During the dynamic process of KT-MT attachment, incorrect attachments such as syntelic and merotelic can occur, which are shown in **Figure 1.7B**. Syntelic KT-MT attachment is when both sister kinetochores are bound to microtubules coming from the same pole. Merotelic KT-MT attachment occurs when one sister kinetochore has connections to microtubules from both spindle poles (Lampson et al., 2017). Proteins of the SAC and other regulators of chromosome segregation detect incorrect attachments and promote an error correction process which allows KTs to disengage from attached KTs and an additional opportunity to establish biorientation (Bunning et al., 2023).

Amphitelic KT-MT attachments of properly bioriented sister KTs generates tension across the centromeric–kinetochore region due to dynamic nature of microtubules. Composed of multiple heterodimers of alpha and beta tubulin, microtubule polymers can assemble and disassemble dynamically by the binding of GTP or hydrolysis of GDP, respectively (Mitchison

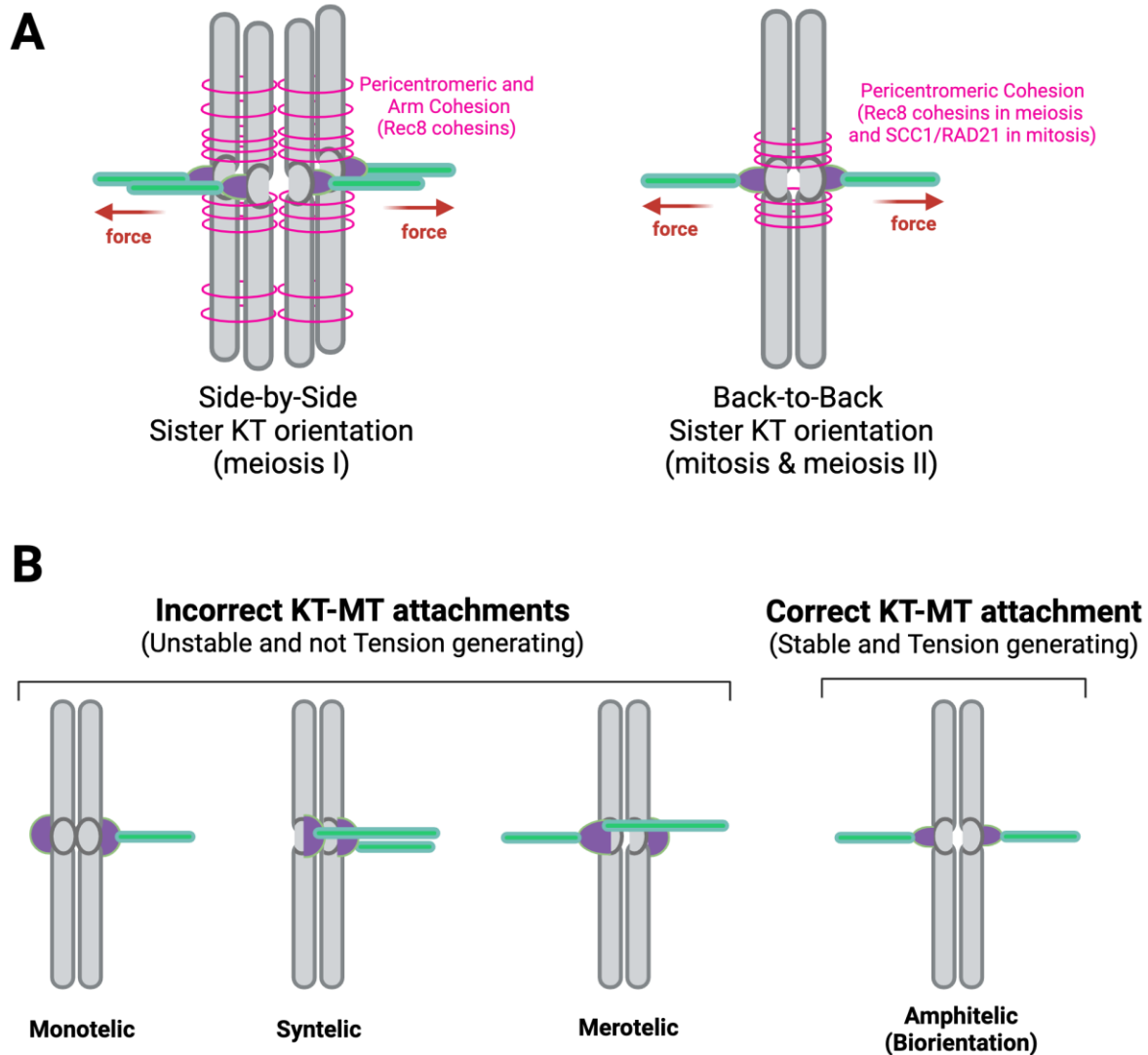


Figure 1.7 Orientation of sister KTs during mitosis and meiosis and types of KT-MT attachments.

A. In meiosis I, sister KTs (purple half circles) mono-orient, adopting a Side-by-Side configuration, and attach to microtubules (MTs) (green lines) from the same pole to allow homologs to biorient. In mitosis and meiosis II, sister KTs biorient, adopting the Back-to-Back configuration, to attach to MTs from opposite poles. **B.** The status of sister KT-MT attachment can either be correct or incorrect with respect to the type of KT-MT attachment required to generate tension across the centromere-KT region for a stable KT-MT attachment. Incorrect attachments can be monotelic (only one sister KT attached to one pole), syntelic (both sister KTs attached to one pole), or merotelic (one sister KT attached to both, opposite poles). Only amphitelic (sister KTs attached to opposite poles in mitosis for example) generate tension and are stable, bioriented KT-MT attachments. Created in <https://BioRender.com>

et al., 1984). These reactions are thermodynamically favorable and play the major role in generating active forces which contribute to chromosome congression and chromosome segregation (Grishchuk et al., 2006); (Asbury, 2017).

In addition to the dynamic behavior of microtubule polymers, microtubule associated proteins such as kinesins or proteins aiding in microtubule bundling also facilitate microtubule-associated chromosomal movements during prometaphase, metaphase, and anaphase (Brouhard et al., 2005); (Bunning et al., 2023). The combination of a kinetochore fiber undergoing polymerization and kinesin activity largely contributes to chromosome congression to the metaphase plate in mitosis (Cochran et al., 2009). Microtubules attached to bioriented sister kinetochores undergo depolymerization. The depolymerization of microtubules accounts for the major force to drive the poleward movement of chromosomes during anaphase. However, depolymerization of microtubules can also aid establishing chromosome alignment on the metaphase plate as well; therefore, does not only occur during anaphase onset. Importantly as shown in **Figure 1.6**, the presence of centromeric cohesins and condensins which link paired sister chromatids until division are critically important for providing resistance to the pulling forces exerted by events of depolymerization (Asbury, 2017).

Tension generated by spindle forces can influence centromere structure, KT-MT attachment strength, Aurora B kinase activity, and the timing of anaphase onset (Bunning et al., 2023). The centromere and surrounding pericentromeric regions of the chromosome are unique to chromosomal arms with respect to chromatin composition and structure. The highly repetitive satellite DNA within the centromere and pericentromere adopts a heterochromatic nature which is enriched with condensins and cohesins. As the kinetochore attaches to microtubules, forces

emitted by the microtubule could easily disrupt sister chromatid pairing by DNA sliding events if the centromeric region was not in a fixed, stabilized form (Paldi et al, 2020). Models such as the “bottlebrush” mechanism propose that the pericentromere adopts a unique structure in which cohesins highly populate this region and tightly link sister centromeres together until their timely removal during chromatid segregation (Lawrimore et al., 2015). This structural organization provides a stiffness to the centromere region which is critical in resisting the forces generated by KT-MT attachments (Harasymiw et al., 2019); (Bunning et al, 2023).

The tension-generating forces produced by KT-MT attachments are sensed by kinases, phosphatases, and the spindle assembly checkpoint (SAC) which importantly function to ensure appropriate timing of chromosome segregation. Aurora B kinase, or Ipl1, has been shown to be required for responding to tensionless kinetochores and for the KT-MT attachment error process (Chen et al., 2021); (Audett et al., 2023). KT-MT bipolar attachments generate high levels of tension and are therefore inherently stable. Conversely, KT-MT attachments which are not bipolar due to improper attachment (i.e. syntelic or merotelic) or lack tension activate the error correction process which is mediated by Aurora B kinase activity. Kinetochore proteins Ndc80 and Spc105 have been shown to be phosphorylated by Aurora B kinase, which modulates KT-MT attachments (DeLuca et al., 2006); (Audett et al., 2023); (Bunning et al, 2023).

How Aurora B kinase specifically targets tensionless KT-MT attachments is not fully understood, but potential mechanisms of its ability to sense the degree of tension across sister kinetochores have been proposed. One mechanism such as the spatial dependent model suggests that Aurora B localization is limited to the inner centromere where it only phosphorylates substrates within its vicinity. As shown in **Figure 1.8**, sister kinetochores with bipolar

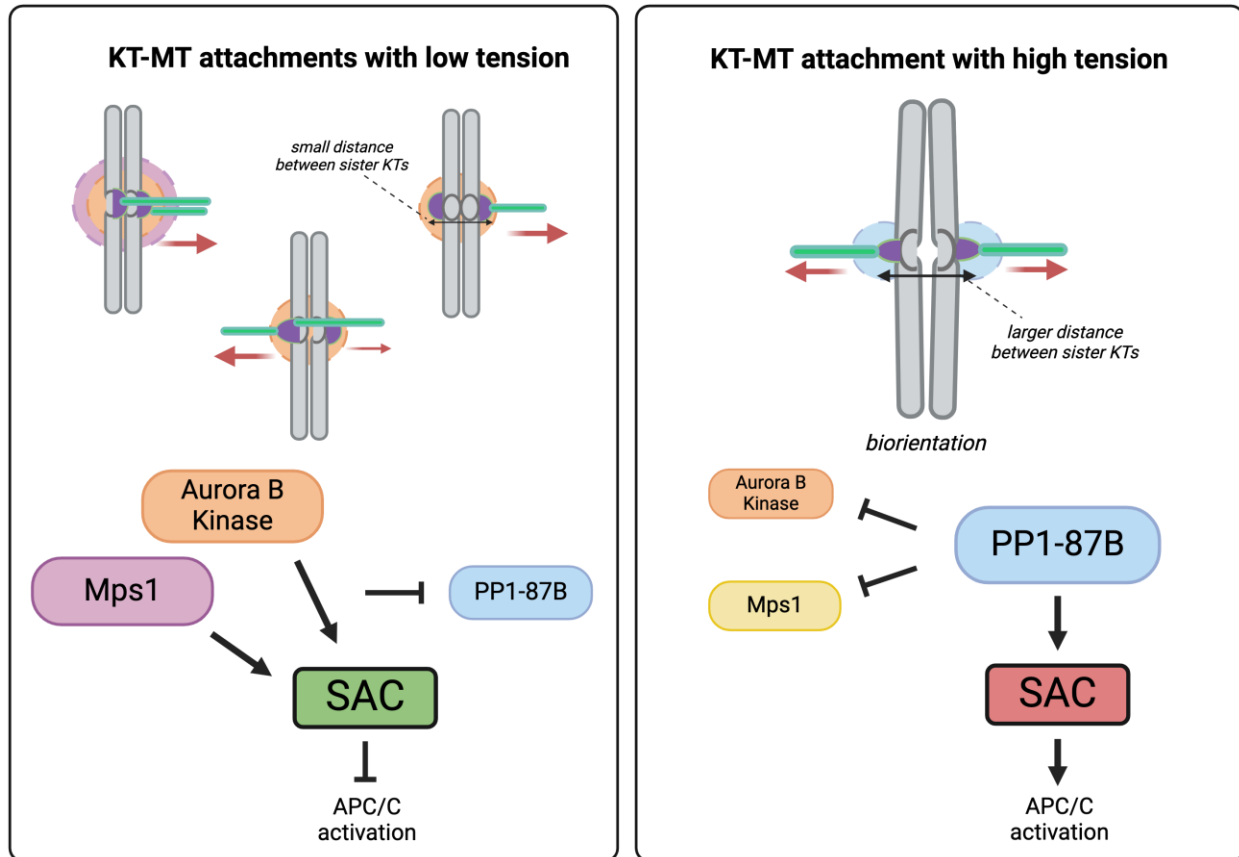


Figure 1.8 Graphic summary of how stability of KT-MT attachments under low or high tension are regulated by kinase, phosphatase, and the spindle assembly checkpoint (SAC) activity. (Left image) Merotelic, syntelic, and monotelic KT-MT attachments are under low (small red arrow) and have unequal tension across sister centromeres. Kinase activity (Aurora B Kinase or Mps1) at KTs leads to phosphorylation of KT proteins and recruitment of SAC proteins. SAC signaling is on, and the APC/C (Anaphase Promoting Complex/Cyclosome) remains inactive. (Right image) Biorientation of sister KTs generates high tension (large red arrows) across the centromere/kinetochore region. Protein phosphatases, like PP1-87B, dephosphorylate KT proteins which leads to loss of SAC protein localization at KTs, which turns SAC signaling off. Biorientation and lack of SAC signaling leads to downstream signaling events which activates the APC/C to permit chromosome segregation. Created in <https://BioRender.com>.

attachment are under high tension, which leads to the separation of sister centromeres and their respective kinetochores within the paired chromatids. This separation distances the sister kinetochores away from the localized pool of Aurora B within the inner centromere, which can stabilize the KT-MT connections. Conversely, if sister kinetochores are under low tension, then sister centromeres and their kinetochores are less separated and still within range of Aurora B kinase activity (Liu et al., 2009). Other models propose that Aurora B kinase activity is regulated either directly or indirectly by the tension status or by the kinetochore itself (Broad et al., 2020).

The spindle assembly checkpoint (SAC) also plays an important role in sensing tension status (**Figure 1.8**). Unattached KTs result in the activation of SAC signaling, which delays anaphase onset. Another kinase, Mps1, has been shown to phosphorylate Spc105 at unattached KTs which promotes KT localization of Bub and Mad proteins of the SAC (Hayward et al., 2022). In somatic cells in *Drosophila*, Mps1 has been shown to localize to syntelic end-on attachments to promote timely error correction. Mps1 activation is required to activate SAC signaling; and conversely, Mps1 inactivation is required to silence SAC signaling. In flies and human cells, phosphatases, PP1-87B and PP1-B56, respectively, counteract Mps1 activity to silence the SAC via their dephosphorylation of kinetochore proteins (Moura et al., 2017); (Hayward et al., 2019).

1.3.3 Unique mechanisms ensure accurate chromosome segregation in meiosis

Meiosis is a crucial process in sexual reproduction, responsible for producing gametes - sperm and eggs - through two rounds of chromosome segregation. Special mechanisms are employed during meiosis to ensure accurate and coordinated segregation of homologous chromosomes and sister chromatids, maintaining the integrity of the genome. These mechanisms involve complex

steps, including homologous recombination, synapsis, and the regulation of checkpoints, which collectively ensure that each gamete receives a complete set of chromosomes. During meiosis I, chromosomes must also adopt unique configurations, such as homolog biorientation and mono-orientation of sister centromeres. Through precise control of these events, meiosis not only reduces the chromosome number by half but also introduces genetic diversity, vital for the formation of healthy offspring (Hughes et al., 2018).

Meiotic recombination is a hallmark event which uniquely differentiates meiosis from mitosis. In most eukaryotic organisms, this process at the fundamental level is what contributes to genetic variation within species. However, the crossing over of nonhomologous sister chromatids within a pair of homologs and production of chiasmata serve as an important linkage to ensure paired homologs align on the metaphase I plate and that homologs segregate accurately (Hunter, 2015). In yeast, female *Drosophila*, and mammals, lack of cross-over events results in the random assortment of homologs during anaphase I (Hughes et al., 2018).

One of the most well studied meiotic specific proteins REC8 is required for meiotic recombination and accurate segregation of both homologs and sister chromatids. In most eukaryotes, REC8 replaces its paralogous alpha kleisin, SCC1/RAD21, in meiotic cohesin complexes. REC8 cohesin complexes populate chromosome arms to ensure non-sister DNAs are used when double stranded breaks are repaired and support the structural maintenance of chiasmata until anaphase I onset. In addition to this, REC8 cohesin complexes at centromeres are retained during anaphase I which is required to maintain sister cohesion until anaphase II onset (**Figure 1.9**). Centromeric REC8 cohesin complexes are provided protection from Separase activity during meiosis I by Protein Phosphatase 2A (PP2A) activity, which prevents REC8

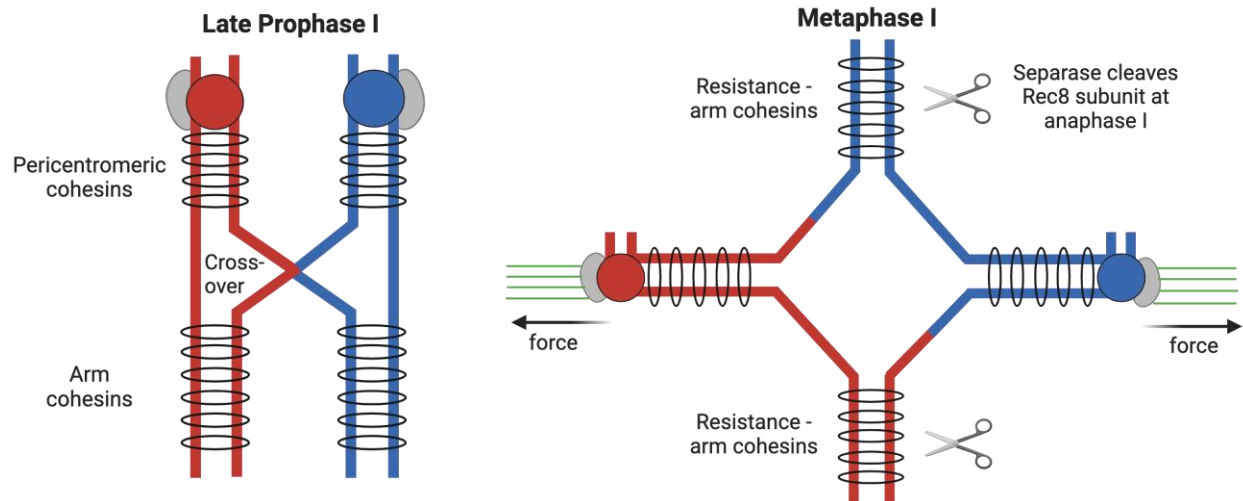


Figure 1.9 Chiasmata structures formed during crossing over links homologs in meiosis I. (Left image) During prophase I, because of meiotic recombination, chiasmata create the physical linkage of homologous chromosomes. The two pools of cohesin rings at the pericentromeric region and on chromosomal arms maintain cohesion of sister chromatids and stabilize the structure of the bivalent. (Right image) During metaphase I, both pools of cohesin rings provide resistance to the force generated by the dynamic nature of depolymerizing microtubules. As a result, tension across paired sister centromeres and kinetochores occurs when homologs are properly bioriented. Proper biorientation of homologs and sensing of tension signals key regulators of cell division to initiate chromosome segregation, such as the APC/C (anaphase promoting complex/cyclosome). The protease Separase is a key enzyme activated by APC/C which is responsible for cleaving cohesin rings at chromosomal arms during metaphase I. Created in <https://BioRender.com>.

phosphorylation and specific localization of Shugoshin proteins at the centromere (Sakuno et al., 2022). The utilization of a REC8 meiotic specific cohesin complex appears to be universally conserved as homologs of REC8 have been identified in yeast, *C. elegans*, and mammals but there are some exceptions. In the case of *Drosophila melanogaster* meiosis, a direct homolog of REC8 is not apparent by genome sequence or bioinformatic analysis of predicted gene products. However, identification of meiotic specific cohesion proteins SOLO, ORD, and SUNN have been identified as *Drosophila* specific products which function much like REC8 cohesin complexes (Bickel et al., 1997); (Yan et al., 2010); (Yan et al., 2013); (Krishnan et al., 2014).

In addition to a meiotic specific cohesion mechanism, the process of sister centromere and kinetochore (KT) co-orientation is a necessity during meiosis I. Co-orientation refers to the fact that sister centromeres and KTs must adopt a side-by-side configuration instead of the back-to-back orientation found in mitosis and meiosis II. The mechanism by which co-orientation occurs is by far the least understood process in meiosis. However, detailed work in budding yeast fission yeast, and mice has shed some light into how sister KTs co-orient (Nasmyth, 2015).

In *S. cerevisiae*, budding yeast, the monopolin complex composed of proteins Csm1, Lrs4, casein kinase I Hrr25, and Mam1 is proposed to act as a molecular clamp to link sister KTs together in metaphase I (Corbett et al., 2012). Interactions of the Csm1 subunit with the MIND/Mis12 complex subunit Dsn1 are essential for recruitment of the complex and co-orientation of sister KTs. In addition to the monopolin complex, the Cdc5 Polo-Like Kinase (PLK) also promotes co-orientation. Cdc5 PLK has been shown phosphorylate the Lrs4 subunit of the monopolin complex, possibly modulating its function, and protect centromeric REC8 cohesin complexes with PP2A and Shugoshin (Watanabe, 2003).

With insights into a mechanism of sister centromere co-orientation in budding yeast, a hypothesis that this mechanism may be conserved in all eukaryotes has been shown to be untrue. Homologs of the monopolin complex found in fission yeast do not promote sister KT fusion. Moreover, outside of fungi monopolin subunit homologs have not been identified. Despite this, studies in fission yeast were monumental in establishing sister centromeric cohesion is essential in meiosis I, and that it is a prerequisite for sister KT co-orientation (Nasmyth, 2015). REC8 mutants in fission yeast show equational homolog segregation, resulting from loss of sister centromeric cohesion. For example, in the case of the XY pair: homologs segregate in an equational manner (XY::XY), instead of segregating in a reductional manner (XX::YY) (Watanabe et al., 1999). Centromeric cohesion provided by REC8 ensures sister centromere proximity so that they mono-orient, which importantly directs their respective sister KTs to establish end-on KT-MT attachments to the same pole during meiosis I. The kinetochore, meiotic specific protein Moa1 in fission yeast was identified as *moa1* mutants show equational segregation in meiosis I like *rec8* mutants (Yokobayashi et al., 2005). Moa1 protein has been shown to physically interact with REC8 and with Polo Kinase (Plo1) to recruit Sgo1 (Shugoshin 1). Moa1 with Plo1 has been shown to lead to the phosphorylation of kinetochore protein Spc7 (KNL1 homolog) which is proposed to act in regulating monopolar KT-MT attachments of paired sister chromatids in meiosis I (Bernard et al., 2001); (Miyazaki et al., 2017).

In mice, the protein MEIKIN, another meiotic and kinetochore specific protein, also has been identified, which shows many similarities to Moa1 function. MEIKIN is required for mono-orientation of sister KTs and protection of centromeric cohesin. Like Moa1, MEIKIN recruits Polo-like Kinase 1 (PLK1), and with PLK1 activity sister centromere cohesion is protected via

Shugoshin localization (Kim et al., 2015). Though these mechanisms of sister centromere co-orientation still remain a mystery, work in these three organisms set the foundation for establishing at least two independent ways in which homologs can be segregated properly in meiosis I: a cohesin-independent manner (via monopolin) and a cohesin-dependent manner (via Moa1 and MEIKIN) **Figure 1.10** represents a summary of each meiotic specific KT protein involved in facilitating sister centromere co-orientation and mono-orientation of sister KTs.

Recent work in *Drosophila* females have identified how sister centromeres co-orient in meiosis I. In comparison to males, meiosis in females entails a major difference in microtubule and spindle dynamics. In *Drosophila* female meiosis, the spindle is acentrosomal. The bipolar spindle is assembled during prometaphase I by microtubule nucleation events occurring around the chromosomes after they have condensed into a single mass, a structure called the karyosome. All homologous centromeres are initially clustered together but then undergo a separation phase so that homologs can biorient during metaphase I (Radford et al., 2015). Sister chromatids maintain cohesion along chromosomal arms distal to cross-over sites, within the pericentromeric heterochromatin and at the core centromeric region via SMC1, SMC3, SOLO, and SUNN, a proposed meiotic specific cohesin complex in *Drosophila* (Gyuricza et al., 2016). As noted above, both fission yeast and mice sister centromere co-orientation requires centromeric cohesion to sufficiently co-orient centromeres. *solo* and *sun* mutant oocytes both result in premature sister centromere separation and random assortment of homologs during metaphase I (Yan et al., 2010); (Yan et al., 2013); (Krishnan et al., 2014); (Gyuricza et al., 2016). This with other evidence supports that a possible mechanism(s) for co-orientation may be more like the ones found in fission yeast and mice.

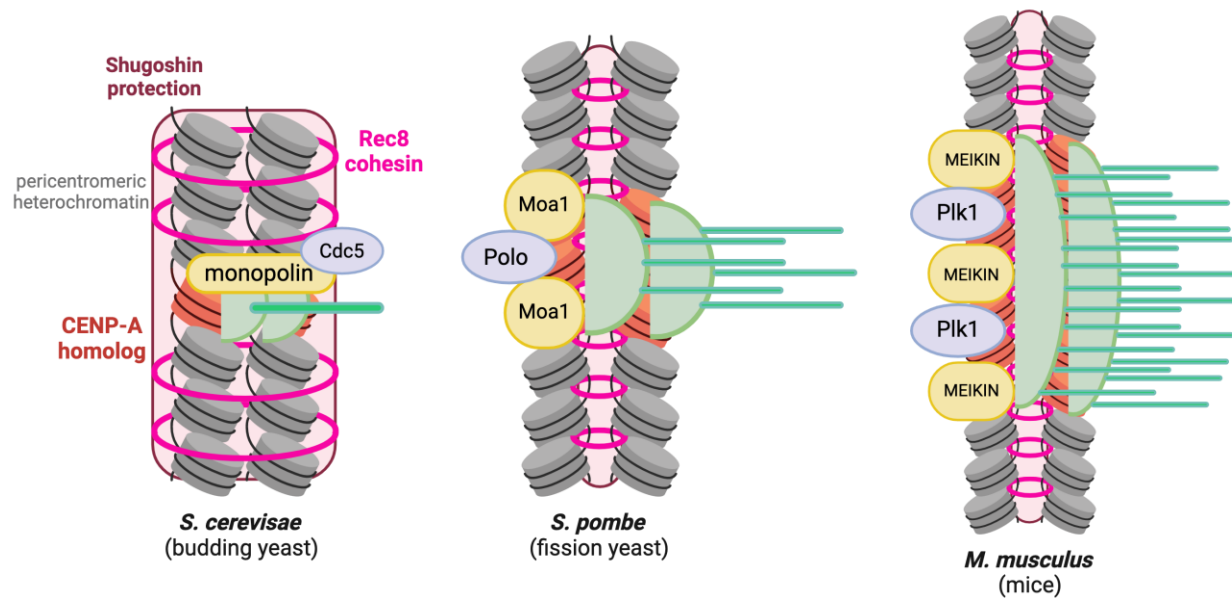


Figure 1.10 Mechanisms of sister centromere co-orientation in meiosis I in budding yeast, fission yeast, and mice.

For each organism, the meiotic-specific kinetochore protein (monopolin in budding yeast, Moa1 in fission yeast, and MEIKIN in mice) is shown in yellow, localized to KT (green half circles) to promote sister centromere co-orientation and monopolar attachment of sister KTs to microtubules (green lines). A key difference between the co-orientation factor in budding yeast when compared to fission yeast and mice is where it is localized. Monopolin has been shown to interact directly with the KT Mis12 complex, which “clamps” sister KTs together. Moa1 and MEIKIN both appear to physically interact with Rec8 cohesin complexes, which supports sister centromere co-orientation. As shown the number of microtubules attached to KTs in each organism and size of centromeric region differs, which may contribute to complexities of the unknown mechanisms utilized to co-orient sister centromeres. This figure was adapted from Hauf et al., 2004 and Yamagishi et al., 2014. Created in <https://BioRender.com>

Interestingly, two proposed mechanisms of sister centromere fusion, or co-orientation, at least involve the protein phosphatase PP1-87B and kinetochore protein Spc105R in two independent manners, a cohesin-independent manner and cohesin-dependent manner, respectively (**Figure 1.11**). PP1-87B activity regulates the formation and stability of end-on KT-MT attachments, which directly influence whether sister centromeres are properly attached to the same pole. How PP1-87B directly does this remains undetermined, data shows that PP1-87B has an antagonistic relationship with Aurora B Kinase, Polo Kinase, BubR1 to regulate spindle MT dynamics and stability of KT-MT attachments. In the second mechanism, Spc105R has been implicated in having a direct role in protecting centromeric cohesion from Separase activity. Depletion of Spc105R in *Drosophila* oocytes leads to the loss of the meiotic specific Shugoshin protein MEI-S332 localization to centromeres and premature sister centromere separation (Radford et al., 2015); (Wang et al., 2019). A role for Spc105R in aiding recruitment of MEI-S332 would be like the findings in fission yeast in which Spc7, the recruits Sgo1 (Bernard et al., 2001); (Miyazaki et al., 2017). Additional work to define other players in these two processes is required and will be exciting to gain a clearer understanding of how sister co-orientation occurs.

1.4 Novel Meiotic Proteins Discovered in *Drosophila*

The identification of meiotic specific gene products required for faithful chromosome segregation in *Drosophila* meiosis came from characterization of ethyl methane-sulfonate (EMS) mutagenized fly lines (Koundakjian et al., 2004). A subset of homozygous mutant lines showed elevated fourth chromosome loss in the paternal germline specifically during meiosis (Wakimoto et al., 2004). Further characterization of these mutant alleles identified two novel mechanisms used to segregate meiotic chromosomes: a homolog conjunction complex (male specific) and a

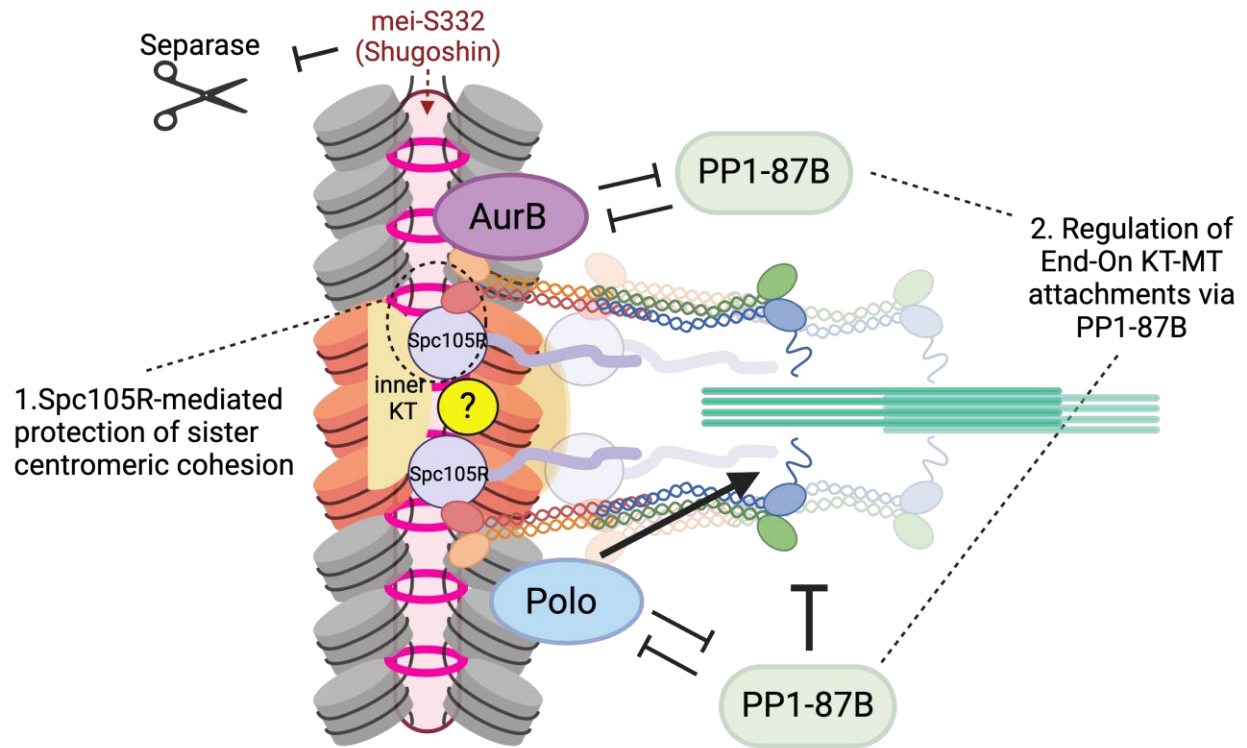


Figure 1.11 Graphic representation of cohesin-independent and cohesin-dependent proposed mechanisms for maintenance sister centromere fusion in *Drosophila* female meiosis I. This figure was generated (Wang et al., 2019). Created in <https://BioRender.com>.

group of sister chromatid cohesion proteins (male and female).

1.4.1 Spermatogenesis in *Drosophila melanogaster*

The *Drosophila* testis as early as the third instar larval stage begins to make sperm. Though the gonad itself matures from an oval-like structure to a spiral-like structure, spermatogenesis begins at the L3 stage. Spermatogenesis accounts for the entire process from an asymmetric division of a germline stem cell, creating the spermatogonium, to the two round of meiosis, all the way to the generation of individualized sperm for sexual reproduction, summarized in **Figure 1.12**.

At the apical tip of the testis (the rounded end of the gonad) resides a hub cell niche, which is composed of about 20 somatic cells. Within the hub, eight germline stem cells (GSCs) are each surrounded by two somatic cyst stem cells (SCSCs). An asymmetrical mitotic division of the GSC and the accompanied SCSCs creates a spermatogonium surrounded by two somatic cyst cells (SCCs). The SCCs at this point will not divide any further but will grow and remain associated with the germline cells through the rest of the spermatogenesis process. The spermatogonium will undergo four more mitotic divisions with incomplete cytokinesis, giving rise to a 16-cell cyst, still associated with the two SCCs. The 16 cells at this point will complete one premeiotic S phase and then initiate meiosis (White-Cooper, 2004).

Prophase I in *Drosophila* male meiosis dramatically differs from female meiosis. Chromosomes in male meiosis are achiasmate and take on specific nuclear morphologies as primary spermatocytes mature. The stages of prophase I are defined as stages S1-S6 in which the decondensed chromosomes gradually form three major territories, two for the major autosomes and one for the sex bivalents, that migrate away from each other and even make contacts with the nuclear envelope (Cenci et al., 1994). During prophase I, primary spermatocytes increase their

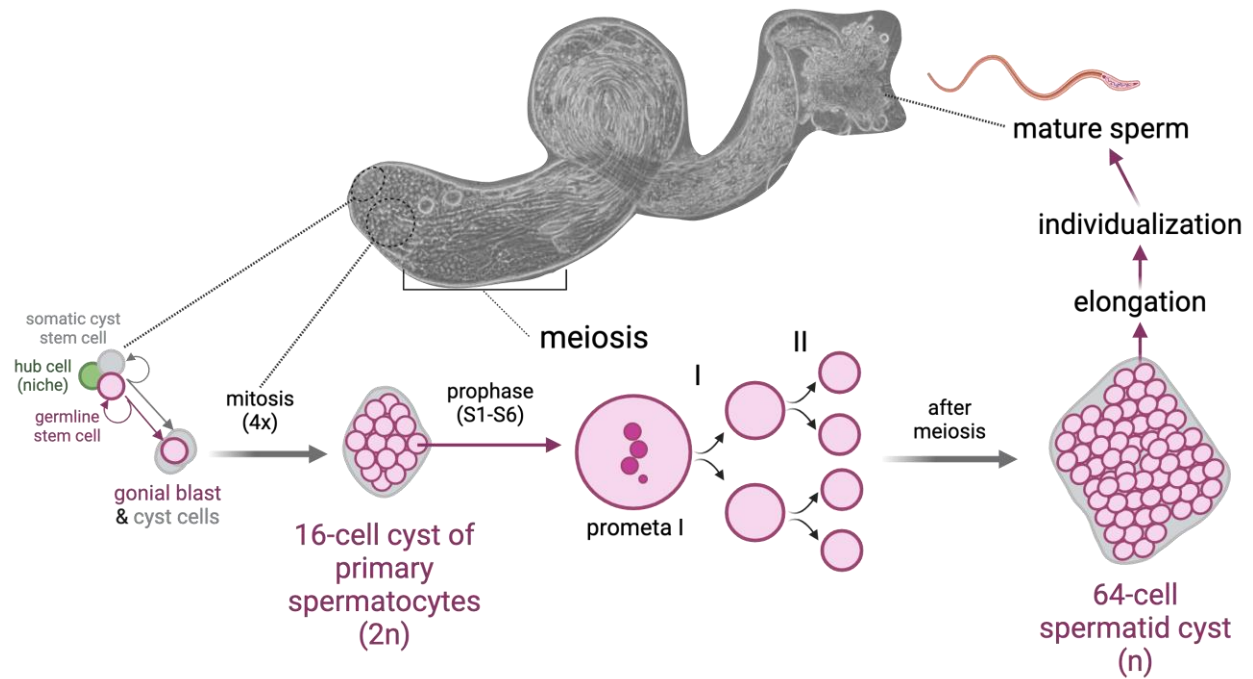


Figure 1.12 Spermatogenesis in *Drosophila melanogaster*.

The hub found at the apical tip of the *Drosophila* testis comprises of about 20 somatic cells (green cell) with 8 germline stem cells (pink cell), each accompanied by two somatic cyst stem cells (light gray cell). Germline cells participate in mitotic divisions and then two meiotic divisions to give rise to spermatids (process shown by pink cells). Location of each cell type in the gonad is generally indicated by the black circles and lines. Figure adapted from White-Cooper, 2014 and Weber et al., 2020. Created in <https://BioRender.com>.

cell volume by a 25-fold, spending 80-90 hours in this stage, generating enough cellular materials to complete spermatogenesis.

Once prophase I is complete, chromosome condensation begins in which each set of paired homologs appear as three to four round DNA masses at the center of each cell in prometaphase I. Full chromosome congression to the metaphase plate is complete when all four homologs appear as a single, round DNA mass at the center of each primary spermatocyte. After anaphase I and telophase I, the cyst contains 32 cells referred to as secondary spermatocytes, which are still associated with their two SCCs. After a short interphase, secondary spermatocytes begin meiosis II, in which stages mimic similar nuclear morphologies that occur in meiosis I just with half as much DNA. At the completion of meiosis, all 64 spermatid nuclei are initially still interconnected within the cyst. Each spermatid nuclei will undergo the process of elongation, which polarizes the sperm cell with the nuclei being referred to as a “head” and a “tail” region being a motile flagella, and the individualization process which leads them to be stored in the seminal vesicle to be used during copulation with a female (White-Cooper, 2004).

1.4.2 Homolog conjunction proteins link homologs during male meiosis I

The term homolog conjunction was coined to referred to the pairing and connectivity of homologous chromosomes during meiosis I specifically in the male germline. Male meiosis in *Drosophila* occurs without meiotic recombination, and meiotic chromosomes lack the localization of synaptonemal complex proteins, which are required for crossing over. As stated earlier, chiasmata structures formed by the meiotic recombination process have been shown to contribute to ensuring proper homolog segregation in many organisms. So, the question of how homologous chromosomes remain together during meiosis I in the male germline remained

unanswered until the identification of genes *snm* (*stromalin in meiosis*), *mnm* (*mod(mdg(4)) in meiosis*), *tef* (*teflon*), and *uno* (*univalents only*).

Mutants of *snm*, *mnm*, *tef*, and *uno* all show loss of autosomal homolog conjunction during prometaphase I and the random assortment of the autosomal univalents during anaphase I. Mutants of *snm*, *mnm*, and *uno* also result in the loss of the sex (XY) homolog conjunction and random assortment of the XY pair during the first division. Localization of the SNM, MNM, and UNO proteins to meiotic chromosomes has been shown during prometaphase I and metaphase I and their disappearance during the metaphase I to anaphase I transition (Thomas et al., 2005); (Weber et al., 2020). The TEF protein has not yet been visualized on meiotic chromosomes, but by previous studies it is assumed to localize and contribute to autosomal homolog conjunction (Tomkiel et al., 2001). How the conjunction proteins function to facilitate the connectivity of homologous is not well understood. By protein-protein interaction studies, we have shown that SNM, MNM and UNO at least physically interact with one another. We hypothesize that they form an alternate homolog conjunction (AHC) protein complex that may perhaps encircle homologous chromosomes, like an evolutionarily diverged cohesin complex (Adams et al., 2020). Though *Dipteran* males have achiasmate chromosomes, homologs still pair and remain linked via the Alternate Homolog Conjunction (AHC) complex, shown in **Figure 1.13**, which is essential for proper homolog segregation in male meiosis (Tomkiel et al., 2001); (Thomas et al., 2005); (Weber et al, 2020); (Adams et al., 2020).

1.4.3 Meiotic specific sister cohesion proteins ensure proper sister chromatid separation

Unlike many eukaryotic organisms in which encode a homolog of the meiotic specific cohesin subunit REC8, *Drosophila* lacks a direct homolog of the specialized alpha-kleisin subunit. As

discussed previously, the presence of such meiotic specific alpha-kleisin subunit is indispensable during meiosis I as it is protected from protease cleavage via Separase activity during anaphase I of most eukaryotes.

However, identification of the *Drosophila* specific, meiotic specific cohesion proteins SOLO (Sisters on the Loose), SUNN (Sisters Unbound), and ORD (Orientation Disruptor), referred to as **SOS** proteins, provided new insights into how sister chromatid cohesion is maintained during both male and female meiosis in the absence of a direct REC8 homolog. Mutations in any of the three genes *solo*, *sun*, or *ord* results in the premature separation of sister chromatids well before the first meiotic division. The premature separation of sister chromatids results in equational segregation during homolog disjunction and high rates of sister chromatid mis-segregation events during meiosis II (Bickel et al., 1997); (Yan et al., 2010); (Yan et al., 2013); (Krishnan et al., 2014). Additionally, in female meiosis meiotic recombination is severely disrupted. Localization of SOLO and SUNN proteins have been shown to localize specifically to centromeres in both males and females and with synaptonemal protein C(3)G, specifically in females (Yan et al., 2013); (Krishnan et al., 2014). Our studies of the SOS proteins have led us to hypothesize that they most likely form a meiotic specific cohesion complex, specific to the *Drosophila* species, along with the known cohesins SMC1 (Structural Maintenance of Chromosomes 1) and SMC3 (Structural Maintenance of Chromosomes 3), as shown in **Figure 1.13**.

Characterization of EMS-induced mutant alleles led to the discovery of novel protein complexes required for proper chromosome segregation in fly meiosis. In addition to this, these studies represent a small example of how powerful a forward genetics approach can be in not

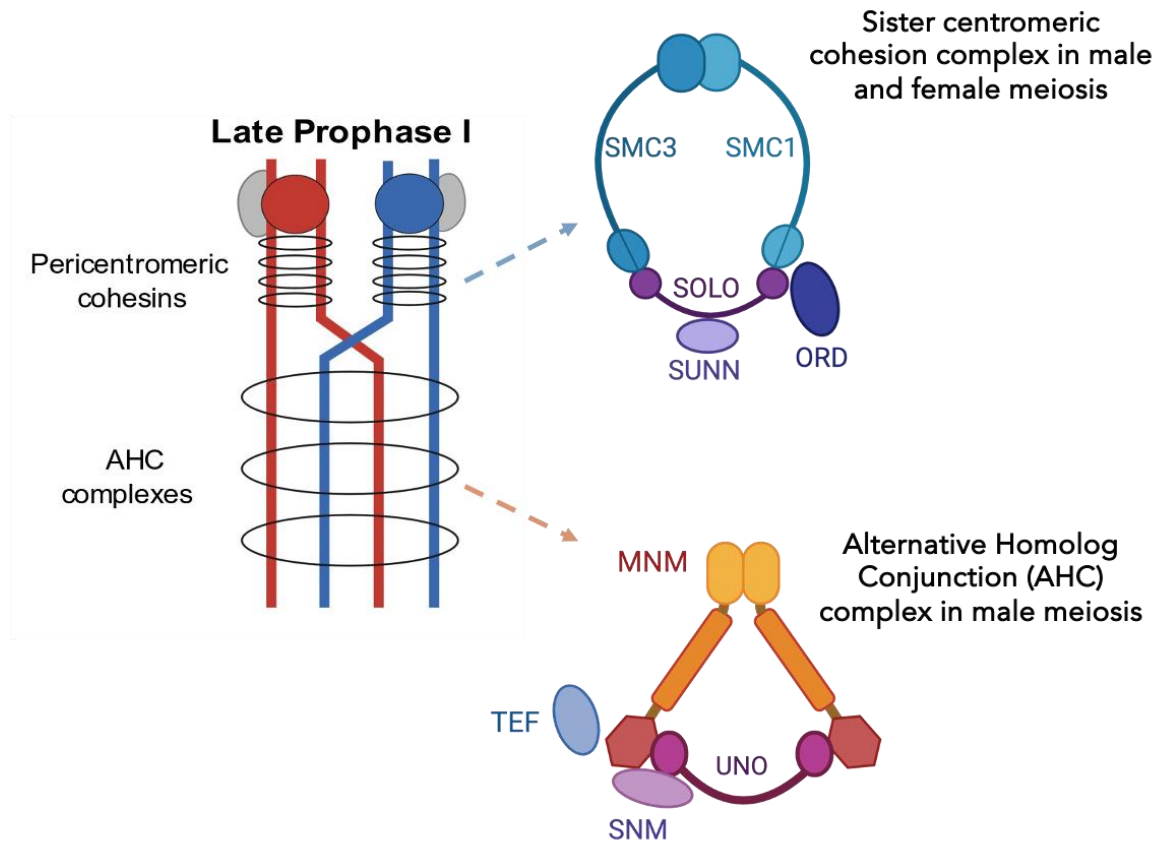


Figure 1.13 Representation of the sister chromatid cohesion complex and the alternate homolog conjunction (AHC) complex in *Drosophila* meiosis. This figure is modified from Adams et al., 2020 and created in <https://BioRender.com>.

only identifying novel genes but also developing new hypotheses about how cells ensure proper chromosome segregation, especially in the unique story of fly meiosis. This dissertation work focuses on the characterization of another EMS-induced mutant allele, originating from the same collection of mutant alleles that led to the discovery of the homolog conjunction complex and SOS proteins, with the desire to further our understanding of how other genes ensure proper meiotic chromosome segregation in fly meiosis.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Fly Lines Used and Fly Culturing

The Z3-3822 was obtained from the EMS-mutagenized third chromosome Zuker-3 (Z3) collection (Wakimoto et al., 2004). All other fly lines obtained from the Bloomington *Drosophila* Stock Center (BCSC) are provided in **Table 2.1** and **Table 2.2**. Flies were raised on the molasses-cornmeal-agar medium or dextrose-cornmeal-agar medium at 25°C or 22°C for expression of UASc-Spc25 transgenic constructs.

2.2 Meiotic Chromosome Nondisjunction (NDJ) Assays by Genetic Crossings

2.2.1 General fly crossing

For each NDJ assay, parents were allowed to mate for up to seven days. Progeny were scored up to 20 days but no later. The crossing schemes for the NDJ assays followed the procedures as described in Thomas et al., 2005.

2.2.2 Sex chromosome nondisjunction cross assay

To measure meiotic sex chromosome (XY) NDJ, $w/B^s Y y^+$ males were crossed singly to 2–3 $C(1)RM, y^2 w^a su(w^a)/O$ females in which the two X chromosomes are attached to a single centromere. The genotypes of the eggs produced by these females are diplo-X and nullo-X at approximately equal frequencies. Fertilization of nullo-X eggs gives rise to $y^+ w$ females and B^s males. These two types of progeny are derived from XX and XY sperm respectively, and are diagnostic of sister chromatid and homolog NDJ, respectively. Fertilization of diplo-X eggs by nullo-XY (O) sperm, which can result from either sister chromatid or homolog NDJ, gives rise $y^2 w^a su(w^a)$ females. The genotypes of gametes produced by the male and female and all possible

Table 2.1 Fly stocks used in this study.

Stock Number	Genotype	Chromosome Break Points
9227	w[1118]; Df(3R)ED5428, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5428/TM6C, cu[1] Sb[1]	3R:9630791;3R:10048611
25696	w[1118]; Df(3R)BSC621/TM6C, cu[1] Sb[1]	3R:1000091010001239;3R:10144754
9082	w[1118]; Df(3R)ED5474, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5474/TM6C, cu[1] Sb[1]	3R:10109412;3R:10350724
24983	w[1118]; Df(3R)BSC479/TM6C, Sb[1] cu[1]	3R:10265599;3R:10854406
7638	w[1118]; Df(3R)Exel6159, P{w[+mC]=XP-U}Exel6159/TM6B, Tb[1]	3R:10638895;3R:10889353
8957	w[1118]; Df(3R)ED5514, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5514/TM6C, cu[1] Sb[1]	3R:10884998;3R:11569253
9084	w[1118]; Df(3R)ED5518, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5518/TM6C, cu[1] Sb[1]	3R:10884998;3R:11619900
24973	w[1118]; Df(3R)BSC469/TM6C, Sb[1] cu[1]	3R:11230695;3R:11949049
8029	w[1118]; Df(3R)ED5577, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5577/TM6C, cu[1] Sb[1]	3R:11828741;3R:12477578
24990	w[1118]; Df(3R)BSC486/TM6C, Sb[1] cu[1]	3R:12430124;3R:13401874
37537	w[1118]; Df(3R)ED5623, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5623/TM2	3R:13259749;3R:13983912
9090	w[1118]; Df(3R)ED5644, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5644/TM6C, cu[1] Sb[1]	3R:14017903;3R:14625709
9086	w[1118]; Df(3R)ED5591, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5591/TM6C, cu[1] Sb[1]	3R:12350531;3R:12720010
27920	w[1118]; Df(3R)BSC847/TM6C, Sb[1] cu[1]	3R:12372864;3R:12562460
7932	w[1118]; Df(3R)Exel7317/TM6B, Tb[1]	3R:12441376;3R:12630599-12630609
3003	Df(3R)T-32, cu[1] sr[1] e[s]/MRS	3R:11246530-11269696;3R:12660496-12673277
7645	w[1118]; Df(3R)Exel6166, P{w[+mC]=XP-U}Exel6166/TM6B, Tb[1]	3R:12679031;3R:12715026-12738206
9087	w[1118]; Df(3R)ED5610, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5610/TM6C, cu[1] Sb[1]	3R:12444016;3R:12995675
7972	w[1118]; Df(3R)Exel7318/TM6B, Tb[1]	3R:12723855;3R:12947274-12975803
7973	w[1118]; Df(3R)Exel8157/TM6B, Tb[1]	3R:13012713;3R:13051785
7646	w[1118]; Df(3R)Exel6167, P{w[+mC]=XP-U}Exel6167/TM6B, Tb[1]	3R:13051457;3R:13259379
25690	w[1118]; Df(3R)BSC615/TM6C, cu[1] Sb[1]	3R:1291878112918812;3R:13258860
9088	w[1118]; Df(3R)ED5612, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5612/TM6C, cu[1] Sb[1]	3R:12719985;3R:13645134

Table 2.2 Additional fly stocks used in this study.

Stock Number	Genotype	General Information
16532	w[1118]; P{w[+mGT]=GT1}CG11226[BG01780]	Used for P-element mapping; located on Chr 3, 80A4, 3L:22837931
12719	w[1118]; P{w[+mGT]=GT1}BG02628	Used for P-element mapping; located on Chr 3, 98F5, 3R:28990887
91734	w[*]; P{w[+mC]=gSpc25-mRFP}II.1	Expresses Spc25 protein with a C-terminal mRFP1 tag under control of Spc25 regulatory sequences.
91729	w[*]; P{w[+mC]=gEGFP-Nuf2}II.1	Expresses Nuf2 protein with an N-terminal EGFP tag under control of Nuf2 regulatory sequences
4414	y[1] w[*]; P{w[+mC]=Act5C-GAL4}25FO1/CyO, y[+]	Expresses GAL4 ubiquitously under control of Act5C promoter (P{AyGAL4} with the y[+] FRT cassette flipped out)
91733	w[*]; PBac{w[+mC]=PB}grsm[c00064] Spc25[c00064]/TM6B, P{w[+mC]=Dfd-EYFP}3, Sb[1] Tb[1] ca[1]	The PBac{PB} insertion maps to 38bp upstream of the Spc25 initiation codon in the 5'UTR of the Spc25 gene.

phenotypes observed in the progeny are shown in **Figure 2.1**. Percentage of sex chromosome NDJ is calculated by percent sister chromatid NDJ/total NDJ = $100 \times (2 \times XX) / ((2 \times XX) + XY)$.

2.2.3 Second chromosome nondisjunction cross assay

To measure meiotic chromosome 2 NDJ, mutant males were singly crossed to two to three C(2)EN *b p* virgins. These virgins produce two types of aneuploid eggs with half of their gametes containing two copies of chromosome 2 (diplo-eggs) and the other half lacking chromosome 2 (nullo-eggs). Viable embryos from such a cross can only occur by fertilization of diplo eggs by nullo-sperm or nullo-eggs by diplo-sperm, shown in **Figure 2.2**. Frequency of chromosome 2 NDJ is obtained by scoring progeny per male.

2.2.4 Fourth chromosome nondisjunction cross assay

To measure meiotic chromosome 4 NDJ, mutant males were singly crossed to two to three C(4)RM *ci ey^R*. Virgins in the C(4)RM *ci ey^R* produce two types of eggs, ones with two copies of chromosome 4 (diplo eggs) or no copy of chromosome 4 (nullo eggs). Progeny from the cross of virgin females are viable as aneuploidy for the 4th chromosome in flies is not detrimental to viability. However, paternal NDJ can only be observed in the progeny by the result of nullo sperm (genotype: O) fertilizing diplo eggs (genotype: 4^{ci ey^R}, 4^{ci ey^R}), shown in **Figure 2.3**. To calculate the percentage of fourth meiotic chromosome NDJ, the number of *ci ey^R* progeny are multiplied by two, divided by the total number of progeny, and then multiplied by 100. The number of *ci ey^R* progeny are multiplied by two as the generation of nullo sperm is expected to be equivalent to the number of diplo sperm generated.

$w / Y^{Bs y+}$  X  $C(1)RM B y^2 w^a su(w^a) / O$		
sperm genotype	egg genotype	
	$X^{B y2}, X^{B y2}$	O
X^w (normal)	lethal	 (Round eyed males)
$Y^{Bs y+}$ (normal)	 (Bar-stone eyed females)	lethal
O (nullo)	 (Bar eyed females)	lethal
$X^w, Y^{Bs y+}$ (homolog NDJ)	lethal	 (Bar-stone eyed males)
X^w, X^w (sister NDJ)	lethal	 (Round eyed females)

Figure 2.1 Sex Chromosome NDJ Assay.

Diagram of possible genotypes of the gametes generated by males and C(1)RM B y2 wa su(wa) females and resulting phenotypes in the F1 progeny. Figure created in <https://BioRender.com>.

		$\frac{b\ pr}{b\ pr}$ 	O
Normal sperm	$+$ 		
	$+$ 		
NDJ sperm	$\frac{+}{+}$ 		 $\frac{+}{+}$
	O	 $\frac{b\ pr}{b\ pr}$	

Figure 2.2 Chromosome 2 NDJ Assay.

Diagram of possible genotypes of the gametes generated by both males and C(2)EN b pr females and resulting phenotypes in F1 progeny. Figure created in <https://BioRender.com>.

w / Y  $w^+ ; C(4)RM \text{ ci } ey^R / O$ 		
X		
sperm genotype	egg genotype	
	$4^{ci, ey^R}, 4^{ci, ey^R}$	O
4^+ (normal)	 $4^+ / 4^+$	lethal
$4^+, 4^+$ (diplo)	lethal	 $4^+ / 4^+$
O (nullo)	 $ci, ey^R / O$	lethal

Figure 2.3 Fourth Chromosome NDJ Assay.

Diagram of possible genotypes of the gametes generated by both males and $C(4)RM \text{ ci } ey^R$ females and resulting phenotypes F1 progeny. Figure created in <https://BioRender.com>.

2.3 Chromosome Mapping

2.3.1 Recombination mapping

After the *Z3-3822* mutant fly stock was introduced to a *white* background for recombination mapping experiments, we used two independent lines containing *P*-elements *P*(98F5) and *P*(80A4) inserted into the tip of 3R (right arm of chromosome 3) and the pericentromeric heterochromatin of 3L (left arm of chromosome 3), respectively. To detect recombination events between the *Z3-3822* allele and each *P*-element, heterozygous *Z3-3822* males (*w/Y*; *Z3-3822* / *TM6B*) were crossed to females carrying the *P*-element (*P*(98F5) or *P*(80A4)) (*PE*). As shown in **Figure 2.4**, in the germline of females, meiotic recombination between nonhomologous sister chromatids can result in recombinant third chromosome chromatids lacking the *PE* and some portion of the mutant chromosome that either contain or do not contain the *Z3-3822* lethal mutant allele (*Z3-3822*^{*l*+} or *Z3-3822*^{*l*-}, respectively).

Heterozygous *Z3-3822/PE* females were then back crossed to *Z3-3822/TM6B* males, and the phenotypes of the progeny were scored to determine if recombination between the *Z3-3822* and *PE* occurred. If recombinant gametes were generated by the heterozygous females, recombinant progeny would be detected as viable, white-eyed flies, shown in **Figure 2.5**. The proportion of viable white-eyed F2 progeny (*w/Y*; *Z3-3822* / *Z3-3822*^{*l*+}) out of the total progeny was used to calculate a map distance between the *PE* and the lethal allele. Any viable recombinant F2 male progeny (*w/Y*; *Z3-3822* / *Z3-3822*^{*l*+}) were then tested for meiotic chromosome NDJ events by the chromosome 2 NDJ assay, explained in **Figure 2.2**, to determine whether the lethal allele and a proposed meiotic mutant allele (*m*⁻) are linked. The proportion of F2 white-eyed males detected to have meiotic NDJ events out of all white-eyed males tested was

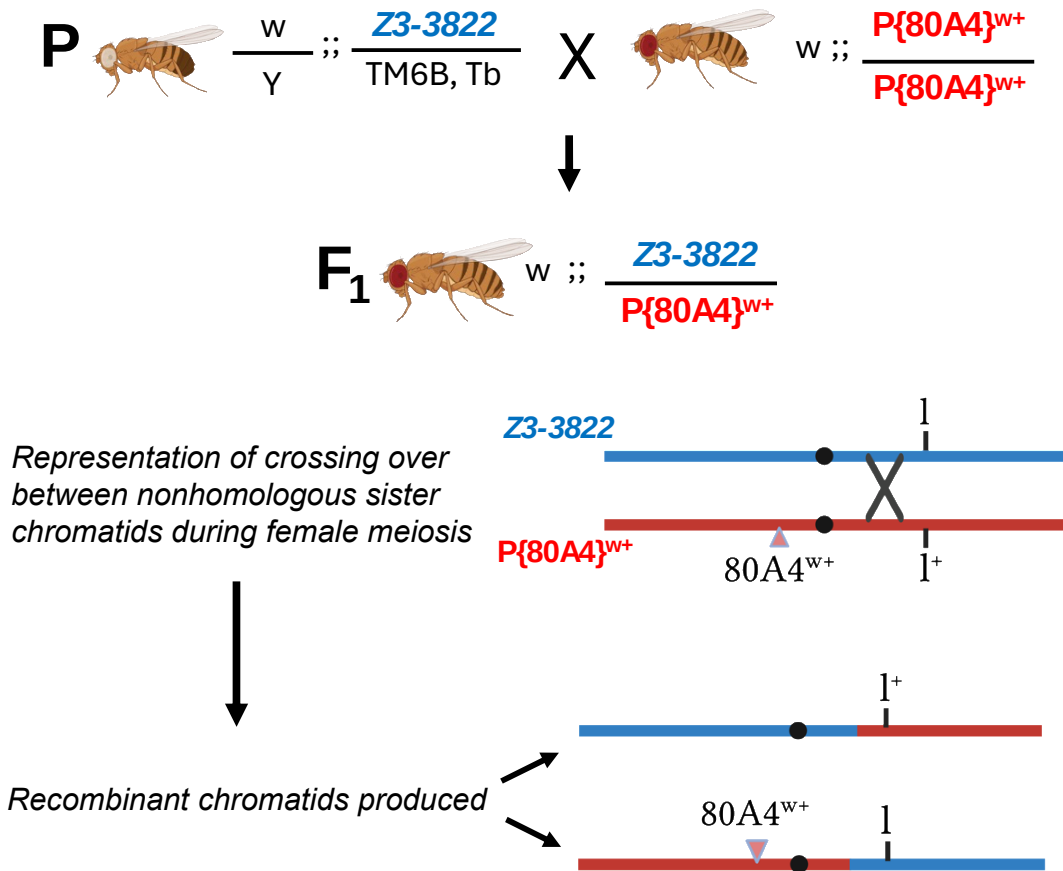


Figure 2.4 Recombination Mapping with P-elements.

Diagram of crossing scheme in recombination mapping experiment to generate heterozygous females where meiotic recombination occurs to produce potential Z3-3822 recombinant chromatids. Figure created in <https://BioRender.com>.

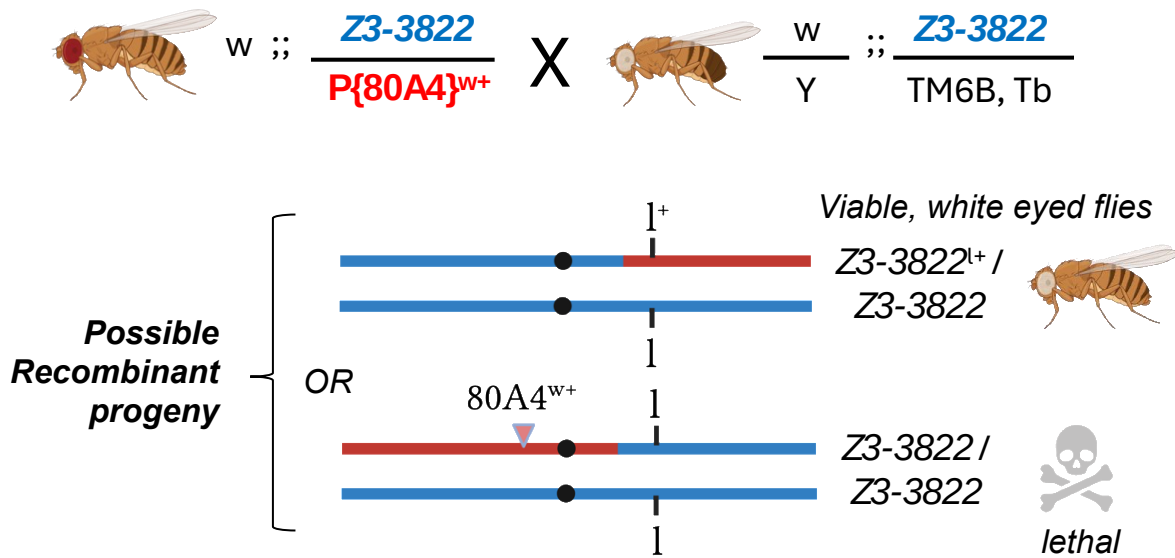


Figure 2.5 Generation of recombinant progeny in recombination mapping.
 Diagram of crossing scheme between heterozygous $Z3-3822 / P(80A4)^{w+}$ females and heterozygous $Z3-3822$ males to obtain F2 progeny. Figure created in <https://BioRender.com>.

used to determine the frequency of recombination between the *l* allele and the *m* allele and to calculate a map distance between the lethal and meiotic mutant alleles.

2.4 Molecular Characterization of Z3-3822 Mutant Allele

2.4.1 Genomic DNA sequencing

To determine the mutation site of Z3-3822 within the *spc25* locus, ten 3rd instar larvae (L3) were homogenized in DNAzol reagent (Invitrogen) to extract the genomic DNA as per user manual. Gene specific primers gSpc25-F and gSpc25-R were used to amplify the entire region of the *spc25* gene. Sequences for gSpc25-F and gSpc25-R are provided in **Table 2.3**. The PCR product was cloned into the pAGM1311 vector and sequenced. The dm6 *D. melanogaster* reference genome sequence obtained from FlyBase was used for comparison.

2.4.2 Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Approximately one hundred L3 of homozygous Z3-3822 and Z3-0317 (a genetic control that came from the same Zuker Z3 Collection) were collected, cleaned with RNase-free water, and then homogenized in 300 µL of Tri-Reagent (Zymo Research, Cat. Reference # R2050-1-50). Homogenates were incubated on ice for 10 min and then centrifuged for 1 min at 11,000g at room temperature. Extracted RNA in the supernatant was then further purified with an RNA clean-up kit (Zymo Research) following the user manual. Quality and quantity of the purified RNA were measured by the Bioanalyzer (TapeStation Analysis). To determine *spc25* transcripts expressed in Z3-3822 and Z3-0317 (control), RT-PCR was conducted with a 2-step RT-PCR protocol (Takara Cat. Reference # R022A), following the user manual. The oligo dT primed cDNA library was first generated for each genotype by the reverse transcription reaction. Gene specific primers Spc25-ex1,2-F and Spc25-ex3-R were used to specifically amplify a 337 bp

Table 2.3 Primers used in this study.

Name	Sequence (5' to 3')	Target region	Purpose
gSpc25-F	CCCTTGGTCTCAACATTACCACCACTAACTGGAAAGCAAA ACAA	Start of <i>spc25</i> 5' UTR	Amplify <i>spc25</i> genomic DNA
gSpc25-R	CCCTTGGTCTCTACAAGGGAGATATATGTATAATTTACAC TGCAC	End of <i>spc25</i> 3' UTR	Amplify <i>spc25</i> genomic DNA
Spc25-ex1,2-F	CCCTTGGTCTCAACATCAGCAATTTGTTGGATGTCAAGG	<i>spc25</i> Exon 1 and Exon 2 junction	Amplify <i>spc25</i> cDNA (RT-PCR)
Spc25-ex3-R	CCCTTGGTCTCTACAATATCAACTGGTCGCGTTTATCG	<i>spc25</i> Exon 3	Amplify <i>spc25</i> cDNA (RT-PCR)
Spc25-F	CCCTTGGTCTCAACATCAAAACAAGCGTCTGGAAAA	Start of <i>spc25</i> 5' UTR	Amplify <i>spc25</i> cDNA from BAC
Spc25-R	CCCTTGGTCTCTACAAGGGAGATATATGTATAATTTACAC TGC	End of <i>spc25</i> 3' UTR	Amplify <i>spc25</i> cDNA from BAC
Spc25-GCA-F	CACCGCAATTATTATGACTGAATCGAG	Spc25 second codon GCA	Clone <i>spc25</i> cDNA into pENTR/D-TOPO
Spc25-TGA-R	TCAGGTGTGGCTCATCGGC	Spc25 stop codon	Clone <i>spc25</i> cDNA into pENTR/D-TOPO
Spc25-D71E-F	CGGGAGGAGTTGGAGAAGCGAGTTTCC	Coding region for D71 residue	Site-directed mutagenesis for UASc-Spc25 ^{D71E}
Spc25-D71E-R	GGAAACTCGCTTCTCCAACCTCCAACCTCCTCCG	Coding region for D71 residue	Site-directed mutagenesis for UASc-Spc25 ^{D71E}
Spc25-RSAI-F	CGAAATCATGGAAAGATCTGCGATTAGCATTACACGCT GTC	Coding region to insert RSAI at Exon 2 – Exon 3 junction	Site-directed mutagenesis to make UASc-Spc25 ^{D71E, RSAI}
Spc25-RSAI-R	GACAGCGTGTGAATGCTAATCGCAGATCTTTCCATGATT CG	Coding region to insert RSAI at Exon 2 – Exon 3 junction	Site-directed mutagenesis to make UASc-Spc25 ^{D71E, RSAI}
Spc25-128-R	CTAAGAAAATCTTAATATGATTATCTTTGACCTAATCGCA GATCTTTCCATG	Coding region of 3' end of exon 2	Site-directed mutagenesis to make UASc-Spc25 ¹²⁸

fragment of *spc25* cDNA. Experimental design of this procedure is summarized in **Figure 2.6**. Sequences for primers are listed in **Table 2.3**. Each PCR fragment was gel excised, cloned into the pAGM1311 vector, and then sequenced.

2.5 Immunohistochemistry of *Drosophila* Tissues

2.5.1 Immunostaining of spermatocytes

Testes were dissected in 1X PBS and immediately held on ice until transfer to the microscope slide. On the slide, testes were ripped open at the tip with a 30-gauge needle to release spermatocytes in 15 μ L of 1X PBS. A Sigmacote-treated coverslip was then placed on top of the tissue and gently squashed. The slide was immediately immersed into liquid nitrogen until bubbling stopped; after another 12-15 sec, the coverslip was snapped off. Slides were incubated in 100% ethanol at -20°C for 10 min and then fixed with 1X PBS, 1% Triton X-100 (PBST), and 4% paraformaldehyde (PBST-PFA). Subsequent steps were done at room temperature, unless stated otherwise. Slides were then incubated in PBST containing 3% Sodium Deoxycholate (PBST-DOC) 2x15 min each and then blocked with PBST plus 3% bovine albumin serum (PBST-BSA) for 30 min. Primary antibodies mouse anti-alpha Tubulin (DM1A) diluted 1:10,000 and rabbit anti-MYC (Cell Signaling 71D10 #2278S) diluted 1:200 in PBST-BSA. Primary antibodies were added to slides in humidifying chamber overnight at 4°C. After incubation, slides were washed 4x15 min each with PBST. Goat, anti-mouse Alexa₅₆₈ or Donkey, anti-mouse Alexa₄₈₈ secondary antibody was diluted 1:1,000 in PBST and added to slides in dark humidifying chamber for 1-2 h. Following incubation with the secondary antibody, slides were washed 4x15 min each with PBST. To visualize the spermatocyte nuclei, 4', 6-diamidino-2-phenylindole (DAPI) (1 μ g/mL) was added to slides and incubated for 10 min in dark a

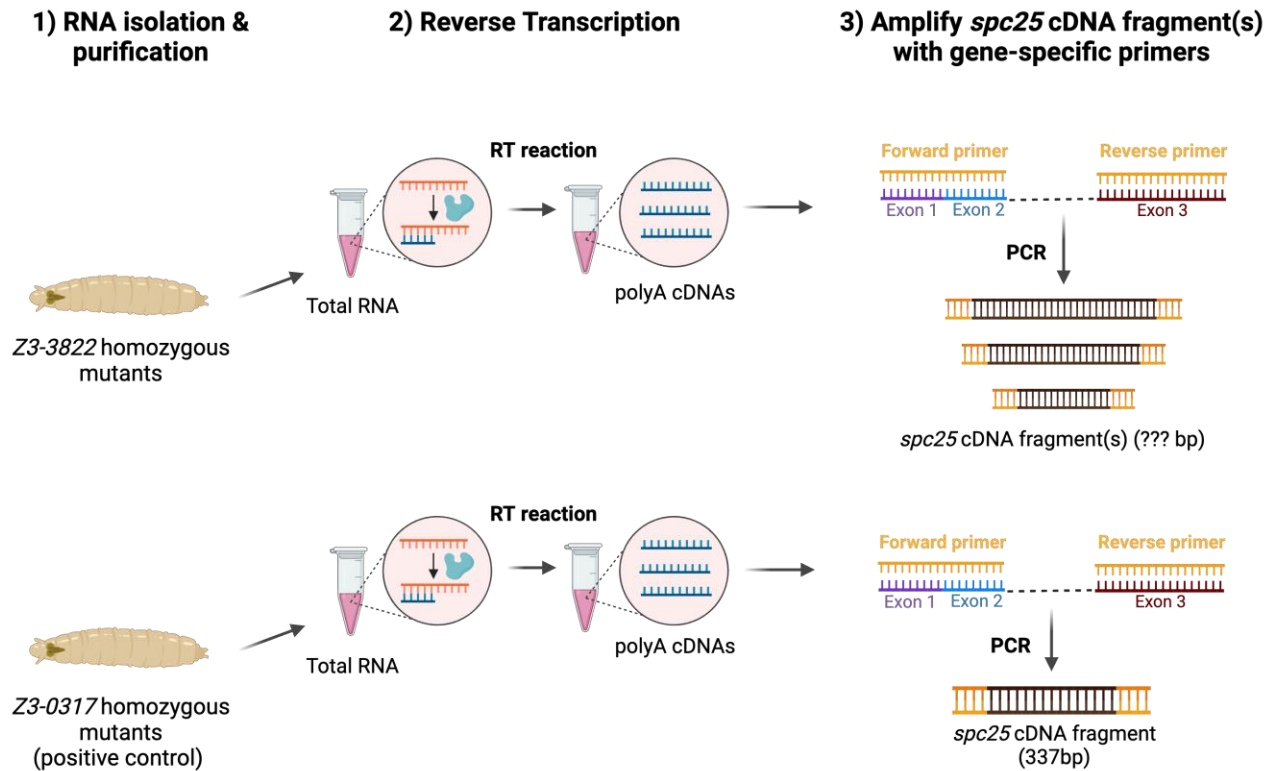


Figure 2.6 Diagram of experimental step-up to characterize mutant *spc25* transcripts with reverse transcription and PCR (RT-PCR).
Figure created in <https://BioRender.com>.

humidifying chamber. Slides were washed in 1X PBS for 10 min and then air-dried for 10 min. Vectashield or house-made mounting medium (2% n-propyl gallate, 80% glycerol, 20% 0.1M Sodium phosphate buffer, pH 7.4) was added onto slides with a coverslip and sealed with clear nail polish.

2.5.2 Immuno-Fluorescence In-Situ Hybridization (FISH) staining of spermatocytes

Immuno-FISH testis squashes were initially prepared the same way as the immunostaining protocol mentioned above up to the completion washing after secondary antibody incubation. An additional fixation with PBST-PFA for 5 minutes in a dark humidifying chamber at room temperature was done. After fixation, Fluorescence In-Situ Hybridization was performed with the modification of starting with the 70% ethanol incubations (Tsai et al., 2011).

2.5.3 Immunostaining of larval, pupal, and adult central nervous system (CNS)

Intact brains were dissected out and placed in a clean well with a fixative solution (200 μ L 1X PBS and 70 μ L of 16% paraformaldehyde) and kept on ice until all brains were dissected. Brains were incubated in the fixative overnight at 4°C. The next day, the brains were washed three times with 1X PBS, the first two washes were done back-to-back and the final PBS wash with slow orbital shaking for 7 min at room temperature. Following washes, the brains were incubated with TNT solution (0.1 M Tris (pH 7.4), 0.3 M NaCl, and 0.5% Triton X-100) for three times, 15 min each, at room temperature with slow orbital shaking. After the TNT solution incubations, the solution was removed, and brains were rinsed with 1X PBS. Brains were then incubated with DAPI (1 μ g/mL in 1X PBS) for 30 min at room temperature with orbital shaking and then washed with 1X PBS (2x7 min each) with orbital shaking. Brains were then incubated with the house made mounting medium for 30 min at room temperature or overnight at 4°C and then mounted

on a microscope slide and covered with a glass coverslip sealed with clear nail polish.

2.6 Generation of Clones for Transgenic Constructs

2.6.1 Cloning of *Spc25* cDNA

Wildtype *spc25* cDNA was obtained within the LD37916 Bacterial Artificial Clone (BAC) from the Drosophila Genomics Resource Center (DGRC Stock 1324398). PCR amplification of full length *spc25* cDNA was performed with primers: Spc25-F and Spc25-R, sequences of primers provided in **Table 2.3**. Purified PCR product was cloned into the pAGM1311 vector. *Spc25* cDNA with the 5' end starting at the second codon (GCA) and ending with the stop codon (TGA) was then PCR amplified from the pAGM1311-*spc25* cDNA clone with the primers Spc25-GCA-F and Spc25-TGA-R, sequences of primers provided in **Table 2.3**. The PCR product was purified and then cloned into the GATEWAY pENTR/D-TOPO entry vector to create a pENTR-*spc25* clone (pENTR manual).

2.6.2 Site-directed mutagenesis (SdM) of *Spc25* cDNA

The pENTR-*spc25* was then used as a template to create pENTR-*spc25*^{D71E} with the QuikChange Lightning Site-Directed Mutagenesis (SDM) kit (Agilent; Catalog #210518). To create the pENTR-*spc25*^{D71E} clone, mutagenic primers Spc25-D71E-F and Spc25-D71E-R were used. To create the pENTR-*spc25*^{D71E,RSAl} clone, pENTR-*spc25*^{D71E} was used as the template and mutagenic primers Spc25-RSAl-F and Spc25-RSAl-R were used to insert the Arg-Ser-Ala-Ile motif between the residues found at the Exon 2 – Exon 3 junction. The pENTR-*spc25*¹²⁸, encoding a predicted truncated peptide of 128 amino acids produced from one of the Z3-3822 mutant *spc25* transcripts, was also constructed. A pENTR-*spc25*^{RSAl} clone, only encoding the four amino acid insertion, was used as a template and the mutagenic reverse primer Spc25-128-R

was used. **Figure 2.7** shows a graphical abstract of all mutant Spc25 pENTR/D-TOPO entry vectors that were generated demonstrating where each mutation was induced. Sequences of mutagenic primers are included in **Table 2.3**.

2.6.3 Generation of expression vectors and transgenic fly lines

Individual pENTR-*spc25* clones were each subject to the bacteriophage lambda (LR) recombination reaction using the Invitrogen Gateway LR Clonase II enzyme mix to insert the *spc25* cDNA into a destination vector according to the protocol. Each *spc25* cDNA was inserted into the destination vector pBID-UASc-MRG, an expression vector that upon expression generates an in-frame fusion Spc25 protein with an amino-terminal tag of three copies of Myc and tRFP (bright Red Fluorescent Protein) (Wang et al., 2012). **Figure 2.8** shows a vector map for the expression vector used. The four expression vectors: 1) pBID-UASc-MR(*spc25*), 2) pBID-UASc-MR(*spc25*^{D71E}), 3) pBID-UASc-MR(*spc25*¹²⁸), and 4) pBID-UASc-MR(*spc25*^{D71E, R5A1}), were generated and injected into embryos from either *y w* P{nos-phiC31/int.NLS}X; P{CaryP}attP40^{y+} or *y w* M{vas-int.Dm}ZH2A; P{CaryP} attP40^{y+} for germline transformation. The transformed flies showing the eye marker mini-white (*mw*⁺) were crossed to *w*; *Noc*^{Sco}/*Cy*; *MKRS/TM6B* for establishment of second chromosome balanced stocks.

2.7 Microscopy and Image Analysis

2.7.1 Microscopy

Spermatocytes were visualized and imaged on a VT-Hawk 2D-array scanning confocal microscope (Visitech intl.) mounted to an IX-83 inverted optical microscope (Olympus). Z-series images with a step interval of 0.3 μ m were collected using a 100X objective lens (Nikon) in MetaMorph (Molecular Devices).

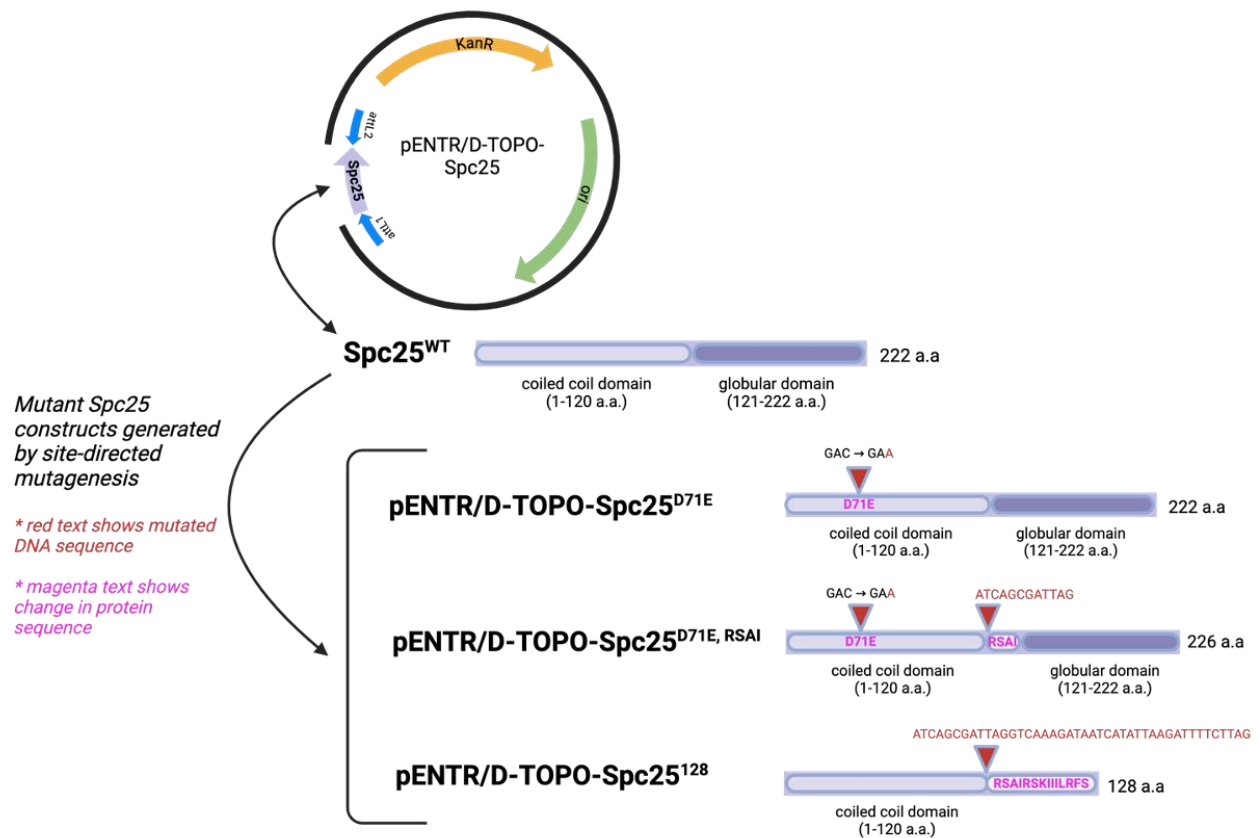


Figure 2.7 Diagram of pENTR/D-TOPO-Spc25 entry vectors generated. Figure created in <https://BioRender.com>.

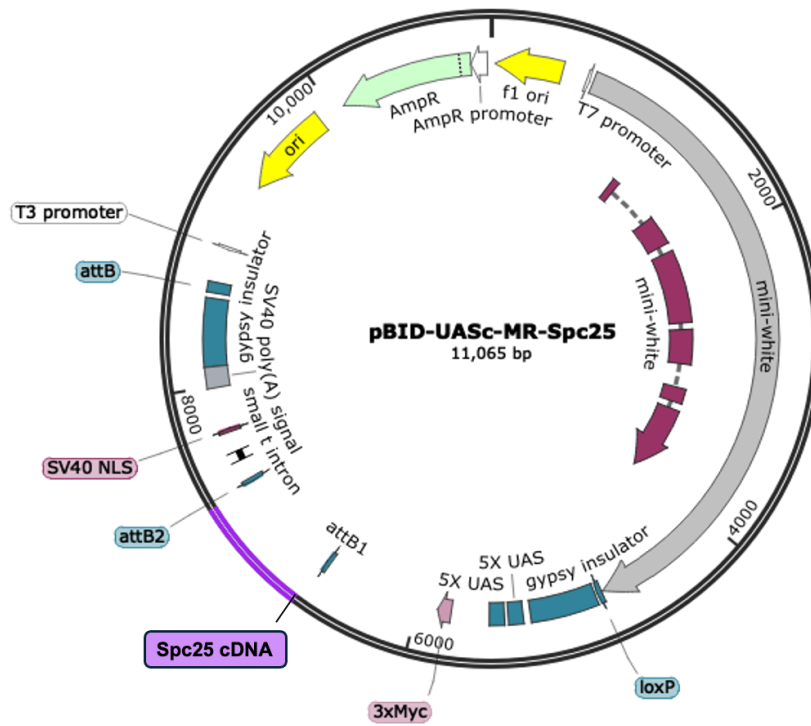


Figure 2.8 Diagram of expression vector used to express Spc25 transgenes. Vector map generated in Snap Gene Viewer.

2.7.2 Image processing and qualitative and quantitative analysis

For image panels, in ImageJ (Fiji), a duplicate of the original image was created and processed by subtracting the background with a rolling ball radius of 50 pixels and a sliding paraboloid. The processed image was then used as 16-bit images for quantification. Maximum intensity projections (16-bit greyscale) were created for Z-series projections for the 405, 488, and 561 channels identified for individual cells using merged tricolor images.

For DAPI intensity quantification of telophase nuclei, in ImageJ (Fiji) maximum intensity projections (16-bit greyscale) images were used to draw a circle around each nucleus to make a Region-Of-Interest (ROI) to save in the Fiji ROI manager. Saved ROIs for each pair of nuclei corresponding to a telophase cell was then projected onto a duplicated 16-bit greyscale image of a Z-series projection of the average intensity within each nucleus. The integrated density of each paired nuclei was calculated within the Image Calculator. T-tests were conducted with Prism (GraphPad) with a Mann-Whitney test with a P-value of greater than 0.05.

For brain lobe size analysis, Z-series stacks were used to make maximum intensity projections of the 405 channel (DAPI staining). Using the draw tool in Fiji, ROIs were drawn around each brain lobe and the total area of each ROI was calculated by Fiji with appropriate scales set.

CHAPTER THREE

RESULTS

3.1 Phenotypic Characterization of Z3-3822 Homozygous Mutants

3.1.1 Homozygous Z3-3822 mutants are semi-lethal

The 3rd chromosomal EMS (ethyl methane-sulfonate) induced mutant fly line Z3-3822 was preliminarily analyzed and categorized within a subset of mutants that show elevated chromosome 4 loss during male meiosis (Koundakjian et al., 2004); (Wakimoto et al., 2004). Several mutants within this collection identified novel proteins directly involved in facilitating proper meiotic chromosome segregation in males (Bickel et al., 1997); (Tomkiel et al., 2001); (Thomas et al., 2005); (Tsai et al., 2011); (Krishnan et al., 2014). Therefore, we hypothesized that the mutant allele Z3-3822 encodes for a gene required in a mechanism that ensures accurate chromosome transmission in male meiosis.

Upon initial analysis of general mutant phenotypes, homozygous Z3-3822 mutants were found to be pupal lethal, with few viable escapers. Viable escapers are physically weak as they have difficulty walking, climbing, and engaging in courtship behavior. Homozygous Z3-3822 females mated with normal males lay eggs that do not develop further. Males appear to be uninterested in courtship behavior. Homozygous males and females do not survive past two to three days following eclosion. The observed sub-normal viability phenotypes were not previously documented. However, reports of several other mutants from this collection were documented to accumulate recessive lethal alleles over several generations, which could have occurred prior to our analysis of the Z3-3822 mutant line (Wakimoto et al., 2004).

3.1.2 Homozygous Z3-3822 mutants show paternal meiotic chromosome nondisjunction

Z3-3822 mutants previously showed phenotypes of disrupted meiotic chromosome segregation (Wakimoto et al., 2004). To estimate the rate of chromosome nondisjunction (NDJ) occurring in Z3-3822 mutants, homozygous mutant larval or pupal testis were dissected, and spermatocytes squash preparations were made to analyze meiotic divisions. Homozygous Z3-3822 spermatocyte preps were subject to immunocytochemistry with an anti-alpha tubulin antibody followed by Fluorescence In-Situ Hybridization (FISH) with the DNA probe Dodeca, labeling the pericentromeric heterochromatin on chromosome 3, shown in **Figure 3.1**. In wild-type primary spermatocytes during prometaphase and metaphase, observation of two Dodeca spots represent the two 3rd homologs each with cohesive pairs of sister chromatids. Then, as homologs segregate during anaphase I only one spot is present in prometaphase II and metaphase II secondary spermatocytes. During anaphase II onset, if each spermatid nuclei receives one Dodeca spot, then 3rd chromosome sister chromatids have separated properly (Tsai et al., 2011). Normal Dodeca spot numbers are shown representatively in each stage of male meiosis in **Figure 3.1**.

An overall rate of meiotic chromosome nondisjunction (NDJ) for chromosome 3 in heterozygous Z3-3822 mutants (Z3-3822 / TM6B) and homozygous mutants was quantified by scoring the number of Dodeca spots per spermatid nuclei. As shown in **Figure 3.1**, normal sperm should only have one spot. If meiotic chromosome NDJ occurs, spermatid nuclei could either have no copies or two copies of chromosome 3, representing spermatids either lacking a Dodeca spot or containing two Dodeca spots, respectively. An overall rate of meiotic chromosome NDJ was calculated by dividing the total number of NDJ spermatids observed by the total number of

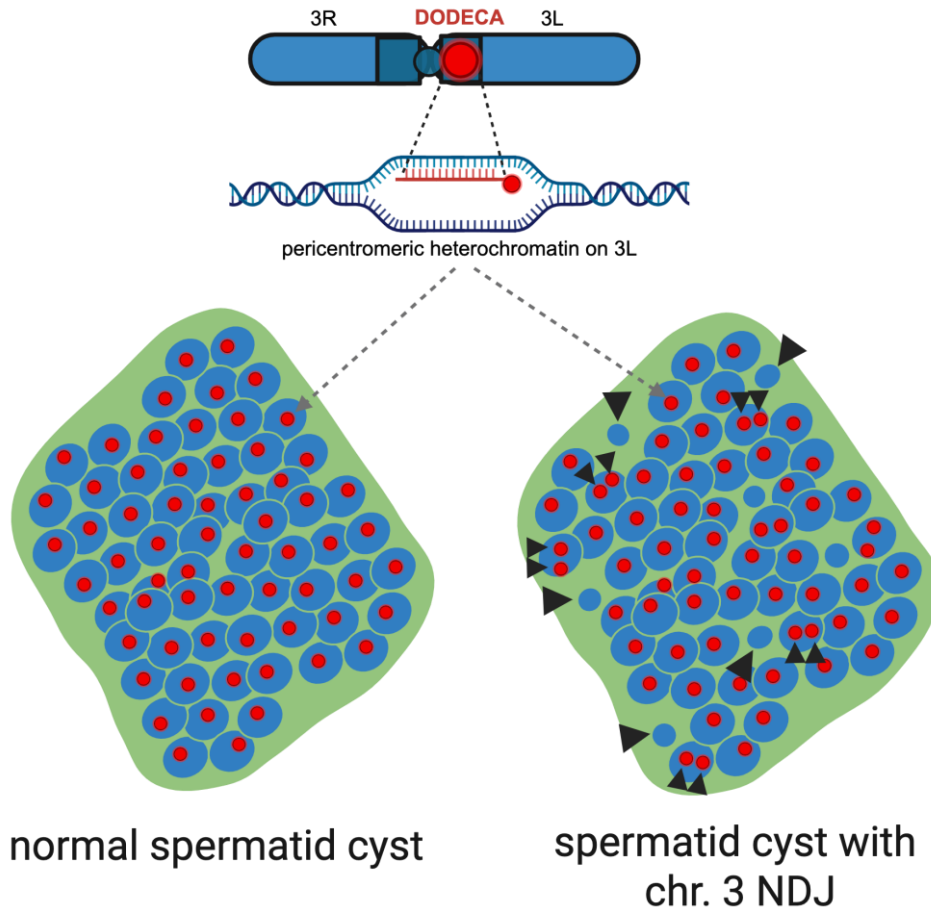


Figure 3.1 Immuno-FISH staining with 3rd chromosome marker, Dodeca, to quantify rate of chromosome 3 meiotic chromosome NDJ in male meiosis. FISH probe Dodeca hybridizes to the pericentromeric heterochromatin of chromosome 3. (Left) Representation of a normal spermatid cyst shows that normal spermatid nuclei (blue circles) only have 1 Dodeca spot (red dot), reflecting only 1 copy of chromosome 3 at the end of meiosis. (Right) Representation of a spermatid cyst that has had chromosome 3 meiotic NDJ events. Single large black arrowheads indicate smaller micronuclei, generated during chromosome NDJ, without a Dodeca spot. Smaller, coupled black arrowheads point to 2 Dodeca spots within one spermatid nuclei, which also result because of chr. 3 NDJ events during meiosis. Figure created in <https://BioRender.com>.

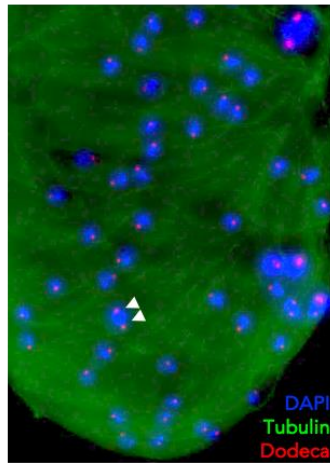
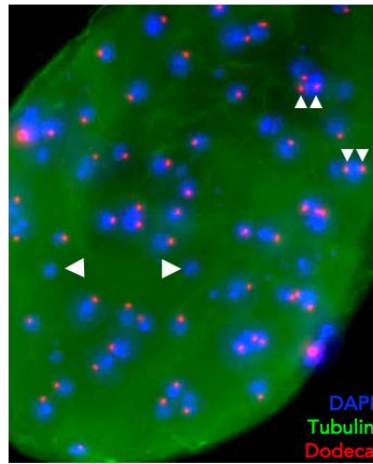
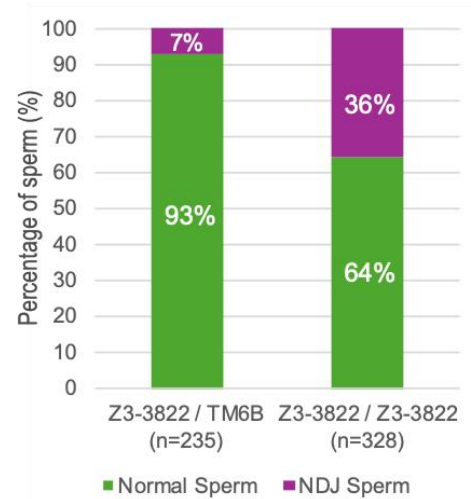
spermatids scored.

Figure 3.2A shows a representative image from both heterozygous and homozygous *Z3-3822* mutant spermatid cysts immunostained with anti-Tubulin, hybridized with the Dodeca probe, and stained with DAPI (4', 6-diamidino-2-phenylindole) to visualize nuclei. As presented in **Figure 3.2B**, heterozygotes (*Z3-3822* / TM6B) had 7% NDJ of chromosome 3; however, homozygotes had 36% NDJ of chromosome 3. Together with the genetic tests, these data strongly suggest that an unknown mutation in the *Z3-3822* fly line is associated with defective chromosome segregation in male meiosis, which became the starting point of the characterization of the mutant line.

3.1.3 Homozygous third instar larvae (L3) Z3-3822 mutants have small brain lobes

Based on the semi-lethality and male meiotic chromosome segregation NDJ phenotypes of the *Z3-3822* mutants, we hypothesized that of the mutants could also have mitotic chromosome NDJ events. To test this hypothesis, we analyzed brain lobe development and growth in third instar larvae (L3). Development of the L3 brain is greatly dependent on postembryonic rapid mitotic divisions of neural epithelial cells, neuroblasts, and progenitor cells to govern the dramatic increase in brain size from the early larval stage to the L3 wandering larval stage (Lee et al., 2024). Hence, disruption of proper mitotic chromosome segregation can lead to aneuploidy and or cell death of neuroblasts and/or progenitor neural cells. Mitotic chromosome loss and cell death events that occur during development severely affects normal brain growth and development. Common phenotypes in null mutations in mitotically essential genes are pupal lethality, small larval brains and small imaginal discs (Schittenhelm et al., 2007).

To address if *Z3-3822* mutants have affected mitotic divisions, we quantified L3 brain

A**Z3-3822 / TM6B****Z3-3822 / Z3-3822****B****Figure 3.2** Analysis of spermatids in homozygous Z3-3822 mutants.

A. Epifluorescent images taken of spermatid cysts from heterozygotes (Z3-3822 / TM6B) and homozygotes (Z3-3822 / Z3-3822) that were stained with anti-tubulin (green), DAPI (blue), and hybridized with the Dodeca probe (red). Normal spermatid nuclei only have one Dodeca spot, reflection of their haploid nature for chromosome 3. White double arrowheads indicate spermatid nuclei that contain two Dodeca spots, reflection of NDJ nuclei with two copies of chromosome 3. Single, larger white arrowheads indicate spermatid nuclei without a Dodeca spot, reflection of NDJ nuclei without a copy of chromosome 3. These two types of NDJ nuclei were scored for both heterozygous and homozygous Z3-3822 mutants. **B.** Percentage of normal sperm and nondisjunction (NDJ) sperm quantified for heterozygotes and homozygotes. Numbers within bar graph are quantified percentages of each sperm type. n = number of spermatid nuclei scored per genotype.

lobe sizes in heterozygous (Z3-3822 / TM6B) larvae and compared their total brain areas to L3 homozygous (Z3-3822 / Z3-3822) total brain areas. To analyze brain lobe sizes, we imaged the frontal midsection of each individual brain lobe with confocal microscopy and then quantified each brain lobe area using Fiji. This section of the brain lobe is the largest area of the lobe and developing structures such as the central brain and optic lobe are easily identifiable (Lee et al., 2024). My prediction was that if mitotic divisions are affected in Z3-3822 mutants, they would show a small brain phenotype and disrupted development of the central brain or optic lobe.

L3 brains were stained with DAPI (4',6-diamidino-2-phenylindole) to visualize the structural integrity of the brain lobe and internal regions specifying the central brain and optic lobe. As shown in **Figure 3.3A**, the general architecture of heterozygous and homozygous brains is normal; however, homozygous Z3-3822 mutant L3 brains are significantly smaller than the L3 Z3-3822 heterozygous brains. Values for the size of brain lobes is shown in **Figure 3.3B**. Overall, homozygous mutants show a 46% size reduction of their brain lobes when compared to heterozygotes. The observation of smaller brains in Z3-3822 homozygous mutants suggests that mitotic divisions are also affected in this mutant fly line.

As meiosis and mitosis are different cellular divisions, the two processes can utilize the same protein complexes and cellular component to facilitate accurate chromosome segregation such as condensins, cytoskeletal polymers and kinetochores for example. Our findings that both meiosis and mitosis are affected by the disrupted gene product in Z3-3822 is not surprising. To further understand our observations, we identified the gene mutated and characterized its molecular lesion to gain insight into the possible mechanism that is disrupted in homozygous Z3-3822 mutants.

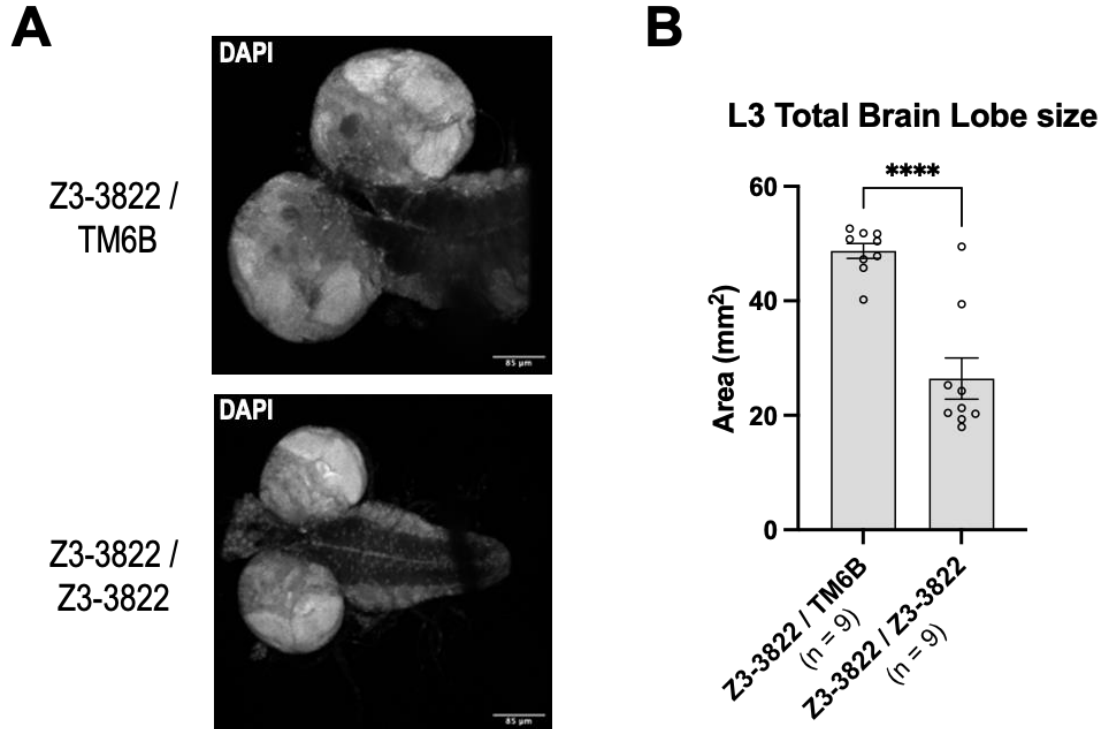


Figure 3.3 Analysis of L3 brains in homozygous Z3-3822 mutants.

A. Confocal images of whole mount L3 brain tissue from heterozygotes (Z3-3822 / TM6B) and homozygotes (Z3-3822 / Z3-3822) stained with DAPI (gray). Scale bar = 85 microns. **B.** Brain lobe sizes for heterozygotes and homozygotes shown by measured area (micrometers squared) of one brain lobe per larvae. n = number of brains analyzed. Unpaired t-test was conducted with a P value of <0.0001.

3.2 Chromosome Mapping of the Z3-3822 Allele

3.2.1 Recombination mapping identifies Z3-3822 mutant allele to be located on 3R

The first step to identify the mutant gene in an EMS-induced mutant fly line was to map the mutation site. EMS mutagenesis induces single nucleotide polymorphisms (SNP) by changing a GC pair to a AT pair in a random manner. Used as a forward genetics approach, the mutagenized nucleotide can cause either a missense or frameshift mutation to generate nonfunctional alleles to study gene function. Though useful, EMS used at an average concentration of 25-50mM can cause a mutation every 150-300 kb, so multiple SNPs are highly likely within a mutagenized chromosome (Bokel, 2008). Therefore, identification of the gene responsible for a phenotype of interest can be challenging with modern techniques like whole genome sequencing. As homozygous Z3-3822 mutants are lethal with few viable escapers, we had to consider that the meiotic mutant phenotype could either be genetically linked or unlinked to the sub-lethal phenotype.

With these considerations, we decided to use recombination chromosome mapping to gauge where the mutant allele is located along the chromosome and if the two phenotypes are linked or unlinked. Transgenic flies encoding a single P-element (PE) with a molecularly defined insertion site on chromosome three were crossed to the Z3-3822 mutant fly line, crossing scheme explained in **Figure 2.4**. Two PEs were used: P(98F5) and P(80A4), with the cytological position along chromosome 3 indicated either at the very tip of the right arm and approximately in the middle of the chromosome in the pericentromeric heterochromatin on the left arm of the chromosome, respectively. Both PEs encodes a transgene with the eye pigment marker *mini white* for easy detection in flies carrying the PE.

Following the same crossing schemes explained in **Figures 2.4 and 2.5** with the PE P(98F5), 422 F₂ viable, white-eyed recombinant progeny were observed out of a total 1078 progeny. These data gave a recombination rate of 39% between the PE P(98F5) and the lethal allele on the Z3-3822 mutant chromosome. The percent of recombination directly demonstrates that there are 39cM (centiMorgans) or map units between P(98F5) and the lethal allele. This determined that the lethal allele on the Z3-3822 mutant chromosome was genetically linked to the right arm of chromosome 3 (3R).

To determine possible linkage between the lethal and meiotic allele, F₂ recombinant, white-eyed males were tested for paternal meiotic chromosome nondisjunction (NDJ) of chromosome 2 by the method explained in **Figure 2.2**. There was a very small frequency of paternal meiotic chromosome 2 NDJ (a frequency of 0.15 for 142 single males tested). This result still supported the two possible scenarios: 1) that the lethal allele and the meiotic mutant allele could be genetically linked or 2) that the lethal allele and the meiotic mutant allele are too far apart from the PE that recombination between the two putative mutant alleles can not be detected with the use of the P(98F5) P-element.

To further identify if the two phenotypes are genetically linked or unlinked, recombination mapping with the second P-element P(80A4) PE was conducted. The use of the PE P(80A4) was advantageous to use as it is proximal to the lethal allele, which could potentially allow us to detect of recombination between the lethal allele and a putative meiotic mutant allele. Females within the F₁ P(80A4) / Z3-3822 progeny were back crossed to the Z3-3822 fly line to detect if recombinant F₂ progeny were generated, explained in detail in **Figure 2.5**. Out of a total of 1,202 F₂ progeny where recombination can occur, 75 of the F₂ viable, white-eyed recombinant

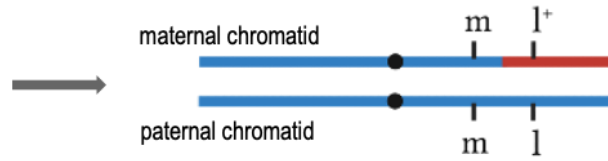
F₂ progeny were recovered in the cross. The number of F₂ viable, white-eyed recombinant F₂ progeny divided by the total progeny and multiplied by 100 gave a 6% recombination rate between the PE P(80A4) and the lethal allele on the Z3-3822 mutant chromosome. The percentage of 6% recombination between these two alleles determined that there was approximately 6cM (centiMorgans) or map units between the lethal Z3-3822 allele and the P(80A4) PE allele.

When F₂ viable, white-eyed males were tested for paternal meiotic chromosome NDJ of chromosome 2 by the Chromosome 2 NDJ assay, a frequency of 2 chromosome 2 NDJ (25 single males tested) was observed. For these 25 males, 7 of them gave rise to progeny and 18 of them had no progeny, meaning that the former males had paternal meiotic chromosome NDJ, and latter males did not. For the 7 F₂ males to be viable, fertile males in the chromosome 2 NDJ, this concluded that their maternally inherited third chromosome had to contain the meiotic mutant allele (*m*) but not contain the lethal allele (*l*) from the Z3-3822 chromatid (**Figure 3.4A**). Conversely, the 18 F₂ males that were not fertile in the chromosome 2 NDJ assay, their maternally inherited third chromosome contained both normal copies of the putative *m* and *l* alleles from the P(80A4) chromatid (**Figure 3.4A**). This data concluded that meiotic recombination at a rate of 28% did occur between the Z3-3822 lethal allele and a separate mutant allele, which is responsible for meiotic NDJ events (**Figure 3.4B**).

The results of the two recombination mapping experiments concluded multiple findings to generate a chromosome map to generally locate the Z3-3822 lethal allele (*l*) and the Z3-3822 meiotic mutant allele (*m*). Our results show that the *l* allele is 6cM (centiMorgans) away from P(80A4) and must be located on the right arm of chromosome 3 (3R). Additionally, since there

A

7/25 males gave rise to progeny; therefore, show meiotic NDJ.



18/25 males had no progeny; therefore, have no meiotic NDJ.

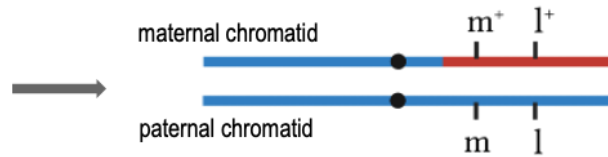
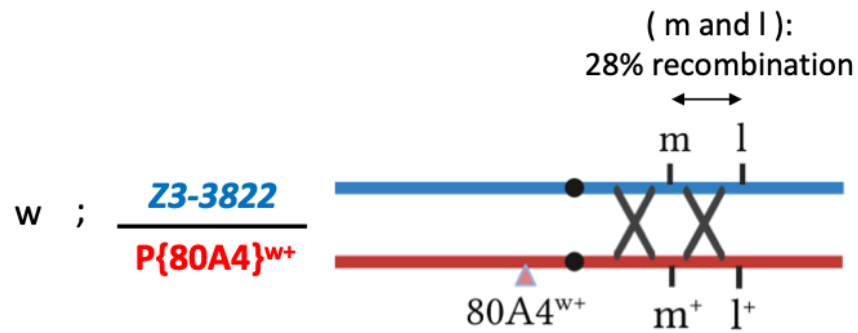
**B**

Figure 3.4 Graphical representation of the summary of results of the paternal meiotic chromosome 2 NDJ assay for F2 recombinant males.
Figure created in <https://BioRender.com>.

was a recombination rate of 28% between *m* and *l*, we calculated a map distance of 2cM between *l* and P(80A4) as 28% of 6 is about 2cM or map units. P(80A4) is at 47cM on the left arm of chromosome 3 (3L). Since there are 4cM between P(80A4) and *m*, the recombination mapping placed *m* at 51cM. This recombination mapping location implements that *m* is located at the cytological location of 87A5 (**Figure 3.5**).

3.2.2 Z3-3822 *m* allele resides at cytological position 87D5 by deficiency mapping

Though recombination mapping was helpful in defining an approximate cytological position where *m* is located, the accuracy of this mapping technique can be limited due to the number of recombination events we only observe. To further refine the genomic location of the *m* allele on the chromosome 3R, deficiency mapping crossings were conducted. Deficiency (Df) mapping employs a set of flies, each carrying chromosomes with specific segments deleted. A cross between a fly carrying the deficiency chromosome with a fly and a wildtype copy of the chromosome results in progeny that are haploid for the set of genes uncovered by the deleted segment (Roote et al., 2012).

Bloomington Drosophila Stock Center (BDSC) has numerous Df stocks with information of exact genomic breakpoints of each deleted segment. The size of deletions within each stock can vary from large deletions (deleting several genes) to small deletions (deleting only one or two genes). Based on the stock information, we obtained a set of stocks in which the defined deletion includes 87AD region where *m* allele is likely to be located, list provided in **Table 2.1**. From each Df stock, virgin females were crossed independently to the Z3-3822 mutant fly line and then viable hemizygous males (Z3-3822 / Df) were singly crossed to *C(2)EN b pr* virgin females to test if the Df complements Z3-3822 for meiotic NDJ (**Figure 2.2**).

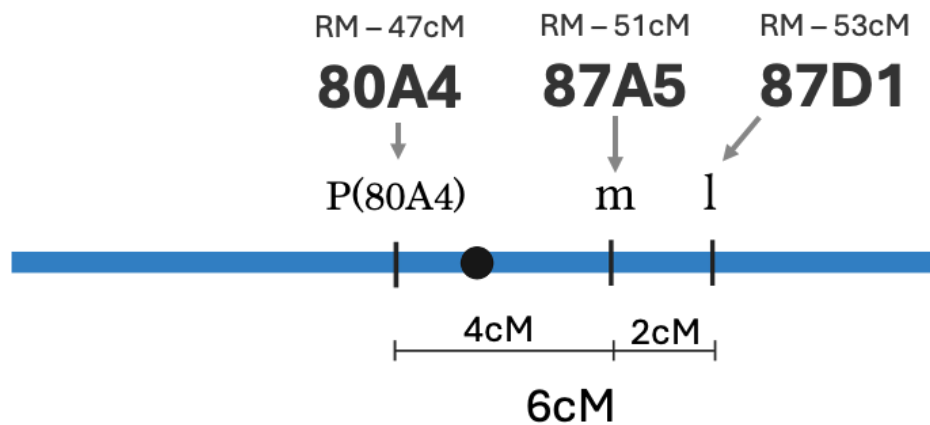


Figure 3.5 Graphical summary of recombination map of the location of m and l alleles on chromosome 3R of the *Z3-3822* mutant chromosome.
Figure created in <https://BioRender.com>.

We found five *Df* lines that failed to complement Z3-3822 for the meiotic chromosome nondisjunction phenotype. **Table 3.1** shows the progeny scored for five groups of Z3-3822 hemizygous (Z3-3822 / *Df*(3R)) males crossed to *C(2)EN b pr* females along with progeny scored for heterozygous (Z3-3822 / TM6B) males as a positive control. As progeny per male correlates to frequency of paternal meiotic chromosome 2 NDJ, all five hemizygous males had a frequency of chromosome 2 NDJ greater than zero when compared to the positive control. To find a genomic region that is commonly deleted from all five *Df* stocks, I examined carefully the overlapping ends of each deficiency line and concluded that *m* resides within the cytological position 87D5 (**Figure 3.6**).

3.2.3 Z3-3822 mutants show paternal meiotic chromosome NDJ for all four chromosomes

To gain more comprehensive NDJ phenotypes, the hemizygous Z3-3822/*Df*(3R) BSC486 (for short, Z3-3822/*Df*) mutant males were tested for their NDJ of all four chromosomes. The mutant show elevated paternal second chromosome NDJ when compared to their heterozygous Z3-3822/TM6B brothers with second chromosome meiotic NDJ frequencies of 3 and 0.07, respectively (**Table 3.1**). The Z3-3822/*Df* males have not only meiotic chromosome 2 NDJ, but also meiotic chromosome NDJ for the sex chromosomes (XY) and the two other autosomes, chromosome 4 and 3. Males with marked Y chromosomes (B^sYy^+) when crossed to *C(1)RM, y^2 w^a su(w^a)/O* virgins tested for XY meiotic NDJ via genetic NDJ cross assay, explained in **Figure 2.1**. Hemizygous Z3-3822/*Df* males when compared to their sibling heterozygous Z3-3822/TM6B brothers show elevated total XY chromosome NDJ, 3.6% and 0.57%, respectively (**Table 3.2**). For the 4th chromosome, Z3-3822 / *Df* mutant males also show elevated NDJ frequencies of 9.6% and 0.2%, respectively (**Table 3.3**).

Table 3.1 Progeny scored for hemizygous and heterozygous Z3-3822 males tested for paternal meiotic chromosome 2 NDJ.

Paternal genotypes (Z3-3822 / TM6B OR Df(3R))	No. single males crossed	Total <i>b pr</i> progeny scored	Total +/- progeny scored	Total progeny scored	Progeny per male
Z3-3822 / TM6B	14	0	1	1	0
Z3-3822 / ED5610	30	108	9	117	4
Z3-3822 / ED5612	30	139	30	169	6
Z3-3822 / Exel7318	28	19	9	28	1
Z3-3822 / BSC615	26	21	8	29	1
Z3-3822 / BSC486	37	70	38	108	3

Table 3.2 Sex (XY) Chromosome NDJ Assay results for Z3-3822 males.

Paternal Genotype	Genotypes of sperm detected by progeny scored					Total progeny scored	% XY NDJ
	X	Y	O	XY	XX		
Z3-3822 / TM6B	553	319	3	0	1	876	0.57
Z3-3822 / Df(3R)BSC486	466	319	23	0	3	811	3.6

Table 3.3 Fourth Chromosome NDJ assay results for Z3-3822 males.

Paternal Genotype	No. Single Males Crossed	No. +/- progeny	No. <i>ci ey^R</i> progeny	Total progeny scored	% 4th Chr. NDJ
Z3-3822 / TM6B	13	945	1	946	0.2
Z3-3822 / Df 3R(BSC486)	55	1423	72	1495	9.6

Chr. 3R

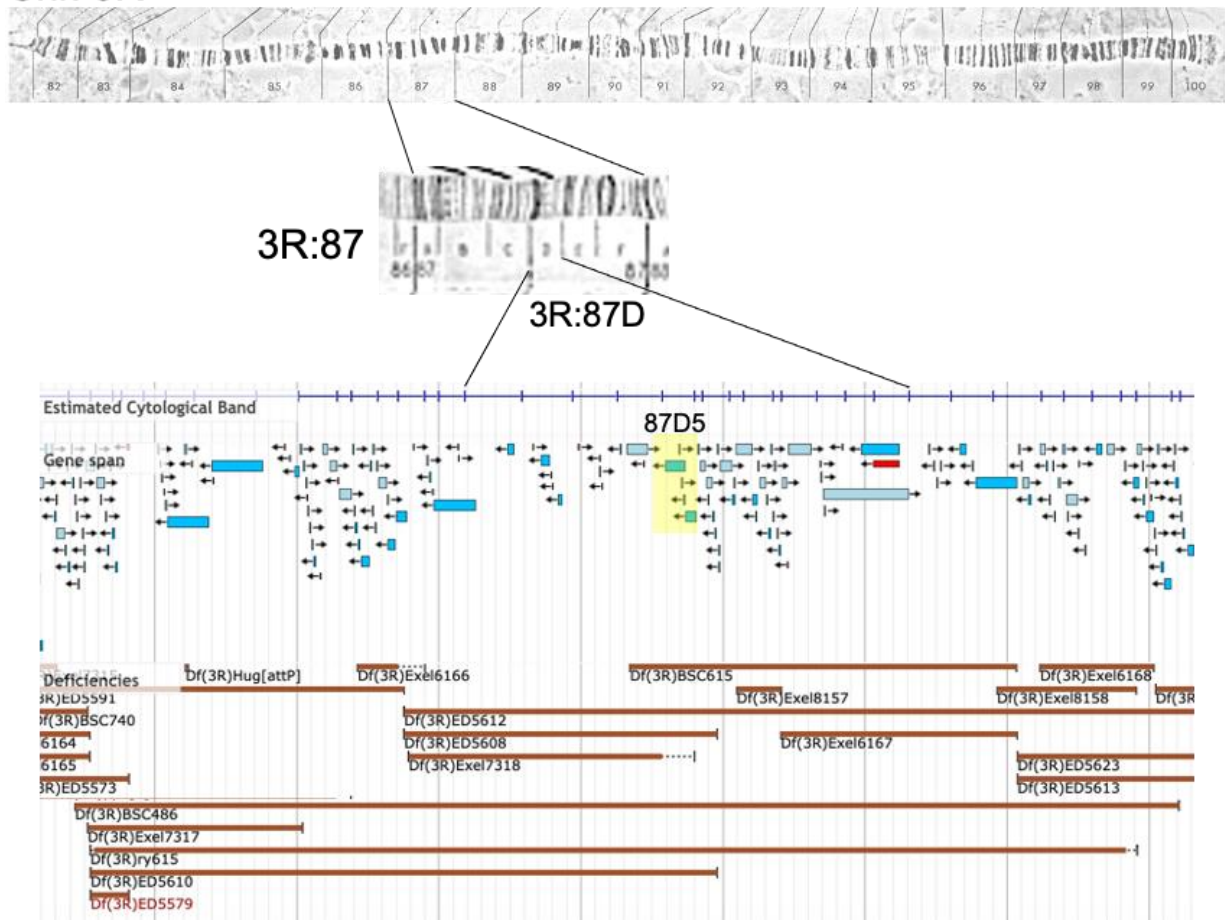


Figure 3.6 Diagrammatic representation of chromosome 3R and deficiencies spanning the cytological position 87D5.

The yellow highlighted region within the gene track shows 5 potential gene candidates found by Df mapping of the Z3-3822 m allele. Gene tracks taken from FlyBase.org.

To determine the rate of meiotic chromosome NDJ for the 3rd chromosome and to further analyze each meiotic division, heterozygous *Z3-3822* / TM6B (control) and *Z3-3822/Df* spermatocytes were analyzed with immuno-FISH with the Dodeca probe by confocal imaging, shown in **Figure 3.7A**. During metaphase I, *Z3-3822/Df* spermatocytes show normal Dodeca spot numbers of two per cell. This observation is consistent of metaphase I nuclei maintaining proper homolog conjunction and sister chromatid cohesion (Tsai et al., 2011). This result shows that the mutated gene does not directly disrupt the homolog conjunction complex or meiotic sister chromatid cohesin complex. However, during anaphase I chromosome laggards are present, as shown in **Figure 3.7A** as a 3rd chromosome homolog remains at the metaphase plate. Despite homologs showing normal homolog conjunction and sister chromatid cohesion, homolog segregation NDJ does occur in *Z3-3822* mutants. Consistent with this, spermatocytes in metaphase II either have one or two Dodeca spots (**Figure 3.7A**). During anaphase II, sister chromatid separation is abnormal in mutants as anaphase bridging occurs and unequal sizes in telophase II nuclei are observed.

Total percent of chromosome 3 NDJ quantified by scored Dodeca spot numbers in spermatid cysts of heterozygotes, homozygotes, and hemizygotes (*Z3-3822* / *Df*) is shown in **Figure 3.7B**. While heterozygotes only show 7% meiotic NDJ of chromosome 3, homozygotes and hemizygotes show similar rates of 36% and 24%, respectively. This data accumulatively shows that a mechanism that ensures proper meiotic chromosome segregation in males is affected by the presence of the *Z3-3822 m* allele, but this mechanism does not involve the process of homolog conjunction or sister chromatid cohesion. To better understand the possible mechanism disrupted in *Z3-3822* mutants, we identified the mutated gene.

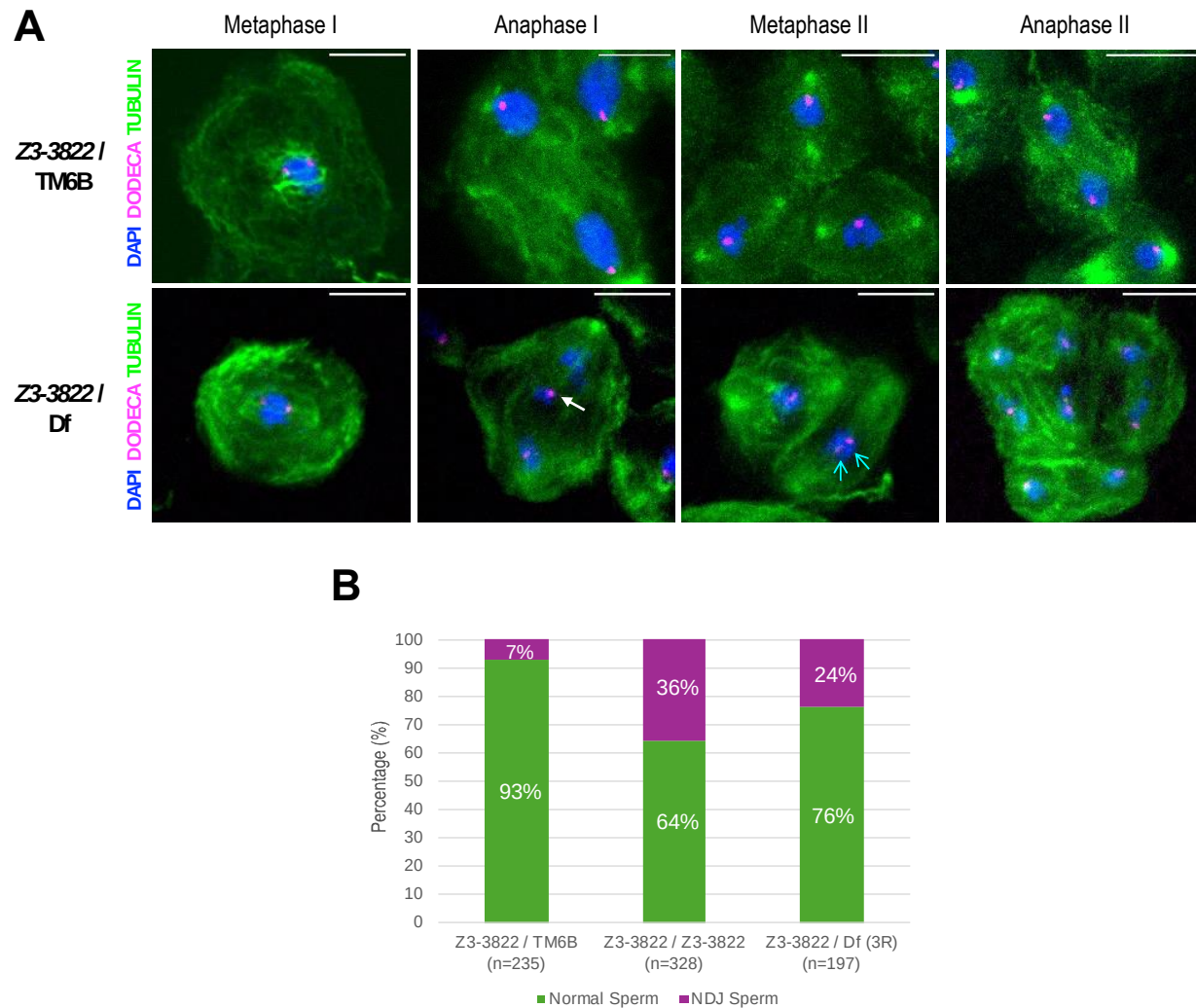


Figure 3.7 Analysis of chromosome 3 homolog and sister chromatid segregation with FISH probe Dodeca in heterozygous and hemizygous Z3-3822 spermatocytes. **A.** Confocal images of heterozygous (Z3-3822 / TM6B) (top panel) and Z3-3822 mutant (Z3-3822 / Df) (bottom panel) spermatocytes in specific meiotic stages (metaphase I, anaphase I, metaphase II, and anaphase II). Chromosome 3 laggard observed during anaphase I in Z3-3822 mutants indicated with white arrow. Z3-3822 mutants have metaphase II spermatocytes with two copies of chromosome 3, indicated by two light blue arrows, which resulted from a meiosis I NDJ event. **B.** Percentage of normal and nondisjunction (NDJ) spermatids scored for heterozygous, homozygous, and hemizygous Z3-3822 mutants. Normal and NDJ sperm were categorized by number of Dodeca spots per spermatid nuclei. Normal spermatids contain only 1 spot. NDJ spermatids contain 0, 2, or 3 spots. n = number of spermatids scored for each genotype.

3.3. Genetic Determination of the Mutant Gene in Z3-3822

3.3.1. Candidate genes responsible for Z3-3822 mutant phenotypes

The foregoing genetic and cytological data of homozygous Z3-3822 / Z3-3822 support that the Z3-3822 *meiotic* (*m*) allele is likely within the 87D5 genomic region. This region contains five genes: *granny smith* (*grsm*), *spc25*, *CG7381*, *Cyp304a1*, and *CG14384*, all shown in **Figure 3.8**.

The five genes encode proteins with a range of roles and amount of information known about them. *Grsm* encodes a leucine aminopeptidase, belonging to a family of proteases involved in several cellular processes (Dorus et al., 2011). *Cyp304a1* encodes a protein within the Cytochrome P450 family, playing a role in the metabolism of hormones (Chung et al., 2009). Genes *CG7381* and *CG14384* are currently uncharacterized genes with no knowledge of their gene products' function. However, of the five genes, *spc25* (*spindle pole body component 25*) was of special interest as its role in chromosome segregation has been well documented. The *spc25* gene encodes the protein Spc25 which is a major component in the kinetochore protein complex Ndc80. *Drosophila*, yeast, humans, mice and many other organisms encode a homolog of Spc25 and the other members of the Ndc80 complex (Przewloka et al., 2007); (Schittenhelm et al., 2007); (Tooley et al., 2011). The genes encoding members of the Ndc80 complex are characterized as mitotically essential as they play a direct role in connecting mitotic chromosomes to microtubules of the spindle (Musacchio et al., 2017). As the kinetochore plays an essential role in ensuring that chromosomes accurately reach their respective poles during cell division, *spc25* was our top gene candidate for possibly being mutated in the Z3-3822 chromosome.

The *D. melanogaster* Spc25-homolog was originally identified and named *mitch* (*mitotic*

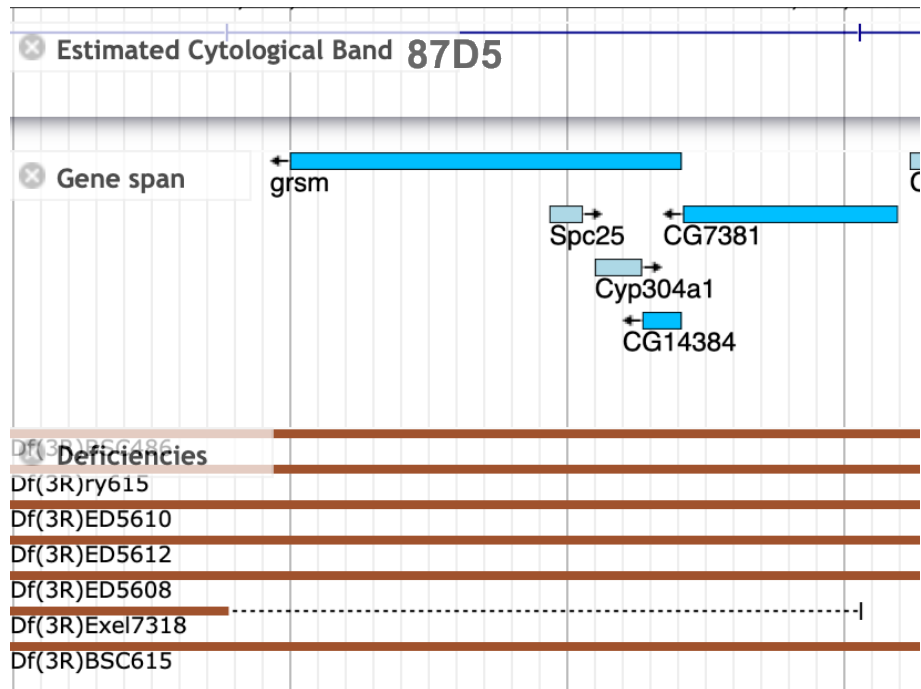


Figure 3.8 Gene candidates identified within cytological position 87D5 on chromosome 3. Following deficiency mapping experiments, the top gene candidate, *spc25*, is highlighted in yellow. Gene track image taken from FlyBase.org.

chaos) through analysis of two EMS generated mutant alleles, *mitch1* and *mitch2*, that either as homozygotes or as trans-heterozygotes were 3rd instar larval (L3) lethal and displayed defective mitotic but also abnormal male meiotic chromosome segregation. Mitotic chromosomes in the mutant brain cells show the over-condensation of chromosomes, lack of chromosome congression in metaphase and unequal distribution of chromosomes during anaphase. Additionally, *mitch* mutant spermatocytes show bivalent misalignment during metaphase I and homolog nondisjunction during anaphase I. Secondary spermatocytes also demonstrate similar phenotypes such as the lack of chromosome congression during metaphase II and sister chromatid nondisjunction (Williams, 2007). In *Drosophila* cell lines or mitotic chromosome spreads from larval brains, localization of Spc25 has been shown to be cell cycle dependent, only localizing to the kinetochore upon entry into prometaphase and remaining at the kinetochore until anaphase (Przewlaka et al., 2007); (Schittenhelm et al., 2007).

3.3.2. Z3-3822 is a mutant *spc25* allele

To identify if the Z3-3822 mutant fly line contains a mutant allele of *spc25* for which is responsible for the meiotic nondisjunction events observed in homozygotes or hemizygotes, we obtained the *spc25* null allele, *spc25^{c00064}*, and crossed it to Z3-3822 for gene complementation analysis. The *spc25^{c00064}* allele has been characterized as a nonfunctional allele of *spc25*, expected to not produce *spc25⁺* transcript due to a 34 basepair (bp) insertion of a PiggyBac transposon in the 5' UTR of the gene (Schittenhelm et al., 2007). Transheterozygous flies (Z3-3822 / *spc25^{c00064}*) are viable and fertile adults. When transheterozygous males are tested for paternal meiotic chromosome 2 NDJ, they had a frequency of 4.5 (**Table 3.4**).

Transheterozygous *spc25^{Z3-3822}* mutant males also show meiotic chromosome NDJ for the XY

and 4th chromosomes with percentages of 4.2% and 2.46%, respectively (**Tables 3.5 And 3.6**).

If Z3-3822 is a *spc25* mutant allele, I performed genomic rescue experiments. For this, transgenic construct *gSpc25::mRFP*, which expresses a wildtype copy of *spc25* under its endogenous promoter, was introduced into Z3-3822 background. As a result, expression of the *gSpc25::mRFP* fully rescues chromosome-2 meiotic NDJ in hemizygous and trans-heterozygous mutant males (**Table 3.4**). These results support that the Z3-3822 allele contains a mutant *spc25* allele which is responsible for the meiotic NDJ phenotypes. From this point, we designated the Z3-3822 allele as a mutant allele of *spc25*, referred to as *spc25*^{Z3-3822}.

To cytologically show that the transgenic construct *gSpc25::mRFP* functions in male meiosis and rescues Z3-3822 mutant meiotic phenotypes we dissected, immunostained, and took confocal images of spermatocytes of hemizygous *spc25*^{Z3-3822} males expressing the rescue construct (**Figure 3.9**). Transgenic expression of *Spc25* under its endogenous promoter tagged with either mRFP or EGFP, shows it to localize in a cell cycle dependent manner, only localizing as distinct foci to the kinetochore region in mitotic cells (Schittenhelm et. al., 2007).

Consistent with this previous report, *Spc25* does not appear to localize to meiotic chromosomes until nuclear envelope breakdown. As shown in **Figure 3.9A**, with expression of *gSpc25::mRFP*, *Spc25* localizes to spermatocyte nuclei as up to eight distinct foci associated with the centromere – kinetochore region during prometaphase I and metaphase I. The intensity of these foci appears to diminish as spermatocytes progress from anaphase I to telophase I. This spatial and temporal localization of *Spc25* repeats itself during meiosis II (**Figure 3.9B**). This localization pattern mimics the localization pattern for *Nuf2*. As *Spc25* and *Nuf2* are two of the four components of the Ndc80 kinetochore complex, this data supports that *Spc25* is a core

Table 3.4 Chromosome 2 NDJ frequencies in *spc25^{Z3-3822}* males.

Paternal Genotype	Total <i>b pr</i> Progeny	Total +/+ Progeny	No. Single Males Crossed	Total Progeny	Progeny Per Male
<i>spc25^{c00064}</i> / TM6B	0	0	22	0	0
<i>Z3-3822</i> / <i>spc25^{c00064}</i>	104	71	39	175	4.5
<i>gSpc25mRFP</i> ; <i>Z3-3822</i> / <i>Df(3R)</i>	1	1	40	2	0.05
<i>gSpc25mRFP</i> ; <i>Z3-3822</i> / <i>spc25^{c00064}</i>	0	0	13	0	0

Table 3.5 Sex chromosome NDJ rates in *spc25^{Z3-3822}* males.

Paternal Genotype	Genotypes of Sperm Detected by Progeny Scored					No. Single Males Crossed	% XY NDJ
	X	Y	O	XY	XX		
<i>Z3-3822</i> / TM6B	553	319	3	0	1	876	0.57
<i>Z3-3822</i> / <i>spc25^{c00064}</i>	920	636	49	9	5	1619	4.2

Table 3.6 Chromosome 4 NDJ rates in *spc25^{Z3-3822}* males.

Paternal Genotype	No. Single Males Crossed	No. +/+ Progeny	No. <i>ci ey^R</i> Progeny	Total Progeny	% 4 th Chr. NDJ
<i>Z3-3822</i> / TM6B	13	945	1	946	0.2
<i>Z3-3822</i> / <i>spc25^{c00064}</i>	37	1523	19	1542	2.46

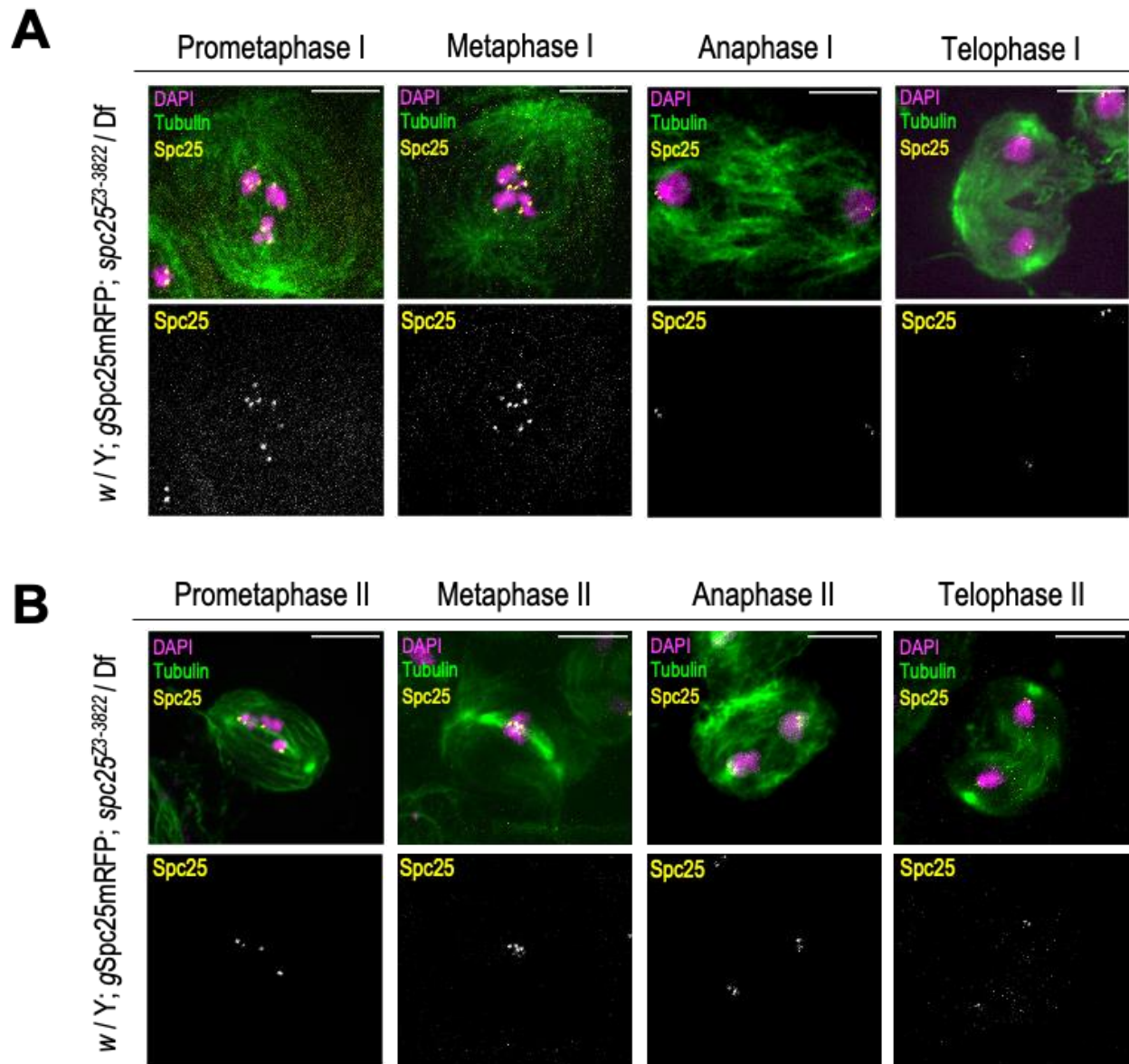


Figure 3.9 Spc25 localizes to the kinetochore / centromere region during male meiosis I and II. Confocal images taken of spermatocytes from males hemizygous (*spc25Z3-3822 / Df(3R)BSC486*) expressing a wildtype copy of Spc25 (*gSpc25::mRFP*) in meiosis I (A) and meiosis II (B). Anti-tubulin staining (green), DAPI staining (magenta), and Spc25::mRFP protein (yellow). Scale bar = 10 microns.

component of the Ndc80 complex in meiosis (Chaurasia et al., 2018).

As shown earlier (**Figure 3.3B**), homozygous *Z3-3822* mutants have smaller total brain lobes. Previous reports of null *spc25* mutants report that they are late larval lethal, and L3 larvae have small brains (Schittenhelm et al., 2007); (Williams et al., 2007). The reported null allele *spc25^{c00064}* homozygous or in trans over a deficiency (hemizygous) expressing the transgenic construct *gSpc25::mRFP* rescues larval lethality (Schittenhelm et al., 2007). However, since *spc25^{Z3-3822}/Df* mutants are viable, I wondered if mitotic chromosome segregation was affected. I dissected *spc25^{c00064}/Df* and *spc25^{Z3-3822}/Df* L3 brains and measured their brain lobe areas. As shown in **Figure 3.10B**, *spc25^{Z3-3822}/Df* do have smaller brain lobes than their heterozygous siblings. However, *spc25^{Z3-3822}/Df* brain lobes are significantly larger than *spc25^{c00064}/Df* larval brains. Importantly, the small brain phenotype in *spc25^{Z3-3822}/Df* larvae is largely rescued by *gSpc25mRFP* (**Figure 3.10**).

Altogether, this led me to conclude that not only meiotic but also mitotic divisions are affected in *spc25^{Z3-3822}* mutants. The result that *spc25^{c00064}/Df* mutants have significantly smaller brains than *spc25^{Z3-3822}/Df* mutants suggests that the *spc25^{Z3-3822}* allele is likely a hypomorphic allele. Like most null or nonfunctional mutant alleles of mitotically essential genes, documented mutant alleles of *spc25* are pupal lethal. In contrast, *spc25^{Z3-3822}* mutants in homozygosity, in trans with a null-allele or hemizygotes, are viable but have paternal meiotic nondisjunction, supporting a hypomorphic nature of the *spc25^{Z3-3822}* allele. The *spc25^{Z3-3822}* allele likely expresses a Spc25 protein, but it may be partially functional during both mitosis and meiosis. To further understand how the mutant Spc25 protein affects chromosome segregation events, I focused on characterizing the *spc25* mutant allele at the molecular level.

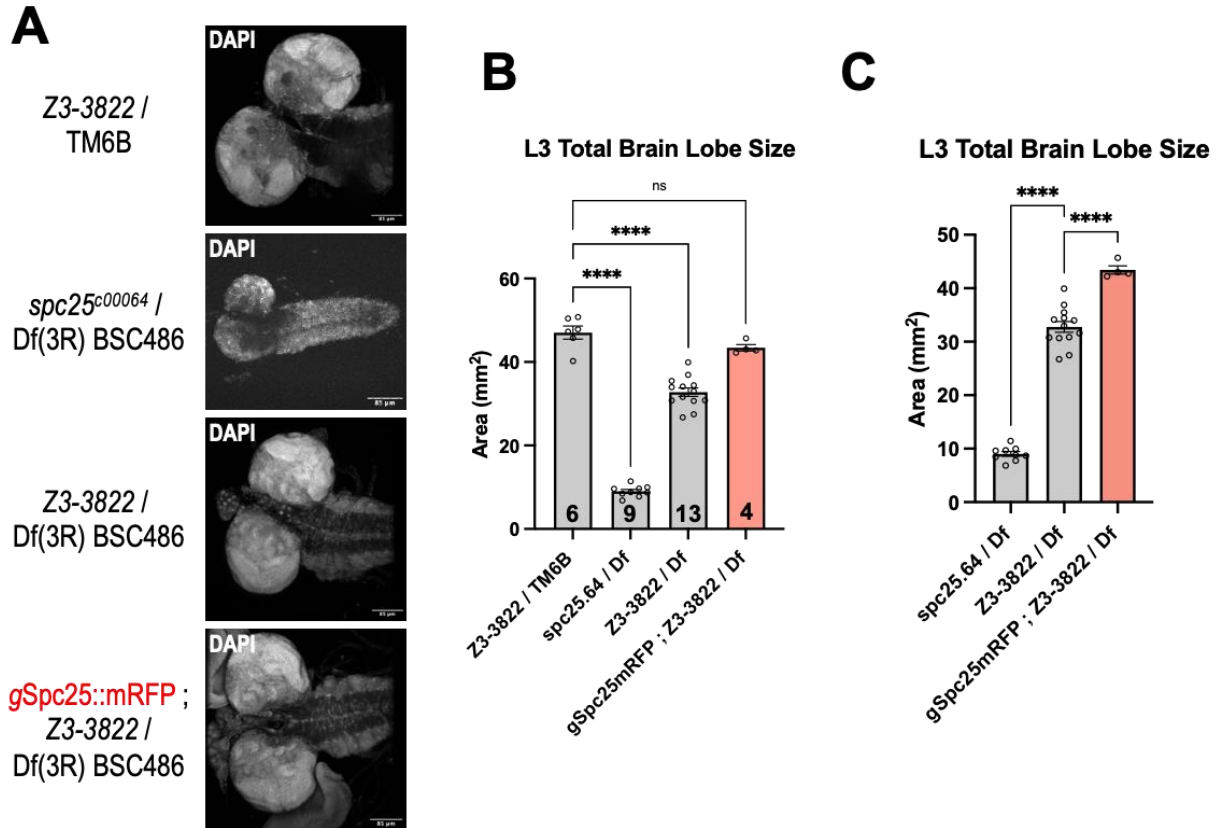


Figure 3.10 Analysis of L3 brain size in Z3-3822 hemizygotes.

A. Confocal images of whole mount L3 brain tissue from Z3-3822 heterozygotes (Z3-3822 / TM6B), *spc25* mutants (*spc25^{c00064}* / Df(3R)BSC486), *spc25*Z3-3822 hemizygotes (Z3-3822 / Df(3R)BSC486), and *spc25*Z3-3822 hemizygotes expressing a wildtype copy of *Spc25* (*gSpc25::mRFP*; Z3-3822 / Df(3R)BSC486) stained with DAPI (gray). Scale bar = 85 microns.

B. & C. Brain lobe sizes for each genotype shown by measured area (millimeters squared) of one brain lobe per larvae. Bold numbers in bar graphs represent the number of brains analyzed. Ordinary One-way ANOVA multiple comparisons conducted **** = P value < 0.0001 and ** = P value < 0.002 **B.** Average brain lobe size for heterozygous Z3-3822 L3 larvae (positive control) when compared to the average brain lobe size of *spc25* null mutant L3 larvae or Z3-3822 mutant L3 larvae are statistically larger. Expression of *gSpc25mRFP* in Z3-3822 mutants rescues the small brain lobe size of Z3-3822 mutants, as the average brain lobe size of *gSpc25mRFP*; Z3-3822 L3 larvae are not statistically different from heterozygous Z3-3822 L3 larvae. **C.** Average brain lobe size of *spc25* null mutant L3 larvae is statistically smaller than the average brain lobe size of Z3-3822 mutant L3 larvae. However, the small brain lobe size of Z3-3822 mutants is statistically smaller than Z3-3822 mutants expressing *gSpc25mRFP*, supporting that the reduced brain lobe size in Z3-3822 mutants is caused by the lack of *Spc25* expression or functional protein.

3.4 Molecular Lesions Identified in the *spc25*^{Z3-3822} Allele

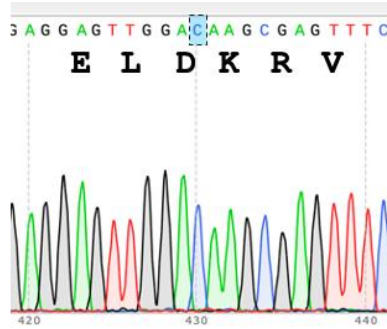
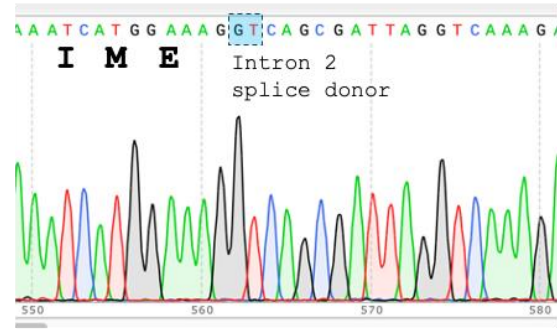
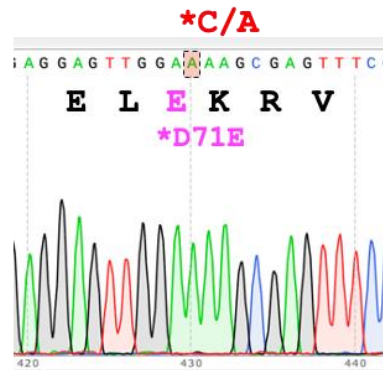
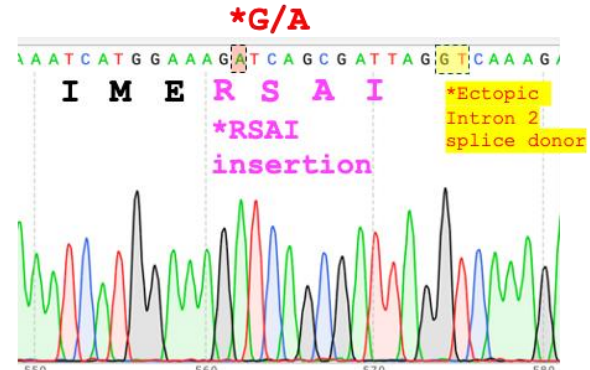
3.4.1. Genomic sequencing identifies two major mutations

The wildtype *spc25* gene consists of three exons and two introns, together spanning 1505 bp, and produces a single transcript of 669 nucleotides in length. All three exons contain protein coding sequence. To determine the molecular lesion present in the *spc25*^{Z3-3822} allele, genomic sequences of the *spc25* locus were compared between Z3-3822 and wildtype *spc25* *Drosophila* reference sequence (dm6) or Z3-0317 (control) line. Though some silent mutations were found commonly in both Z3 lines, two mutations were found only in Z3-3822.

The first was a nucleotide substitution (C/G to A/T) that causes a missense mutation encoding glutamate instead of aspartate (D71E), shown in **Figure 3.11Ai**. The second was a nucleotide substitution (G/C to A/T) at the 5' splice site of the second intron (**Figure 3.11Bii**). This disruption would be expected to cause an alteration in the normal splicing pattern of intron 2, which then would cause the generation of one or more mutant transcripts from *spc25*^{Z3-3822} allele. Interestingly, there is a GT site in intron 2 12bp downstream of the wildtype intron 2 splice donor. It is likely that this GT site might be used as an alternative splice donor in the *spc25*^{Z3-3822} allele. If so, the consequence of this would be an addition of in-frame four amino acid insertion of arginine, serine, alanine, and isoleucine (RSAI), shown in **Figure 3.11Bii**. To verify this hypothesis, I characterized the transcripts derived from the *spc25*^{Z3-3822} allele.

3.4.2 Transcript characterization of *spc25*^{Z3-3822}

A splicing junction mutation causes aberrant splicing events, producing mutant transcripts that may encode for one or multiple mutant isoforms of a protein. To test this, cDNAs were generated from homozygous *spc25*^{Z3-3822} and *snm*^{Z3-0317} mutant larvae for PCR

Ai**Wildtype *spc25* allele****Bi****Wildtype *spc25* allele****Aii****Z3-3822 *spc25* allele****Bii****Z3-3822 *spc25* allele****Figure 3.11** Genomic sequencing of homozygous Z3-3822 mutants.

Ai & Bi Sanger sequencing reads for the *spc25* allele from homozygous Z3-0317 larvae (positive control). **Ai**. Blue highlighted region shows wildtype (WT) nucleotides encoded by the template strand to code for amino acid residue D71. **Bi**. Blue highlighted region shows the GT nucleotides within the template strand that will encode the WT 5 prime intron 2 splice donor. **Aii & Bii** Sanger sequencing reads for the *spc25* allele from homozygous Z3-3822 larvae. **Aii**. Pink highlighted region shows the C/G to A/T nucleotide substitution (red text) identified in the *spc25*Z3-3822 allele, changing the encoded codon from GAC to GAA which encodes the D71E mutation (magenta text). **Bii**. Pink highlighted region shows the G/C to A/T nucleotide substitution (red text) which disrupts the WT 5 prime splice donor of intron 2. Disruption of the intron 2 splice site would at least result in the incorporation of the amino acids R, S, A, I (magenta text). Putative ectopic splice donor for intron 2 identified is highlighted in yellow.

amplification of a portion of the *spc25* transcripts. Using a forward primer spanning the exon-1 and exon-2 junction and a reverse primer within the exon3. RT-PCR would produce a 334 bp (base pair) fragment within the cDNA for the wildtype *spc25* allele. As expected, RT-PCR of the genetic control, *snm*^{Z3-0317}, produced a single 334 bp fragment. The sequence of this fragment showed 100% match when aligned with the reference *spc25* sequence. In contrast, as shown in **Figure 3.12A**, two fragments were generated from the *spc25*^{Z3-3822}, one slightly larger than the wildtype one (transcript 1) and the other at around 900bp (transcript 2). Cloning and sequencing of the transcript 1 revealed that the transcript-1 is nearly the same as the wildtype one but contains an additional 12 bp (**Figure 3.12B**). Except for the G/A substitution, this sequence perfectly matches the first 12 bp of intron 2, suggesting that it is derived from intron 2. Such 12 additional nucleotides would not cause reading frameshift, resulting in the incorporation of the four amino acid residues, Arg-Ser-Ala-Ile (RSAI), within the Spc25 protein between amino acids encoded by exon 2 and exon 3 (**Figure 3.12B**). Moreover, the two nucleotides following these 12 bp are 'GT', which likely plays a role as an ectopic splice donor site for removal of the remaining intron 2, corresponding to the *spc25*^{Z3-3822} transcript 1 shown in **Figure 3.12C**.

After cloning and sequencing of transcript 2, we identified that it contains the entire sequence of intron 2, indicating that the ectopic slicing donor site mentioned earlier is not used for this mRNA. Within transcript 2 retaining intron 2, if read in frame, a stop codon occurs after 42 nucleotides, which would translate into a Spc25 protein truncated after 128 amino acids. As a result, the *spc25*^{Z3-3822} transcript 2 would produce a mutant Spc25 protein encoding a unique carboxy-terminal (CT) region of 14 amino acids but lacking the rest of the wildtype CT region (**Figure 3.12C**). In summary, *spc25*^{Z3-3822} mutant allele likely generates two mutant transcripts

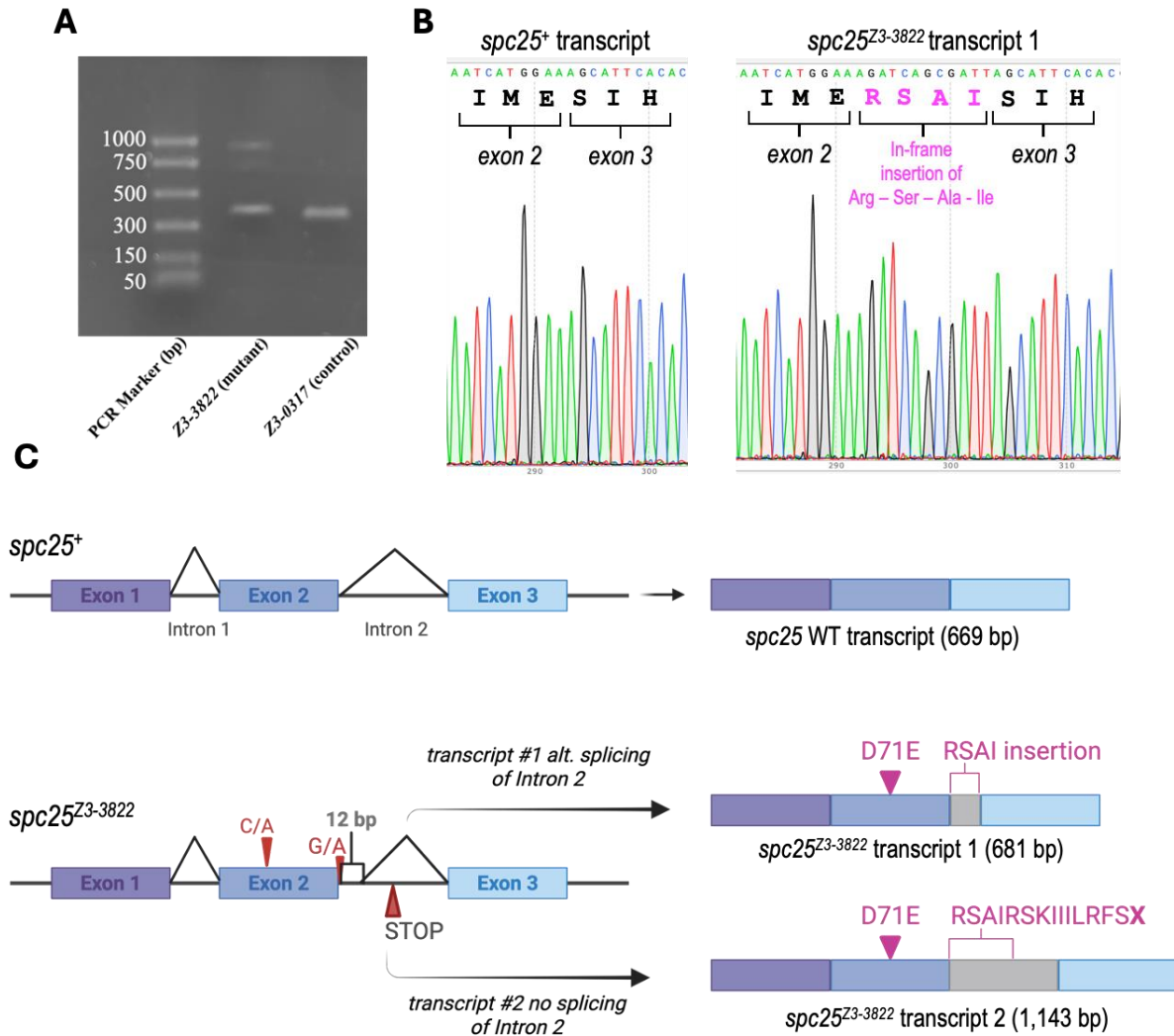


Figure 3.12 Analysis of *spc25*^{Z3-3822} mutant transcripts.

A. Gel electrophoresis post Reverse Transcription and PCR (RT-PCR) of a partial fragment of the *spc25* cDNA from Z3-3822 homozygotes and Z3-0317 homozygotes. Details of the expected fragment is explained in Figure 2.6. The expected fragment for wildtype (WT) *spc25* cDNA is 337 base pairs (bp). Left lane shows the PCR marker with expected placement per molecular weight of products labeled with white numbers. Middle lane shows the two partial *spc25* cDNA products from Z3-3822 mutants. Right lane shows the single, expected partial *spc25* cDNA product from Z3-0317 mutants (positive control). **B.** Partial Sanger sequencing reads from the WT *spc25* cDNA produced from Z3-0317 mutants (*spc25*⁺) (left) and from the *spc25* cDNA produced from Z3-3822 mutants (*spc25*^{Z3-3822} transcript 1) (right) at the exon 2 – exon 3 junction site. Magenta text shows that the amino acid motif RSAI is inserted between exon 2 and exon 3. **C.** Graphic representation of both the WT *spc25* allele (top) and Z3-3822 mutant *spc25* allele (bottom). The WT *spc25* allele produces a single transcript of 669bp. The *spc25*^{Z3-3822} mutant allele encodes for two mutant transcripts. Transcript 1 contains a 12bp insertion, originating from intron-2. Transcript 2 is 1,143bp in length. Retention of intron 2 is expected to lead to truncation of the carboxy terminus of the Spc25 protein due to the presence of a stop codon. Figure created in <https://BioRender.com>.

which are potentially expressed as two different Spc25 mutant proteins, one with the D71E mutation and RSAI insertion (Spc25^{D71E, RSAI} or referred to as Spc25^{ER} later) and the other also containing the D71E but truncated after 128 amino acids (Spc25¹²⁸) (**Figure 3.12C**).

3.5 Functional Characterization of Mutant Proteins Encoded by the *spc25*^{Z3-3822} Allele

3.5.1 *spc25*^{Z3-3822}-encoded proteins lead to chromosome NDJ during male meiosis

Our foregoing data suggest that the meiotic phenotypes of *spc25*^{Z3-3822} mutants are associated with expression of the two mutant proteins. To test this, we constructed four UAS-*spc25* plasmids in which expression of the *spc25* is under the control of UAS promoter. Two constructs, UASc-Spc25^{WT} and UASc-Spc25^{D71E}; the former encodes full length wildtype copy of Spc25 cDNA (positive control) while the latter contains D71E mutation that I found earlier from the *spc25*^{Z3-3822} mutant transcripts. The third one, UASc-Spc25^{ER} also contains the full length Spc25 cDNA, but includes both the D71E mutation and the internal insertion of RSAI in frame, recapitulating the transcript 1 from the *spc25*^{Z3-3822} allele. Since this allele contains these two mutations, we saw it necessary to test each of these two mutations to further understand which of the two mutations could be responsible for any of the mutant phenotypes. The fourth construct, UASc-Spc25¹²⁸, encodes the first 114 residues of the wildtype Spc25 protein followed by the additional 14 residues, recapitulating the transcript 2 gene product. Schematics of the mutant Spc25 proteins are shown in detail in **Figure 2.7**. Each of these constructs was inserted into *att40* genomic landing site on chromosome 2. Transgenic flies were confirmed by mini-white eye color and then established as stocks (see **Chapter 2** for detailed procedures).

Each construct was expressed with the UAS-GAL4 system using *actin*-GAL4 as a ubiquitous driver in a *spc25* null background (*spc25*^{c00064}/Df). The Spc25 proteins expressed by

the expression vector pBID-UASc-MRG fuses them to an in frame amino terminal three tandem copies of MYC fused to the tRFP fluorescent tag for visualization. An expression vector map of the Spc25 transgenic constructs is shown in **Figure 2.8**.

Expression of the UASc-Spc25^{WT}, UASc-Spc25^{D71E}, and UASc-Spc25^{ER} with *actin-GAL4* in *spc25^{c00064}/Df* all produced viable adult flies. Initially however, rearing flies at 25°C gave rise to some adults with subtle developmental phenotypes such as lack of halteres and small testes. I found that rearing flies expressing each UASc-Spc25 construct at 22°C did not cause such developmental phenotypes. Overexpression of mitotically essential genes can cause detrimental phenotypes such as unregulated cell cycle regulation, tumorigenesis or even apoptosis (Diaz-Rodriguez et al., 2008); (Nath et al., 2015). The UAS-GAL4 system is an ectopic, temperature sensitive expression system; higher temperatures lead to increased expression do lower temperatures. Hence, subtle developmental defects seen at 25°C might be due to an undesirable effect by excessively produced Spc25 proteins.

Next, I analyzed subcellular localization of Spc25^{WT}, Spc25^{D71E} (Spc25^E), and Spc25^{D71E, R5AI} (Spc25^{ER}) during male meiosis. Testes from males expressing each UASc-Spc25 construct in the *spc25*-null background were dissected, immunostained with an anti-MYC and anti-alpha-tubulin, and imaged with confocal microscopy. All three Spc25 proteins properly localize to kinetochores during prometaphase, metaphase, and anaphase during both meiosis I and II, resembling endogenous Spc25 localization pattern (**Figure 3.9**).

As shown in **Figure 3.13A-C**, the UASc-Spc25 protein remains localized to the kinetochore region during anaphase and telophase. To determine if chromosomes are segregated accurately or not, we quantified the DAPI intensity of telophase I or telophase II daughter nuclei

by calculating the integrated density of DAPI staining for each nucleus in the respective cell stage. The integrated density accounts for both the area of the nucleus and intensity of DAPI staining. If chromosomes are segregated equally, there should be no significant difference between the integrated densities of DAPI staining for paired nuclei. If chromosomes are unequally segregated, there will be a significant difference between the integrated densities of DAPI staining as sizes of nuclei and/or the intensity of the DAPI staining of paired nuclei will significantly differ. UASc-Spc25 spermatocytes in telophase I show accurate homolog segregation as daughter nuclei show no significant difference in DAPI intensities, quantified in **Figure 3.13A**. Although Spc25^E and Spc25^{ER} mutant proteins still associate with anaphase I and telophase I daughter nuclei, unequal homolog segregation occurs in the presence of both mutant Spc25 proteins (**Figure 3.13B-C**).

Assessment of the three Spc25 proteins during meiosis II showed a similar pattern as during meiosis I. UASc-Spc25 shows the same spatial and temporal localization pattern as the *gSpc25mRFP* construct for secondary spermatocytes and normal sister chromatid separation during telophase II (**Fig. 3.14A**). Both UASc-Spc25^E, **Figure 3.14B**, and UASc-Spc25^{ER}, **Figure 3.14C**, show normal localization during prometaphase II and metaphase II. However, I observed lagging chromosomes during early anaphase II and unequal segregation of sister chromatids when either of two mutant Spc25 proteins are expressed (**Figure 3.14B-C**).

In addition to analyzing the function of three UASc-Spc25 protein in male meiosis cytologically, *spc25*⁻ mutant males expressing each UASc-Spc25 transgene were tested by genetic cross assay to determine a rate of meiotic chromosome nondisjunction (NDJ). In **Table 3.7**, the percentage of 4th chromosome meiotic NDJ for males expressing UASc-Spc25, UASc-

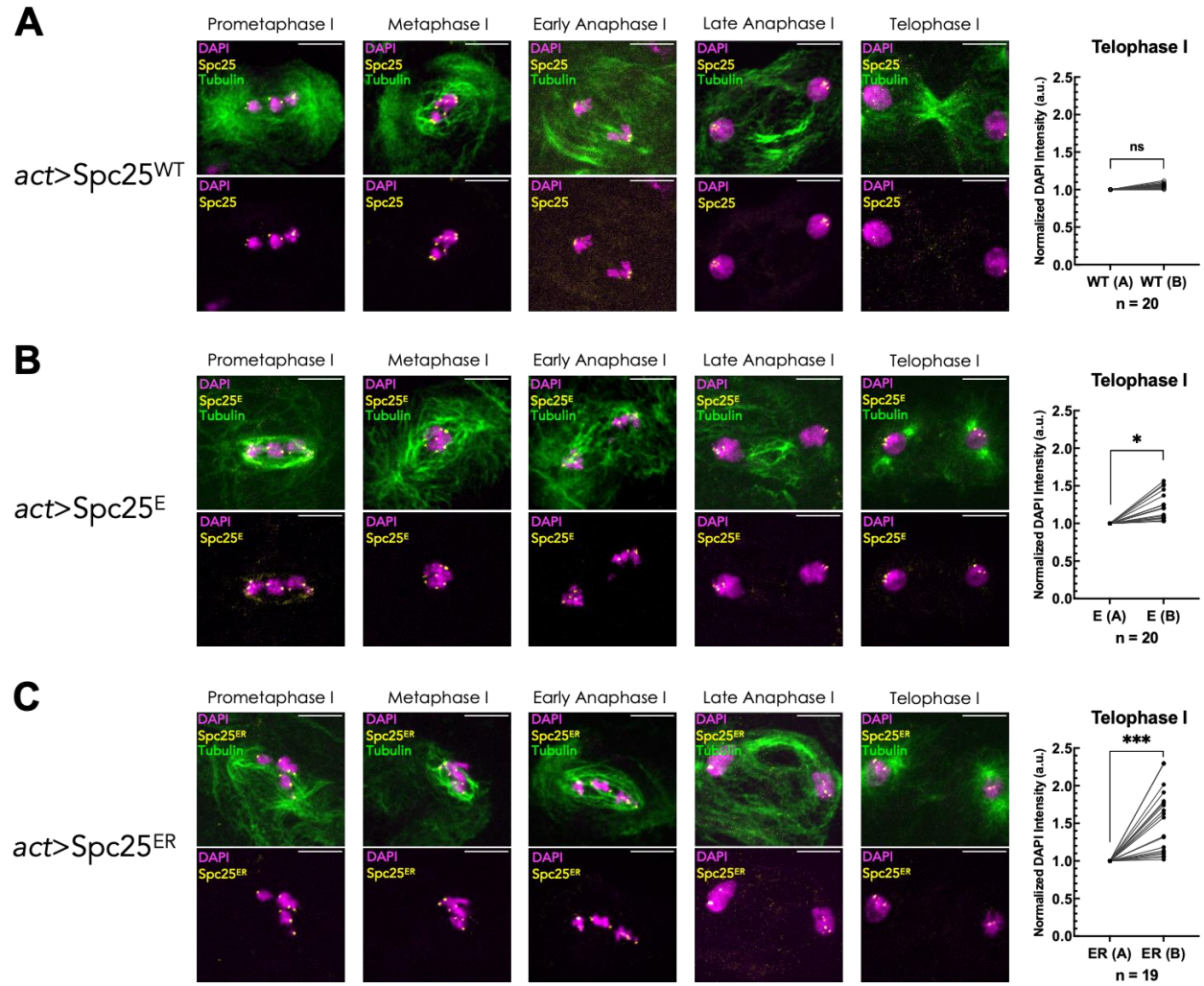


Figure 3.13 D71E amino substitution in the Spc25 protein causes homologous chromosome mis-segregation during male meiosis I.

A-C. Confocal images of *spc25* null spermatocytes expressing Spc25^{WT}, Spc25^E, or Spc25^{ER} transgene from prometaphase to telophase during meiosis I. Genotype of males: w/Y; UASc-Spc25/*act*GAL4; *spc25*^{c00064}/Df, simplified in panels A-C with *act>Spc25*. Anti-tubulin staining (green), DAPI staining (magenta), and anti-MYC staining labels each UASc-Spc25 transgenic protein (yellow). Scale bar = 10 microns. Graphs shown plot the fold change of normalized DAPI intensities (y-axis) for a pair of telophase I daughter nuclei (A and B on x-axis). Significance determined by comparing the mean DAPI intensity for group of A and B daughter nuclei. n = number of paired nuclei scored. Mann-Whitney test for each comparison was conducted. *** = P value < 0.0002, * = P value < 0.02

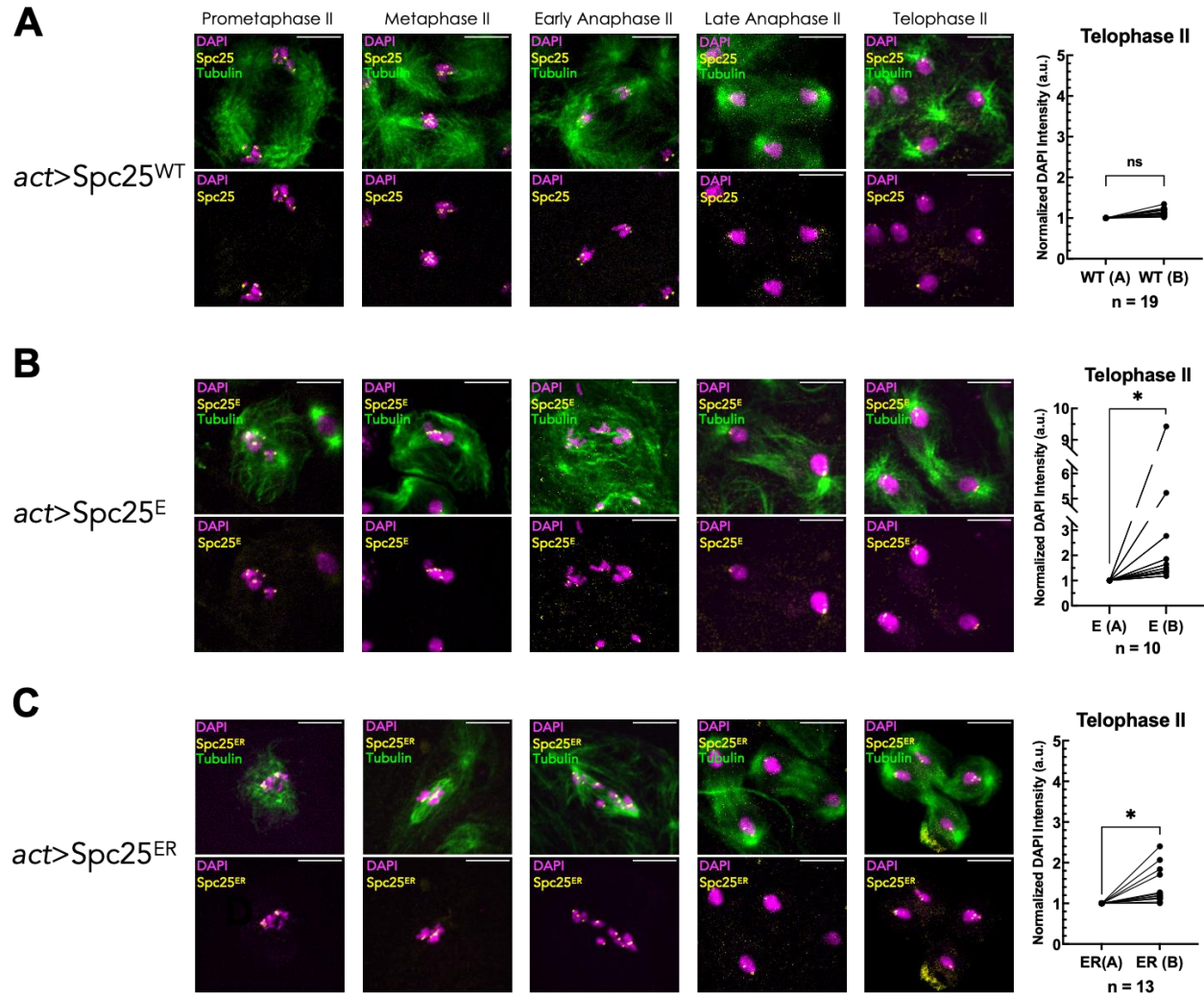


Figure 3.14 D71E amino substitution in the Spc25 protein causes mis-segregation of sister chromatids during male meiosis II.

A-C. Confocal images of *spc25* null spermatocytes expressing Spc25^{WT}, Spc25^E, or Spc25^{ER} transgene from prometaphase to telophase during meiosis II. Genotype of males: w/Y; UASc-Spc25/*actGAL4*; *spc25^{c00064}*/Df, simplified in panels A-C with *act>Spc25*. Anti-tubulin staining (green), DAPI staining (magenta), and anti-MYC staining labels each UASc-Spc25 transgenic protein (yellow). Scale bar = 10 microns. Graphs shown plot the fold change of normalized DAPI intensities (y-axis) for a pair of telophase II daughter nuclei (A and B on x-axis). Significance determined by comparing the mean DAPI intensity for group of A and B daughter nuclei. n = number of paired nuclei scored. Mann-Whitney test for each comparison was conducted. * = P value < 0.03.

Table 3.7 Rate of 4th chromosome meiotic chromosome nondisjunction (NDJ) in viable *spc25* null mutants expressing each *Spc25* transgene.

Paternal Genotype	No. Single Males Crossed	No. +/+ Progeny	No. <i>ci ey^R</i> Progeny	Total Progeny	% 4 th Chr. NDJ
<i>act-GAL4 / + ; spc25⁺ / Df</i>	16	384	2	386	1
<i>act>Spc25^{WT} / actGAL4; spc25⁻</i>	44	489	8	497	3.2 ^{n.s.}
<i>act>Spc25^E / actGAL4; spc25⁻</i>	41	320	23	343	13.4*
<i>act>Spc25^{ER} / actGAL4; spc25⁻</i>	20	96	4	100	8*

X² (Chi-Squared) Analysis test for significance was conducted; X² > X² at P(0.05) indicated within the table with () for meiotic chromosome 4 NDJ percentages (%) that are statistically higher than the calculated expected % of 4th Chr. NDJ defined by the positive control, (*act-GAL4/+ ; spc25⁺ / Df*).

Spc25^E, and UASc-Spc25^{ER} in a *spc25* null background is shown. Males expressing the WT copy of Spc25 (UASc-Spc25), had a 4th chromosome NDJ rate of 3.2; however, the rate is not a significantly different when compared to the NDJ rate observed in control males. However, males expressing either UASc-Spc25^E and UASc-Spc25^{ER} have a significantly higher rate of 4th chromosome NDJ of 13.4% and 8%, respectively, when compared to UASc-Spc25 expressing males and control males. Importantly, the observed rates of 4th chromosome NDJ in males expressing each UASc-Spc25 are consistent with the cytological analysis shown in **Figure 3.13** and **Figure 3.14**. Moreover, the rates 4th chromosome meiotic NDJ in males expressing either mutant protein Spc25^E or Spc25^{ER} are similar to the observed rate of 9.6% 4th chromosome NDJ in *spc25*^{Z3-3822} hemizygous mutant males.

The 4th chromosome nondisjunction (NDJ) rates observed in mutant males are comparatively low to the observed rate of chromosome 3 NDJ determined in *spc25*^{Z3-3822} hemizygous mutants, shown in **Figure 3.7B**. However, rates of NDJ genetic cross assays observed may underrepresent the total number of NDJ events occurring in meiosis. Gametes with autosomal aneuploidy from multiple chromosomes undergoing NDJ could lead to their lack of survival and thus elimination from the genetic data.

Since expression of either Spc25^E or Spc25^{ER} does not rescue mutant meiotic phenotypes observed in *spc25*^{Z3-3822} mutants, the missense mutation D71E in the Spc25 protein is most likely responsible for causing chromosome NDJ events in meiosis.

3.5.2. Essential role of the C-terminal region for Spc25 function

Expression of the fourth construct, UAS-Spc25¹²⁸, which represents the second mutant protein expressed from the *spc25*^{Z3-3822} allele, in a *spc25* mutant background showed more severe

phenotypes than the other three constructs. Flies expressing the truncated Spc25 protein in a *spc25*⁺ background are viable and fertile adults. However, when the truncated protein is expressed in a *spc25* null background they develop into the pharate phase but are not viable adults (**Figure 3.15A**). Developmental abnormalities in the cuticle of the pupal casing were observed such as underdevelopment of the spiracular papillae and posterior spiracles, and irregularities in the ventral, posterior abdominal segments. Developed pharates do not eclose, but if removed from the pupal casing, they appear phenotypically normal but have smaller abdomens.

To determine if the truncated protein could localize to meiotic chromosomes, testes from pharates expressing the UASc-Spc25¹²⁸ protein in a *spc25*⁺ and *spc25*⁺ were dissected and prepared for confocal imaging. Both UASc-Spc25¹²⁸/*actin*GAL4; *spc25*^{c00064}/TM6B and UASc-Spc25¹²⁸/*actin*GAL4; *spc25*^{c00064}/Df showed lack of protein localization to chromosomes or kinetochores during meiosis (**Figure 3.15B-C**). Primary spermatocytes from males expressing the truncated protein in a *spc25* null background show lagging chromosomes and unequal homolog segregation. Consistent with this, secondary spermatocytes in metaphase show cells with large or very small chromatin masses, consistent with major unequal chromosome segregation of homologs. During anaphase II, lagging chromosomes are present and telophase II daughter nuclei with unequal sizes, resulting from unequal segregation of sister chromatids. These phenotypes have been observed in *spc25* null mutants (Williams et al., 2007). Previous reports that show the Spc25 protein utilizes its C-terminal region to localize to the kinetochore and interact with the inner kinetochore Mis12 complex (Venkei et al., 2011); (Venkei et al., 2012). Lack of a functional Spc25 would lead to the absence of the Ndc80 complex at the

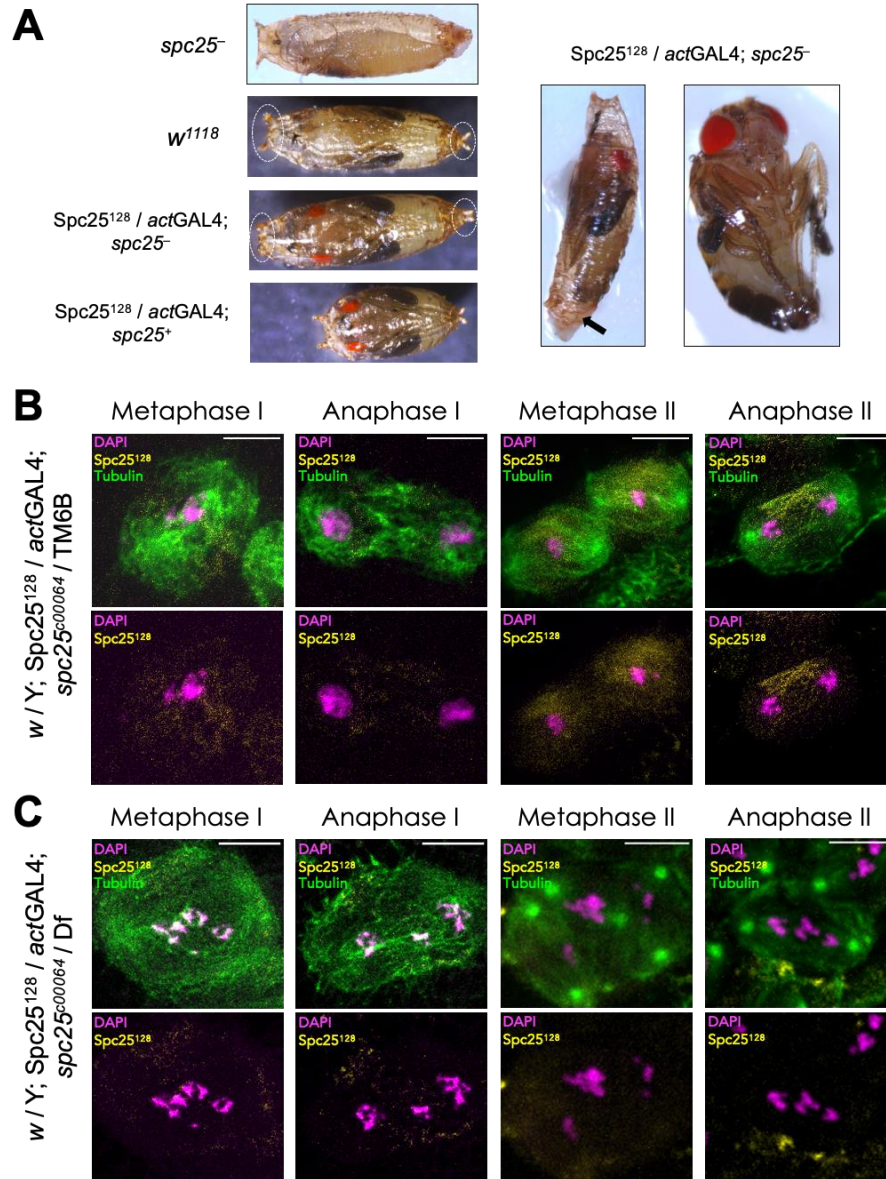


Figure 3.15 The CT region of Spc25 is essential for Spc25 protein function.

A. (Right panel) Ventral images of a *spc25* null pupae (*spc25^{c00064}/Df*), control pharate (*w¹¹¹⁸*), pharate expressing UASc-Spc25¹²⁸ in a *spc25⁺* background (*spc25^{c00064}/TM6B*), and pharate expressing UASc-Spc25¹²⁸ in a *spc25⁻* background (*spc25^{c00064}/Df*). Developmental abnormalities in the spiracular papillae and posterior spiracles in *spc25⁻* pharates expressing UASc-Spc25¹²⁸ are indicated (white outlined circles). (Left panel) Irregularities in the cuticle is indicated by the black arrow on the right. Image of a pharate expressing the UASc-Spc25¹²⁸ protein is shown on the right. **B-C.** Confocal images of spermatocytes in meiotic stages: metaphase I, anaphase I, metaphase II, and anaphase II in *spc25⁺* males expressing the UASc-Spc25¹²⁸ transgene, which eliminates the CT region of Spc25 (*w/Y; UASc-Spc25¹²⁸ / actGAL4; spc25^{c00064} / TM6B*). Expression of UASc-Spc25¹²⁸ in a *spc25* null background (*w/Y; UASc-Spc25¹²⁸ / actGAL4; spc25^{c00064} / Df*(3R)BSC486) Anti-tubulin staining (green), DAPI staining (magenta), and anti-MYC staining which identifies the UASc-Spc25 transgenic protein (yellow). Scale bar = 10 microns.

kinetochore (Schittenhelm et al., 2007); (Przewloka et al., 2007). Without the Ndc80 complex to establish end-on KT-MT attachments during meiosis would be responsible for the phenotypes observed in spermatocytes from flies expressing the truncated Spc25 protein (UASc-Spc25¹²⁸) in a *spc25* mutant background.

3.5.3 spc25^{Z3-3822} mutants show premature loss of Nuf2 during anaphase I and II

In mitotic cells, localization of both Nuf2 and Ndc80 to the kinetochore region has been shown to require stable localization of Spc25 (Przewloka et al., 2007). If the mutant Spc25 proteins expressed from the *spc25^{Z3-3822}* allele affects the localization and/or stability of the Ndc80 complex, we might observe less or lack of Nuf2 on meiotic kinetochores in *spc25^{Z3-3822}* mutants. To test if the Spc25 mutant proteins affect functioning of the Ndc80 complex during meiosis, I analyzed the localization pattern of Nuf2 in *spc25^{Z3-3822}* mutants. The spatial and temporal localization pattern of Nuf2 has been shown previously during male meiosis (Chaurasia et al., 2018). To analyze Nuf2 localization in *spc25^{Z3-3822}* mutants, the transgenic construct *gEGFP::Nuf2*, which expresses a WT Nuf2 protein with an NT EGFP tag under its endogenous promoter was expressed in a *spc25^{Z3-3822}* hemizygous background (*spc25^{Z3-3822}/Df*).

As shown in **Figure 3.16**, Nuf2 can localize and stay associated with the kinetochore in *spc25^{Z3-3822}* mutants during prometaphase, metaphase, and early anaphase, like what is observed in wildtype spermatocytes (Chaurasia et al., 2018). Nuf2 does localize to KTs and remains there during key stages of meiosis when end-on KT-MT attachments are made. This data supports that in *spc25^{Z3-3822}* mutants, a Spc25 protein is expressed, is capable of localization, and supports formation of a Ndc80 complex during meiosis. This conclusion is consistent with the analysis of protein localization of the mutant proteins UASc-Spc25^E and UASc-Spc25^{ER} but not the UASc-

Spc25¹²⁸ protein when expressed. Therefore, we hypothesize that in *spc25*^{Z3-3822} mutants, the mutant transcript 1, shown in **Figure 3.12A**, does express a Spc25 protein, but this Spc25 protein would contain both the D71E and four amino acid insertion motif RSAI (Spc25^{ER}). Thus, analysis of EGFP::Nuf2 protein function and localization in *spc25*^{Z3-3822} mutants tests if the Spc25^{ER} mutant protein has an effect on the function and stability of the Ndc80 complex and establishment of end-on KT-MT attachments.

Localization of Nuf2 and its maintenance on chromosomes in prometaphase (PM), metaphase (M) and early anaphase (E.A) in conclude that in *spc25*^{Z3-3822} mutants, the mutant Spc25^{ER} protein does not affect the formation of the Ndc80 complex. However, *spc25*^{Z3-3822} spermatocytes show lagging chromosomes in early anaphase I and loss of Nuf2 protein at KTs during late anaphase (L.A) I (**Figure 3.16A**). In the *spc25*^{Z3-3822} mutant background, 63% of late anaphase spermatocytes showed weak or complete loss of Nuf2 protein on both nuclei or only one daughter nuclei (**Figure 3.16C**). In the control, 90% of late anaphase I spermatocytes show gEGFP::Nuf2 foci on both daughter nuclei and only 10% had either lost Nuf2 foci on both or only one daughter nuclei. As additionally shown in **Figure 3.16A**, unequal segregation of homologous chromosomes is observed in late anaphase and telophase in *spc25*^{Z3-3822} mutants.

During meiosis II, *spc25*^{Z3-3822} mutant spermatocytes also show EGFP::Nuf2 localization in PMII and MII, revealing that the Ndc80 complex is formed on sister KTs. However again, during L.A., premature loss of Nuf2 foci on daughter nuclei was observed in 91% of *spc25*^{Z3-3822} mutant spermatocytes (**Figure 3.16D**). Unequal sizes of the daughter nuclei observed reflect sister chromatid nondisjunction events during meiosis II in *spc25*^{Z3-3822} mutants (**Figure 3.16B**).

These data conclude that the mutant protein, Spc25^{ER}, can localize and support the

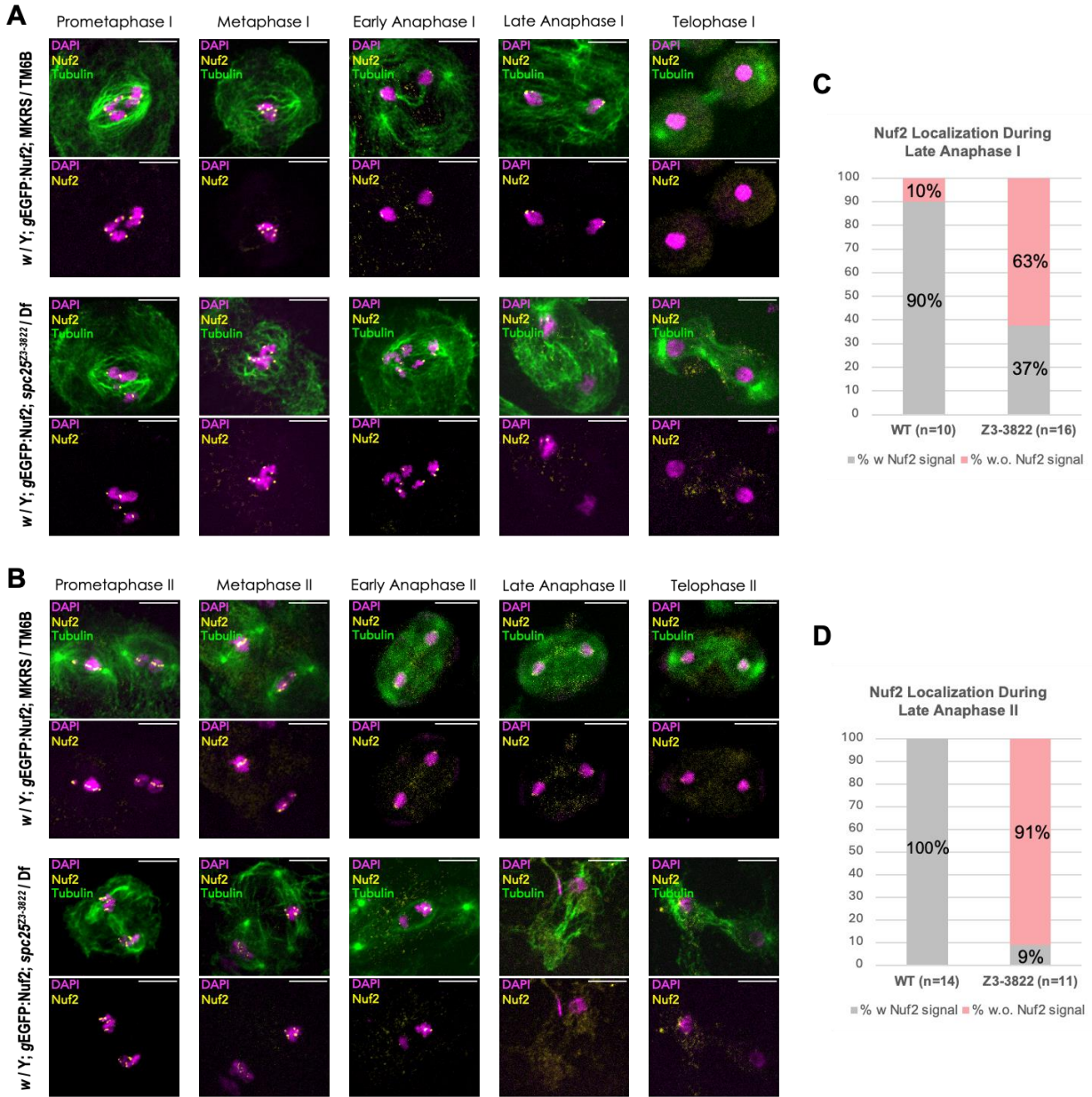


Figure 3.16 Nuf2 expression during meiosis I and II in wildtype and *spc25^{Z3-3822}* mutant males. Confocal images of the localization of Nuf2 during male meiosis I (**A**) and meiosis II (**B**) in wildtype (w/Y; gEGFP::Nuf2; MKRS/TM6B) and *spc25^{Z3-3822}* mutants (w/Y; gEGFP::Nuf2; *spc25^{Z3-3822}/Df*). DAPI staining is labeled in magenta, Tubulin is labeled in green, and EGFP::Nuf2 is labeled in yellow. Scale bar is 10um. Quantification of the presence or absence of EGFP::Nuf2 localization on (**C**) late anaphase I nuclei and (**D**) late anaphase II nuclei is provided as percentages of cells (% w Nuf2 signal) and cells without (% w.o. Nuf2 signal) in WT (w/Y; gEGFP::Nuf2; MKRS/TM6B) and *spc25^{Z3-3822}* mutants (w/Y; gEGFP::Nuf2; *spc25^{Z3-3822}/Df*). n = number of anaphase cells scored. Scale bar is 10 microns.

formation of the Ndc80 complex during meiosis. However, as chromosomes progress to spindle poles, the loss of Nuf2 protein at kinetochores during chromosome transmission can be explained by partial functioning of the mutant Spc25^{ER} protein. The presence of the Spc25^{ER} affects the ability of the Ndc80 complex to maintain KT-MT end-on attachments when spindle forces are exerted onto the kinetochores, via rapid depolymerization of MTs, after chromosome segregation has been initiated in meiosis I and meiosis II. A proposed model for how the mutant Spc25^{ER} protein may affect the Ndc80 complex (Ndc80c) in male meiosis is shown in **Figures 3.17 and 3.18**.

3.5.4 *spc25^{Z3-3822}* mutants generate two mutant Spc25 proteins that differentially affect mitosis

As shown earlier, phenotypic characterization of *spc25^{Z3-3822}* hemizygotes showed that third instar larvae (L3) have smaller brain lobes than their heterozygous siblings (**Figure 3.10**). These results suggested that mitotic chromosome segregation events are affected by the expression of the *spc25^{Z3-3822}* allele. To determine how the two mutant Spc25 proteins affect mitotic chromosome segregation, we analyzed adult brains from flies expressing each UASc-Spc25 construct in the *spc25* null background. We dissected, fixed, DAPI stained and mounted whole brains from five to seven-day old adult flies (**Figure 3.19**) or pharates (**Figure 3.20**). To analyze the brain lobe sizes quantitatively, we imaged the frontal midsection of each individual brain lobe with confocal microscopy and measured the area of one brain lobe per brain. As shown in **Figure 3.19C**, there was no significant difference in the brain lobe sizes of adult male flies independently expressing UASc-Spc25, UASc-Spc25^{D71E}, and UASc-Spc25^{ER}. These results suggest that the Spc25^{ER} mutant protein, although disrupts meiotic chromosome segregation, does not affect mitotic chromosome segregation.

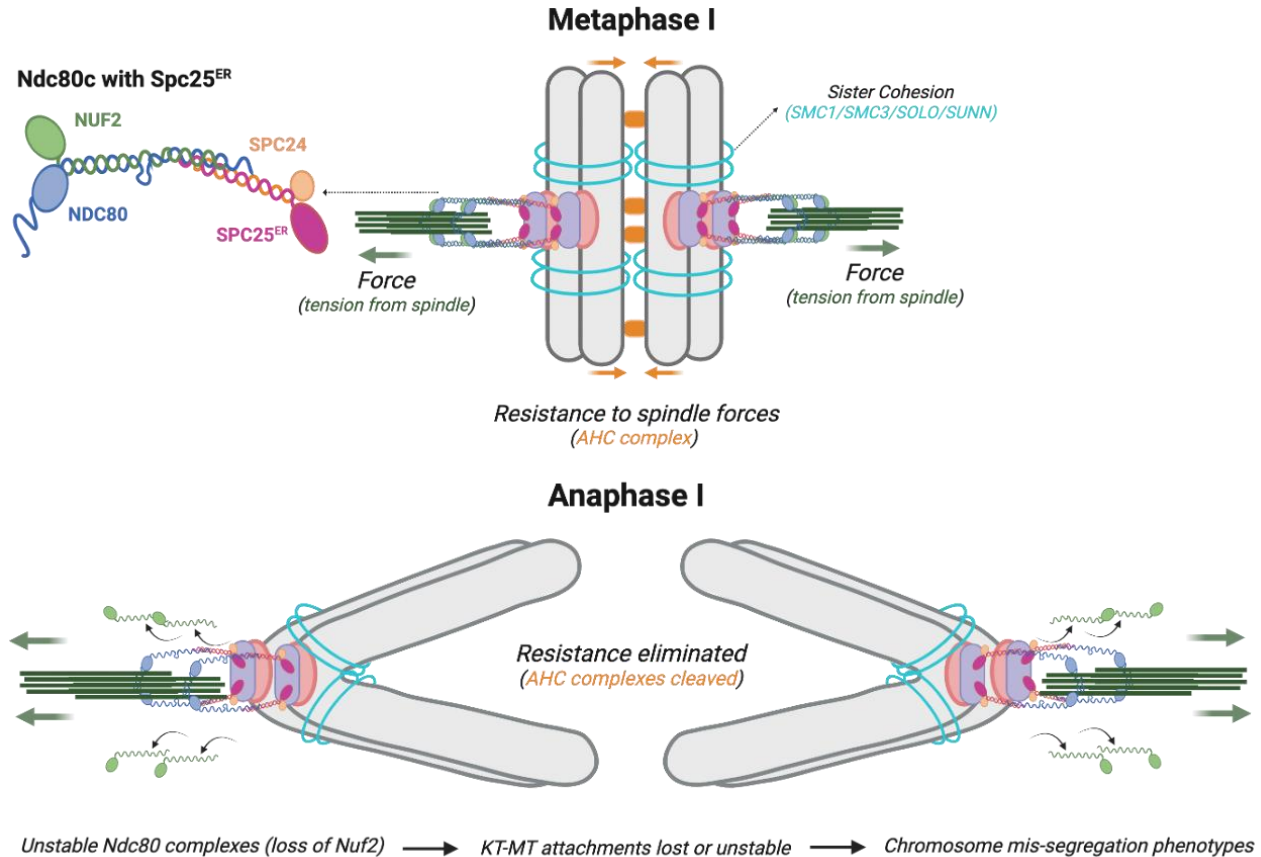


Figure 3.17 Model for how Spc25^{ER} protein incorporation into the Ndc80c affects kinetochore function during male meiosis I.

The mutant Spc25^{ER} protein expressed in *spc25*^{Z3-3822} mutants can localize to the inner kinetochore (KT) and support recruitment of the Ndc80/Nuf2 heterodimer to form a functional Ndc80c up to the stage of metaphase. Homolog biorientation is achieved in metaphase I by establishment of end-on KT-MT attachments. Tension generated by spindle forces is exerted onto sister KTs, but resistance to pulling forces is mediated by homolog connections, provided by the AHC complex. During anaphase I, the AHC complex is cleaved by Separase activity which eliminates resistance to spindle forces and forces homologs to be pulled to opposite poles. However, the force emitted onto the Ndc80c in the presence of the Spc25^{ER} destabilizes the complex's attachment to MTs, which causes loss of Nuf2 during anaphase. Loss of stable end-on attachments provided by the Ndc80 complex result in chromosome mis-segregation phenotypes such as chromosome lagging and unequal segregation.

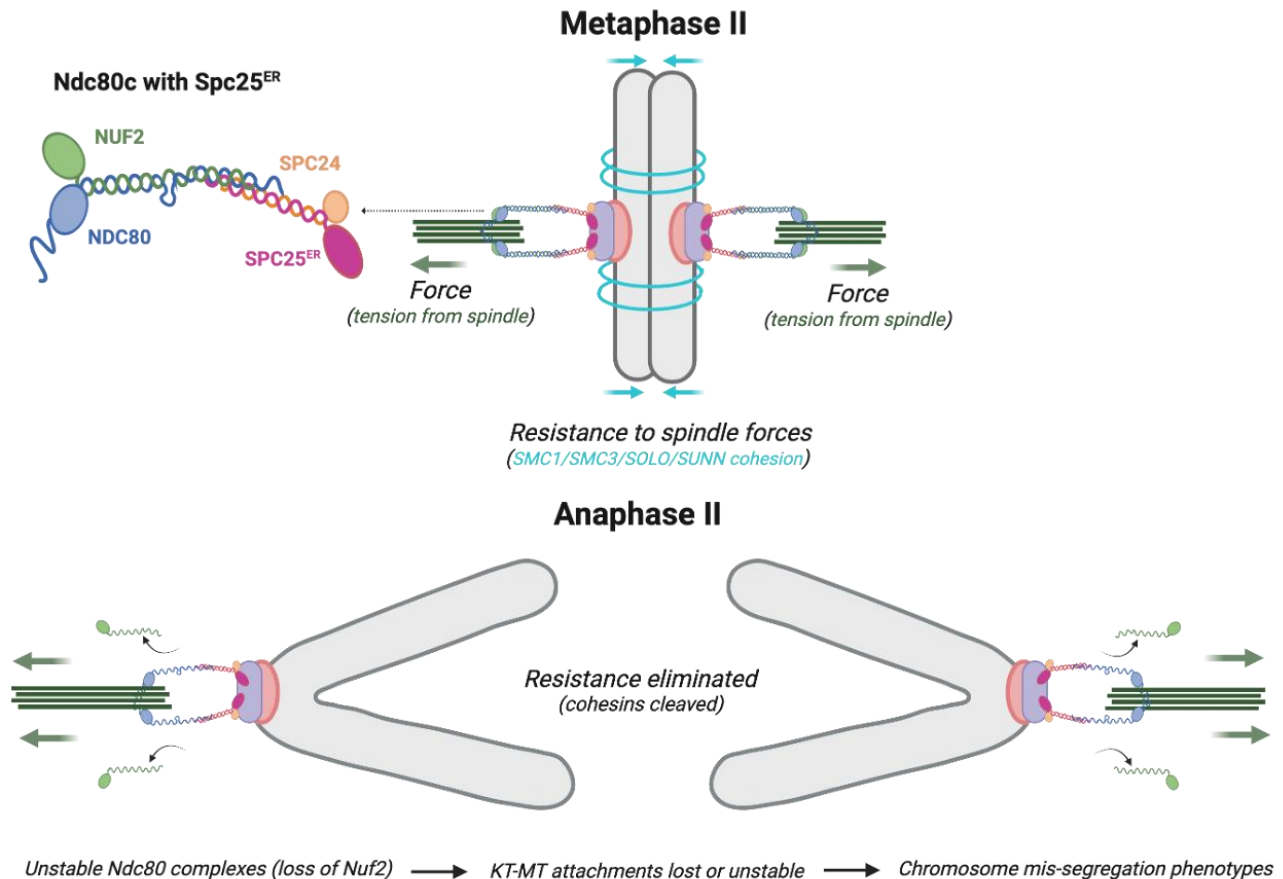


Figure 3.18 Model for how Spc25^{ER} protein incorporation into the Ndc80c affects kinetochore function during male meiosis II.

The mutant Spc25^{ER} protein expressed in *spc25*^{Z3-3822} mutants can localize to the inner kinetochore (KT) and support recruitment of the Ndc80/Nuf2 heterodimer to form a functional Ndc80c up to the stage of metaphase. Sister KT biorientation is achieved in metaphase II by establishment of end-on KT-MT attachments. Tension generated by spindle forces is exerted onto sister KTs, but resistance to pulling forces is mediated by cohesion of sister centromeres, provided by the meiotic cohesin complex (SMC1/SMC3/SOLO/SUNN). During anaphase II, the meiotic cohesin complex is cleaved by Separase activity which eliminates resistance to spindle forces and forces sister chromatids to be pulled to opposite poles. However, the force emitted onto the Ndc80c in the presence of the Spc25^{ER} destabilizes the complex's attachment to MTs, which causes loss of Nuf2 during anaphase. Loss of stable end-on attachments provided by the Ndc80 complex result in chromosome mis-segregation phenotypes such as chromosome lagging and unequal segregation.

Since the sizes of the brain lobes cannot accurately distinguish subtle mitotic defects, if there are, I investigated more microscopic phenotypes in the adult brains by immunostaining them with the anti-chaoptin antibody which labels the projections of the photoreceptor neurons within the optic lobe (**Figure 3.19B**). There are around 750 ommatidia within each eye and each ommatidium contains a core of eight photoreceptor neurons (Klamt, 1997). The formation and architecture of these elements responsible for fly vision is reliant on coordinated mitotic cellular divisions and precise cell signaling during development (Yu et al., 2010); (Otsuki et al., 2018). Any disruptions during development, such as lack of essential signals or inaccuracies in mitosis, can result in severe defects in the structural integrity of the visual system. Hence, I hypothesized that if mitotic chromosome segregation events are compromised in the dividing cells during the formation of the visual structures, consequences of such like cellular apoptosis would severely affect proper development. The result of defective development would be shown by abnormal organization or presence of these photoreceptors. However, when I analyzed the optic lobe of adult flies expressing UASc-Spc25^{WT}, UASc-Spc25^E, or UASc-Spc25^{ER}, I found no apparent deviations in the organization of the photoreceptor neurons nor lack of them (**Figure 3.19B**). These results additionally support that expression of the Spc25^{ER} does not affect mitotic chromosome segregation, and most likely can function normally at the mitotic kinetochore.

I further analyzed the photoreceptor architecture in flies expressing Spc25¹²⁸. Since the expression of Spc25¹²⁸ in the *spc25* mutant background causes pharate adult lethality (see section 3.5.2), I prepared whole mount brains from pharates. While expression of UASc-Spc25¹²⁸ in a *spc25*⁺ background support normal brain lobe size and optic lobe development, expression of UASc-Spc25¹²⁸ in *spc25* null mutants shows defective optic lobe development (**Figures 3.20**).

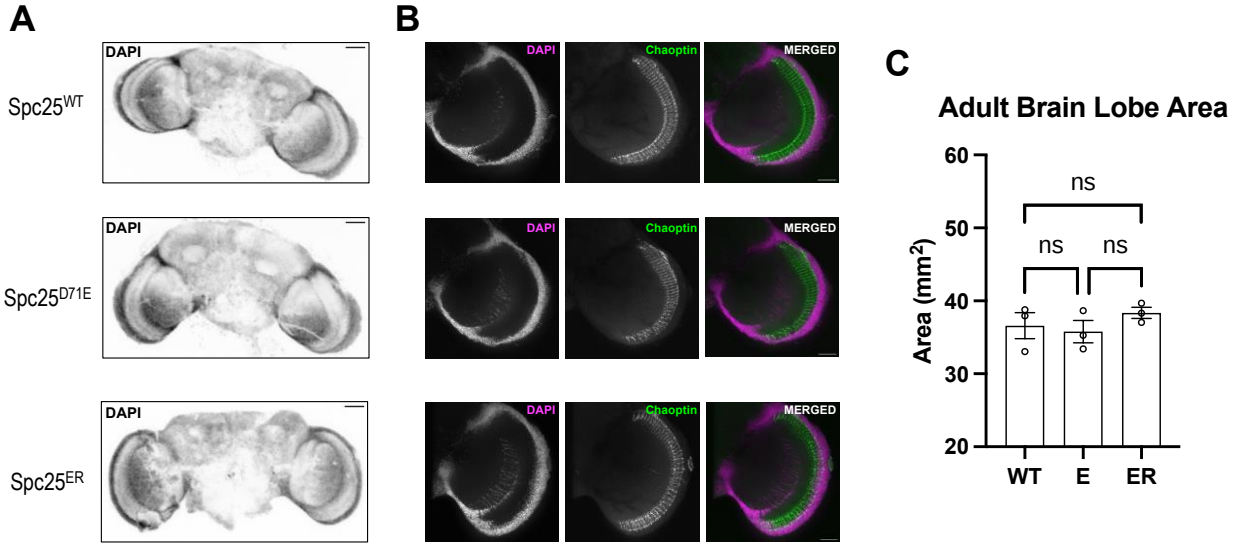


Figure 3.19 Expression of UASc-Spc25 constructs effect on adult brain development. **A.** Whole mount immunostaining of 5-7 day old adult males expressing *UASc-Spc25 (Spc25^{WT}) (Top), *UASc-Spc25^{D71E} (Middle), or *UASc-Spc25^{ER} (Bottom) in a spc25 null background. Genotypes: w/Y; *UASc-Spc25 construct / *actGAL4*; *spc25*^{c00064} / Df(3R)BSC486; *Specifying specific Spc25 construct, respectively. DAPI staining (black). Scale bar = 50 microns. **B.** Whole mount immunostaining of the right brain lobe of 5-7 day old adult males expressing *UASc-Spc25 (Spc25^{WT}) (Top), *UASc-Spc25^{D71E} (Middle), or *UASc-Spc25^{ER} (Bottom). From left to right in each panel: DAPI staining, anti-chaoptin (24B10) staining, and merged images showing DAPI in magenta and anti-chaoptin in green. Scale bar = 10 microns. **C.** Measured total brain lobe areas in micrometers² for adult males expressing UASc-Spc25 (WT), UASc-Spc25^{D71E} (E) and UASc-Spc25^{ER} (ER) in a spc25 null background. Ordinary one-way ANOVA with multiple comparisons was conducted comparing the mean brain lobe area for each genotype.

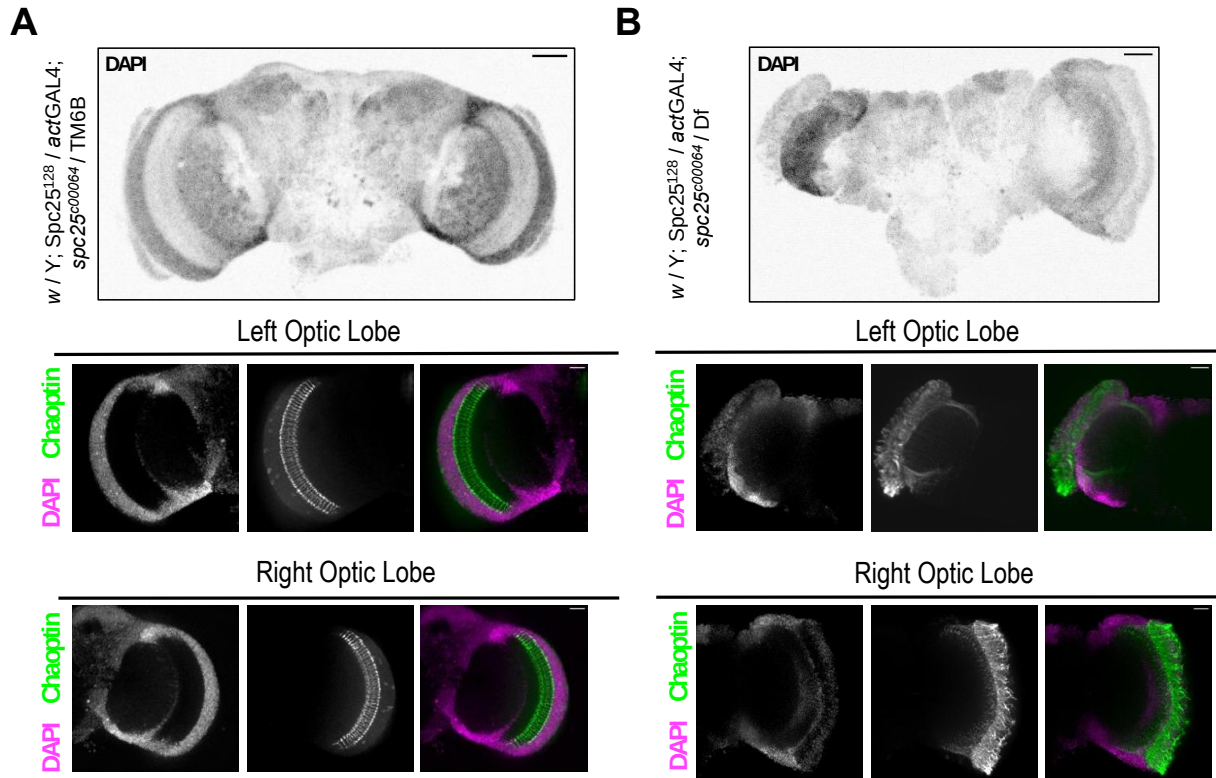


Figure 3.20 Expression of UASc-Spc25128 effect on pharate brain development.

A. (Top) Whole mount immunostaining of a pharate brain from a male expressing UASc-Spc25¹²⁸ in a spc25 wildtype (WT) background; Genotype: w/Y; UASc-Spc25¹²⁸ / actGAL4; spc25^{c00064} / TM6B. DAPI staining (black). Scale bar = 50 microns. (Middle and Bottom) Left brain lobe and right brain lobe of the pharate brain, respectively immunostained with DAPI (magenta) and anti-chaoptin (24B10) (green). Scale bar = 10 microns. **B.** (Top) Whole mount immunostaining of a pharate brain from a male expressing UASc-Spc25¹²⁸ in a spc25 null background; Genotype: w/Y; UASc-Spc25¹²⁸ / actGAL4; spc25^{c00064} / Df(3R)BSC485. DAPI staining (black). Scale bar = 50 microns. (Middle and Bottom) Left brain lobe and right brain lobe of the pharate brain, respectively immunostained with DAPI (magenta) and anti-chaoptin (24B10) (green). Scale bar = 10 microns.

Brains of pharates expressing UASc-Spc25¹²⁸ in the *spc25* null background show asymmetric optic lobe growth. We found that the development of pharate brains varied with regards to size and the development of associated structures within the optic lobe as the organization of the retinal axonal projections were disrupted with respect to their organization. These results suggest that expression of the second mutant transcript which would encode a truncated Spc25 protein, Spc25¹²⁸, could be the major cause of the small brain phenotype observed in *spc25*^{Z3-3822} hemizygous mutants.

CHAPTER FOUR

DISCUSSION, CONCLUSIONS & FUTURE DIRECTIONS

Identification of a unique mutant allele of *spc25*, *spc25^{Z3-3822}*, led us to gain additional insights into the importance of specific regions of the protein. Spc25 is one of four components of the Ndc80 complex (Ndc80c), which functions within the core kinetochore. Proper function of the Ndc80c is required for proper biorientation and segregation of chromosomes in both mitotic and meiotic divisions. Knockdown by RNAi or null mutation of genes encoding Ndc80c proteins cause severe chromosome mis-segregation phenotypes, which leads to pupal lethality in *Drosophila*. Interestingly, hemizygous *spc25^{Z3-3822}* flies are adult viable and fertile. However, our analysis of adult mutant males shows they produce aneuploid sperm because of meiotic chromosome NDJ events. These data suggested that *spc25^{Z3-3822}* is a hypomorphic allele, which we hypothesized could be useful for studying meiotic specific functioning of Spc25. Hypomorphic alleles can be valuable in understanding the function of specific domains and/or amino acid residues within a protein due to their partially functional nature.

Molecular characterization of the *spc25^{Z3-3822}* allele led us to identify that two mutant transcripts are produced, which encode two different mutant Spc25 proteins: Spc25^{ER} and Spc25¹²⁸. We show that expression of either mutant protein negatively effects meiotic chromosome segregation accuracy. Our analysis of spermatocytes expressing the mutant Spc25^{ER} protein suggest it is a partially functional Spc25 protein. Spc25^{ER} localizes in a similar pattern to endogenous Spc25 during meiosis I and II. However, homolog and sister chromatid NDJ events occur when Spc25^{ER} is expressed in a *spc25* null background. These results support previous studies that show Spc25 function within the Ndc80c is required to facilitate the

poleward movement of chromosomes during cell division. On the other hand, the truncated Spc25 mutant protein, Spc25¹²⁸, appears to be a nonfunctional protein as it does not localize to the kinetochore. Lack of Spc25 localization to the kinetochore would prevent formation of the Ndc80 complex, as localization of the other components, Ndc80 and Nuf2, depend on prior localization of Spc25 via its CT region (Przewloka et al., 2007).

Interestingly, our analysis of flies expressing the two mutant proteins suggest they have different effects on mitotic cells. We show that Spc25^{ER} expression in a *spc25* mutant background rescues *spc25* mutant phenotypes of pupal lethality as flies are adult viable and fertile. In addition to this, *spc25* mutant flies expressing the Spc25^{ER} transgenic protein have normal brain growth and development, suggesting that mitotic divisions are normal. Conversely, *spc25* mutant flies expressing Spc25¹²⁸ are adult lethal, surviving up to the pharate stage. Developmental abnormalities at the pupal and pharate stages are observed and spermatocytes of pharates show severe meiotic NDJ phenotypes when Spc25¹²⁸ is expressed. These data closely relate to *spc25* null phenotypes, concluding that Spc25 protein lacking its CT region is nonfunctional during both mitosis and meiosis (Schittenhelm et al., 2009); (Venkei et al., 2012); (Feijao et al., 2016); (Musacchio et al., 2017). Our observation that Spc25¹²⁸ expressed in *spc25* null mutants partially rescues viability suggests that the mutant protein may have some function. Though we could not observe a stable localization of Spc25¹²⁸ to kinetochores, we cannot rule out that the protein may transiently localize to the kinetochore region. In addition to this, as shown in our characterization of the Spc25¹²⁸ protein, it contains the NT and middle region that encodes for the coiled-coil region. It could be possible that this mutant protein still associates with Spc24, transiently localizes to the kinetochore region and permits formation of some Ndc80

complexes. However, if possible, we expect that this Ndc80 complex with the Spc25¹²⁸ protein would be highly unstable and lead to severe problems with establishment of stable end-on attachments and thus chromosome mis-segregation events. This hypothesis would be consistent with our analysis of flies expressing the Spc25¹²⁸ protein. Further studies to address the ability of the Spc25¹²⁸ protein to localize transiently and Ndc80 complex formation in its presence would be interesting to address this hypothesis.

Our analysis of transgenic flies expressing the two Spc25 mutant products encoded by *spc25^{Z3-3822}* transcripts suggest that the Spc25^{ER} protein is responsible for the meiotic specific chromosome NDJ events observed in *spc25^{Z3-3822}* mutants. We show that Spc25^{ER} is capable of normal localization to the KT region and thus should be able to support formation of the Ndc80 complex during meiosis. To address this, we expressed Ndc80 component *gEGFP::Nuf2* in *spc25^{Z3-3822}* mutants. Indeed, we show that Nuf2 properly localizes to the kinetochore during prometaphase and metaphase. However, along with chromosome mis-segregation events, we show that Nuf2 protein localization is prematurely lost during anaphase in both meiotic divisions in *spc25^{Z3-3822}* mutants.

Our data suggests that during chromosome segregation the loss of Ndc80 component Nuf2 reflects loss of the Ndc80 complex in spermatocytes of *spc25^{Z3-3822}* mutants. We hypothesize that during anaphase, the Ndc80 complex becomes unstable in the presence of the mutant Spc25^{ER} protein. The Ndc80c is required to establish and maintain end-on KT-MT attachments, which mediates proper chromosome transmission during anaphase. We hypothesize that due to the instability of the Ndc80c, stable KT-MT attachments are lost during poleward movement of chromosomes which is responsible for the meiotic chromosome NDJ events

observed in *spc25*^{Z3-3822} mutants (**Figures 3.17 & 3.18**).

Our functional analysis of two Spc25 mutant proteins, one with only the D71E mutation (Spc25^E) and the other with both the D71E substitution and RSAI motif insertion (Spc25^{ER}), suggests that the D71E substitution is responsible for the instability of the Ndc80 complex during anaphase. The ability of the Ndc80 complex with the mutant Spc25^{ER} protein to support stable, end-on attachments was not directly analyzed, but it is likely that the complex at least supports the formation of some end-on KT attachments. Null mutants of *spc25* or *ndc80* which completely lack formation of the Ndc80 complex and therefore ability to form end-on KT-MT attachments. Null mutants therefore show more severe phenotypes such as pupal lethality and higher rates of chromosome NDJ in both mitosis and meiosis (Przewłoka et al., 2007); (Schittenhelm et al., 2007); (Williams et al., 2007); (Yamagishi et al., 2014).

The time at which the Ndc80 complex (Ndc80c) becomes unstable in the presence of the Spc25^{ER} protein is curious. During anaphase onset, chromosomes no longer have cohesion and must succumb to the pulling force of spindle microtubules. The poleward movement of chromosomes occurs due to stable KT-MT attachments established by the Ndc80c (Tooley et al., 2011); (Tanaka, 2010). Our data suggests that the D71E substitution within Spc25^{ER} causes instability of the Ndc80c within meiotic KTs specifically during anaphase. Interestingly, the D71E substitution within the *Drosophila* Spc25 resides within its predicted coiled-coil region. In structural studies of the yeast and human Ndc80c, the coiled coils of Spc25 and Spc24 form a two-chain (2C) coiled coil (CC), which stabilizes their dimerization. In addition to this, the coiled coils of both Ndc80 and Nuf2 join the 2C CC of the Spc25/Spc24 dimer to ultimately form the heterotetrameric complex (Musacchio et al., 2017). The tetramerization region of the

complex is composed of a four-chain CC (Ndc80/Nuf2/Spc25/Spc24) and three-chain CC (Ndc80/Spc25/Spc24), which provides rigidity and stability of the overall Ndc80c. The tetramerization region importantly links the MT-binding end to the inner KT/centromeric binding end of the complex (Valverde et al., 2016). Thus, the structural integrity of this region is critical for complex function as it ensures chromosomes can establish attachment to the spindle for their proper orientation and transmission during division.

Understanding the interactions among the CCs of the four components important for formation and stability of the Ndc80c in *Drosophila* is challenging. KT proteins in *Drosophila* (*D.*) show the highest levels of evolutionary divergence. For example, only particular regions of *Drosophila* Ndc80 and Nuf2 proteins share only 40% and 54% sequence identity with respective homologs (Meraldi et al., 2006). For Spc25 homologs, there is low sequence similarity across the entire primary sequence when aligned as sequence homology ranges from 15% to 30%. For *D. melanogaster* Spc25, 99 residues within the middle region and 35 residues at its CT, however does share a higher percentage of sequence similarity, 38% and 35% respectively, to corresponding regions of the human Spc25 homolog. The predicted coiled coil region of *Drosophila* and human Spc25 proteins fall within this region, but the coiled-coil regions do not exactly overlap (Przewloka et al., 2007).

In *D. melanogaster* and their most closely related species *D. sechellia* and *D. simulans*, the Spc25 orthologs do share a high sequence identity. Using the NCBI Protein BLAST tool, the predicted Spc25 proteins in *D. sechellia* and *D. simulans* share approximately 91% sequence identity with *D. melanogaster* Spc25. Relative species such as *D. yakuba* and *D. erecta* share a moderate percentage of sequence identity to *D. melanogaster* of 86% and 83%, respectively. The

predicted Spc25 proteins in more distantly related species only share approximately 30-47% sequence identity with the *D. melanogaster* Spc25 protein. Analysis of *Drosophila* predicted orthologs of Spc24, Nuf2, and Ndc80 also share similar trends in their percentage sequence identity shared. Protein alignments of these kinetochore proteins within *Drosophila* species and among other eukaryotes show how rapidly the sequences for the genes are evolving.

To determine if any particular regions of the Spc25 protein is conserved in *Drosophila*, a sequence alignment of Spc25 *Drosophila* orthologs with Clustal Omega Multiple Sequence Alignment tool was generated and shown in **Figure 4.1**. Surprisingly, the region of the protein that begins to have at least 50% shared sequence similarity starts approximately at the residue D71 and ends at residue 215. This region includes the predicted coiled-coil and CT regions of the Spc25 protein. In *D. melanogaster*, *D. sechellia* and *D. simulans* their Spc25 proteins all encode aspartic acid at residue 71. However, in all the other distantly related *Drosophila* species their Spc25 orthologs encode glutamic acid at residue 71.

Our analysis showing that the Spc25 D71E substitution does affect the function of Spc25 strongly suggests the amino acid change may influence the protein's overall structure or interaction with other Ndc80c components. The amino acid substitution could affect interactions within the Spc25/Spc24 dimer and/or interactions at the tetramer junction of the four subunits specifically in *Drosophila melanogaster* and possibly in the other closer related *Drosophila* subspecies. The Spc25/Spc24 dimer is not only responsible for recruiting the Ndc80/Nuf2 dimer but also acts as the anchor to maintain the Ndc80c localized to the KT. Disruption of Ndc80/Nuf2 interactions with the Spc25/Spc24 dimer and/or instability within the Spc25/24 dimer itself could ultimately influence end-on KT-MT attachments in a detrimental way.

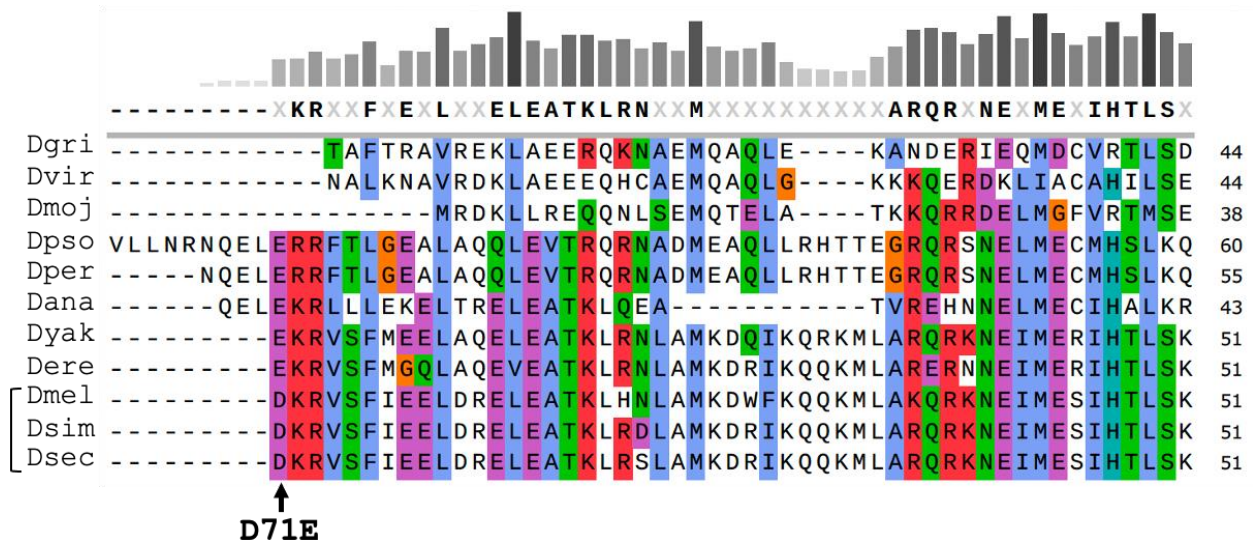


Figure 4.1 Multiple sequence alignment of Spc25 orthologs in *Drosophila*.

Clustal Omega results of the multiple sequence alignment of Spc25 orthologs in *Drosophila* (D) subspecies amino acid residues 62-121. *Drosophila* species name abbreviated with first three letters of the species name. Gray bars on top represent level of sequence similarity found with larger, darker bars being more similar and smaller, lighter bars being less similar. Bold, black letters below are the consensus residue found at that position. Colors within the sequence alignment are colored by shared chemical properties of amino acids. The numbers shown on the right next to each alignment represent the number of residues found to share sequence similarity within the 62-121 amino acid region. The D71E missense mutation found in the *spc25*^{Z3-3822} allele is indicated below the multiple sequence alignment.

While the sequence similarities of Ndc80c components are not well conserved, the secondary structure of subunits is generally thought to be maintained within the complex. Most structural studies of yeast and human Ndc80c subunits have expressed recombinant proteins in bacteria or insect cell lines to study formation of the CT globular domains of both the Spc24/Spc25 and Ndc80/Nuf2 dimers. In most studies, the recombinant proteins studied either have their coiled region shortened (Ndc80c^{dwarf}) or are fusion proteins of two components (Ndc80c^{bonsai}) (Ciferri et al., 2008); (Valverde et al., 2016). Within these studies, though some interactions among the subunits are identified, the junction site where the helices of the Spc25/Spc24 and Ndc80/Nuf2 heterodimers would join endogenously are mostly eliminated. Thus, our current knowledge of the residues essential for tetramerization is lacking.

Structural work in budding yeast has set a foundation for how the Ndc80c components take form. The tetramer junction is composed of two parallel coiled coils, in which involve the two NT regions of the Spc24/Spc25 dimer which interact with the CT regions of the Ndc80/Nuf2 dimer, based on localization patterns and protein interaction studies. Though some thought the ends of the two subcomplexes might just “meet” together end to end; however, the junction site shows a higher complexity. At the center, the helices of the subunits form a four-chain coiled coil (cc), incorporating all subunits, and then closer to the CT of Ndc80 form a three-chain cc, incorporating Ndc80, Spc24, and Spc25. Key findings from X-ray crystallography of the Ndc80c^{dwarf} protein show that the tetramerization region contains a nonpolar surface, aromatic side chain stacking within the center of the junction, and a network of polar contacts focused within the Spc24 alpha helix, which may provide binding sites for other KT proteins and/or regulators (Valverde et al., 2016).

The pairing of alpha helices to form a coiled-coil domain can either be in an antiparallel or parallel orientation and either be formed within the same protein or between two different monomers. For example, the coiled-coil regions of the SMC proteins are formed within the same protein chain in which the protein folds back upon itself via its hinge domain (Petela et al., 2021). Motor proteins also contain coiled-coils, such as myosin-X and myosin-II which form an antiparallel cc and parallel cc, respectively (Li et al., 2003); (Lu et al., 2012). Coiled coils are defined as helices that wrap around each other in a “knobs to holes” geometry, in which amino acid side chains occur at the interface. The term “knob” refers to one residue of a helix that fits into a position surrounded by four other residues of the second helix, providing the “holes”. For two or more helices to adopt these specific interactions, the helices must be formed by repeats of a heptad with an organization of “a-g”, where “a” and “d” are nonpolar residues (leu, ile, val, or ala) and “e” and “g” are typically polar or charged residues. When two helices come together, the nonpolar residues at the “a” and “d” positions of each helix forms a hydrophobic core interface. Charged residues such as glu (E) or lys (K) found at the “e” and “g” positions can interact by electrostatic interactions, which contributes to stability of the dimer (Lupas et al., 2017).

Along with the progress of understanding the Ndc80c cc domains in yeast, more work with the human Ndc80c components has added to our understanding of interactions found at the junction site. Purification of human Ndc80c components that were then subject to cross-linkage, trypsin digestion and LC/MS-MS identified unique fragments of Ndc80, Nuf2, Spc25 and Spc24 that represent the coiled coil core of the tetramer junction. **Figure 4.2A** shows a summary of their findings that show several lysine residues are involved in human Ndc80c (hsNdc80c) protein interactions within the tetramerization domain (Maiolica et al., 2007).

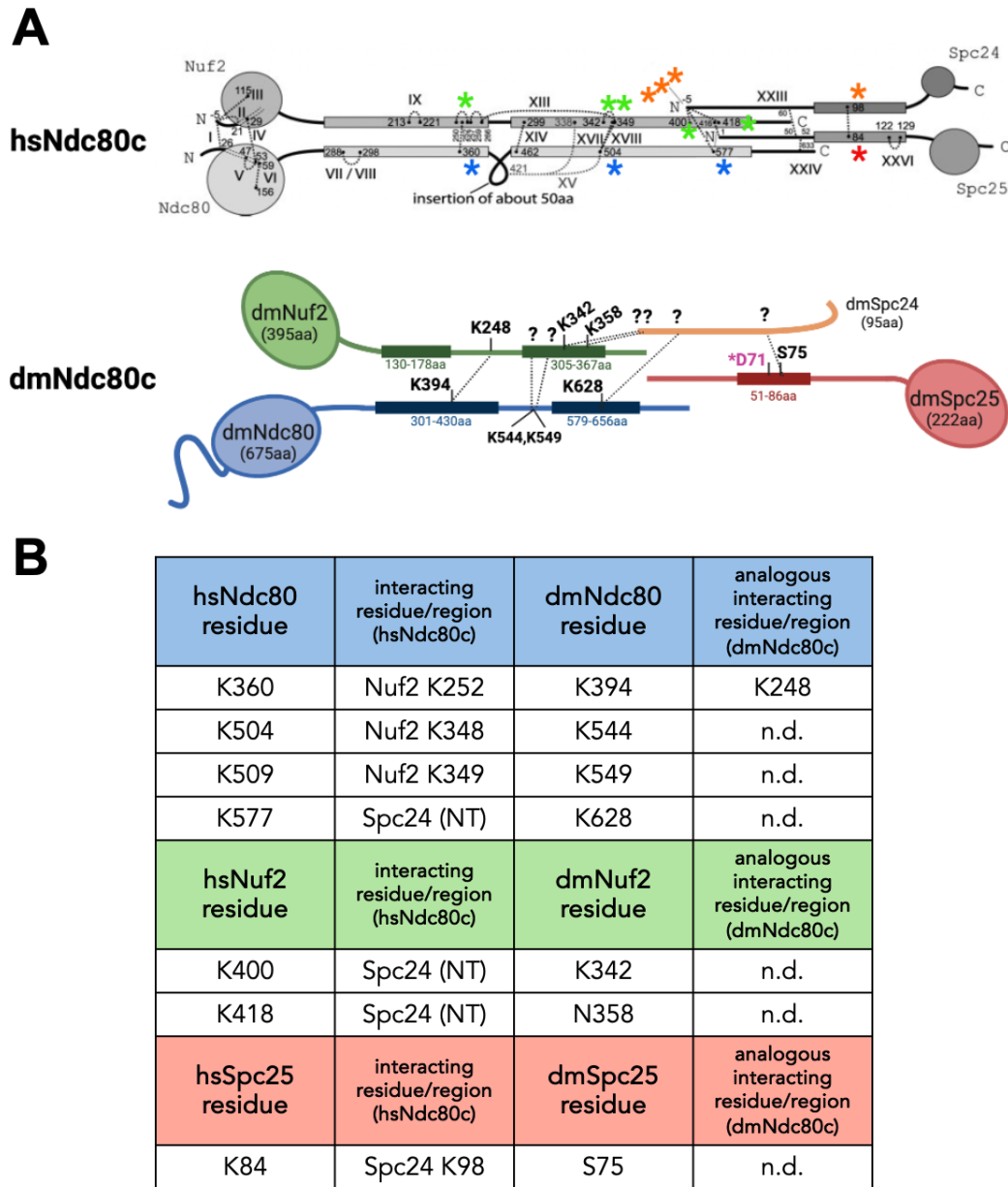


Figure 4.2 Residues involved in the tetramer junction of the Ndc80 complex in humans (hsNdc80c) and possible residues involved in the *Drosophila* Ndc80c junction site. A. Adapted figure from Maiolica et al., 2007 (Results, Figure 6) that shows lysine residues involved in protein-protein interactions among Ndc80c components. Colored stars are provided for comparison proposed model of *Drosophila* Ndc80c analogous residues identified by Clustal Omega Multiple Protein Alignment of hsNdc80c, dmNdc80, dsecNdc80c, and dsimNdc80c proteins. The D71E substitution found in the spc25^{Z3-3822} allele characterized in this study is shown in magenta for context. B. Table provided lists K residues found in the hsNdc80c proteins and conserved residues identified in dmNdc80c proteins. n.d. = not determined.

To identify if any residues within the human Ndc80c (hsNdc80c) proteins important for tetramerization may relate to residues within *Drosophila melanogaster* Ndc80 proteins, we did an alignment of hsNdc80c proteins with *D. melanogaster*, *D. sechellia* and *D. simulans* Ndc80c proteins with Clustal Omega. Shown in **Figure 4.2B**, we identified some residues are conserved, either completely or shared residue similarity. It is interesting that the K84 residue in hsSpc25, that interacts with Spc24, aligns with the S75 residue in the *Drosophila melanogaster* Spc25 protein. This residue resides closely with the D71 residue, which is mutated in our *spc25*^{Z3-3822} allele and by our results affects stability of the *Drosophila* Ndc80c. Structural studies to determine how the D71E substitution in the *Drosophila* Spc25 protein could affect interactions between Spc25, Spc24, Ndc80, and Nuf2 would be required and exciting to further understand the tetramer junction in the fly Ndc80c. Moreover, additional studies to identify putative unknown protein interactors with Spc25 and/or Ndc80c components will be needed to understand a meiotic specific role the D71 residue may be involved in during male meiosis.

One remarkable difference between *Drosophila* Ndc80c proteins and the Ndc80c homologs in other eukaryotes is the length of Spc24. The *spc24* gene in *Drosophila* is predicted to encode two isoforms, which only differ by the presence or absence of two amino acids. The lengths of these two proteins are 95 and 93 amino acids, while the human Spc24 protein and other Spc24 homologs contain approximately 100 more amino acids. The hsSpc24 and hsSpc25 proteins are of similar length, 197 and 224 respectively. In *Drosophila*, all Spc24 orthologs are around 95 amino acids, while the other Spc24 homologs all have similar lengths to their Spc25 homologs of 222 amino acids. In the structural studies of both human and yeast Ndc80c proteins, Spc24 and Spc25 appear to be equally involved in contributing to interactions with the

Ndc80/Nuf2 dimer through their long NT domains (Ciferri et al., 2008); (Valverde et al., 2016). Since the *Drosophila* Spc24 protein is much shorter and its NT region shares no homology with any Spc24 protein, it is hard to predict how well it is incorporated into the tetramer junction. It is possible that Spc25 in *Drosophila* may have adapted a more essential role in stabilizing the Ndc80/Nuf2 heterodimer since its length has been maintained over evolutionary time. Very little work has established requirements and/or functional domains for the *Drosophila* Spc24 protein since identification of the gene. The dmSpc24, originally named Kmn2, protein has been shown to localize to mitotic KTs in S2 cells (Schittenhelm et al., 2007). Additional work to characterize the functional roles of dmSpc24 will be needed to fully understand its essential roles in both mitosis and meiosis in flies.

In this study, we characterized a novel mutant allele of *spc25* and identify meiotic and mitotic mutant phenotypes attributed to two mutant transcripts encoded by the hypomorphic allele. The data concludes that the meiotic mutant phenotypes of unequal homolog and unequal sister chromatid separation in *spc25*^{Z3-3822} mutants is caused by a D71E missense mutation. Interestingly, the D71E substitution does not affect brain growth, shown in **Figure 3.17**. This data supports a hypothesis that the D71E substitution negatively effects meiotic Ndc80 complexes but not mitotic Ndc80 complexes. These results suggest that there may be fundamental differences between the mitotic and meiotic kinetochore.

At the structural level, differences in mitotic and meiotic KT-MT attachments have been observed in many reports. The earliest analysis of meiotic kinetochores in *Drosophila* males elegantly showed by electron microscopy that only four to six microtubules embed into the kinetochore of primary spermatocytes in metaphase (Goldstein et al., 1981). Analysis of mitotic

S2 mitotic cells show that mitotic kinetochores incorporate 11 to 15 microtubules per kinetochore (Maiato et al., 2006). Differences in the number of microtubules per kinetochore (MT/KT) could be attributed to cell-type specific requirements. In mitosis, sister kinetochores configure in the back-to-back orientation, creating bipolar attachment of sister chromatids. However, during meiosis I sister KT's are in the side-by-side configuration so that paired sister chromatids are attached to the same pole.

The fact that a single KT in mitosis incorporates a higher number of MT/KT than paired of sister KT's is surprising yet interesting. It can be hypothesized that different cell-types implement cell-specific regulation of cell division. Consequences of chromosome nondisjunction in mitosis fundamentally differs from the consequences of NDJ in meiosis. As mitosis is the primary cell division process responsible for the generation and differentiation of multiple cell types and maintain tissue homeostasis, accurate chromosome segregation is of utmost importance to ensure proper cellular functioning. In mitosis, each kinetochore may incorporate more than enough microtubules to ensure proper chromosome transmission. On the other hand, meiosis is a unique cell division process that only gives rise to one cell type, sperm or eggs. It could be hypothesized that over evolutionary time, meiotic cells may have changed or eliminated certain regulatory signaling involved in chromosome segregation. This hypothesis is consistent with reports that have analyzed SAC (spindle assembly checkpoint) protein function in meiosis. In *Drosophila*, the SAC protein MAD2 is not required for accurate chromosome segregation during meiosis (Chaurasia et al, 2018). However, MAD2 is also not required for viability and fertility when other SAC components such as Bub3 or the RZZ complex are essential in mitotic chromosome segregation (Buffin et al., 2007). Canonical SAC functioning in meiosis in

Drosophila has not been well investigated. Regardless of having a functional SAC or not, in male meiosis chromosomes display rapid efficiency in chromosome congression and biorientation utilizing a more tension sensing centered mechanism in meiosis I and II to initiate chromosome segregation (Chaurasia et al, 2018).

Our results are consistent with a hypothesis that since chromosome biorientation is achieved quickly and efficiently, meiotic chromosomes may have become more dependent on KT assembly and, specifically, the Ndc80c to establish KT-MT attachments in preparation for chromosome segregation. Studies in male meiosis have shown that once meiosis starts, primary spermatocytes take approximately one hour to complete the first division and likewise for the second division (Cenci et al., 1994); (Chaurasia et al., 2018). Compared to the timing of prophase, which can last from 80-92 hours, the time of meiotic divisions in spermatocytes is significantly lower (Cenci et al., 1994). For spermatocytes to be proficient, it can be hypothesized that any monitoring or regulation of segregation events may already be directly involved in the stages leading up to the division. As mentioned earlier, studies in *Drosophila* female meiosis have shown essential role for protein phosphatase PP1-87B in regulating the process of end-on KT-MT attachment during meiosis I for proper homolog biorientation and segregation. Studies to understand the roles of PP1-87B and if it plays a similar role during male meiosis would be interesting to investigate in the future.

Establishment of end-on KT-MT attachments are essential for accurate chromosome segregation. Though chromosomal movements within cells can be mediated by lateral KT-MT attachments or dynamic behavior of interpolar MTs, establishment and maintenance of end-on KT-MT attachments is crucial to properly orient chromosomes toward the correct pole and

ensure they reach opposite poles. The Ndc80c plays a center role in forming stable, end-on attachments to the spindle while other KT proteins and/or complexes support KT-MT attachment formation and stability. In addition to KT localized proteins, other protein complexes in other organisms also play important roles in ensuring chromosome transmission. In budding yeast, the Dam1 complex forms a ring around the end-on attached MT and interacts directly with the Ndc80c to strengthen MT-binding of Ndc80c (Kim et al., 2017). Similarly, in humans the Ska complex binds MTs and the Ndc80c during end-on KT-MT attachment formation. The Ska complex, composed of three Ska proteins, directly interact with Ndc80c components within the coiled-coil regions (Helgeson et al., 2018). In addition to the Ska complex, another Ndc80c interactor Astrin also has been shown to mediate stabilization of end-on attachments via Astrin recruitment of PP1 to the KT in a tension-dependent manner (Conti et al., 2019). Ska and Astrin proteins are proposed to be conserved but homologs of either have not yet been identified in *Drosophila*. It would be interesting to determine if homologs and/or similar proteins are expressed during the cell cycle of mitosis and meiosis in flies, and particularly if Ska proteins or Astrin might have meiotic specific binding co-factors that might help stabilize sister KT mono-orientation.

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VITA

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