A Thesis

Entitled

A Visual Screen for Centriolar Mutants in *Drosophila melanogaster*

by

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The centrosome is a conserved organelle that functions as the cell’s main microtubule organizing center and to template formation of cilia. Defects in centrosomes lead to cancer and a type of human developmental diseases called ciliopathies. While the importance of studying this organelle is clear, how it forms and functions is not fully understood. In particular, understanding of the mechanisms of centriole elongation is lacking. *Drosophila melanogaster* is an excellent model to study centriole elongation since centrioles in its testes are atypically long, and many tools exist to study localization of fluorescently-tagged centrosomal proteins under endogenous control. Centriolar genes in *Drosophila* were identified in the past through reverse genetic, genomic, and molecular approaches. Additional *Drosophila* centriolar genes were uncovered in forward genetic screens for mechanosensation and meiotic function defects. Here, we devised a novel visual screen of centrioles in *Drosophila* testes using the centriolar marker Ana1-GFP. A total of 44 centriolar mutants were identified from the 1436 mutants screened. Previously described centriolar phenotypes including short centrioles, long centrioles, and abnormal
PCL comprised 8 of these mutants. Intriguingly, 34 of these mutants displayed a novel centriolar phenotype characterized by Ana1-labeling of the cilia. None of the mutants exhibit the distinct behavioral phenotype common in centriolar mutants, however many display a novel mild form of uncoordination. The identification of a large collection of centriolar mutants, a new “Ana1 leak” phenotype, and a new behavioral phenotype associated with these mutants suggests that visual screens are an effective way to identify new types of centriolar mutations.
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**List of Abbreviations**

Asl………………………..Asterless
CyO………………………Curly Wings (Marker Mutation/Balancer Chromosome)
Dr………………………Drop (Eye Marker Mutation)
EMS……………………...Ethyl Methanesulfonate
F-actin…………………Filamentous Actin
GC………………………..Giant Centriole
Hu………………………Humeral (Bristle Marker Mutation)
IC………………………..Individualization Complex
MKRS…………………….Balancer
MT………………………..Microtubule
PCL………………………Proximal Centriole-Like
PCM……………………..Pericentriolar Material
PTM……………………..Post-translational Modification
Tb………………………Tubby (Pupa Marker Mutation)
TM6B………………….Balancer
Chapter One

Introduction

1.1 Defects in Centrosomes Underlie Human Disease Prompting Better Understanding of Centrosome Formation and Function.

The centrosome is a dynamic eukaryotic organelle that classically consists of 2 microtubule-based centrioles surrounded by an amorphous protein matrix termed the pericentriolar-material (PCM). The PCM serves to nucleate astral microtubules that aid in cytoskeletal organization (Figure 1). In addition to its cytoskeletal role, the PCM also nucleates spindle microtubules essential for proper division of chromatin during mitosis. (Bettencourt-Dias and Glover, 2007). Centrosomal mutations can give rise to cellular defects including aneuploidy and formation of dysfunctional cilia, which respectively contribute to tumorigenesis and a category of human developmental diseases called ciliopathies (Nigg and Raff, 2009). Further study of the mechanisms of centrosome biology is required to characterize the sources of these serious diseases. Therefore, our lab uses Drosophila melanogaster as a model to better characterize the roles of known
proteins in centrosome biology, as well as to uncover new proteins that will help complete our understanding of centrosome formation and function.

Figure 1 - The centrosome is composed of 2 microtubule-based centrioles (green) surrounded by an amorphous protein matrix called the PCM (orange). The PCM nucleates formation of astral microtubules (black lines).

Figure 1: The Eukaryotic Centrosome

1.2 *Drosophila melanogaster* and Its Testes Are Effective Models for Studying Centrosomes.

*Drosophila melanogaster*, commonly known as the fruit fly, has emerged over the years as an excellent model to study conserved biological processes. One advantage *Drosophila* provides is a genome well-conserved in humans (Fortini et al., 2000). In addition, genetic manipulation is powerful in this system due to the availability of a vast open-source toolkit and short generation time.

*Drosophila melanogaster* testes are a well-established model for the study of many cellular and developmental processes (Fuller, 1993; Fabian and Brill, 2012). This model organ is useful to study centriolar mutations via standard fluorescence microscopy for several reasons. First, most of the proteins essential for centrosome biology in *Drosophila* testes are conserved among eukaryotes, allowing for potential insight to be gained into centrosome biology in higher organisms like humans. Importantly, *Drosophila* is one of
the few established models that can be used to study the effects of normally embryonic-lethal centrosomal mutations in a fully developed organism. While centrosomes are essential for early embryonic development, sperm with null centrosomal mutations can be rescued by maternal contribution of all the necessary centrosomal proteins for early development. Then, once all the maternally contributed proteins are depleted by pupa stage, centrosomes are no longer essential for development (Avidor-Reiss et al., 2012). In addition, fluorescently-tagged centrosomal proteins can be expressed under endogenous control. Lastly, sperm development occurs in chronological order along the length of the testes, allowing for rapid identification of different developmental stages (Basiri et al., 2012). We therefore believe that looking for mutants in the testes using fluorescently-tagged centrosomal proteins will be an effective way to identify new centriolar mutations.

1.3 A Diversity of Centrosomal Processes Underlie Drosophila Spermatogenesis

As the sperm develops along the length of the testes, the sperm centrioles undergo many changes including duplication, elongation, separation, and reduction (Avidor-Reiss et al., 2012; Basiri et al., 2012) (Figure 2). Spermatogenesis begins at the testis tip where 7-10 stem cells divide asymmetrically to form a spermatogonium and another stem cell copy (Losick et al., 2011). The spermatogonia, surrounded by two cyst cells, then differentiate and divide synchronously within their cyst. The spermatogonium undergoes 4 symmetrical divisions resulting in formation of 16 spermatocytes all containing short centrioles, which is followed by an extended G2 when cell size and centriole length dramatically increase. In addition, the centrioles in these cells form a primary cilium (Gottardo et al., 2013; Riparbelli et al., 2013; Basiri et al., 2014). Spermatocytes then enter meiosis I and II, which require centrioles for successful division (Bonaccorsi et al.,
Meiosis results in 64 round spermatids each containing 1 centriole. Each cyst then begins to differentiate during a process called spermiogenesis (Figure 2).

Figure 2 - Spermatogenesis occurs in chronological order along the testes beginning at the tip with the stem cells giving rise to spermatogonial divisions (left). Then spermatocytes are formed after an extended G2 (2nd to left) followed by spermatid development involving nuclear/mitochondrial reshaping, and centrosome reduction (2nd to right). This results in spermatozoa with a reduced centriole and PCL (right). Note that the mitochondria still persist leading to sperm maturity, but are only labeled in one stage because it is a marker for this developmental stage. In addition, the dimensions of the testes and all cells in this figure are not to scale.

Figure 2: Drosophila Spermatogenesis
During spermiogenesis, the spermatids undergo numerous morphological changes roughly in parallel. The initially round nucleus becomes leaf, canoe, and ultimately needle-shaped at maturity (Fabian and Brill, 2012). Alongside nuclear morphogenesis, the mitochondria similarly change from an initial round shape to an elongated needle shape at maturity. Around the onset of nuclear and mitochondrial morphogenesis, the centriole migrates from the plasma membrane pulling the cilium with it to dock to the nucleus. A group of distal centriolar and ciliary transition zone proteins, further detailed in the next section, then migrate away from the centriole leaving a unique cytoplasmic cilium in its wake (Enjolras et al., 2012; Basiri et al., 2014; Malicki and Avidor-Reiss, 2014). Shortly after the cilium begins to elongate, an unusual centriolar precursor called the proximal centriole-like (PCL) forms near the pre-existing centriole. The pre-existing centriole is referred to as the giant centriole (GC) due to the uniquely long centrioles present in Drosophila testes. The PCL eventually serves as the second zygotic centriole (Blanchon et al., 2009; Blanchon et al., 2014). Over the course of mid and late spermiogenesis, the PCM and many centriolar proteins are removed from the centriole and PCL in a mechanistically uncharacterized process called centrosome reduction (Avidor-Reiss et al., 2015; Khire et al., Submitted) (Figure 2). Once the spermatids have fully differentiated, they are invested with their own plasma membranes in a process called individualization. This process is characterized by formation of an individualization complex (IC) around the spermatid nuclei consisting of 1 F-actin-containing investment cone around each spermatid. Once formed, the complex synchronously migrates along the cilia to invest each cell with its own plasma membrane (Tokuyasu et al., 1972). Upon completion of individualization, the mature sperm cells are finally ready to be stored in the seminal vesicle (Figure 2).
The host of centrosomal processes that occur during spermatogenesis in *Drosophila* makes the testes an attractive model to study centrosome biology. By inducing point mutations through EMS (ethane-methanosulfonate treatment), we expect to uncover a diversity of centriolar phenotypes using fluorescent visualization of the centrioles.

1.4 Two Categories of Proteins Have Roles in Regulating Centriole Length.

The dimensions of eukaryotic centrioles typically hover around 200 nm in diameter and 500 nm in length, suggesting that regulation of centriole size is important in the cell. With that said, for centrioles to achieve such specific dimensions, their elongation must be tightly regulated. The mechanisms that underlie centriole elongation are poorly understood, however the proteins involved in the process to date can be roughly categorized into two groups: centriolar proteins and distal centriolar/transition zone proteins. Defects in proteins within these groups could potentially perturb centriole morphology, and therefore be identified in the visual screen we are proposing.

For the purposes of this section, the category of centriolar proteins broadly consists of those that localize to the centriole, but not specifically to its distal end. Many proteins fit this general description, but few have been implicated in centriole elongation. Before elongation can begin, CPAP (Centrobin-centrosomal protein 4.1-associated protein) must be recruited to the distal end of a centriolar precursor. There, CPAP’s interaction with γ-Tubulin among other core centriolar proteins has been implicated in elongation and stabilization of centriolar MT’s (Avidor-Reiss and Gopalakrishnan, 2013). In contrast to cytoplasmic MT’s, centriolar MT’s contain many PTM’s like acetylation and polyglutamylation, they are very stable, and their final length is strictly regulated. (Kochanski and Borisy, 1990; Bettencourt-Dias and Glover, 2009). Overexpression of
mammalian CPAP in cell culture leads to centriole over-elongation (Kohlmaier 2009; Schmidt 2009; Tang 2009). Bld10/CEP135 and Poc1/POC1 are centriolar proteins recruited to the centriole around the same time as Sas4/CPAP. These proteins stabilize centrioles likely through direct binding of centriolar MT tubulins (Pearson 2009; Bayless 2012; Carvahlo-Santos 2012). *Drosophila bld10, poc1<sup>c06059</sup>, and poc<sup>k245</sup> mutants exhibit short centrioles (Blanchon 2009; Mottier-Pavie 2009). In addition, overexpression of mammalian POC1 leads to over-elongation of centrioles (Keller 2009). However, unlike CPAP, their localization is not restricted to the centriole’s proximal end (Blanchon 2009; Mottier-Pavie 2009). Mammalian Centrobin is a centriolar protein that prevents degradation of CPAP. Therefore mutations in human Centrobin indirectly cause short centrioles through loss of centriolar CPAP (Gudi 2015). *Drosophila* Centrobin directly interacts with Sas4, suggesting a common function (Januschke 2013). Note that the genes discussed in this section were limited to those on the 3<sup>rd</sup> chromosome in *Drosophila*. Due to limitations in the genetic manipulation that can be done for one screen in this model, we are choosing to target the 3<sup>rd</sup> chromosome. While targeting the 1<sup>st</sup> or 2<sup>nd</sup> chromosome could also potentially lead to identification of novel centriolar mutations, we chose the 3<sup>rd</sup> because it contains the highest % of predicted essential genes (Koundakjian et al., 2004). With that said, it can be predicted that new alleles of these genes will be identified in our 3<sup>rd</sup> chromosomal screen through observation of abnormally short centrioles.

Distal centriolar/transition zone proteins consist of those that comprise the transition zone or localize near it at the mother centriole’s distal end. Only the mother centriole serves as the basal body to template cilia formation. The transition zone is a conserved region in ciliated cells that serves as a selective gate making the ciliary compartment distinct from the cytosol. It strictly regulates traffic in and out of the ciliary compartment, and many of
its components are essential for cilia formation and/or stability (Williams et al., 2011; Basiri et al., 2014; Malicki and Avidor-Reiss, 2014)(Figure 3). Proteins in this category have been implicated in regulating restriction of centriole elongation rather than promotion. While transition zone proteins have been extensively studied in other systems including humans, only one has been studied in *Drosophila*. The conserved gene that codes for this protein, *Cep290/CEP290*, is essential for cilia formation and maintaining proper centriole length in *Drosophila* (Basiri et al., 2014). Human *CEP290* mutations lead to a host of ciliopathies (Baala et al., 2006; den Hollander et al., 2006; Valente et al., 2006), while *Drosophila Cep290* mutations lead to abnormally long centrioles and severe uncoordination caused by ciliary defects (Basiri et al., 2014). Among the distal centriolar/transition zone genes studied in *Drosophila*, *Cep290* is the only located on the 3rd chromosome. However, since the coding region of this gene spans over 4 kb, and long centrioles should be easy to identify with our visual approach, we anticipate identification of novel alleles of the gene. Also, given the large number of proteins that comprise the transition zones in other organisms, our screen has the potential to uncover novel *Drosophila* transition zone genes through observation of long centrioles.
Together, the studies performed on regulators of centriole elongation and length restriction suggest the mechanisms of elongation may be conserved in *Drosophila* and other eukaryotes. By fluorescently labeling centrioles in *Drosophila* testes, we equip ourselves with the unique ability to detect differences in centriole length using light microscopy. Hence, we expect to find mutations in known and novel centriole elongation genes through observation of abnormally long or short centrioles.
1.5 A Visual Genetic Screen of Centrioles in *Drosophila* Testes Expects to Identify Novel Centriolar Mutations.

In the past, the centrioles in *Drosophila* were systematically studied through reverse genetic, genomic, or molecular approaches (Bettencourt-Dias et al., 2005; Basto et al., 2006; Rodrigues-Martins et al., 2007; Dobbelaere et al., 2008). Centriolar mutants in this model organism have also been discovered during forward genetic screens aiming to find mechanosensation and meiotic defect mutants (Bonaccorsi et al., 1998; Baker et al., 2004; Blanchon et al., 2009). However, performing these types of screens to specifically identify centriolar mutations would be insufficient since they only visualize potential downstream effects of centriolar mutations. Therefore, we chose to devise a novel approach to screening that visualizes the centrioles of developing sperm in *Drosophila* testes. This EMS-based screen is expected to generate milder mutations that will disrupt centriole morphology, but not necessarily have serious functional consequences. We therefore anticipate that novel centriole morphology phenotypes caused by centriolar mutations will be identified.
Chapter Two

Materials and Methods

2.1 Fly Stocks

All flies were cultured on standard media (Roote and Prokop, 2013). Flies used to generate mutants in the screen were all cultured at 25°C with the exception of the 24 hour cross 2 incubation periods at 22°C. Unhealthy mutant lines tested for behavioral defects and/or sent for genomic sequencing were cultured at either 22°C or 18°C to prolong development and ensure survival of homozygote adults for analysis/collection.

The FRT82 and HS-Hid-Dr stocks were ordered from the Bloomington Stock Center (#2035 and #7758, respectively). The construct for Ana1-GFP was made in the Avidor-Reiss lab and delivered to BestGene to generate transgenic lines containing Ana1-GFP under endogenous control. Avidor-Reiss lab stock 2283 was used for characterization of the timing of Ana1-reduction and as the control for centriole length mutants. Stocks used for complementation tests were also from the Avidor-Reiss lab and include stock #1624 (poc1^{+} mutant), #1629 (bld10 mutant), and #1838 (cep290^{MecH}).
2.2 EMS treatment

Males 24 – 48 hours old were collected and starved for approximately 18 hours in a chamber with damp filter paper. After approximately 18 hours, the males were moved to a chamber with filter paper dampened with a 25 mM EMS solution composed of 1% sucrose and food coloring for 8 – 10 hours. Flies were then scored for green abdomens indicating that the EMS solution was ingested. Flies without green abdomens were disposed of and those with green abdomens were moved to a fresh food vial overnight to recover before being crossed (adapted from (Koundakjian et al., 2004)).

2.3 Virgin Collection

Females used for the screen were collected in a vial every 12 hours. Those with no meconium were separated, labeled as “M-“, and were considered virgin if no larva were produced after 3 – 4 days while sitting at 22°C. Those that had a meconium were separated, labeled as “M+”, and could be used right away. Females used for complementation analysis of leaky centriole and behavioral mutants were collected every 3 hours to ensure they were all virgin.

2.4 Screen Crossing Scheme

For cross 1 (C1), EMS-treated males were crossed en masse to females containing the centriolar transgene Ana1-GFP coupled to its endogenous promoter at an exogenous locus on the 2\textsuperscript{nd} chromosome; this transgene was phenotypically marked by the presence of dominant red eyes. These females also contained the apoptotic gene Hid coupled to a heat-inducible promoter on the 3\textsuperscript{rd} chromosome; this was phenotypically marked by the dominant marker mutation Drop (Dr).
Cross 1 (C1) male progeny containing Ana1-GFP over the balancer Cyo on the 2nd chromosome and a mutated FRT over the balancer TM6B on the 3rd chromosome were then collected for use in cross 2 (C2). Cyo and TM6B are phenotypically marked by dominant curly wings and the dominant marker mutation Humeral (Hu), respectively. One male was crossed to 3-4 of the same females used for cross 1 (C1). Heat shock was performed to let only progeny with identical genotypes survive. Cross 2 (C2) progeny were scored for absence of the Hid-associated drop eye (Dr) phenotype to check for successful heat shock. These flies were then transferred to a new vial to mate (cross 3 (C3)), and their progeny were scored for lethality after 10 days (Figure 4).

![Screen Crossing Scheme](image)

**Figure 4:** Screen Crossing Scheme

### 2.5 Lethality Scoring

The phenotypic markers associated with the 3rd chromosomal TM6B balancer were used to score lethality of the EMS-induced mutations. The absence of the dominant Tubby (Tb) and Humeral (Hu) markers, denoted by a (+), indicated flies homozygous for the
mutated 3rd chromosome. Vials with no Tb+ pupa indicated either an embryo- or larva-lethal phenotype. Vials with Tb+ pupa that did not develop enough to determine sex were considered to be pupa-lethal. If vials contained Tb+ pupae developed enough to determine sex, but failed to eclose, they were determined to be pharate adult-lethal. If Tb+ pupa were observed, but no live Hu+ flies could be collected, they were adult-lethal. Vials were considered to have viable mutations if both Tb+ were observed and live Hu+ flies could be collected.

2.6 Heat Shock

Cross 2 vials were allowed to mate for 23-25 hours at 22°C, at which time they were moved to new vials to mate for another 23-25 hours. The empty vials containing embryos were then placed in a 37°C incubator for 1 hour to kill embryos of all undesired progeny. After the second mating period, the same procedure was repeated, except flies were discarded instead of moved to new vials before heat shock. Note that this heat shock must be performed before progeny develop past embryo stage. After the second heat shock, the 2 resulting copies of each cross 2 line were placed in a 25°C incubator to mate for 12 to 14 days. Progeny were then scored for absence of the Drop (Dr) marker mutation to ensure the heat shock was successful. Any lines containing flies with this marker were discarded.

2.7 Dissections and Staining Protocols

All screening and centriole length analysis dissections were performed on any pupae with discernable sex combs as described in (Basiri et al., 2013) with the following changes: the testes were cut into 2 pieces for higher quality staining, and incubated in the stain
(1:1000 µg DAPI or Hoescht) for 10 minutes. Dissections performed to confirm and characterize centriole leak phenotypes were done the same way as screening/centriole length analysis dissections, but were not stained after fixation.

Dissections performed to characterize the timing of Ana1-GFP reduction were performed like mentioned above, but differed after the fixation step. Once fixative was wicked off with Whatman paper, testes were washed with 1X PBS. The wash was immediately wicked off followed by addition of 7 µL of 0.01 % Triton X-100 (Sigma-Aldrich Inc. #93443) in 1X PBS for 10 minutes. Triton was then wicked off followed by PBS wash, immediate removal of wash, then stained with 7 µL of 1 µg/ml DAPI and 70 nM Rhodamine-Phalloidin (Cytoskeleton Inc. #PHDR1) in 1X PBS for 10 minutes. The stain was then removed followed by addition of 7 µL of 1X PBS. Samples were then smashed with a coverslip, sealed with nail polish, and left to sit for 1 hour to allow for degradation of any background staining.

2.8 Microscopy

Screening of the mutant testes, and phenotype confirmation/characterization were done using a Leica SP5 fluorescent microscope with a 100X objective. Mutant behavioral imaging was performed using an Olympus MVX10 microscope with a 1X objective.

2.9 Complementation Tests

Complementation tests were performed against mutants exhibiting similar recessive centriolar phenotypes to previously characterized 3rd chromosomal mutants. Flies heterozygous for the 3rd chromosome with unknown mutations were crossed to flies
heterozygous for the 3rd chromosome containing the known centriolar mutation. Then the pupa of progeny transheterozygous for the unknown and known 3rd chromosomal mutations were dissected and analyzed using the Leica SP5 fluorescent microscope. If the centrioles of progeny displayed the mutant phenotype, the mutations failed to complement one another. Mutations that failed to complement were suggested to be new alleles of the known gene tested. If the centrioles of progeny displayed a WT phenotype, the mutations succeeded to complement one another. Mutations that succeeded to complement were suggested to represent mutations in a new gene causing the previously characterized centriolar phenotype.

2.10 Motility Assays

Sperm motility was determined using males 24 – 48 hours old. Their testes were dissected and placed in 3µL of PBS and crushed with a glass coverslip. Slides were analyzed using a Leica SP5 microscope and phase contrast light with a 100x objective for spermatozoa with motile tails.

2.11 Behavioral Assays

Mutants that exhibited either long or leaky centrioles were all assayed for behavioral defects. At least 20 Tb+ pupae from each mutant were plated on a plastic dish lined with a piece of damp Whatman paper. If the paper glistened in the light, it was considered to be too wet. Paper that is too wet can cause flies to have difficulties in exiting their pupae leading to injury that could falsely be interpreted as a behavioral defect. Once pupae are plated, a lid was placed on the dish to prevent newborn flies from escaping. Dishes are periodically checked over the next 2 days for flies at least two hours old. Those flies are
assayed for any behavioral defects including: inability to stand or walk, slow walking with splayed legs, slow clumsy walking, and inability to climb the walls of the dish. Once assayed flies are discarded, the dish is rewet to prevent Whatman paper from drying out. This is important once again to prevent injury of the flies as they exit their pupae.

2.12 Centriole Length Quantification and Phenotype Dominance Analysis

Testes from control and mutant testes were dissected and stained as described above. Both a control containing one and two copies of the Ana1-GFP transgene were quantified to ensure measured centriole length didn’t vary when one or two copies of Ana1-GFP were present. Once prepared, samples were visually scanned for leaf-stage spermatid bundles by visualizing DAPI. Then, Ana1-GFP was visualized in the same field and immediately imaged using a Leica DFC450 C camera. Centrioles in focus and parallel to the apparent plane of view were manually measured using the Leica Application Suite Version 4.4.0 software program. Measurements were performed by aligning the inner edge of the bracket with the apparent edge of Ana1-GFP labeling on either side of the centriole. At least 10 centrioles were measured for each mutant and control. N = 3 for control, heterozygous mutant, and/or homozygous mutant dissections performed for each quantification trial.

Two-tailed unpaired t-tests were performed with Microsoft Excel to determine whether mutant centriole length was significantly different from control. While no significant difference was detected between controls with one copy of Ana1-GFP and those with two copies, only the data from controls with two copies of Ana1-GFP was used for the t-tests. This is because only mutants containing two copies of Ana1-GFP were used for tests to ensure robust labeling for imaging purposes.
The first T-test included a comparison of control to the homozygote mutant. If the lengths were significantly different, a t-test comparing control to heterozygote mutant was performed. If that test yielded no significant difference, the mutant was determined to be recessive. If that test did yield a significant difference, an additional t-test was performed to compare heterozygote mutant to homozygote mutant. If there was no significant difference, the mutant was determined to be dominant. If there was a significant difference, the mutant was determined to be semi-dominant.

2.13 Qualitative Confirmation of Centriole Leak Phenotype and Determination of Phenotype Severity and Dominance.

Testes from 1 control, 3 heterozygote mutants, and 3 homozygote mutants were dissected as described above. The control was examined closely for any leak of Ana1-GFP followed by close examination of all spermatid bundles in all 3 homozygote mutant samples. The same process was then repeated for the 3 heterozygote samples. If no leak was observed in any heterozygote samples and a leak was observed in all homozygote samples, the mutant was determined to exhibit a recessive leak and vice versa.

To assess phenotype severity, a qualitative scale was created: severe > moderate > mild. In addition, to assess phenotype expressivity, a rough quantitative scale was created: (< 50% of centrioles), (> 50% of centrioles), (> 75% of centrioles), and (100% of centrioles). These varying degrees of severity and expressivity were characterized during phenotype confirmation and dominance determination for the leak mutants. Since all leak phenotypes were recessive, 3 homozygote samples were analyzed for phenotype severity and expressivity for each mutant.
2.14 Collection of Mutant Flies For Genomic Isolation

Fully coordinated flies homozygous for the mutant 3rd chromosome were collected from culture vials ranging from 10-20 days old to increase ratio of young flies collected. Since mutants with behavioral defects often get stuck in food and die, pupae from these lines were plated as previously described in section 2.11. Only very late pupae were collected simultaneously en masse to ensure flies in one dish eclosed at the same time. Mutants with and without behavioral defects were transferred to empty culture vials, starved for 2.5 hr., then transferred to a new empty vial for another 2.5 hr. of starvation. The starvation step rids flies of yeast from their food, which prevents DNA contamination. Flies from different mutant lines were then inspected for cleanliness and transferred to their own 1 mL microcentrifuge tube followed by immediate storage at -80°C. Collection of ~25 flies/mutant line in one homogenization tube is recommended since it helps minimize contamination during genomic isolation. This is because the isolation protocol calls for 25 flies in each of 2 homogenization tubes to get desired yield for each mutant. If flies are collected in numerous tubes before isolation, they must eventually be transferred to only two tubes, increasing the chance of contamination with every additional transfer. Lastly, leave flies at -80°C until they are ready to be homogenized.

2.15 Isolation of Genomic DNA from Control and Mutant Lines.

The Genomic-tip 20/G (QIAGEN Inc. 10223) and Genomic DNA Buffer Set (QIAGEN Inc. 19060) were used and contained all required reagents (excluding the Proteinase K and DNase-free RNase A) to complete genomic isolations. Genomic DNA was isolated from 25 fly lines. The control line contained the isogenized FRT 3rd chromosome from which the 24 EMS-induced mutant lines were derived.
50 flies from each line (25 in each of two 1 mL homogenization tubes) were removed from -80°C freezer and put on ice followed by addition of 100 µL of G2 buffer containing 20 mM EDTA, 100 mM NaCl, 1% Triton® X-100, 500 mM Guanidine-HCl, 10 mM Tris, pH 7.9 to each tube. Flies were then homogenized with a motorized pestle followed by addition of 900 µL of G2 buffer to each tube. Each tube was then supplemented with 20 µg/mL of DNase-free RNase A and incubated at 37°C for 30 min. Then 0.8 mg/mL of Proteinase K was added to each tube, mixed by inverting, and incubated at 50°C for 2 hr. with gentle agitation (thermomixer @ 400rpm is recommended, but periodically mixing by inverting was also effective). After the incubation, tubes were centrifuged for 20 min. at 20,000xg to pellet insoluble debris at RT. During the last 5 min. of centrifuging, 1 Genomic-tip/mutant was equilibrated with 1 mL of QBT buffer. Clarified lysate from the two tubes for each mutant was then transferred to its respective column (Use one 1 mL tip/tube and be careful not to take up supernatant too quickly; 600 µL supernatant/mutant yielded 6-10 ug DNA). Columns were then washed 2x with 2 mL of QC buffer followed by elution with 0.8 mL QF buffer. Next, 0.7 volumes of RT isopropanol were added to each tube and mixed by inverting to precipitate DNA (precipitate most often was not visible). Samples were then centrifuged for 20 min at 20,000xg to pellet DNA at RT followed by three 15 min. washes with 70% ethanol at RT. The last wash was then removed, and each sample was air-dried and resuspended in 35 µL of sterile water. Samples were stored at 4°C overnight to dissolve DNA, then were nanodropped for concentration. All 25 samples were sent to the University of Baylor College of Medicine for genomic sequencing and mutational analysis.
Chapter Three

A Visual Screen For Centriolar Mutants

3.1 Screen Statistics

At the screen’s conclusion, we attempted to generate 7466 mutant lines, of which 2361 survived. The lines that survived were scored to determine lethality at different developmental stages. Among the 1124 lines that exhibited lethality, 887 were embryo- or larva-lethal, 38 were pupa-lethal, and 199 were pharate-adult- or adult-lethal. All lethal lines were discarded except the pharate-adult and adult-lethal lines since they produced pupae that could be screened. In total, the EMS concentration used in the screen resulted in 1124 lethal lines out of the 2361 generated, a 48 % lethality rate. Among the 2361 lines that survived, a total of 925 lines were discarded giving a total of 1436 lines that were screened (Table 1, Figure 5).

The 3\textsuperscript{rd} chromosome was targeted in this screen due to the tools we had in the lab, but also because it carries a higher % of essential genes than the others. The 3\textsuperscript{rd} chromosome of Drosophila contains ~6000 genes with 4300 estimated to be essential (Koundakjian et al., 2004). We calculated a rate of 2.86 mutations induced per chromosome by assuming
a Poisson distribution (Pollock and Larkin, 2004), which gave a total of ~4107 mutations screened. A Poisson distribution was used because it can compare the lethality rate to the number of predicted essential genes on the 3rd chromosome to approximate how many mutations per chromosome the mutagen induced (Table 1, Figure 5).

Table 1: Screen Outcome Statistics

<table>
<thead>
<tr>
<th>Lines Attempted</th>
<th>7466</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Lines Generated</td>
<td>2361</td>
</tr>
<tr>
<td>Early Lethal</td>
<td>887</td>
</tr>
<tr>
<td>Pupa Lethal</td>
<td>38</td>
</tr>
<tr>
<td>Pharate Adult/Adult Lethal</td>
<td>199</td>
</tr>
<tr>
<td>Total Lethal</td>
<td>1124</td>
</tr>
<tr>
<td>Viable Lines</td>
<td>1237</td>
</tr>
<tr>
<td>Lines Screened</td>
<td>1436</td>
</tr>
<tr>
<td>Estimated Mutations/Chromosome</td>
<td>2.86 (48% lethality)</td>
</tr>
<tr>
<td>Total mutations screened</td>
<td>2.86 x 1436 = 4107</td>
</tr>
</tbody>
</table>

Table 1: Statistics generated at the conclusion of the screen including number of lines generated, number and type of lethal mutations scored, number of lines screened, and estimated number of mutations screened based on Poisson Distribution.
3.2 Abnormal Testes Morphology Mutants

During the screen, a total of 54 mutants were identified that exhibit some degree of a recessive testes deformity. This phenotype is characterized by varying degrees of “bulb-tipped” testes. Some exhibited a slight bulge at the testes tip, while the most severe mutants exhibited “ball-shaped” testes that were completely detached from the seminal vesicle (Figure 6). Another example of testes deformity was characterized by small, underdeveloped testes (data not shown).
3.3 Nuclear Defect Mutants

Mutants from the screen with defects in nuclear morphology exhibit various phenotypes. In total, 16 mutants displayed some degree of recessive nuclear abnormality. Among these, 10 displayed nuclear reshaping arrests, and 8 exhibited curved mature nuclei. The most common nuclear reshaping defect was characterized by a reshaping arrest between the leaf- and canoe-shaped nuclear stages. This phenotype was partially expressive in that ~ half of the nuclei within each spermatid bundle were affected. The arrested nuclei can be described as amorphous in appearance with inconsistent degrees of elongation. (Figure 7). Another category of nuclear defect is characterized by failure of any nuclei to begin reshaping, referred to in the mouse and human system as Globozoospermia. The remaining 8 mutants displayed curved nuclei at maturity (data not shown).
3.4 Developmental Mutants

A subset of recessive phenotypes, comprised by 21 mutants, was identified that affects proper localization of cells at different developmental stages of spermatogenesis. Certain developmental stages are typically confined to specific areas of the testes. For example, stem cells and spermatocytes are always found at the tip of the testes, while meiotic cells are found along the outer edge of the testes just before the testes begin to spiral (Figure 8). However, spermatocytes and meiotic cells are found where nearly mature cells are normally found in a number of developmental mutants (Figure 8). It is possible that the abnormal increase in the number of spermatocytes in these mutants could be the reason they are so abnormally localized. Since meiotic defect mutants identified in *Drosophila* can slow meiosis down, it’s possible that a slower or defective meiosis could be leading to a buildup of pre-meiotic cells (spermatocytes) in our mutants (Courtot et al., 1992). We have also identified a mutant that appears to have too many stem cells in its hub (not shown).

Figure 8 - Control testes (left) exhibit proper localization of spermatocytes (dotted line). Testes from *UT850* (right) contain an atypically large number of spermatocytes with abnormal localization to the base of the testes usually reserved for nearly mature spermatid bundles (dotted line). Note: SV = seminal vesicle.

Figure 8: Developmental Defect Mutants
3.5 Short Centriole Mutants

A total of 2 mutants were identified in the screen that exhibit short centrioles (Figure 9). As previously mentioned, short centrioles can be caused by mutations in the centriolar genes \textit{bld10} and \textit{poc1} (Mottier-Pavie and Megraw, 2009). One allele of \textit{bld10} and 2 alleles of \textit{poc1} are known, all of which are viable and fully coordinated. The \textit{bld10} allele exhibits both recessive short centrioles and sperm immotility, while both \textit{poc1} alleles exhibit semi-dominant short centrioles and lack Ana1-labeling of the PCL (Blanchon et al. 2009; Mottier-Pavie and Megraw 2009). The two short centriole mutants identified in the screen exhibit recessive short centrioles, but differ in length (Figure 9, Table 2). Both mutants displayed Ana1-labeling of the PCL, and complemented the \textit{poc1} length phenotype, suggesting they are not \textit{poc1} alleles (data not shown). Both mutants exhibit fully coordinated behavior as homozygotes.

Mutant \textit{UT737} failed to complement both the \textit{bld10} short centriole (1.85±0.03 compared to 2.51±0.14, N≥20) and sperm immotility phenotypes, suggesting that \textit{UT737} is either a new allele of \textit{bld10} or it genetically interacts with \textit{bld10}. Over the course of the screen, \textit{UT737} acquired a recessive pupa-lethal phenotype, however flies transheterozygous for \textit{UT737} and \textit{bld10} are viable. The acquired lethality of our mutant may be due to a 3\textsuperscript{rd} chromosomal mutation in a gene other than \textit{bld10} that is recessive pupa-lethal.

Mutant \textit{UT5580} complemented the short centriole phenotypes of both \textit{bld10} and \textit{poc1}, but it failed to complement the sperm immotility phenotype of \textit{bld10}. This suggests that \textit{UT5580} may also be a new allele of \textit{bld10}. 

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3.6 Long Centriole Mutants

During the screen, we were able to identify a total of 19 mutants with long spermatid centrioles. No lines were recessive, 2 lines were semi-dominant, and 17 lines were dominant (Table 2).

Long centrioles can be caused by mutations in the transition zone gene cep290 (Basiri et al. 2014), and the distal centriolar genes cp110 (Franz et al. 2013), and klp10a (Delgehyr et al. 2012). Since cep290 is the only gene of those 3 located on the 3rd chromosome in Drosophila, it means all of our long centriole mutants are candidate cep290 mutants. Hence, we decided to characterize the dominance of the long centriole phenotype of cep290\textsuperscript{MecH}, which is the only allele studied in Drosophila (Basiri et al. 2014). Like two of the mutants identified in the screen, cep290\textsuperscript{MecH} exhibits semi-dominant long centrioles. Cep290\textsuperscript{MecH} also displays a recessive unc-type uncoordination characteristic of many centriolar mutants. Interestingly, both of our semi-dominant long centriole mutants exhibit a recessive mild uncoordination characterized by slow walking with splayed legs (Table 2). However, these 2 semi-dominant long centriole mutants complemented the long centriole phenotype and the uncoordination phenotype of cep290\textsuperscript{MecH}, suggesting that our semi-dominant long centriole mutants may contain mutations in genes functionally related to cep290.

The remaining 17 long centriole mutants identified exhibit dominant long centrioles, however 16 of them display at least 1 recessive phenotypic characteristic of a centriolar mutant. Seven of the mutants display the recessive mild uncoordination described earlier, and 14 of the mutants display a novel recessive “leak” of Ana1-GFP into the ciliary axoneme (detailed further in section 3.7) (Table 2, Figure 9).
The sheer volume of mutants identified with abnormally long centrioles demonstrates the ease with which these phenotypes can be observed in Drosophila testes. Whereas other systems must rely on electron microscopy quantification to identify elongation defects, our system allows for simple visual identification of these phenotypes. These differences in centriole length can then be confirmed with much more efficient and extensive quantification than electron microscopy would permit. Therefore, we believe this system could be used in future screens to expedite the study of centriole elongation mechanisms.
Figure 9 - (A-C) All images represent the mean centriole length for each mutant/control from leaf-stage spermatids (het=heterozygote; hom=homozygote; GC=giant centriole). All graphical data indicate mean, SD of centriole length (dark gray=het; light gray=hom). Refer to Methods for detailed statistics and Table 4 for detailed length quantification. (D) Shown here are representative examples of spermatids (top) and spermatocytes (bottom) from mutants that exhibit either a mild (left), moderate (middle), or severe (right) leak of Ana1-GFP. *** in the graphs indicate a significant difference in centriole length between mutants and control.

Figure 9: Centriole Length and Leak Mutants
Table 2: Centriole Length Mutants

<table>
<thead>
<tr>
<th>Line #</th>
<th>Centriole Length (n≥10 centrioles) (µm)</th>
<th>Ana1-GFP Leak</th>
<th>Behavior (n=30 flies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>2.59±0.12 (Het) 2.51±0.14 (Hom)</td>
<td>No</td>
<td>Coordinated</td>
</tr>
<tr>
<td>MecH</td>
<td>2.99±0.04 (Het)(p&lt;0.0001) 3.84±0.05 (Hom)(p&lt;0.0001)</td>
<td>No</td>
<td>unc-type</td>
</tr>
<tr>
<td></td>
<td><strong>Recessive Short Centrioles (2 lines)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>737</td>
<td>2.61±0.06 (Het)(p&lt;0.0001) 1.53±0.03 (Hom)(p&lt;0.0001)</td>
<td>No</td>
<td>Coordinated</td>
</tr>
<tr>
<td>5580</td>
<td>2.56±0.03 (Het)(p&lt;0.0001) 1.96±0.08 (Hom)(p&lt;0.0001)</td>
<td>No</td>
<td>Coordinated</td>
</tr>
<tr>
<td></td>
<td><strong>Semi-dominant Long Centrioles (2 lines)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3152</td>
<td>2.98±0.09 (Het)(p&lt;0.0001) 3.39±0.03 (Hom)(p&lt;0.0001)</td>
<td>No</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>6599</td>
<td>2.89±0.03 (Het)(p&lt;0.0001) 3.08±0.04 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, all stages, severe, 100% of centrioles)</td>
<td>Splayed legs</td>
</tr>
<tr>
<td></td>
<td><strong>Dominant Long Centrioles (17 lines)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2207</td>
<td>2.81±0.14 (Het)(p&lt;0.0001) 2.98±0.20 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, severe, 100% of centrioles)</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>2211</td>
<td>2.82±0.15 (Het)(p&lt;0.0001) Nuclear Morph* (Hom)</td>
<td>Yes; (recessive, Nuclear Morph*, severe, 100% of centrioles)</td>
<td>Coordinated</td>
</tr>
<tr>
<td>3844</td>
<td>2.81±0.12 (Het)(p&lt;0.0001) 2.74±0.14 (Hom)(p&lt;0.0001)</td>
<td>No</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>5370</td>
<td>2.84±0.09 (Het)(p&lt;0.0001) 2.78±0.1 (Hom)(p&lt;0.0001)</td>
<td>Yes; (Semi-dom, all stages, severe, Het: 50%; Hom: &gt;90% of centrioles)</td>
<td>Coordinated</td>
</tr>
<tr>
<td>5964</td>
<td>2.84±0.12 (Het)(p&lt;0.0001) 2.79±0.11 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, all stages, severe, 100% of centrioles; pp)</td>
<td>Coordinated</td>
</tr>
<tr>
<td>4229</td>
<td>2.84±0.15 (Het)(p&lt;0.0001) 2.82±0.14 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, all stages, mild, &lt;50% of centrioles)</td>
<td>Coordinated</td>
</tr>
<tr>
<td>6040</td>
<td>2.85±0.13 (Het)(p&lt;0.0001) 2.85±0.09 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, all stages, severe, 100% of centrioles)</td>
<td>Coordinated</td>
</tr>
<tr>
<td>5902</td>
<td>2.82±0.03 (Het)(p&lt;0.0001) 2.90±0.02 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, all stages, severe, 100% of centrioles)</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>6849</td>
<td>2.84±0.13 (Het)(p&lt;0.0001) 2.94±0.09 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, all stages, severe, &gt;90% of centrioles)</td>
<td>Coordinated</td>
</tr>
<tr>
<td>4866</td>
<td>2.86±0.03 (Het)(p&lt;0.0001) 2.91±0.17 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, all stages, extreme, 100% of centrioles)</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>7138</td>
<td>2.87±0.03 (Het)(p&lt;0.0001) 3.0±0.14 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, severe in spermatocyte, 100% of centrioles;)</td>
<td>Coordinated</td>
</tr>
<tr>
<td>6035</td>
<td>2.89±0.1 (Het)(p&lt;0.0001) 2.81±0.11 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, all stages, severe, &gt; 75% of centrioles)</td>
<td>Coordinated</td>
</tr>
<tr>
<td>7133</td>
<td>2.91±0.05 (Het)(p&lt;0.0001) 2.86±0.13 (Hom)(p&lt;0.0001)</td>
<td>No</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>6534</td>
<td>2.86±0.09 (Het)(p&lt;0.0001) 2.92±0.06 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, all stages, mild, &gt;50% of centrioles)</td>
<td>Coordinated</td>
</tr>
<tr>
<td>5643</td>
<td>2.92±0.08 (Het)(p&lt;0.0001) 2.83±0.08 (Hom)(p&lt;0.0001)</td>
<td>No</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>3128</td>
<td>2.94±0.12 (Het)(p&lt;0.0001) 2.79±0.09 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, all stages, severe, &gt;80% of centrioles)</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>5972</td>
<td>3.11±0.03 (Het)(p&lt;0.0001) 2.82±0.09 (Hom)(p&lt;0.0001)</td>
<td>Yes; (recessive, all stages, moderate, in &gt;80% of centrioles)</td>
<td>Coordinated</td>
</tr>
</tbody>
</table>
Table 2 – Centriole length mutants with/without additional centriolar phenotypes displayed including length quantification, leak phenotype description, and behavioral phenotype description. *Mutants that do not include quantification or leak staging data had nuclear shaping defects preventing these analyses. Leak severity scale is: mild < moderate < severe. NOTE: All behavioral phenotypes were recessive and bolded mutant lines were sent for genome sequencing analysis.

Table 3: Leaky Centriole Mutants

<table>
<thead>
<tr>
<th>Mutant Line #</th>
<th>Spermatocyte Leak</th>
<th>Spermatid Leak</th>
<th>Phenotypic Severity</th>
<th>Behavior (n≥20 flies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6797</td>
<td>Yes</td>
<td>Yes</td>
<td>Severe; all centrioles</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>6959</td>
<td>Yes</td>
<td>Yes</td>
<td>Severe; all centrioles</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>7294</td>
<td>Yes</td>
<td>Yes</td>
<td>Severe; &gt; 90% of centrioles</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>6561</td>
<td>No</td>
<td>Yes</td>
<td>Severe; &gt;90% of centrioles</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>3642</td>
<td>Yes</td>
<td>Yes</td>
<td>Severe; &gt;75% of centrioles</td>
<td>Coordinated</td>
</tr>
<tr>
<td>6578</td>
<td>Yes</td>
<td>Yes</td>
<td>Severe; &gt;75% of centrioles</td>
<td>Coordinated</td>
</tr>
<tr>
<td>2306</td>
<td>Yes</td>
<td>No</td>
<td>Moderate; all centrioles</td>
<td>Splayed legs</td>
</tr>
<tr>
<td>6108</td>
<td>Yes</td>
<td>Yes</td>
<td>Moderate; all centrioles</td>
<td>Coordinated</td>
</tr>
<tr>
<td>3477</td>
<td>Yes</td>
<td>Yes</td>
<td>Moderate; &gt;75% of centrioles</td>
<td>Coordinated</td>
</tr>
<tr>
<td>7085</td>
<td>Yes</td>
<td>Yes</td>
<td>Moderate; &gt;75% of centrioles</td>
<td>Coordinated</td>
</tr>
<tr>
<td>5412</td>
<td>No</td>
<td>Yes</td>
<td>Mild; all centrioles</td>
<td>Coordinated</td>
</tr>
<tr>
<td>6523</td>
<td>Yes</td>
<td>No</td>
<td>Mild; all centrioles</td>
<td>Coordinated</td>
</tr>
<tr>
<td>6553</td>
<td>Yes</td>
<td>Yes</td>
<td>Mild; all centrioles</td>
<td>Coordinated</td>
</tr>
<tr>
<td>7140</td>
<td>Yes</td>
<td>Yes</td>
<td>Mild; all centrioles</td>
<td>Coordinated</td>
</tr>
<tr>
<td>7142</td>
<td>Yes</td>
<td>Yes</td>
<td>Mild; &gt;75% of centrioles</td>
<td>Coordinated</td>
</tr>
<tr>
<td>7100</td>
<td>Yes</td>
<td>Yes</td>
<td>Mild; &gt;75% of centrioles</td>
<td>Coordinated</td>
</tr>
<tr>
<td>5966</td>
<td>Yes</td>
<td>Yes</td>
<td>Mild; &gt;50% of centrioles</td>
<td>Coordinated</td>
</tr>
</tbody>
</table>

Table 3 – The centriole length mutants w/ or w/out behavioral phenotypes are displayed including leak specificity to different developmental stages, leak dominance, leak severity/expressivity, and description of behavioral phenotype. NOTE: Mutant lines in bold were send for genome sequencing analysis.
3.7 Leaky Centriole Mutants

During the screen, we identified a surprisingly large number of mutants that exhibit a novel centriole-labeling phenotype. This phenotype, termed the “Ana1-GFP leak”, is characterized by a distinct trail of Ana1-GFP that leaks from the apparent distal end of the centriole along the ciliary axoneme (Figure 9). Since these mutants displayed different phenotypic severities and degrees of expressivity, they were characterized as, mild, moderate, or severe with an associated approximate % of spermatids affected (Figure 9, Table 3). Interestingly, this phenotype was most often more severe in spermatids than spermatocytes. This could have something to do with the different types of cilia formed between the two cell types. Spermatocytes form a non-motile primary cilia compartmentalized by a transition zone. On the other hand, spermatids form motile cilia that are not compartmentalized by a transition zone thereby exposing them to the cytoplasm (Basiri et. al. 2014).

Among the 34 leak mutants identified in the screen, 31 displayed recessive leaks and 3 displayed semi-dominant leaks. Further, 15 of the leak mutants also exhibit long centrioles, while the remaining 19 have normal centriole length (Figure 9, Table 2, Table 3). Importantly, 7 of the leak mutants without long centrioles also display the previously described mild uncoordination phenotype (Figure 10). This suggests that the mutated gene(s) in our uncoordinated leak mutants may be functionally related to those mutated in our uncoordinated long centriole mutants.
3.8 Mild Uncoordination Phenotype

As previously mentioned, the semi-dominant long centriole mutants were originally assayed for behavioral defects to determine how phenotypically similar they were to the \textit{cep290}^{mecH} mutant. These mutants did not display the classic \textit{unc}-type uncoordination, shared by many centriolar and ciliary mutants, that is characterized the inability to stand up with wings erect (Figure 11b). However, they did display a distinct and novel recessive uncoordination characterized by slow walking with splayed legs, and the occasional inability to inflate their wings after eclosion (Figure 11c). To our surprise, 14 additional mutants that exhibit long and/or leaky centrioles also display this distinct recessive behavioral phenotype (Figure 11, Table 2, Table 3).
Figure 11: Behavioral Phenotype Examples

Figure 11 – (A) Shown is a control fly able to stand upright and move around swiftly. (B) The *cep290* fly exhibits *unc*-type uncoordination with wings erect and inability to stand. (C) Shown are examples of mutants that share the mild uncoordination phenotype characterized by spread out legs, slow and clumsy walking, and the occasional inability to inflate their wings as in *UT4866*. (A-C) All images were captured looking down upon flies in covered dishes either (A,C) standing upright or (B) laying on their side.

Figure 11: Behavioral Phenotype Examples
Chapter Four

Complementation and Genomic Sequencing Analysis

4.1 Genetic Complementation Analysis

Most of the Ana1-leak and behavioral phenotypes we identified were determined to be recessive, allowing us to test them for genetic complementation. All combinations of the 19 recessive Ana1-leak mutants were tested as well as all combinations of the 15 mutants with recessive behavioral phenotypes. Also, many combinations of control crosses were made to test for specificity of the experimental allele interactions. Examples of these controls include: mutant with no recessive phenotype crossed to mutant with recessive centriolar phenotype; crosses in which neither mutant exhibits recessive centriolar phenotype; mutants with different recessive centriolar phenotypes crossed to one another. The goal of these experiments was to place mutants into different complementation groups, however we were ultimately unable to do so (data not shown). This outcome was unexpected considering the large number of mutants we identified displaying the same recessive phenotypes. Further, it is very unlikely that none of the mutants would fall into complementation groups if we operate under the assumption that the genetics underlying
their phenotypes are Mendelian. Therefore, we must consider the possibility that the genetics of these phenotypes are non-Mendelian, in which case interpretation of complementation data becomes more complicated. Instances of intragenic complementation and non-allelic non-complementation could partly explain these complex interactions, but genetic experimentation alone would be insufficient to fully interpret our complementation results (Hawley and Gilliland, 2006).

4.2 Genomic Sequencing Analysis

Although the results of the leak and behavioral complementation tests were inconclusive, we took advantage of a collaborative opportunity to have genomic sequencing performed on our centriolar mutants. Altogether, genomic samples from 24 of our most interesting centriolar mutants and the control line from which they were derived were sent out for genomic sequencing (Table 2, 3). Mutants with recessive centriolar phenotypes took priority over others because the probability of identifying their responsible mutations is higher. This is because we expect that the same base on each chromosome copy to contribute to the recessive phenotypes. On the other hand, dominant mutations will be much more difficult due to the abundance of non-related point mutations contained in our mutants. We have received the results from the control line and one mutant that exhibits dominant long centrioles, but no leak or behavioral defect. The results of this comparison identified 8 protein-affecting mutant-specific homozygote variants (data not shown). We can predict from this low number that identification of responsible genes for the rest of the mutants will be relatively simple. However, none of the 8 protein-affecting mutations were in genes with characterized or implicated roles in centrosome biology.
Chapter Five

Characterization of Ana1 Reduction Timing

5.1 Spermatid Individualization and Nuclear Morphology can be used Together to Precisely Characterize the Timing of Ana1 Reduction.

At the beginning of the screen, we developed an assay to help identify subtle defects in the timing of the centrosome reduction of Ana1-GFP. This assay was created to increase the chances of finding a centrosome reduction mutant, which was the initial, but ultimately unsuccessful goal of our screen (more on initial screen objective in Chapter 6).

Throughout spermiogenesis, the spermatid nuclei change shape, which can be used as a marker for their developmental stage (Fabian and Brill, 2012). In addition, the previously mentioned process of individualization occurs at the end of spermatid development around the time that Ana1-GFP is reduced. Since Ana1 reduction occurs towards the end of spermatid development like individualization, we decided to use the timing of investment cone formation and spermatid nuclear shape to precisely characterize the timing of Ana1 reduction.
Four stages of Ana1 reduction were characterized by costaining control testes with DAPI to mark spermatid nuclei and Rhodamine-Phalloidin to mark the individualization complex. The first stage was characterized by accumulation of F-actin at the base of the leaf-stage spermatid nuclei. At this point, Ana1 reduction has not begun (Figure 12a). Then, once the individualization complex (IC) is formed by the late-elongated nuclear stage, Ana1-GFP intensity begins to dwindle from the GC, but not the PCL (Figure 12b). When the nuclei reach needle-stage, and before the IC has moved away from the nuclei, Ana1-GFP becomes completely undetectable in the GC, but not in the PCL (Figure 12c). Last, Ana1-GFP becomes undetectable in the PCL when the IC has begun to migrate (Figure 12d). From these data, we can conclude that Ana1-GFP is completely reduced from the GC before the PCL. These results will be used in the future as a template to test centrosome reduction mutants we identify in proteins other than Ana1 for defects in Ana1-reduction.

Figure 12 - (A-D) Images display DAPI (blue) and Rhodamine-Phalloidin (red) staining of spermatids from control flies that express Ana1-GFP (green). (E) Quantification data displaying specificity of nuclear shape and degree of IC assembly/movement to degree of Ana1 reduction from the GC and PCL (n>10 spermatid bundles imaged for each of the 4 stages).

**Abbreviations:** GC = giant centriole; PCL = proximal centriole-like; IC = individualization complex; N = nucleus.

Figure 12: Characterization of Ana1 Reduction Timing
Chapter Six

Discussion and Future Directions

The initial objective of our EMS-based screen was to discover genes with roles in a process for which nothing about its mechanisms is known. This well-conserved male germline-specific process, termed centrosome reduction, involves stepwise removal of the majority of a sperm’s centrosomal components over the course of its development (Avidor-Reiss et al., 2015). Our lab already had the tools to track the disappearance of a number of centrosomal proteins removed during this process by using fluorescently-tagged expression at endogenous levels. Since emission of the centriolar wall protein, Ana1-GFP is completely absent from mature sperm, we hypothesized that screen mutants in the reduction of Ana1-GFP would reveal themselves in the form of fluorescence emission in mature sperm.

Unfortunately, this approach to study centrosome reduction failed to identify any mutants. We believe it may have been unsuccessful for a couple of reasons. First of all, only 1 centrosomal marker was used to probe for mutants. The probability of finding mutants could have been increased through incorporation of more centrosomal proteins
coupled to tags with different colors. It is also possible that Ana1 reduction is less sensitive to mutation than reduction of other centrosomal proteins. Indeed, our lab has identified a single domain of the Asterless (Asl) protein that is essential for Asterless to be reduced by Plk4 (Khire et al., 2015). Because of our recent finding that the reduction of Asterless is controlled by a kinase called Plk4, we plan to propose an RNAi-based centrosome reduction screen targeting all known kinases and phosphatases in the Drosophila genome.

At its conclusion, our novel visual screen spanning 2 years screened ~4107 mutations, which covered ~2/3 of the genes located on the 3rd chromosome (Table 1). Among the 1436 lines screened, a total of 44 centriolar mutants were identified and characterized (Table 4). This novel visual screen afforded a number of advantages contributing to identification of such a high number of putative centriolar mutants. The long centrioles of the testes in Drosophila provided us with the unique opportunity to observe subtle defects in centriole elongation without the need for electron microscopy. This resulted in identification of 27 mutants with defective centriole length, making ours the largest collection of this type of mutant to date. Another reason this screen was so effective at finding centriolar mutants was the fluorescent marker used to label the centrioles. Because Ana1-GFP is such a robust centriolar marker, we were able to identify the novel Ana1-GFP leak phenotype in a total of 34 mutants. Interestingly, we also found that many of the long and leaky centriole phenotypes were associated with a novel behavioral phenotype. This further suggested the centriolar nature of these mutants considering centriolar mutations are often associated with behavioral defects. As previously discussed, cep290MecH is the only transition zone mutant studied in Drosophila, which happens to cause long centrioles and unc-type uncoordination. Considering how
unstudied the transition zone is in *Drosophila*, we can suggest that at least some of the Ana1-GFP leak and/or long centriolar mutants have underlying transition zone mutations.

Table 4: Centriolar Phenotype Categories

<table>
<thead>
<tr>
<th>Group</th>
<th>Phenotype (44)</th>
<th>Complementation Groups and Lines In Each Category</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Centriole Length</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short GC (2)</td>
<td>5580</td>
<td>Bld10 complementation group: 737</td>
</tr>
<tr>
<td>Long GC (4)</td>
<td>3152, 3844, 5643, 7133</td>
<td></td>
</tr>
<tr>
<td>Ana1-GFP Leak (19)</td>
<td>2306, 3477, 3642, 3894,</td>
<td>5412, 5966, 6068, 6108, 6523, 6553, 6561, 6578, 6797, 6959, 7085, 7100, 7140, 7142, 7294.</td>
</tr>
<tr>
<td>Long GC and Ana1-GFP</td>
<td>2207, 2211, 3128, 4229,</td>
<td>4866, 5370, 5902, 5964, 5972, 6035, 6040, 6534, 6599, 6849, 7138.</td>
</tr>
<tr>
<td><strong>Spermatocyte Specific</strong></td>
<td>Centriole Separation (2)</td>
<td>2110, 4334</td>
</tr>
<tr>
<td><strong>PCL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No PCL (1)</td>
<td>Poc1 Complementation Group: 1446</td>
<td></td>
</tr>
<tr>
<td>Disengagement (1)</td>
<td>2718</td>
<td></td>
</tr>
</tbody>
</table>

Table 4 – The centriolar mutants from the screen are comprised by 40 centriole length mutants, 2 spermatocyte-specific centriole separation mutants, 1 with no PCL-labeling, and 1 with a PCL disengagement phenotype.

Research on the topic of centriole elongation is still in its infancy, and very few genes have been implicated in this process to date. Thus, we anticipate the sequencing results for both our length and leak mutants will reveal new mutations in both known and novel centriole elongation genes. The sequencing results will be the main deciding factor for whether our lab will study these mutants any further. Interpretation of the genetic data has proven complicated since it appears that the mutant alleles are interacting in a non-Mendelian way. With that said, it won’t be worth the time it will take to understand these potentially complex genetic interactions if the sequencing results don’t produce any hits relevant to centrosome formation and/or function.
On the other hand, if the sequencing results do indicate that centrosomal/centriolar genes are mutated in the lines we sent, we would certainly like to study them further. One set of experiments we would like to perform involves testing the fertility of the males with centrosomal mutations. These males would be mated with WT females followed by assaying the ratio of eggs laid to embryos formed. A reduction or complete ablation of the ability of eggs to be fertilized would suggest that the males are infertile. If any degree of infertility is observed, we would proceed to assay sperm motility. If the sperm is immotile, we can suggest that the products of the centrosomal genes mutated in these males cause defects in ciliary function. Indeed, this would make sense considering these mutants also display uncoordination and/or long centrioles, which are also displayed by the ciliary mutant $cep290^{MecH}$. Furthermore, many of these mutants display the Ana1-GFP leak phenotype, which contains abnormal protein localization in the region of different types of cilia. If the sperm of these mutants proves to be immotile, we would have sufficient evidence to go forward and perform pulldown studies to test whether the products of the genes mutated in our mutants can bind to Cep290. If any of these proteins are able to pull down Cep290, deletion studies would be performed to determine whether certain domains are essential for this binding. Alongside the pulldown experiments, we would perform behavioral and sperm motility assays on these deletion mutants regardless of their ability to bind Cep290. This would be done to generate evidence that failure to bind Cep290 is associated with ciliary phenotypic manifestations.

The other line of experiments we would be interested in pursuing involves adding other fluorescently tagged centrosomal proteins under endogenous control to the sequenced lines that exhibit the Ana1-GFP leak with underlying centrosomal mutations. These flies
would then be used as tools to test whether other centrosomal proteins also localize
abnormally into the ciliary axoneme in testes germ cells.
References


