

# Identifying Genes Required for Cell Division in the Early *C. elegans* Embryo

By Hayley Standage


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Hayley Standage for the degree of Bachelor of Science

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The oocyte meiotic spindle and the mitotic spindle are necessary to proper cell division and subsequent development of the zygote. The spindle facilitates chromosome segregation to properly distribute genetic information to newly formed daughter cells. Four *C. elegans* temperature-sensitive mutants are examined in this paper that have defects in spindle formation or function. *or1592* and *or854* have defective oocyte meiotic spindles, while *or1269* and *or987* have dysfunctional mitotic spindles. Analysis of these mutants suggests each may be a novel allele of a gene essential to early embryonic development. Further study of these mutants will be carried out to identify the mutation and characterize their specific role in the spindle throughout cell division.

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## **Introduction**

Cell division initiates and sustains life in all organisms. Humans begin as a single cell—a fertilized egg—and through the process of cell division become complex multicellular organisms that exceed one trillion cells. Cell division is necessary for many different functions including embryonic development, organogenesis, and regeneration of damaged tissue. Meiosis and mitosis are two types of cell divisions. Meiotic cell divisions generate haploid gametes by separating homologous chromosomes during Meiosis I and sister chromatids during Meiosis II. This produces haploid sperm and egg cells that are essential for fertilization and formation of the diploid zygote. Once a zygote is formed, it begins the ongoing process of DNA synthesis followed by mitosis. Unlike meiosis, mitosis generates two diploid daughter cells with identical sets of chromosomes. Chromosomes must first be duplicated in a process called DNA synthesis. Mitosis then segregates identical sister chromatids and the parent cell divides to form two new cells. This is a frequent and repetitive process that maintains diploidy and supports the growth and maintenance of an organism's system.

Both meiosis and mitosis progress by using a highly organized intracellular structure called the spindle, which facilitates chromosome movement. The spindle is a network of polymeric fibers called microtubules that connect to chromosomes to move them throughout cell division. Correct formation and organization of the spindle is necessary for proper chromosome segregation during meiosis and mitosis.

Although meiosis and mitosis are the driving force of life in multicellular organisms, these processes can also have harmful effects. For instance, Down syndrome is a genetic disorder that results from aneuploidy, a condition in which cells contain too many or too few chromosomes following meiosis. A disruption in spindle assembly can prevent homologous pairs of chromosomes from separating properly during meiosis (called nondisjunction) and lead to an abnormal number of chromosomes in the new cells. Cancer is another condition that may result from a disturbance of mitotic machinery and lead to uncontrolled cell division. In the Bowerman lab, we are interested in examining the irregularities that can occur in meiotic and mitotic spindle assembly, which will ultimately contribute to a better understanding of genetic disorders and cancer.

### *Genes: the Foundation of Organisms*

Genes are a set of instructions that guide protein and RNA synthesis necessary to the growth and maintenance of the organism. The proteins and RNA produced from genes greatly influence the structure and function of the organism. Thus, genes provide the foundation from which an organism develops. This genetic information, inherited through cell division, is composed of individual nucleotides, which are monomers made up of different organic elements. A set of nucleotides is a single gene that codes for a specific trait of the organism. In diploid organisms, an individual has two versions of the same gene, or two alleles in which one allele comes from each parent. Both alleles are expressed, but whether the allele is dominant or recessive will influence its expression in the corresponding trait.

Dominant alleles are expressed while recessive alleles are only expressed in absence of the dominant counterpart. Some alleles may be codominant, and the trait is a combination of the two alleles. The combination of different types of alleles makes up the genotype of the organism and is the genetic designation for traits. Genotypes control phenotypes, which are the physical characteristics that make up the organism. All genes in an organism make up its DNA and are separated onto chromosomes, which are single-stranded duplexes of complementary sequences of DNA [1].

The number of chromosomes between species varies. Humans have 46 chromosomes while *Caenorhabditis elegans* have only six chromosomes [1,6]. Both species, however, are diploid (denoted as  $2n$ ) because there are two copies of each chromosome, one from the mother and one from the father. Each set from either parent is haploid (denoted as  $n$ ), or a single set of chromosomes. With the exception of haploid egg and sperm cells, all cells in a single organism are diploid and have identical genetic information [1].

### *Deducing Gene Function*

The connection between genes and protein synthesis allows us to manipulate genes as a means to determine protein function. Loss-of-function mutants are a valuable tool in genetics to study gene function because analysis of these mutants can provide information about the type of protein the gene encodes and the protein's function. Loss-of-function mutants are those in which the nucleotide sequence of the gene is mutated to result in complete inactivation of the

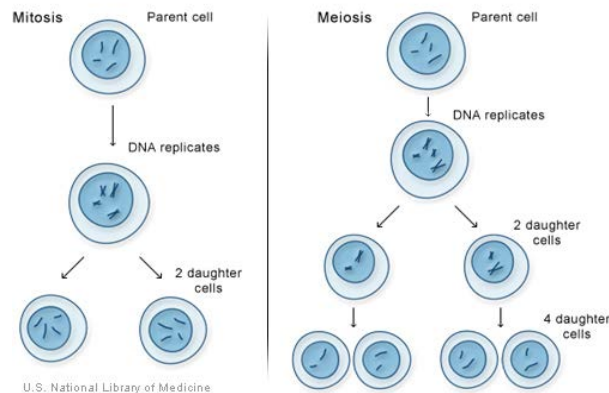


gene and a failure to produce any gene product or an unstable gene product that cannot carry out its specified role. Subsequently, any processes that rely on proper protein function are also disrupted. Thus, the resulting mutant phenotypes can help us infer gene function. In the Bowerman Lab, loss-of-function mutants are used to study the function of genes important in the early stages of cell division.

### *The Products of Cell Division*

Mitosis and meiosis are two types of cell division that pass on genetic information to a new generation of cells. However, the products of these processes are different. Mitosis is the division of somatic cells, or body cells that contribute to the growth and development of the zygote. After a single round of division, a parent cell splits into two diploid daughter cells. An identical set of DNA is passed from the parent cell to each of these daughter cells. Meiosis is a specialized type of cell division that produces gametes (egg and sperm). Two sequential meiotic cell divisions produce four haploid gametes from a single diploid precursor cell [1]. The general scheme of mitosis and meiosis are depicted in Figure 1. My work in the Bowerman lab is focused mainly on meiotic cell division.

**Figure 1. Products of Mitosis and Meiosis**



unique haploid daughter cells.

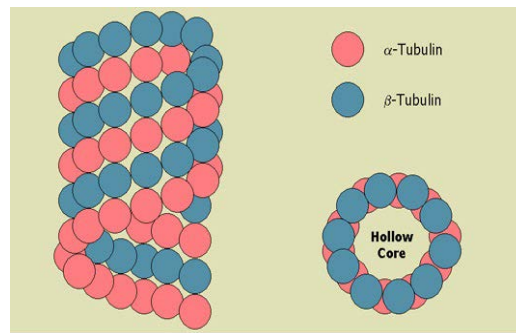
ster chromatids and forms four genetically

Source: <http://ghr.nlm.nih.gov/handbook/illustrations/mitosismeiosis>

### *Components of the Spindle*

The spindle is an important intracellular structure that facilitates chromosome segregation during meiosis and mitosis. Spindle fibers are made up of microtubules, which are polymers of two closely related proteins called alpha and beta tubulin. These proteins bind each other to form heterodimers, and the heterodimers assemble (alpha/beta, alpha/beta, and so forth) to form a single microtubule protofilament. The pattern of repeating heterodimers within individual microtubules establishes polarity because there is a distinct alpha (minus) end and a distinct beta (plus) end of each dimer. The structure of a microtubule is achieved by addition of heterodimers to generate a hollow cylinder (Figure 2).

**Figure 2. Microtubule Structure**



of microtubules (bottom right).

hollow cylindrical structure

Source: [http://www.agron.iastate.edu/Courses/Agron317/Cell\\_Div\\_Inhibitors.htm](http://www.agron.iastate.edu/Courses/Agron317/Cell_Div_Inhibitors.htm)

Growth or shrinkage of microtubules occurs by the addition or removal of heterodimers, respectively, at the plus end. Dynamic instability, the ability of microtubules to grow or shrink, is an important feature of the spindle because it can conform to the needs of the cell based on the phase of division or cell type. Growth is needed to reach the chromosomes and attach to them at a point called the centromere, which enables microtubules control chromosome movement. Shrinkage may be necessary if microtubules have extended beyond the chromosomes toward the opposing side the cell [1,8].

Microtubules polymerize from structures called microtubule-organizing centers (MTOCs). Mitotic spindles having MTOCs called centrosomes from which microtubules radiate. Microtubules from the centrosome exhibit polarity; minus ends of the microtubules are anchored to the centrosome while the plus ends grow out to surround and capture chromosomes. One centrosome nucleates approximately 2,000 microtubules in the one-cell *C. elegans* embryo. In mitosis, two centrosomes form and migrate to opposing sides of the cell beginning in

prophase. Each centrosome is composed of a pair of centrioles, which are two tube-like structures within the centrosome that are necessary for its formation. The centrioles within a centrosome are positioned orthogonally (perpendicular) to each other. In *C. elegans*, a centriole is a central tube surrounded by nine individual microtubules. These microtubules also exhibit polarity, but cannot grow or shrink like cytoplasmic microtubules. The centrioles are wrapped in pericentriolar material (PCM), which help bind microtubules to centrosomes [1,8].

### **The model organism *Caenorhabditis elegans***

#### *Features of C. elegans*

*Caenorhabditis elegans* is a nematode, or microscopic roundworm, that is a well-studied model organism. It is an ideal organism for studying cell division because of its rapid generation time, transparent anatomy, and the powerful genetic tools that have been developed. Female worms are hermaphrodites that can quickly and easily generate a population through self-fertilization or mating with males. Hundreds of small worms can be produced on a single growth plate with each life cycle lasting about three days. The transparent bodies of the worms make it possible to observe cell divisions within a living organism and within the embryos of adult hermaphrodites.

The early *C. elegans* embryo is especially useful for studying embryonic cell division. Embryonic cells are also transparent, and the one-cell embryo is large relative to most animal cells. These qualities allow us to document embryonic cell

division using DIC video microscopy [7]. Unlike other model organisms, the embryonic meiotic and mitotic spindles are assembled relatively quickly within the same cytoplasm (approximately one hour). Both meiotic and mitotic spindle assembly can be viewed with live-cell imaging due to the transparency of the worms and their embryos [8].

### *Studying the Genes of C. elegans*

The *C. elegans* genome can be manipulated to study gene function. With relative ease, we can knock down genes and observe the effects. RNA-interference (RNAi) is an effective method used to directly target and inhibit gene function of a random gene or a pre-selected gene of interest. Conditional alleles are another method used to examine the effects of a loss of gene function. Unlike RNAi knockdowns, conditional alleles are a particularly powerful genetic tool to examine gene function because we can knock down genes at specific time points during development. This allows us to bypass earlier requirements necessary for growth. However, constructing these alleles can be difficult. Conditional alleles are discovered after random DNA mutagenesis and a screen through thousands of candidate mutants.

Transgenic strains are another form of gene manipulation that can be easily done in a laboratory to investigate how genes work. For example, mCherry and Green Fluorescent Protein (GFP) are fluorescent markers that tag specific proteins within the cell, such as histone in chromosomes or tubulin in microtubules. Spinning disc confocal microscopes are used to track these cellular features

throughout cell division. Genetic recombination methods in test tubes (genetic engineering) allow us to fuse DNA sequences that encode mCherry or GFP to the coding sequences of any protein. Such recombinant genes can then be introduced into the genome of an oocyte, and upon fertilization, a transgenic worm develops and expresses the fusion protein in early embryonic cells. These techniques allow us to study the genetic requirements of cellular processes, which make *C. elegans* a powerful genetic tool to gain information about fundamental processes that are conserved throughout the animal kingdom.

#### *C. elegans Embryonic Development to the Four-Cell Stage*

Our study of *C. elegans* early embryonic development is limited to cell division the one to four-cell stage (Figure 3). We focus on early embryonic cell divisions because these cells are large and thus it is easier to image the division with microscopes. The one-cell embryo, or the P0 cell, has two pronuclei on opposing sides of the cell. A pronucleus is a haploid nucleus. There are two in the P0 cell; a maternal pronucleus contributed by the oocyte and a paternal pronucleus from the sperm that fertilized the oocyte. The maternal pronucleus migrates toward the paternal pronucleus to meet in the posterior hemisphere of the cell in order to merge the two genomes prior to the first mitotic division. Pronuclear meeting occurs here because the maternal pronucleus moves first and travels at a faster rate. Upon meeting, chromosomes condense and the spindle begins to assemble. The pronuclei then move together to the center of the cell and rotate approximately 90 degrees. Microtubules extend out from centrosomes to

attach to chromosomes at the centromeres. In anaphase, the spindle microtubules pull apart chromosomes and cytokinesis forms a two-cell embryo made up of the AB cell and P1 cell. These cells divide again with AB cell division preceding that of the P1 cell. AB cell division produces the ABa and ABp cells, and P1 division gives rise to the EMS and P2 cells [5,6].

**Figure 3. *C. elegans* Embryonic Development to the Four-Cell Stage**



## Meiosis

Meiosis produces haploid gametes after their precursor genomes have undergone genetic recombination. Two of the gametes that result from this process, one egg and one sperm, unite later on during fertilization to form a zygote. Genetic recombination is a result of crossing over in which segments from homologous chromosomes physically exchange with each other early in meiosis. As copies of genes on chromosomes from each parent become recombined, recombinant chromosomes form with some genes from one parent and some from the other. This process promotes genetic variation. Random assortment of the homologous parental chromosomes during meiosis also generates genetic diversity. Chromosome pairs from the maternal set line up in the cell next to non-

homologous pairs of chromosomes from the paternal set. There is no specific pattern or mechanism that determines how pairs from each set align next to each other. As there are multiple pairs of homologous chromosomes and meiotic cell division randomly distributes one copy of each pair to each daughter, different oocytes end up receiving different combinations of recombinant parental chromosomes [1].

### *Oocyte Meiosis*

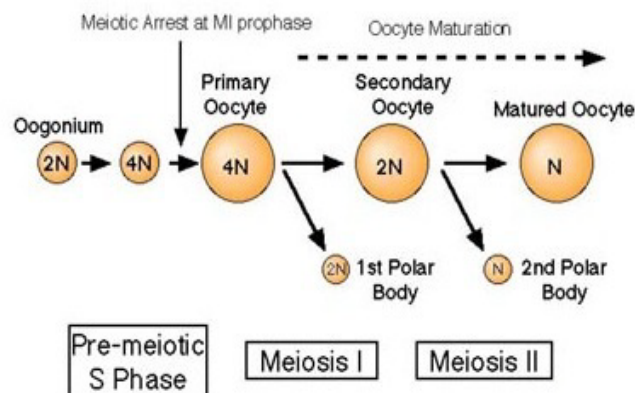
Oocyte meiosis occurs in two rounds of cell division, Meiosis I and Meiosis II, to produce a haploid oocyte. Prior to meiosis, each chromosome in the diploid parent cell is replicated and the cell becomes  $2(2n)$ . These replicates, or sister chromatids, are joined together at a point called the centromere. Pairs of non-identical sister chromatids are referred to as homologs.

In prophase of Meiosis I, the homologous chromosomes condense and the spindle forms and attaches to the centromeres of the sister chromatids. In metaphase, the spindle moves the sister chromatids to line up along an axis in the cell called the metaphase plate. Microtubules pull apart homologous chromosomes toward opposite sides of the cell in telophase. Sister chromatids remain connected at the centromere. In cytokinesis, a haploid set of homologous chromosomes remains within the cell's cytoplasm and participates in another round of meiotic divisions. The other set is extruded from the cell to form a polar body, which is a small amount of cytoplasm containing genetic content [10,13]. A polar body is not a cell and does not go on to divide. Polar body formation is a highly asymmetric



division because the polar body is significantly smaller than the embryonic cell. This helps maintain the size and contents of the cytoplasm that the cell will need in subsequent divisions [19]. The process repeats itself in Meiosis II, but sister chromatids are instead pulled apart from each other. A haploid set of sister chromatids is extruded from the cell to form a second polar body. The remaining set of chromosomes within the cytoplasm forms the oocyte pronucleus, which subsequently goes on to meet a haploid paternal pronucleus. Overall, only one viable cell results from oocyte meiosis. The products of oocyte meiosis are shown in Figure 4.

**Figure 4. Oocyte Meiosis**



pronucleus.

rnal

Source: <http://www.biochem.s.u-tokyo.ac.jp/yamamoto-lab/E/Emeiosis-f.html>

Oocyte meiosis is distinct from sperm meiosis because it produces a single haploid egg and polar bodies, while sperm meiosis produces four haploid sperm cells and no polar bodies [10,13]. My work in the Bowerman lab focuses primarily on oocyte meiosis.

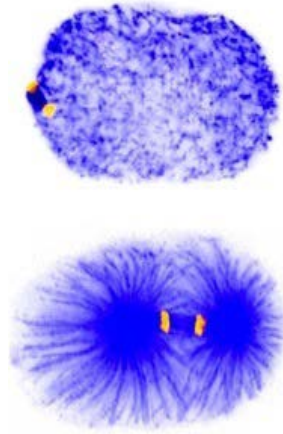
### *Oocyte Meiotic Spindle Assembly*

One focus of research in the Bowerman lab is to study how meiotic spindles separate chromosomes throughout meiosis. In oocyte meiosis, two spindles form from the polymerization of microtubules, but their polymerization is initiated by chromosomes rather than by the centrosomes that nucleate microtubule assembly during mitosis. The centrioles that direct the formation of centrosomes disappear as the oocyte matures, and thus meiotic spindles form in absence of centrosomes [8,16]. This is apparent after the oocyte is fertilized in the spermatheca when microtubules begin to accumulate around chromosomes [17]. The reasons for an acentrosomal meiotic spindle are not known, but previous research has indicated that this may be advantageous. Most notably is the anastral nature of the meiotic spindle, which may be important for the meiotic spindle's small size. A small spindle leads to the production of small polar bodies. A small polar body enables the cell to maintain a large cytoplasm that is important for nourishing and enabling the early development of an embryo after fertilization. It is poorly understood how meiotic spindles become bipolar in the absence of centrosomes at each pole, and this is a major focus of research in the Bowerman lab and of my project.

The differences between the meiotic and mitotic spindles are compared in Figure 5. The top cell shows an oocyte meiotic spindle. The structure is positioned at the anterior edge of the cell, which facilitates an asymmetric division that aids in the formation of a small polar body. The meiotic spindle is anastral, or microtubules span out from its source in one direction toward the chromatin. The cell beneath shows a mitotic spindle in a dividing one-cell embryo. The spindle is

larger relative to the oocyte meiotic spindle and is more centrally located in the cell to form two daughter cells of equivalent size. In contrast to the meiotic spindle, the mitotic spindle is astral and microtubules extend out in all directions from the centrosomes until they are captured by chromosomes or meet the cell wall [8,16].

**Figure 5. Meiotic and Mitotic Spindle**



Fixed embryo staining of meiotic spindle (top) and mitotic spindle (bottom, mitotic division at one-cell stage) during anaphase. Tubulin is marked blue and chromosomes are marked yellow. The meiotic spindle is positioned near the edge of the anterior of the cell for an asymmetric division. Microtubules span out only toward the chromosomes. The mitotic spindle is larger relative to the oocyte meiotic spindle and is located toward the middle of the cell for a more symmetric division. Microtubules radiate out in all directions from the centrosomes.

Source: Bowerman lab, personal contribution

The oocyte meiotic spindle first forms during prophase as the nuclear envelope breaks down and adopts a tight barrel shape in metaphase. Following this, the spindle moves to position with its pole-to-pole axis parallel to the cell cortex. It then rotates to orient itself orthogonal to the plasma membrane. The orthogonal orientation of the spindle allows the pole closest to the membrane to be extruded into the polar body and maintains the other pole within the cell. This orientation ensures that chromosome sets are equally distributed during polar body formation [10,19].

## Genes Required for Oocyte Meiosis in *C. elegans*

*mei-1 and mei-2 form an enzyme complex required for spindle length regulation*

The proteins MEI-1 and MEI-2 together form a katanin complex that is required for proper assembly and orientation of the oocyte meiotic spindle. Katanin is a widely conserved heterodimeric enzyme that has microtubule severing activity. *mei-1* encodes the p60 catalytic subunit of katanin. The C-terminal region of MEI-1 binds microtubules and the N-terminal region of MEI-1 binds MEI-2 and microtubules. MEI-1 has been previously shown to require the activity of MEI-2 [10,15,16]. In oocyte meiosis, the katanin complex localizes to the polar ends of meiotic chromatin and microtubules of the oocyte meiotic spindle [8]. Together, MEI-1 and MEI-2 shorten microtubules to regulate spindle length during metaphase. This helps maintain a sufficient amount of cytoplasm within the egg that the embryo needs after polar body extrusion [15,16].

*mei-1(-)* mutants have apolar oocyte meiotic spindles. Chromosomes and microtubules are disorganized beginning in Meiosis I, and chromosomes fail to align on the metaphase plate. Oocyte chromosomes are completely ejected from the cell, and there is no maternal contribution of chromosomes to the zygote. Conversely, chromosomes may fail to be extruded and break apart into several pieces that result in multiple maternal pronuclei [10]. *mei-2(null)* mutants have disorganized microtubules and apolar spindles. Partial loss of *mei-2* results in long microtubules because of a lack of microtubule severing activity [10,15,16].

Chromosomes do not separate normally during anaphase and a random grouping of chromosomes is expelled into the polar bodies [15].

*aspm-1 is necessary for proper oocyte meiotic spindle rotation and positioning*

*aspm-1* encodes a microtubule scaffolding protein that is required for proper oocyte meiotic spindle orientation. ASPM-1 is recruited to the spindle by MEI-1 to interact with the proteins dynein and LIN-5, which rotates the spindle and positions it orthogonal to the plane of the plasma membrane. The Bowerman lab has recently shown that ASPM-1 is also required for spindle end focusing and chromosome segregation. *aspm-1* TS mutants fail to rotate the spindle orthogonally, which leads to disorganized meiotic chromosomes and an inability to extrude chromosomes into the polar bodies. Although the spindle is bipolar, spindle poles are unfocused and microtubules fail to join at a common point. Multiple maternal pronuclei are observed in the one-cell embryo following meiotic divisions [10,13,14].

*klp-18 is required for oocyte meiotic spindle bipolarity*

*klp-18* encodes a motor protein with a N-terminal kinesin-related motor domain that is similar to Klp2 kinesins. As a plus-end directed protein, KLP-18 is thought to carry antiparallel microtubules toward each other. The protein brings the plus ends of microtubules oriented in opposite directions toward each other to the spindle equator. This movement produces the sliding motion of microtubules, which is a function similar to that of KLP-18 homologs in other organisms. KLP-18

is localized to oocyte meiotic spindle poles in prometaphase, metaphase, and anaphase, and migrates to the interzone of separating chromosomes in the latter stages of meiosis.

*klp-18(-)* mutants initially have misaligned chromosomes that fail to segregate properly. However, the chromosomes eventually migrate to a small area, which signifies that chromosomes were captured and moved by microtubules that radiated from a single spindle pole. Fluorescent markers in *klp-18(-)* mutants confirmed that the spindle is monopolar, which is attributed to the lack of motor protein not being able to move microtubules in the opposite direction. As a result, mutants extrude all chromosomes during polar body formation and early embryos have no maternal pronucleus, or chromosomes are disorganized and result in extra female pronuclei. Remnants of these pronuclei lead to multiple nuclei seen in mitosis. Thus, KLP-18 is necessary for bipolarity of the oocyte meiotic spindle in order to facilitate proper chromosome segregation, but is not essential to oocyte meiotic spindle assembly [11,17].

*zyg-9 is essential for microtubule organization during meiosis and mitosis*

*zyg-9* encodes a protein that is required for microtubule organization and function in both meiosis and mitosis. ZYG-9 is highly localized to meiotic spindle poles and is found in lesser amounts at the central portion of the spindle in metaphase of meiosis. Unlike the previously discussed genes, ZYG-9 activity persists in mitosis in order to increase the length of microtubules. Despite the need for shorter microtubules of the meiotic spindle to preserve the cytoplasm, ZYG-9

may have similar activity in meiosis to offset the microtubule severing activity of MEI-1. It has been previously found that ZYG-9 activity is dependent upon MEI-1 activity. The antagonistic relationship between ZYG-9 and MEI-1 could stimulate the subsequent rearrangement of the microtubules into a more organized bipolar array and promote greater stability of the spindle. *zyg-9(-)* mutants have disorganized spindles and clusters of microtubules in the cytoplasm due to a lack of microtubule organization. Subsequent pronuclear migration fails [8,9,17,18].

## **Screening for *C. elegans* Mutants**

### *Temperature-Sensitive Mutants*

In the Bowerman lab we screen for temperature-sensitive (TS) alleles of genes involved in meiotic and mitotic cell division in *C. elegans*. TS mutations are conditional alleles that permit us to study early embryonic development with greater ease. Many genes have multiple essential functions that are necessary for growth of the embryo into a fertile adult. These genes are known as essential genes. There are approximately 2500 essential genes out of 20,000 total genes in *C. elegans*. These genes are difficult to study because when mutated and inactivated, they fail to support development. The worms may die early in development or result in sterile adults, both of which eliminates the ability to examine early embryogenesis. Such problems arise when methods like RNAi are used to knockdown gene function.

TS mutants, however, allow us to bypass early requirements for oocyte development. We use fast acting and heat-sensitive TS mutants. Mutants are grown at the low permissive temperature where they develop normally. Only when the worms are shifted to the higher restrictive temperature is the mutation induced in early embryos. Thus, TS mutations are valuable for the study of early embryonic development because viable worms are produced and the gene can then be inactivated when desired [2,4].

#### *Primary Screen Identifies Temperature-Sensitive Mutations*

We use ENU to randomly mutagenize the DNA and then search for worms that die as early embryos at the restrictive temperature (26 degrees Celsius). To identify worms from a mutagenized population that have TS mutations in essential genes, a strain with a mutation in a gene called *lin-2* is used. The *lin-2* mutants are viable but have defects in vulva development that prevent egg-laying. Thus, these worms produce embryos but cannot lay them. Embryos accumulate in the uterus and eventually hatch and survive within the worm (killing the parent and then escaping to mature into fertile adults). The *lin-2* mutation is helpful because the embryos that die prematurely will remain within the gonad of the mother. In the primary screen following mutagenesis, mutant worms are grown for two generations at the permissive temperature (15 degrees Celsius) and then shifted to the restrictive temperature. Adults that fill with dead embryos are isolated and shifted back down to the permissive temperature. If a few embryos are then produced and hatch, that signifies that the worms have a TS mutation [4].



### *Secondary Screen Determines Cell Division Defects in Temperature-Sensitive Mutants*

After we identify a TS mutation, an allele number is assigned to this mutation to distinguish it from other mutations. The allele number begins with “or” (referring to Oregon) and is followed by numbers that signify the order that the allele was added to the lab collection. For example, *or1592* is the 1592<sup>nd</sup> TS allele that has been added to the Bowerman lab collection. A strain number is also used to refer to the genomic background that the mutation is in, such as the fluorescent markers or the *lin-2* mutation. The strain number is designated by “EU” (referring to Eugene) followed by a number. One “or” number may have several different “EU” numbers because the same mutation can be put into several different genomic backgrounds based on how we want to study the mutation.

Once TS embryonic-lethal mutants are isolated, mutant embryos are examined during early embryonic cell division and sorted into a few general phenotypic classes using Nomarski optics to observe the mutant phenotype. About 10-15% of TS mutants showed detectable defects during the early stages of cell division, and these are the mutants the Bowerman lab focuses on in their research.

Several Nomarski movies of the early embryonic cell divisions are then made to determine penetrance of the mutant phenotype. Penetrance explains how consistently the same phenotype or phenotypes appear within the same mutant. Highly penetrant strains repeatedly exhibit the same visible defects whereas weakly penetrant strains exhibit defects a couple of times. Only highly penetrant strains are submitted for further study because there is stronger evidence that the mutant phenotype is due to a gene that is directly involved in a particular process

and is not downstream or a secondary effect. Penetrant TS mutants that are selected are then tested to determine whether the affected gene is dominant or recessive. If dominant, these mutants are set aside for later study because these are gain-of-function mutants from which gene function cannot be inferred. Only mutants with a loss-of-function mutation, or a recessive gene, can provide information about gene function [4]. For example, if a recessive mutant fails to assemble a meiotic spindle, then you know that gene is somehow required for meiotic spindle assembly. Gain of function, or dominant, mutations can result in a mutant protein that causes defects unrelated to its normal requirements. Although dominant mutations can be useful for investigating gene function, most geneticists give higher priority to the study of recessive mutants.

## **Methods**

### *Phenotypic Analysis*

The forward genetics approach is an important tool used to identify genes that are required for cell division. TS mutants obtained from the screening process are kept at the permissive temperature, and the EL mutation is induced by shifting L4 female mutants to the restrictive temperature for eight to twelve hours. The worms are then removed from the incubator and a few worms containing embryos are suspended in M9 solution. Worms are cut open to release the embryos, and embryos are mounted on agar pads. Nomarski movies, or time-lapse images using DIC video microscopy, document the mutant phenotypes during the embryonic

one-cell to four-cell stage. Phenotypic analysis of these movies allows preliminary conclusions to be made regarding the type of gene that may be affected.

#### *Dominance Tests and Outcross with him-5 Males*

Penetrant strains, or those that continually show the same mutant defects, are tested to determine if the mutant gene is dominant or recessive. Only recessive genes are submitted for further study. Recessive mutant hermaphrodites are outcrossed with *him-5* males to eliminate background mutations (random mutations within the genome that don't contribute to the phenotype-causing mutation). Outcrossing also ensures that there is only one mutation contributing to the phenotypes. The *lin-2* mutation is removed through this process to allow females to lay eggs again, which can otherwise inhibit future genetic crosses. Additional Nomarski movies are made after the outcross to confirm that the mutant phenotypes are still present. If embryonic cell division appears to be wild type, no further work-up is done because the mutation of interest was lost during the outcross.

#### *Complementation Tests*

The phenotypes are assessed, and if the phenotype resembles that of other previously identified mutants, complementation tests are done to identify if the mutations of interest is an allele of a known gene. Outcrossed mutants are mated with a strain that contains a mutation in the known candidate gene. F1 female progeny are transferred to the restrictive temperature, and plates are scored for

dead eggs and live larvae. A majority of dead eggs represents a failure to complement, which indicates that the candidate allele and mutant allele are of the same gene. Both are defective copies of the same gene that when mutated cannot support embryonic development. If a majority of eggs did hatch, the candidate allele and mutant allele complemented each other, and therefore are alleles of different genes. Both had an unaffected copy of the other's mutated gene that allowed the embryos to survive even at the restrictive temperature.

#### *Single Nucleotide Polymorphism Mapping and Whole Genome Sequencing*

If the mutant does not complement any known candidate genes or has a novel phenotype, the identity of the mutation is found through positional cloning by Hawaiian outcross. Single nucleotide polymorphism (SNP) mapping can narrow down the mutated gene to a one-chromosome interval. SNP-mapping uses Hawaiian *C. elegans* strains, which are morphologically wild type, but contain SNPs that are uniformly distributed throughout the genome at an average density of about 1/1,000. These SNPs are small changes in the nucleotide sequence that serve as genomic markers and aid in narrowing down the location of the mutation. Whole genome sequencing (WGS) can then identify the mutated sequence. The combination of these two methods has recently been proven to be a rapid and effective technique for identifying mutations in the *C. elegans* genome. The location of the phenotype-causing mutation can be acquired with relative ease; a single cross is required, individual mutants can be utilized, and one WGS run is needed in place of extended SNP-mapping [12].

The positional cloning process begins with a polymorphic Hawaiian strain crossed with the mutant strain. A small fraction of the F2 progeny will have recombined chromosomes that contain the mutation of interest and SNPs. The F2 recombinant worms bearing the mutation of interest are isolated by identifying embryonic lethality at the restrictive temperature. After isolation, approximately 50 F2 recombinants, homozygous for the TS mutations, are selected and allowed to self-fertilize. Equivalent samples from each of the F3 populations are pooled together, and their DNA is prepared for WGS. The information collected from WGS allows us to find the region of the genome where there is a higher incidence of N2 (wild-type) sequences. Regions flanking the mutation will be almost 100% N2 DNA because we selectively chose F2 worms with the TS mutation. The remainder of the genome will contain 50% N2 DNA and 50% Hawaiian DNA.

### *Transgenic Gene Constructs*

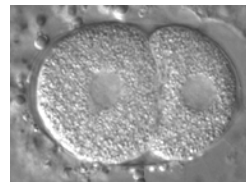
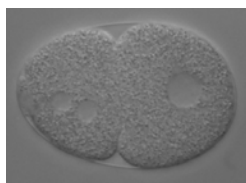
After discovering gene identity, transgenic strains can be constructed to study the mutant defects more closely. Such constructs are often made with mCherry::H2Bhistone and GFP:: $\beta$ -tubulin, which marks histone red, a protein found in chromosomes (denoted by mCherry) and tubulin, or microtubules, green (denoted by GFP). Mutants are crossed with strains that contain these fluorescent markers, and fluorescent and DIC video microscopy will verify that the progeny from this cross contains both the fluorescent markers and the mutant phenotype. Fluorescent markers allow us to visualize intracellular components, like chromosomes and the spindle. By crossing the mutants into this transgenic strain

we can make observations as to how the spindle morphology is impacted when there is a loss of this gene. This is particularly informative once we identify the gene.

## Results

### ***or1592* shows multiple maternal pronuclei indicating meiotic spindle assembly defects**

Nomarski movies using DIC video microscopy of *or1592* were completed and phenotypically analyzed for defects during early embryogenesis. Mutant phenotypes included multiple maternal pronuclei, large polar bodies, and multiple nuclei at the two-cell and four-cell stages (Figure 6). Multiple maternal pronuclei were the most penetrant defect and occurred in four of six of Nomarski movies.



A dominance test found that the gene of interest was recessive, and the strain was outcrossed with *him-5* males to eliminate background mutations. Nomarski movies made of outcrossed *or1592* revealed that the penetrant phenotypes were maintained after the outcross.

The most penetrant defect, multiple maternal pronuclei, suggests that *or1592* may have defects in chromosome segregation during meiosis. Multiple maternal pronuclei are often a result of deficient meiotic spindles in which the spindle does not assemble properly and chromosomes segregation is defective. Chromosomes tend to be disorganized throughout the cell and are not extruded properly, which leads to many maternal pronuclei instead of a single maternal pronucleus. This could affect polar body size because excess cytoplasm could be lost in place of genetic material and cause the polar body size to be greater than normal. These problems are attributed to improper meiotic spindle assembly. Multiple nuclei seen in later mitotic divisions could be remnants of multiple pronuclei.

The penetrant phenotype of multiple maternal pronuclei is similar to those of already identified genes *mei-1*, *mei-2*, *aspm-1*, and *zyg-9*. These genes were candidates for complementation test because they have previously been shown to be defective in meiotic spindle assembly and specifically result in mutant phenotypes such as multiple maternal pronuclei. *klp-18* was not selected for a complementation test because *klp-18(-)* mutants typically show no maternal pronucleus as opposed to the multiple maternal pronuclei that *or1592* shows. If *or1592* failed to complement one of these known genes, *or1592* would be an allele

of that known gene. Cross progeny of *or1592* and the candidate gene were scored for total hatched embryos. Results are shown in Table 1.

**Table 1: *or1592* Complementation Test Results**

| Genotype                      | Total # Scored | Total # Hatched |
|-------------------------------|----------------|-----------------|
| <i>or1592</i> x <i>mei-1</i>  | 929            | 895             |
| <i>or1592</i> x <i>mei-2</i>  | 294            | 270             |
| <i>or1592</i> x <i>aspm-1</i> | 342            | 339             |
| <i>or1592</i> x <i>zyg-9</i>  | 76             | 65              |

Table 1 shows that 96%, 92%, 99%, and 86% of embryos, respectively, hatched at the restrictive temperature when *or1592* was crossed with each of the genes mentioned above. This ability to complement demonstrates that *or1592* is not an allele of a known gene because the cross did not produce embryonic lethal progeny. Therefore, the mutation in *or1592* was complemented by an unaffected version in the known gene and allowed embryos to develop at the restrictive temperature. The results of these tests are exciting because it indicates that *or1592* is likely a mutant of an unidentified gene involved in meiosis.

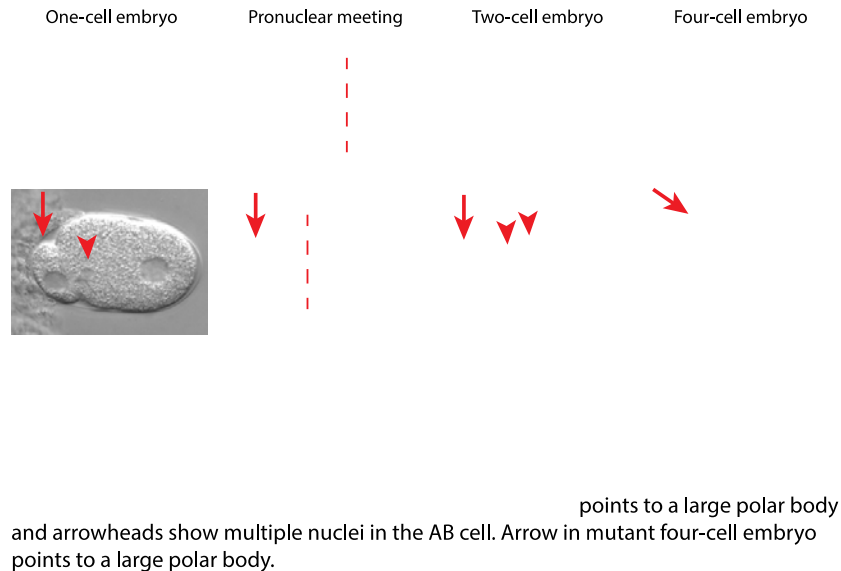
SNP-mapping and WGS were then employed to narrow the genomic interval of the phenotype-causing mutation and identify the sequence mutation. A cross was set up with *or1592* and a polymorphic Hawaiian strain. Results from SNP-mapping and WGS are still being analyzed. An *or1592* mCherry::H2Bhistone, GFP:: $\beta$ -tubulin strain was also constructed to further study the defective meiotic spindle and possibly identify the part of the spindle that led to multiple maternal pronuclei.



***or854* has a large polar body suggesting oocyte meiotic spindle positioning defects**

Nomarski movies for *or854* were made, and the most prominent mutant phenotype was a very large polar body in the one-cell, two-cell, and four-cell embryo. Other defects included pronuclear meeting in the middle of the P0 cell instead of in the posterior and multiple nuclei at the two-cell stage (Figure 7).

**Figure 7. *or854* Cell Division Defects**



*or854* Imaging credit: Amy Connolly

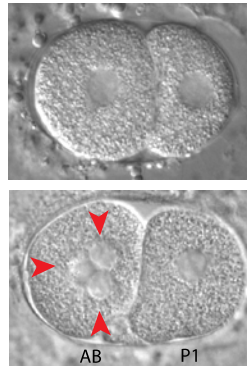
A dominance test revealed that the gene of interest was recessive, and an outcross of *or854* and *him-5* males was completed to eliminate background mutations. Following the outcross, Nomarski movies showed that the mutant phenotypes persisted.

Mutants with abnormal sized polar bodies typically suggest that there are deficiencies with the oocyte meiotic spindle leading to a greater extrusion of

genetic material. However, the polar bodies observed for *or854* are so large that it is likely that the polar body is not entirely chromosomal material. This may be due to defects with oocyte meiotic spindle positioning. This phenotype did not appear to resemble that of other known genes, and therefore no complementation tests were completed for *or854*. A cross with the mutant and a polymorphic Hawaiian strain was started for SNP-mapping and WGS to determine gene identity. These experiments are still being conducted. A transgenic strain with mCherry::H2B histone, GFP:: $\beta$ -tubulin was constructed to better visualize spindle defects during meiosis.

***or1269* has abnormal pronuclear meeting, large polar bodies, and multiple nuclei at the two-cell and four-cell stages implicating mitotic spindle assembly defects**

Nomarski movies of *or1269* showed many penetrant defects throughout embryonic division (Figure 8). Pronuclear meeting in the center of the cell and multiple nuclei at the two-cell and four-cell stage were detected most often. A small portion of Nomarski movies showed large polar bodies.

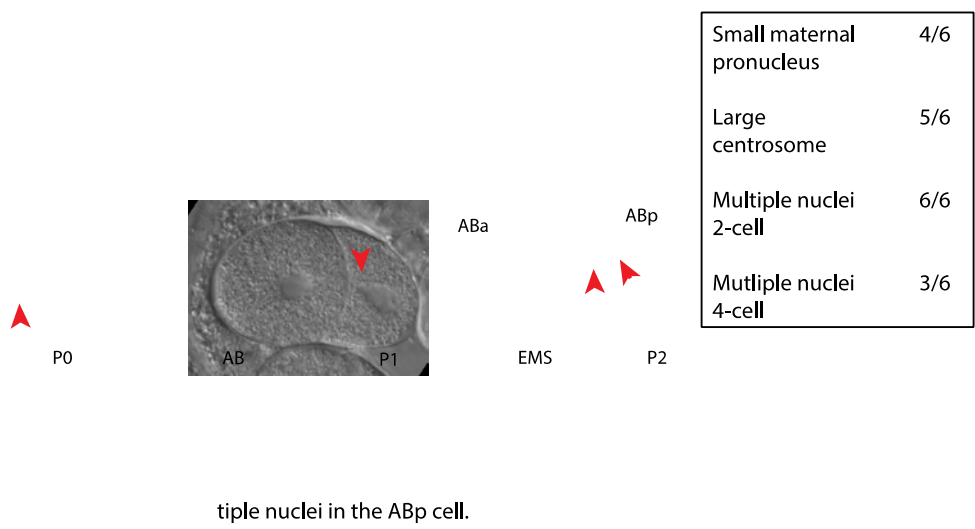


*or1269* was submitted to a dominance test and it was found that the mutation was recessive. *or1269* was outcrossed with *him-5* males to eliminate background mutations, and Nomarski movies made after the outcross showed that the mutant phenotypes still existed.

The presence of multiple nuclei at the two-cell and four-cell stages suggests that there are defects with chromosome segregation during mitosis. These phenotypes were compared to that of other known genes important in mitotic spindle assembly and they did not resemble that of previously identified genes. No complementation tests were completed with *or1269*. Positional cloning by outcross with a Hawaiian strain was started for *or1269* to identify the location of the gene. Results are still being analyzed.

***or987* has a small maternal pronucleus, large centrosomes, and multiple nuclei at the two-cell and four-cell stage indicating mitotic spindle defects**

The phenotypes observed in Nomarski movies of *or987* suggested that oocyte meiosis proceeded properly but mitotic cell division was defective. Multiple nuclei at the two-cell stage were detected in each of six Nomarski movies while half of the movies also had multiple nuclei at the four-cell stage. Large centrosomes and a small maternal pronucleus were also highly penetrant. Defects are shown in Figure 9.



These phenotypes indicate that the mitotic spindle is defective. Despite the interesting phenotypes that *or987* presents, time restraints limited my work with this mutant. However, the Bowerman lab will follow through with the same workup as the previous mutants discussed.

## Discussion

Four *C. elegans* TS mutants were examined in this paper, each of which has results that suggest the discovery of a previously unidentified mutation. Two of the mutants discussed, *or1592* and *or854*, showed mutant phenotypes that are attributed to defects in oocyte meiotic cell division. *or1592* was highly penetrant for multiple maternal pronuclei, which indicate that a defective oocyte meiotic spindle may have led to improper chromosome segregation during meiosis. A large polar body observed in *or854* is also an implication of defective oocyte meiotic division because polar body extrusion is exclusive to oocyte meiosis. *or1269* and *or987* had normal oocyte meiotic cell divisions but showed defects in early embryonic mitotic cell division. Multiple nuclei at several stages of mitosis in *or1269* and *or987* suggest that there was improper chromosome segregation during mitosis.

Further study of these mutants will contribute to previous research that has identified genes necessary to early embryogenesis and help find novel genes that influence early stages of embryonic development. Each of the mutants discussed here are novel alleles that have not been previously identified. The workup with *or1592* found that it failed to complement four candidate genes, which provides strong evidence that *or1592* is a novel gene. SNP mapping and WGS will be completed in order to locate the mutation and identify the mutated sequence. The multiple maternal pronuclei observed in *or1592* indicate that the female meiotic spindle is disrupted, and the chromosomes fail to segregate properly during meiosis to result in multiple pronuclei. This could be a consequence of several

factors, such as a dysfunctional kinesin or dynein protein that cannot aid in microtubule assembly. Proteins that localize kinesin or dynein could also be defective upon which spindle assembly would not be able to proceed properly.

The large polar bodies observed in *or854* suggest that this may also be a novel gene because it is not a phenotype seen in previous mutants. SNP mapping and WGS will also be used to identify the mutation. The abnormal polar bodies in *or854* implicate an oocyte meiotic spindle defect. Mutants with abnormally sized polar bodies often have disorganized microtubules that cannot sufficiently segregate chromosomes. This could lead to a greater extrusion of genetic material during polar body formation. However, the polar bodies in *or854* are much larger than that of previously studied mutants. Thus, it is likely that the large polar bodies include more than just genetic content. Such a defect could be attributed to defective oocyte meiotic spindle positioning that is occurring at the middle of the cell rather than the anterior.

Finally, the multiple nuclei at the two-cell and four-cell stage in *or1269* are likely mitotic defects. No complementation tests were completed for *or1269* because these phenotypes have not been seen in other known mutants. The multiple nuclei observed at several cell stages in *or987* also suggest improper chromosome segregation in mitosis that would lead to improper chromosome distribution between the new daughter cells. Positional cloning will be carried out to identify the mutations in both *or1269* and *or987*. Although these mutants have interesting phenotypes and may present further information important to the study of mitotic cell division, the Bowerman lab is more interested in oocyte

meiotic spindle assembly. Further work will be done with these mutants but mitotic-defective mutants currently have less of a priority.

### *Future Directions*

The forward genetics approach provides an interesting and effective way to study gene function in *C. elegans*. Through elimination of one component in the process of cell division we can infer gene function by observing the resulting phenotype. Although the mutants discussed in this paper may lead to the discovery of a few of the unknown essential genes in *C. elegans*, more research will be needed to fully characterize the gene upon finding its location in the genome. Transgenic strain construction is an important step in the process of identifying the gene's specific role. For the mutants examined here, transgenic strains allow us to examine spindle morphology and determine the gene's specific role in the spindle. Epistatic analysis of double mutants is also an effective method to decipher how genes interact and affect one another. Double mutant phenotypes may resemble one of the mutants or have a novel phenotype; this allows us to place the two genes into a pathway deciphering which gene regulates the other or to determine if they are in two independent pathways. Additional transgenic markers could also be utilized to study the mutant, such as MEI-1 or other known genes that are important to the division process. Presence and localization of these known proteins could be defective in these mutants, which would offer further information about the role of the mutant gene.

Identifying and understanding such genes can provide further insight about the function of conserved genes in other organisms and their possible role in human health [3]. However, these conclusions only present a few answers about gene function during early embryogenesis. Further research will be needed to characterize the cause of mutations in genetic disorders and cancer and how these mutations lead to the severe health defects that are observed in such patients.



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