ANALYZING EARLY EMBRYONIC CELL DIVISION DEFECTS IN *CAENORHABDITIS ELEGANS*

by

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A THESIS

Presented to the Department of Biology
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

June 2014

An Abstract of the Thesis of

Sheetal Krishnakumar for the degree of Bachelor of Science
in the Department of Biology to be taken June 2014

Title: Analyzing Early Embryonic Cell Division Defects in *Caenorhabditis Elegans*

Approved:

Bruce Bowerman

Searching for and analyzing novel mutant phenotypes in early cell division of *C. elegans* allows researcher to infer how the wild-type gene/ protein normally functions after observing defects. To investigate these phenotypes, three different temperature sensitive (ts) mutants (*or1786*ts; *lin-2 (e1309), or1700*ts; *lin-2 (e1309), and or1578*ts; *lin-2 (e1309)) were analyzed.* Using Nomarski/ DIC videomicroscopy, phenotypic analysis was completed to record the mutant embryos from the 1-cell to 4-cell embryo stage. After following up the phenotypic analysis with outcrosses and re-analysis, the mutants showed to have several defects prolonged durations within stages of the cell cycle. This could be a result of mutations in DNA replication machinery or of cell cycle regulator. Further tests can be done to isolate the origin of the mutation and permit a better understanding of the genes in which the mutations occurred.

Acknowledgements

I would like to thank Dr. Bruce Bowerman for letting me have the opportunity to do research in his lab for my thesis project. I would like to thank my mentor Josh Lowry, for teaching me almost everything I know about *C. elegans* research, and always being there to guide me every step of the way. I express the sincerest gratitude for all the time he spent working with me in the last two years. I want to thank the members of the Bowerman lab, for always being approachable and willing to help me. The other undergraduate students in the lab were peers I could go to for anything, specifically Ava Klein, a fellow undergraduate in the lab for giving me one of her *C. elegans* strains to do work on. I'd like to thank my honors college advisor, Professor Mark Carey, thesis coordinator, Miriam Jordan and the rest of the Clark Honors College staff for always being on top of things and guiding me through the process and answering most of my questions before I even asked the. I want to thank all my professors, who taught me so much about biology and my time with a big workload. I cannot express how grateful I am for my loving family who always supported me and all my goals, and my close friends and roommates who always motivated me and accompanied me in my long days and nights of work both in and outside of the lab. I would not have been able to do this without the help of all these people, and I very much appreciate all they have done.

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I. Introduction

**** Bolded terms in the body of the text and notation are included in the glossary in section VI.*

Human genetics is a very important topic to study because genes dictate all of the developmental events that lead a single cell to become a full-grown adult human. Genetic diseases are very common, and caused by genetic mutations. But studying that one mutation in a gene that is causing disease is difficult in humans. Since it is impractical to study with humans, researchers study with different model organisms. In the Bowerman lab we look for novel mutant **phenotypes** in cell division of *C. elegans* cells so that we can infer how the wild-type gene/protein normally functions based on the observed defects. The mutations studied typically reduce or eliminate gene function; the absence of normal function is what causes the defect. Studying cell cycle mutations can help us understand their effect on development, and developmental defects in growing organisms. Recently more research is being done on the mechanism of the cellular and molecular machinery involved, so that it can be understood how alterations to this machinery can change the progression of the cell cycle. *C. elegans* is one of the two model organisms that have been well used to study the coupling of cell cycle and development.[1] Since these organisms share conserved genes with humans, by studying them, we can learn more about how certain genes required for cell division work in humans, to be able to make drugs that regulate them to work against specific diseases.

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[1] Budirahardja Y, Gönczy P. (2009). Coupling the cell cycle to development. Development. 136(17):2861-72.

One such disease is cancer, the problem with cancer is that the cells are growing and replicating too fast—but by learning more about cell growth and division we can identify the specific genes involved. And once we know which genes work in the specific processes, new targets for cancer drugs can be found.

II. Background

a. Model Organism- *Caenorhabditis elegans*

The research in my lab uses the nematode, *Caenorhabditis elegans (C. elegans*) as a model organism. A model organism is a living organism that is used for research purposes in laboratories, where working on humans is not a possibility. Specific model organisms are usually used because they have some correlation to humans. Experiments are done on the model organism to then apply the finding to humans. *C. elegans* makes a good model organism because they are transparent, have short lifespans, are easy to store, cost effective to manage, share genes in common with humans, and their entire genome has already been mapped. This means we know where all the genes are located on the genome. We don't necessarily know what the genes are and how they interact with each other though. *C. elegans* belong to the phylum Nematoda. They are found in any nutrient and bacteria rich environment. *C. elegans* feed on bacteria; for my experiments, I use the bacteria E. coli plated into petri dishes to feed my worms.

b. *C. elegans* Anatomy and Life Cycle

C. elegans are fairly simple organisms in terms of their body type and anatomy. There are hermaphrodites, which can self fertilize and produce self-progeny, but they can also mate with males and produce cross progeny. Both hermaphrodites and males have an identifiable head, mouth and pharynx, germline, and somatic gonad. As shown in figure 1, the hermaphrodite has two sets of ovaries, oocytes, and spermatheca (even though it is not labeled on both sides). The spermetheca is the location in which the sperm are stored. As the oocytes pass through the spermatheca on both sides and come

towards the center, they become fertilized eggs in the uterus. The center of the uterus is the vulva; this region slightly protrudes (noticeable in determination of sex) and is where the egg passes through the worm body, as the worm lays it. The male anatomy is different, as expected. It mostly contains the testis, sperm, seminal vesicle, and vas deferens. The cloaca, spicule, rays and fan are located on the posterior end of the worm and make a "spade-like" structure. The male is distinguished by its thinner body shape, clear ventral gonad, and the "spade-shaped" distinctive tail.[2] The hermaphrodites are also easily noticeable when they reach the adult stage because they get filled with eggs fairly fast.

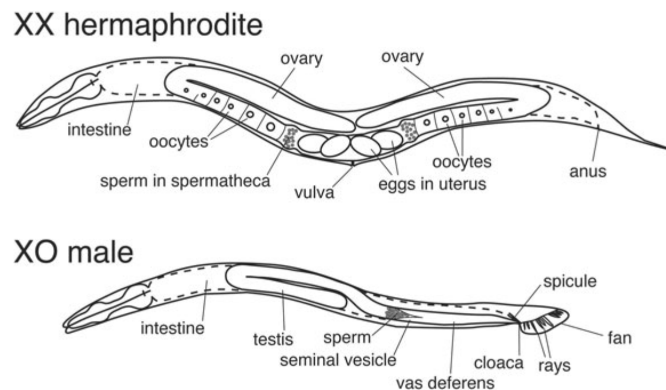

Figure 1. Anatomy of hermaphrodite and male *C. elegans*. Shows various anatomical structures that differentiate the hermaphrodite from the male.[3]

C. elegans have different stages of development which are important to know and be able to identify in order to use them for different experiments. Figure 2 shows the different stages of the nematode from the single-cell stage to adult stage. Once the

[2] Lints, R. and Hall, D.H. 2009. Male introduction. In *WormAtlas*. doi:10.3908/wormatlas.2.1 Edited for the web by Laura A. Herndon. Last revision: July 11, 2012.

[3] Zarkower, D. Somatic sex determination (February 10, 2006), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.84.1, http://www.wormbook.org.

egg hatches it is at the L1 (larval 1) stage. If there is no food present before the L1 stage, the larvae will arrest at the L1 stage. If there is food supply after the L1 stage, but not between the L3 and L4 stages, it will arrest at the dauer stage.[4] The dauer stage is classified by darker lines outlining the thin, starved worm body. Dauer worms can be rescued by being transferred on to plates with more food. In the presence of food, the worm progresses into the L2, L3, and L4 stages. The L4 stage is an important stage to be able to identify because it is the stage right before adulthood, when eggs begin to develop. The L4 stage can be identified by an empty semicircle shape in the region of the vulva, which marks the developing vulva. The next stage about 10 hours after is the first adult stage.

[4] Cassada RC, Russell RL. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Dev Biol. 46(2):326-42.

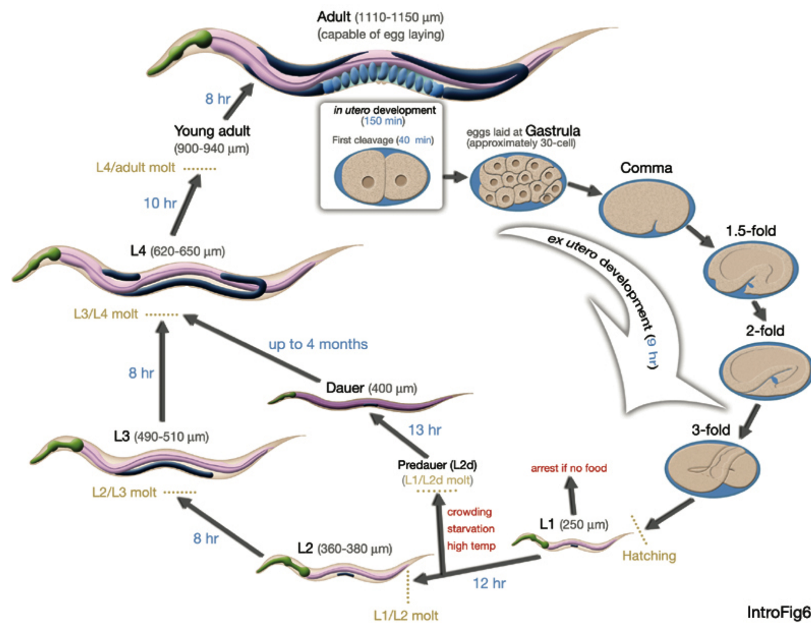

Figure 2. Lifecycle of *C. elegans* from cell stages to adult. Includes average time intervals between stages.[5]

c. *C. elegans* Cell Division in N2 strain

The N2 worm strain was originally isolated in Bristol England in 1965.[6] This strain has been designated as wild type. It is considered to have a normal, standardized phenotype, which all other mutant phenotypes are compared to.

Cell division of *C. elegans* embryos begins soon after fertilization of the oocyte by the sperm. The embryo begins at the one cell stage with 2 distinct clearings at the anterior and posterior ends of the cell (figure 3a); these are the maternal **pronucleus** and the paternal pronucleus. There is a membrane envelope surrounding the whole cell and it has a larger separation from the cell body on one side. On this side, there is also a cell mass called a polar body, which sits at the edge of the cell, inside the membrane. The

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[5] Introduction to C. elegans Anatomy- *Caenorhabditis elegans as a Genetic Organism.* 2006. http://www.wormatlas.org/ver1/handbook/anatomyintro/anatomyintro.htm accessed 2/28/2014.

[6] Riddle DL, Blumenthal T, Meyer BJ, et al., editors. (1997). Origins of the Model - C. elegans II. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press

side with the polar body is the side of the maternal pronucleus, which will become the anterior side of the animal. A pseudo-cleavage furrow beings to form at the superior and inferior edge of the cell (figure 3a). The maternal pronucleus slowly begins to move towards the paternal pronucleus. As it crosses the pseudo cleavage furrow, the furrow goes away. The maternal pronucleus then meets the paternal pronucleus on the posterior side of the cell (figure 3b). During this process, the combined nuclei migrate back towards the center of the cell. At the center, spindles begin to form, and the nucleus rotates to form a mitotic spindle to divide the cell anteriorly and posteriorly (figure 3c). The two pronuclei combine and the nuclear envelope breaks down. The genetic material begins to pull apart in anaphase, as two-cleavage furrow form at the superior and inferior edges of the cell. The furrows come together as the nucleus has split into two separate nuclei. The cell separates into two cells as the first round of cytokinesis occurs. The anterior cell is called AB and it is bigger than the posterior cell, which is called P1 (figure 3d). Within each cell, mitotic spindles begin to form once again, starting with the AB cell. Division in the AB cell starts to occur while the mitotic spindle starts to form on the P1 cell (figure 3e). The AB cell splits into ABa (anterior) and ABp (posterior). The P1 cell division follows soon after, it splits into the EMS(inferior) and P2(posterior.) In a wild type embryo, the AB cell always divides before the P1 cell; this is called asynchronous cell division. The cells continue to divide further in a similar fashion, before the larvae begins to form. The various stages of N2 cell division are shown in images I've taken, presented in figure 3. For the purposes on my research, I only pay attention to what is happening in the embryo between the 1-cell and 4-cell stage.

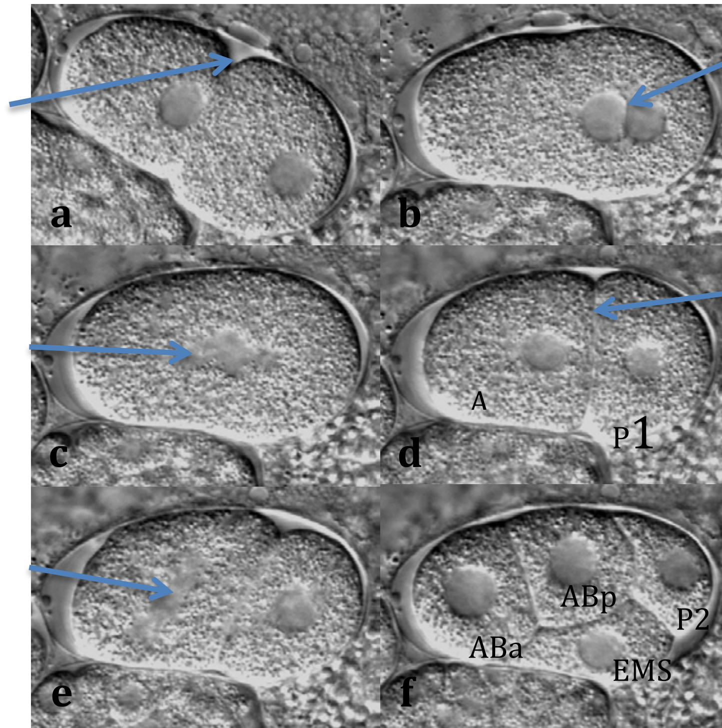

Figure 3. Shows different stages of cell division of an N2 worm, from 1-cell to 4-cell. (a) The arrow point to the pseudo cleavage furrow that originally forms, then goes away when the maternal pronucleus crosses the center. (b) Shows the joining of the maternal and paternal pronuclei. (c) The arrow points to the mitotic spindle expanding out. (d) First cell division, resulting in AB and P1 cells. (e) The arrow shows the cell division of the AB cell in progress, and highlights that this occurs before the division of the P1 cell. (e) Show the cell at the 4-cell stage. The 4 different cells are, ABa, ABp, EMS, and P2.

d. The Temperature Sensitive Screen

A couple years ago, the Bowerman lab did a screen for temperature sensitive mutants using the methods described in a review by Erik Jorgensen and Susan Mango in Nature Reviews Genetics in 2002. The egg laying defective **wild type** mutant hermaphrodite was soaked in the mutagen. Under 15°C conditions, the worm was allowed to self fertilize. The progeny (F1 generation) contained mostly progeny similar to the parents (wild type) while some of the progeny were mutated and were “maternal

effect lethal" (mel). The plates were then moved into a 26°C incubator. The majority of the worms (F2 generation), which were wild type, produced self-progeny that were the same, were "bagged out" due to the temperature. For a worm to bag out, it means that the worm cannot lay eggs, so the eggs hatch into larvae while still inside the parent's body. The body keeps bagging out with larvae, until the larvae break through the body. Other worms of the F2 generation had two possible phenotypes. The first is "bagged out", which comes from two different **genotypes**: wild type, or **heterozygous** for a **recessive mutation**. The second phenotype was the worm filling with dead eggs (F3 generation). Overall, only 10-25% of the singled F2 worms produced live larvae at 15 ^{o}C. These worms had a genotype that was **homozygous** for mel, and since it was a recessive mutation, it displayed the dead egg phenotype. The worms that had the dead eggs were then singled out and moved back into 15°C. Once in the 15°C, the worms were able to continue producing eggs, and under these conditions, the eggs were viable. Each singled out plate became the start of a new strain created, and these are the strains we use in the Bowerman lab today, as temperature sensitive embryonic lethal mutants.

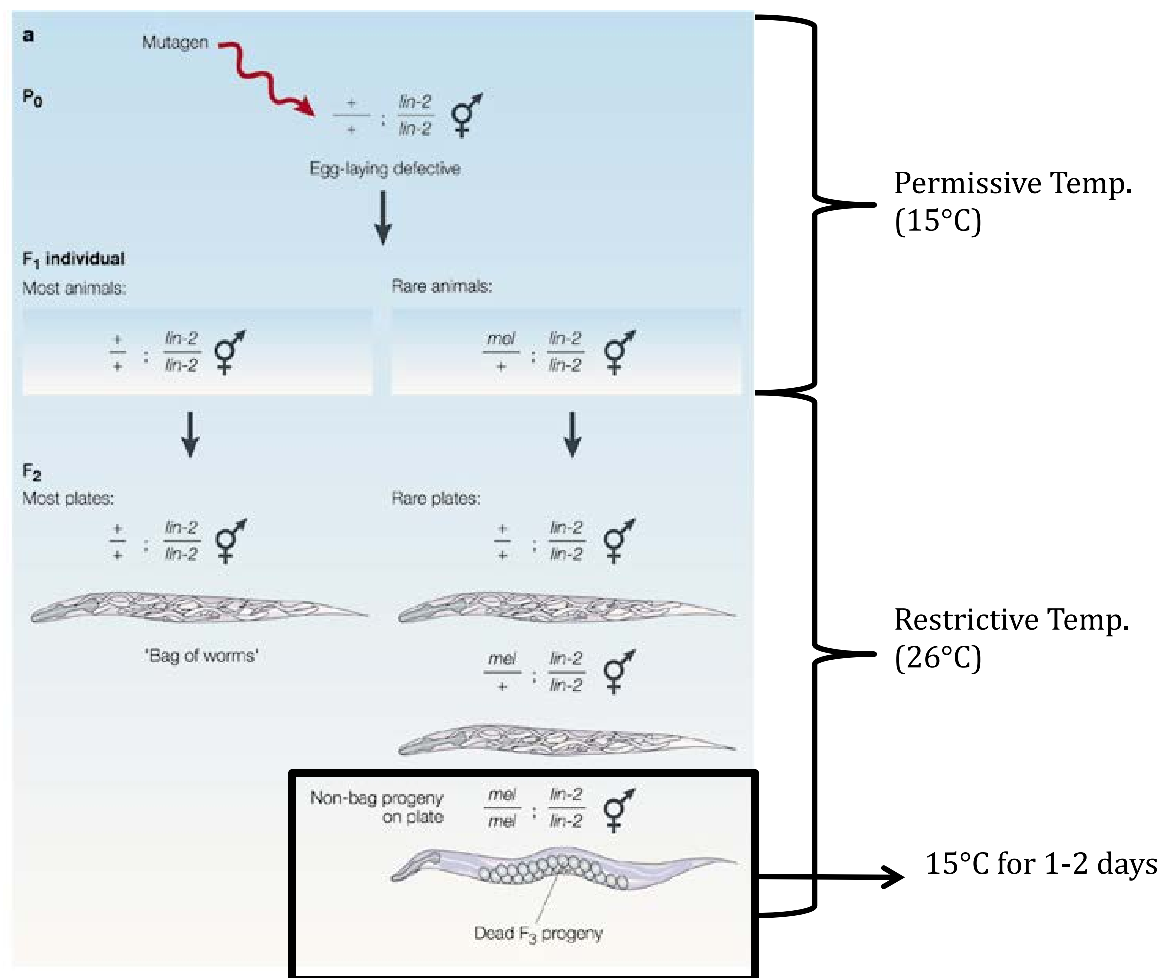

Figure 4. A screen that is similar to the process done to collect the mutants that are used in the Bowerman lab today. This screen image was modified from Erik M. Jorgensen & Susan E. Mango's *The art and design of genetic screens: Caenorhabditis elegans* with modified labels.[7]

e. Cell Cycle and DNA Replication

The cell cycle of a eukaryotic organism consists of 4 distinct phases, synthesis (S) phase, mitosis (M) phase, and two gap (G1, G2) phases as shown in figure 5. The M phase of the cell cycle typically stays constant in length, but there can be varying time

[7] Jorgensen EM1, Mango SE. (2002). The art and design of genetic screens: caenorhabditis elegans. Nat RevGenet. 3(5):356-69.

durations for the other phases. Mutations in the cell cycle can prolong a cell's time in the gap phases because the cell is not ready to move on to the next phase. The cell can become arrested in the G1 phase for a long time, until the cell is ready to replicate its contents in S phase—or even exit the cell cycle if needed at G0 if there are unfavorable growth conditions. During each stage of the cell cycle, there exist cyclins and cyclin-dependent kinases (Cdk), which are complexes that regulate each cell cycle transition.[8] Different cdks and cyclins are specific to each phase. The phases of the cycle can be prolonged if more time is needed to accumulate a certain amount of cyclin protein.

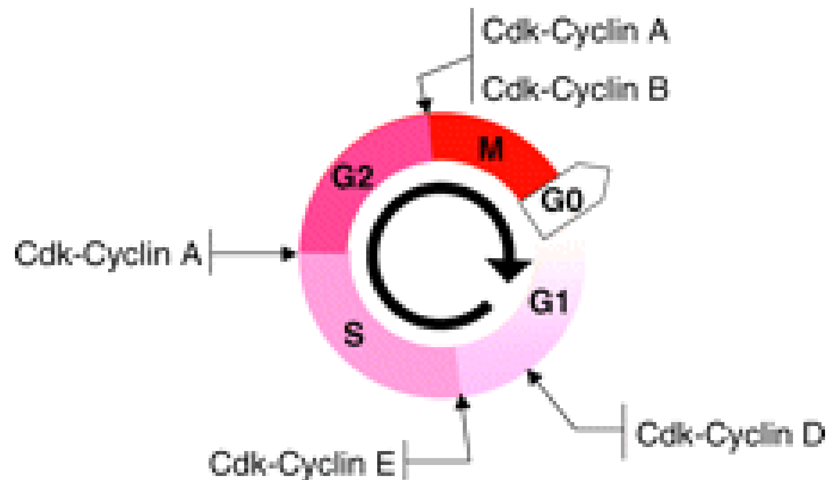

Figure 5. The cell cycle showing where specific Cdk-Cyclin complexes function. This image comes from Yemima Budirahardja and Pierre Gönczy's review, *Coupling the cell cycle to development*.[9]

DNA is replicated in the S phase of interphase during the cell cycle. Mutations in the genes that allow replication of both the DNA and the parts of the cell required for mitosis can prolong the cell cycle in G2. Defects in proteins that control both cell cycle

[8] Budirahardja, 2009.
[9] Budirahardja, 2009.

timing and DNA replication can play a role in prolonging stages of the cycle. One common phenotype that can be caused by of either of these defects is a P1 delay. The P1 cell takes a longer time to begin dividing even after the AB cell has completed cytokinesis. One mutant that expresses similar phenotypic conditions is the *div-1* mutant. "*Div-1* is a gene mutation that encodes for the B subunit of a predicted DNA polymerase ALPHA-primase complex. Defects in DNA replication in *div-1* mutants delay cell divisions, and the delays are accompanied by defects in the early asymmetric cleavages that produce founder cells."[10] The *div-1* mutation can be explained as the following: "In *div-1* mutant embryos, an increased time interval between the divisions of P1 and AB result in a prominent persistent 3-cell stage."[11] Encalada's study also mentioned that these delays could be defined by the length of interphase and mitosis. These are possible mutants to watch out for with mutations that cause cell division delays.

f. Current interest

There is always research being done with cell division defective mutants in *C. elegans*. The research stems from underlying questions in genetics, such as: 1) what is causing the mutant phenotype of a certain strain? 2) Is it a mutation in the genes required for cell cycle timing or DNA replication? Lots of research has been done with mutations in the genes controlling DNA replication, and a lot of research is also done using yeast since they are a much simpler organism- making them easier to work with.

[10] Encalada SE, Martin PR, Phillips JB, Lyczak R, Hamill DR, Swan KA, Bowerman B. (2000). DNA replication defects delay cell division and disrupt cell polarity in early Caenorhabditis elegans embryos. Dev Biol. 228(2):225-38.

[11] Encalada, 2000.

It is more valuable to use *C. elegans* to research defects in the cell cycle because those mutations relate more to developmental factors. The multicellularity of C. elegans add a level of complexity to the cell cycle, that can’t be studied in yeast.

III. Methods

a. Preparation of the Strain

To gather my data, I follow a routine method, with follow up tasks and crosses based on the initial phenotype I identify. I first take the strain of worms I desire to use, let the worms grow until I find a good amount of worms in the L4 stage. I move the L4 worms to a fresh petri dish and transfer them into the 26 ^{o}C incubator. It is important to find the worm at the L4 stage because it is before eggs have begun to develop. These worms have temperature sensitive mutations, so the mutations only become expressed in the eggs if they are fertilized under the restrictive temperature of 26 ^{o}C. The mutations could be changes in the sequence of the gene—a protein with a single amino acid difference from wild-type, due to the mutation changing a codon sequence. At lower temperatures, the mutant protein is stable, but at higher temperatures, there are subtle changes in the amino acid that result in protein unfolding, rendering them non-functional because of a greater thermal motion. The proteins can be made in the germline and gonads before the oocytes are formed or fertilized, or sometimes translated after fertilization. This makes it important to move them to the restrictive temperature early, because the temperature the protein is exposed to is important; the higher temperature destabilizes the mutant protein, inactivating it. I leave them at 26 ^{o}C overnight, or at least 6-10 hours before beginning movies through time-lapse video microscopy.

b. Mounting Early Embryos for Microscopy

Once the worms have grown from L4s to adult worms, with eggs in their body cavity, I take them out and plate them as fast as I can so the change in temperature does not affect the mutation. I pick about five worms, which I can visibly see eggs in, and put them on a cover slip with a drop of M9 on it. M9 is a buffering salt solution that mimics the environment inside the mother worm. This way when the embryos are exposed, they will develop as they would in a maternal environment. I then take a scalpel and cut the worms, aiming to cut them in half so that the eggs pour out. I cover the coverslip with a microscope slide, which had been mounted with agar. Once the slide is complete, I take it under a Nomarski/differential interference contrast (DIC) microscope and look for an egg that looks very early in development under the 4x magnification. Once I think I can see one, I raise the magnification to 60x. Once I confirm that the egg has not begun dividing, I start the movie and let it record until 2 cell divisions are complete (1-cell to 4-cell), with a video frame recorded every 2 seconds. If the egg has already begun its first division, I start looking for a different egg. This process can sometimes be tedious if the worms are older and most of the eggs are developing very fast, therefore from the moment the worms are taken out of 26 ^{o}C to the when they are put under the microscope, the time period must be minimal.

c. Videomicroscopy

The movies record the first two rounds of cell divisions, from the 1-cell to 4-cell stage of development with a video frame recorded every 2 seconds. After recording the movies and editing them for direction of the image, brightness, and contrast I go through each one step-by-step and record abnormal phenotypes that I notice. I repeat

this process so I have made at least 6 movies for each strain I work with. When I compare the movies of the same strain to each other, there are usually phenotypes that seem to be common in most of the movies, and I use this to determine if the phenotype is a known, or common phenotype, or a brand new one. If it is known, and studied excessively, I stop working on the strain at that point, after categorizing it. If it is a new phenotype, which is unrecognized, I begin doing further analysis on them, to see what can be determined about them, starting with an outcross.

d. Outcross

An **outcross** is done following phenotypic analysis. When the worms of the parent strain were originally soaked in the aqueous solution with the mutagen dissolved within it during the screen, the mutagen caused various different mutations in the genome. The purpose of an outcross is to get rid of the background mutations and get the strain down to only the mutation that causes the phenotype. An outcross is performed by mating mutant hermaphrodites with wild-type males and isolating homozygous F2 progeny. Along with trying to get rid of the induced mutation, an outcross also helps to get rid of the *lin-2* mutation, which causes the worms to be egg-laying defective. We use *lin-2* worms for the screen because even though they are egg-laying defective, they still produce ~5% of worms that are capable of laying eggs, allowing us to start crosses. By getting rid of the *lin-2* during the outcross, it makes it easier to perform genetics with because it makes it easier for the worms to mate and lay eggs. I will now go through a step-by-step process of the work I would do with one strain, along with the possible outcomes.

IV. Results

This section includes the phenotypic analysis and various crosses and tests that I have done for some of the strains I have worked with. The following strains have presented interesting phenotypes that are worth pursuing, due to not recognizing their phenotypes or not knowing the origin of the mutation. Strains usually have a varying number of mutant phenotypes, but it is usually the most **penetrant** ones that are the most interesting and focused on.

a. *or1786*ts; *lin-2 (e1309)*- Phenotypic analysis and crosses

During the phenotypic analysis of *or1786*ts; *lin-2 (e1309)*, I noticed several different phenotypes, with one phenotype that was much more penetrant than the others: a P1 delay. A P1 delay is a phenotype in which the AB cell has completed cytokinesis, and the nuclear envelope of the P1 cell has yet to break down to begin cell division. In a WT (N2) embryo, both the AB cell and the P1 cell would have an overlapping period of anaphase, whereas a P1 delay embryo completes the AB cell division before beginning the P1 cell division. This phenotype is shown in figure 6(g-l). In 6(j), it is visible that cytokinesis of the AB cell has completed, while the nucleus of the P1 cell is circular and intact. When compared to the WT images in 6(d) and (e), they show that the P1 cell begins to divide in frame d before cytokinesis of AB occurs, and once cytokinesis of AB does occur in frame e, the P1 cell is well on its way through mitotic cell division. In the figure, each column shows frames captured at similar stages of cell division of the embryo, but the time at which they are captured is very different. By the time it take N2 to reach the 2-cell stage in 6(c), the nucleus of *or1786*ts; *lin-2 (e1309)* P0 is still in the

process of cell division. By the time the N2 strain has reached the 4-cell stage, *or1786*ts; *lin-2 (e1309)* is only at the 2-cell stage. This shows that the whole cell cycle of the mutant has been significantly slowed down.

I also observed some other phenotypes in my movies of the embryos of *or1786*ts; *lin-2 (e1309)*, which were not very penetrant; these are presented in table 1. A large polar body is when the polar body, which is a small cell containing cytoplasm that is located on the maternal side of the cell, is enlarged. Uneven pronuclei are when the maternal and paternal pronuclei are different sizes; this means that there are uneven amounts of genetic material from the paternal side and the maternal side. Cytoplasmic clearings are clearings that are visible in the cytoplasm of the cell; they sometimes look like nuclei, but are not confined to a particular shape. These other phenotypes are not displayed in figure 6.

Table 1. Frequency of phenotypes in *or1786*ts; *lin-2 (e1309)* and *or1786*ts *(outcrossed)*

	Frequency in *or1786*ts; *lin-2 (e1309)*	**Frequency in *or1786*ts *(uncrossed)***
P1 delay	5/6	2/7
Large polar body	1/6	–
Uneven pronuclei	1/6	1/7
Cytoplasmic clearing	1/6	–
Multinucleated cells	–	1/7
WT	–	4/7

I completed an outcross for this strain twice and found segregation frequencies of 3/39 (7.7%) and 7/37 (18.9%). The outcross was repeated for a second time because the first frequency was very low compared to an expected 25%. Repetition of the experiment determined if indeed the result was real, or if there was error in the procedure. The expected segregation frequency is about 1/4 (25%) because with a recessive mutation,

heterozygote parents would be expected to make 1/4 of their offspring homozygous recessive and visibly show the embryonic lethality. The second outcross revealed a segregation frequency closer to what was expected. Next I had to select homozygous mutant animals that did not carry the lin-2 mutation. I singled out ~20 L4 hermaphrodites from one of my plates which produced embryonic lethal mutants at the restrictive temperature. After monitoring the plates over a couple days, I picked out a plate that produced no egg laying defective worms, which could be identified by worms that were "bagged-out". The plate I picked was what I used as the outcrossed or1786ts; *lin-2 (e1309)*, and I completed phenotypic analysis to see if the penetrant phenotype would hold up.

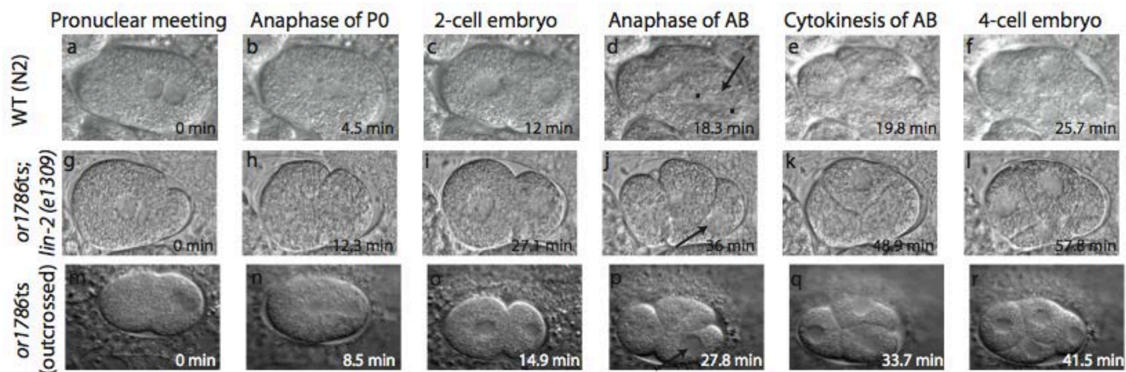

Figure 6. Different stages of cell division for the mutant *or1786*ts; *lin-2 (e1309)*, *or1786*ts *(outcrossed)* and compares them to WT. The figures listed for the two or 1786 mutants only show frames from embryos that depict P1 delays.

b. *or1786*ts (outcrossed)- Phenotypic analysis

In examining the phenotypes after the outcross, my phenotypic analysis of *or1786*ts *(outcrossed)* showed that the initially penetrant P1 delay was not the most penetrant phenotype, but it still carried through the outcross. The other phenotypes observed were uneven pronuclei, multinucleated cells that are multiple nuclei in the

place of a single nucleus at different cell stages, and WT. The frequencies are listed in table 1. A completely WT phenotype was the most penetrant outcome post-outcross, but the P1 delay still held up in two of the movies. Reasons for this result could be that there were multiple mutations in the original strain, which were not present in the outcrossed strain. The combination of mutations in the original strain may have, in combination, caused the P1 delay phenotype more often than in the outcrossed strain, which had fewer mutations. The P1 phenotype is shown in figure 6 (p) where the P1 nucleus has not yet begun to divide when the AB cell has completed cytokinesis. Similar to *or1786*ts; *lin-2 (e1309)*, the timing of the cycles for *or1786*ts *(outcrossed)* is delayed in comparison to the WT (N2) movie. This shows that after the outcross, the prolonged cell cycle exists, suggesting that the mutation is in a gene controlling the cell cycle and extending one of the stages.

c. *or1700*ts; *lin-2 (e1309)* - Phenotypic analysis and crosses

The *C. elegans* strain with the allele *or1700*ts; *lin-2 (e1309)* is an interesting mutant that displayed an array of phenotypes, and some were highly penetrant. The phenotypes I recorded for this strain are shown in Table 2, which lists each of their frequencies. The most penetrant phenotype for this strain was kissing nuclei. This phenotype is evident immediately following cytokinesis; the two parts of the nucleus that had already divided were still trying to stay together. This phenotype can be seen in figure 7(k). The kissing nuclei phenotype, along with multinucleated cell phenotype are characteristic of a chromosome segregation defect. Other phenotypes were also present, such as failure in cytokinesis as shown in figure 7(k and l), abnormal cleavage furrows, which are cleavages appearing at abnormal times in abnormal locations (not during

cytokinesis), and P1 delay. Figure 7 shows images of an *or1700*ts; *lin-2 (e1309)* embryo with P1 delay. Although the P1 delay in figure 7(j) was not the most penetrant phenotype, it was interesting that it appeared along with the chromosome segregation defect. Similar to the strains of *or1786*ts, *or1700*ts; *lin-2 (e1309)* has a slowed overall cycle. At the time the WT (N2) embryo has reached the 4-cell stage, the mutant P1 cell is still undergoing anaphase. The time difference is not quite as extreme as *or 1786*ts, but there is still a clear difference. *or1700*ts; *lin-2 (e1309)* could be a *div* mutant with the given phenotype. “While chromosome segregation in *div* mutants has not been directly examined, in some *div* mutants mitosis is defective due to incomplete DNA replication. Consistent with this possibility, segregation defects have been reported in budding yeast mutants that fail to replicate DNA but nevertheless undergo mitosis.”[12]

Table 2. Frequency of phenotypes in *or1700*ts; *lin-2 (e1309)* and *or1700*ts *(outcrossed)*

	Frequency in *or1700*ts; *lin-2 (e1309)*	**Frequency in *or1700*ts *(outcrossed)***
P1 delay	1/6	5/6
Kissing nuclei	6/6	–
Multinucleated cells	5/6	1/6
Abnormal cleavage furrow	2/6	–
Failure in cytokinesis	2/6	–
Uneven pronuclei	–	1/6

I did further testing on this strain, after outcrossing it I found a 7/38 (18 %) segregation frequency. I would expect to have around a 1/4-outcross segregation frequency, suggesting that only a single gene is affected—so the 7/38 segregation frequency was within a range. Now that the background mutations had been removed, I

[12] Encalada, 2000.

screened to find a plate without the *lin-2* mutation and gave it a new strain name. This strain would only have the mutation represented by the phenotype (homozygous *or1700*ts.)

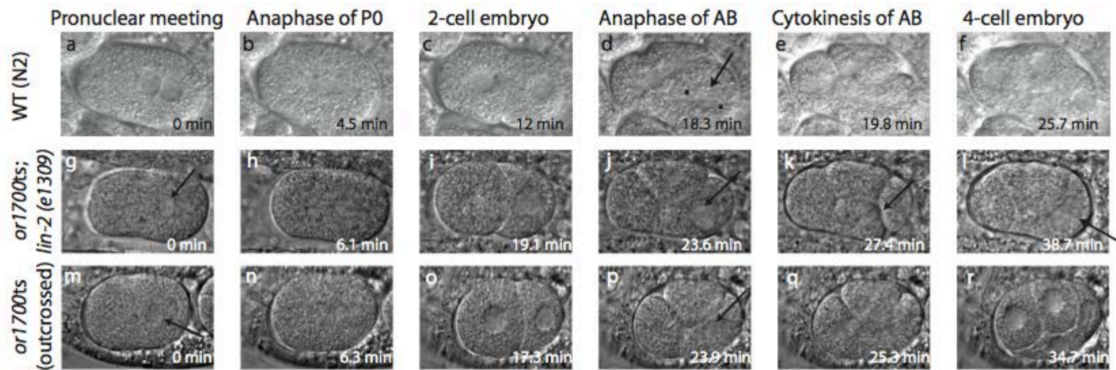

Figure 7. Stages of cell division for the mutant *or1700*, *or1700 (outcrossed)* and compares them to WT. The figures listed for the two *or 1700* mutants only show frames from embryos that depict P1 delays. The figures for *or1700* also show a failure in cytokinesis in frames k and l.

d. *or1700*ts (outcrossed) – Phenotypic analysis

After renaming the strain following the outcross, I had to repeat phenotypic analysis to make sure the outcross had kept the mutation that expressed the exhibited phenotype. Surprisingly, the chromosome segregation defects did not persist after the outcross, but the P1 delay became very penetrant. The multinucleated cell phenotype also was present after the outcross but it was a lot less penetrant, and a new uneven pronuclei phenotype appeared. The lack of the chromosome segregation defects, and strong penetrance of the P1 delay could be due to the fact that the original strain contained a double mutant. The multiple mutations in the original strain could have interacted to cause a different phenotype, which would not be present if there were only one mutation. Though there were some differences, *or1700*ts, both before and after

outcrossing, had prolonged time duration. While the WT (N2) embryo has reached the 4-cell stage, the mutant *or1700*ts P1 cell is still undergoing anaphase. The outcross could have gotten rid of one mutant while keeping the other. To be able to determine an explanation, the strain will need to be re-outcrossed.

e. *or1700*ts – Next steps

A future step for this strain is to determine the cause of the P1 delay. This requires looking at spinning disk confocal movies under fluorescence in a two-color background. The term "two-color" background refers to using two different fluorescent protein markers to label specific cell structures in living animals. In this case, we would be using mCherry:Histone and GFP:ß-tubulin. Histone markers are located on the nucleus, and the ß-tubulin makers are on the spindles. It helps to have them on two different colored backgrounds to easily differentiate between the two. This will help me determine the nature of the P1 delay to show which stage of the cell cycle is being affected. During the phenotypic analysis, the evaluation of the phenotype was based on qualitative observation with a phenotype that is hard to confirm without looking at specific markers. Looking at the markers allows me to determine how the P1 delay happened in a more quantitative manner. After checking the markers in my *or1700*ts *(outcrossed)* strain, I found that it only had the histone marker on it under GFP. I found a strain in the database that had mCherry:ß-tubulin, which I then started to cross with my strain. By crossing these strains, I will eventually get both markers and the mutation all in one strain. The markers and mutations are all assumed to be on different chromosomes, so there will be a very small number of progeny at the F3 generation.

Unfortunately, there was not enough time to complete this cross, so it will be a future direction to take with this strain.

f. *or1578*ts; *lin-2 (e1309)*- Phenotypic analysis and crosses

Phenotypic analysis of *or1578*ts; *lin-2 (e1309)* presented a different set of phenotypes. The most penetrant phenotype was a lack of pseudo-cleavage. In a WT (N2) embryo, there is a pseudo-cleavage at the 1-cell stage that goes away once the maternal pronucleus crosses the center of the cell. Other phenotypes the mutant had in addition to the lack of pseudo-cleavage are included with frequencies in Table 3. There was a misshapen maternal pronucleus; instead of an abnormal size, the shape was skewed. The late P0 rotation refers to the rotating of the nucleus once the two pronuclei have combined; this "lateness" could also extend the cell cycle duration. The failure in P1 rotation refers to the nucleus of the P1 cell not rotating, and as a result causing cytokinesis along the horizontal axis instead of the vertical—though it does not refer to this strain, figure 7 (p and q) show this. Unlike the strains *or1786*ts and *or1700*ts, the *or1578*ts; *lin-2 (e1309)* strain does not extend the duration of the phases quite as long. In the figures, the timing for AB cell cytokinesis and P1 cell cytokinesis seem fairly close to WT. Scoring individual movies of *or1578*ts; *lin-2 (e1309)* shows that 4/6 embryos (shown in table 4) have cytokinesis of the AB cell and cytokinesis of the P1 cells within less than a minute of each other.

Table 3. Frequency of phenotypes in *or1578*ts; *lin-2 (e1309)* and *or1578*ts *(outcrossed)*

	Frequency in *or1578*ts; *lin-2 (e1309)*	**Frequency in *or1578*ts *(outcrossed)***
Pseudo-cleavage furrow	7/9	4/6
Misshapen pronuclei	1/9	–

Late P0 rotation	1/9	1/6
Failure of P1 rotation	2/9	3/6
Arrest at 2-cell	2/9	–
Pronuclei meeting in center	–	2/6
Synchronous 2nd division	–	3/6
WT	1/9	1/6

The strain was then outcrossed, and resulted in having an 18/60 (30%) segregation frequency. It was a little higher than the 1/4 expected segregation frequency, so I repeated the last part of the outcross with the 18 plates. One plate was misplaced in the process, so I found that 13/17 plates had dead embryos, making the new segregation frequency 13/59 (22%). Next I screened the 13 plates for the lin-2 mutation, so the plate did not carry it (had no worms that were egg-laying defective).

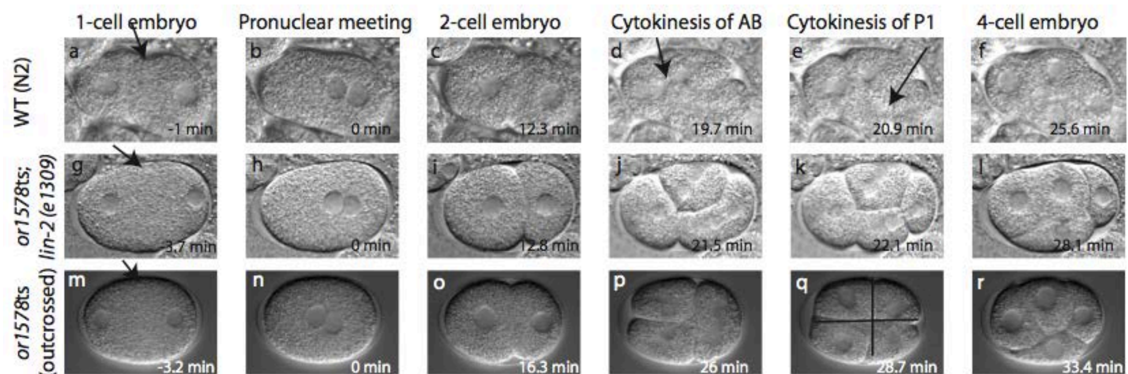

Figure 8. Shows the stages of cell division for the mutant *or1578*ts; *lin-2 (e1309)*, *or1578*ts *(outcrossed)* and compares them to WT. The figures listed for the two *or1578*ts mutants show the lack of pseudo-cleavage furrow phenotype. The figures of *or1578*ts*(outcrossed)* show the failure in P1 rotation in frames p and q.

g. *or1578*ts (outcrossed)- Phenotypic analysis

After completing the outcross, I did phenotypic analysis for movies of the embryos again and found that some phenotypes remained very penetrant, some disappeared, and new ones surfaced. The phenotypes and their frequencies are

displayed in Table 3. The most penetrant phenotype was still the lack of pseudo-cleavage. The failure of P1 rotation phenotype also persisted and became more penetrant, and is shown in figure 8 (p and q). A new phenotype was the pronuclei meeting at the center. In a WT (N2) embryo, the pronuclei meet on the posterior side, towards the paternal side. Once they meet, together they migrate towards the center; the phenotype observed can suggest there is some mutation in the genes coding for the mitotic spindles. The last new phenotype observed was a synchronous 2nd division. In WT (N2) the AB cell begins to divide first, and P1 cell division begins shortly after when the AB cell is still in anaphase. In a synchronous division, it looks like the AB and P1 cells are dividing at the same time with not much lag between them. This could mean that there is a mutation that is causing the AB cell to lag, or the P1 cell to speed up. The timing of the individual movies of *or1578*ts *(outcrossed)* shows that 2/6 embryos (shown in table 4) have cytokinesis of both the AB cell and the P1 cell within less than a minute of each other—close to synchronous cell division. The extra phenotypes that came up after the outcross could be due to other mutations that were in the original strain were influencing the phenotype. The outcross could have gotten rid of the those mutations, causing the temperature sensitive mutations which are still causing lethality to show a different effect due to the difference in genotype at other locations in the genome. The individual mutant phenotypes in these strains have mostly tended to change duration of the cycles. Those changes are also important to look at while trying to determine the mutation behind the phenotype.

Table 4. Time duration till cytokinesis at P0, AB, P1 in mutants, *or1578*ts; *lin-2 (e1309) and or1578*ts *(outcrossed).* (Bolded rows are those with AB and P1 divisions within a minute of each other.) T=0 starts at pronuclear meeting. Bolded numbers show the synchronous embryos.

	Time (min) to cytokinesis in *or1578*ts; *lin-2 (e1309)*			**Time (min) to cytokinesis in *or1578*ts *(outcrossed)***		
	P0	**AB**	**P1**	**P0**	**AB**	**P1**
1	**8.5**	**14.5**	**15.2**	10.7	15.6	18
2	**11.4**	**14.2**	**15.1**	13.1	**15.6**	**16.4**
3	**9.7**	**11.5**	**12.4**	11.4	14.3	17.5
4	11	12.9	14.9	14.6	15.6	17.5
5	9.4	**13.8**	**14.8**	11	12.5	14.3
6	11.7	16.4	17.6	12.4	**15.5**	**16.5**

h. Time duration

A common theme throughout all these mutants is cell cycle timing. The time duration for particular stages in the mutants seems to be extended over all, while comparing within mutant durations get longer or shorter. The durations between different checkpoints are displayed in figure 9.

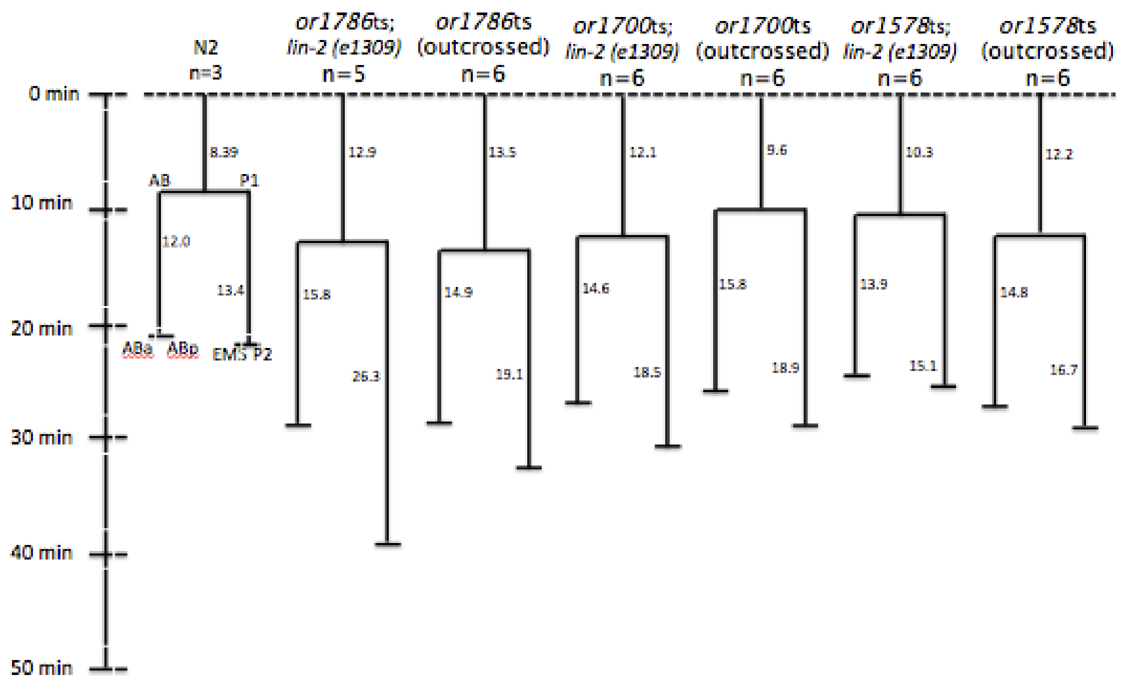

Figure 9. Displays the cell lineages of wild type and mutant embryos. Time 0 is at the moment the maternal and paternal pronuclei first meet. The first vertical line determines the length from pronuclear meeting to the first cell division (cytokinesis), and the next set of vertical lines further down represent the length of time till the seconds divisions are complete.

In the figure, the first vertical line represents the time from pronuclear meeting (t=0) to cytokinesis of P0. The vertical line on the left is the time from cytokinesis of P0 to cytokinesis of AB, and the line on the right is cytokinesis of P0 to cytokinesis of P1. From observing the figure, it is evident that both the *or1786*ts and the *or1700*ts mutants have very clear P1 delays. The *or1578*ts mutants, though they have an overall longer duration from pronuclear meeting to the 4-cell embryo, have closer periods in time for cytokinesis of AB and P1—similar to WT (N2). The *or1578*ts mutants were ones that had closer to a synchronous 2nd division in some of the phenotypic movies, but since it is an averaged value, the embryos with the more symmetric divisions are not as evident in the figure.

V. Future Directions

Of the mutants discussed many of them are P1 delay mutants. Phenotypic analysis and crosses have shown that the P1 delay has been a fairly penetrant mutation that has held up in the strain. The next steps for these mutants, *or1758*ts and *or1700*ts, are to determine the reason for the P1 delays witnessed. Further crosses will be done with these strains to add visual fluorescent markers that can be used to determine the movement of individual parts of the cell that are not visible with Nomarski video microscopy. The P1 delays could have been result of mutant genes that encode known components of the multiprotein complex DNA Polymerase, which replicate DNA, or encode a known cell cycle regulator—making the defect due to defects in cell cycle regulation and not DNA replication. If it is neither of those, it could be a gene of an unknown function, meaning that a new requirement for a gene's function that was not previously understood could be discovered. Since the penetrant phenotype in *or1786*ts did not hold up very well after outcrossing, this mutant may be put aside for a while or re-outcrossed to see if it results in a more penetrant phenotype—making it more worthwhile to continue working with. The other strain, *or1578*ts, which had synchronous second divisions, will be analyzed more with different crosses to help determine the nature of the mutation. With *or1578*ts, there are many other phenotypes that are more significant which other members of the lab will look at more closely. These other phenotypes are not associated with immediately recognizable mutants, but could be determined after more testing.

For the strains that had penetrant phenotypes after outcrossing; *or1700*ts and *or1578*ts, cloning the gene will be the top priority. This will allow us to identify what

gene in the genome is being mutated and responsible for the defect, allowing one to understand the role of the gene in the affected process. Understanding the gene in the affected process will allow one to ultimately infer the normal role of the gene; the initial purpose of these studies. By understanding normal function, the genes responsible for function in cell division can be found and used as targets for future cancer drug research.

VI. Glossary

Phenotype- The physical appearance of an organism, determined by genetic composition and environmental factors.

Pronucleus- The genetic material from the hermaphrodite (mother) and male (father) separately.

Wild type- An organism/ gene which has characteristics of the species that are naturally occurring in a population.

Genotype- An organism's genetic make up, a result of the alleles received from each parent.

Heterozygous- A combination of two different alleles in a genotype.

Recessive mutation- A mutation which occurs only when both alleles in the genotype are the recessive allele.

Homozygous- A combination of two od the same alleles in a genotype.

Outcross- Cross between the mutant and wild type organism to get rid out background mutations.

Embryonic lethality- Observing dead eggs.

Penetrant- Refers to the phenotype which appears a majority of the time.

P1 delay- A phenotypic mutation in which cytokinesis has finished between the ABa and ABp cells before mitotic spindles have even formed in the P1 cell.

or1xxxts; *lin-2 (e1309)*- This is the notation for the allele with the *lin-2* mutation (before outcrossing).

*or1xxx*ts- This is the notation for the allele without the *lin-2* mutation (after outcrossing).

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