Identifying Genes Required for *Caenorhabditis Elegans* Embryonic Morphogenesis

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Introduction

During embryogenesis of all multicellular organisms, cells must propagate, undergo differentiation, and migrate to give rise to tissues, organs, and the general body plan. The three tenets of development (cell growth, differentiation and morphogenesis) are imperative to produce a complete organism. Morphogenesis, originating from the Greek word meaning "beginning of shape," refers to coordinated tissues movements including migration, contraction, folding, and fusion to shape an embryo and form its organs. Disruption of morphogenesis causes a variety of birth defects in humans, including vascular irregularities, neural tube closure, and limb developmental defects (Roberts et al., 2004; Sabapathy et al., 1999;

Dudley et al., 1995). Therefore, understanding the genes and signaling pathways required for morphogenesis to properly occur is of great importance.

Caenorhabditis elegans is a small nematode that for a number of reasons is an excellent genetic model for studying morphogenesis. Perhaps the most compelling reason to view *C. elegans* as a powerful model organism for genetic studies of genome function is that it has high genetic conservation with humans: 83% of the worm proteome is homologous to that of humans (Lai et al., 2000). Second, the worms can be easily grown in a petri dish. The nematode has two sexes, hermaphrodites and males. Hermaphrodites produce both sperm and eggs; therefore, they procreate by either self-fertilization or cross-fertilization. Self-fertilization can simplify the maintenance of stocks because hermaphrodites can give rise to an entire population without the need of males. Further, *C. elegans* has large brood sizes, and a single hermaphrodite can give rise to about 300 progeny. These worms can be stored as frozen stocks making them cheap and easy to maintain. Not only are the worms easy to cultivate and require low maintenance, they also have quick generation times and rapid life cycles, taking only 4 days at 20°C to go from a fertilized egg to a reproductive adult. This rapidity reduces

the waiting periods necessary to obtain reproductive adults, and the rapid generation time allows for faster genetic results. They are also completely transparent, another key virtue that allows us to easily image and analyze morphogenesis. All these features make *C. elegans* a powerful model for genetic dissection of morphogenesis, which relates directly to human health and disease (Ahringer, 2006).

The tenet of morphogenesis is the process of cell shape changes and migration. Cells can change shape, migrate, or rotate, and each change alters the way tissues are organized. Thus, small changes at the cellular level can lead to large changes at the tissue level in forming the shape of an organism's body and organs. Although morphogenesis in *C. elegans* remains only partially understood, many of the basic processes have been characterized and are shared with other organisms, including epiboly, cell intercalation, and apical constriction. Development of the epidermis has been the main tissue studied to understand morphogenesis in *C. elegans* (Andrew and Hardin, 2005), which is broken into three main stages: gastrulation, ventral closure of the epidermis, and elongation (Figure 1).

During early animal development, most embryos go through a process called gastrulation (Figure 1). During gastrulation, cells divide and invaginate to create a tiny blastopore (Wassarman, 2011);

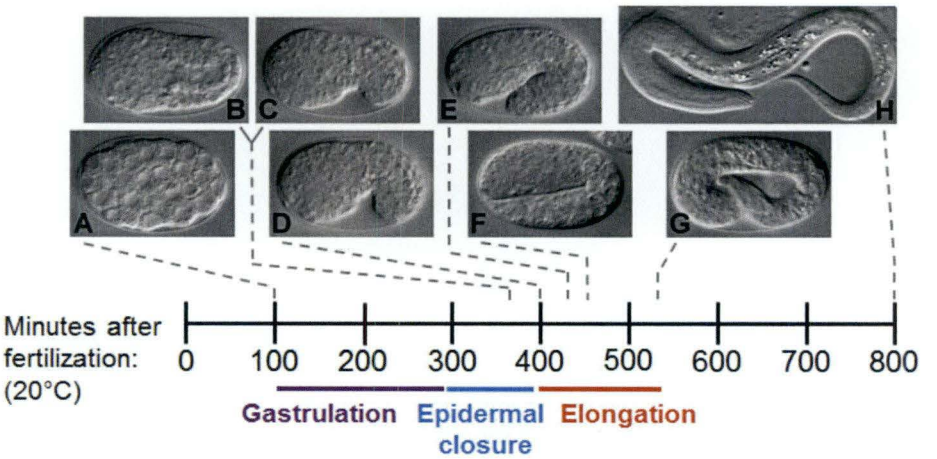

Figure 1: Morphogenesis stages during embryogenesis at 20° C. (A) 26-cell embryo, (B and C) bean stage [1.25-fold], shown ventrally (B) and laterally (C), (D) comma stage, (E) tadpole stage [1.5-fold], (F) plum stage [2-fold], (G) pretzel stage [3-fold], and (H) fully elongated L1 worms that hatched from their eggshell. Timeline based on Sulston et al (1983).

thus, cell movement is essential for gastrulation. Transcription factors are necessary to specify tissue precursors and activate actin cytoskeleton regulating genes. (Wassarman, 2011). Gastrulation in *C. elegans* involves the specification of three germ layers that are the initial tissue layers of the embryo: (1) ectoderm that gives rise to hypodermis and neurons, (2) mesoderm that generates pharynx and muscle, and (3) endoderm that gives rise to germ line and intestine (Wassarman, 2011).

Following gastrulation is epidermal morphogenesis, which is broken down into two events: dorsal cell intercalation and ventral

enclosure (Figure 1), both of which require actin microfilaments and microtubules for cell shape changes. The first morphogenetic event to occur in the epidermis is intercalation of dorsal epidermal cells. Epidermal cells are born at the end of gastrulation as a group of cells on the posterior and dorsal surface of the embryo and form two cellular rows (Heid et al., 2001; Schierenberg et al., 1983). These dorsal cells become wedge-shaped as they begin intercalation, eventually adopting a "ladder like" arrangement in the dorsal midline. After completion of dorsal intercalation, the lateral epidermal cells on each side of the embryo migrate toward the ventral midline and fuse to enclose the embryo in epidermis. Ventral enclosure takes place in two phases (Raich et al., 1999). In the first phase, the two most anterior epidermal cells on each side of the embryo, called the "leading cells," migrate by extending long protrusions toward the ventral midline. They eventually meet and establish contact. In the second phase, the posterior cells form a continuous actin-rich border around the "ventral pocket." The pocket cells become wedge-shaped and initiate contractions to fully enclose the epidermis through a "purse-string" mechanism.

Once ventral enclosure is completed, the embryo elongates from the lima bean shape to the characteristic worm shape, resulting in

a fourfold increase in length (Figure 1). Elongation is the least understood of the three morphogenetic developmental stages in *C. elegans.* During elongation, epidermal cells modify their shape. About midway through elongation, muscles start to contract, an change that is essential because muscle-defective embryos are paralyzed and their development arrested at the 2-fold stage (Waterson, 1989; Williams and Waterson, 1994). Elongation can be divided into an early and late phase relative to the beginning of muscle contractions, with each stage mediated by distinct molecular pathways. During early elongation, genetic and molecular data suggest that actomyosin contractility is essential, and that actin filaments are necessary for cell shape changes and include the actin cytoskeleton as well as proteins that regulate actin dynamics, such as Rac, WASP, and Arp2/3 (Andrew and Hardin, 2005). During late phase elongation, muscle cells play a central role, and actomyosin contractibility in the epidermis is thought to be less important (Wassarman, 2011). Muscle contractions create a longitudinal tension that is transmitted to the epidermis, causing the dorsal and ventral cells to become locally compressed and stretched longitudinally (Wassarman, 2011), and allowing the body shape of the worm to lengthen.

One reason that morphogenesis in *C. elegans* remains only par-

tially understood is that many genes have dual roles in its development. If a gene has a requirement earlier in development before morphogenesis occurs, that gene may be hard to link to morphogenetic processes. Although RNAi has provided a rich tool for conditionally knocking down genes to study morphogenesis, that method can be inefficient and create weak phenotypes. Furthermore, forward genetic screens that are labor intensive have likely not been saturated. (Andrew and Hardin, 2005). Thus, there is a lack of conditional, loss-of-function, stable genetic mutants in the field.

Bruce Bowerman's lab is performing a forward genetic screen for temperate-sensitive (TS) mutant alleles that are powerful tools for elucidating gene function. These TS alleles are conditional, based on temperature and loss-of-function genetic mutations. Worms grown at the permissive temperature of 15°C develop and reproduce like wild-type worms, but worms grown at the restrictive temperature of 26°C lay embryos that fail to hatch. Simply by controlling the temperature of the TS mutant alleles, researchers can induce a mutant phenotype at different stages of development. In this manner, early essential requirements can be by-passed by growth at a permissive temperature, with later essential functions then studied after temperature increases. About 1000 TS mutant alleles have already been

generated by chemical mutagenesis in the Bowerman Lab and given a unique *or* number, such as *or1330*ts. This collection has been successfully screened for early cell division mutant alleles (Connolly et al., 2014, Connolly et al., 2015) and for germline membrane and eggshell defective mutant alleles (Lowry et al., 2015).

By screening the Bowerman Lab's TS mutant collection for morphogenetic mutant alleles, I aim to achieve two goals: (1) to discover new alleles of genes known to affect morphogenesis, and (2) to discover new genes that have never been identified to affect morphogenesis. Currently, I have found two morphogenetic genes: *rib-1* (*or1193*ts) that functions in heparan sulfate proteoglycan biosynthesis and *emb-4* (*or1330*ts) that may have a role in regulating transcription. These findings may contribute to the endeavor to better understand the genes and pathways that control morphogenesis.

Materials & Methods:

Worm maintenance and generation of mutants

All strains were maintained following standard procedures (Brenner, 1974). The TS alleles were generated in a *lin-2* (*e1309*) mutant strain using chemical mutagenesis (ENU) as previously described (Kemphues et al., 1988; Encalada et al., 2000; O' Rourke et

al., 2011). Mutant *lin-2* (*e1309*) worms produce a defective vulva and are unable to lay eggs, thus leading to a classic "bag of worms" phenotype (Kenyon et al., 1983). This mutation makes it easy to decipher TS embryonic lethal strains at the restrictive temperature of 26°C.

Phenotypic analysis

Morphogenetic alleles were screened and mapped to genes according to Lowry et al. (2015). Briefly, I first screened the mutant collection for alleles that exhibit defective morphogenesis at the restrictive temperature of 26°C using Nomarski optics. The L4 worms were upshifted to 26°C for a minimum of eight hours. Eggs were harvested and 1-8 cell-stage embryos were mounted on 4% agarose pad slides before being returned to 26°C. About 16 hours later, embryos were scored for developmental arrest and penetrant alleles were kept for genetic testing.

Genetic Tests

Standard genetic tests were performed to identify recessive and mendelian segregating alleles of each strain. Genetic tests were performed by setting a cross between homozygous mutant hermaphrodites and N2 (Bristol) wild-type males (Figure 2A). This cross is

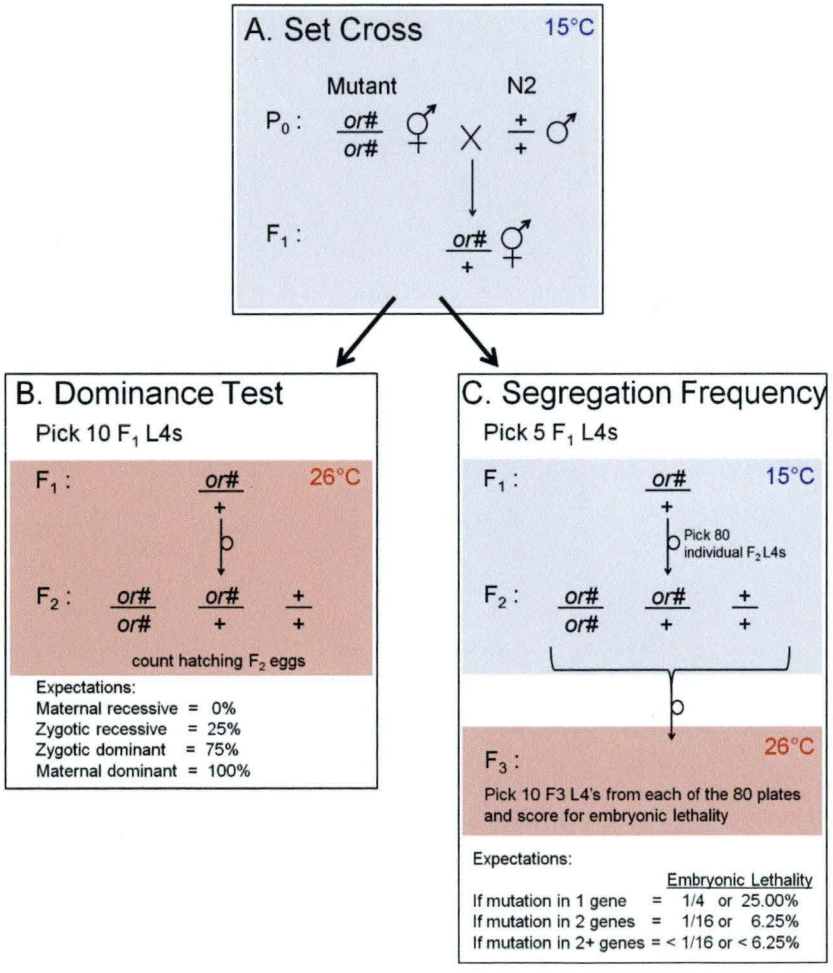

Figure 2: Crossing scheme for genetic characterization. (A) For both the dominance test (B) and the segregation frequency (C), and initial cross is set between a homozygous mutant hermaphrodite and a wild-type (N2) male to generate heterozygous F_1 hermaphrodites. (B) Dominance test. 10 heterozygous F_1 hermaphrodites are upshifted to 26°C and embryonic lethality is scored. Expectations of embryonic lethality for recessive, dominant, maternal and zygotic requirements are listed. (C) Segregation frequency. 5 heterozygous F_1 hermaphrodites are picked and allowed to self at 15°C. 80 individuals F_2 L4s are picked from a single F_1 and allowed to self at 15°C. 10 F_3 L4s are picked from each F_2 parent, upshifted to 26°C, and scored for embryonic lethality. Homozygous F_3 mutants will have 100% embryonic lethal progeny. Expectations for mutation at a single locus versus at multiple loci are listed

important for three reasons: (1) it allowed the leaky *lin-2* (*e1309*) mutation to be removed from the genetic background of the TS allele, (2) it permitted the identification recessive versus dominant alleles (Figure 2B), and (3) it distinguished strains that have one mutant allele instead of multiple mutant alleles (Figure 2C). After crossing to N2, I confirmed that the mutant phenotype of the allele was maintained by scoring embryonic lethality at 15°C and 26°C.

Identification of causative genes

After the genetic testing, I mapped the causative gene for each TS allele by whole genome sequencing according to Lowry et al (2015). Homozygous mutant hermaphrodites were outcrossed to *CB4856* males, a strain derived from Hawaii (Hw) with a unique array of single nucleotide polymorphisms (SNPs) compared to the N2 (Bristol) strain (Figure 3). About eighty F_2 worms were singled out and homozygous mutants identified by picking ten F_3 L4 hermaphrodites from each F_2 plate and incubating at 26°C for three days. Synchronized L1s were obtained by pooling gravid adults from homozygous mutant F_2 plates and destroying contaminant bacteria DNA by hypochlorite treatment. These L1 worms were used for genomic DNA extraction (Qiagen DNeasy Kit). Libraries were prepared using the KAPA Hyper Plus Library Preparation Kit

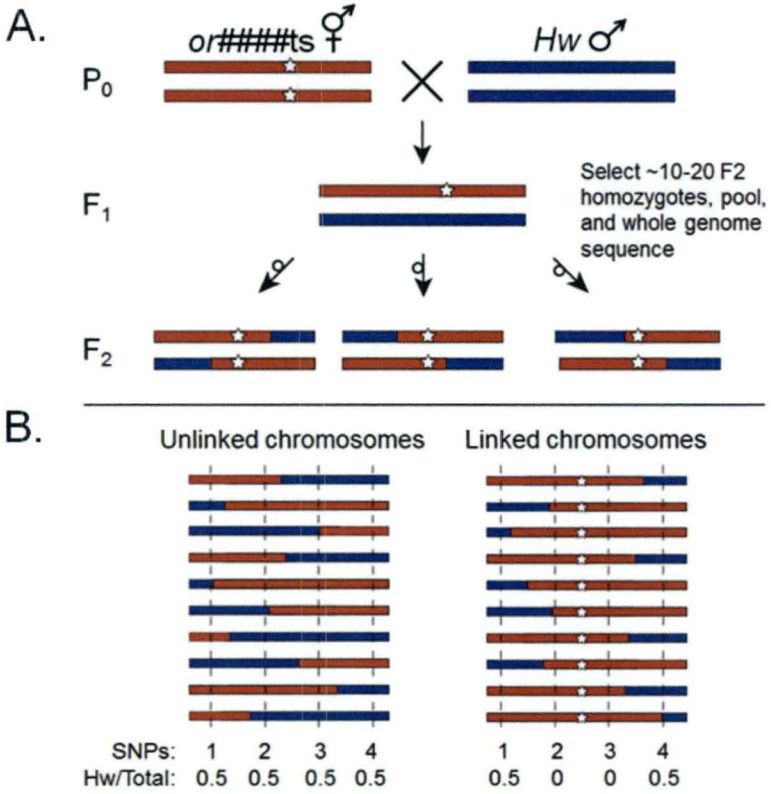

Figure 3: Crosses scheme and analysis for whole genome SNP mapping. (A) A hermaphrodite harboring a homozygous mutation (star) in a wild-type (N2) background (red chromosome) is mated with a Hawaiian (Hw; blue chromosome) male. P_0 will yield F_1 worms that are heterozygous for all chromosomes. The F_1 worms will then self-fertilize, and during meiosis, recombination will occur randomly to give rise to the F_2 generation. We select for 10-20 homozygous F_2 worms and send their progeny for whole genome sequencing. (B) Interpretation of mutationally unlinked and linked chromosome analysis. In an unlinked chromosome, we will see 50% Hw/Total SNPs. In our linked chromosome, we will see 0% Hw/Total SNPs (i.e., where the mutation of interest is located).

and Illumina TruSeq Adapters. Sequencing was performed by using the Illumina HiSequation 2000 at the Genomics and Cell Characterization Core Facility at the University of Oregon. Alleles were mapped using the Galaxy platform (Giardine et al., 2005; Goecks et al., 2010; Menevich et al., 2012; Langmead and Salzberg, 2012; DePristo et al., 2011; Cingolani et al., 2012; Quinlan and Hall, 2010). Candidate genes were identified as the causative genes by classic complementation genetic tests with null deletion alleles whenever possible.

Results

Terminal phenotypes

Morphogenesis refers to the development of structure and form of the body and organs of multicellular eukaryotes. Because morphogenesis remains partially understood, I sought to identify temperature-sensitive (TS) morphogenetic mutants as a tool for the community. The first step involved screening the Bowerman Lab collection of mutants with defective morphogenesis by analyzing embryos and scoring the stage at which they arrest at the restrictive temperature of 26°C. Most mutants arrest early during morphogenesis with no elongation past the 1-fold stage (Figures 4, 5). When

greater than 70% of the embryos produced by a mutant exhibit a consistent morphogenesis phenotype, the mutant is classified as penetrant. Thus far, penetrant mutants include *or870*ts, *or896*ts, *or922*ts *or1006*ts, *or1193*ts, or*1214*ts *or1330*ts, *or1354*ts *or1404*ts, or1742ts and *or1755*ts; most of these alleles arrest with no elongation while a smaller number of alleles arrest with some elongation (Figures 4, 5A). Some alleles have a variable phenotype, where greater than 50% but less than 70% of embryos exhibit a single embryonic terminal phenotype. Alleles *or893*ts *or1164*ts, *or1300*ts, *or1395*ts, or1672ts arrest at multiple stages of morphogenesis (Figures 4, 5B).

There are three categories of terminal phenotypes that warrant expulsion of the mutant from further study. First, alleles that can survive and fully elongate through the restricted temperature of 26°C are not considered morphogenetic mutants (data not shown). Second, *or1167*ts is an example of an allele taken out of our future studies because it is too variable and did not have a definite phenotype (Figures 4, 5C). Third, alleles *or1257*ts and *or1424*ts display large undifferentiated cells and are not considered morphogenetic mutants but are likely weak cell division mutants (Figures 4, 5C). We initially categorized *or1424*ts incorrectly as a mutant arresting with no elongation, and as a result, we performed the genetic test

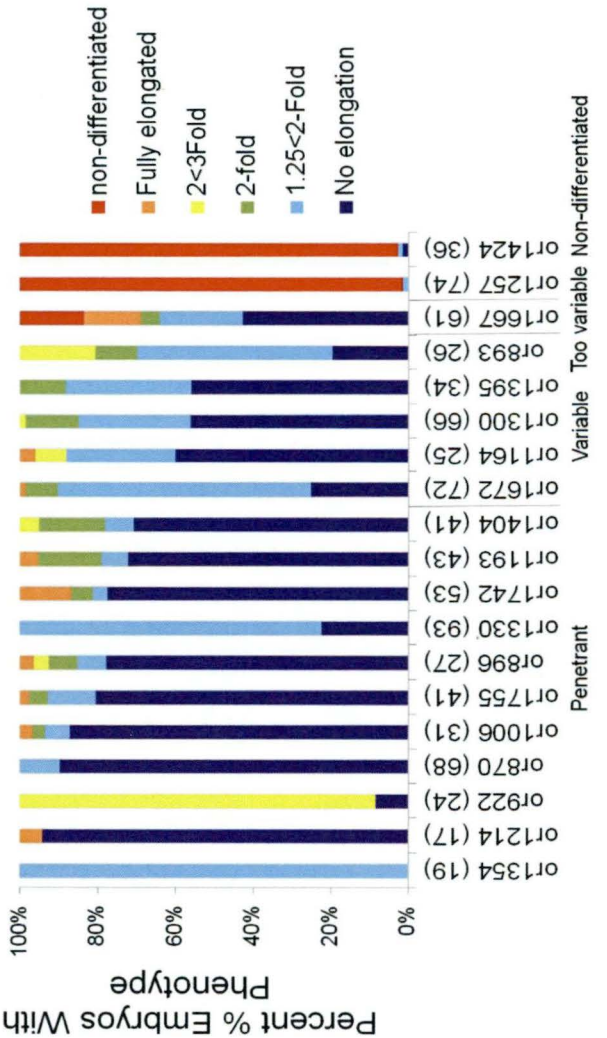

Figure 4: Quantification of morphogenetic terminal phenotypes percent of embryos that arrest at specific stages are color coded (see key). Alleles categorized as penetrant display a single phenotype 70% of the time or more. Alleles categorized as variable display 50%-70% of a single phenotype. Alleles categorized as too variable show all phenotypes less then 50%. Alleles categorized as non-differentiation mutants do not produce differentiated cell types (such as skin, pharynx, gut, etc.) and arrest with large undifferentiated cells. Temperature sensitive alleles are listed on the x-axis in order of decreasing penetrance from left to right. The total numbers of embryos scored are in parenthesis.

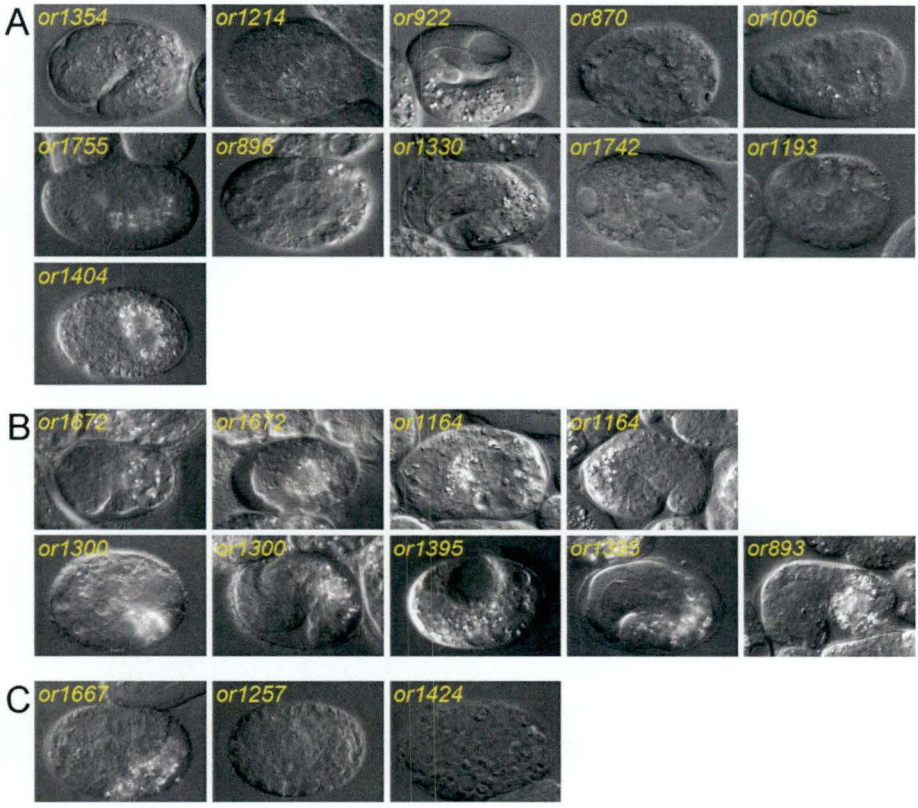

Figure 5: Nomarski images of terminally differentiated mutant embryos. (A) Penetrant alleles, (B) variable alleles, and (C) too variable and undifferentiated alleles as defined in Figure 4. Alleles are organized in decreasing penetrance from left to right (as in Figure 4).

and mapping crosses. Although *or1424*ts is not a morphogenetic mutant, its data is included in Table 1 below. In our study, we focused on penetrant morphogenetic alleles because these phenotypes are most likely caused by mutations in essential genes.

Table 1. Genetic characterization and embryonic lethality for temperature-sensitive alleles

			Embryonic Lethal	
Allele	**Dom. Test (n)**	**Seg. Freq (n)**	**15°C (n)**	**26°C (n)**
or870	7.4% (269)	27.5% (80)	0 % (91)	99.2% (130)
or896	2.0% (147)	25.0% (80)	1.9% (262)	90.8% (412)
or922	4.8% (272)	17.5% (80)		
or1193	0.9% (473)	21.8% (78)	1.9% (782)	91.8% (538)
or1330	1.2% (330)	10.0% (80)	46% (791)	100% (847)
or1354	3.3% (456)	22.5% (80)		
or1424	1.1% (533)	23.8% (80)	0.4% (272)	97.2% (671)
or1672	4.1% (291)	22.5% (80)	14.9% (341)	100% (456)
or1742	1.2% (450)	20.0% (80)	1.82% (177)	80.0% (235)
or1755	7.2% (454)			

Genetic Characterization

The TS conditional mutants can reproduce at the permissive temperature of 15°C and exhibit embryonic lethality only when L4 parents are upshifted to the restrictive temperature of 26°C. Therefore, using complicated genetics, such as balancers, is unnecessary to maintain the alleles. As genes that influence morphogenesis were

identified, mutants were characterized for being recessive and occurring at a single locus (Mendelian segregation).

First, a dominance test provided a general and superficial characterization of whether a gene is recessive or dominant and whether it has a maternal or zygotic requirement. We wish to work with only recessive mutants to study the "normal" function of a gene. Recessive mutations tend to be loss-of-function null or hypomorphic alleles, whereas dominant mutations tend to be gain-of-function or neomorphic alleles and do not provide insight into a gene's normal function. Moreover, early embryos contain genes expressed from two distinct origins: those expressed from the mother's genome and deposited in the oocytes (maternal) and those expressed from the embryo's genome after fertilization (zygotic). *C. elegans* may have essential morphogenetic genes that have multiple requirements during development from either or both pools. Whether a gene is required maternally, zygotically, or both, analysis of progeny from upshifted homozygous recessive mutants allows us to bypass earlier requirements for a gene and reduce both maternal and zygotic functions. To perform a dominance test, a mutant hermaphrodite is mated to wild-type (N2) males (Figure 2A). The resultant F_1 heterozygous L4 hermaphrodites are upshifted to the restrictive temperature and

the F_2 embryos scored for lethality (Figure 2B). I expected the following: (1) 0% embryonic lethality for recessive, maternal effect mutations, (2) 25% embryonic lethality for recessive, zygotic mutations, (3) 75% embryonic lethality for dominant, zygotic mutations, and (4) 100% for dominant, maternal effect mutations. Alleles that have embryonic lethality under 25% are considered recessive, while alleles with embryonic lethality over 25% are considered dominant. Therefore, alleles *or870*ts, *or896*ts, *or922*ts *or1006*ts, *or1193*ts, *or1330*ts, *or1354*ts *or1404*ts, *or1672*ts or1742ts, and *or1755*ts display close to 0% embryonic lethality, characterizing them as recessive and likely maternal-effect mutations (Table 1).

Second, to evaluate whether each allele represents a mutation at a single locus versus at multiple loci, I scored each mutant's segregation frequency. When investigating the function of a gene, it is important to study the gene in isolation so that the mutant phenotype can be attributed to a single gene. In studying a double mutant, it would be difficult to distinguish how each gene contributes to the phenotype. To measure the segregation frequency, a homozygous recessive mutant hermaphrodite was mated to wild-type (N2) males, and the embryonic lethality of the F_3 progeny of 80 upshifted F_2 L4 hermaphrodites was scored (Figure 2A, C). If the homozygous re-

cessive allele is a mutant in a single gene, about 1/4 (or 25%) of the F_2 parents will have embryonic lethal progeny at 26°C. If, however, the homozygous recessive allele is a double mutant, 1/16 (or 6.25%) of the F_2 parents will have embryonic lethal progeny at 26°C. Alleles *or870*ts, *or896*ts, *or922*ts, *or1193*ts, *or1354*ts, *or1424*ts, *or1672*ts, and *or1742*ts have a segregation frequency around 25%, falling between the acceptable range of 15-35% (Table 1), indicating these alleles are single genetic mutants. When a segregation frequency is below 10%, the strain is likely a double mutant. Therefore, it is unclear whether *or1330*ts is a double mutant or whether the frequency is low due to early picking of single F_2 worms, which can cause an overabundance of phenotypically wild-type worms (Table 1). Since *or1330*ts has a penetrant morphogenetic phenotype, the analysis moved forward with all nine alleles.

After the dominance and segregation frequency analyses, I needed to ensure that the N2-outcrossed strain has maintained the TS mutant phenotype of embryonic lethality, similar to the original stain in the *lin-2* (*e1309*) mutant background. I performed a test scoring for percent of embryonic lethality at 15°C and 26°C, which should give 0% and 100% embryonic lethality, respectively. Alleles *or870*ts, *or896*ts, *or1193*ts, *or1330*ts, *or1424*ts, *or1672*ts,

and *or1742*ts have acceptable embryonic lethality compared to the expected levels (Table 1). Although alleles *or1330*ts and *or1672*ts display embryonic lethality higher than the expected 0% at the permissive temperature of 15°C, there is still a drastic increase in embryonic lethality from 15°C to 26°C for these alleles. Therefore, the high embryonic lethality at 15°C is acceptable and simply indicates these mutants will be slow growers at the permissive temperature. Therefore, all these alleles are acceptable for genetic mapping analysis because they are homozygous recessive, single mutant alleles that exhibit high embryonic lethality 26°C. I will perform the same genetic tests on the remaining alleles: *or922*ts, *or1006*ts, *or1214*ts, *or1354*ts, *or1404*ts, and *or1755*ts. The results gained from performing genetic characterization (dominance test, segregation frequency, and mutant maintenance) give a preliminary characterization of the mutants to determine which strains to map.

Mapping and Gene Identification

To study morphogenesis, the causative gene needs to be identified for each mutant allele. The process of identifying candidate genes in a chromosomal location uses whole-genome, single nucleotide polymorphism (SNP) sequencing by mating homozygous mutant N2-outcrossed hermaphrodites to Hawaiian (Hw; *CB4856*) males, a

strain with a unique array of SNP's roughly spaced at 1kb intervals across its genome compared to the N2 wild-type strain (Figure 3). The cross yields an F_1 heterozygous generation in which the worms each have an N2 and Hw chromosome for each chromosomal pair. Meiotic recombination will occur randomly in the genomes of the F_1 heterozygous generation, and the F_1 worms will self-fertilize to give rise to an F_2 generation with a recombined N2-Hw genome. From that F_2 generation, homozygous mutants are selected by picking F_3 L4s, upshifting them to 26°C, and screening for embryonic lethality three days later. Ten to twenty homozygous mutant F_2 worms are isolated and their progeny sent for whole genome sequencing. Analysis using the Galaxy platform (Giardine et al., 2005; Goecks et al., 2010; Menevich et al., 2012; Langmead and Salzberg, 2012; DePristo et al., 2011; Cingolani et al., 2012; Quinlan and Hall, 2010) compares the N2 and Hw SNPs across all chromosomes of the genome (Figure 3). An N2 chromosome that is unlinked to the mutation will have random recombination with the Hw chromosome for every SNP; therefore, I expect the ratio of Hw over total SNPs to be about 50% across the entire unlinked chromosome. An N2 chromosome that is linked to a mutation will have low recombination with the Hw chromosome at SNPs physically close to the location of the

mutation; therefore, I expect the ratio of Hw over total SNPs to be 0% at the site of the mutation. This technique allows us to identify the chromosomal region where the mutation lies and also gives rise to a list of gene candidates.

Mapping was completed, and candidates were identified for two alleles: *or1193*ts and *or1330*ts. Allele *or1193*ts maps to the 5-12 Mb region on chromosome IV, and *or1330*ts maps to the 11-21 Mb region on chromosome V (Figure 6). Two potential gene candidates were identified on chromosome IV for *or1193*ts: (1) *rpn-1*, which encodes a subunit of the 26S proteastome (Takahashi et al., 2002), and (2) *rib-1*, which encodes an N-acetylglucosamine (GlcNAc) transferase, important for heparan sulfate proteoglycan (HSPG) synthesis (Franks et al., 2006; Kitagawa et al., 2007). Three potential gene candidates were identified on chromosome V for *or1330*ts: (1) *emb-4*, which encodes a nuclear protein with a putative ATPase domain (Checchi and Kelly, 2006); (2) *Y39B6A.25,* which is a ring finger protein and involved in body morphogenesis during embryo development; and (3) *F10A3.16,* which regulates *csr-1*, which in turn encodes an Argonaute protein involved in RNAi, and *sir-2.1*, which encodes a deacytalase protein involved in the metabolism (Harris et al., 2014).

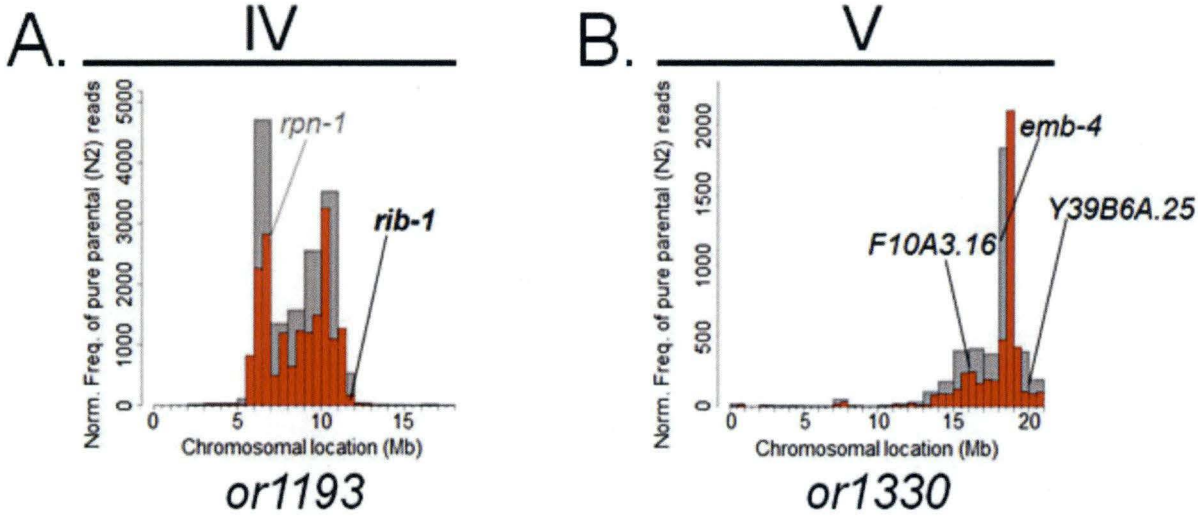

Figure 6: Genomic location of temperature sensitive mutants. Normalization frequencies of Hawaiian/Total SNPs across chromosomes IV and V for mutants or1193ts (A) and or1330ts (B), respectively. Candidate genes and their location are indicated.

Once candidate genes are identified, complementation tests using existing, known alleles identify the causative gene for the mutation. To determine the causative gene for *or1193*ts, complementation tests were performed by mating *or1193*ts hermaphrodites to *rpn-1* (*ok259*) and *rib-1* (*ok556*) deletion allele males and scoring for embryonic lethality at 26°C. The *rpn-1* (*ok259*) gene complemented *or1193*ts, with only 2.7% (n = 990) embryonic lethality, indicating *rpn-1* is not the causative gene for *or1193*ts (Table 2). Alternatively, *rib-1* failed to complement *or1193*ts, with 97.1% (n = 1514) embryonic lethality, indicating *or1193*ts is a mutant allele of the gene *rib-1* (Table 2).

Whole genome sequencing identified the nucleotide change of *or1193*ts to be an A to T transition, resulting in the non-synonymous substitution of amino acid 126 from a histidine (H) to a leucine (L) in the gene *rib-1* (Table 2; Figure 7A). There are two existing alleles of *rib-1*: (1) *ok556* causing an in-frame deletion of 55 amino acids, and (2) *tm516* in which 468 bp are deleted and 32 bp are inserted into *rib-1*, causing an immediate premature stop. A comparison of the terminal phenotype of the isolated allele of *rib-1*, *or1193*ts, to the previously published results for the two existing alleles revealed that *or1193*ts mutant embryos primarily arrest with no elongation

Table 2. Gene identification among *or1193*ts and *or1330*ts.

Allele	Gene Tested (allele)	% Emb. Leth (26° C)	(n)	Sequence change (genome/protein)	Human ortholog	Gene function
or1193ts	rpn-1 (ok2259)	2.7%	990	N/A	RPN-1	Non-ATPase subunit of the 26S proteasome
or1193ts	rib-1(ok556)	97.1%	1518	A>T/H126L	EXT1	GlcNAc transferase for synthesis of heparin sulfate proteoglycans
or1330ts	emb-4 (hc60ts)	100%	1111	atc>aAt/ I967N; this frameshift causes a premature stop at amino acid 1004	Aquarius	Nuclear protein with putative AAA. ATPase domain

(72%, n = 42; Figure 7B). Similarly, *tm516* mutant embryos arrest primarily at the 1 to 1.25-fold stage (68%, n = 92; Kitagawa et al., 2007; Figure 7B). Alternatively, *ok556* mutant embryos primarily arrest at a later stage compared to *or1193*ts and *tm516* so that 57% of embryos arrest at the 1 to 1.25-fold and 19% embryos arrest at 3-fold or as hatched larva (n=37%; Franks et al., 2006; Figure 7B).

To determine the causative gene for *or1330*ts, I performed a complementation test. Of the three gene candidates, *emb-4* is the best option for *or1330*ts because previous studies indicated that *emb-4* is a morphogenetic mutant (Checchi and Kelly, 2006). The other two alleles, *Y39B6A.25* and *F10A3.16*, have no existing alleles, and prior analysis was performed using RNAi. For the complementation test, the *emb-4* (*hc60*ts) mutant hermaphrodite was crossed with wild-type (N2) males to generate heterozygous F_1 males. Then, a homozygous mutant *or1330*ts hermaphrodite was crossed to *hc60*ts/+ males to generate cross progeny of two expected genotypes, *or1330*ts/*hc60*ts and *or1330*/+, and scored for embryonic lethality at 26°C. If *or1330*ts is a mutation of the *emb-4* gene, half of the cross progeny (those of the genotype *or1330*ts/*hc60*ts) should fail to complement and display 100% embryonic death at 26°C. Alternatively, if *or1330*ts is not a mutation of the *emb-4* gene,

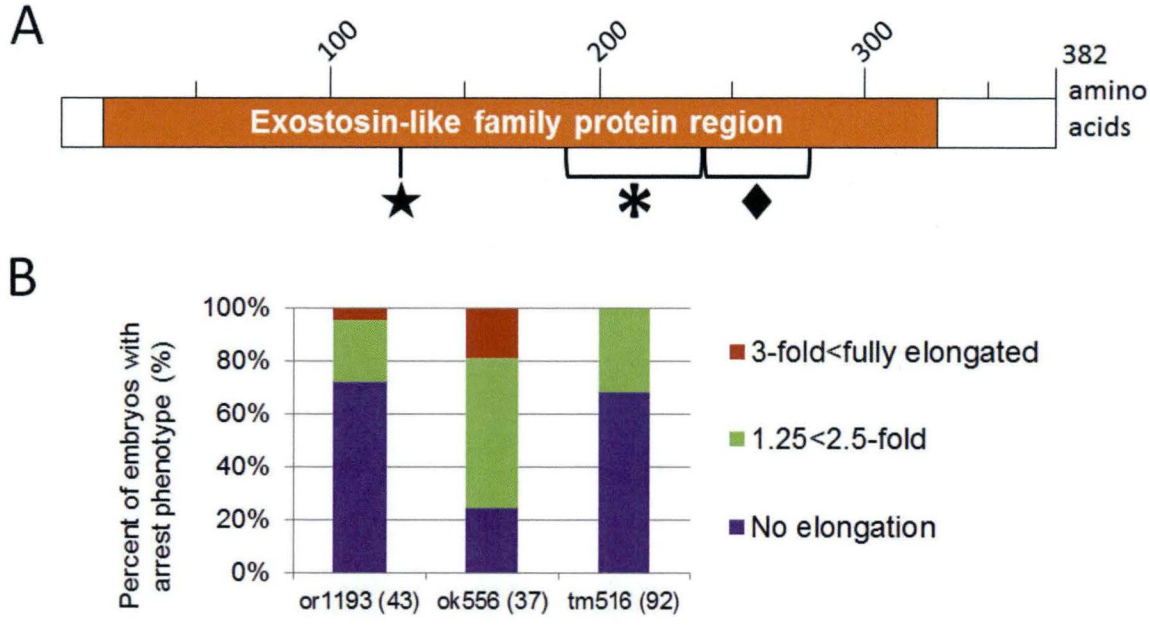

Figure 7: Comparison of the rib-1 alleles: or1193ts, ok556, and tm516. (A) Protein structure of RIB-1. or1193ts (star) mutation is a non-synonymous change between a histidine to a leucine in amino acid 126 . ok556 (asterisk) is a 55 amino acid in-frame deletion of amino acids 189-243. tm516 (diamond) is a complex mutation of a 35 amino acid deletion amino acids (244-278) and a 32 bp insertion causing a frame shift and a premature stop at amino acid 245 (B) Terminal phenotypes of or1193ts compared to ok556 and tm516 (ok556 data from Franks et al., 2006: tm516 data from Kitagawa et al., 2007).

all of the cross progeny should yield 100% hatched larva at 26°C. Results showed that *emb-4* (*hc60*ts) failed to complement *or1330*ts, and that 35/78 cross progeny had 100% embryonic lethality (n = 1111 embryos) indicating that *or1330*ts is a mutant allele of the gene *emb-4* (Table 2).

Whole genome sequencing identified *or1330*ts to be an insertion of a single adenine in codon 967 (atc to aatc) that causes a frame shift and premature stop at codon 1004 (Table 2; Figure 8A). Previous studies have indicated there are other existing mutant alleles of *emb-4*, the strongest and most studied being *hc60*ts. The nature of the mutation of *hc60*ts has not been well curated, although Checchi and Kelly (2006) indicate that there is a non-synonymous change of an amino acid to a premature stop somewhere in exon 5 (Figure 8A). I compared the embryonic lethality and terminal phenotypes of alleles *emb-4*, *or1330*ts to the previous pre-existing allele, *hc60*ts. Both alleles have about 50% embryonic lethality at 15°C, 100% embryonic lethality at 26°C, and both terminally arrest primarily in the 1.25 to 2 fold stage (Figure 8B, C).

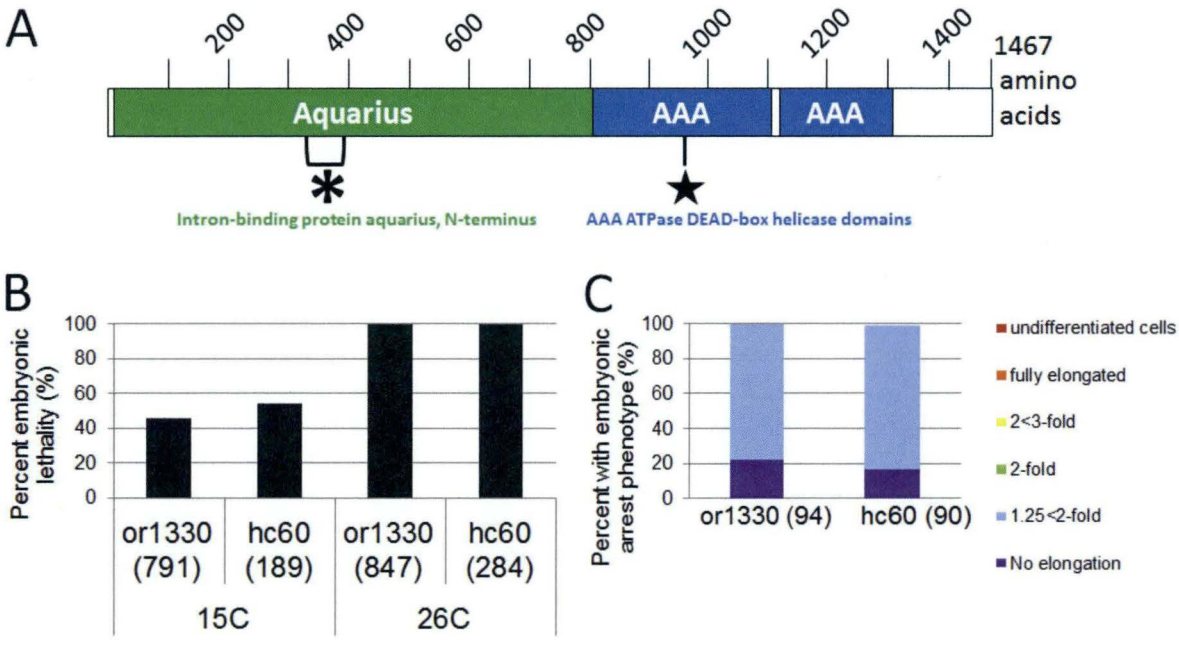

Figure 8: Comparison of the emb-4 alleles: or1330ts and hc60ts. (A) Protein structure of EMB-4. or1330ts (star) is a single nucleotide insertion in codon 967 (atc to aatc) causing a frame shift and premature stop at amino acid 1004. hc60ts (asterisk) is a non-synonymous change of an amino acid located somewhere in exon 5 (amino acids 321-395) to a stop. (B) Embryonic lethality at 15°C and 26°C. (C) Terminal phenotypes of 1330ts compared to hc60ts.

Discussion

The basic genetic and molecular changes that occur at the cellular basis and result in gross tissue morphogenesis are only partially understood. One challenge in studying morphogenesis is that genes often have multiple roles during their development; thus, if a gene has an earlier role in embryogenesis before morphogenesis occurs, its role in morphogenesis often remains unstudied. In order to study morphogenesis, I have screened for conditional, temperature-sensitive (TS), morphogenetic mutants in the model organism *C. elegans* seeking to identify alleles with penetrant defects in morphogenesis and identify their genetic mutations. I have discovered several penetrant alleles with severe morphogenetic defects, including *or870*ts, *or896*ts, *or922*ts *or1006*ts, *or1193*ts, *or1214*ts, *or1330*ts, *or1354*ts *or1404*ts, *or1742*ts, *or1755*ts. Currently, I have mapped two morphogenetic mutant alleles, *or1193*ts and *or1330*ts, and identified one to be the gene *rib-1* and one to be the gene *emb-4*.

*Allele or1193*ts *is the gene rib-1, known to affect sugar modification biosynthesis*

We have identified the allele, *or1193*, to be a mutation in the gene *rib-1*. The human ortholog of *rib-1* is EXT1, a member of the Exostosin (EXT) gene family of glycosyltraferases. The EXT1 gene

functions in heparan sulfate proteoglycan (HSPG) biosynthesis. In their turn, HSPGs are composed of heparan sulfate (HS) chains, alternating glucoronic acid and *N*-acetlyglucosamine (GlcNAc) residues, that are covalently linked to a core protein at serine amino acids (reviewed in Poulain and Yost, 2015). EXT1 is the enzyme responsible for the addition of GlcNAc sugar molecules to the elongating HS chain. The structure of HS chains is highly diverse because the glucuronic acid and GlcNAc residues can undergo extensive sulfation and epimerization (Lindahl et al., 1998; Esko and Selleck, 2002; Bulow and Hobert, 2006; Bishop et al., 2007; Kreuger and Kjellen, 2012). The HS chains are attached to proteins located at the cell surface (receptors) or in the extracellular space (ligands); thus, HSPGs have been implicated in modulating signal transduction (including Wnt, Hh, Dpp, EGF, and FGF signal pathways), important for cell survival, division, migration, and differentiation in vertebrates and invertebrates (Poulain and Yost, 2015; Humphreys et al, 2013). According to the "sugar code" hypothesis (Holt and Dickson, 2005), HS modifications within core proteins in specific tissues at specific times within the embryo control signaling pathways and coordinate morphogenesis and development. Loss of *EXT1* gene function in mice does, in fact, lead to embryonic death and gastrulation defects

(Lin et al., 2000; Mitchell; et al., 2001; Shimokowa et al., 2011), along with neurogenesis defects (Inatani et al., 2003). Furthermore, deletion of *EXT1* in humans has been linked to mental disorders and autism (Lie et al., 2002), and disruption of *EXT* genes result in hereditary multiple exostoses, a rare medical disorder in which boney spurs develop in embryos/fetuses, children and adults (Ahn et al.,1995).

Characterization of *rib-1* in *C. elegans* has identified a role for HS modification in embryonic morphogenesis. Like EXT1, RIB-1 likely functions as glycosyltransferase in HS biosynthesis because loss of *rib-1* in embryos results in decreased levels of HS (Franks et al., 2006; Kitagawa et al., 2007). Analysis of two previously existing alleles, *ok556* and *tm516*, indicates that both result in maternal effect embryonic lethality and exhibit variable penetrance for developmental arrest (Franks et al., 2006; Kitagawa et al., 2007). In addition to body elongation defects, *ok556* also exhibits pharyngeal isthmus elongation defects. These data taken together suggest that *rib-1* is required for morphogenetic events. By comparing terminal phenotypes of the *rib-1* allele to the two preexisting alleles, I found that *or1193*ts is stronger than *ok556* and as strong as *tm516*. Intuitively we would expect the deletion alleles to cause a more penetrant phe-

notype, but my data indicate that the single amino acid substitution in *or1193*ts causes a phenotype as strong as the complex mutation in *tm516*. This result is a surprise because deletions usually result in stronger alleles than single nucleotide substitutions causing a non-synonymous amino acid change. Allele *or1193*ts is the only known temperature sensitive allele for gene *rib-1* and, therefore, may provide a better tool for studying morphogenesis.

Allele or1330 is the gene emb-4, a known morphogenetic gene with unclear function

We identified the allele, *or1330*ts, to be a mutation in the gene *emb-4* that encodes a nuclear protein with a helicase-like domain, meaning it could potentially affect the unwinding of the DNA or RNA (Checchi and Kelly, 2006). Studies by Katic and Greenwald (2006) confirmed that *emb-4* is homologous to the Aquarius family. The *emb-4* gene is conserved within a wide array of species other than *C. elegans*, including Arabidopsis, *Saccharomyces pombe*, Drosophila, mice, and humans. The function of *emb-4* is still unclear and has yet to be identified; thus, *emb-4* has multiple listed roles and potential mechanisms. First *emb-4* is involved in the regulation of the notch-signaling pathway post-embryonically in vulva development in *C.elegans* (Katic and Greenwald, 2006). Second, the *S.*

pombe emb-4 ortholog, Cwf11p, co-purifies with Cdc5p, which is a member of the pre-mRNA splicing complex (Katic and Greenwald, 2006). Therefore, *emb-4* could potentially have a role in RNA splicing. Moreover, the best line of evidence suggests that *emb-4* regulates histone modification. Specifically, *emb-4* suppresses H3K4 methylation, which marks transcriptionally competent chromatin, in the germ line lineage cells Z2/Z3 (Checchi and Kelly, 2006). Therefore, it is most likely that *emb-4* function globally to suppress transcription. Any one of the three proposed functions is a potential mechanism by which *emb-4* could control gene expression, perhaps by regulating the cytoskeleton or cell adhesion genes important to cell shape changes and/or migration.

The *emb-4* gene is a known morphogenetic gene because although its cell fate patterning is fine, its cell placement is impaired in mutants. Specifically, E cell lineage cells were found to divide 33% slower in *emb-4* mutants than in wild-type embryos (Schierenberg et al., 1980). Furthermore, E lineage cells mark by ETL-7::GFP indicated that the number of gut cells specified were not different from wild-type, but their location within the embryo was altered, and in general, many tissues were poorly organized (Checci and Kelly, 2006). Taken together, these data indicate that *emb-4* is

a gene required for morphogenesis. In comparison to the preexisting allele *hc60*ts, our allele *or1330*ts behaves similarly; both have strong TS embryonic lethality and penetrant terminal phenotype. While both alleles result in premature stop, the mutation in *hc60*ts truncates the protein at an earlier location than *or1330*ts; therefore, we would have expected *hc60*ts to be a stronger mutant. However, both mutations have equally strong phenotypes. Because *emb-4* is clearly an important morphogenetic gene, the use of either of the TS alleles may help better understand regulation of cell shape changes and migration important to morphogenetic events.

Conclusion

Alleles *or1193*ts and *or1330*ts show great promise as morphogenetic mutants. Both alleles have interesting phenotypes that could allow us to better investigate cell shape changes and migration. Additionally, genes identified by my forward genetic screen aid our basic understanding of morphogenesis in *C. elegans* and may also be applicable to our understanding of morphogenesis in other species, such as humans. These studies may help to eventually provide insight and suggest therapeutic approaches to morphogenetic defects during human embryogenesis. Furthermore, the alleles and genes identified by this forward genetic screen will be shared with

the research community through the Caenorhabditis Genetic Center (CGC) to more broadly help the community study questions regarding morphogenesis.

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