Negative regulation of cell fate specification by the *lin-15* locus during vulva induction in *Caenorhabditis elegans*

Thesis by

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I dedicate my thesis to my husband and son, Fred and Joey Serricchio. They made me laugh and feel I could do anything I put my mind to.

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Abstract

We have visualized extrachromosomal arrays by targeting the green fluorescent protein (GFP) to a specific DNA sequence (*lac* operator) incorporated into *Caenorhabditis elegans'* transgenes. This system can be used to determine polyploidy and to investigate chromosome segregation. This technique also allows rapid, accurate determination of spontaneous loss of an array, thereby allowing high-resolution mosaic analysis. We carried out genetic mosaic analysis on *lin-3* (epidermal growth factor) using the GFP-LacI + *lacO* method. This methodology confirmed *lin-3*'s site of action for vulval induction is at the anchor cell. This result also proved this technique works.

We used both the GFP-LacI + $lacO_{256}$ system as well as the *ncl-1* gene as genetic mosaic markers to determine the site of action of *lin-15A* and *lin-15B*. Both markers indicate that *lin-15A* gene function is required within the vulval precursor cells (VPCs) to prevent an excessive number of VPCs from generating vulval progeny. The mosaic expression pattern for *lin-15B* is broad therefore, proven difficult to pinpoint a site of action.

The products of the *lin-15* gene were first defined genetically as negative regulators of the vulval induction pathway. It encodes two novel hydrophilic proteins, LIN-15A and LIN-15B. According to antibody stainings and GFP expression patterns, both proteins are nuclear and present in almost all the cells. *lin-15* is part of the synthetic multivulva (synMuv) set of genes which are comprised of two classes, A and B. Mutation of both an A and a B gene is required to obtain a multivulva (Muv) phenotype. Further characterization of the *lin-15* locus reveals an effect on fertility.

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Chapter 1

The power of genetics

Genetic analysis is a powerful tool used to clarify how genes specify complex structures and behaviors in higher organisms. It is used to order genes in functional pathways as well as to define in which cells a gene product is required (genetic mosaic analysis).

***Caenorhabditis elegans:* A model system for genetics**

The experimental organism chosen for my genetic study is the small (1mm long) nematode, *Caenorhabditis elegans*. *C.elegans* has several features that make it particularly amendable to genetic analysis. These features include a short generation time of 3 1/2 days and an invariant pattern of cell divisions. Also, differentiation and morphorgenesis can be viewed with single-cell resolution in intact living animals. These self-fertilizing hermaphrodites allow new mutations to become homozygous, without the additional generation consequential to sibling mating (Brenner, 1973; Brenner, 1974; Kenyon, 1988; Riddle *et al.* 1997; and Wood, 1998).

One way to identify potentially interesting alleles of genes involved in complex structures and behaviors is by chemical mutagenesis. Potent mutagens such as ethyl methanesulphonate (EMS) penetrate the animal easily and effectively. EMS (at 50mM) induces point mutations (G/C→A/T transitions) at frequency of $7x10^{-6}$ per mutagenized G/C base pair resulting in loss of function, reduction of function alleles or gain of function, and is therefore commonly used. Many of the mutants isolated from EMS mutagenesis are lethal. Mutants isolated have been selected and categorized on the basis

of morphological abnormalities (i.e., size or shape), deviation from the normally smooth sinusoidal movement of the nematode's body on the agar surface, and mating behavior (Brenner, 1973; Kenyon, 1988; Riddle *et al.* 1997; and Wood, 1998). Once the mutants are placed into a broad category, they are genetically characterized.

Recently, the *C. elegans* genome has been sequenced. This not only allows for easy cloning, but most importantly, finding functional homologs within its genome as well as other genomes that have been completed (i.e., human, *Drosophila*, and *Saccharomyces cerevisiae*).

Deciphering functional pathways by genetics

The dissection of developmental pathways in *C. elegans* begins with thorough comprehension of the relevant phenotypes associated with the genes involved. Double mutant analysis can be used to determine the epistatic relationship between mutations (Avery and Wasserman, 1992). Epistasis is the masking of one mutant phenotype by a second mutant phenotype that maps to another locus. Epistasis analysis can order gene action of two types but the order depends on the type of pathways: the substrate-dependent pathway and the switch regulation pathway. The distinction between switch regulation pathway versus substrate-dependant pathway depends on the phenotype studied or the event assayed, respectively (Avery and Wasserman, 1992; and Huang, 1995).

The substrate-dependent pathway involves a substrate initiating a required series of events to generate a final outcome. In *C. elegans'* vulval development, there are a

series of genes that are necessary for the development of the 12 "Pn.p" cells (P1.p-P12.p). A subset of which (P3.p-P8.p) is competent to be vulval precursor cells (VPCs). Three of the VPCs (P5.p-P7.p) adopt vulval cell fates (Sulston and Horvitz, 1977; Sulston and White, 1980; Sternberg and Horvitz, 1986; Ferguson, Sternberg and Horvitz, 1987; Sternberg, 1988; Ferguson and Horvitz, 1989; Huang, Tzou, and Sternberg, 1994; and Huang, 1995). Mutant alleles of *lin-26* (*lin* = *lin*eage abnormal), *lin-39* and *let-23* (*let*= lethal) result in a vulvaless phenotype. *lin*-26 (zinc finger transcription factor) is responsible for the formation of the Pn.p cells and if mutated, the Pn.p cells are absent. *lin-39* (homeoprotein) is responsible for P3.p-P8.p adopting VPCs fate while *let-23* (EGF receptor) determines which Pn.p cell will actually differentiate into vulval tissue. Animals with mutations in *lin-39* have no VPCs generated, while animals' with mutations in *let-23* have undifferentiated VPCs. The Pn.p cells are the substrate to the activities of *lin-26, lin-39,* and *let-23*. The developmental status or fate of the Pn.p cells by these genes' activity determines the order of action of the three genes (Sulston and Horvitz, 1977; Sulston and White, 1980; Sternberg and Horvitz, 1986; Ferguson, Sternberg and Horvitz, 1987; Sternberg, 1988; Ferguson and Horvitz, 1989; Huang, Tzou, and Sternberg, 1994; and Huang, 1995).

Switch regulation pathway is dependent upon distinct phenotypic states like in the case of *C. elegans*, multivulva (Muv) versus vulvaless (Vul) versus wildtype vulva (wt). Epistasis tests are done between two mutations in different loci with opposite phenotypes in order to define the given switch regulation pathway.

Analysis of the vulval induction pathway was done using comprehensive double-mutant analysis. The inductive signal that initiates *C.elegans* vulval differentiation occurs between the anchor cell (AC), a cell in the gonad, and six equipotential cells in the ventral epidermis or VPCs, P3.p-P8.p (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston and White, 1980; Sternberg and Horvitz, 1986; and Thomas *et al.* 1990). As mentioned previously, 3 of the 6 VPCs adopt vulval fate while the other three cells adopt non-vulval, non-specialized epidermal fates. The induced VPCs may adopt either a 1° or 2° vulval fate, which can be distinguished by subsequent cell division patterns and morphology (Sulston and White, 1980; Kimble, 1981). If the AC is eliminated by laser ablation, the inductive signal, LIN-3 protein, is not produced and therefore its receptor, LET-23, can not initiate the vulval induction pathway, resulting in all 6 VPCs adopting non-vulval, non-specialized epidermal fates (Vul) (Kimble, 1981; Hill and Sternberg, 1992). Other mutations that cause this Vul phenotype are: *let-23* (EGFR), *lin-3*(EGF), *let-60* (Ras protein), and *lin-45* (Raf kinase). Mutations that result in the opposite phenotype or Muv are *lin-15* (novel proteins) and *lin-1* (ETS domain protein, transcription factor) (Ferguson, Sternberg, and Horvitz, 1987; Han, Aroian and Sternberg, 1990; Hill and Sternberg, 1992; and Sternberg, 1993). According to the double mutant analysis *lin-1* is epistatic to all Vul genes affecting VPC specification. The other double mutants yield the following result: *let-23* epistatic to *lin-15; lin-15* epistatic to *lin-3; let-60* and *lin-45* are epistatic to *lin-15* (Avery and Wasserman, 1992; Ferguson, Sternberg,

and Horvitz, 1987; Han, Aroian and Sternberg, 1990; Hill and Sternberg, 1992; Sternberg, 1993; Lackner *et al.* 1994). The double mutant analysis with the Muv and Vul genes resulted in this preliminary pathway: *lin-3* —| *lin-15* —| *let-60, let-23, lin-45* —| *lin-1* —| vulval fates (Avery and Wasserman,1992; Ferguson, Sternberg, and Horvitz, 1987; Hill and Sternberg, 1992; Sternberg,1993, Lackner *et al.* 1994). Inference of this pathway by epistasis was misleading because not all the genes used are complete loss of function. Also, most pathways are not linear and simple as epistasis results imply. Interpretation of epistasis can lead to an incorrect order of a pathway if the description of phenotype is rudimentary. For example, the double mutant *lin-15*(Muv); *n300* (Vul) is Vul as in n300 therefore suggesting that *n300* is downstream of *lin-15*. However, *lin-15* is downstream of *n300* because the cause of the Vul phenotype is the absence of the VPCs (Ferguson *et al.* 1987 and Huang, 1995).

Genetic redundancy also complicates the interpretation of epistasis. If two or more genes can perform the same function, then inactivation of one of these genes often has little to no effect on the biological process (Nowak *et al.* 1997). There are examples of genetic redundancy in many studies of developmental biology, immunology, neurobiology and the cell cycle and it seems to dominate in the genomes of higher organisms (Nowak *et al.* 1997). The synthetic multivulva (synMuv) pathway is an example of a genetically redundant pathway in *C.elegans* (Ferguson and Horvitz, 1989; Huang, Tzou, Sternberg, 1994 and Clark, Lu, Horvitz, 1994 and Nowak *et al.* 1997). This functionally redundant pathway consists of two classes of gene types, A and B class, that affect vulval induction. A mutation in either an A or a B class gene or genes has no

phenotypic result on the vulva. If both A and B class genes are mutated, the hermaphrodite is Muv (Ferguson and Horvitz, 1989; Huang, Tzou, Sternberg, 1994 and Clark, Lu, Horvitz, 1994; and Huang, 1995). The four class A genes (*lin-8, lin-15A, lin-38, and lin-56*) and the ten class B genes (*lin-9, lin-15B, lin-35, lin-36, lin-37, lin-51, lin52, lin-53, lin-54*, and *lin-55*) encode negative regulators of vulval induction (Horvitz and Sulston, 1980; Ferguson *et al.* 1987; Ferguson and Horvitz, 1989; Huang *et al.* 1994; and Lu and Horvitz, 1998). The absence of the synMuv activity causes P3.p, P4.p and P8.p to adopt vulval fates resulting in the Muv phenotype of the animal.

Positive and negative regulators during vulval induction

Vulval induction in *C. elegans* occurs between the AC and P3.p – P8.p (VPCs) cells (Sternberg and Horvitz 1986; Sulston and White 1980). The anchor cell secretes the LIN-3 (EGF-like protein) to the VPCs, causing 3 of the 6 cells to adopt vulval fates since LIN-3 binds and activates its LET-23 (receptor tyrosine kinase). The other 3 cells will adopt an epidermal fate resulting in one round of division. There are two different vulval fates, 1° and 2° that can be distinguished by the pattern of the subsequent divisions and morphology adopted by the granddaughter cells of the induced VPCs (P5.p-P7.p) (Katz *et al.* 1995; Sternberg and Horvitz 1986). During induction in intact wild-type hermaphrodite, P6.p will adopt the 1° fate since it is closest to the anchor cell while P5.p and P7.p adopt 2° vulval fate (Sulston and Horvitz 1977). Lateral signaling from P6.p to

P5.p and P7.p elicit the invariant pattern of cell fates (Greenwald *et al.* 1983; Katz *et al.* 1995; Koga and Ohshima 1995b; Sternberg and Horvitz 1986; Sternberg and Horvitz 1989). Once LET-23 is stimulated by LIN-3, the effector proteins downstream (SEM-5, LET-341 SOS, LET-60 RAS, LIN-45 RAF) alter the transcriptional factors to specify the cell to adopt a vulval fate. This positive regulative pathway for vulval induction utilizes a member of the epidermal growth factor (EGF) receptor (EGFR) family of receptor tyrosine kinases (RTKs) (Aroian and Sternberg 1991; Eisenmann and Kim 1994; Greenwald and Broach 1990; Gutch *et al.* 1998; Hill and Sternberg 1992; Kayne and Sternberg 1995; Koga and Ohshima 1995b; Moghal and Sternberg 1999; Simske *et al.* 1996). Acting on this EGF/EGFR pathway, there are five negative regulatory pathways affecting vulval development (Clark *et al.* 1994; Ferguson and Horvitz 1989; Galisteo *et al.* 1995; Huang *et al.* 1994; Jongeward *et al.* 1995; Lesa and Sternberg 1997; Yoon *et al.* 1995). Two of the five pathways, synMuv A and synMuv B, appear distinct from the other 3 negative regulators (*sli-1, unc-101*, and *rok-1*) which are dependent on and act directly on LET-23 (Clark *et al.* 1994; Galisteo *et al.* 1995; Huang *et al.* 1994; Jongeward *et al.* 1995; Lesa and Sternberg 1997; Yoon *et al.* 1995).

The functionally redundant synthetic multivulva pathways comprise two classes, the synMuv class A and the synMuv class B. The Muv strain CB1322 was found first to require mutations in two unlinked genes, *lin-8* and *lin-9* (Horvitz and Sulston 1980). An additional 5 synMuv mutations were inadvertently found after mutagenizing a strain with an undetected class A mutation (Ferguson and Horvitz 1989). Eventually, mutagenesis were done on the known single mutants of class A and B, each phenotypically wild-type,

which led to further isolation of synMuv mutants (Ferguson and Horvitz 1989). Hermaphrodites carrying an A class and a B class mutation result in all 6 Pn.p cells adopting either a primary or secondary VPCs fate (i.e., A Muv phenotype). Hermaphrodites with one or two mutations of the same class have a wild-type vulva phenotype (Clark *et al.* 1994; Ferguson and Horvitz 1985; Ferguson and Horvitz 1989; Horvitz and Sulston 1980; Huang *et al.* 1994; Lu and Horvitz 1998). The synMuv genes encode negative regulators of vulval induction (Clark *et al.* 1994; Ferguson and Horvitz 1985; Ferguson and Horvitz 1989; Horvitz and Sulston 1980; Huang *et al.* 1994; Lu and Horvitz 1998). There are 4 class A genes (*lin-8, lin-15A, lin-38, and lin-56*) and 10 class B genes (*lin-9, lin-15B, lin-35, lin-36, lin-37, lin-51, lin-52, lin-53, lin-54, and lin-55* (Clark *et al.* 1994; Ferguson and Horvitz 1985; Ferguson and Horvitz 1989; Horvitz and Sulston 1980; Huang *et al.* 1994; Lu and Horvitz 1998).

lin-15 is a complex locus with two independent mutable activities, A and B class (Clark *et al.* 1994; Ferguson and Horvitz 1985; Ferguson and Horvitz 1989; Horvitz and Sulston 1980; Huang *et al.* 1994; Lu and Horvitz 1998) and it acts independently of two known signals controlling the fates of the vulval precursor cells (VPCs), the anchor cell (AC) inductive signal and a lateral signal among the VPCs (Herman and Hedgecock 1990). Each of the two cistrons of the *lin-15* locus encodes a hydrophilic protein, LIN-15A and LIN-15B, which have no obvious similarity to each other or to other known proteins, so far. Previous mosaic analysis indicated that *lin-15* acts in cells other than AC and the VPCs (i.e., non-autonomous) (Herman and Hedgecock 1990). The *lin-15* locus is

also involved in cell fate specification of the *C. elegans* male tail (Chamberlin and Sternberg 1993; Chamberlin and Sternberg 1994)

Mosaic Analysis

Once a gene is characterized, genetic mosaic analysis can be used to determine in which cells a particular gene product is required to rescue a certain phenotype or event. *C. elegans* transparent body and its invariant cell lineage allows one to score at the single cell level for the presence of a given gene activity by using non-autonomous single cell marker plus the gene of interest (Herman, 1984, 1987, and 1989).

Determining which cells the gene product is needed for a given function is more biologically relevant than simply knowing where the gene is transcribed, or where the protein is found. Mosaic analysis is informative when the cells are genotypically mutant for a given gene while another set of cells is genotypically wild type in a given animal. Then one asks what is the phenotypic event for the animal (Herman, 1984, 1987, and 1989; and Hedgecock and Herman, 1995). Mosaic analysis of a gene can be done only with the knowledge about the phenotype of the mutant or null animal. One can perform mosaic analysis on a mutant phenotype and not focus on cellular abnormalities such as dumpy worm" (Dpy) in the case of *C. elegans.* Mosaics are generated to determine which cells, either hypodermal or muscles cells, for example, need the wild-type *dpy* gene to rescue the Dpy phenotype. The mutant phenotype is rescued only when the responsible cells contain wild-type copies of the given gene regardless of the other cell's genotype. These data imply the site of action of the gene among all the cells in the animal (Herman, 1984, 1987, and 1989; and Hedgecock and Herman, 1995).

Genetic mosaics are generated by the loss of the wild-type gene from a progenitor cell during development thus generating a lineage of mutant cells. This type of analysis addresses the question of whether the phenotype of the affected cell is dependant upon the genotypes of other cells due to cellular interactions (cell non-autonomous) or the cellular abnormality is solely dependent on the genotype of the affected cell (cell autonomous) (Herman, 1984, 1987, and 1989; and Hedgecock and Herman, 1995). The wild-type protein that was synthesized in the progenitor cell or its grand or great-grand parent cells prior to gene loss might persist and be transmitted to its descendants even though the gene is not present. Such persistence is called perdurance (Garcia-Bellido and Merriam, 1971; Herman, 1984, 1987, and 1989; Villeneuve and Meyer, 1990; and Hedgecock and Herman, 1995). The persistence of the gene product even after the gene is lost masks the site of action. *sdc-1* is a gene involved in both sex determination and dosage compensation. Genetic mosaic analysis of *sdc-1* reveals neither AB(-) nor P_1(-) (symbolize loss of wild-type copy of the gene) mosaics in the cell lineage of *sdc-1* exhibited a wild type phenotype (Villeneuve and Meyer, 1990).

Another form of perdurance is maternal effect. One type of maternal effect is maternal rescue. Maternal rescue occurs when homozygous mutant progeny from heterozygous mother has a less severe phenotype than the homozygous mutant progeny from a homozygous mutant mother. *lin-15* demonstrates maternal effect of the Muv phenotype. A homozygous mutant for a *lin-15* mutant is Muv as its progeny would be Muv. If the *lin-15* mutant were a heterozygote, then the progeny that is homozygous mutant for *lin-15* would have wild-type vulvae (Ferguson and Horvitz, 1989; Villeneuve

and Meyer, 1990; Huang, Tzou, Sternberg, 1994 and Clark, Lu, Horvitz, 1994; and Huang, 1995).

Gene characterization, pathway analysis and its site of action in an organism can be determined with the power of genetics. As mentioned previously, *lin-15* is a member of a set of negative regulators of vulval development in which appropriate mutation combinations result in a multivulva (Muv) phenotype (Ferguson and Horvitz, 1989). *lin-15* is a complex locus with two independent, mutable activities, A and B (Ferguson and Horvitz, 1989; Huang, Tzou and Sternberg, 1994; Clark, Lu and Horvitz , 1994; Lu and Horvitz,1998) and it acts independently of two known signals controlling the fates of the VPCs, the AC inductive signal and a lateral signal among the VPCs (Herman and Hedgecock, 1990). The two cistrons of the *lin-15* locus each encode a hydrophilic protein, LIN-15B and LIN-15A, which have no obvious similarity to each other or to other known proteins.

In this thesis, I develop a powerful new technique, GFP-LacI + $lacO_{256}$, which is used for a variety of genetic tests. I demonstrate its utility by analyzing the site of action of *lin-3* for vulval induction. Also, I use GFP-LacI + $lacO_{256}$ as a single cell marker for mosaic analysis on the *lin-15* locus in order to determine its site of action for both gene activities. Moreover, I further characterize *lin-15A* by localizing its expression to certain tissues of the hermaphrodite. Finally, I identify vital domains in the gene for proper protein activity.

Chapter 2

Visualization of *C. elegans* extrachromosomal arrays by GFP: A single cell marker for mosaic analysis and its use demonstrating *lin-3* functions in anchor cell

Abstract

We have visualized extrachromosomal arrays by targeting the green fluorescent protein (GFP) to a specific DNA sequence (lac operator) incorporated into *C.elegans'* transgenes. Cells containing this system have nuclear-localized bright spots of GFP-LacI bound to *lacO,* and diffuse nuclear fluorescence corresponding to nuclear localized GFP-LacI. A chromosomal-integrated array of lac operators allows detection of nematode chromosomes in living animals. This technique allows rapid, accurate determination of spontaneous loss of an array, thereby allowing high-resolution mosaic analysis. This system can also be used to determine polyploid and to investigate chromosome segregation. We carried out genetic mosaic analysis on *lin-3*, which encodes an epidermal growth factor family member, using the GFP-LacI + *lacO* system. The anchor cell is the site of action for induction of the vulva by *lin-3.*

INTRODUCTION

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has been used extensively for observation in vivo of gene expression and cell morphology in *C.elegans* (Chalfie *et al*. 1994; Cubitt *et al*. 1995; Heim *et al*. 1994; Heim and Tsien 1996). GFP has also been targeted to specific subcellular structures by fusing GFP to various proteins (Chalfie *et al*. 1994; Cubitt *et al*. 1995; Heim *et al*. 1994; Heim and Tsien 1996). A technique utilizing a chimeric protein of GFP (S65T) and the *E. coli* lac repressor (LacI) along with lac operator (*lacO*) makes the visualization of chromosomes

possible (Chalfie *et al.* 1994; Cubitt *et al.* 1995; Heim *et al.* 1994; Heim and Tsien 1996; Robinett *et al.* 1996; Straight *et al.* 1996; Webb *et al.* 1995). This fusion protein has the DNA-binding capability of LacI and the fluorescent properties of GFP. The fusion protein is capable of binding to the *lacO,* thus localizing GFP expression at the DNA repeat. Such localization allows direct visualization of segregating chromosomes during mitosis.

We have applied the GFP-LacI technique to *C. elegans*. We show that the GFP-LacI + *lacO* repeat technique allows visualization of extrachromosomal arrays, viewing chromosomal segregation and determining polyploidism in *C. elegans*. We have also applied the GFP-LacI + *lacO* technique to mosaic analysis.

Genetic mosaics in *C. elegans* are typically generated by the spontaneous somatic loss of an extrachromosomal array or free duplication (Hedgecock and Herman 1995; Herman 1984; Herman 1989; Herman 1995). When the free duplication or extrachromosomal array containing a wild-type cell-autonomous marker gene (usually *ncl-1*; enlarged nucleoli) and a gene of interest is lost from one of the daughter cells during mitosis, it gives rise to a lineage of cells lacking wild-type activity of the marker gene and the gene of interest (Hedgecock and Herman 1995; Herman 1984; Herman 1989; Herman 1995). This "loss" allows the determination of which cells are required for proper gene function. Extra-chromosomal arrays are mitotically unstable such that the mosaic pattern is complex (Hedgecock and Herman 1995; Herman 1984; Herman 1989; Herman 1995; Miller *et al.* 1993), and thus a precise method of scoring individual cells under fluorescence microscopy would be useful.

The LIN-3 protein affects hermaphrodite fertility. It is also an inductive signal for vulval differentiation, animal viability, as well as the specification of anterior cell fates in male B cell lineage (Ferguson and Horvitz 1985; Ferguson *et al.* 1987; Hill and Sternberg 1992; Horvitz and Sulston 1980; Liu *et al.* 1999; Sulston and Horvitz 1981). *lin-3* gene encodes a protein of the epidermal growth factor (EGF) family (Hill and Sternberg 1992; Liu *et al.* 1999). The EGFs family is involved in animal development to promote cell proliferation and differentiation. LIN-3 has an extracellular domain with one EGF motif, a transmembrane domain and a cytoplasmic domain. We focus on LIN-3 inductive signal to initiate vulva differentiation in this paper.

The progeny of three vulva precursor cells (VPCs) forms the vulva. The VPCs are the posterior daughters (P3.p-P8.p) of the 12 P cells that are present since hatching (Sternberg and Horvitz 1986; Sulston and Horvitz 1977). During vulval induction, the AC secretes the epidermal growth factor LIN-3 protein (Hill and Sternberg 1992; Katz *et al.* 1995; Sternberg and Horvitz 1986). The VPC (P6.p) that is nearest to the AC will adopt the primary fate since it has more of its receptor tyrosine kinases occupied by the LIN-3 protein. P5.p and P.7.p cells are induced to adopt the secondary fate while the VPCs (P3.p, P4.p. and P8.p) further from the AC adopt a tertiary or non-vulval fate. The fates adopted by the VPCs indicate the number of cell descendants. The primary and secondary cell fates generate 8 and 7 cell descendants, respectively. Together, these cells form the vulva while the tertiary fates generate 2 non-vulval descendants that fuse with

the hyp7 epidermis (Horvitz 1988; Horvitz and Sternberg 1991; Kimble and Hirsh 1979; Koga and Ohshima 1995b; Liu *et al.* 1999; Simske *et al.* 1996; Simske and Kim 1995; Sternberg and Horvitz 1986; Sulston and White 1980). Evidence from genetic and laser ablation studies and structural comparison with other EGFs suggest LIN-3 is secreted from the AC to activate the induction pathway resulting in three VPCs to take vulval fate (Aroian *et al.* 1990; Hill and Sternberg 1992; Hill and Sternberg 1993; Katz *et al.* 1996; Liu *et al.* 1999; Simske *et al.* 1996; Sternberg and Horvitz 1986). We have confirmed via mosaic analysis using GFP-LacI + *lacO*, that *lin-3* signal is required solely in the anchor cell for proper vulval induction.

MATERIALS AND METHODS

Nematode methods: Growth and handling of *C. elegans* strain N2 were according to Brenner (1974) and Sulston and Hodgkin (1988). All experiments were performed at about 20°C unless otherwise stated. The genetic and cellular nomenclature of *C. elegans* was followed according to Horvitz *et al.* (1979) and Sulston *et al.* (1983), respectively.

Strains: The standard wild-type N2 strains and other mutant strains used *ncl-1(e1865)* and *lin-5(e1348) dpy-10(e128) II* were obtained from the *Caenorhabditis* Genetics Center (USA). The *lin-15* null allele *e1763* was used for *lin-15A* mosaic analysis (Ferguson and Horvitz 1985).

The transgenes *syEx*[*dpy-20(+)* (20ng/μl) + *dpy-30::S65T*LacI* (100ng/μl*)* + *pPD49-78GFP-LacI* (100ng/μl) and *syEx(dpy-20(+)* (20ng/μl) + *pPD49-78 GFP-LacI* + *lacO* (50ng/μl)] were integrated into *ncl-1(e1865)III; dpy-20(e1282)IV* and *dpy-20(e1282)IV* strains via X-ray irradiation (Fire 1986), respectively, to yield PS2441 (*syIs39)* and PS2442 (*syIs44)* (Table 1).

GFP-LacI fusion plus 256 *lacO* repeat: The GFP-LacI fusion protein plus the *lacO* repeat (Robinett *et al*. 1996; Straight *et al*. 1996; Webb *et al*. 1995) was graciously given to us by Dr. Andrew Belmont. We placed GFP-LacI under the transcriptional control of an hsp16 promoter/enhancer element pPD49-78 (Fire and Xu 1995; Mello and Fire 1995; Perry *et al*. 1993). The fusion protein was inserted into KpnI/SacI site of heat shock vector pPD49-78. pPD49-78 is expressed very well in the neural and hypodermal cells, as well as in the gut, muscles, and pharynx but not in the germline (Mello and Fire 1995; Perry *et al*. 1993). The GFP-LacI was also placed under the transcriptional control of the *dpy-30* promoter graciously given to us by Dr. Barbara Meyer. The *dpy-30* promoter directs expression throughout the animal, including the germline. The GFP was replaced with GFP(S65T) from the vector pPD93-65, which contains introns, in order to increase translation of the fusion protein (Mello and Fire 1995). The GFP (now designated S65T*) is inserted in the KpnI/EcoRI site of the *dpy-30*::GFP-LacI, now called *dpy-30*::S65T*-LacI.

Germline-mediated transformation by microinjection: Microinjection was performed according to Mello *et al.*, (1991), which was modified from Fire (1986). Young adult hermaphrodites were placed live on pads of 2% agarose under an inverted differential contrast-interference (Nomarski) microscope (Carl Zeiss, Oberkochen, West Germany) and the DNA was injected into the gonad using an Eppendorf micro injector 5242 (Eppendorf Gertebau Netheler, Hamburg, West Germany). For the Ncl vs. GFP-LacI and polyploid experiments, the plasmid pRF4, containing the *rol-6(su1006)* mutant gene (Mello *et al.* 1991), was used as a dominant transformation marker at a concentration of 40ng/µl). The injection mixture for the Ncl vs. GFP-LacI experiment contained pPD49-78GFP-LacI (100ng/µl), 256 repeat *lacO* array (50ng/µl), cosmid C33C3 (rescues the Ncl-1 mutant phenotype (Miller *et al.* 1996) (50ng/µl) and pBluescript II SK+ (Stratagene) as carrier DNA (5ng/µl). This mixture was injected into *ncl-1(e1865)* gonads. The injection mixture for the polyploidism experiments contained pPD49-78::GFP-LacI (100ng/µl), 256 repeat *lacO* array (50ng/µl), and pBluescript II SK+ (Stratagene) as carrier DNA (5ng/µl). This injection mixture was injected into the gonads of *lin-5(e1348) dpy-10(e128) II* to yield *syEx207* in the strain PS2629.

The transgenic lines obtained from each experiment were heat-shocked for 30 minutes at 33°C to elicit GFP-LacI expression. Expression of the GFP-LacI can be seen as early as 30 minutes after heat-shock, and as late as 24 hours.

Mosaic analyses *lin-3* gene function in vulval induction: Mosaic animals were obtained from a somatic loss of the extrachromosomal array *syEx345[lin-3(+), lacO,*

myo-2::GFP(pPD118-33)] from the vulvaless strain *syIs46; lin-3(n378) let-59(s49) unc22(sy7)/ lin-3(n1059)unc-24(e138)* (Table 1). The point of loss is determined by the absence of the fluorescent spot. We used L3-L4 wildtype, multivulva, and vulvaless animals with their pharynx fluorescing, due to MYO-2::GFP, for mosaic analysis to ensure the array is present (Okkema *et al.* 1993). The nuclei observed to identify mosaic animals are: AC, P3.p, P4.p, P5.p, P6.p, P7.p and P8.p.

Microscopy and Photography: Animals were anesthetized with 2mM levamisole on 5% Noble agar pads. Photographs were taken on Kodak Ektachrome, ASA 160 or Fuji Provia, ASA 400 on a Zeiss Axioplan with Chroma High Q GFP LP filter set (absorption band 450nm and 505nm emission) at 100X optics or by Confocal photomicrography for strain PS2442.

Results

Visualization of Chromosomes: To test whether the GFP-LacI + *lacO* system could be used to visualize the DNA of extrachromosomal arrays in *C. elegans*, we engineered a GFP-LacI fusion protein under the control of the heat-shock promoter in vector pPD49-78 (hsGFP-LacI). We then microinjected a DNA mixture containing hsGFP-LacI, the *lacO* repeat and *dpy-20* rescuing DNA (pMH86) into the gonad of an adult *dpy-20 (e1282)* hermaphrodite. After a 30-minute heat-shock at 33°C, transformants were

found to express nuclear GFP and intense foci of subnuclear fluorescence, presumably corresponding to the DNA of the extrachromosomal arrays. DAPI co-staining confirmed that the GFP-LacI + *lacO* system has nuclear expression and association with the DNA. We first detected expression in embryos at early gastrulation (~24 cell stage). Larvae and adults express GFP broadly.

DNA molecules injected into the *C. elegans* gonad syncytium assemble into arrays; extrachromosomal arrays consist of many rearrangements of the DNA injected (Mello *et al*. 1991). Figure 1A and Figure 1B demonstrate, respectively, the fusion protein bound to the *lacO* repeat resulting in one to two bright spots per nucleus as well as the unbound fusion protein resulting in nuclear diffuse fluorescence ("haze"). Mitotic loss of these mixed extrachromosomal arrays in a single founder cell resulting in a clone of cells lacking the activities of all genes in the array (Herman 1984; Herman 1989; Miller *et al*. 1996). Figure 1B also demonstrates the mitotic instability and the ease of determining whether a loss occurred (AB loss).

In order to test the reliability and consistency of the GFP-LacI + *lacO* as a detection method for transgenes, non-Dpy transformants were X-irradiated to integrate the transgenic array into the genome, yielding *syIs44* in the strain PS2442. In this strain, we observed that most nuclei had one or more spots of fluorescence (see Figure 1C). Detection of chromosomes was efficient. For example, we observed two pairs of sister cells in each of the 20 animals, the embryonic sisters F and U and the postembryonic sisters P8.pa and P8.pp, and found that all 80 cells had one or two fluorescent spots. Therefore, we are able to detect a transgene in every cell.

GFP-LacI + *lacO* as a single cell marker

The *ncl-1(e1865)* mutation results in enlarged nucleoli (Ncl phenotype) of a large number of cell types. Since *ncl-1* acts in a cell autonomous fashion, it is useful as a cell lineage marker (Hedgecock and Herman 1995).

The cosmid clone (C33C3) rescues the *ncl-1* phenotype and has been used as a cell lineage marker on extrachromosomal arrays (Koga and Ohshima 1995b; Miller *et al.* 1996; Yochem *et al.* 1998). To test the utility of the GFP-LacI + *lacO* technique as a single cell marker, we compared it with *ncl-1.* A DNA mixture composed of the *lacO* repeat, C33C3, and the *lin-15B(+)* DNA was injected into the gonads of *syIs46; ncl-1(e1865); dpy-20(e1282); lin-15(e1763)* animals (Table 2). The isolated transgenic mosaic animals were then heat shocked (See Materials and Methods). We scored the Ncl phenotype in the nucleoli of 22 different cells (m2, m3L, m3VL, m4, m3R, m3VR, m3DR, m3DL, hyp7 dorsal head, hyp7 ventral head, P/I, p3.p, P4.p, P5.p, P6,p, P7.p, P8.p, B, F, U, hyp7 ventral tail, hyp7 anus) per mosaic animal (n=37)(Table 2). The presence of the spot is unequivocal evidence that the transgene is present. The existence of Ncl(-) cells with spots indicate a false negative by Ncl-1. Conversely, non-Ncl cells with no spots may indicate the perdurance of the NCL-1 protein (i.e., false positive) or an early loss of the *lacO* array. According to Table 2, both markers agree in scoring approximately 80%, while false negatives of Ncl-1 or (N, +) occur about 16%. The occurrence of false positives of Ncl-1 or (W, -) is 2%. However, neither marker is perfect. The Ncl marker is undetectable in the germline, intestinal nucleoli and endogenously large nucleoli such as some hyp cells and muscle cells. The GFP-LacI

+ *lacO* system has a higher apparent loss rate per cell division of 15.9% (Table 3)(discussed later) as well as fading of the GFP.

One possibility for the lack of intense fluorescent spots is that the expression of GFP-LacI is insufficient. We thought this possibility likely since the expression depended on the GFP filter combination used, and the light source (200 watt vs. 100 watt). To increase the sensitivity, we engineered a GFP-LacI under the control of the ubiquitously expressed *dpy-30* promoter/enhancer (Hsu *et al.* 1995). Another possibility is that non-Ncl nuclei without spots reflect perdurance of NCL-1.

The combined use of the cell lineage marker *ncl-1* and the GFP-LacI + *lacO* would increase the accuracy and ease of mosaic analysis. In order to use both markers, the double mutant *ncl-1; dpy-20* was injected with the DNA mixture (pMH86 (*dpy-20(+)*) + pPD49-78::GFP-LacI + *dpy-30*::GFP-LacI). Once the non-Dpy transformants were isolated, the extrachromosomal array was integrated into the genome by X-ray irradiation to yield strain PS2958 *syIs46* [pMH86 + pPD49-78::GFP-LacI + *dpy-30::*S65T*LacI] *II; ncl-1 III; dpy-20 IV.* This strain became the base for our genetic mosaic analysis of *lin-15A* and *lin-15B;* the results of which will be discussed in a later journal.

Polyploidy

To test whether our method can detect polyploidy, we injected into *lin-5(e1348)* mutant, which fails to undergo mitosis (Albertson *et al.* 1978), a DNA mixture

consisting of a fusion protein under the control of the heat-shock promoter pPD49-78, the *lacO* repeat, plus *rol-6(su1006)* dominant, mutant transforming marker. The Rol segregants of strain PS2629 confirmed that GFP-LacI +*lacO* can assay for polyploid cells. Multiple dots have been seen when viewing polyploid cells (i.e., intestines and hypodermal cells; Figure 6). We conclude that the GFP-LacI +*lacO* method is useful in determining which cells are polyploid. However, the number of spots within the cell cannot unambiguously determine the extent of ploidy. Diploid cells have 1-3 spots while polyploid cells express more than 4 spots.

GFP-LacI + *lacO* as a mosaic marker: The GFP-LacI + *lacO* as a mosaic marker confirms *lin-3*'s site of action for vulval induction is in the AC. The strain we chose to use contains to mutant alleles of *lin-3* in trans and *syIs46* in the background. The mutant alleles we chose for our purpose are: *n378* that is defective only in vulval development, i.e., vulvaless hermaphrodites, and *n1059* that resembles a genetic null and causes L1 lethality (Hill and Sternberg 1992; Liu *et al.* 1999). This strain *syIs46; lin-3(n378) let-59(s49) unc-22(sy7)/lin-3(n1059) unc-24(e138)* was then injected with *lin-3(+)* (20ng/µl), *lacO* (50ng/µl), transformation marker pPD118-33 (*myo-2::GFP*)(16ng/µl) (Okkema *et al.* 1993), and carrier DNA BSK+II (120ng/µl).

We examined animals with fluorescent pharynx due to *myo2*::*gfp*. These chosen animals have the transgene in either the AB lineage or P1 lineage or both lineages

(Okkema *et al*. 1993). We picked L3-L4 animals expressing *myo-2::GFP* by viewing them under a dissecting microscope with a GFP filter. These animals were then heat-shocked for 30 minutes in a 33 degree water-bath followed by a one-hour recovery period in a 20 degree incubator.

We examined a total of 114 animals prescreened by the dissecting microscope. We considered three possibilities: *lin-3* acts in the AC, it acts in the VPCs or both. We therefore scored the AC and the VPCs of W.T., Vul and Muv *lin-3* transgenic animals. Of the 114 animals, 91 were wild-type vulva, 15 animals were vulvaless and 8 animals were multivulva. 88 animals with the array present in both the AC and VPCs were wt and Muv. Eight animals lacking the array in both the AC and VPCs were Vul. 11 animals had the array in the AC but not in the VPCs were wt (n=10) and a Muv (n=1) (Figure 2, 3, and 5). Seven animals had the array in the VPCs but not in the AC were all Vul (Figure 2 and 4). We conclude *lin-3* acts in the AC.

Discussion

Visualization of arrays: We have demonstrated that GFP-LacI + *lacO* can be used to visualize arrays transgenes, either as extrachromosomal arrays or integrated into a chromosome, in living *C. elegans.* We have also shown that GFP-LacI + *lacO* is a useful *in vivo* marker for ploidy determination. We also show that this direct visualization allows high-resolution mosaic analysis using transgenes. We examined this method by comparing its efficiency with Ncl-1 as a single cell marker for mosaic

analysis. We have demonstrated the utility of GFP-LacI + *lacO* as a mosaic marker by locating *lin-3* site of action at the AC for vulval induction.

Single cell marker: The comparison between *ncl-1(e1865)* and the GFP-LacI + *lacO* indicate that this new marker is comparable in its reliability to *ncl-1* as a cell lineage marker for the presence of a transgene (Table 2). The main advantage of using the fusion protein GFP-LacI + *lacO* rather than *ncl-1* is that scoring the mutant Ncl phenotype is typically more difficult than scoring cells with the bound GFP-LacI fusion protein. Also, GFP-LacI can be used in cells such as intestinal cells and germline for which *ncl-1* is not applicable. We find that the GFP-LacI method (spot or not spot) is much easier than scoring nucleolus size. Such ease of scoring may also be used for more accurate mosaic analysis. The comparison between the GFP-LacI + *lacO* and *ncl-1* as a single cell marker as well as its loss rate per cell division (Table 2 and 3) confirms that using both the *ncl-1* and the integrated GFP-LacI together will increase the accuracy for mosaic analysis. An alternative mosaic marker, SUR-5GFP(NLS), demonstrates the ease and speed of scoring cells by fluorescence comparative to the Ncl-1 marker (Yochem *et al.* 1998). In this technique, one is able to rapidly screen with a dissecting microscope for rare mosaic animals (Yochem *et al.* 1998), unlike the GFP-LacI + *lacO* methodology that requires a compound microscope.

The high loss rate of GFP-LacI + *lacO,* 15.9%, may be due to the destabilization of the arrays formed such as the early loss of the marker gene possibly with the of the gene of interest expression continuing or there may be silencing of neighboring genes due to heterochromatic regions formed by the *lacO* repeat. The latter case is more likely than the former because multiple repeats due have a tendency to condense affecting the genes nearby. The early loss of the array with the expression of the gene of interest continuing is the result of the transgenic array breaking. This situation can be circumvented by having more than one stable transgenic line to score for mosaics in order to secure no irregularity of the results obtained. The chances having three or more stable transgenic line such unstability are nearly zero.

Site of action of *lin-3*: We used the PS2958 strain for the *lin-3* mosaics. This strain is an excellent base for performing mosaic analysis for any gene. Any null mutation for the gene of interest can be placed in the background in the PS2958 strain. Then inject into the new strains' gonad the remaining components of the mosaic markers, *ncl-1(+)* and the *lacO* repeat, the rescuing DNA of the gene of interest and a transformation marker (if needed). *lin-3* is required at multiple time points for several aspects of *C.elegans* development. It's required throughout all larval stages for viability. It is required during mid-larvae stages for vulval induction and male spicule development, and in the later stages for fertility (Aroian *et al*. 1990; Hill and Sternberg 1992; Hill and Sternberg 1993; Katz *et al*. 1996; Liu *et al*. 1999; Simske *et al*. 1996; Sternberg and Horvitz 1986). We focus on *lin-3*'s requirement in vulval induction. One mutant allele *lin-3(e1417)* has a

mutation in the promoter region, which affects the expression of *lin-3* in the AC. This allele has the VPCs adopting non-vulval fates with no other developmental defects. This was confirmed genetically by constructing the strain *e1417/n1059.* If the *e1417* allele specifically affects the vulva, the phenotype of *e1417/n1059* should be Vul but have no other *lin-3* mutant phenotype (Liu *et al.* 1999). If *e1417* has low levels of activities in all tissue, the phenotype of *e1417/n1059* would not only be Vul but also have pleotropic phenotypic effects of *lin-3* loss (Liu *et al.* 1999). The resulting phenotype of *e1417/n1059* is solely Vul, therefore, specifically affecting the vulva due to non-expression of *lin-3* in the AC. Of the 114 animals scored during the *lin-3* mosaics, 18 mosaic animals (Figure 2) further confirm *lin-3* function is required in the AC to induce vulval differentiation (Figure 3, 4, 5). The 10 animals with wild-type vulva, loss the array (no bright spot) in the Pn.p cells but all had the array present (bright spot) in the AC (Figure 2 and 3) signifying that *lin-3* is not required in the VPCs to have vulva development but in the AC. The 7 vulvaless animals had the array (bright spot) in the Pn.p but all loss the array (no bright spot) in the AC (Figure 2 and 4) signifying that the AC is the site of *lin-3* expression for vulval induction. One multivulva animal loss the array (no bright spot) in all the Pn.p cells but present (bright spot) in the AC again signifying that *lin-3* is not required in the VPCs to have vulva development but required in the AC for vulval induction (Figure 2 and 5).

Acknowledgments: We greatly thank Andrew Belmont for the fusion protein GFP-LacI and *lacO* repeat constructs as well for invaluable help developing this system, and Linda

Huang for suggesting its use in mosaic analysis. We also thank Barbara Meyer for the *dpy-30* promoter. We appreciate Shahla Gharib's assistance during the strain integration. We also thank the members of our lab for vital input into this manuscript. The USPHS (grant HD23690 to PWS) supported this work. A.S.G-S. is a Howard Hughes Medical Institute Graduate Fellow. P.W.S. is an Investigator with the Howard Hughes Medical Institute. The *Caenorhabditis* Genetic Center provided some strains.

Figure 1. Visualization of *C. elegans* extrachromosomal arrays by GFP

a) One to two bright spots are seen per nucleus when the fusion proteins are bound to the *lacO* array. b) If the fusion proteins remain unbound, then the nucleus has diffuse fluorescence. Embryos, *dpy-20; syEx* [pMH86, pPD49-78::GFP-LacI, *lacO*], were mounted on 5% Noble agar and examined under Nomarski optics and epifluorescence at 100X. c) A confocal photomicrograph of *syIs44* L4 hermaphrodite's pharynx. Arrowheads point to unbound GFP-LacI. Arrows point to bound GFP-LacI to *lacO.*

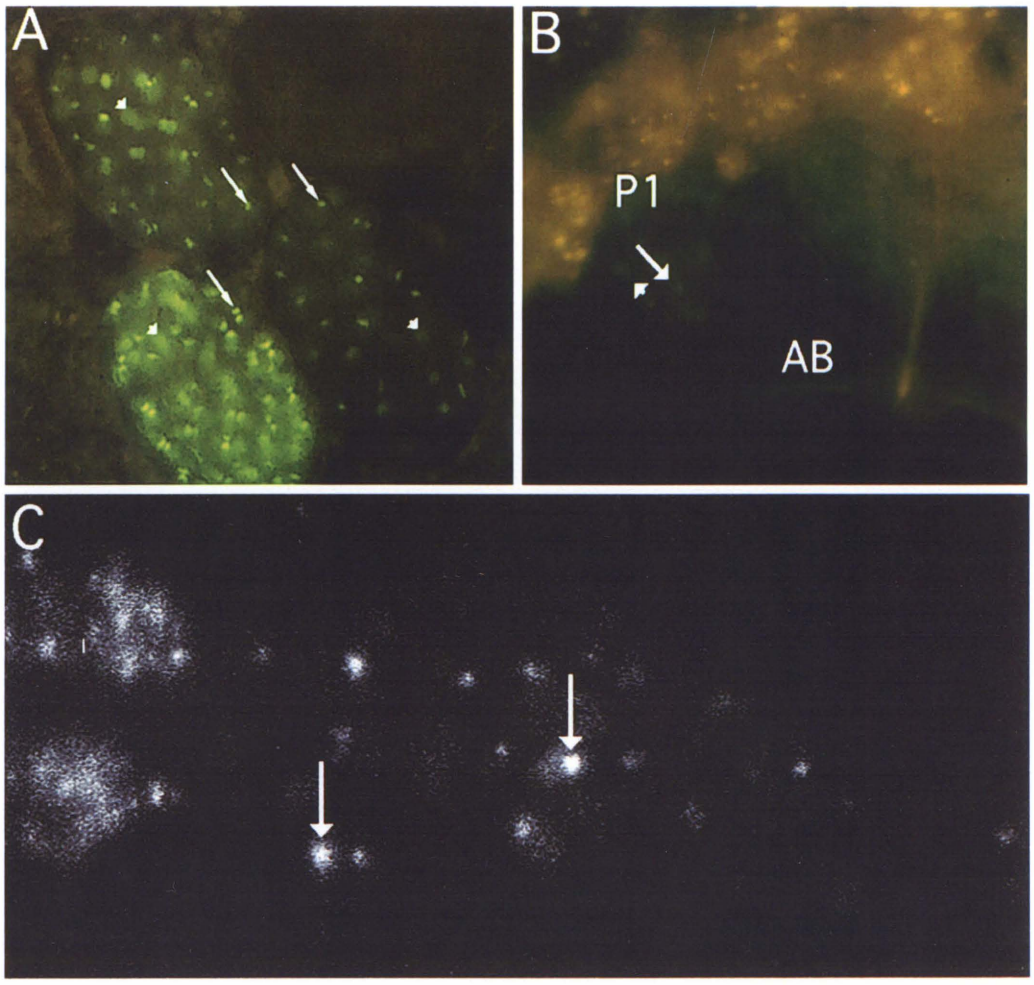

A
B
P1
AB
C

Figure 2. Mosaic analysis of *lin-3* with GFP-LacI + *lacO*

Mosaic analysis of *lin-3(n378)/(n1059))* animals using GFP-LacI + *lacO* as the marker reveals that the wild-type *lin-3* gene is required in the AC for normal vulval induction. +: GFP-LacI• *lacO*, fluorescent spot; -: no fluorescence. Letters correspond to vulval status: **W**= normal vulva; **M**= multivulva; **V** = vulvaless. The numbers in the Pn.p column indicates the number of nuclei with bright spots of fluorescence. The asterisks indicate where pseudovulvae formed in Muv animal.

Worm	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	Anchor Cell
W.1	-	-	-	-	-	+2	+ inferred
W.26	-	+	+3/7cells	-	+5/7cells	+	+
W.36	+	+2	+2/7cells	-8	+2/6cells	+2	+
W.39	-	-2	-7	-8	-7	-2	+
W.53	+	-2	+4/7cells	-8	+2/7cells	-2	+
W.58	-	+1	-7	-8	-7	-2	+
W.64	-	+1	+5/7cells	-8	+6/7cells	-2	+
W.84	-	-2	+2/4cells	-4/4cells	-4/4cells	-2	+
W.89	-	-2	-7	-8	-7	-2	+ inferred
W.91	-	-2	-7	-8	-7	-2	+
V.2	-	-	-	-	-	+	-
V.3	+	+	+	+	+	+	-
V.7	+	+	-	+	-	-	-
V.9	+	+	+	+	+	+	-
V.10	-	+	+	+	+	+	-
V.12	+	+2	+2	+2	+2	+2	-
V.15	+	+	+	+	+	+	-
M.5	-	-2	-*	-*	-*	-2	+

Figure 3. *lin-3* mosaic animal: wild-type vulva a) *lin-3* mosaic animal at L4 stage with normal vulval development. Anchor cell labeled with arrow. b) Same *lin-3* mosaic animal at L4 stage with normal vulval development. The anchor cell expresses the transgenic array (*lin-3(+) + lacO + pPD118-33*) or bright-localized spot. The vulval precursor cells do not express the transgenic array or no bright-localized spot.

lin-3 mosaics: AC= +; VPCs= -
Wildtype Vulva

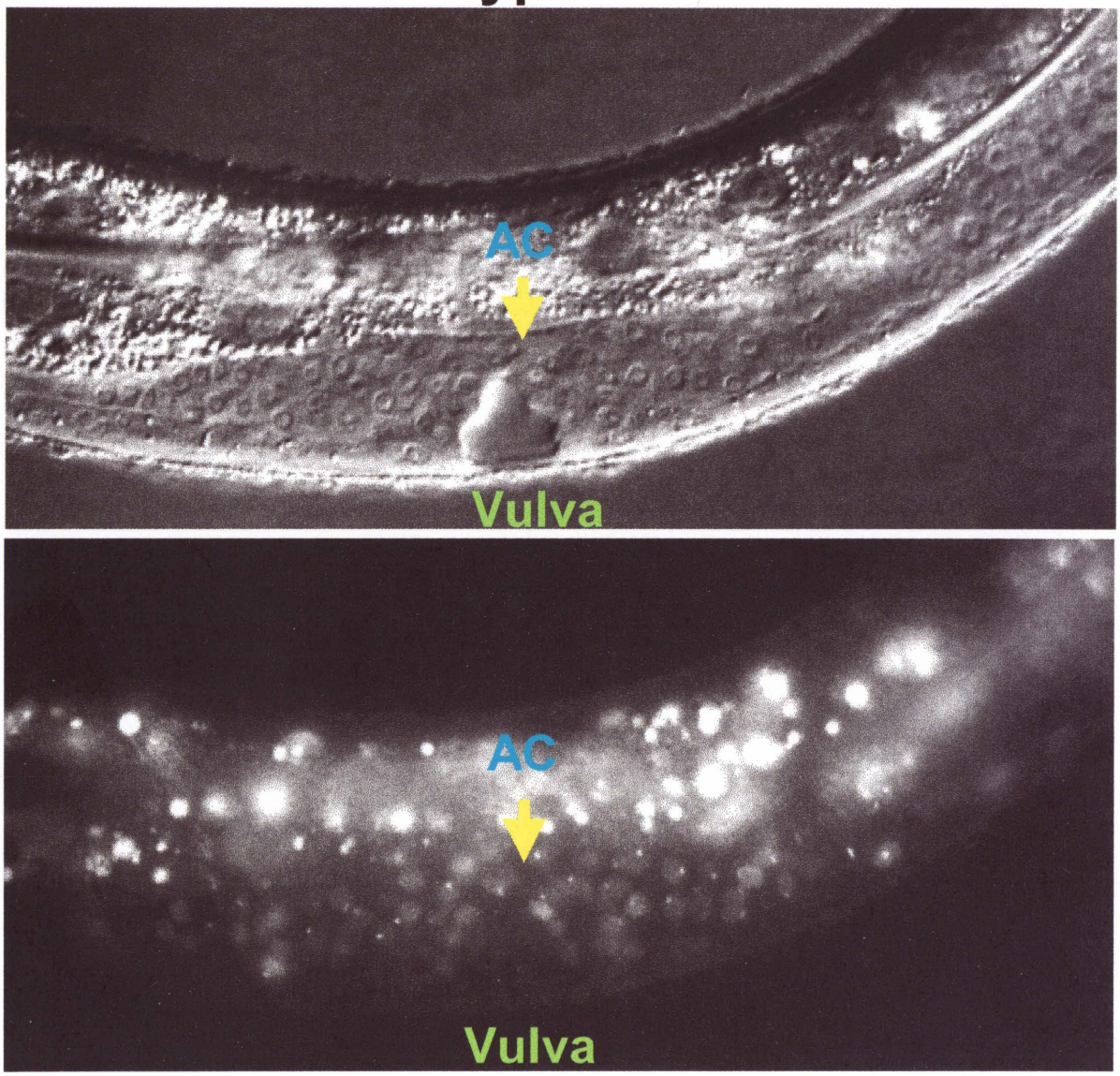


Figure 4. *lin-3* mosaic animal: vulvaless

a) lin-3 mosaic animal at L4 stage with no Pn.p cells adopting vulval fate. Anchor cell labeled with yellow arrow. Pn.p cells labeled with white arrow. b) Same *lin-3* mosaic animal at L4 stage with no Pn.p cells adopting vulval fate. The anchor cell does not express the transgenic array (*lin-3(+) + lacO + pPD118-33*) or bright-localized spot. The Pn.p cells do express the transgenic array or bright-localized spot.

lin-3 mosaics: AC= -; VPCs= + Vulvaless

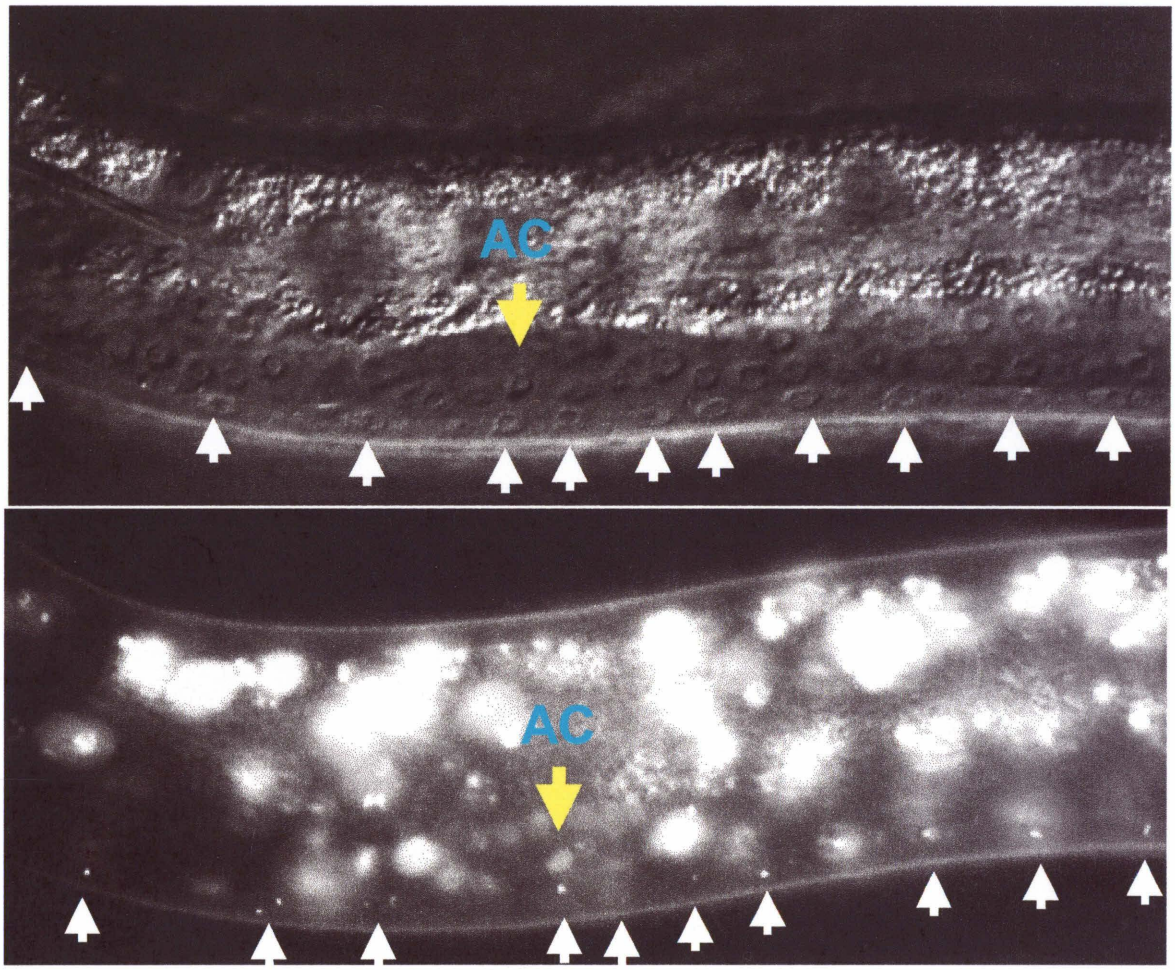


Figure 5. *lin-3* mosaic animal: multivulva vulva

a) lin-3 mosaic animal at L4 stage with multivulva phenotype. Anchor cell labeled with arrow. b) Same *lin-3* mosaic animal at L4 stage with multivulva phenotype. The anchor cell expresses the transgenic array (*lin-3(+)* + *lacO* + *pPD118-33*) or bright-localized spot. The vulval precursor cells do not express the transgenic array or no bright-localized spot.

lin-3 mosaics: AC= +; VPCs= -
Multivulva

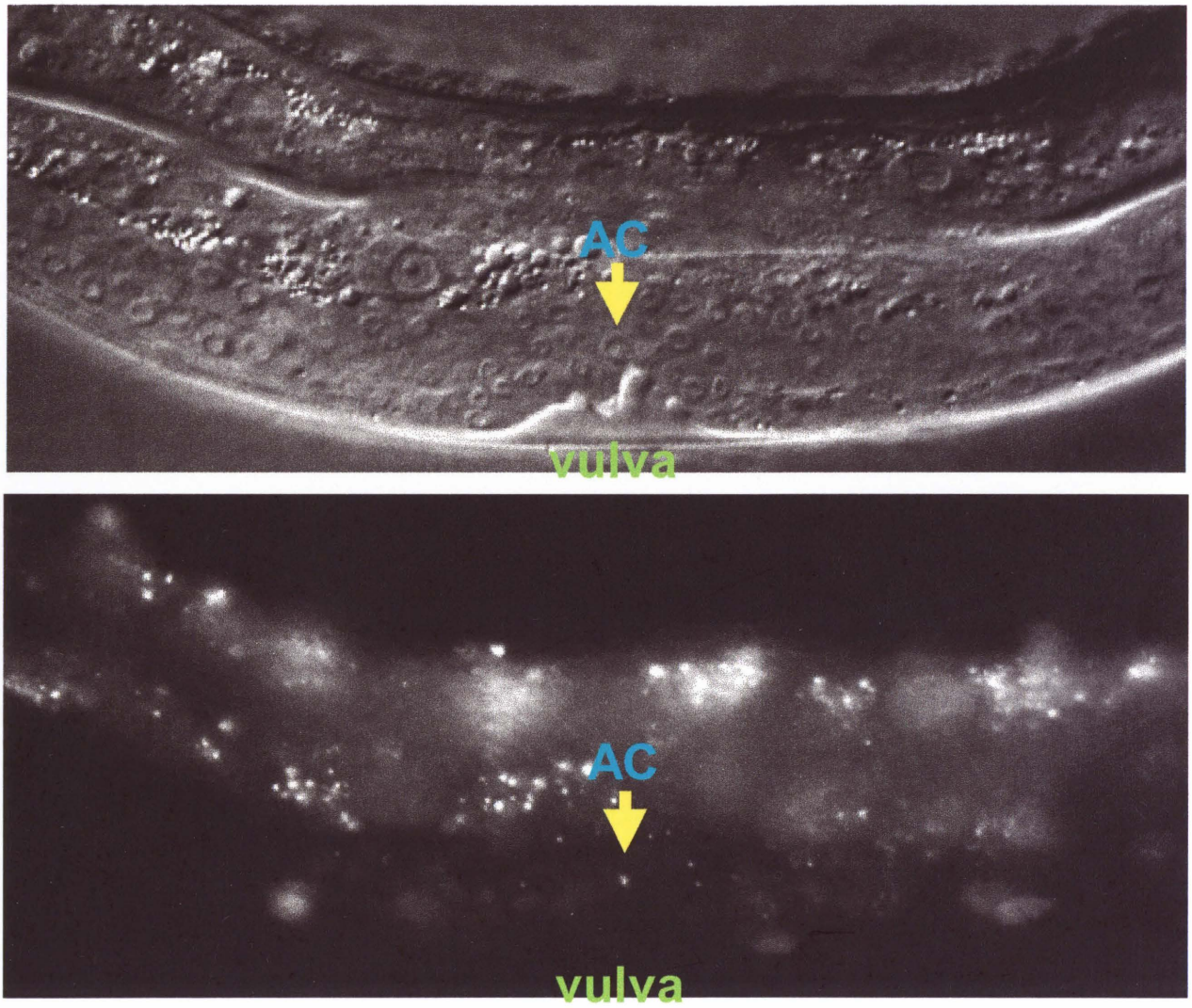
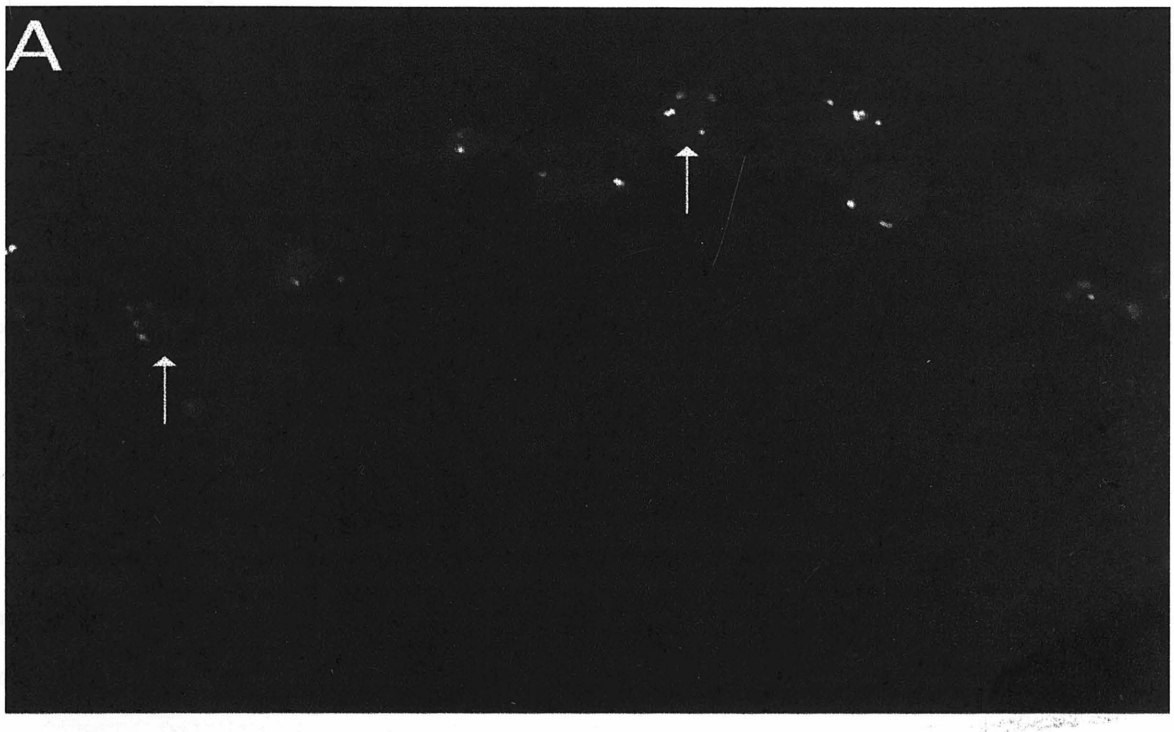


Figure 7. Assay for Polyploidy of nuclei

Comparison of body muscle between *syIs44* [*dpy-20(+)*+pPD49-78::GFPlacI+*lacO*]; *dpy-20* and *lin-5(e1348), dpy-10(e128) II*; *syEx207* [pRF4 *(rol-6(su1006)*) +pPD49-78GFP-LacI+*lacO*]. Animals were mounted on 5% Noble agar containing ~100nM levamisole and examined under Nomarski microscopy and fluorescence at 100X. a) *lin-5(e1348), dpy-10(e128) II*; *syEx207* [pRF4 *(rol-6(su1006))*+pPD49-78GFP-LacI+*lacO*] L4 hermaphrodite. b) *syIs44* [*dpy-20(+)* +pPD49-78::GFP-LacI+*lacO*]; *dpy-20* L4 hermaphrodite. Arrowheads point to unbound GFP-LacI. Arrows point to bound GFP-LacI to *lacO.*


A

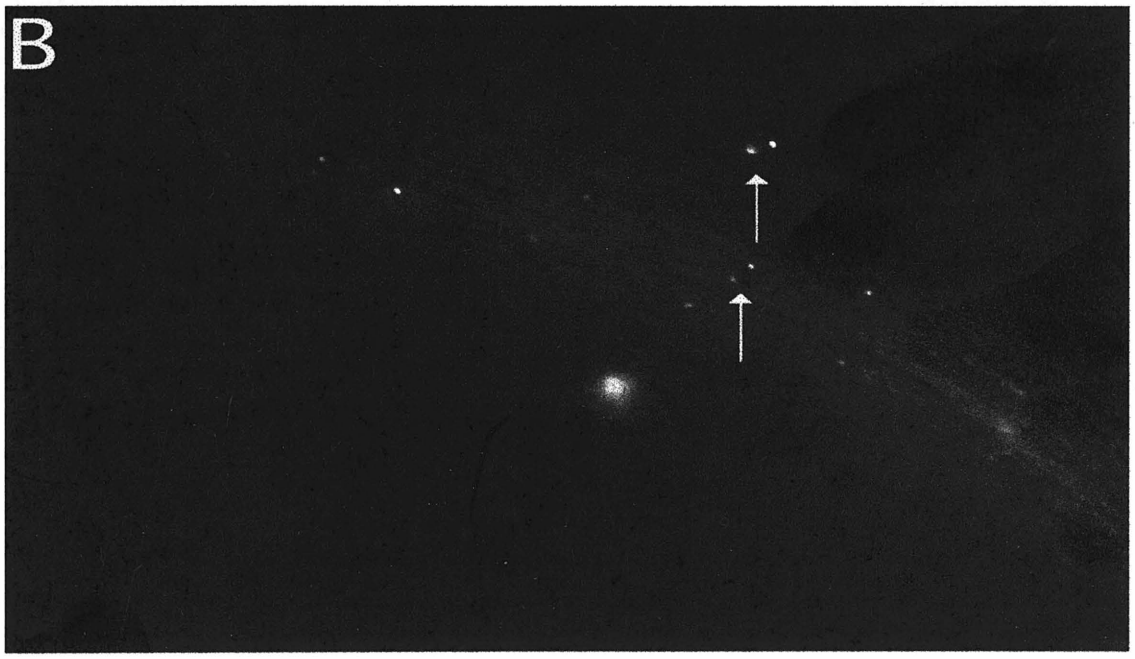

B

Table 1. Transgenes and strains

PS2442: *syIs44* [pMH86 (*dpy-20(+)*), pPD49-78GFP-lacI, *lacO*]; *dpy-20(e1282)*

PS2381: *syEx154* [pRF4 *(rol-6(su1006)*), C33C3, pPD49-78GFP-lacI, *lacO*]; *ncl-1(e1865) III*

PS2629: *lin-5(e1348), dpy-10(e128) II*; *syEx207* [pRF4 *(rol-6(su1006)*), pPD49-78GFP-LacI, $lacO_{256}$]

PS2958: *syIs46* [pMH86 (*dpy-20(+)*), dpy-30S65T*LacI, pPD49-78GFP-LacI] *II; ncl-1(e1865) III; dpy-20(e1282) IV; him-5(e1490) V*

PS3047: *syIs46; ncl-1(e1865); dpy-20(e1282); lin-15(e1763); syEx272* [*lin-15B* (+), *lacO*, C33C3]

PS3427: *syIs46; lin-3(n378) let-59(s49) unc-22(sy7)/ lin-3(n1059) unc-24(e138); syEx345* [*lin-3* (+), *lacO,* pPD118-33]

Table 2. *ncl-1* versus GFP-LacI + *lacO* as markers for mosaic analysis. *lin-15B* mosaic animals (n=37) used to determine the occurrence of the Ncl phenotype and GFP-LacI + *lacO.* Scored by cell from anterior to posterior of the worm: m2, m3L, m3VL, m4, m3R, m3VR, m3DL, m3DR, hyp7(dorsal, head), hyp7(ventral head), P/I, P3.p, P4.p, P5.p, P6.p, P7.p, P8.p, B, F, U, hyp7(tail), hyp7(anus). +: GFP-LacI bound to the *lacO*, fluorescent spot; -: no fluorescence, **W**: normal nucleolus size, **N**: enlarged nucleolus and ?: undetermined.

Cell	**W, + or N,-**	**W, -**	**N, +**	**?**
m2	32(86.5%)	0	3 (8%)	2
m3L	31(83.8%)	2(5.4%)	4 (10.8%)	
m3VL	31(83.8%)	1(2.7%)	5 (13.5%)	
m4	28(75.7%)	0	9 (24.3%)	
m3R	31(83.8%)	2(5.4%)	4 (10.8%)	
m3VR	30 (81%)	1(2.7%)	6 (16.2%)	
m3DR	29(78.4%)	0	8 (21.6%)	
m3DL	30 (81%)	0	7 (18.9%)	
hyp7(D. head)	30 (81%)	1(2.7%)	6 (16.2%)	
hyp7 (V.head)	32(86.5%)	0	5 (13.5%)	
P/I	33(89.2%)	1(2.7%)	3 (8.1%)	
P3.p	30 (81%)	1(2.7%)	3 (8.1%)	3
P4.p	29(78.4%)	2(5.4%)	4 (10.8%)	2
P5.p	30 (81%)	2(5.4%)	4 (10.8%)	1
P6.p	32(86.5%)	0	4 (10.8%)	1
P7.p	33(89.2%)	0	3 (8.1%)	1
P8.p	26(70.3%)	1(2.7%)	9 (24.3%)	1
B	22(59.5%)	1(2.7%)	14(37.8)	
F	31(83.8%)	1(2.7%)	5 (13.5%)	
U	29(78.4%)	2(5.4%)	6 (16.2%)	
hyp7 (V.tail)	29(78.4%)	0	7 (18.9%)	1
hyp7 (anus)	21(56.8%)	1 (2.7%)	8 (21.6%)	7
Mean %	80%	2%	7%	2%
Standard Dev	0.08	0.02	0.07	0.04

Table 3. Apparent loss rate per cell division: FU sister cells. -/- : cell had lost the array; +/- : loss at this cell division; +/+: no loss at this cell division. The determination of the loss rate for each marker. Loss rate is [+/-]/[(+/+) +(+/-)] Strains used PS3427 (Table 1).

Integrated GFP-LacI: n=67 animals [PS3427: *lin-3* mosaics]

	FU #	FU %	Loss Rate
+ +	37	55.2%	15.9%
- -	23	34.3%	
+ -	7	10.4%	

Chapter 3

Mosaic analysis of *lin-15* gene function in vulval induction

Abstract

The products of the *lin-15* gene were first defined genetically as negative regulators of the vulval induction pathway. The *lin-15* locus encodes two novel hydrophilic proteins, LIN-15A and LIN-15B. *lin-15A* and *lin-15B* belong to the synthetic multivulva (synMuv) set of genes which are comprised of two classes, A and B. Mutation of both an A and a B gene is required to obtain a multivulva (Muv) phenotype. We used both, the GFP-LacI + $lacO_{256}$ system as well as the *ncl-1* gene (abnormally large nucleoli, used as a single cell marker), as genetic mosaic markers to determine the site of action of *lin-15A* and *lin-15B*. Both markers indicate that *lin-15A* gene function is required within the vulval precursor cells (VPCs) to prevent an excessive number of VPCs from generating vulval progeny. *lin-15B* has a broad focus making it difficult to pinpoint a site of action. Further mosaics for *lin-15B* were done using the SUR-5::GFP as an additional mosaic marker and again proved difficult to pinpoint a site of action. We concluded either LIN-15B is required at the hypodermis or the perdurance of the LIN-15B protein is confounding the mosaic analysis result. Previous mosaics with a free duplication and a mutation that eliminate function of both *lin-15A* and *B* (*lin-15(n309)*) (Herman and Hedgecock 1990) indicated that at least one *lin-15* function acts non-autonomously. Our results indicate *lin-15A* gene activity in the VPCs is sufficient to rescue the Muv phenotype. This result is strengthened by the rescue of the *lin-15* null phenotype by localizing *lin-15A* expression solely at the VPCs.

Introduction

Vulval development

Four different pathways are involved to form the hermaphrodite vulva: the receptor tyrosine kinase LET-23 (RTK)/Ras pathway, the LIN-12 Notch pathway and two functionally redundant synthetic multivulva pathways known as synMuv class A and synMuv class B (Figure 1) (Greenwald and Broach 1990; Greenwald *et al.* 1983; Horvitz 1988; Sternberg and Horvitz 1986; Sternberg and Horvitz 1989). The initiating signal for vulva patterning and morphogenesis is from the anchor cell (AC).

The AC decision is determined by lateral signaling. Two gonadal cells Z1.ppp and Z4.aaa have an equal chance of becoming an AC, with the remaining cell becoming a ventral uterine precursor (VU). Factors involved in this decision are the *lin-12* (receptor) and *lag-2* (ligand) (Greenwald and Broach 1990; Greenwald *et al.* 1987; Greenwald *et al.* 1983). LIN-12 protein is a member of the Notch protein family which are predicted transmembrane proteins with epidermal growth factor (EGF) motif, 3 LNR (LIN-12 /Notch repeat) motifs in their extracellular domains and 6 ankrin repeats in their cytoplasmic domains. *lin-12* activity is also seen in lateral signaling between the VPCs, which will be described later (Greenwald *et al.* 1983; Sternberg and Horvitz 1989).

LET-23/Ras pathway

The VPCs are the posterior daughters (P3.p-P8.p) of the 12 P cells that are present since hatching (Sulston and Horvitz 1977). During vulval induction, the AC secretes the

epidermal growth factor LIN-3 protein (Hill and Sternberg 1992). The VPC (P6.p) that is nearest to the AC will adopt the primary fate since it receives more LIN-3 protein or receives it earlier. P5.p and P.7.p cells are induced to adopt the secondary fate via LIN-12 while the VPCs (P3.p, P4.p. and P8.p) further from the AC adopt a tertiary or non-vulval fate. The fates adopted by the VPCs indicate the number of cell descendants. The primary and secondary cell fates generate 8 and 7 nuclei descendants, respectively, but also differ in their morphogenesis. Together, these cells form the vulva while the tertiary fates generate 2 non-vulval descendants that fuse to the hyp7 epidermis (Horvitz 1988; Horvitz and Sternberg 1991; Kim 1995; Kimble and Hirsh 1979; Koga and Ohshima 1995b; Simske *et al.* 1996; Sulston *et al.* 1980; Sulston and Horvitz 1977). There is also lateral signaling during vulval induction, involving the LIN-12/Notch protein. The primary cell signals the lateral cells in order to prevent them adopting the primary fates. *lin-12* affects the VU/AC decision before vulval induction as well as VPCs fates. In a *lin-12* null animal, there are additional primary cell fates which replaces the secondary cell fates (Ferguson *et al.* 1987; Greenwald *et al.* 1983; Sternberg and Horvitz 1989; Wilkinson and Greenwald 1995).

Synthetic multivulva pathway

Two additional pathways involved in vulva development are the functionally redundant synthetic multivulva pathways. There are two classes of pathways involved: the synMuv class A and the synMuv class B. Animals with an A class and a B class mutation result in a Muv phenotype while animals with one or two mutations of the same

class have a wild-type vulva phenotype (Clark *et al.* 1994; Ferguson and Horvitz 1985; Ferguson and Horvitz 1989; Horvitz and Sulston 1980; Huang *et al.* 1994; Lu and Horvitz 1998). The synMuv genes encode negative regulators of vulval induction (Clark *et al.* 1994; Ferguson and Horvitz 1985; Ferguson and Horvitz 1989; Horvitz and Sulston 1980; Huang *et al.* 1994; Lu and Horvitz 1998). There are 4 class A genes and 10 class B genes (Refer to Chapter A)(Clark *et al.* 1994; Ferguson and Horvitz 1985; Ferguson and Horvitz 1989; Horvitz and Sulston 1980; Huang *et al.* 1994; Lu and Horvitz 1998).

lin-15 is a member of a set of negative regulators of vulval development in which appropriate mutation combinations result in a multivulva (Muv) phenotype (Ferguson and Horvitz 1989). It has two independent mutable activities, A and B class (Clark *et al.* 1994; Ferguson and Horvitz 1985; Ferguson and Horvitz 1989; Horvitz and Sulston 1980; Huang *et al.* 1994; Lu and Horvitz 1998) and it acts independently of two known signals controlling the fates of the vulval precursor cells (VPCs), the anchor cell (AC) inductive signal and a lateral signal among the VPCs (Herman and Hedgecock 1990). *lin-15* locus encodes novel hydrophilic proteins, LIN-15A and LIN-15B. Antibodies staining against both proteins show each protein present in all cells, including oocytes (Huang 1995). As we shall see, GFP expression under the control of the *lin-15* promoter shows fluorescence in all cells except early germline cells. Since both antibody staining and GFP expression pattern are broad, the site of action for each gene for repressing vulval induction could not be determined. *lin-15*'s site of action during vulva induction can be determined by genetic mosaic analysis. Previous mosaic analysis indicated *lin-15* acts in cells other than AC and the VPCs (i.e., non-autonomous)

(Herman and Hedgecock 1990). We have expanded this mosaic analysis by performing mosaic studies on each cistron (*lin-15A* and *lin-15B*) separately using GFP-LacI + *lacO* as well as *ncl-1* as mosaic markers. We describe the analysis that shows *lin-15A* gene site of action is at the VPCs.

The localization of the LIN-15A protein to the VPCs further complements the mosaic result of *lin-15A*. VPCs localize *lin-15A* is sufficient to rescue the Muv phenotype of the null *lin-15* allele unlike its localized expression in the hypodermis and intestines.

Materials and Methods

Nematode methods

Growth and handling of *C. elegans* strain N2 were according to Brenner (1974) and Sulston and Hodgkin (1988). All experiments were performed at about 20°C unless otherwise stated. The genetic and cellular nomenclature of *C. elegans* was followed according to Horvitz et al., (1979) and Sulston et al. (1983), respectively. (Brenner 1974; Horvitz *et al.* 1979; Sulston *et al.* 1983)

Strains

The standard wild-type N2 strains and *ncl-1(e1865)* were obtained from the Caenorhabditis Genetics Center (USA). The *lin-15* null allele *e1763* was used for *lin-15A* mosaic analysis (Ferguson and Horvitz 1985).

The transgenes *syEx*[*dpy-20(+)* (20ng/μl) + *dpy-30::S65T*LacI* (100ng/μl) + *pPD49-78GFP-LacI* (100ng/μl) was integrated into *ncl-1(e1865)III; dpy-20(e1282)IV* (Fire, 1986), respectively, to yield PS2958 (*syIs46*). (Table 1, Chapter 2).

Molecular determination of *lin-15A* lesions

Genomic DNA was purified from strains carrying known *lin-15A* mutations*: lin-15*(*sy197)*; *lin-15*(*n744B, sy211)*; and *lin-15*(*n744B, sy212).* The *lin-15* coding region and the regions of introns near the splice sites were amplified using the polymerase chain reaction (PCR), and the sequences of theses PCR products were determined using an automated ABI 373A cycle sequencer by the California Institute of Technology sequencing facility (Applied Biosystems, Foster City, CA).

We also amplified *lin-15A* genomic clone (pBLH51) (Huang 1995; Huang *et al.* 1994) by PCR in order to introduce premature stop codons at the end of exon 4(K304Amber), exon 5(S360Amber) and in the middle of exon 6(G579Amber). Each clone with a premature stop codon was injected into the gonads of *lin-15(e1763)* animals in order to determine Muv rescue. The injection mixture consisted of the premature stops in pBLH51 (100ng/μl), the transformation marker pRF4 (40ng/μl) and 80ng/μl of N2 genomic DNA (*PvuII* digested) as carrier. The *lin-15A* genomic clone, pBLH51, has a 330 bp deletion from the fifth exon in the *lin-15B* transcript. This construct is only able to rescue *lin-15A* mutant alleles not *lin-15B* mutant alleles (Huang *et al.* 1994).

Construction of *lin-15::GFP* reporter

The *lin-15* promoter region (~559bp) was amplified with primers AS59 5' CTCCACCGCGGTGTCGACCGCTCTAGAAC 3' end AS60 5' GCGTTTGCATAGGTACCTGAAAATA 3' flanked with restriction sites, *SalI*/*Kpn*I, using PCR. The PCR product was digested and then ligated into a promoterless GFP reporter vector, pPD95-81, which contains a *C.elegans unc-54* 3' end and a fluorescence enhancing S65C mutation (A. Fire, personal communication).

The *lin-15::GFP* construct was injected into the hermaphrodite's gonads of *unc-119(ed4).* The injection mixture consisted of 100ng/µl of *lin-15::GFP*, 40ng/µl of unc-119(+) DNA and 80ng/µl of N2 genomic DNA (*PvuII* digested) as carrier. 12 rescued lines were isolated after germline transformation of *unc-119(ed4)* (Maduro and Pilgrim 1995). The transformed animals from the 12 rescued lines were viewed under fluorescence.

Localized expression of *lin-15A*

The native promoter of *lin-15A* genomic DNA was placed under the transcriptional control of the *vit-2* promoter (Spieth *et al.* 1988; Spieth *et al.* 1991) and the *col-10* promoter (Lu and Horvitz 1998) and the LIN-31 promoter [Miller, 1996; Eisenmann, 1994; Miller, 1993]. Each construct was co-injected at a concentration of 100ng/µl) with pRF4 (*rol-6(su1006)* mutant gene) at a concentration

40ng/μl) (Mello *et al.* 1991) into the *lin-15* null allele *e1763.* 3 stable Rol-Muv transgenic lines were isolated after *vit-2::lin-15A*genomic germline transformation. two stable Rol-nonMuv transgenic lines were isolated after *col-10::lin-15A* genomic germline transformation. Two stable Rol-nonMuv as well as 3 non-Rol nonMuv transgenic lines were isolated after *lin-31::lin-15A*genomic germline transformation.

Germline-mediated transformation by microinjection

Microinjection was performed according to Mello et al. (Mello *et al.* 1991) which was modified from Fire (Fire 1986). Young adult hermaphrodites were placed live on pads of 2% agarose under an inverted differential contrast-interference (Nomarski) microscope (Carl Zeiss, Oberkochen, West Germany) and the DNA was injected into the gonad using an Eppendorf micro injector 5242 (Eppendorf Gertebau Netheler, Hamburg, West Germany).

For the *lin-15A* and *B* mosaics, the plasmid containing the *lin-15A(+) or lin-15B(+)* DNA, depending on the mosaic, was also used as the transformation marker at a concentration of 100ng/μl. Also, included in the injection mixtures of both mosaics are: 256 repeat *lacO* (50ng/μl), *ncl-1(+)* (50ng/μl) and pBluescript II SK+ (Stratagene) (5 ng/μl). The mixtures were injected into the gonads of *syIs46; ncl-1(e1865); dpy-20(e1282); lin-15(e1763).*

The transgenic lines obtained from each experiment were heat-shocked for 30 minutes at 33°C to elicit GFP-LacI expression. Expression of the GFP-LacI can be seen as early as 30 minutes after heat-shock, and as late as 24 hours.

Mosaic analyses

Mosaic animals of *lin-15A* gene function and *lin-15B* gene function are obtained from a somatic loss of an extrachromosomal array *syEx273* [*lin-15A(+)* + *lacO* + C33C3] and *syEx272* [*lin-15B(+)*+ *lacO* + C33C3] from *syIs46; ncl-1(e1865); dpy-20(e1282); lin-15(e1763)*. C33C3 is the rescuing cosmid for *ncl-1*. The point of loss is determined by the absence of the fluorescent spot as well as enlarged nucleoli. We used L3-L4 Muv animals for mosaic analysis that ensures that *lin-15(e1763)* is homozygous. The nuclei observed to identify mosaic animals are: pharyngeal cells: m2L, m3L, m3VL, m4R, m3R, m3VR, m3DL, m3DR, pharyngo-intestinal valve cells, hyp7; Tail cells: hyp7, F, U; VPCs (Pn.p cells); and intestines (Refer to Figures 3 and 4 for lineage).

Mosaic analysis of *lin-15B* gene function was also done with SUR-5GFP(NLS) as the mosaic marker (Yochem *et al.* 1998). Mosaic animals are obtained from a somatic loss of an extrachromosomal array *syEx316* [*lin-15B(+)* + *lacO* + *sur-5GFP(NLS)* + C33C3] from *syIs46; ncl-1(e1865); dpy-20(e1282; lin-15(e1763*) (Table 1, Chapter 2). The nuclei observed are the same as the previous mosaic (Figure 5).

Microscopy

Animals were anesthetized with 2mM levamisole on 5% Noble agar pads. The fluorescence was viewed with the Zeiss Axioplan with Chroma High Q GFP LP filter set (absorption band 450nm and 505nm emission) at 100X optics. The animals were placed live in 3 microliters of S basal + cholesterol on 5% Noble agar pads (Wood 1985).

Results

lin-15::GFP reporter is expressed broadly

We observe expression of GFP driven by the *lin-15* promoter in all cells except in the gonadal cells in L1 and L2 stage animals. In the later larval stages as well as in adult, the germline cells do not express GFP. There is embryonic expression as early as "comma"- stage embryo (400min after fertilization) (Figure2).

lin-15A Mosaics

We crossed the *lin-15* null allele *(e1763)* into the PS2958 strain to create PS3048, and then injected the mosaic markers, *ncl-1(+)* + *lacO*, and *lin-15A(+)*. We checked for fluorescent spots as well as nucleolar size in the nucleus of the cells scored. A fluorescent spot and/or normal nucleolar size indicates the presence of the *lin-15A* gene. We observed by Nomarski and fluorescence microscopy, 120 non-Muv animals and 52 Muv animals. The mosaic non-Muv (n=5) animals lose both of the mosaic markers (i.e., GFP and Ncl-1) in every lineage except those leading to the VPCs.
In mosaic Muv animals (n=8), the mosaic markers are present in every lineage except the VPCs (i.e., AM2.5 and AM3.24) (Figure 3).

Figure 3 shows the key mosaic animals (n=13) that indicate that *lin-15A* has a site of action in the VPCs. Of the 5 non-Muv animals, the VPCs P3.p, P4.p and P8.p are *ncl-1(-)* and *lacO(-)* raising the possibility of local non-autonomy. The Muv animal, AM3.19, had the array present, according to the *lacO* marker, at P3p, therefore further

supporting local non-autonomy of *lin-15A* among the VPCs. According to Figure 3, F and U cells might be another site for *lin-15A*. The other 159 animals scored do not support the pattern of *lin-15A* function at F and U (data not shown). We conclude that *lin-15A* function is required in the VPCs to rescue the Muv phenotype.

***lin-15B* Mosaics**

lin-15B mosaics were done in a similar manner as with *lin-15A* except that the rescuing DNA was *lin-15B(+)*. We observed 37 animals using Nomarski optics and fluorescence microscopy. Out of 37 animals, 3 were non-Muv. Figure 4A and B show no obvious pattern of *lin-15B* function. These results are suggesting that *lin-15B* is needed broadly for proper function (i.e., hypodermis) or perdurance of the protein skew the site of action since we were not able to determine in the cell lineage where *lin-15B(+)* is needed to rescue the multivulva phenotype.

We also performed the *lin-15B* mosaics using SUR-5GFP(NLS) as the mosaic marker (Yochem *et al*. 1998). The differences between the latter mosaic versus this mosaic with SUR-5GFP(NLS) are: the stability of the transgenic line *syIs46;syEx272* which is 30-40%, compared to the stability of the transgenic line *syIs46;syEx316* is 80-90%. We scored only Muv animals that expressed SUR-5GFP in order to eliminate foci that do not require *lin-15B* gene expression for multivulva rescue.

Of the12 animals observed by Nomarski and fluorescence microscopy, 5 were non-Muv and 7 were Muv. Animal WT.5 and M3.6 indicate that P3.p, P4.p and P8.p do

not require *lin-15B* gene expression for rescue. We observed fluorescing hyp7 nuclei around the vulva in animal WT.6 (data not shown). In Muv animals, it was difficult to determine which are hyp7 nuclei around the vulva nonetheless if it fluoresces or Ncl. It is possible that the maternal effect of *lin-15B* may be affecting the determination of the focus of action of *lin-15B* when examined by mosaic analysis (Figures 4 and 5).

Molecular determination of *lin-15A* lesion

We used PCR to amplify *lin-15* DNA from mutant synMuv class A alleles to determine the sequence changes in these strains. *lin-15(sy197)* has a splice-site point mutation (base addition) at the splice acceptor of the fifth exon in the A transcript resulting in a frame-shift. We were able to determine the molecular lesion of the *lin-15B* allele *n744* because it was in the background of two *lin-15A* alleles we sequenced: *lin-15*(*n744B, sy211)* and *lin-15*(*n744B, sy212). lin-15(n744)* has a transition mutation, (CΔT) resulting in a missense mutation of the amino acid, S124F. *lin-15(sy211)* has a transition mutation (GΔA) at the splice acceptor of exon 5 in the A transcript. *lin-15(sy212)* has a transition mutation, (CΔT) resulting in a nonsense mutation, Q116Amber (Table 1).

In order to determine how much of the *lin-15A* gene is needed to rescue the Muv phenotype of *lin-15* null allele, we placed an amber codon at the end of the fourth and fifth exon as well as an amber codon in the middle of the sixth exon (Table 2). The *lin-*

15A truncation, 5ATAG, is sufficient to rescue the Muv phenotype. 4ATAG and 6ATAG did not rescue the Muv phenotype. The transgenic lines obtained from the 4ATAG injection did not remain stable. An array containing 4ATAG DNA may be lethal since there were dead L1 larvae and unhatched eggs on the plate level.

lin-15A localize expression to tissues in hermaphrodites

In order to confirm LIN-15A protein activity is needed in the VPCs for Muv rescue of a *lin-15* null allele, we drove *lin-15A* gene expression with tissue specific promoters. *lin-15A* expression is localized to the intestines (*vit-2* promoter) (Spieth *et al.* 1988; Spieth *et al.* 1991), the Pn.p cells (*lin-31* promoter) (Miller *et al.* 1993; Miller *et al.* 1996) and hypodermis and Pn.p cells (*col-10* promoter) (Lu and Horvitz 1998). Each promoter drive the expression of a promoterless *lin-15A* genomic DNA since *lin-15A* cDNA does not rescue the Muv phenotype of *lin-15* mutant alleles. I have viewed many transformed animals after the germline transformation for each tissue-specific construct. The 3 transgenic lines of the *vit-2::lin-15A* genomic were transmitting Rol animals that were Muv. The 2 transgenic lines of the *col-10::lin-15A*genomic were transmitting roller non-Muv animals. The 5 transgenic lines of the *lin-31::lin-15A* genomic were transmitting either roller non-Muv or only non-Muv animals, never roller Muv animals. Of the three constructs injected into the gonads of the null *lin-15(e1763)* allele hermaphrodites, *col-10::lin-15A*genomic and *lin-31::lin-15A* genomic

rescued the Muv phenotype. *vit-2::lin-15A*genomic did not rescue the Muv phenotype. Since the *col-10::lin-15A*genomic and *lin-31::lin-15A*genomic rescue the Muv phenotype, we conclude *lin-15A* expression at the VPCs is sufficient to rescue the *lin-15(-)* phenotype (Figure 6).

Discussion

Previous mosaics suggested the *lin-15* locus act non-autonomous. Herman and Hedgecock hypothesized that there is a negative signal sent by the product of the *lin-15* locus is from the hypodermis preventing vulval differentiation (Herman and Hedgecock 1990). It was then determined that the *lin-15* locus encodes two uni-directional transcripts. The two proteins that it encodes are not similar to any currently identified proteins and contain no significant homology with known protein motifs (Clark *et al.* 1994; Huang *et al.* 1994). We performed genetic mosaic analysis with *lin-15A* and *lin-15B* genes using 2 mosaic markers, *ncl-1* and GFP::LacI + *lacO.* We determine the site of action for *lin-15A* gene expression is at the VPCs (i.e., autonomous) while the *lin-15B* remains unclear due to maternal effect or it may act at the hypodermis.

Site of action of *lin-15A*

Figure 3 shows the key mosaic animals (n=13) that indicate that *lin-15A* has a site of action in the VPCs. We conclude that *lin-15A* function is required in the VPCs to rescue the Muv phenotype. This result is in contrast to previous *lin-15* mosaics done by Herman and Hedgecock (1990), which suggested *lin-15* acts within the hypodermis.

They concluded it had a hypodermal focus because loss of the array, which contained *osm-1(+), unc-3(+),* and *sup-10(+)* in every cell lineage no matter if the animal was Muv or non-Muv (Herman and Hedgecock 1990). They scored mosaicism by whole animal phenotype unlike our cell-by-cell method.

In order to confirm LIN-15A protein activity is needed in the VPCs for Muv rescue of a *lin-15* null allele, we drove *lin-15A* gene expression with tissue specific promoters. *lin-15A* expression is localized to the intestines (*vit-2* promoter) (Spieth *et al.* 1988; Spieth *et al.* 1991), the Pn.p cells (*lin-31* promoter) (Eisenmann and Kim 1994; Miller *et al.* 1993; Miller *et al.* 1996) and hypodermis and Pn.p cells (*col-10* promoter) (Lu and Horvitz 1998) (Figure 6). Since the *col-10::lin-15A*genomic and *lin-31::lin-15A*genomic rescues the Muv phenotype but *vit-2::lin-15A*genomic do not rescue the Muv phenotype, we conclude *lin-15A* expression at the VPCs is sufficient to rescue the *lin-15(-)* phenotype. This result further supports the hypothesis that *lin-15A* acts in the VPCs to negatively regulate the vulval induction pathway preventing P3.p, P4.p and P8.p adopting either 1° or 2° vulval fate.

Synthetic multivulva genes

Synthetic multivulva (synMuv) genes encode negative regulators of vulval induction (Clark *et al.* 1994; Ferguson and Horvitz 1989; Horvitz and Sulston 1980; Huang *et al.* 1994). In the absence of synMuv activity, P3.p, P4.p, and P8.p generate vulval cells. As mentioned previously, the Muv phenotype is the result of mutations in each class, referred to as A and B, which represent two functionally redundant pathways.

There are four class A genes (*lin-8, lin-15A*, *lin-38*, and *lin-56*) and ten B class genes (*lin-9*, *lin-15B*, *lin-35*, *lin-36*, *lin-37*, *lin-51*, *lin-52*, *lin-53*, *lin-54*, and *lin-55*) (Ferguson and Horvitz 1989; Horvitz and Sulston 1980; Lu and Horvitz 1998). *lin-36, lin-15A* and *lin-15B* each encode novel proteins. *lin-35* encodes a protein related to Rb, and *lin-53* encodes a protein similar to the Rb-binding protein, p48 (Lu and Horvitz 1998). So far, all proteins examined are nuclear proteins [Lu, 1998; Huang, DeModena, and Sternberg, unpublished observations).

Based on mosaic analysis, the B class gene *lin-37* acts cell non-autonomously, possibly in the *hyp7* hypodermis (Hedgecock and Herman 1995) while the B class gene *lin-36* acts cell autonomously in the VPCs cells (Thomas and Horvitz 1999). Lu and Horvitz (1998) argue that synMuv gene activity acts in the VPCs from the results of triple mutants carrying either *lin-35* or *lin-53* mutation. They also hypothesize that LIN-35 and LIN-53 act cell autonomously due to localization of the 2 proteins in P(3-8).p cells as detected by the antibody and a GFP transgene, respectively. As a result of our data, we hypothesize class A synMuv function in the Pn.p cells. Protein interaction mapping by yeast two-hybrid reveal interaction sequence tags clusters for synMuv gene products (Walhout et al., 2000). The informative clusters are: LIN-53/LIN-37/LIN-36/EGR-1/LIN-53; LIN-36/LIN-15A/LIN-33 and LIN-35 (pRB)/ LIN-53 (RbAp48)/HAD-1/LIN-35 (Walhout *et al.* 2000). Also, RNA-mediated interference (RNAi) experiments to further study the function of *egr-1* brought the possibility of the recruitment of a nucleosome remodeling and histone deacetylase (NURD) complex by both synMuv pathways to repress vulval development target genes by local histone deacetylation

[Solari, 2000; Solari, 1999]. The information obtained about the potential protein interaction of the synMuv genes from the ISTs clusters plus *C.elegans* gene homologues to the NURD complex, mosaic data and protein localization, may lay the foundation of a synMuv class B complex (similar to Rb pathway) and the synMuv class A pathway activities are initiating or maintaining the NURD complex to alter chromatin structure of vulval development genes to antagonize the Ras-mediated vulval induction (Figure 7).

The different foci of action for *lin-15A* and *lin-15*B are puzzling since they are expressed as part of an operon (Clark *et al.* 1994; Huang *et al.* 1994). Antibody studies of LIN-15A and LIN-15B proteins are broadly expressed and nuclear (L. Huang, J. DeModena and P. Sternberg, personal com.). GFP driven by the *lin-15* promoter further show expression in all cells except early germline (Figure 2). It is possible that the differential requirement for A and B is quantitative. For example, both proteins might act in all cells, non-autonomously, but there is a greater requirement for *lin-15A* function at the VPCs than in nearby cells. Another possibility is that the different functions respond differently to mosaic analysis: greater perdurance of B than A could also lead to different apparent foci of action.

Figure 1. Model for vulval induction

This pathway summarizes the work of many people (as referenced in Moghal *et al*. 1999; Sternberg *et al*. 1993).

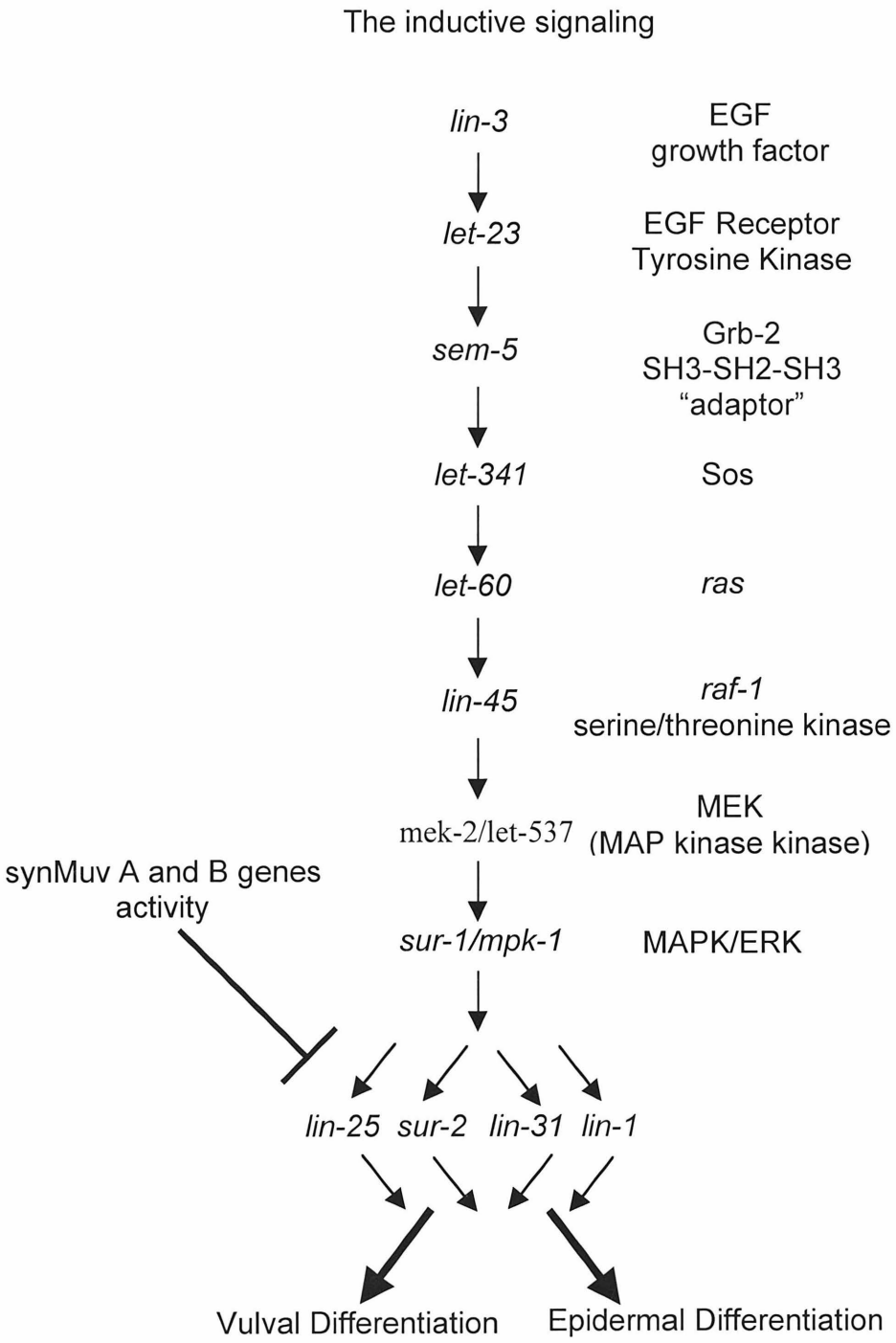


Figure 2. *lin-15::GFP* expression pattern

In a *unc-119(ed4)* background, there is a broad fluorescent pattern of GFP indicating where the *lin-15* promoter drives expression. The GFP fluorescence is not nuclear. **A.** Embryos approximately 400 minutes after fertilization. **B.** L1 larvae. **C**. Mid-L4 hermaphrodite with the VPCs and the utse tissue fluorescing. Anterior is right; dorsal is up. **D.** Spermatheca. **E**. Intestines. Photomicrographs taken using Nomarski optics at 100X.

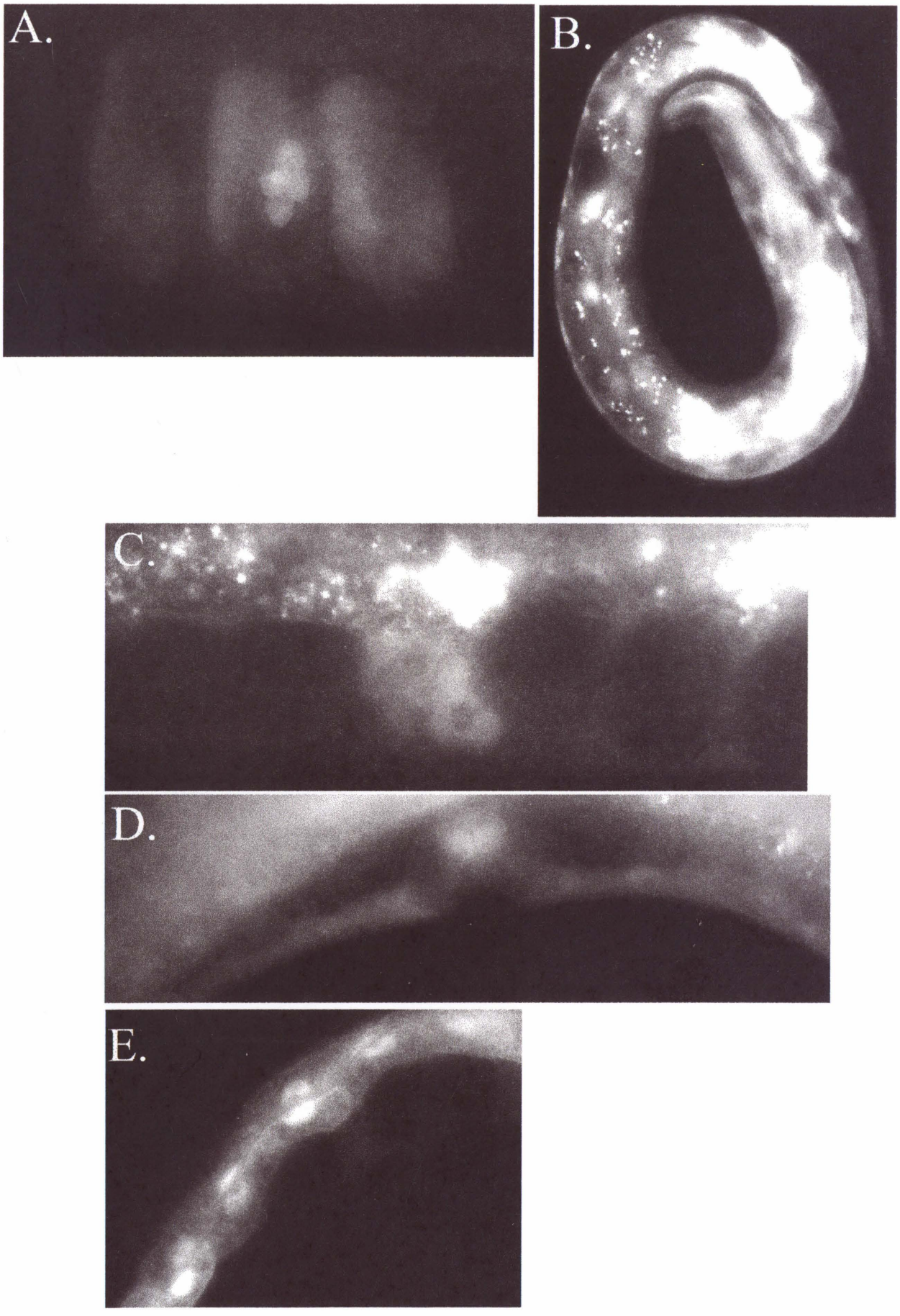
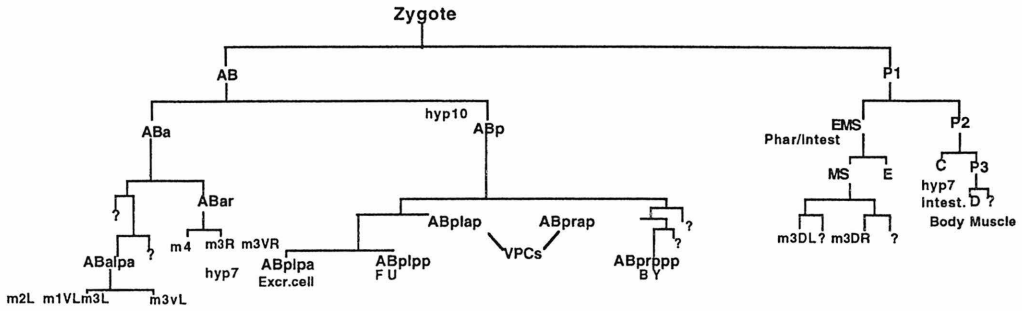

A.
B.
C.
D.
E.

Figure 3. Mosaic Analysis of the *lin-15A* gene with the GFP-LacI + *lacO* and *ncl-1*

Mosaic analysis of *lin-15(e1763)* reveals that the wild-type *lin-15A* gene is required in the VPCs for normal negative regulation of the *lin-3/let-23* pathway. The data shown are the key mosaic animals from the 172 animals viewed that indicate the *lin-15A* focus of action. L3-L4 Muv and non-Muv animals were examined. +: GFP-LacI bound to the *lacO*, fluorescent spot; -: no fluorescence, W: normal nucleolus size and N: enlarged nucleolus. Letters correspond to vulval status: AW: normal vulva; AM2. #: Muv animal with 2 pseudovulvae; AM3. #: Muv animal with 3 pseudovulvae; AM4.#: Muv animal with 4 pseudovulvae. The numbers in the Pn.p column indicates the number of nuclei with the bright fluorescent spot or *ncl-1*(+/-). Under the "ventral tail; head hyp7" heading, "T" stands for tail while "H" stands for head.

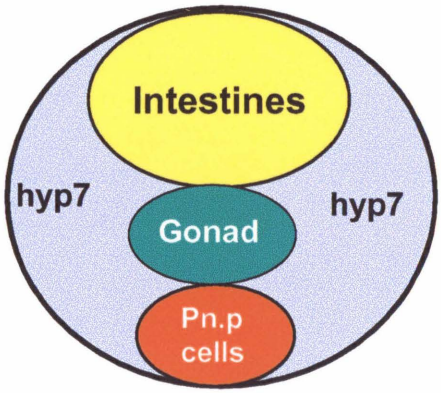


Worm	m2	m3L	m3VL	m4	m3R	m3VR	hyp7	F	U	P3p	P4p	P5p	P6p	P7p	P8p	B	hyp7	P/l	m3DL	m3DR	Int	hyp7
AW11	W2	W	W	W6	N	W	N2	W	W	W	W2	W7	W8	W7	W2	N	T:N2 H:N2	N	N	N	?	"N"
AW43	W4	W	W	N6	W	W	N2	W	W	W	W2	?7	?8	?7	W1	N	T:N2 H:W2	N	N	N	?	?
AW63	?	N	N	W5	W	W	W2	W	W	W	W2	W7	W8	W7	W2	W	T:W2 H:W2	W	W	W	?	W
AW70	W4	W	W	N6	W	W	N2	W	W	N	N2	W4	W8	W4	W2	W	T:W2 H:N2	N	N	N	?	N
AW80	N4	N	N	W6	N	W	W1	N	N	N	W2	W7	W8	W7	N2	N	T:W2 H:W2	W	N	N	?	W
AW11	+2	+	+	+1	+	+	-2	-	+	+	+2	+7	+8	+7	+2	-	T:-2 H:-2	-	-	-	?	-
AW43	-4	-	+	-6	+	-	-2	-	-	-	-2	-7	+4	-7	+1	-	T:-2 H:+2	-	-	-	+4	-
AW63	?	-	-	+5	+	+	+2	+	+	+	+2	-7	+8	-7	+2	+	T:+2 H:+2	+	+	+	+5	+
AW70	+4	+	+	-6	+	+	-2	+	+	-	-2	+3	+8	+3	+1	+	T:-2 H:-2	-	-	-	+	+
AW80	-4	-	-	+6	+	+	+2	-	+	-	+2	-7	+8	-7	+2	-	T:+2 H:+2	+	+	+	+	+
AM2.2	W4	N	N	N6	N	N	N1	N	N	N	N2	N*	N8	N7	N*	N	T:N2 H:N2	N	N	N	?	N
AM2.5	W4	W	W	W6	W	W	W2	N	N	N	N*	N7	N8	N7	N*	N	T:W2 H:W2	W	W	W	?	N
AM2.2	-4	-	-	-6	-	-	-2	-	-	-	-2	-*	-8	-7	-*	-	T:-2 H:-2	-	-	-	-	-
AM2.5	+4	+	+	+6	+	+	+2	-	-	-	-*	-7	-8	-7	-*	-	T:-2 H:+2	+	+	+	+	-
AM3.19	N4	N	N	W6	N	N	W2	N	N	W	N*	N7	N*	N7	N*	N	T:N2 H:N2	W	W	W	?	W
AM3.20	W4	N	N	N6	N	N	N2	N	N	N	N*	N7	N*	N7	N*	N	T:N2 H:N2	N	N	N	?	N
AM3.2	N4	N	N	W6	N	N	W2	N	N	N	N*	N7	N*	N7	N*	N	T:N2 H:W2	N	N	N	?	W
AM3.7	?	W	W	W4	W	W	N2	N	N	N	N*	N7	N*	N7	N*	N	T:N2 H:N2	N	W	W	?	N
AM3.24	V W2	W	W	W6	N	N	"W2"	N	N	N	N**	N7	N8	N7	N*	N	T:N2 H:W2	W	W	W	?	N
AM3.2,7,20	-4	-	+	+3	-	-	+2	-	-	-	-*	-7	-*	-7	-*	-	T:-2 H:-2	+	+	+	+	+
AM3.19	-4	-	+	+3	-	-	+2	-	-	+	-*	-7	-*	-7	-*	-	T:-2 H:-2	+	+	+	+	+
AM3.24	V+2	+	+	+5	-	-	+2	-	-	-	-**	-7	-8	-7	-*	-	T:-2 H:+2	+	+	+	+	-
AM4.9	N4	N	N	W4	N	N	W2	N	N	N	N2	N**	N8	N7	N**	W	T:W2 H:W2	W	W	W	?	N
AM4.9	-4	-	-	+4	-	-	+2	-	-	-	-2	-**	-8	-7	-**	+	T:-2 H:+2	+	+	+	+2	-

Figure 4. Tissue specific promoters localizing *lin-15A* expression

lin-15A expression is driven by tissue specific promoters in order to determine which tissue expressing *lin-15A* is sufficient to rescue the muv phenotype of the *lin-15* null. *lin-15A* expression is localized to the intestines (*vit-2* promoter) , the Pn.p cells (*lin-31* promoter) and hypodermis and Pn.p cells (*col-10* promoter). Each construct was injected into *lin-15(e1763)* animal. The cross section of a hermaphrodite reveal the relative distance of the intestines, gonad and hypodermis to the Pn.p cells.

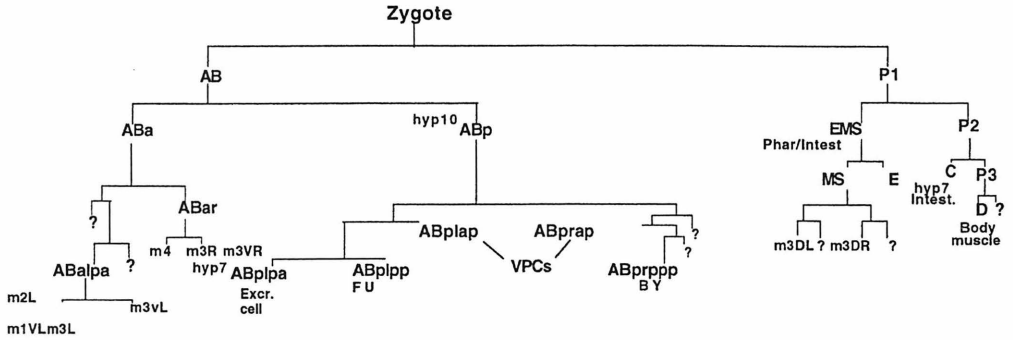
Localized expression of *lin-15A*



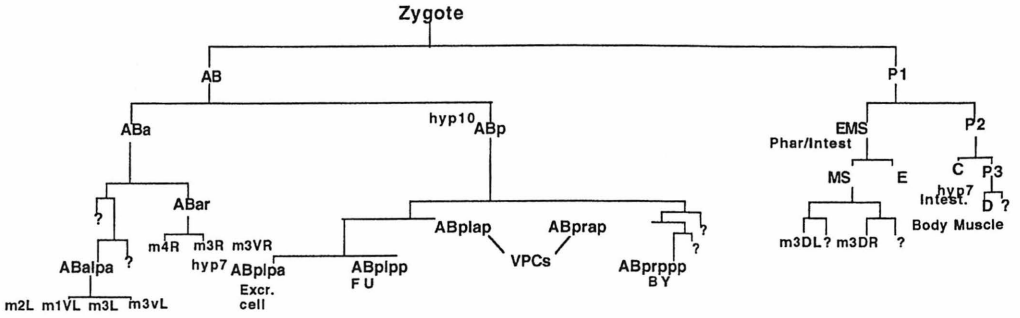
Promoter	Expression pattern	Rescue Muv phenotype	# transgenic lines	# transformed animals viewed
col-10	hypodermis and Pn.p cells	YES	2	Many
vit-2	intestines	NO	3	Many
lin-31	Pn.p cells	YES	5	Many

Figure 5A and B. Mosaic Analysis of the *lin-15B* gene with GFP-LacI + *lacO* and *ncl-1*

Mosaic analysis of *lin-15(e1763)* animal's reveal that the wild-type *lin-15B* gene may be required ubiquitously for normal negative regulation of the *lin-3/let-23* pathway. The data shown below are the key mosaic animals from the 37 animals viewed that determined *lin-15B* foci of action. L3-L4 Muv and non-Muv animals were examined. +: GFP-LacI bound to the *lacO*, fluorescent spot; -: no fluorescence, W: normal nucleolus size and N: enlarged nucleolus. Letters correspond to vulval status: BW: normal vulva; BM1. #: Muv animal with 1 pseudovulvae; BM2. #: Muv animal with 2 pseudovulvae; BM3. #: Muv animal with 3 pseudovulvae; BM4. #: Muv animal with 4 pseudovulvae. The numbers in the Pn.p column indicates the number of nuclei with the bound fusion protein/spot or *ncl-1*(+/-). Under "ventral tail; head hyp7" heading, T: tail while H: head.



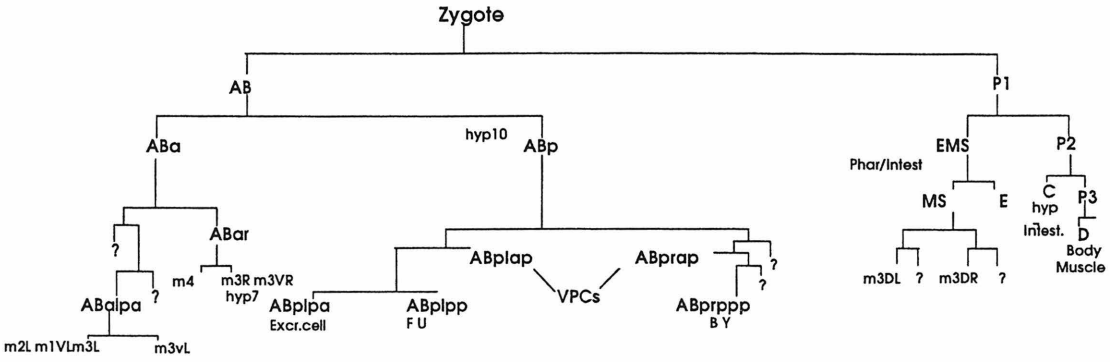
Worm	m2	m3L	m3VL	m4	m3R	m3VR	hyp7	F	U	P3p	P4p	P5p	P6p	P7p	P8p	B	hyp7	P/I	m3DL	m3DR	Int	hyp7
BW.1	+4	+	+	+6	+	+	+2	+	+	+	+2	+7	+8	+7	+2	+	T:+2 H:+2	+	+	+	+	+
BW.2	+2	-	-	+2	-	-	+2	+	+	+	+2	+7	+8	+7	+2	+	T:+2 H:+2	-	-	-	+2	-
BW.3	+4	+	-	+6	+	+	+2	+	+	+	+2	+7	+8	+7	+1	+	T:+2 H:+2	+	+	+	+5	+
BM1.1	+4	+	+	+6	+	+	+2	+	+	+	+*	+3	+4	+3	+2	+	T:+1 H:+2	+	+	+	+	+
BM1.2	?4	+	+	+4	+	+	+2	+	+	+	-*	-7	+7	+1	+2	+	T:+2 H:+2	+	+	+	-	+
BM1.3	+4	+	+	+6	+	+	+2	+	+	+	+*	+7	+8	+7	+2	+	T:+2 H:+2	+	-	-	+	+
BM1.4	+4	+	+	+6	+	+	+2	+	+	+	+*	+1	+6	-7	+2	+	T:-2 H:+2	+	+	+	-	+
BM1.5	+2	+	+	+6	+	+	+2	+	+	+	+*	+7	+8	+7	+2	+	T:+2 H:+2	+	+	+	-	+
BM1.6	+4	+	+	+6	+	+	+2	+	+	+	+2	+7	+8	+7	+*	+	T:+2 H:+2	+	+	+	+	+
BM1.7	+4	+	+	+6	+	+	+2	+	+	+	+*	+7	+8	+7	+2	+	T:+2 H:+2	+	+	+	-	+
BM1.8	+4	+	+	+6	+	+	+2	+	+	+	+*	+7	+8	+7	+2	+	T:+2 H:+2	+	+	+	-	+
BM2.1	+4	+	+	+6	-	+	+2	+	+	+	+*	+*	+8	+7	+2	+	T:+2 H:+2	+	-	-	+	+
BM2.2	+4	+	+	+6	+	+	+2	+	+	+	+**	+7	+8	+7	+2	+	T:+2 H:+1	+	+	+	-	+
BM2.3	+2	+	+	+6	+	+	+2	+	+	+	+*	+7	+8	+7	+*	+	T:+2 H:+2	+	+	+	+	+
BM2.4	+4	+	+	+6	+	+	+2	+	-	+	+*	+2	+8	+4	-*	+	T:+2 H:+2	+	+	+	+	+
BM2.5	-4	-	-	+5	-	-	-2	+	+	-	-*	+3	-8	-7	-*	+	T:+2 H:+2	-	-	-	-	-
BM2.6	+4	+	+	+6	+	+	+2	+	+	-	+*	+7	+8	+7	+*	-	T:+2 H:+1	+	+	+	-	+
BM2.7	+3	-	-	+6	-	+	+2	-	+	-	-*	-7	+5	-7	+*	-	T:+2 H:+2	+	-	-	-	-
BM2.8	+2	+	+	+6	-	-	-2	+	+	-	-*	-7	-8	-7	+*	+	T:+1 H:-2	-	+	-	-	+
BM2.9	+4	-	-	-6	-	-	-2	+	+	+	+**	+7	+8	+7	+1	-	T:+1 H:-2	-	-	-	-	-
BM2.10	+4	+	+	+6	+	+	+2	+	+	+	+*	+7	+8	+7	+*	+	T:+2 H:+2	+	+	+	+2	+
BM2.11	+4	+	+	+6	+	+	+2	+	+	+*	+*	+7	+8	+7	+2	+	T:+2 H:+2	+	+	+	-	+
BM3.1	+2	+	-	+5	+	+	+2	+	+	+	+**	?7	?8	?7	+*	+	T:+2 H:+2	+	+	+	-	+
BM3.2	+2	+	+	+3	+	+	+2	+	+	?	+**	+7	+8	+7	+*	+	T:+2 H:+2	-	-	+	+	+
BM3.3	+2	+	-	+3	+	+	+2	+	-	+	+**	+4	+8	-7	+*	+	T:+1 H:+2	+	+	+	+	+
BM3.4	?4	+	+	+6	+	+	+2	+	+	+	-**	+4	+3	+7	+*	+	T:+2 H:+2	+	+	+	+4	+
BM3.5	-4	+	+	+3	+	+	-2	-	+	+	+**	+1	+5	+3	-*	+	T:+2 H:+2	+	+	+	+4	-
BM3.6	-4	+	-	+3	-	-	-2	-	-	-*	-**	-7	-8	-7	-2	+	T:-2 H:+1	-	-	-	-	-
BM3.7	+1	+	-	-6	+	-	+1	-	-	+	-**	-7	+3	-7	+*	+	T:+2 H:+1	-	-	-	-	+
BM3.8	-4	+	+	+6	-	-	+2	-	-	-	+**	-7	-8	-7	-*	-	T:-2 H:+2	+	+	+	-	+
BM3.9	+4	+	+	+6	-	+	+2	+	+	-	+**	+7	+8	+7	+*	+	T:+1 H:+2	+	+	+	+2	?
BM3.10	+3	+	+	+3	+	+	-2	-	-	+	+*	+7	+8	+7	+*	-	T:+1 H:+2	+	-	+	+4	+
BM3.11	+4	+	-	+6	+	+	-2	+	+	+	+**	+7	+8	+7	+*	+	T:-2 H:-2	+	+	+	+2	+
BM3.12	+2	+	+	+6	-	-	+2	-	-	-*	-*	+7	+8	+7	-*	+	T:-2 H:-2	+	+	+	-	+
BM3.13	+4	+	+	+3	-	-	+2	+	+	+*	+*	+7	+8	+7	+*	+	T:+2 H:+2	+	+	+	+2	+
BM4.1	+4	+	+	+6	+	+	-2	+	+	+*	+**	+5	+8	+3	+*	-	T:+2 H:+1	+	+	+	+2	+
BM4.2	-4	+	-	-6	-	-	-2	-	-	-*	-**	-7	-8	-7	-*	-	T:-2 H:-2	-	-	-	-	-



Worm	m2	m3L	m3VL	m4	m3R	m3VR	hyp7	F	U	P3p	P4p	P5p	P6p	P7p	P8p	B	hyp7	P/I	m3DL	m3DR	Int	hyp7
BW.1	W4	W	W	W4	W	W	W1	W	W	W	W2	W7	W8	W7	W2	W	T:W2 H:W2	W	W	W	?	N
BW.2	N4	N	N	W2	N	N	N2	W	W	W	W2	W7	W8	W7	W2	N	T:W2 H:N2	W	W	N	?	N
BW.3	W4	W	W	W6	W	W	W2	W	W	W	W2	W7	W8	W7	W2	W	T:W2 H:W2	W	W	W	?	W
BM1.1	W4	W	"W"	N6	N	"W"	"N2"	W	W	W	W*	W7	W8	W6	N2	W	T:"N2" H:N2	N	N	N	?	W
BM1.2	?	N	N	W5	W	W	"W2"	W	W	W	W*	W7	W8	W7	W2	W	T:W2 H:W2	W	W	W	?	W
BM1.3	W4	W	W	W6	W	W	W2	W	W	W	W*	W7	W8	W7	W2	W	T:W2 H:W2	W	N	N	?	W
BM1.4	W4	W	W	W6	W	W	W2	W	W	W	W*	W2	W8	N7	W2	W	T:?2 H:W2	W	W	W	?	W
BM1.5	W2	W	W	W6	W	W	W2	W	W	W	W*	W7	W8	W7	W2	W	T:W2 H:W2	W	W	W	?	W
BM1.6	W4	W	W	W6	W	W	W2	W	W	W	W2	W7	W8	W7	W*	W	T:W2 H:W2	W	W	W	?	W
BM1.7	W4	W	W	W6	W	W	W2	W	W	W	W*	W7	W8	W7	W2	W	T:W2 H:W2	W	W	W	?	W
BM1.8	W4	W	W	W6	W	W	W2	W	W	W	W*	W7	W8	W7	W2	W	T:W2 H:W2	W	W	W	?	W
BM2.1	W4	W	N	N6	N	N	N2	N	N	?	?*	W*	W8	W7	N2	N	T:"W2" H:N2	W	N	N	?	N
BM2.2	W4	W	W	W6	W	W	W2	W	W	W	W**	W7	W8	W7	N2	N	T:N2 H:W2	W	N	N	?	?
BM2.3	W2	W	W	W6	W	W	W2	W	W	W	W*	W7	W8	W7	W*	W	T:W2 H:W2	W	W	W	?	W
BM2.4	W4	W	W	W6	W	W	W2	W	W	W	W*	W7	W8	W7	N*	N	T:W2 H:W2	W	W	W	?	N
BM2.5	N4	N	N	N6	N	N	N2	N	N	N	N*	N7	N8	N7	N*	N	T:W2 H:W2	N	N	N	?	N
BM2.6	W4	W	W	W6	W	W	W2	W	W	N	W*	W7	W8	W7	W*	W	T:W2 H:W2	W	W	W	?	W
BM2.7	W4	N	N	W6	N	N	W2	N	N	N	N*	W3	W5	W7	W*	N	T:N2 H:W2	W	N	N	?	N
BM2.8	W2	N	N	N6	N	N	N2	W	W	N*	N*	N7	N8	N7	N*	N	T:W2 H:N2	N	N	N	?	N
BM2.9	W4	N	N	N6	W	N	N2	W	W	W	W**	W7	W8	W7	W2	N	T:W2 H:N2	N	N	N	?	N
BM2.10	W4	W	W	W6	W	W	W2	W	W	W	W*	W7	W8	W7	W*	N	T:N2 H:W2	W	W	W	?	W
BM2.11	W4	W	W	W6	W	W	W2	W	W	W*	W*	W7	W8	W7	W2	W	T:W2 H:W2	W	W	W	?	W
BM3.1	W4	W	N	N6	N	N	N2	N	N	?	?**	W7	W8	W7	N*	N	T:"W2" H:N2	W	N	N	?	N
BM3.2	W2	+	+	N4	W	N	N2	N	N	?	N**	N7	N6	N7	N*	N	T:N2 H:W1	N	N	N	?	N
BM3.3	W2	"W"	"W"	W6	"W"	"W"	W2	W	N	N	N**	W4	W8	N7	W*	N	T:W2 H:W2	W	W	W	?	W
BM3.4	W2	+	+	W6	W	"N"	W1	N	N	W	N**	W4	W8	W7	N*	N	T:N2 H:W2	W	W	N	?	N
BM3.5	N4	+	+	W6	W	W	N2	N	W	W	W**	W7	W8	W7	N*	W	T:W2 H:N2	W	N	N	?	W
BM3.6	N4	+	-	N6	N	N	W1	N	N	N*	N**	N7	N8	N7	N*	W	T:N2 H:W1	N	N	N	?	N
BM3.7	W4	+	-	N6	W	N	W1	N	N	N	N**	N7	N8	N7	N*	N	T:N2 H:W1	N	N	N	?	W
BM3.8	N4	+	+	W6	N	N	N2	N	N	N	W**	N7	N8	N7	?*	N	T:N2 H:N2	W	N	N	?	N
BM3.9	W4	+	+	W6	N	W	W2	W	W	N	W**	W7	W8	W7	W*	N	T:W2 H:W2	W	W	W	?	?
BM3.10	N4	+	+	N6	W	W	N2	N	N	N	N**	N7	N8	N7	N*	N	T:N2 H:N2	N	N	W	?	N
BM3.11	N4	+	-	N6	N	N	N2	W	W	W	N**	N7	N8	N7	W*	N	T:N2 H:N2	N	N	N	?	N
BM3.12	W4	+	+	W6	N	N	W2	W	W	W*	W*	W7	W8	W7	W*	W	T:W2 H:W2	W	W	W	?	?
BM3.13	W4	+	+	W6	W	W	W2	W	W	W*	W*	W7	W8	W7	W*	W	T:N2 H:W2	W	W	W	?	?
BM4.1	W4	W	W	W6	W	W	N2	W	W	W*	W**	W7	W8	W7	W*	N	T:W2 H:W1	W	W	W	?	W
BM4.2	N4	N	N	N6	N	N	N2	N	N	N*	N**	N7	N8	N7	N*	N	T:N2 H:N2	N	N	N	?	N

Figure 6. Mosaic Analysis of the *lin-15B* gene done with SUR-5GFP and *ncl-1*

Mosaic analysis of *lin-15(e1763)* animal's reveal that the wild-type *lin-15B* gene maybe required ubiquitously for normal negative regulation of the *lin-3/let-23* pathway. The data shown below are the mosaic animals from the 12 animals viewed that determined *lin-15B* foci of action. Young adult Muv and L4 non-Muv animals were used for this study. S+: SUR-5GFP(NLS) bright fluorescence; S-: no bright fluorescence, W: normal nucleolus size and N: enlarged nucleolus. Letters correspond to vulval status: WT: normal vulva; M1. #: Muv animal with1 pseudovulvae; M2. #: Muv animal with 2 pseudovulvae; M3. #: Muv animal with 3 pseudovulvae. The numbers in the Pn.p column indicates the number of nuclei with the bound fusion protein/spot or *ncl-1*(+/-). Under "ventral tail; head hyp7" heading, T: tail while H: head. Animals WT.1 to WT.4 and M3.1 to M3.3 were heat-shocked at 33°C for 30 minutes for further analysis.



worm	m2	m3L	m3VL	hyp6	hyp7	m3VR	m3R	m4	Ex.Cell	F	U	P3p	P4p	P5p	P6p	P7p	P8p	B	hyp6	hyp7	m3DL	m3DR	P/I	hyp7	Int
WT.1	W4	W	W	?	W	W	W	W6	?	N	N	?	?	?	?	?	?	N	?	?	W	W	?	?	?
WT.2	W4	W	W	W4	W2	W	W	W6	?	W	W	W	W	W	W	W	W	N	W2	W2	W	W	N	N	?
WT.3	W4	W	W	W4	W2	W	W	W6	?	N	N	N?	N?	W7	N8	W6	W2	W	W2	W1	W	W	W	N	?
WT.4	N4	N	N	?	W2	N	N	N6	?	N	N	N?	N?	W7	W8	W7	W	N	?	?	N	N	W	N	?
WT.5	W2	W	W	W2	N2	W	W	W6	N?	W	W	?	?	?	?	?	?	N	N2	W2	W	W	N	W	?
WT.1	S+4	S+	S+	S+4	S+2	S+	S+	S+6	S+	S+	S+	S+	S+2	S+7	S-8	S+7	S+	S+	S+2	S+4	S+	S+	S+	S+	S+
WT.2	S+4	S+	S+	S+4	S+2	S+	S+	S+6	S+	S+	S-	S+	S+2	S+7	S-8	S+7	S+	S-	S+2	S+4	S+	S+	S-	S-	S+
WT.3	S-4	S-	S+	S+4	S+2	S+	S+	S+6	S+	S-	S-	?	?	?	S-8	?	?	S-	S+2	S+2	S+	S+	S+	S-	S+
WT.4	S+4	S+	S+	S+4	S+2	S+	S+	S+6	S+	S-	S-	S-	S-	S+7	S+8	S+7	S+	S-	S+2	S+2	S+	S+	S+	S-	S+
WT.5	S+2	S+	S+	S+2	S-2	S+	S+	S+6	S-	S+	S+	S-	S-2	S-7	S+8	S-7	S-	S-	S-2	S-2	S+	S+	S+	S+	S+
M2.1	N	N	N	N	N	N	N	N	N	N	N	N	N**	N	N	N	N	N	W2	N	N	N	N	N	?
M2.1	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-**	S-	S-	S-	S-	S-	S+1	S-	S-	S-	S-	S-	S-
M3.1	N	N	N	N	N	N	N	N	N	N	N	N	N**	N	N	N	N*	N	N	N	N	N	N	N	?
M3.2	N	N	N	N	N	N	N	N	N	N	N	N	N**	N	N	N	N*	N	N	N	N	N	N	N	?
M3.3	W4	W	W	N	N	N	N	W6	N	N	N	N	N**	N	N	N	N*	W	N	N	N	W	N	N	?
M3.4	N	N	N	N	N	N	N	N	N	N	N	N	N**	N	N	N	N*	N	N2	N	N	N	N	N	?
M3.5	W4	W	W	W4	W2	W	W	W6	N	N	N	N	N**	N	N	N	N*	N	W2	N2	W	W	W	N	?
M3.6	W4	W	W	W4	W2	W	W	W6	W	W	W	W	W**	W	W	W	W*	W	W2	W2	W	W	W	W	?
M3.1	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-**	S-	S-	S-	S-*	S-	S-	S-	S-	S-	S+	S-	S-
M3.2	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-**	S-	S-	S-	S-*	S-	S-	S-	S-	S-	S-	S-	S+
M3.3	S+4	S-	S+	S-	S-	S-	S-	S+6	S-	S-	S-	S-	S-**	S-	S-	S-	S-*	S+	S-	S-	S+	S-	S+	S-	S+
M3.4	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-	S-**	S-	S-	S-	S-*	S-	S-	S-	S-	S-	S-	S-	S-
M3.5	S+4	S+	S+	S+4	S+2	S+	S+	S+6	S-	S-	S-	S-	S-2*	S-7	S-8	S-7	S-*	S-	S+2	S-2	S+	S+	S+	S-	S+
M3.6	S+4	S+	S+	S+4	S+2	S+	S+	S+6	S+	S+	S+	S+	S+2[4]	S+5	S+2	S+3	S+*	S+	S+2	S+2	S+	S+	S+	S+	S+

Figure 7. Positive and negative regulation of vulval induction: model

A possible model for specifying Pn.p cell fate. The lack of LIN-3 protein result in no strong activity of the EGFR pathway allowing the 2 synMuv pathway in recruiting the NURD complex to halt VPC specifying genes from being expressed due to an alteration of its chromatin structure (i.e., P3p, P4.p and P8.p). If LIN-3 is secreted from the AC, then the target proteins of synMuv pathway A and B may be phosphorylated resulting in no recruitment of the NURD complex to repress vulval development genes (i.e., P7.p, P6.p and P5.p).

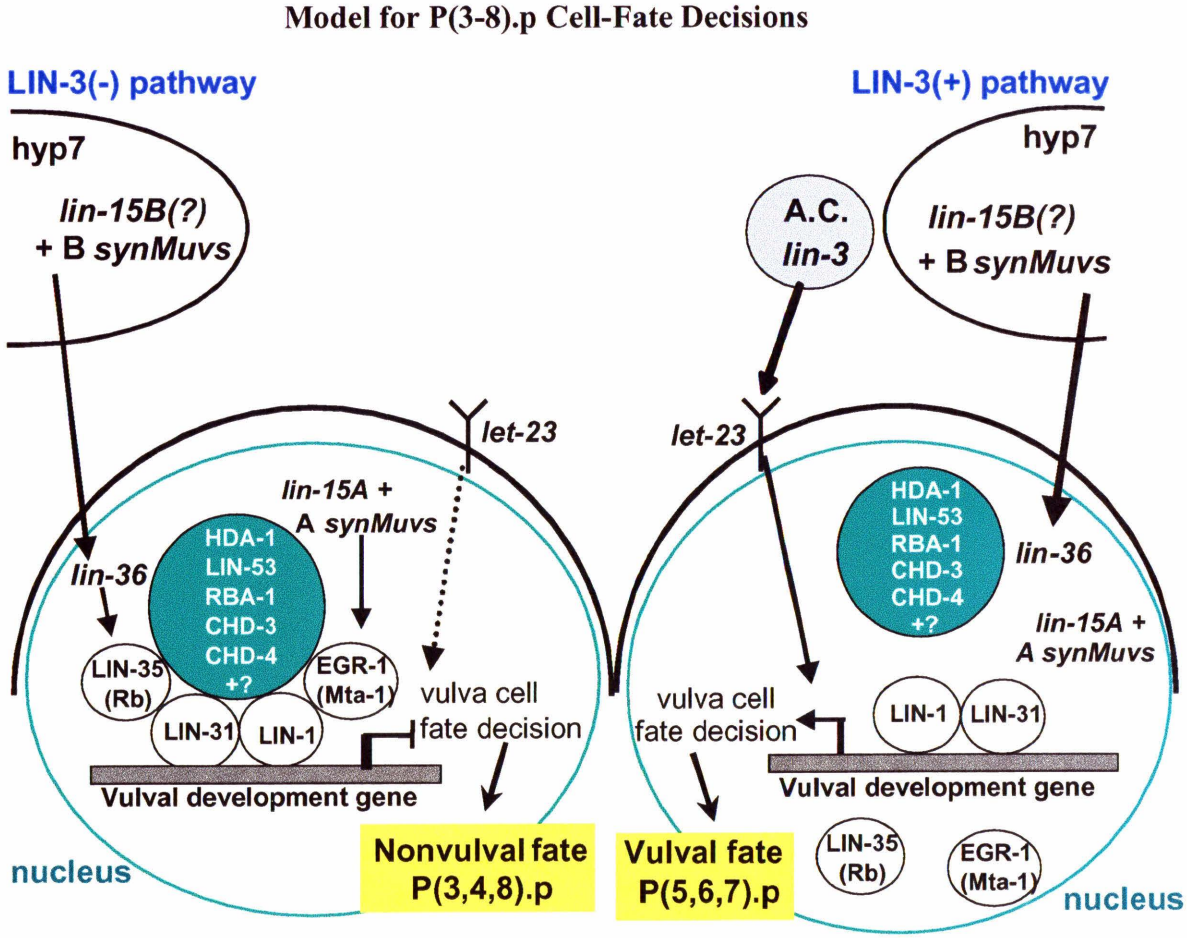


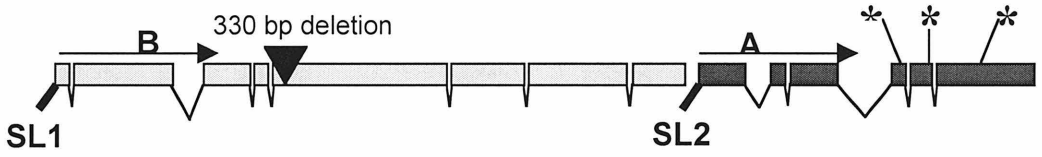
Table 1. Sequences of *lin-15* mutations

Uppercase letters signify exon sequences and the lowercase letters signify intron sequences. The C.elegans consensus splice acceptor site is wwtttcag/NNN, where W is A or T, N is any nucleotide (Fields 1990). Amino acid substitutions are shown as wild-type residue identity, residue number, and predicted mutant residue.

Allele	Wild-type sequence	Mutant sequence	Substitution or splice-site change
sy197	aatgttttaag/AGA	aatgtttctaag/AGA	Exon 5 acceptor in *lin-15A* transcript
n744	TCT	TTT	S124F in *lin-15B* transcript
sy211	aatgttttaag/AGA	aatgttttaaa/AGA	Exon 5 acceptor in *lin-15A* transcript
sy212	CAG	TAG	Q116amber in *lin-15A* transcript

Table 2. Truncation of LIN-15A protein

The *lin-15A* genomic clone, pBLH51 (Huang *et al.* 1994), has 330 bp deleted from the fifth exon in the *lin-15B* transcript. This construct is only able to rescue *lin-15A* mutant alleles not *lin-15B* mutant alleles (Huang *et al.* 1994). An amber codon (TAG) is introduced at the end of exon 4 (pBLH51:K304amber), exon 5(pBLH51:S360amber), and in the middle of exon 6 (pBLH51:G579amber) in the *lin-15A* transcript in order to truncate the LIN-15A protein. Each construct was injected into *lin-15* null hermaphrodites. SL1 signifies SL1 trans-splice leader and SL2 signifies SL2 trans-splice leader (Huang and Hirsh 1989). Asterisk denotes premature stop codon, TAG.



Construct	Rescue Muv Phenotype	# lines examined
4ATAG: K304amber	No (lethal)	2(died)
5ATAG: S360amber	Yes	11
6ATAG: G579amber	No	2

Appendix 1

Mosaic analysis of *let-23*

Abstract

The *C. elegans* epidermal growth factor receptor homologue LET-23 has multiple functions during development. In order to determine where *let-23* is required for vulval induction, mosaic analysis was performed. Mosaics determine its site of action at the VPCs for proper vulval development (Koga and Ohshima 1995b; Simske and Kim 1995). In order to test the efficacy of the GFP::LacI + *lacO* as a single cell marker, we perform mosaic analysis on *let-23*. The GFP::LacI + *lacO* confirmed *let-23* functions at the VPCs for proper vulval development.

Introduction

LET-23 protein

LET-23 is the *C. elegans* homologue member of the epidermal growth factor receptor family (Aroian *et al*. 1990; Lesa and Sternberg 1997). The extracellular portion of LET-23 contains two ligand-binding domains and two cysteine-rich domains. The cytoplasmic region contains a tyrosine kinase domain and a carboxyl-terminal tail. The tail has tyrosines which defines eight putative SH2-binding sites which specifies multiple functions: viability, vulval differentiation, and fertility (Aroian *et al*. 1990; Aroian *et al*. 1994; Aroian and Sternberg 1991; Lesa and Sternberg 1997). Mosaic analysis indicates *let-23* act cell autonomously in the VPCs to promote vulval differentiation (Koga and Ohshima 1995b; Simske and Kim 1995) while mosaic analysis for viability indicates *let-23*'s site the action is at the excretory cell (Koga and Ohshima 1995b). *let-23* may also

function in the gonad for fertility since mutations in *let-23* results in defects in ovulation (Lesa and Sternberg 1997).

We use the GFP::LacI + *lacO* system in order to verify its accuracy as a mosaic marker by confirming *let-23* site of action for vulval differentiation is at the VPCs. We also indirectly determined the site of lethality of *let-23(sy17)* to be in the ABala lineage.

MATERIALS AND METHODS

Nematode methods: Growth and handling of *C. elegans* strain N2 were according to Brenner, 1974 and Sulston and Hodgkin (1988). All experiments were performed at about 20°C unless otherwise stated. The genetic and cellular nomenclature of *C. elegans* was followed according to (Horvitz *et al.* 1979; Sulston *et al.* 1983), respectively.

GFP-LacI fusion plus 256 *lacO* repeat: The GFP-LacI fusion protein plus the *lacO* repeat (Robinett *et al.* 1996; Straight *et al.* 1996; Webb *et al.* 1995) was graciously given to us by Dr. Andrew Belmont. We placed GFP-LacI under the transcriptional control of an hsp16 promoter/enhancer element pPD49-78 (Fire and Xu 1995; Mello and Fire 1995; Perry *et al.* 1993). The fusion protein was inserted into KpnI/SacI site of heat shock vector pPD49-78. pPD49-78 is expressed very well in the neural and hypodermal cells, as well as in the gut, muscles, and pharynx but not in the germline (Fire and Xu 1995; Mello and Fire 1995; Perry *et al.* 1993).

Germline-mediated transformation by microinjection

For the *let-23* mosaics, the plasmid pMH86, containing the *dpy-20(+)* gene, was used as a transformation marker at a concentration of 15ng/μl (Han and Sternberg 1991)). Also, included in this injection mixture for the mosaic analysis of *let-23* are: pPD49-78 GFP-LacI (100ng/μl), 256 repeat *lacO* array (50ng/μl) *let-23* genomic DNA pk7-13.8 (50ng/μl) (Aroian *et al.* 1990) and pBluescript II SK+ (Stratagene) as carrier DNA (5ng/μl). *syEx214* was created from this mixture injected into *let-23(sy17) unc-4(e120)/mnC1; dpy-20* gonads to yield PS2642.

The transgenic lines obtained from each experiment were heat-shocked for 30 minutes at 33°C to elicit GFP-LacI expression. Expression of the GFP-LacI can be seen as early as 30 minutes after heat-shock, and as late as 24 hours.

Mosaic analyses

Mosaic analysis of *let-23* gene function in vulval induction (Koga and Ohshima, 1995) was repeated to test the reliability of the GFP-LacI plus *lacO* as a mosaic marker. Mosaic animals are obtained spontaneously from a somatic loss of an extrachromosomal array *syEx214* [*let-23(+)* + *dpy-20(+)* + pPD49-78::GFP-LacI + *lacO*] from *let-23(sy17) unc-4/ mnC1; dpy-20* parents. The point of loss was determined by the absence of fluorescent spots. We used L3-L4 Unc worms for mosaic analysis that ensures that *let-23(sy17)* is homozygous. The nuclei observed to identify mosaic animals are: pharyngeal

cells: m2L, m1VL, m3L, m3VL, m4R, m3R, m3VR, m3DL, m3DR, pharyngointestinal valve cells, excretory cell; Tail cells: hyp8, hyp9, F, U, B, Y; VPCs (Pn.p cells); intestines; body wall muscles derived from D. (Refer to Figure 1 for lineage).

Results

let- 23 site of action

To test the use of GFP-LacI + *lacO* as a mosaic marker, we repeated mosaics with *let-23* that was done with *ncl-1* (Koga and Ohshima 1995b). Then as described below, we tested it with new mosaic analysis. The strain PS1484 includes a null allele of *let-23* linked to a recessive *unc-4* mutation balanced by *mnC1*[*dpy-10(e128) unc-52(e444)*], and *dpy-20* as a transformation recipient marker. Homozygous *let-23(null)* animals die as young larvae (Aroian and Sternberg 1991; Ferguson and Horvitz 1985; Sigurdson *et al.* 1986) while *mnC1/mnC1* worms are immobile, and semisterile (Herman 1978). This strain was used to repeat the mosaic analysis of Koga and Ohshima, (1995) and Simske and Kim, (1995) using the GFP-LacI + *lacO* repeat as the mosaic marker instead of *ncl-1(+)* . The DNA mixture for injection consists of the *let-23* genomic clone, pk7-13.8, pPD49-78 GFP-LacI + *lacO* and the injection transformation marker pMH86.

Mosaic analysis using *ncl-1* as the marker revealed that *let-23*(+) gene function is required in a vulval precursor cell to adopt the 1° vulval fate (Koga and Ohshima 1995b; Simske and Kim 1995). We examined a total of 53 *let-23(sy17) unc-4* animals, all of

which were mosaic (Figure 1). Of these, 33 had complete vulvae, eight had incomplete vulvae, eleven were vulvaless, and one was multivulva.

The 33 animals with complete vulva and the multivulva animal had partial or total loss in the ABalpa, ABar, ABplpa, ABplpp, MS, C and D lineages, indicating that complete vulval differentiation is not dependent on these foci. In contrast, the VPCs consistently had the array present, especially P6.p. The marked exception, animal B11.3, has a complete vulva but there was no detectable expression of GFP-LacI + *lacO* in the ABp lineage: this is either due to perdurance of *let-23(+)* or is a false negative. For the 11 vulvaless animals, they had partial or total loss in the ABalpa, ABar, ABplpa, ABplpp, MS, C and D but the VPCs had no array present (no spot:-), especially P6.p. *let-23* is required for vulval induction in the ABp lineage, more specifically for the Pn.p cells (Figure 1) (Koga and Ohshima 1995b; Simske and Kim 1995). The incomplete vulva animals' VPC mosaicism was not analyzed because it was not vital for determining the effectiveness of the GFP-LacI + *lacO* technique.

Homozygous *let-23* null alleles (*mn23* and *sy17*) die as L1 larvae (Aroian and Sternberg 1991; Herman 1978; Koga and Ohshima 1995a). Mosaic analysis of *let-23* gene function for animal survival was previously determined in a *mn23* background. This study revealed somatic loss of the array was extremely rare in 2 lineages, ABal (except ABalpa) and ABplp (Koga and Ohshima, 1995). In a *let-23(sy97)* reduction of function background, mosaics in the AB-AB.p-AB.pl lineages were as rare as in the *mn23* background. The *let-23(sy97)* focus of lethality is in the ABplp but not ABal

(Koga and Ohshima,1995). Vulvaless and lethal phenotypes resulting from the *sem-5* and *let-60* mutations are similar to the *let-23* mutations. Genetic epistasis tests using *let-60* and *let-23* gain of function alleles show that the 3 genes act in a common (Beitel *et al.* 1990; Clark *et al.* 1992; Han and Sternberg 1990; Katz *et al.* 1996; Lesa and Sternberg 1997). Since *let-23* stimulates viability and vulval differentiation via SEM-5 and LET-60, we indirectly scored. In the *let-23(sy17)* null background, no "all +" or "all -" animals were observed. We can definitely conclude that the foci of lethality of *let-23(sy17)* is not in the ABplp lineage and ABalpa because 30/53 animals lost the array at the ABplp lineage (cells scored excretory cell, hyp8, hyp9, F and U) therefore inconsistent with *sy97* foci of lethality. We cannot conclude anything about the ABal lineage (except ABalpa) since it was not scored with our technique. We believe the focus of viability is in the ABala lineage since it derives neurons, especially the vital neurons CANL and CANR for animal survival. (Chalfie and White, 1988; and Koga and Ohshima,1995).

Discussion

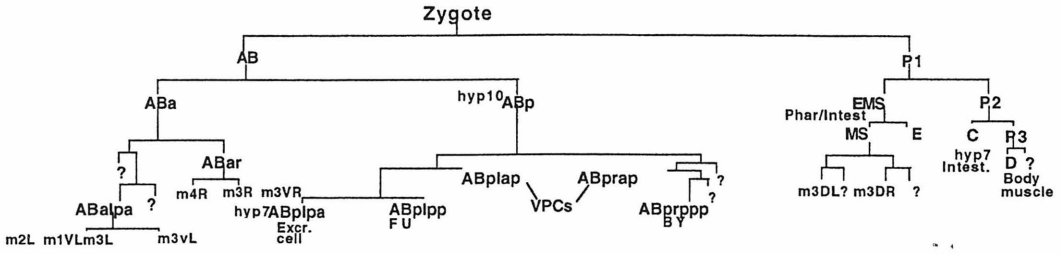
let- 23 site of action

Koga and Ohshima (1995) carried out mosaic analysis of *let-23* using *ncl-1* and found a focus in the VPCs. We repeated the *let-23* mosaics using GFP-LacI + *lacO*. Overall, GFP-LacI + *lacO* mosaic data are consistent with a focus of the Vul rescue be *let-23* in the VPCs. However, animal N.7 (Figure 1) contain no fluorescent spots but the presence of *let-23(+)* since the animal has a wild-type vulva. Another possibility for this

animal's lack of fluorescence is fading of the GFP. Conservatively, this method unambiguously indicates which cells have an array present.

Figure 1. Mosaic analysis of the *let-23* gene re-done with GFP-LacI + *lacO* as the marker

a) Mosaic analysis of *let-23(sy17)* animals using GFP-LacI + *lacO* as the marker, reveal that the wild-type *let-23* gene is required in the ABp lineage for normal vulval induction. Also, the locus of lethality is found in ABalp and ABplp lineages. L3-L4 Unc animals (n=53) were used for this study. +: GFP-LacI bound to the *lacO*, fluorescent spot; -: no fluorescence; H: only GFP-LacI present, presumably the array is lost; ND: not determined; F= fading; " ": cannot positively identify the cell but can determine if H, +, or -. Letters correspond to vulval status: V.1 to V.32= complete vulva; MV.1= multivulva; IV.1 to IV.8 = incomplete vulva; V.3 to V.12 = vulvaless. The numbers in the Pn.p column indicates the number of nuclei with the bound fusion protein or spot.



worm	m2	m1VL	m3L	m3VL	m4R	m3R	m3VR	Excr	hyp8	hyp9	F	U	P3p	P4p	P5p	P6p	P7p	P8p	B	Y	P/I	m3DL	m3DR	Int	b.m.
N.1	-	-	-	+	+	-	-	+	-	-	+	H	-	-	H	H	H	H	+	+	+	+	+	+	+
N.2	-	+	+	+	-	-	+	ND	-	-	+	-	+	+2	+7	+8	+7	+2	+	-	-	+	+	+	+
N.3	-	+	+	+	+	+	+	-	-	-	-	+	+	+2	+7	+8	+7	+2	-	+	+	+	+	+	-
N.4	+	ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
N.5	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+7	+8	+7	-	-	ND	+	+	+	+	-
N.6	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+2	+5	-7	ND	-	-	-	+	+	+	-
N.7	-	+	+	+	+	+	+	+	-	-	+	-	-	-	+4	+4	+4	-	+	-	ND	+	+	+	-
N.8	+	+	+	+	+	+	+	-	-	-	-	-	+	-	+4	-7	-7	ND	-	-	+	+	+	+	-
N.9	+	-	+	ND	-	ND	+	+	-	-	-	-	-	-	+1	+4	-7	ND	-	-	+	-	+	+	-
N.10	+	ND	+	+	ND	+	+	ND	-	-	ND	ND	ND	-	+3	+3	ND	ND	-	ND	ND	+	+	+	ND
N.11	-	+	+	+	+	+	+	-	-	-	-	-	-	-	H7	+4	+4	-2	+	-	-	+	+	-	-
N.12	-	-	+	+	+	-	+	+	+	+	H	H	-	-	+7	+8	+7	-2	+	H	+	+	+	+	+
N.13	+	-	+	+	+	+	+	-	-	-	-	-	-	-	+7	+4	+3	+2	-	-	+	+	+	+	-
N.14	-	-	+	+	-	+	+	F	+	H	H	+	-	+2	H	H	H	-2	+	+	H	+	ND	-	-
N.15	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+1	+3	-7	-2	-	-	-	-	+	-.	-
N.16	-	-	+	+	-	+	+	-	-	-	-	-	-	-	+3	+3	+3	-2	H	-	+	+	+	+	-
N.17	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-7	+2	-7	-2	+	-	-	-	+	-	+
N.18	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-7	+2	-7	+2	+	-	+	+	-	+	-
N.19	-	-	+	+	+	+	+	-	-	-	+	-	-	-	-7	+8	-7	-2	+	+	+	+	+	+	-
N.20	-	-	+	+	+	+	+	-	-	-	+	-	-	-	+3	+8	+5	ND	+	-	+	+	-	+	+
N.21	-	"-"	+	+	+	+	+	ND	-	-	-	-	-	-	-7	+6	-7	-2	-	-	-	+	+	+	-
N.22	+	+	+	+	+	+	+	+	-	-	-	-	-	-/+	+3	+2	+2	-2	+	-	+	+	+	+	-
N.23	+	+	+	+	+	+	+	H	+	+	+	+	-	-	+4	+4	+4	-2	+	+	+	+	+	+	-
N.24	+	"+"	+	+	-	+	+	H	-	-	+	-	+	+2	+7	+8	+7	-2	+	-	+	+	+	+	-
N.25	ND	-	+	+	+	-	+	-	-	-	-	-	+	+2	+7	+8	+7	+2	-	+	+	+	+	+	+
N.26	+	+	+	+	+	+	+	+	-	-	+	+	-	-	H7	+8	+7	+2	+	-	+	+	+	+	+
N.27	ND	ND	+	+	ND	+	+	ND	+	+	+	ND	-	-	+3	+8	+2	-2	+	ND	+	+	+	+	+
N.28	+	+	+	+	+	+	+	+	+	+	+	-	+	+2	+4	+4	+4	+2	+	-	+	+	+	+	+
N.29	+	H	+	+	+	"+"	+	-	-	-	ND	-	+	+2	+7	+8	+7	+2	-	-	+	+	+	+	-
N.30	+	-	-	+	+	+	+	+	-	-	ND	-	-	+2	+1/H2	+4	+4	+/-	+	-	-	+	+	-	-
N.31	+	"+"	+	+	+	+	+	+	+	+	+	+	+	+2	+7	+8	+7	+2	+	+	+	+	+	+	+
N.32	ND	ND	+	+	ND	+	+	ND	-	-	-	ND	+/-	+2	+7	+8	+7	+2	"+"	ND	ND	+	+	ND	ND
MV.1	+	H	+	+	+	+	+	-	+	-	-	-	-	-	+7	+6	+7	-3	+	-	+	+	+	+	+
IV.1	+	H	+	+	+	+	+	-	-	-	+	-	-/+	-/+	+	+3	"+3"	-/+	+	+	+	+	+	+	ND
IV.2	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-/+	+	-	+	-	-	+	+
IV.3	-	+	+	+	-	+	+	+	-	-	+	+	-	-	-	-	-	+	+	-	+	+	+	+	-
IV.4	+	-	+	+	+	+	+	+	-	-	H	-	-	-	-	-	-	-	H	-	+	-	+	+	+
IV.5	-	ND	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	-	+	+2	+2	+2	-	ND	ND	ND	"+"	"-"	ND	ND
IV.6	-	-	+	+	-	-	-	-	-	-	ND	ND	-	-	-	+4	-	-	+	-	-	+	-	-	-
IV.7	-	-	H	+	-	+	+	-	-	-	-	-	-	ND	-	-	-	-	-	H	+	+	+	+	-
IV.8	+	-	+	+	+	+	H	-	-	"+"	-	-	-/+	-	+	+	+4	-	+	-	+	H	+	+	+
V.1	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
V.2	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-	-	-/+	-	-	-	+	+	+	-
V.3	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	-
V.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
V.5	-	-	+	+	-	+	+	+	H	H	-	-	-	-	-	-	-	-	H	+	-	+	-	+	-
V.6	ND	-	+	H	+	-	+	-	-	+	-	-	-	-	-	-	-	-	+	-	+	+	+	+	ND
V.7	ND	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+2	-	-	+	+	+	+	+
V.8	-	ND	+	-	ND	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	-
V.9	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
V.10	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
V.11	"+"	-	+	+	+	+	H	-	+	+	-	+	-	-	-	+?	+?	+?	-	+	ND	+	+	+	+
V.12	+	-	+	+	+	+	+	-	+	+	+	-	-	-	-	+?	+?	+?	+	-	ND	+	+	+	-

Chapter 4

Additional characterization of LIN-15 proteins

Abstract

Further characterization of the *lin-15* locus reveals an effect on fertility. Hermaphrodites with *lin-15B* mutant alleles have a considerable lower broodsize in comparison with wild type animals and *lin-15A* mutant animals at 20°C . *lin-15* null animals at 25°C are completely sterile.

Introduction

The descendants of three of six tripotent form the *C.elegans* vulva hypodermal cells (VPCs). The fate of each of these 6 VPCs is determined by an intercellular signal generated by the AC that initiates positive and negative regulators of the EGF/EGFR pathway for vulval induction [Aroian, 1991; Beitel, 1990; Chamberlin, 1994; Chamberlin, 1993; Clark, 1994; Ferguson, 1989; Ferguson, 1987; Ferguson, 1985; Hill, 1992; Huang, 1994; Jongeward, 1995; Kayne, 1995; Moghal, 1999].

There are 14 identified genes involved in one or two negative regulative pathways out of the five that negative regulate the EGF/EGFR pathway for vulval induction. These 14 genes are involved in the synMuv pathways. Synthetic multivulva (synMuv) genes encode negative regulators of vulval induction (Clark *et al.* 1994; Ferguson and Horvitz 1989; Horvitz and Sulston 1980; Huang *et al.* 1994). In the absence of synMuv activity, P3.p, P4.p, and P8.p generate vulval cells. As mentioned previously, the Muv phenotype is the result of mutations in each class, referred to as A and B, which represent two functionally redundant pathways. There are four class A

genes (*lin-8, lin-15A*, *lin-38*, and *lin-56*) and ten B class genes (*lin-9*, *lin-15B*, *lin-35*, *lin-36*, *lin-37*, *lin-51*, *lin-52*, *lin-53*, *lin-54*, and *lin-55*) (Ferguson and Horvitz 1989; Horvitz and Sulston 1980; Lu and Horvitz 1998) *lin-36, lin-15A* and *lin-15B* each encode novel proteins. *lin-35* encodes a protein related to Rb, and *lin-53* encodes a protein similar to the Rb-binding protein, p48 (Lu and Horvitz 1998).

The Muv strains share two characteristics: heat-sensitivity and maternal effect. When synMuv animals are grown at high temperatures, 25°C, they display three common phenotypes: a longer generation in comparison to wildtype; adult hermaphrodite's body size decreases; and high incidence of sterility. Some, however, show a heat-sensitive decrease in viability. This heat-sensitivity may be reflecting the loss-of-function or reduction of function of the silent Muv genes (Ferguson and Horvitz 1989).

The second characteristic is maternal effect. The penetrance of the Muv phenotype in hermaphrodites with both an A and B class mutation that are progeny of heterozygous hermaphrodites is lower than the penetrance of the Muv phenotype with both an A and B class mutation that are progeny of homozygous A and B class mutation for hermaphrodites. It has not been determined if the maternal effect is due to the combinatorial relationship between the 2 classes of synMuv mutations in the strain or is it due to one of the two mutations in the strain. The results from Ferguson, 1989 show evidence that heat-sensitivity decrease viability and fertility may be the influence of the B silent synMuv mutation. Phenotypic observations at the plate level between synMuv class A strains versus synMuv classB strains reveal while both classes are non-Muv,

synMuv class B strains are in general thinner and less fertile [Ferguson, 1989; my observation].

lin-15 is a member of a set of negative regulators of vulval development in which appropriate mutation combinations result in a multivulva (Muv) phenotype (Ferguson and Horvitz, 1989). *lin-15* is a complex locus with two independent, mutable activities, A and B (Ferguson and Horvitz, 1989; Huang, Tzou and Sternberg, 1994; Clark, Lu and Horvitz, 1994; Lu and Horvitz,1998) and it acts independently of two known signals controlling the fates of the VPCs, the AC inductive signal and a lateral signal among the VPCs (Herman and Hedgecock, 1990). We quantified the broodsize of the *lin-15* null *e1763* at 20°C and 25°C. At 20°C, *lin-15(e1763)* have a reduced broodsize by 6 fold compared to wild-type. At 25°C, *lin-15(e1763)* is sterile. We saw the rescue of sterility of *lin-15(e1763)* at 25°C when the double mutant was built with *lfe-1* and *lfe-2*. LFE-1/ITR-1, an inositol 1,4,5-triphosphate receptor (IP3R) homologue, acts as a ras independent positive effector of LET-23, while LFE-2/IP3 kinase acts as a negative effector to mediate fertility (Clandinin *et al*. 1998; Lesa and Sternberg 1997). We believe *lin-15* may be playing a role in fertility via *let-23* mediated fertility not involving the *ras* pathway.

Materials and Methods

Nematode methods

Growth and handling of *C. elegans* strain N2 were according to Brenner (1974) and Sulston and Hodgkin (1988). All experiments were performed at about 20°C unless otherwise stated. The genetic and cellular nomenclature of *C. elegans* were followed according to Horvitz et al. (1979) and Sulston et al. (1983), respectively.

Strains

The standard wild-type N2 strains was obtained from the *Caenorhabditis* Genetics Center (USA). Below is a list of alleles used in this work. The source of alleles other than from Brenner (1974) or the Caenorhabditis Genetics Center are also indicated. *lfe-1(sy290), unc-24(e120)* IV and *unc-38(e264), lfe-2(sy326)* I (Clandinin *et al.* 1998), *lin-9(n112)* III, *lin-15(e1763)*X, *lin-15(n765)*ABts, and *lin-9(n112)* III; *lin-15(n749)*X (Ferguson and Horvitz 1989; Huang *et al.* 1994), *unc-4(e120) let-23(sy10)/mnC1[dpy-10 unc-52], unc-4(e120)(Aroian et al. 1990; Koga and Ohshima 1995b; Lesa and Sternberg 1997; Simske et al. 1996)* and *let-23(sy10)/mnC1[dpy-10 unc-52], lin-15(e1763)* (Ferguson and Horvitz 1989; Huang 1995; Huang *et al.* 1994).

We crossed in *lin-15(e1763); syEx[lin-15(+)]* males into *lfe-1(sy290), unc-24(e120)* IV and *unc-38(e264), lfe-2(sy326)* I to obtain *lfe-1(sy290), unc-24(e120)*IV; *lin-15(e1763)* and *unc-38(e264), lfe-2(sy326)*I; *lin-15(e1763).* We crossed in *lin-15(n765)* into *lfe-1(sy290), unc-24(e120)* IV and *unc-38(e264), lfe-2(sy326)* I to obtain

*lfe-1(sy290), unc-24(e120)*IV*; lin-15(n765)* and *unc-38(e264), lfe-2(sy326)* I*; lin-15(n765).*

Broodsize quantification at 20°C and 25°C

All manipulations were done with the ambient temperature at 18°C. We place 10 L1 hermaphrodites each on their own plate for each strain for this study. The hermaphrodites were grown in their respective temperature (20°C and 25°C) for 3 1/2 - 4 days then their progeny were counted. We performed two temperature assays concurrently with all strains involved. Since *let-23(sy10)* animals are sterile, *let-23(sy10)* and *let-23(sy10); lin-15(e1763)* animals were picked as L1 Unc animals segregating from an *unc-4(e120) let-23(sy10)/mnC1[dpy-10 unc-52], unc-4(e120)* and *let-23(sy10)/mnC1 [dpy-10 unc-52], lin-15(e1763)* parent, respectively, in order to perform the assay accurately. Since *lfe-1* and *lfe-2* have no phenotype on there own, they are linked *to unc-24(e120)* and *unc-38(e264),* respectively, in order to score for their presence.

Results

Broodsizes at 20°C

After 4 days growing in an incubator set for 20°C, L1 larvae become adults with young progeny (n=10 for each strain). The total number of strains involved are 13: N2, *lfe-1(sy290), unc-24(e120)* IV and *unc-38(e264), lfe-2(sy326)* I (Clandinin *et al.* 1998), *lin-9(n112)* III, *lin-15(e1763)*X, *lin-15(n765)*ABts, and *lin-9(n112)* III*; lin-15(n749)*X

(Ferguson and Horvitz 1989; Huang *et al.* 1994), *unc-4(e120) let-23(sy10)/mnC1[dpy-10 unc-52]* [Aroian, 1990; Lesa, 1997; Simske, 1996; and Koga, 1995] and *unc-4(e120) let-23(sy10)/mnC1[dpy-10 unc-52], lin-15(e1763)* (Ferguson and Horvitz 1989; Huang 1995; Huang *et al.* 1994); *lfe-1(sy290), unc-24(e120)*IV*; lin-15(n765)* and *unc-38(e264), lfe-2(sy326)* I*; lin-15(n765);* and *lfe-1(sy290), unc-24(e120)*IV; *lin-15(e1763)* and *unc-38(e264), lfe-2(sy326)*I*; lin-15(e1763).* We calculated the average broodsize as well as percent fertility per each strain. N2 animals average broodsize at 20°C is ~300 (n=many). *let-23(sy10)* and *let-23(sy10); lin-15(e1763)* are sterile (n=10 for both). The average broodsizes from most to least are*: unc-38(e264), lfe-2(sy326)* = 182.7; *lfe-1(sy290), unc-24(e120)* = 158.5; *lin-9(n112)* = 139.7; *lin-15(n765) = 119.6; unc-38(e264), lfe-2(sy326); lin-15(n765) = 95.9; lin-9(n112); lin-15(n749)A = 82.4; lin-15(e1763)* = 55.4; *lfe-1(sy290), unc-24(e120); lin-15(n765)* = 47.5; *unc-38(e264), lfe-2(sy326); lin-15(e1763) = 27.8*; and *lfe-1(sy290), unc-24(e120); lin-15(e1763)* = 21.4 (Table 1). A reduction of broodsize occurred when both alleles of *lin-15, e1763* (null) and *n765*ABts, were used to build triple mutants with *lfe-1(sy290), unc-24(e120)* and *unc-38(e264), lfe-2(sy326).* The triple mutants *lfe-1(sy290), unc-24(e120)*IV*; lin-15(n765)* and *unc-38(e264), lfe-2(sy326)* I*; lin-15(n765)* as well as *lfe-1(sy290), unc-24(e120)*IV; *lin-15(e1763)* and *unc-38(e264), lfe-2(sy326)*I*; lin-15(e1763)*, dropped 2 fold in broodsize compared to its parent strains. All the strains have 100% fertility except *lfe-1 (sy290), unc-24 (e120)* IV; *lin-15 (e1763) and* unc-*38 (e264), lfe-2(sy326)*I*; lin-15(e1763)* which each had 80% fertility (Table 2).

Broodsizes at 25°C

After 4 days growing in an incubator set for 25°C, the L1 hermaphrodites are now adults with young progeny (n=10 for each strain). The total numbers of strains involved are 13. We calculated the average broodsize as well as percent fertility per each strain. N2 animals average broodsize at 25°C is ~300 (n=many). *let-23(sy10)* and *let-23(sy10); lin-15(e1763)* are sterile (n=10 for both). The average broodsizes are*: unc-38(e264), lfe-2(sy326)* = 42.8; *lfe-1(sy290), unc-24(e120)* = 48.7; *lin-9(n112)* = 9.5; *lin-15(n765)* = 2.3*; unc-38(e264), lfe-2(sy326); lin-15(n765)* = 19*; lin-9(n112); lin-15(n749)A* = 5.2*; lin-15(e1763)* = 0.1; *lfe-1(sy290), unc-24(e120); lin-15(n765)* = 19; *unc-38(e264), lfe-2(sy326); lin-15(e1763)* = 12.4; and *lfe-1(sy290), unc-24(e120); lin-15(e1763)* = 13.8 (Table 1). An increase of broodsize occurred when both alleles of *lin-15, e1763* (null) and *n765*ABts, were used to build triple mutants with *lfe-1(sy290), unc-24(e120)* and *unc-38(e264), lfe-2(sy326).* The triple mutants, *lfe-1(sy290), unc-24(e120)*IV*; lin-15(n765)* and *unc-38(e264), lfe-2(sy326)* I*; lin-15(n765)*, increased broodsize by 5 fold for *lin-15(n765).* Triple mutants, *lfe-1(sy290), unc-24(e120)*I; *lin-15(e1763)* and *unc-38(e264), lfe-2(sy326)*I*; lin-15(e1763)* rescue *lin-15(e1763)* sterility. *unc-38(e264), lfe-2(sy326)* I*; lin-15(n765), lfe-1(sy290), unc-24(e120)* IV and *unc-38(e264), lfe-2(sy326)* have 100% fertility. *lin-9(n112)* and *lfe-1(sy290), unc-24(e138); lin-15(n765)* are 60% fertile while *lin-9(n112); lin-15(n744), lfe-1(sy290), unc-24(e120)*IV; *lin-15(e1763)* and *unc-38(e264), lfe-2(sy326)*I*; lin-15(e1763)* are 70%, 80% and 40% fertile, respectively (Table 2).

Discussion

SynMuv A and B proteins, all nuclear, may either recruit or activate, via their own pathways, a complex that binds to vulva genes in order to block the cell from adopting the vulva fate (Ferguson and Horvitz 1989; Huang 1995; Huang *et al.* 1994; Kelly and Fire 1998; Lu and Horvitz 1998; Solari and Ahringer 2000; Thomas and Horvitz 1999; Walhout *et al.* 2000). The disruption in both pathways results in all 6 VPCs cells adopting vulval fates therefore the animal will have a Muv phenotype fate (Ferguson and Horvitz 1989; Huang 1995; Huang *et al.* 1994; Kelly and Fire 1998; Lu and Horvitz 1998; Solari and Ahringer 2000; Thomas and Horvitz 1999; Walhout *et al.* 2000). A disruption in one of either A or B synMuv pathway is sufficient to suppress the vulval fate in a given VPC therefore a wild-type vulva but broodsize is noticeably reduced especially if the disruption is in the synMuvB pathway (Ferguson and Horvitz 1989). *lin-9,* a B class gene, has alleles (*n942* and *n943*) that are sterile. There is a drastic reduction in brood-size when both synMuv pathways are disrupted and grown at 25°C as we have seen (Table 1 and 2). Animals with the null allele of *lin-15* have broodsizes one-sixth lower than the standard wild-type strain, N2 at 20°C and at 25°C, *lin-15(null)* animals are sterile (Table 1 and 2). *lin-15(n765)*ABts animals also have broodsizes one-third lower than N2 strains at 20°C while at 25°C it is almost sterile. Both alleles of *lin-15* were viewed under Nomarski optics and we observed a reduced number of mature sperm and oocytes. We see high expression in the spermatheca by GFP driven by the *lin-15* promoter indicating possible activity in spermathecal

contractions (Chapter 3, Figure 2). *lin-15* may be involved in germline development due to low numbers of mature sperm and oocytes, but further investigation is needed.

The receptor tyrosine kinase LET-23 is required for normal development five distinct tissues so far: the vulva, the male tail, the hermaphrodite's posterior ectoderm, the hermaphrodite gonad as well as an essential focus in L1 larvae. A RAS/MAP kinase cascade in all of these tissues except one, hermaphrodite's gonad, mediates LET-23 function. LET-23 activity in the gonad is RAS independent (Clandinin *et al.* 1998; Lesa and Sternberg 1997). Clandinin *et al.,* (1998), identified tissue-specific effectors of *let-23* which revert sterility but not the vulval or lethal defects associated with reduced pathway activity. Two loci were identified, *lfe-1* and *lfe-2*, and they appear to function downstream of *let-23* in the hermaphrodite gonad. They encode proteins involved in the regulation of intracellular calcium levels via inositol phosphate metabolism. Mutations in each gene alone are phenotypically silent but *lfe-1; lfe-2* double mutant animals are sterile, demonstrating involvement in ovulation of *let-23(+)*animals. The gonad of *lfe-1; lfe-2* double mutant animals differentiate normally but ovulation of mature oocytes is abnormal.

lin-15 fertility is dependent on the LET-23 receptor since it is not able to suppress *let-23(sy10)* sterility as a double mutant, *let-23(sy10); lin-15(e1763),* at 20°C. Both *lfe-1* and *lfe-2* are able to rescue *lin-15* sterility at 25°C as a double mutant (Table 1). At 20°C, the double mutants have a synergistic effect resulting in a lower brood-size as

compared to the broodsizes as single mutants (Table 1). These results suggest that *lin-15* is involved in another pathway that does not involve the Ras pathway via the *let-23* receptor. We propose LET-23 affects intracellular calcium levels in the hermaphrodite somatic gonad, perhaps as part of the mechanism that regulates sheath and spermathecal contractions during ovulation, regulated by the *lfe-1* and *lfe-2* (Clandinin *et al.* 1998), as well as germline development and/or spermatheca contractions, regulated by synMuv pathways (possibly solely B synMuv pathway) (Ferguson and Horvitz 1989).

Figure 1. Average Broodsizes at 20°C and 25°C

The broodsizes shown is the average of 10 progeny events of each strain used. Ten L1 stage animals for each strain used were place on a plate, one per plate, for a 3-4 day incubation at 20°C and 25°C. The progeny were then counted and averaged for each strain at both temperatures

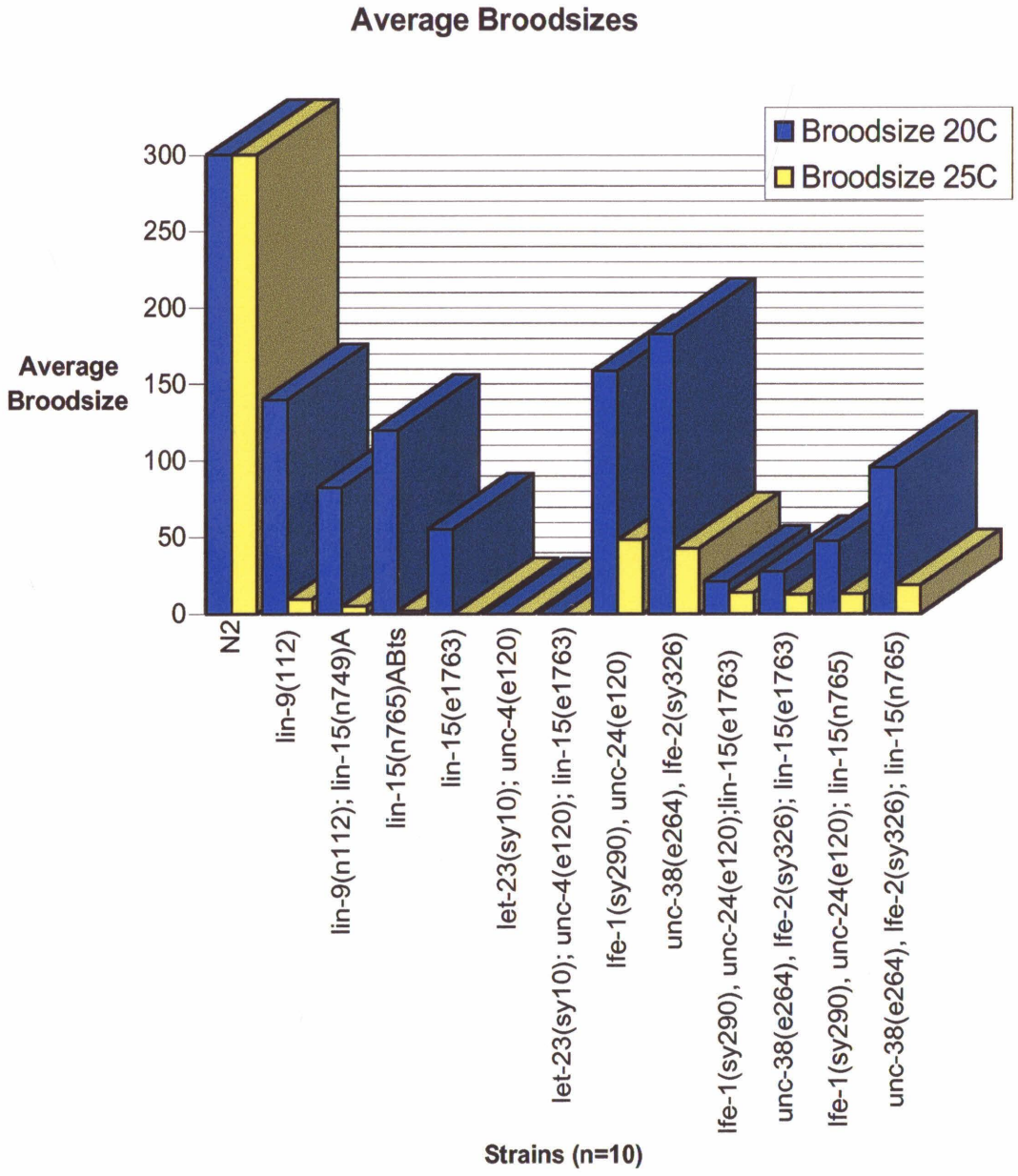
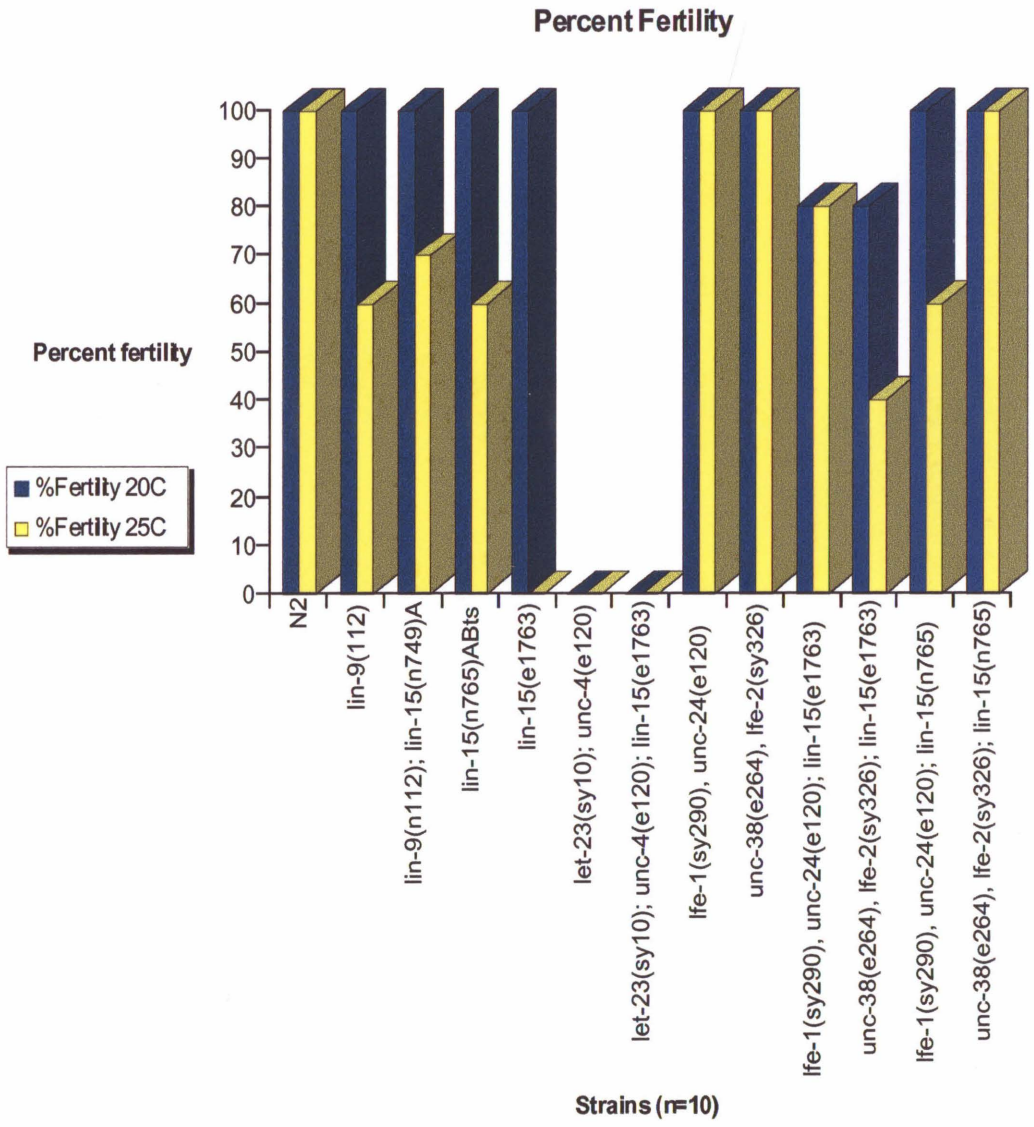

Average Broodsizes
Broodsize 20C
Broodsize 25C
Average Broodsize
300
250
200
150
100
50
0
N2
lin-9(112)
lin-9(n112); lin-15(n749)A
lin-15(n765)ABts
lin-15(e1763)
let-23(sy10); unc-4(e120)
let-23(sy10); unc-4(e120); lin-15(e1763)
lfe-1(sy290), unc-24(e120)
unc-38(e264), lfe-2(sy326)
lfe-1(sy290), unc-24(e120);lin-15(e1763)
unc-38(e264), lfe-2(sy326); lin-15(e1763)
lfe-1(sy290), unc-24(e120); lin-15(n765)
unc-38(e264), lfe-2(sy326); lin-15(n765)
Strains (n=10)

Figure 1. Percent fertility at 20°C and 25°C

Out of the 10 L1 stage animals for each strain, how many had progeny at 20° and 25°C 3-4 days later.


Percent Fertility
Percent fertility
100
90
80
70
60
50
40
30
20
10
0
%Fertility 20C
%Fertility 25C
N2
lin-9(112)
lin-9(n112); lin-15(n749)A
lin-15(n765)ABts
lin-15(e1763)
let-23(sy10); unc-4(e120)
let-23(sy10); unc-4(e120); lin-15(e1763)
lfe-1(sy290), unc-24(e120)
unc-38(e264), lfe-2(sy326)
lfe-1(sy290), unc-24(e120); lin-15(e1763)
unc-38(e264), lfe-2(sy326); lin-15(e1763)
lfe-1(sy290), unc-24(e120); lin-15(n765)
unc-38(e264), lfe-2(sy326); lin-15(n765)
Strains (n=10)

Chapter 5

Summary

In my graduate work, I have investigated the mechanisms used in *C.elegans* vulval differentiation through molecular and genetic analysis of the *lin-15* locus. The *lin-15* locus has two separate activities, A and B, which act in the negative regulation of the inductive event in concert with synMuv class A genes and synMuv class B genes (Ferguson and Horvitz 1985; Ferguson *et al.* 1987; Huang 1995; Huang *et al.* 1994; Sternberg and Horvitz 1986). As mentioned in previous chapters, animals carrying mutations in either a class A gene or a class B gene have no phenotype. A double mutant animals defective in both A and B function display the Multivulva phenotype characteristic of all VPCs cells adopting vulval fate (excessive vulval differentiation) (Ferguson and Horvitz 1985; Ferguson *et al.* 1987; Huang 1995; Huang *et al.* 1994; Sternberg and Horvitz 1986).

LIN-15A and LIN-15B proteins are nuclear with a broad expression pattern (Huang 1995). *lin-15::GFP* also has a broad expression patterns. Both the antibodies and the GFP result signifying involvement in other developmental events, not only vulva differentiation. There is strong evidence that the synMuv pathways, perhaps more so in the synMuv class B pathway, affect fertility (Ferguson and Horvitz 1989). Phenotypic observations at the plate level between synMuv class A strains versus synMuv classB strains reveal synMuv class B strains are in general thinner and less fertile [Ferguson, 1989; personal observation]. *lin-15* fertility is dependent on the LET-23 receptor since it is not able to suppress *let-23(sy10)* sterility as a double mutant, *let-23(sy10); lin-15(e1763),* at 20°C. Both *lfe-1* and *lfe-2* are able to rescue *lin-15* sterility at 25°C as a

double mutant (Chapter 4). At 20°C, the double mutants have a synergistic effect resulting in a lower brood-size. These results suggest that *lin-15* is involved in another pathway for fertility that does not involve the Ras pathway via the *let-23* receptor. We believe LET-23 affects intracellular calcium levels in the hermaphrodite somatic gonad, as part of a mechanism controling sheath and spermathecal contractions during ovulation, regulated by the *lfe-1*, *lfe-2* (Clandinin *et al.* 1998) and *lin-15*. SynMuv pathways (possibly solely B synMuv pathway) may regulate germline development, via LET-23 due to the sterility *lin-9* alleles *n942* and *n943* and the reduced number of mature sperm and oocytes as seen in the *lin-15* null allele.

We wanted to create an assay that can be used as a single cell marker, plus a variety other uses (Chapter 2), for mosaic analysis. The GFP::LacI + *lacO* system (Straight *et al.* 1996; Webb *et al.* 1995; Webb *et al.* 1997) provides easy scoring (spot vs. no spot), in all cells including germline unlike the previous mosaic marker, *ncl-1,* which is limited to certain cells and the scoring is more challenging. We confirmed GFP::LacI + *lacO* system effectiveness as a single cell marker for mosaic analysis by unequivocally determining *lin-3* site of action for vulval induction is at the AC as well as *let-23* site of action for vulval induction is at the VPCs (Chapter 2 and Appendix 1).

The yeast two-hybrid of the synMuv proteins (Walhout *et al.* 2000) plus *C.elegans* gene homologues to the NURD complex (Solari and Ahringer 2000), mosaic data and protein localization (Lu and Horvitz 1998; Thomas and Horvitz 1999) lays the foundation of a synMuv class B complex (similar to Rb pathway) and the

synMuv class A pathway activities are initiating or maintaining the NURD complex to alter chromatin structure of vulval development genes to antagonize the Ras-mediated vulval induction. We further strengthen the model by confidently using the GFP::LacI + *lacO* to perform mosaic analysis on *lin-15A* and *lin-15B*. *lin-15A* site of action is at the VPCs. It was then further confirmed by localizing its expression within the VPCs (Chapter 3). Unlike the *lin-15A* mosaic, *lin-15B* was not as clear. The expression pattern of the mosaic marker did not reveal *lin-15B* site of action, which is due to the maternal effect of the LIN-15B protein (Chapters 1 and 3). In order to bypass the maternal effect of LIN-15B protein, tissue localized expression of *lin-15B* can determine where *lin-15B* activity is sufficient to rescue the Muv phenotype of *lin-15* null allele.

lin-15 is part of a new mechanism of receptor regulation. Further understanding its role in the A and B class synthetic Multivulva pathways and its association with the NURD complex that affects chromatin structure will further increase the importance of chromatin regulation in developmental decisions. The link between *lin-15* to the NURD complex and its negative regulation to the EGF- receptor, will further elucidate molecularly and genetically how cancer cells adopt such a fate via its chromatin structure. Hopefully in the near future, individuals with carcinomas may be diagnosed early and be treated effectively due to our continuing knowledge on cell fate specification via the positive and negative regulation on the EGF- receptor/ MAP kinase signaling pathway.

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