Genetic and Molecular Analysis of Synthetic Multivulva Genes in *Caenorhabditis elegans*

by
Jeffrey H. Thomas

B. A. Biology
University of Virginia, 1989

Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the degree of

DOCTOR OF PHILOSOPHY

at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 1997

© 1997 Jeffrey H. Thomas. All rights reserved.

The author hereby grants to MIT permission to reproduce and to distribute publicly paper and electronic copies of this thesis document in whole or in part.

Signature of Author: _____
Department of Biology
May, 1997

Certified by: _____
H. Robert Horvitz
Professor of Biology
Thesis Supervisor

Accepted by: _____
Richard Young
Chairman of the Graduate Committee
Department of Biology

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
JUN 0 2 1997
LIBRARIES
Science

Genetic and Molecular Analysis of Synthetic Multivulva Genes in *Caenorhabditis elegans*

by

Jeffrey H. Thomas

Submitted to the Department of Biology in May, 1997 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

ABSTRACT

A synthetic multivulva phenotype results from mutations in interacting genes that negatively regulate the *ras*-mediated intercellular signaling system of *Caenorhabditis elegans* vulval induction. The interaction of two synthetic multivulva mutations in two different classes of genes, class A and class B, is required to produce the multivulva phenotype in which all six vulval precursor cells adopt vulval fates thereby forming ectopic vulval tissue in addition to a normal vulva. Mutations in either class alone do not cause a vulval phenotype.

To further characterize these pathways, mutations in both classes of synthetic multivulva genes were isolated. A total of 49 mutations were isolated and characterized. The analysis of some of these mutations and some previously isolated mutations allowed the identification of five new class B genes and one new class A gene.

The class B gene *lin-36* was characterized in greater detail. *lin-36* mutations alone did not cause vulval lineage fate transformations, but in conjunction with a class A mutation, caused all vulval precursor cells to adopt vulval fates. *lin-36* was cloned and shown to encode a novel protein. Genetic mosaic analysis suggests that *lin-36* is required cell autonomously, and a *lin-36* reporter is expressed in the nuclei of vulval precursor cells. The interaction between synthetic multivulva genes and genes that positively regulate the *ras*-mediated signaling system was studied.

Thesis Supervisor: H. Robert Horvitz
Title: Professor of Biology

Acknowledgments

It is a daunting task to thank all of the myriad people who have influenced the writing of this thesis. It is impossible to thank them all. However, several stand out as having had a particular impact on this work.

Bob Horvitz has been invaluable as my advisor for this thesis. His rigorous standards for experimental interpretation and presentation will always remain with me.

The members of my thesis committee have provided useful guidance and perspective. In particular, Ruth Lehmann has been the source of excellent scientific and career advice.

My fellow lab members have contributed immensely to the experience this thesis represents, especially my former baymates, Kerry Kornfeld and Michel Labouesse. Other members of the lab, notably Michael Stern, Scott Clark, Erik Jorgensen, Cori Bargmann, and Sander van den Heuvel were influential.

Shai Shaham deserves special mention. A great friend and a powerful intellect, the Horvitz lab has never been the same without him.

The friendship of Greg Beitel, Brad Hersh, Gordon Campbell, Tory Herman, and Zheng Zhou has been a source of great happiness over the years.

Xiaowei Lu and Craig Ceol have made the past two years in the lab intellectually exciting.

Robert Grainger is in large part responsible for my interest in developmental biology and for my interest in the problem this thesis addresses. Mary Franklin Fulghum showed me how exciting biology was and bears some responsibility for my study of this field. Frances Dameron knew I would write this long before anyone else.

My parents, Melvin and Rebecca, have encouraged me in this as they have in all things. Their limitless love and support has always been the foundation in my life. I cannot imagine better parents. My sister Lesley has always been a source of encouragement and sound advice.

I thank all of these people.

Table of Contents

Chapter 1

Antagonistic Signals in the Control of Development and Cell Proliferation

Jeffrey H. Thomas

Howard Hughes Medical Institute, Massachusetts Institute of Technology
Cambridge, MA 02139

Summary

Many complex biological processes are regulated by both positive and negative signals which act antagonistically with respect to each other. Positive and negative inputs allow a cell to effectively respond to its environmental context. The synthetic multivulva genes act antagonistically to the anchor cell signal in *Caenorhabditis elegans* vulval fate specification. A number of other examples of signals that act antagonistically to regulate development and cell proliferation are now known. These signals interact by a variety of different mechanisms.

Antagonistic signals can provide information effectively in complex processes

Many complex biological processes are controlled by multiple signals. These signals may act in concert and reinforce each other or they may act in opposition and antagonize each other. In both cases, multiple signals can provide more information to a cell about its environment than a single signal, allowing the cell to respond to environmental conditions more effectively. Often the environment of the cell is defined by the time and place of the cell in development. The proper interpretation of its developmental context by a cell is crucial to its adoption of the proper fate, be it in terms of identity, relative spatial position, growth, or role in further development. In highly complex biological processes such as development, there is a requirement for highly specific information. Highly specific information is readily produced by multiple signals, each of which provide only some of the information needed, but together provide all of the information needed.

In some respects, accurate information about the environmental context of a cell can be more readily provided by antagonistic signals rather than by cooperative signals. Inhibitory information can be provided directly, rather than through the absence of additional stimulatory information. This point can be illustrated by several simple scenarios. In the case of two positive signals acting to regulate a biological process, a threshold of response is necessary to distinguish between receiving both positive signals versus receiving just one. Alternatively, each positive signal may be distinct; neither one type nor the other is sufficient to elicit the proper response. In this case, both types of signal must be received and must be distinguished. In the case of a positive signal and purely inhibitory signal, no threshold is required. The positive signal need merely be received in the absence of the inhibitory signal, which would negate it or act to prevent its reception. Thus situations can be imagined whereby antagonistic signals provide information more effectively and parsimoniously than cooperative signals.

Potential mechanisms of positive and negative signal interaction

A negative signaling system can act in a variety of ways to antagonize the action of a positive signaling system. The cases documented here are all cases in which negative regulation is exerted through a distinct signaling system. Only examples of negative regulation that clearly involve both a negative and a positive signaling system are described. This negative signaling system may exert its effect at any of several levels. It may negatively regulate the positive signal upstream of the positive signal transduction system, it may directly regulate the signal transduction apparatus of the positive signal, or it may act in the nucleus, downstream of positive signal transduction.

An inhibitory signal that acts upstream of the stimulatory signal transduction apparatus may act via several mechanisms. It may directly inhibit the cell that secretes the stimulatory signal from either producing the positive signal or secreting it. The negative system may prevent the transcription of the stimulatory ligand or the molecules necessary for producing it. The negative signal may prevent the proper processing of the stimulatory ligand or its secretion. The inhibitory signal may titrate out the stimulatory signal by binding it and rendering it inoperative. The negative signal may be a competitive inhibitory ligand which prevents the positive signal from binding its receptor by occupying, and thus blocking access to, the ligand binding domain of the receptor.

The inhibitory signal may exert its negative regulation by directly modulating the activity of the signal transduction system that transduces the positive signal within the responding cell. The possible mechanisms for this type of regulation are numerous. The negative signal may prevent receptor dimerization by binding to the extracellular domain of the receptor and altering its conformation or by occluding receptor association. It may also prevent receptor dimerization by activating a competing receptor subunit which prevents propagation of a signal, or by means of an intracellular factor which occludes dimerization or alters receptor conformation. The negative signal may activate a phosphatase that dephosphorylates the receptor or a downstream target of a kinase. The negative signal may activate another enzymatic activity that counters one found in the positive signal's transduction system. An example of such an activity is a GTPase activating protein (GAP) activity in a Ras-mediated pathway. The negative signal may stimulate a kinase which phosphorylates a negative regulatory site on a signal transduction protein. The negative signal may activate an inhibitory protein which binds an essential signal transduction substrate and inhibits its activity or thwarts its ability to bind to its target or form an active complex. These are some of the many conceivable ways to inhibit a signal transduction pathway.

The negative signaling system may exert its influence in the nucleus. It may activate a molecule that prevents the transport of a signal transduction molecule such as mitogen-activated protein (MAP) kinase into the nucleus. It may activate a nuclear protein that binds to a transcription factor activated by the positive signaling pathway and prevent its binding to DNA,or prevent it from interacting with an essential cofactor. The negative signaling system may activate a DNA-binding factor that blocks the binding site of a transcription factor activated by the positive signal, thereby preventing its action. Transcription factors activated by the negative signal may simply bind promotor regulatory sites that act in opposition to those promotor regulatory sites upon which transcription factors stimulated by the positive signal bind. The negative signal may also

inhibit the transcription of necessary components of the positive signal trasnsduction system if these components are limiting and their production is positively controlled by the activation of the positive signaling system (a positive feedback loop). Of course, it is possible that the negative signal activates the transcription of an inhibitory protein that acts in any of the myriad ways described previously in this section.

As described later in this chapter, many of these mechanisms have been found to act in various regulatory schemes in different biological processes. In this chapter, examples of these interacting positive and negative systems are described in detail. These systems provide a framework to think about how a group of negative regulatory genes may act in *Caenorhabditis elegans* vulval development act.

Antagonistic signaling systems in the specification of cell fates during *Caenorhabditis elegans* vulval development

The focus of this work is on the antagonistic signaling systems that determine the cellular fates of the vulval equivalence group of *Caenorhabditis elegans*. These signaling systems provide information to each member of this group of equipotent cells. This information is integrated by the cells and each responds according to the information it receives by adopting a specific fate. Antagonistic signals allow cell fates to be specified in the proper spatial pattern, allowing development of the vulva to be coordinated with the development of the uterus and the hypodermis.

The vulval equivalence group, P(3-8).p, is a set of six cells that have the potential to adopt either one of two vulval fates (1° or 2°) characterized by a distinctive division pattern and morphology, or a nonvulval hypodermal fate (3°) (Sulston and White, 1980; Kimble, 1981; Sternberg and Horvitz, 1986). In wild type, the hermaphrodite vulva of *C. elegans* is formed from the descendents of three hypodermal blast cells, P(5,6,7).p. Specifically, P5.p and P7.p adopt 2° cell fates in mirror image symmetry about P6.p, which adopts a 1° cell fate. P(3,4,8).p adopt 3° cell fates such that they produce component nuclei of the hypodermal syncytium (Sulston and Horvitz, 1977).

These cell fates are specified by several cell interactions (for review, see Horvitz and Sternberg, 1991; Eisenmann and Kim, 1994; Sundaram and Han, 1996). The gonadal anchor cell signals the nearest Pn.p cells to adopt vulval fates. This signal has a limited range, and cannot act over the entire field of the vulval equivalence group. A signal from the anchor cell is at least partly responsible for establishing the proper pattern of vulval fates (Kimble, 1981; Sternberg and Horvitz, 1986; Thomas et al., 1990; Simske and Kim, 1995; Katz et al., 1995). In the absence of the anchor cell, no vulval tissue is formed in wild-type animals (Sulston and White 1980; Kimble, 1981). In addition to the influence

of the gonadal anchor cell signal on the pattern of vulval cell fates, another signal is propagated amongst the induced Pn.p cells to prevent the formation of adjacent primary cells. This serves to reinforce the proper (2°-1°-2°) pattern (Sternberg, 1988).

Many genes have been identified and shown to encode proteins implicated in the transduction of the inductive signal from the anchor cell. These genes include: *lin-3*, an EGF-like molecule (Hill and Sternberg, 1992), *let-23*, a receptor tyrosine kinase (Arioan et al., 1990), *sem-5*, an adaptor molecule (Clark et al., 1992a), *let-60*, a *ras* GTPase (Han and Sternberg, 1990, Beitel et al., 1990), *lin-45*, a *raf* serine/threonine kinase (Han et al., 1993), *mek-2*, a mitogen activated protein (MAP) kinase kinase (Kornfeld et al., 1995a, Wu et al., 1995), *mpk-1/sur-1*, a MAP kinase (Lackner et al., 1994, Wu and Han, 1994), and *ksr-1*, a protein kinase (Kornfeld et al., 1995b). These genes constitute the core of the anchor cell-mediated signaling pathway. Other genes have been identified, some of which act in an auxillary role to positively regulate the anchor cell signaling pathway. These include *lin-2*, *lin-7* and *lin-10*, genes which have been implicated in the localization of the LET-23 receptor to the Pn.p cell junction (Ferguson et al., 1987; Kim and Horvitz, 1990; Hoskins et al., 1996; Simske et al., 1996).

Another group of genes acts in opposition to the genes described above. These genes likely encode the components of a signaling system since some have been shown to act cell nonautonomously. The hypodermal syncytium postulated to be the source of this signal since it is the tissue that most clearly fits criteria of an anatomical focus of activity suggested by the genetic mosaic data (Herman and Hedgecock, 1990). This is a negative signal, acting antagonistically to the positive signal propagated from the anchor cell. This signal, as discussed below, is probably not a single signal but rather a pair of functionally redundant signaling systems, either of which is sufficient to mediate the negative regulation of vulva cell fates in wild type (Ferguson and Horvitz, 1989). Note that this case the "negative signal" probably consists of two functionally redundant signaling systems which are both responsible for negative regulation. It is likely that the anchor cell overrides this inhibitory signal to induce the vulva from the cells nearest to the uterus.

Several multivulva (Muv) strains have been identified which disrupt these negative signaling systems. The Muv phenotype of these mutant strains results from the interaction of two different mutations (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985; 1989). The mutations that interact to produce such a synthetic multivulva (synMuv) phenotype fall into two classes: A and B. Animals carrying both a class A and a class B mutation have a Muv phenotype. Animals which carry mutations in a single class have a wild-type vulval phenotype. Ferguson and

Horvitz (1989) have proposed that the synMuv genes encode the components of two functionally redundant pathways which negatively regulate vulval development. Four class A genes (*lin-8*, *lin-15* A, *lin-38* and *lin-56*) and ten class B genes (*lin-9*, *lin-15* B, *lin-35*, *lin-36*, *lin-37*, *lin-51*, *lin-52*, *lin-53*, *lin-54*, *lin-55*) have been identified (Horvitz and Sulston, 1980; Ferguson and Horvitz, 1989; Chapter 2). Of these genes, *lin-15* is particularly notable in that it is a complex locus with genetically separable class A and class B activities encoded by two nonoverlapping transcripts (Ferguson and Horvitz, 1989; Clark et al., 1994; Huang et al., 1994). Four genes, *lin-15* A, *lin-15* B, *lin-9* and *lin-36* have been cloned and shown to encode novel proteins (Clark et al., 1994; Huang et al., 1994; Beitel and Horvitz, in preparation; Chapter 3).

SynMuv mutants in which the anchor cell has been ablated, thereby eliminating the positive signal, still display a Muv phenotype (Ferguson et al., 1987; Chapter 3). This suggests that in the absence of synMuv gene activity, the Pn.p cells do not require the anchor cell signal to adopt vulval cell fates. Thus the anchor cell is not absolutely required for the specification of vulval cell fates, but is required for the proper pattern and localization of cell fates in the vulval field.

Genetic mosaic analyses suggest that both *lin-15* AB and *lin-37* act cell non-autonomously. Loss of *lin-15* AB activity in either the AB or P_1 lineages can result in a Muv phenotype. Loss in either lineage is sufficient to produce a phenotype. This suggests that *lin-15* AB acts cell nonautonomously since *lin-15* AB activity is required in both AB and P_1. The only tissue that both AB and P_1 lineages give rise to is the syncytial hypodermis. This is likely to be the focus of *lin-15* AB activity. Loss of *lin-37* activity in AB, but not in its daughter cell, AB.p, which gives rise to the Pn.p cells infrequently produces a Muv phenotype. Thus, *lin-37* acts cell nonautonomously. This result, though different from the *lin-15* AB result, is consistent with a hypodermal focus for *lin-37* activity. The difference in these results is probably a result of a differing dose requirements for these genes (Herman and Hedgecock, 1990; Hedgecock and Herman, 1995). The non-autonomous focus of these genes suggests that the synMuv genes encode the components of signaling systems that inhibit the adoption of vulval fates by the Pn.p cells. Genetic mosaic analysis of *lin-36* suggests that this gene acts cell autonomously in the Pn.p cells (Chapter 3). Thus, for the class B pathway, the negative signaling system has components within the responding cells, eliminating models which involve a negative ligand binding the extracellular domain of the LET-23 receptor and exerting its influence directly.

Reduction-of-function mutations in genes known to be involved in inductive signal transduction, *let-23*, *sem-5*, *let-60 ras*, and *lin-45 raf*, are epistatic to synMuv

mutations (Ferguson et al., 1987; Beitel et al., 1990; Han et al., 1990, Clark et al., 1992; Han et al., 1993; Huang et al., 1994; Chapter 3). These results suggest that the inductive signal transduction system is required for the adoption of the Muv phenotype in the absence of inhibitory synMuv gene activity even though the anchor cell signal itself is not. The phenotype of mutations in the *lin-2*, *lin-7* and *lin-10* receptor localization system and the synMuv phenotype are coexpressed in triple mutants (Ferguson et al., 1987; Chapter 3). Pn.p cells can still adopt vulval cell fates in animals in which the anchor cell signal, the negative signaling systems and the localization of the receptor have been eliminated but the positive signal transduction cascade is intact (Chapter 3). This suggests that the unstimulated activity of the *let-23* pathway is necessary for the adoption of vulval fates.

Together, these data suggest the following model. The six equipotent vulval precursors are predisposed to adopt vulval fates by virtue of the unstimulated activity of the *let-23* signal transduction pathway. In the absence of this pathway, the cells are incapable of adopting vulval fates. This predisposition to adopt vulval fates is inhibited through the action of two functionally redundant signaling pathways from the hypodermis. These pathways act to downregulate the unstimulated activity of the *let-23* signal transduction pathway. This changes the cells' inherent tendency to adopt vulval cell fates to a tendency to adopt nonvulval cell fates. The inductive signal from the anchor cell stimulates the *let-23* pathway in the cells nearest it, leading them to adopt a vulval cell fate. It is likely that this stimulation acts by overriding the effect of the two inhibitory pathways but may act by relieving the Pn.p cells of inhibition by the synMuv genes. These possibilities differ in the means by which inhibition is overcome. The signal transduction system may override the negative regulation of the synMuv genes by being activated so strongly that the inhibitory activity of the synMuv genes is insufficient to reduce the activity of the fully activated pathway below the threshold for a response. Alternatively, activation of the inductive signaling pathway may activate a secondary pathway which inhibits the activity of the synMuv genes thereby relieving the inhibition exerted by the synMuv genes. At present, there are no compelling observations that allow these two possibilities to be distinguished.

Examples of antagonistic signals are found in other biological processes

A number of biological processes are known in which antagonistic signaling pathways function. In each of these examples, a "positive" signaling system activates a process such as specification of a certain cell fate. This process is modulated by a second signaling system that operates in opposition to the first; this signaling pathway is referred

to as the "negative" signaling system. The information delivered by positive and negative signals is then integrated by the responding cell.

Several examples of such antagonistic signaling systems are described in the following sections. In each case, negative regulation has been shown to involve signaling between cells rather than cell-intrinsic negative regulation. Only examples in which this is known to be the case are described in the following sections. The examples are primarily drawn from studies of cell growth and development where biological control must be exerted precisely. In many cases, a molecular mechanism for the negative regulation is known. In some of these examples, similar molecular mechanisms are used to effect negative regulation of the positive signaling system in different processes. In other examples, very different mechanisms are used. These processes and their modes of negative regulation provide a framework to think about negative regulation of vulval development by the synMuv genes.

The mating pheromone signal transduction pathway acts to inhibit cell proliferation in *Saccharomyces cerevisiae*

Antagonistic signals act to control cell proliferation in the yeast *Saccharomyces cerevisiae*. Nutrients serve as a positive signal for cell growth. Mating pheromone serves as a negative signal, preventing cell division and allowing the cell to mate. Mating pheromone exerts its negative influence through the inhibition of the cell cycle (Table 1).

Cell division and growth in *S. cerevisiae* are regulated by nutrient avalability and by the presence or absence of mating factor for haploid cells. The availability of sufficient nutrients acts as a positive signal to begin cell division. This is assessed at "Start," the major control point of the yeast cell cycle which occurs at the end of G1. If the cell passes Start, it is committed to divide. This entails several processes which can be defined mutationally. The gene *CDC28* has been found to perform an essential role at Start and is required for all the different processes involved in cell division (Hartwell et al., 1974; Reed, 1980; Hartwell and Pringle, 1981).

In addition to *CDC28*, several cyclin homologues have been isolated. These cyclin genes, *CLN1*, *CLN2* and *CLN3* were identified and shown by loss-of-function and gain-of-function mutations to control cell proliferation (Hadwiger et al., 1989). Other dominant mutations define a third cyclin homologue, *CLN3* (*DAF1/WHI1*). *CLN3* apparently acts as an activator of Start (Cross, 1988; Nash et al., 1988). These three cyclins are redundant (Richardson et al., 1989). As expected for cyclins, *CLN1* and *CLN2* mRNAs oscillate throughout the cell cycle as do the proteins they encode. *CLN3*, however, does not oscillate throughout the cell cycle (Nash et al., 1988; Cross, 1990;

Wittenberg et al., 1990; Tyers et al., 1993). Immunoprecipitation experiments have shown that these cyclins can associate with Cdc28; these complexes have kinase activity (Wittenberg et al., 1990; Tyers et al., 1992; 1993). Tyers et al. (1993) have proposed that *CLN3* acts as an upstream positive regulator of the other G1 cyclins. Both *CDC28* and the *CLN* genes are necessary for the accumulation of *CLN1* and *CLN2* mRNAs in a positive feedback loop (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991).

Mating pheromone acts as a negative signal; cell division is arrested upon exposure of the haploid cells to mating pheromone. The effect of mating pheromone is mediated by a distinct signal transduction system within the responding cell (Marsh et al., 1991). Mating pheromone is one of the signals that is integrated by the cell at Start and has been thought to interact with components of the cell cycle machinery (Hartwell et al., 1974; Cross, 1988; Nash et al., 1988; Richardson et al., 1989; Wittenberg et al., 1990; Marsh et al., 1991). This negative signaling system is activated by a-factor in α cells and α-factor in a cells. In each case, a specific (a or α) seven transmembrane domain receptor binds the appropriate peptide mating factor. A heterotrimeric G protein transduces this signal (Marsh et al., 1991). Downstream of the G protein, the components of a MAP kinase signaling cascade have been identified (Elion et al., 1990; Marsh et al., 1991; Cairns et al., 1992; Gartner et al., 1992; Stevenson et al., 1992; Errede et al., 1993). Deletion of *FUS3*, a MAP kinase homologue, causes a failure to arrest in G1. This disruption of cell division control can be suppressed by a *cln3* mutation, suggesting that *FUS3* regulates *CLN3* (Elion et al., 1990). In addition to negatively regulating *CLN1* and *CLN2* through its negative regulation of *CLN3*, *FUS3* downregulates *CLN1* and *CLN2* transcript levels independently of *CLN3*. Thus *FUS3* acts to cause cell cycle arrest in G1 by negatively regulating all three of the G1 cyclins (Elion et al., 1991). However, it is not the direct action of *FUS3* that is responsible for the negative regulation of the yeast cell cycle.

FUS3 does not seem to act directly on the cyclins but rather through another gene, *FAR1*. *far1* mutants have been isolated in screens for mutants that grow in the presence of α-factor and transcribe *lacZ* from a *FUS1* promotor (Chang and Herskowitz, 1990) and in a multicopy suppressor screen of *fus3* mutants (Elion et al., 1993). *far1* null mutants fail to arrest in response to mating pheromone; although they show some physiological changes reminescent of mating, they fail to mate and continue to divide (Chang and Herskowitz, 1990). *FAR1* encodes a novel 780 amino acid protein. Its expression is elevated four- to five-fold in the presence of pheromone (Chang and Herskowitz, 1990). *cln2* loss-of-function mutants suppress the *far1* defect, but not completely. Together *cln2* and *cln3* mutations suppress the *far1* defect, allowing arrest at physiological α-factor

concentrations (Chang and Herskowitz, 1990). *FAR1* has been shown to be necessary for the negative regulation of *CLN1* and *CLN2* transcript levels in response to α-factor (Valivieso et al., 1993). However, the effect of *FAR1* is not mediated solely through the downregulation of message levels; it is also mediated post-transcriptionally. Both Cln1 and Cln2 protein levels are regulated by *FAR1* independently of its effect on transcription (Valdivieso et al., 1993).

As alluded to in the previous paragraph, *FUS3* is necessary for phosphorylation of *FAR1* which, in turn, is necessary for *FAR1* activity. *FAR1* is phosporylated in response to α-factor. Thus, *FAR1* is controlled by the mating pheromone signal transduction pathway (Chang and Herskowitz, 1992). *FAR1* is not phosphorylated in deletion mutants of pheromone signal transduction components. Fus3 immunoprecipitated from cells exposed to α pheromone can phosphorylate the N terminal domain of Far1 *in vitro*, whereas Fus3 isolated from untreated cells cannot (Elion et al., 1993; Errede et al., 1993; Peter and Herskowitz, 1993). Thus, *FAR1* is positively regulated by the MAP kinase cascade controlled by mating pheromone.

Far1 has also been shown to form a complex with Cdc28 and each of the cyclins, Cln1, Cln2 and Cln3. These associations, shown by coimmunoprecipitation experiments, are α-factor dependent and require the phosphorylation of Far1 by Fus3. It is noteworthy that the ability of truncations of the Far1 protein to bind the Cdc28-Cln2 complex correlate with the ability of these truncated products to mediate G1 arrest in response to α pheromone stimulation (Peter et al., 1993; Tyers and Futcher, 1993). Peter and Herskowitz (1994) argue that Far1 binding to the Cln-Cdc28 complex is necessary for inhibition. In α-factor treated cells, immunoprecipitated complexes have reduced histone H1 kinase ability compared to untreated, dividing cells. This reduction of kinase activity is dependent on *FAR1* activity as shown by the observation that complexes isolated from *far1* cells treated with α-factor have levels of histone H1 kinase activity similar to those observed in untreated cells. An inducible constitutively active form of Far1 stimulated the kinase activity of Cdc28-Cln1 and Cdc28-Cln2 complexes in a dose-dependent manner whereas a weakly binding Far1 mutant protein could inhibit kinase activity only at very high doses. These results suggest that the binding of Far1 to the Cdc28-Cln complexes is necessary to inhibit the activity of these complexes and thereby prevent cell division (Peter and Herskowitz, 1994). Thus, the negative regulation of cell division in yeast by mating pheromone is mediated through a cyclin-kinase inhibitor protein activated by the pheromone signal transduction cascade (Figure 1).

A notable characteristic of mating pheromone inhibition of cell growth is that the neagtive signal is received and propagated via its own signal transduction system. This

nagativeregulatory pathway of yeast cell division consists of a complete signal transduction apparatus. The mechanism of inhibition is also noteworthy. A protein is activated by the negative signaling system which directly binds and inhibits a complex essential for cell cycling. Thus, inhibition occurs directly on the molecules necessary for cell growth, the cell cycle machinery, the same molecules stimulated by the positive signaling system.

TGF-β acts to inhibit mitogen-stimulated cell proliferation in some types of mammalian cells

Antagonistic signals also control proliferation of mammalian cells. Various mitogens, the positive signal, stimulate cell division. TGF-β, in many cases, acts as an anti-mitogenic signal. TGF-β, like yeast mating pheromone, exerts its effects on the cell cycle machinery (Table 1).

Mammalian cells are subject to regulation of proliferation in the G1 phase of the cell cycle. Mitogens are activators of the cell cycle; they promote cell proliferation. These growth signals are transduced into the cell where they act to positively regulate the components of the cell cycle machinery to cause cycling. Such mitogens include epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin, and insulin-like growth factor I (IGF-I). Often, several mitogens are required at different subphases of the G1 phase to activate the cell cycle (Pardee, 1989; Aaronson, 1991).

Transforming growth factor-β (TGF-β) has been shown to inhibit the proliferation of a wide variety of mammalian cell types. Picomolar quantities have been shown to inhibit the proliferation of many melanoma, lung carcinoma and breast carinoma cell lines. Often TGF-β acts antagonistically to EGF, which has a proliferative effect. However, it is notable that in the same cell line, under different growth conditions, TGB-β can act as an antagonist of EGF for proliferation (anchorage-independent conditions) or act synergistically with EGF or PDGF to promote proliferation (anchorage-dependent conditions) (Roberts et al., 1995). TGF-β has been shown to inhibit growth and DNA synthesis in non-transformed cells in a dose-dependent manner(Masui et al., 1986). The inhibitory effect of TGF-β has also been shown *in situ*. Ethylene vinyl acetate copolymer implants which allow the slow release of TGF-β can completely inhibit the growth of normal mammary gland tissue when implanted in the mammary end buds of pre-adult mice. Removal of the implants restores growth (Silberstein and Daniel, 1987). This indicates that TGF-β can play a role in the inhibition of normal developmental growth as well as in tumorigenic growth. TGF-β signaling is mediated through a heterodimeric

receptor complex (Carcamo et al., 1994, Wrana et al., 1994). Thus, it, like yeast mating pheromone, has its own signal transduction system.

Evidence suggests that cyclins and cyclin-dependent protein kinases (cdks), which are required for progress through the cell cycle and tumor proliferation, are targets for the TGF-β-mediated signal transduction system. It is the cyclins and Cdks that specifically act in the G1 phase of the cell cycle to control progress into the S phase that are subject to negative regulation. These are the cyclin E-Cdk2 complex and the Cyclin D-Cdk4 complex. These complexes are active when their components are associated and display a histone H1 kinase activity when purified from cycling cells (Pardee, 1989; Sherr, 1993; Matushime et al. 1994). The cyclin D-Cdk4 and cyclin E-Cdk2 complexes regulate the retinoblastoma (Rb) protein which acts at the restriction point of G1 to regulate the cell's decision to divide or remain quiescent. The cyclin D-Cdk4,6 and cyclin E-Cdk2 complexes are responsible for the hyperphosphorylation of Rb seen in proliferating cells (Sherr, 1993; Weinberg, 1995). The addition of TGF-β to cycling cells inhibits Rb activity and prevents its hyperphosphorylation (Laiho et al., 1990). Thus, TGF-β acts by inhibiting the activation of the cell cycle at the cyclin-cdk level by means of cyclin kinase dependent inhibitory proteins.

Studies in Mv1Lu cells, human keratinocyte cell lines, and non-immortal cultured breast epithelial cells have revealed that cyclin-Cdk complexes are a target of TGF-β inhibition (Ewen et al., 1993; Geng and Weinberg, 1993; Koff et al., 1993; Slingerland et al., 1994). Cyclin E mRNA levels as well as Cdk2 and Cdk4, but not cyclin D1, D2, or D3, mRNA levels are reduced in response to TGF-β (Ewen et al., 1993; Geng and Weinberg, 1993; Slingerland et al., 1994). Mv1Lu cells that continuously express Cdk4, but not cells that continuously express Cdk2, can bypass the TGF-β induced arrest. However, entry into S phase is still somewhat delayed, suggesting that TGF-β inhibition relies on mechanisms other than just control of the transcription of genes encoding the cyclins and Cdks (Ewen et al., 1993).

Cyclin E and Cdk2 associate in a complex as shown by co-immunoprecipitation from Mv1Lu cells. As these cells pass through the G1 phase, Cdk2 protein is increasingly phosphorylated. This phosphorylation is associated with cyclin E-Cdk2 histone H1 kinase activity. Addition of TGF-β prevents the accumulation of the phosphorylated form of Cdk2, the formation of cyclin E-Cdk2 complexes and cyclin E associated kinase activity. No reduction in protein levels of cyclin E or Cdk2 were observed (Ewen et al., 1993; Koff et al., 1993; Slingerland et al., 1994).

In the G1 phase, the cyclin D-Cdk and cyclin E-Cdk complexes regulate the decision whether to divide or not. These complexes are regulated by a group of

molecules known as the cdk inhibitors. These molecules are the likely means by which TGF-β exerts its negative influence on cell proliferation.

The cdk inhibitory protein 1 (CIP1) which encodes a novel 21 kDa protein with a cysteine-rich region and a nuclear localization sequencewas identified as a molecule that interacts with Cdk2 and cyclin D (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al, 1993). Overexpression of the *cip1* gene suppresses growth of several cell lines (El-Deiry et al., 1993; Xiong et al., 1993). p21 has been shown to directly associate with various cyclin-Cdk complexes *in vitro*, including cyclin D-Cdk4 and cyclin E-Cdk2 complexes. The association of p21 with these complexes inhibits their histone H1 and retinoblastoma (Rb) kinase activity (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). Addition of TGF-β to the human keratinocyte cell line HaCaT leads to the rapid induction of *cip1* transcription independent of p53. Correlated with transcript increase is the association of p21 with Cdk2 and cyclin D as shown by immunoprecipitation experiments (Datto et al., 1995). These data suggest that p21 is a promiscuous cyclin-Cdk inhibitor that acts in response to TGF-β.

The cdk inhibitory protein 1 (Kip1) was identified as a 27 kDa protein, similar to CIP1, which associates with the cyclin E-Cdk2 and the cyclin D-Cdk4 complex (Polyak et al., 1994a; 1994b, Toyoshima and Hunter, 1994). $p27^{Kip1}$ can bind cyclins D1, D2, D3, and Cdk4 strongly, and cyclin E and Cdk2 less strongly (Polyak et al, 1994a, Toyoshima and Hunter, 1994). In *in vitro* assays, p27 can inhibit the histone H1 kinase and Rb kinase activities of cyclin E-Cdk2 and cyclin D-Cdk4 (Polyak et al., 1994a; 1994b; Toyoshima and Hunter, 1994). Overexpression of $p27^{Kip1}$ leads to cellular arrest in the G1 phase. However, mRNA levels of Kip1 are equivalent in both arrested and proliferating cells, suggesting that p27 acts post-transcriptionally to inhibit cell proliferation (Polyak et al., 1994b; Toyoshima and Hunter, 1994). In extracts from inhibited cells, addition of cyclin D2-Cdk4 complexes, including ones with an inactive Cdk4, restore the activity of cyclin E-Cdk2 complexes. This may indicate a role for cyclin D2-Cdk4 in regulating p27-induced inhibition of cyclin E-Cdk2 (Polyak et al., 1994a).

Another class of inhibitory molecule has been found which associates with Cdk4. Serrano et al. (1993) isolated a gene which encodes a 16 kDa inhibitor of cdk4 (Ink4). This novel protein immunoprecipitates with Cdk4 in a 1:1 molar ratio, but does not associate with Cdk2. The addition of p16 to cyclinD-Cdk4 inhibits complex phosphorylation of Rb. However, p16 does not inhibit cyclinD-Cdk2 phosphorylation of Rb (Serrano et al. 1993). Injection of p16 mRNA into normal breast epithelial cells and fibroblasts leads to growth arrest. Injection into normal fibroblasts from Rb^{-}/Rb^{-} mice

did not produce any effect, suggesting that p16 acts to promote cell cycle arrest through an Rb-dependent mechanism (Lukas et al., 1995b; Medema et al., 1995). Hannon and Beach (1994) observed that the immunoprecipitation of Cdk4 and closely related Cdk6 from TGF-β-arrested keratinocytes pulls down a 15 kDa protein as well as p16. The $p15^{Ink4b}$ protein shows similarity to $p16^{Ink4a}$. *In vitro* binding studies reveal an association with Cdk4 and Cdk6 but not with Cdc2, Cdk2 or Cdk5. p15, like p16, can inhibit the kinase activity of cyclin D-Cdk4 and cyclin D-Cdk6 complexes. Addition of TGF-β to HaCaT (a human keratinocyte derived cell line) leads to a large increase in *ink4b* mRNA levels as well as an increase in Cdk4 and Cdk6 association with p15. This occurs in the absence of a reduction in Cdk4 protein and mRNA levels (Hannon and Beach, 1994).

Reynisdottir et al. (1995) have proposed a model in which the concerted action of Kip1/Cip1 proteins and Ink4 proteins mediates the TGF-β-induced arrest of cell proliferation. They show that for both Mv1Lu cells and HaCaT cells there is a complete loss of Cdk2 activity shortly after treatment with TGF-β. This treatment also leads to the rapid loss of p27-Cdk4 and p27-Cdk6 association. In Mv1Lu cells, this corresponds to an increase of Cdk2-bound p27. A rapid increase in p15 mRNA levels is also observed in both Mv1Lu and HaCaT cells. A rapid induction of *cip1* mRNA, independent of p53 activity, is seen in HaCaT cells and is correlated with a rise in p21-Cdk2 association as well as Cdk activity inhibition. No increase in p27-Cdk2 complexes is observed in these cells (Reynisdottir et al., 1995). Reynisdottir et al. (1995) propose that in Mv1Lu cells TGF-β treatment increases transcription of $p15^{Ink4b}$ which, when transcribed, binds Cdk4. This binding displaces cyclin D from Cdk4 thereby rendering the cyclin D-Cdk4 complex inactive and liberating p27 from the now defunct complex The liberated p27 binds to the cyclin E-Cdk2 complex, inactivating it. The process is similar in HaCaT cells, except that the displaced p27 does not bind cyclin E-Cdk2. Instead, a rapid rise in *cip1* mRNA levels leads to the association of p21 with cyclin E-Cdk2, inactivating the complex. Thus, TGF-β-induced cell proliferation arrest is strongly correlated with the inhibition of the activities of various classes of cdk inhibitors (Figure 2).

As expected for the components of a signaling pathway that acts negatively on mammalian cell proliferation, mutations in these components have been observed in various tumors. These, again as expected, tend to be of the tumor suppressor variety. Loss of TGF-β expression has been observed in mouse skin carcinomas (Glick et al., 1993). Lack of, or defects in, the TGF-β receptors have been noted in gastric cancer cell lines, lymphomas, and certain colon cancer cell lines (Park et al., 1994; Kadin et al., 1994; Markowitz et al., 1994). Several melanomas have been found to carry a mutant

version of Cdk4. These mutations involve a change in arginine 24 such that the mutant Cdk4 protein cannot form stable complexes with $p16^{Ink4a}$ or $p15^{Ink4b}$ nor can these cyclin-dependent kinase inhibitors inhibit the mutant Cdk4 protein. This effect is specific; mutant Cdk4 protein can form active complexes with cyclin D1 and can be inhibited by p21 and p27 (Wolfel et al., 1995). A number of melanomas, breast tumors, leukemias, and non-small cell lung tumors have been shown to carry deletions in human chromosomal region 9p21, the region in which the genes encoding the Ink4 proteins p15 and p16 are located. Melanoma cell lines often carry point mutations in the $p16^{Ink4a}$-encoding gene (Hannon and Beach, 1994; Kamb et al., 1994; Nobori et al., 1994). Rb mutations are, of course, common in several tumor types. These include retinoblastomas, small lung cell carinoma cell lines, bladder carcinoma, and breast carcinoma cell lines (Horowitz et al., 1990; Weinberg, 1990).

TGF-β-mediated inhibition involves a negative signal transduction cascade like yeast mating pheromone and acts on the cell cycle machinery as does yeast mating pheromone signaling. The mechanism of inhibition is similar as well: inhibitory factors bind cyclin-kinase complexes. The activation of p27 inhibitor by TGF-β is post-transcriptional, as is the activation of Far1 by yeast mating pheromone. However, the activation of the p21 and p15 inhibitors requires the transcription of the genes encoding these proteins. Thus TGF-β inhibition requires the transduction of the negative signal to the nucleus of the responding cell to activate the transcription of molecules that inhibit the biologuical process. This provides an example of another variation of negative signaling that may serve as a mechanism for the action of the synMuv genes.

Antagonistic signaling systems are involved in *Xenopus* dorso-ventral pattern formation

Antagonistic signals act to specify dorso-ventral polarity in *Xenopus laevis*. BMP-4 acts as a ventralizing factor and has arbitrarily been designated the "positive signal." Chordin and Noggin act as dorsalizing factors and have been designated "negative signals." These negative signals act directly on the positive signal without requiring signal transduction cascades to mediate their effect (Table 1).

Bone morpogenetic protein-4 (BMP-4), a secreted member of the TGF-β family was found in searches for known activin related molecules involved in *Xenopus* development (Koster et al., 1991, Dale et al., 1992). BMP-4 was found to be expressed in the gastrula; expression progressively extends through the ventral marginal zone to include all of the equatorial region with the exception of the dorsal marinal zone: an expression pattern complementary to that of *goosecoid*, a dorsal marker. Later, the BMP-

4 transcripts are excluded from the presumptive neural plate ectoderm, and finally are found only at the ventral side of the slit blastopore and in a ventral anterior patch (Cho et al., 1991; Fainsod et al., 1994; Schmidt et al., 1995b).

BMP-4 can act as a ventralizing factor. Injection of BMP-4 RNA can ventralize both embryos and explanted tissue (Koster et al., 1991; Dale et al., 1992; Jones et al., 1992; Schmidt et al., 1995b). Thus the addition of ventralizing BMP-4 signal can override the endogenous dorsalizing activity. Ectopic expression of a dominant negative BMP-4 receptor construct dorsalized *Xenopus* embryos (Graff et al., 1994; Suzuki et al., 1994; Schmidt et al., 1995b). Antisense BMP-4 RNA can also dorsalize embryos (Steinbiesser et al., 1995) as can dominant negative BMP-4 ligands that cannot be cleaved to form an active dimer (Hawley et al., 1995). BMP-4 can alter the expression of genes involved in patterning and positively autoregulate itself (Fainsod et al., 1994; Jones et al., 1992; Schmidt et al., 1995b). BMP-4 can block the neuralization of dissociated animal cap cells *in vitro* (Wilson and Hemmati-Brivanlou, 1995). Likewise, dominant negative BMP-4 ligand expression induces neural tissue (Hawley et al., 1995). Thus BMP-4 has been implicated in the ventralization of embryonic mesoderm and the epidermalization of the ectoderm. It plays an active role as a positive signal. These results are further supported by observations that in mice in which BMP-4 has been knocked out, defects occur in the formation of posterior and ventral mesoderm (Hogan et al., 1994).

noggin was isolated as a clone with dorsalizing activity from LiCl-treated dorsalized *Xenopus* gastrulae (Smith and Harland, 1992). *noggin* encodes a novel protein that is secreted and acts as a dimer (Smith and Harland, 1992; Smith et al., 1993). Dorsalizing activity has been demonstrated in both uv-treated ventralized embryos and normal embryos. Zygotic transcription of *noggin* occurs in the dorsal lip region of the Spemann organizer, a region responsible for neural induction and capable of dorsalizing ventral mesoderm. *noggin*-conditioned media can dorsalize gastrula stage ventral marginal zone mesoderm in explant. This activity is predicted of a mesoderm dorsalizing agent from the Spemann organizer (Smith and Harland, 1992; Smith et al., 1993). *noggin* also fulfills another function of the Spemann organizer, neural induction. Purified Noggin protein can induce neural markers in blastula to mid-gastrula stage animal cap ectoderm explants cultured *in vitro*. It can induce neural tissue without first inducing mesodermal tissue, implying that it directly, rather than indirectly through the agency of mesoderm, induces neural tissue (Lamb et al., 1993). These results are all consistent with a role for *noggin* as a dorsalizing agent for mesoderm and a neural inducer from the Spemann organizer in *Xenopus*.

BMP-4 and *noggin* act antagonistically to pattern the mesoderm. Re'em-Kalma et al. (1995) have proposed a model that involves a competition between BMP-4 and *noggin* to regulate the dorsalization of marginal zone mesoderm. Noggin emanating from the Spemann organizer acts to turn off or inhibit BMP-4 in nearby marginal zone mesoderm allowing dorsalization. BMP-4 is present in more ventral domains in high enough concentration to inhibit the expansion of the dorsalization process while allowing ventral mesoderm formation. The authors provide several lines of evidence to support this model. BMP-4 RNA injections induce an increase of ventral mesodermal markers in animal caps and reduce the ability of treated Spemann organizer to induce muscle from ventral marginal mesoderm. BMP-4 can also inhibit *noggin*-mediated dorsalization of mesoderm in a concentration-dependent manner (Re'em-Kalma et al.,1995). All of these data point to a BMP-4-Noggin antagonistic interaction

The work of Zimmerman et al. (1996) elucidates the molecular mechanism of the *noggin*-BMP-4 antagonism. They show that noggin can block BMP-4 induced alkaline phosphatase activity of a murine bone marrow stromal cell line in a 1:1 molar ratio. They show that Noggin specifically binds BMP-4 directly with high affinity (higher than the affinity of BMP-4 to its own receptor). Noggin-BMP-4 complexes cannot bind BMP-4 receptor types I or II. Similarly, antibodies that block Noggin activity also block Noggin-BMP-4 binding. From this evidence, the authors propose that Noggin mediates the Spemann organizer function by blocking ventralizing BMP-4 signaling by directly binding and titrating out BMP-4 (Zimmerman et al., 1996) (Figure 3).

In vivo evidence from a heterologous system complements the biochemical evidence for a direct negative regulation of BMP-4 by *noggin*. Injection of *noggin* mRNA can block dorsal fates and promote ventral fates in a dose-dependent manner in dorsalized *Drosophila* embryos just as injected BMP-4 mRNA can dorsalize lateralized embryos (Padgett et al., 1993; Holley et al., 1996). Note that the dorsal and ventral axes are reversed in arthropods relative to mammals. Despite the reversal of axis orientation, the mechanisms and characteristics of the dorso-ventral pattern are similar (DeRobertis and Sasai, 1996; Holley et al., 1996). Coinjection experiments of *noggin* mRNA with *decapentaplegic* (a *Drosophila* TGF-β-like dorsalizing agent, see below), BMP-4, activated *thick veins* (type I receptor) or wild-type *punt* (type II receptor) showed that *noggin* can block the dorsalizing activity of *decapentaplegic* or BMP-4 ligands but not the activities of the injected receptor RNA. This suggests that *noggin* acts upstream of the receptor (Holley et al., 1996).

Thus *noggin* acts by binding TGF-β-like molecules and thereby preventing the binding of these TGF-β-like molecules to their receptors. Another gene, *chordin*,

behaves similarly. The role of these other molecules suggests that the dorso-ventral patterning system may be highly redundant or specific.

Chordin, another molecule implicated in the action of the Spemann organizer, acts as an antagonistic signal. *chordin* (*chd*) was identified as a clone enriched in dorsalized *Xenopus* embryos in a differential cDNA screen. It encodes a novel secreted protein (Sasai et al., 1994; Piccolo et al., 1996). *chordin* is expressed in the Spemann organizer of the dorsal marginal zone just before and at early gastrula stages (Sasai et al., 1994) and in the mesodermal mantle which lies under the presumptive neural plate at the gastrula stage (Sasai et al., 1995). The expression of *chd* and the expression of *noggin* in the dorsal lip is probably regulated differently (Cho et al., 1991; Sasai et al., 1994; Taira et al., 1994).

Chordin is capable of dorsalizing mesoderm. *chd* mRNA can dorsalize embryos and explanted ventralized mesoderm but cannot induce mesoderm (Sasai et al., 1994). In explant experiments, *chd* mRNA can induce neural tissue in the absences of mesodermal tissue (Sasai et al., 1995). Thus, Chordin dorsalizes, but does not induce, mesoderm and induces neural tissue.

BMP-4 is capable of antagonizing *chd*-induced dorsalization of mesoderm when the two mRNAs are injected into different cells suggesting that this antagonism is cell-nonautonomous. BMP-4 also acts to eliminate *chd*-mediated neuralization of ectoderm, but cannot act to eliminate the neuralization caused by injection of mRNA encoding a dominant negative BMP-4 receptor. A similar antagonism between BMP-4 and Chd is seen in *in vitro* explant experiments (Sasai et al., 1995; Piccolo et al., 1996). Biochemical work has shown that *chd* can inhibit the alkaline phosphatase activity of the 10T1/2 cell line induced by BMP-4 but not that induced by retinoic acid. A direct physical interaction is suggested by observations that Chordin is capable of competing BMP-4 away from its receptors and of immunoprecipitating BMP-4. Chd appears to bind BMP-4 in a 1:1 Chd to BMP-4 dimer ratio with high affinity (Piccolo et al., 1996) (Figure 3).

noggin and *chordin* play similar, and probably somewhat redundant, roles in mesoderm dorsalization and neural induction in *Xenopus*. The molecules are unrelated and differ greatly in size; *noggin* is much smaller than *chordin* (Harland and Smith, 1992; Sasai et al., 1994). *noggin* expression and *chordin* expression are under different controls. *chd* is active at similar concentrations in both dorsalization and neuralization whereas noggin is a more potent dorsalizer that neuralizer. These two molecules may act synergistically in subtly different ways to antagonize BMP-4 and thus finely control dorsalization and neuralization in the developing *Xenopus* embryo.

Anti-dorsalizing morphogenetic protein (ADMP), another TGF-β family member, has been identified in *Xenopus* embryos. ADMP is expressed in the Spemann organizer and later forms a gradient of expression through the neuroectoderm extending anteriorly. The expression of this gene is reduced in uv-irradiated ventralized embryos and increased in LiCl-treated dorsalized embryos as expected for a gene expressed in the organizer. Paradoxically, the injection of ADMP mRNA into dorsal blastomeres inhibits axis formation, most particularly the formation of the most anterio-dorsal structures (Moos et al., 1995). This result is similar to that seen in experiments in which BMP-4 mRNA is injected dorsally resulting in the decrease of *noggin* and *goosecoid* expression (Fainsod et al., 1994). Moos et al. (1995) argue that ADMP serves to modulate the dorsalizing activity of the Spemann organizer. Presumably, ADMP acts through the same mechanism as BMP-4 and seems to more finely regulate the distribution of active dorsalizing molecules since it acts at the source of these dorsalizing molecules. ADMP may act as a negative regulator of the negative (dorsalizing) signal.

The antagonisticinteractions involved in *Xenopus* dorso-ventral polarity specification are noteworthy in two particiulars. The positive and negative signals are mutually antagonistic; either can override the other depending on relative dose. This is likely a necessary function of polarity specification in which gradients define polarity. Such gradients are discussed in the next section. The other notable characteristic is the mechanism. The negative signal titrates out the positive signal. The inhibition of the positive signal by the negative signal occurs upstream of the positive signal transduction apparatus. The negative signal has no signal transduction system to mediate its antagonistic effect; it acts directly on the positive signal itself. This may be a possible mechanism of action for the class A synMuv genes for which there is no evidence for cell autonomous action.

Antagonistic signals act in the establishment of dorso-ventral polarity in *Drosophila* embryos

The determination of dorso-ventral polarity in the *Drosophila melanogaster* embryo is similar to that in *Xenopus*, only the axes are reversed. The work in *Drosophila* more clearly illustrates the regulation of activity gradients in antagonistic systems. The general mechanism is the same, and the molecules involved are homologues (Table1).

In *Drosophila*, *decapentaplegic* (*dpp*) null mutations produce a ventralized embryo. *dpp* acts strictly zygotically and the null allele is haploinsufficient for lethality (Irish and Gelbart, 1987). *dpp* encodes a member of the TGF-β peptide growth factor family (Padgett et al., 1987) that is closely related to BMP-4 (Gelbart, 1989). Expression

studies using probe from the two-exon haploinsufficient region that is present in all *dpp* transcripts show embryonic expression just after nuclear division 11, reaching its peak at the early cellular blastoderm stage. This initial expression is seen in the dorsal-most 40 % of the embryo (St. Johnston and Gelbart, 1987). The early dorsal expression is eliminated in embryos from mutant mothers carrying the maternal ventralizing alleles of *Toll* (*Tl*), *easter*, and *cactus*. In dorsalized embryos produced by *dorsal* (*dl*) mothers, *dpp* expression is expanded throughout the entire dorso-ventral axis of the embryo (Ray et al., 1991).

The *dpp* ventralized phenotype is incompletely epistatic to the maternal dorsalized phenotype produced by dorsalizing alleles of *Tl*, *dl* and *pelle* (*pll*). Double mutants (homozygous *dpp* animals derived from mothers homozygous for the maternal effect mutation) with *dl* and *pll* were lateralized whereas double mutants with the weaker *Tl* allele were ventralized (Irish and Gelbart, 1987). Zygotic ventralizing mutations, however, do not affect the initial *dpp* espression pattern (Ray et al., 1991). These results suggest that *dpp* expression is regulated by the maternal localization gradient of *dl* (Roth et al, 1989; Ray et al., 1991). The observation that *dpp* mutations produce the strongest ventralized phenotype of any of the zygotic ventralizing mutations, and the observation that these other mutations cannot enhance the *dpp* phenotype, suggest that *dpp* is the primary determinant of dorsal pattern in the *Drosophila* embryo (Irish and Gelbart, 1987; Ray et al., 1991; Arora and Nusslein-Volhard, 1992).

dpp acts in a graded fashion to specify dorso-ventral polarity. *dpp* loss-of-function and reduction-of-function mutations form a single allelic series in which the extent of polarity defects correlates with the extent of embryonic lethality. The dorsal-most structures are lost in mutants carrying the weakest alleles whereas defects in increasingly ventral tissues are seen in strains carrying stronger alleles. This suggests that the *dpp* gradient specifies amnioserosa at its highest activity level, and dorsal epidermis and ventral epidermis at decreasing activity levels (Arora and Nusslein-Volhard, 1992; Wharton et al., 1993). Dosage studies can be conducted with duplications carrying dpp^+. Increasing the dpp^+ dose in wild type increases the extent of amnioserosa, the dorsal-most tissue (Wharton et al., 1993). Increasing the dose of dpp^+ in animals carrying mutations in the zygotically acting ventralizing genes can bypass the ventralizing effect of these mutations (Ferguson and Anderson, 1992b). However, these effects are seen only in the dorsal and lateral regions of the embryo. In the absence of ventralizing activity in *dl* embryos, increased dpp^+ dose can lead to the expansion of the amnioserosa to encompass the entire circumference of the embryo (Wharton et al., 1993).

In addition to these genetic data, RNA microinjection data support the contention that *dpp* acts in a graded fashion to specify dorsal fates. Ferguson and Anderson (1992a) report that small amounts of *dpp*$^+$ RNA injected into the ventral side of a syncytial embryo can expand the dorsal domain and that large amounts can lead to the formation of amnioserosa along the entire dorso-ventral axis except the ventral mesodermal region. Injection of *dpp*$^+$ RNA into lateralized embryos, which do not express zygotic dorso-ventral genes, from *snake* (*snk*) *Tl* mothers show that *dpp*$^+$ RNA can cause a dose-sensitive dorsalization of the embryo. *dpp*$^+$ RNA can organize elements of the dorso-ventral pattern at the site of the injection, pattern the dorsoventral axis from amnioserosa to ventral epidermis, and allow germband extension to occur (Ferguson and Anderson, 1992a). Since *dpp* is expressed at uniform concentrations in the dorsal-most 40 % of the embryo (St. Johnston and Gelbart, 1987), it is likely that the *dpp* gradient is post-transcriptional; that is, based on its activity rather than expression (Ferguson and Anderson, 1992a; Wharton et al., 1993).

Several gene products seem to enhance the activity of *dpp*. *screw* (*scw*) and *tolloid* (*tld*) mutations lead to ventralization of *Drosophila* embryos but do not result in as severe a phenotype as do *dpp* null mutations (Ray et al., 1991; Arora and Nusslein-Volhard, 1992). Allelic series of *scw* and *tld* mutations show increasing loss of amnioserosa with increasing allele strength (Arora and Nusselein-Volhard, 1992; Ferguson and Anderson, 1992; Arora et al., 1994). Thus, allelic series of these genes are similar to the *dpp* allelic series, though not as severe. *scw* and *tld* mutations enhance each other but even together do not produce a phenotype as strong as that produced by strong *dpp* alleles (Arora and Nusslein-Volhard, 1992). Increased doses of *dpp*$^+$ can at least partially bypass the requirement for *scw* and *tld* (Ferguson and Anderson, 1992b; Arora et al., 1994).

scw is expressed along the entire dorsoventral axis at the same level just before gastrulation, but expression of *scw*$^+$ under the control of a *tld* promotor which expresses only in the dorsal region is sufficient to rescue the *scw* phenotype. *scw* has been cloned and shown to encode a TGF-β homologue and has been hypothesized to act as a heterodimer with Dpp to control the formation of the dorsal-most amnioserosa cells (Arora et al., 1994). *tld*, like *dpp*, has antimorphic alleles that are stronger than deficiencies. Elevated *dpp*$^+$ expression can partially bypass *tld* but elevated *tld*$^+$ activity cannot bypass *dpp*, suggesting that *tld* acts to elevate the level of *dpp* activity (Ferguson and Anderson, 1992b). *tld* is expressed at peak levels during early cellularization stages in the dorsal-most 50 % of the embryo, overlapping the *dpp* expression domain (Shimell et al., 1991). *tld* encodes a protein similar to bone morphogenetic protein-1 (BMP-1), a

protease that copurifies with the TGF-β-like BMPs and has bone morphogenetic activity (Wozney et al., 1988; Shimell et al., 1991). It has been postulated that the binding ability and protease activity of *tld* act to enhance the activity of *dpp* and *scw* (Shimell et al., 1991; Arora et al., 1994; Childs and O'Connor, 1994). Thus the *dpp* gradient is positively modulated by other, secreted proteins.

short gastrulation (*sog*) mutants were originally characterized as having a weak ventralized phenotype similar to that of the weakest *dpp* alleles (Zusman et al., 1988; Ray et al., 1991; Wharton et al., 1993). Germline mosaic results demonstrate that *sog* acts strictly zygotically (Zusman et al., 1988). *sog* does not affect the ventral expression of *dpp* but eliminates the later refinement of the *dpp*, *tld* and *zen* expression patterns. Ventral genes are unaffected (Ray et al., 1991; Fracoise et al., 1994). A closer inspection of the phenotype of null alleles of *sog* reveals that the primary defect in *sog* mutants is the failure to subdivide the dorsal region into amnioserosa and dorsal ectoderm domains (Francoise et al., 1994). Mosaic analysis of *sog* gynandromorphs suggests that *sog* is required in the ventral region of the embryo (Zusman et al., 1988). This coincides with the expression domain of *sog* as determined by *in situ* hybridization; *sog* is expressed at early cellularization in the ventral region of the embryo. Transcripts are found in the apical regions of cells and their expression pattern is not altered in zygotic ventralization mutants. The domain of *sog* expression is directly adjacent to the domain of *dpp* expression as seen in co-staining experiments (Francoise et al., 1994).

sog acts antagonistically to the dorsalizing activity of *dpp* and *tld*. Heterozygous *sog* can suppress antimorphic alleles of *tld* and the haploinsufficiency of strong *dpp* alleles (Ferguson and Anderson, 1992b; Francoise et al., 1994). Duplications which increase the dose of dpp^+ in *sog* mutants enhance the *sog* phenotype. The neurogenic pattern is altered and fates shift to more ventral positions; i.e. the embryo becomes more dorsalized. Thus, *sog* appears to inhibit *dpp* activity in the neurogenic ectoderm and in the ventral domain (Ferguson and Anderson, 1992b; Biehs et al., 1996). Results from experiments involving the dorsal injection of *sog* mRNA support this conclusion. Such injections block amnioserosa formation and ventralize embryos (Holley et al., 1995).

Recent evidence suggests that *dpp* is epistatic to *sog*. *sog; dpp* double mutants show marker expression similar to that of *dpp* mutants (Biehs et al., 1996). Biehs et al. (1996) have shown that the range at which *sog* can affect the expression of dorsal markers varies directly with the dose of sog^+ activity. These results provide additional support to the evidence presented above by Zusman et al. (1988) and Francoise et al. (1994) that *sog* acts over a distance to modulate cell fate specification. These results lend

support to the model of Ferguson and Anderson (1992b), that *sog* acts in an inhibitory manner to define the *dpp* activity gradient (Figure 3).

As has been noted above, the *Drosophila* dorso-ventral patterning system has many mechanistic parallels to the *Xenopus* dorso-ventral patterning system. The general patterning mechanism in which at least one TGF-β-like molecule defines one pole and an antagonistic signaling mechanism defines the other pole with respect to the first signal is similar in both organisms. The secreted signaling molecules that act as antagonists, *chordin* and *sog*, are homologous (Francois and Bier, 1995). Many of the molecules in these two organisms have been shown to be functionally interchangable. (Padgett et al., 1993; Holley et al., 1995; Schmidt et al., 1995a; Holley et al., 1996). However, *dpp*/BMP-4 acts in ventral specification in *Xenopus* and dorsal specification in *Drosophila* whereas *sog*/*chd* and *noggin* act in dorsal specification in *Xenopus* and ventral specification in *Drosophila*. Thus, the poles of these axes seem to be reversed despite the conservation of the mechanisms of specification. Several workers have proposed that these results support the contention of Etienne Geoffrey Saint-Hilaire that the dorso-ventral axis of arthropods and chordates are homologous, but inverted with respect to each other (Holley et al., 1995; DeRobertis and Sasai, 1996; Ferguson, 1996). Thus, the vertebrate and arthropod dorso-ventral patterning systems seem to be homologous. In light of the similarity between these two systems, the conclusions drawn from *Drosphila* dorso-ventral polarity can be considered to be the same as those drawn from the studies of *Xenopus* dorso-ventral polarity determination. The *Drosophila* work does serve to point out the sophisticated regulation of activity gradients possible in antagonistic signaling systems.

clr-1 acts antagonistically to *egl-15* in *C. elegans* development

Antagonistic signaling systems may directly inhibit the transduction of the positive signal within the cytoplasm of the responding cell. In the case of *egl-15* and *clr-1* in C. elegans developnent, this is not known to be the case, but is the simplest model. The EGL-15 receptor propagates the positive signal whereas the CLR-1 receptor propagates the negative signal. CLR-1 is postulated to directly inhibit EGL-15-mediated signal transduction (Table 1).

In addition to a role in sex myoblast migration, *egl-15* (egg-laying defective) mediates an essential function in *C. elegans* development. Complete loss-of-function alleles in *egl-15* lead to developmental arrest in early L1 larvae, shortly after hatching. Many Egl-15 larvae die shortly after this cessation of development (Stern and Horvitz, 1991; DeVore et al., 1995). *egl-15* encodes a fibroblast growth factor (FGF) receptor,

suggesting that it acts to transduce a signal that is necessary for continued development of the L1 larva (DeVore et al., 1995).

The *clr-1* (clear) gene has also been shown to play a role in early larval development. Mutations in *clr-1* produce a Clear phenotype that is characterized by a fluid-filled pseudocoelom. Scrawny, dumpy, uncoordinated, sterile and lethal phenotypes are also produced by mutations in the gene (Hedgecock et al., 1990; Clark et al. 1992a; 1992b; DeVore et al., 1995). *clr-1* can suppress the *egl-15* lethal phenotype and *egl-15* can suppress the *clr-1* lethal, clear and other phenotypes (Clark et al., 1992b; DeVore et al., 1995). This mutual suppression constitutes genetic evidence for an antagonistic interaction between these genes in early larval development.

clr-1 has been cloned and found to encode a receptor tyrosine phosphatase. The extracellular domain has been found to be required for rescue, suggesting that CLR-1 may respond to a signal. This has led to the proposal that CLR-1 dephosphorylates some component of the *egl-15* FGF receptor signal transduction pathway, possibly Egl-15 itself, in response to the antagonistic signal. This dephosphorylation negatively regulates the *egl-15* pathway (M. Kokel and M. Stern, personnal communication). Although the exact developmental process that these genes regulate is not well defined, it is clear that it is mediated by opposing signaling systems (Figure 4).

The model for *egl-15* and *clr-1* antagonism is noteworthy for several reasons. First, the effect of the negative signal is mediated by a short signal transduction system, the receptor itself. No further signal transduction need occur. Second, the negative signal acts by directly inhibiting the transduction of the positive signal within the responding cell. Third, the postulated biochemical mechanism is intriguing. The negative signaling system undoes the the activating phosphorylation of a molecule caused by the positive signal. Thus, this system serves as an example of direct negative regulation of the positive signal transduction system itself. The variations on this theme are enormous and well suited for a mechanism of synMuv gene activity.

Antagonistic signals act to specify cell fates in *Drosophila* ommatidia

Antagonistic signals act in the specification of ommatidial cell fates in the developing eye of *Drosophila*. The positive signal is Spitz, the ligand of the *Drosophila* EGF receptor at this time in the developing eye. It is countered by the negative signal Argos. This system provides another example of a regulatory system that acts upstream of the signal transduction system, albeit in a different manner (Table 1).

Drosophila eye development provides an excellent system for the analysis of cell signaling mechanisms. The eye is composed of a crystalline array of self-contained,

identical ommatidia. Each ommatidium is composed primarily of an outer group of pigment cells which surround a group of four cone cells, which in turn surround a cluster of eight photoreceptors and two "mystery cells." These photoreceptors differentiate in a set sequence: R8, R2 and R5, R3 and R4, R1 and R6, and finally, R7 (reviewed by Tomlinson, 1988). In particular, the determination of the R7 cell has been intensively studied. R7 determination is primarily directed by the *sevenless* (*sev*) receptor tyrosine kinase and a ligand encoded by *bride of sevenless* (*boss*). Genetic analysis has revealed that a *ras* -mediated signal transduction pathway transduces the *sev* signal (reviewed by Simon, 1994). This signal transduction pathway is required for the development of all photoreceptors, not just R7 (Simon et al., 1991; Fortini et al., 1992). The phenotypes caused by mutations in these signal transduction genes resemble those caused by *Drosophila* EGF receptor (DER) mutations in adults (Diaz-Benjumea and Hafen, 1994).

Ellipse (*Elp*) mutations provided evidence for the involvement of DER in *Drosophila* eye development. The eyes of *Elp* flies are irregular and have few ommatidia. To the extent tested, *Elp* acts as a hypermorph. Revertants of *Elp* mutations fail to complement *faint little ball* (*flb*) mutations in the *Egfr*, demonstrating that Elp mutations are alleles of *Egfr*, the gene encoding the DER. In x-ray-induced mosaic clones of recessive revertant alleles and *flb* alleles, the mutant cells did not survive. This suggests that *Egfr* plays a role in eye disc cell proliferation and ommatidial formation (Baker and Rubin, 1989). *In situ* expression data in which the *Egfr* is found uniformly expressed throughout the eye disc is consistent with this model (Schejter et al., 1986).

Viable *torpedo* (*top*) alleles of *Egfr* as homozygotes and *trans*-heterozygotes with null alleles did not affect the number or spacing of ommatidia but led to the development of eyes containing ommatidia with aberrant morphology or fewer photoreceptors than wild type. These results suggest that DER may be playing multiple roles in eye development (Baker and Rubin, 1992). Genetic mosaic analysis using the FLP/FRT system enabled Xu and Rubin (1993) to analyze the role of *Egfr* in eye development more precisely. They confirmed the role of *Egfr* in eye disc cell proliferation in early third instar larval eye discs. Clones induced posterior to the morphogenetic furrow contained cells which did not differentiate into photoreceptors as judged by basal nuclei and lack of expression of neuronal markers. This result suggested a role in photoreceptor determination in addition to the role seen in earlier cell proliferation (Xu and Rubin, 1993). These results were further extended through the use of dominant negative DER constructs under various promotors. Expression of the dominant negative receptor in cells behind the morphogenetic furrow blocked the determination of photoreceptors other than R8 and R2/5. Heat shock-controllable promotors allowed the production of

ommatidia in which the photoreceptors being recruited at the time of the heat shock are not found. Later heat shock produced the same result for cone cells and pigment cells. It was also noted that R7 determination required DER activity (Freeman, 1996).

The role of the DER in the determination of photoreceptor identity is mediated by the gene *spitz* (*spi*). *spitz* encodes a molecule with a signal sequence, a transmembrane domain, a dibasic proteolytic clavage site, and a single EGF domain. In this sense, it is similar to TGF-α, which has only a single EGF repeat (Rutledge, et al., 1992). *In vitro* experiments in Schneider S2 cells show that secreted Spi can lead to the autophosphorylation of DER whereas the membrane-bound form of Spi cannot (Schweitzer et al., 1995b). These authors suggest that *Star* and *rhomboid* play a role in processing the Spi ligand (Schweitzer et al., 1995b). Weak, viable, partial loss-of-function alleles of *spi* show a reduced number of photoreceptor cells (Tio et al., 1994). Genetic mosaic analysis reveals that *spitz* is required for most photoreceptor determination. R8 absolutely requires *spi* activity in order to lead to the formation of additional photoreceptors. If R8 lacks *spi* activity, the ommatidium fails to form. *spitz* activity in R8 is sufficient to lead to photoreceptor development. R2/5 show a strong, but not absolute, requirement for *spitz* activity. This mosaic analysis shows that *spitz* is required for photoreceptor determination but not precursor cell proliferation. These results suggest that R8 does not require *spitz* activity to be determined but that all other photoreceptors do. The current model holds that R8 secretes Spitz protein which determines R2/5 photoreceptors which also secrete Spitz protein. The secreted Spitz protein induces the other photoreceptors in sequence. Clonal analysis suggests that the *spitz* activity range is quite limited in that it can act within an ommatidial precursor but not between ommatidial precusors in photoreceptor determination (Freeman, 1994b; Tio et al., 1994; Tio and Moses, 1997). Preliminary expression data for *spitz* is consistent with this interpretation (Tio et al., 1994; Tio and Moses, 1997).

Another gene, *argos* (*aos*), mediates an inhibitory signaling pathway for photoreceptor development. *argos* mutants have rough eyes, reduced viability, and one or two extra photoreceptor cells in many of their ommatidia (Freeman et al., 1992; Kretschmar et al., 1992; Okano et al., 1992). The so-called "mystery cells," whose ultimate fate in the developed eye is as yet unknown, differentiate as photoreceptors in *argos* mutants. Later defects are also observed in the developing eye. More cone cells form (an average of nine compared with four in wild type), as do more primary pigment cells (an average of five rather than two). Thus the Argos gene product appears to inhibit the adoption of successive cell fates in the ommatidia (Freeman et al., 1992). Complete loss-of-function alleles are lethal, but mosaic clones of these alleles in the developing eye

show defects similar to those of weaker alleles (Freeman et al., 1992; Kretzschmar et al., 1992). This mosaic analysis suggests that *argos* acts cell non-autonomously. The extra cells in ommatidia need not be mutant. Small or narrow clones have few defects and ommatidia on the border of large mutant clones are often wild-type. These defects suggest that *argos* activity has a range of 1-2 ommatidia (Freeman et al., 1992). Overexpression of *argos* under the control of a heat shock promotor in early pupae leads to missing photoreceptors, fewer cone cells, and fewer primary pigment cells in anterior to posterior positions, respectively, suggesting that the time of overexpression of *argos* is important to the production of the mutant phenotypes in these cells (Freeman, 1994a).

argos has been shown to interact genetically with many alleles of genes involved in the DER signaling pathway. Reduction-of-function *argos* phenotypes are suppressed by heterozygous loss-of-function mutations of DER signaling pathway components and of *spitz*. The increased activity of *argos* by heat shock overexpression is enhanced in the genetic backgrounds described above which suppress *argos* reduction-of-function phenotypes. In early development, *Egfr* hypomorphic phenotypes are similar to phenotypes caused by overexpression of *argos* while reduction-of function alleles of *argos* produce a phenotype similar to that produced by *Elp* gain-of-function alleles of *Egfr*. Notably, the phenotypes produced by overexpression of *argos* and *Elp* alleles of *Egfr* mutually supress each other. These genetic interactions support a model in which *argos* acts as an inhibitor of the *Egfr* signaling pathway (Schweitzer et al., 1995a).

argos encodes a novel 444 amino acid protein with a signal sequence, but no transmembrane domain. It also contains a single six cysteine domain with some similarity to the concensus EGF repeat (Freeman et al., 1992; Kretschmar et al., 1992; Okano et al., 1992). One loop in particular is larger than that seen in other EGF motifs (Schweitzer et al., 1995a). *argos* can be expressed in cultured cells which secrete Argos protein into the media, thus suggesting that Argos acts as a secreted protein (Freeman, 1994a). *lacZ* expression studies were conducted using an enhancer trap line in which *lacZ* is under the transcriptional control of the *argos* promotor. These expression studies show expression behind the morphogenetic furrow. *lacZ* is expressed strongly in all developing photoreceptors in order of differentiation: R8, R2/5, R3/4, R1/6, R7. The mystery cells only express *lacZ* weakly.

Argos has been shown to directly inhibit the DER pathway. Argos acts as a competitive inhibitor of DER activation by Spitz protein in experiments in which the autophosphorylation activity of DER expressed in S2 cells is assayed. Preincubation of these cells with Argos prevents activation by Spitz (Schweitzer et al., 1995a). The observation that Argos can rapidly reduce the ligand-independent autophosphorylation

activity of overexpressed DER suggests that Argos does not act to titrate out Spitz but acts on the receiving cell itself, presumably directly binding to the receptor in a fashion similar to Spitz protein. In this regard, it is notable that both Argos and Spitz contain a single EGF-like domain (Schweitzer et al., 1995a) (Figure 5).

These results have led to the proposal of a model for the sequential determination of photoreceptors, cone cells, and pigment cells in the developing ommatidia. Spitz is produced by the first photoreceptor R8 and subsequently by R2 and R5. The Spitz ligand activates DER in the other presumptive photoreceptor cells in sequence. It also activates Argos expression (Freeman, 1996; 1997). Golembo et al., (1996) showed that DER activity was necessary for *argos* expression both *in vivo* and in cell culture experiments. Argos then inhibits the induction of more distant cells since its range is significantly greater than that of Spitz. Higher levels of Spitz can overcome Argos-mediated inhibition of DER. After the photoreceptors are determined in sequence, the cone cells , which are radially more distant from the initially determined R8 cell of the ommatidium, are formed. Later still, the primary pigment cells are induced, followed by the secondary and tertiary pigment cells. Thus the Freeman model involves remote inhibition, and the limitation of the spreading of Spitz. This reduction of Spitz activity spread coupled with a postulated clock that determines cell competence to adopt different fates permits the proper determination of ommatidial components and radial pattern (Freeman, 1996; 1997).

The Spitz/Argos system provides an example of a simple mechanism of inhibition. The positive and negative signals each bind to the same receptor. The receptor acts to propagate the positive signal. However, the negative signal can bind to the receptor ligand binding site but cannot activate the receptor. By blocking the access of the positive ligand to the receptor, the negative ligand inhibits the positive signaling system. The negative ligand presumably acts as a competitive inhibitor of the positive ligand. This mechanism is more fully developed in the folloing section. This example demonstrates how a positively acting receptor can be negatively regulated directly. Other variations are possible, such as a negative signal molecule binding to the receptor at a site other than the positive ligand binding site and preventing receptor dimerization or the conformation changes necessary for signal transduction. In these cases, the negative signal would be expected to act as a noncompetitive inhibitor. These mechanisms of negative regulation of a positively acting receptor are good possibilities for the class A synMuv genes since few molecules are required to effect negative regulation in this manner.

Inhibition of IL-1 receptor activity by a negative regulatory ligand

The mechanism by which Argos acts as an antagonist of DER may be similar to the mechanism by which the interleukin-1 receptor antagonist (IL-1ra) acts on the interleukin-1 (IL-1) receptor. A detailed biochemical analysis of the mechanism of Argos inhibition of DER has not yet been conducted. However, more detailed biochemical data on the negative regulation of the IL-1 receptor by an inhibitory ligand are available. This data provides some insight into a possible mechanism of Argos action.

IL-1ra was purified from media conditioned by IgG-stimulated monocytes which also secrete IL-1 α and IL-1β, the stimulatory ligands of the IL-1 receptor. IL-1ra was cloned and shown to encode a small protein containing a signal sequence with a limited similarity to IL-1 α (26 %) and IL-1 β (19 %). IL-1ra fails to stimulate the IL-1 receptor even at high concentrations. IL-1ra was shown to not bind IL-1, but to bind the IL-1 receptor. Binding studies indicate that IL-1ra binds to the IL-1 receptor with an affinity similar to that of IL-1 α and IL-1β, and that it acts as a competitive inhibitor of IL-1 binding to receptor (Carter et al., 1990; Eisenberg et al., 1990; Hannum et al., 1990). It is noteworthy that the same cell type secrets both the stimulatory ligand and the antagonist, but that the mRNA encoding IL-1ra does not include the potential RNA stability motifs found in the mRNAs encoding IL-1 α and IL-1 β. The differential stability of the positive and negative ligands may play a role in the modulation of IL-1 receptor activation by the stimulatory cell (Carter et al., 1990; Eisenberg et al., 1990).

Recent results reveal more about the mechanism of antagonistic action by IL-1ra. Co-crystallization of IL-1β and IL-1 receptor extracellular domain and co-crystalization of IL-1ra and IL-1 receptor extracellular domain have allowed the nature of the binding of these ligands to receptor to be determined at a high level of resolution. IL-1ra does not interact with one of the receptor sites that IL-1β does. IL-1 receptors in which this site has been deleted bind IL-1 α and β with much reduced affinity but continue to bind IL-1ra with high affinity (Schreuder et al., 1997; Vigers et al., 1997). These results suggest that an antagonist that acts as a competitive inhibitor prevents binding of the stimulatory ligand to the receptor by binding to the ligand interaction site but not interacting with site necessary to stimulate activity by the receptor. Presumably, Argos acts in a similar manner on the DER (Figure 5).

Different mechanisms are used to antagonize stimulatory signals

The preceeding examples illustrate not only the great diversity of different processes in which antagonistic signals act, but also the great diversity of mechanisms by which these signaling systems exert their effects. Many of these antagonistic regulatory

pathways act directly on the positive signaling system to inhibit or modulate the activity of the stimulatory pathway. In these cases, the antagonism manifests itself as a downregulation or a complete inhibition of the stimulatory pathway.

Direct action on the stimulatory signaling system can either be mediated upsteam of the signal transduction cascade or on the signal transduction cascade itself. Examples of inhibitory regulators acting upstream of the stimulatory signal transduction pathway are *chordin* and *noggin*, *sog*, *argos*, and IL-1ra. Chordin, Noggin and Sog exert their effects by directly binding the stimulatory ligand and rendering it incapable of activating its receptor. Thus, these molecules act to titrate out the positive signal. Argos and IL-1ra act as inhibitory ligands for the receptor of the stimulatory ligand. These inhibitory ligands compete for the ligand binding domain, preventing the stimulatory ligand from binding. The bound inhibitory ligand precludes stimulatory ligand binding of the receptor, but does not activate the receptor itself.

Other antagonistic signaling systems act directly on the stimulatory signal transduction system. These include the TGF-β-mediated and yeast mating pheromone-mediated negative regulation of cell proliferation and the *clr-1* negative regulation of larval developmental progression in *C. elegans*. The cell proliferation inhibitory pathways consist of a receptor and signal transduction cascade which transduce the negative signal in the responding cells . These systems activate a molecule or molecules, cdk inhibitors in mammals or Far1 in yeast, which directly inhibit the cell cycle machinery activated by the stimulatory pathway. In these cases, the stimulatory and inhibitory signals are transduced to a common target for regulation. The other example cited of a mediator of a stimulatory signal transduction cascade involves the Clr-1 receptor phosphatase. Although its target is unknown, it likely acts by dephosphorylating the stimulatory Egl-15 receptor, or another downstream molecule in the Egl-15 signal transduction cascade. This downregulates Egl-15-mediated signaling.

Although there are no examples presented in which the negative regulatory pathway acts indirectly at the level of transcriptional control, this is theoretically possible. In this scenario, the stimulatory and inhibitory signals are transduced to the nucleus of the responding cell. There, they control the transcription of genes necessary for the process which is being regulated. The inhibitory system may negatively regulate transcription factors stimulated by the positive signaling system or may stimulate negatively acting transcription factors which would interact with positive transcription factors at the promotor. Such a scenario does not differ conceptually from the positive and negative regulation of cyclin-cdk complexes. In this case, the targets of the positive and negative signaling systems are the promotors of genes necessary for the process being regulated

rather than a cyclin-cdk complex. Since *lin-36* may encode a nuclear protein, it is possible that the class B synMuv genes negatively regulate vulval development at the promotor level or by modulating the activity of transcription factors involved in this process.

References

Aaronson, S. A. (1991). Growth factors and cancer. *Science* **254**, 1146-1153.

Arioan, R. V., Koga, M., Mendel, J. E., Oshima, Y. and Sternberg, P. W. (1990). The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature* **348**, 693-699.

Arora, K. and Nusslein-Volhard, C. (1992). Altered mitotic domains reveal fate map changes in *Drosophila* embryos mutant for zygotic dorsoventral patterning genes. *Development* **114**, 1003-1024.

Arora, K., Levine, M. S. and O'Connor, M. B. (1994). The *screw* gene encodes a ubiquitously expressed member of the TGF-β family required for specification of dorsal cell fates in the *Drosophila* embryo. *Genes Dev.* **8**, 2588-2601.

Baker, N. E. and Rubin, G. M. (1989). Effect on eye development of dominant mutations in *Drosophila* homologue of the EGF receptor. *Nature* **340**, 150-153.

Baker, N. E. and Rubin, G. M. (1992). *Ellipse* mutations in the *Drosophila* homologue of the EGF receptor affect pattern formation, cell division, and cell death in eye imaginal discs. *Dev. Biol.* **150**, 381-396.

Beitel, G. J., Clark, S. G. and Horvitz, H. R. (1990). *Caenorhabditis elegans ras* gene *let-60* acts as a switch in the pathway of vulval induction. *Nature* **348**, 503-509.

Biehs, B., Francois, V. and Bier, E. (1996). The *Drosophila short gastrulation* gene prevents Dpp from autoactivating and suppressing neurogenesis in the neuroectoderm. *Genes Dev.* **10**, 2922-2934.

Cairns, B. R., Ramer, S. W. and Kornberg, R. D. (1992). Order of action of components in the yeast pheromone response pathway revealed with a dominant allele of *STE11* kinase and the multiple phosphorylation of the *STE7* kinase. *Genes Dev.* **6**, 1305-1318.

Carcamo, J., Weis, F. M. B., Ventura, F., Wiesener, R., Wrana, J. L., Attisano, L. and Massugue, J. (1994). Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor β and activin. *Mol. Cell. Biol.* **14**, 3810-3821.

Carter, D. B., Deibel Jr., M. R., Dunn, C. J., Tomich, C-S. C., Laborde, A. L., Slightom, J. L., Berger, A. E., Bienkowski, M. J., Sun, F. F., McEwan, R. N., Harris, P. K. W., Yem, A. W., Waszak, G. A., Chosay, J. G., Sieu, L. C., Hardee, M. M., Zurcher-Neely, H. A., Reardon, I. M., Heinrikson, R. L., Truesdell, S. E., Shelly, J. A., Essalv, T. E., Taylor, B. M. and Tracey, D. E. (1990). Purification, cloning, expression and biological characterization of an interleukin-1 receptor antagonist protein. *Nature* **344**, 633-637.

Chang, F. and Herskowitz, I. (1990). Identification of a gene necesary for cell cycle arrest by a negative growth factor of yeast: *FAR1* is an inhibitor of a G1 cyclin *CLN2*. *Cell* **63**, 999-1011.

Chang, F. and Herskowitz, I. (1992). Phosphorylation of*FAR1* in response to α-factor: a possible requirement for cell-cycle arrest. *Mol. Biol. Cell* **3**, 445-450.

Childs, S. R. and O'Connor, M. B. (1994). Two domains of the *tolloid* protein contribute to its unusual genetic interaction with *decapentaplegic*. *Dev. Biol.* **162**, 209-220.

Cho, K. W. Y., Blumberg, B., Steinbeisser, H. and DeRobertis, E. M. (1991). Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene *goosecoid*. *Cell* **67**, 1111-1120.

Clark, S. G., Stern, M. J. and Horvitz, H. R. (1992a). *Caenorhabditis elegans* cell-signalling gene *sem-5* encodes a protein with SH2 and SH3 domains. *Nature* **356**, 340-344.

Clark, S. G., Stern, M. J. and Horvitz, H. R. (1992b). Genes involved in two *Caenorhabditis elegans* cell-signaling pathways. *Cold Spring Harbor Symp. Quant. Biol.* **57**, 363-373.

Clark, S. G., Lu, X. and Horvitz, H. R. (1994). The *Caenorhabditis elegans* locus *lin-15*, a negative regulator of a tryrosine kinase signaling pathway, encodes two different proteins. *Genetics* **137**, 987-997.

Cross, F. R. (1988). *DAF1*, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **8**, 4675-4684.

Cross, F. R. (1990). Cell cycle arrest caused by *CLN* gene deficiency in *Saccharomyces cerevisiae* resembles START-I arrest and is independent of the mating-pheromone signalling pathway. *Mol. Cell. Biol.* **10**, 6482-6490.

Cross, F. R. and Tinkelenberg, A. H. (1991). A potential positive feedback loop controlling *CLN1* and *CLN2* gene expression at the start of the yeast cell cycle. *Cell* **65**, 875-883.

Dale, L., Howes, G., Price, B. M. and Smith, J. C. (1992). Bone morphogenetic protein 4: a ventralizing factor in early *Xenopus* development. *Development* **115**, 573-585.

Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y. and Wang, X-F. (1995). Transforming growth factor β induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc. Natl. Acad. Sci. USA* **92**, 5545-5549.

DeRobertis, E. M. and Sasai, Y. (1996). A common plan for dorsoventral patterning in Bilateria. *Nature* **380**, 37-40.

DeVore, D. L., Horvitz, H. R. and Stern, M. J. (1995). An FGF receptor signaling pathway is required for the normal cell migration of the sex myoblast in *C. elegans* hermaphrodites. *Cell* **83**, 611-620.

Diaz-Benjumea, F. J. and Hafen, E. (1994). The *sevenless* signalling cassette mediates *Drosophila* EGF receptor function during epidermal development. *Development* **120**, 569-578.

Dirick, L. and Nasmyth, K. (1991). Positive feedback in the activation of G1 cyclins in yeast. *Nature* **351**, 754-757.

Eisenberg, S. P., Evans, R. J., Arend, W. P., Verderber, E., Brewer, M. T., Hannum, C. H. and Thomson, R. C. (1990). Primary structure and functional expression from complementary DNA of a human interleukin-1 receptor antagonist. *Nature* **343**, 341-345.

Eisenmann, D. M. and Kim, S. K. (1994). Signal transduction and cell fate specification during *Caenorhabditis elegans* vulval development. *Curr. Opinion Genet. Develop.* **4**, 508-516.

El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. and Vogelstein, B. (1993). *WAF1*, a potential mediator of p53 tumor suppression. *Cell* **75**, 817-825.

Elion, E. A., Grisati, P. L. and Fink, G. R. (1990). *FUS3* encodes a *cdc2*$^{+}$/*CDC28*-related kinase required for the transition from mitosis into conjugation. *Cell* **60**, 649-664.

Elion, E. A., Brill, J. A. and Fink, G. R. (1991). *FUS3* represses *CLN1* and *CLN2* and in concert with *KSS1* promotes signal transduction. *Proc. Natl. Acad. Sci. USA* **88**, 9392-9396.

Elion, E. A., Satterberg, B. and Kranz, J. E. (1993). *FUS3* phosphorylates multiple components of the mating signal transduction cascade: evidence for *STE12* and *FAR1*. *Mol. Biol. Cell* **4**, 495-510.

Errede, B., Gartner, A., Zhou, Z., Nasmyth, K. and Ammerer, G. (1993). MAP kinase-related *FUS3* from *Saccharomyces cerevisiae* is activated by *STE7 in vitro*. *Nature* **362**, 261-264.

Ewen, M. E., Sluss, H. K., Whitehouse, L. L. and Livingstone, D. (1993). TGFβ inhibition of Cdk4 synthesis is linked to cell cycle arrest. *Cell* **74**, 1009-1020.

Ferguson, E. L. (1996). Conservation of dorsal-ventral patterning in arthropods and chordates. *Curr. Opinion Genet. Develop.* **6**, 424-431.

Ferguson, E. L. and Anderson, K. V. (1992a). *decapentaplegic* acts as a morphogen to organize dorsal-ventral pattern in the *Drosophila* embryo. *Cell* **71**, 451-461.

Ferguson, E. L. and Anderson, K. V. (1992b). Localized enhancement and repression of the activity of the TGF-β family member, *decapentaplegic*, is necessary for dorsal-ventral pattern formation in the *Drosophila* embryo. *Development* **114**, 583-597.

Ferguson, E. L. and Horvitz, H. R. (1985). Identification and genetic characterization of 22 genes that affect the vulval cell lineages of the nematode *Caenorhabditis elegans*. *Genetics* **110**, 17-72.

Ferguson, E. L. and Horvitz, H. R. (1989). The Multivulva phenotype of certain *Caenorabditis elegans* mutants results from defects in two functionally redundant pathways. *Genetics* **123**, 109-121.

Ferguson, E. L., Sternberg, P. W., and Horvitz, H. R. (1987). A genetic pathway for the specification of the vulval cell lineages of *Caenorhabditis elegans*. *Nature* **326**, 259-267.

Fortini, M. E., Simon, M. A. and Rubin, G. M. (1992). Signalling by the *sevenless* protein tyrosine kinase is mimicked by Ras1 activation. *Nature* **355**, 559-561.

Francois, V. and Bier, E. (1995). *Xenopus chordin* and *Drosophila short gastrulation* genes encode homologous proteins functioning in dorsal-ventral axis formation. *Cell* **80**, 19-20.

Francois, V., Solloway, M., O'Neill, J. W. and Bier, E. (1994). Dorsal-ventral patterning of the *Drosophila* embryo depends on a putative negative growth factor encoded by the *short gastrulation* gene. *Genes Dev.* **8**, 2606-2616.

Freeman, M. (1994a). Misexpression of the *Drosophila argos* gene, a secreted regulator of cell determination. *Development* **120**, 2297-2304.

Freeman, M. (1994b). The *spitz* gene is required for photoreceptor determination in the *Drosophila* eye where it interacts with the EGF receptor. *Mech. Dev.* **48**, 25-33.

Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. *Cell* **87**, 651-660.

Freeman, M. (1997). Cell determination strategies in the *Drosophila* eye. *Development* **124**, 261-270.

Freeman, M., Klambt, C., Goodman, C. S. and Rubin, G. M. (1992). The *argos* gene encodes a diffusible factor that regulates cell fate decisions in the *Drosophila* eye. *Cell* **69**, 963-975.

Gartner, A., Nasmyth, K. and Ammerer, G. (1992). Signal transduction in *Saccharomyces cerevisiae* requires tyrosine and threonine phosphorylation of *FUS3* and *KSS1*. *Genes Dev.* **6**, 1280-1292.

Gelbart, W. M. (1989). The *decapentaplegic* gene: a TGF-β homologue controlling pattern formation in *Drosophila*. *Development Suppl. 1989*, 65-74.

Geng, Y. and Weinberg, R. A. (1993). Transforming growth factor β effects on expression of G_1 cyclins and cyclin-dependent protein kinases. *Proc. Natl. Acad. Sci. USA* **90**, 10315-10319.

Glick, A. B., Kulkarni, A. B., Tennenbaum, T., Hennings, H., Flounders, K. C., O'Reilly, M., Sporn, M. O., Karlsson, S. and Yuspa, S. H. (1993). Loss of expression of transforming growth factor β in skin and skin tumors is associated with hyperproliferation and a high risk for malignant conversion. *Proc. Natl. Acad. Sci. USA* **90**, 6076-6080.

Glick, A. B., Lee, M. M., Darwiche, N., Kulkarni, A. B., Karlsson, S. and Yuspa, S. H. (1994). Targeted deletion of the TGF-1β gene causes rapid progression to squamous cell carcinomas. *Genes Dev.* **8**, 2429-2440.

Golembo, M., Schweitzer, R., Freeman, M. and Shilo, B-Z. (1996). *argos* transcription is induced by the *Drosophila* EGF receptor pathway to form an inhibitory feedback loop. *Development* **122**, 223-230.

Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J. and Melton, D. A. (1994). Studies with a *Xenopus* BMP receptor suggest that ventral mesoderm-inducing signal override dorsal signals *in vivo*. *Cell* **79**, 169-179.

Gu, Y., Turck, C. W. and Morgan, D. O. (1993). Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. *Nature* **366**, 707-710.

Hadwiger, J. A., Wittenberg, C., Richardson, H. E., De Barros Lopes, M. and Reed, S. I. (1989). A family of cyclin homologs that control the G_1 phase in yeast. *Proc. Natl. Acad. Sci. USA* **86**, 6255-6259.

Han, M. and Sternberg, P. W. (1990). *let-60*, a gene that specifies cell fates during *C. elegans* vulval induction, encodes a ras protein. *Cell* **63**, 921-931.

Han, M., Golden, A., Han, Y., and Sternberg, P. W. (1993). *C. elegans lin-45 raf* gene participates in *let-60 ras*-stimulated vulval differentiation. *Nature* **363**, 133-140.

Hannon, G. J. and Beach, D. (1994). $p15^{INK4B}$ is a potential effector of TGF-β-induced cell cycle arrest. *Nature* **371**, 257-261.

Hannum, C. H., Wilcox, C. J., Arend, W. P., Joslin, F. G., Dripps, D. J., Heimdal, P. L., Armes, L. G., Sommer, A., Eisenberg, S. P. and Thompson, R. C. (1990). Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. *Nature* **343**, 336-340.

Harper, J. W., Adam, G. R., Wei, N., Keyomarsi, K. and Elledge, S. J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. *Cell* **75**, 805-816.

Hartwell, L. H., Culotti, J., Pringle, J. R. and Reid, B. S. (1974). Genetic control of the cell division cycle in yeast. *Science* **183**, 46-51.

Hawley, S. H. B., Wunnenberg-Stapleton, K., Hashimoto, C. Laurent, M. N., Watanabe, T., Blumberg, B. W. and Cho, K. W. Y. (1995). Disruption of BMP signals in embryonic *Xenopus* ectoderm leads to direct neural induction. *Genes Dev.* **9**, 2923-2935.

Hedgecock, E. M., Culotti, J. G. and Hall, D. H. (1990). The *unc-5*, *unc-6*, and *unc-40* genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. *Neuron* **4**, 61-85.

Hedgecock, E. M. and Herman, R. K. (1995). The *ncl-1* gene and genetic mosaics of *Caenorhabditis elegans*. *Genetics* **141**, 989-1006.

Herman, R. K. and Hedgecock, E. M. (1990). Limitation of the size of the vulval primordium of *Caenorhabditis elegans* by *lin-15* expression in the surrounding hypodermis. *Nature* **348**, 169-171.

Hill, R. J. and Sternberg, P. W. (1992). The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans*. *Nature* **358**, 470-476.

Holley, S. A., Jackson, P. D., Sasai, Y., Lu, B., DeRobertis, E. M., Hoffman, F. M. and Ferguson, E. L. (1995). A conserved system for dorsal-ventral patterning in insects and vertebrates involving *sog* and *chordin*. *Nature* **376**, 249-253.

Holley, S. A., Neul, J. L., Attisano, L., Wrana, J. L., Sasai, Y., O'Connor, M. B., DeRobertis, E. M. and Ferguson, E. L. (1996). The *Xenopus* dorsalizing factor *noggin* ventralizes *Drosophila* embryos by preventing DPP from activating its receptor. *Cell* **86**, 607-617.

Horowitz, J. M., Park, S-H., Bogenmann, E., Cheng, J-C., Yandell, D. W., Kaye, F. J., Minna, J. D., Dryja, T. P. and Weinberg, R. A. (1990). Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human cells. *Proc. Natl. Acad. Sci. USA* **87**, 2775-2779.

Horvitz, H. R. and Sternberg, P. W. (1991). Multiple intercellular signalling systems control the development of the *Caenorhabditis elegans* vulva. *Nature* **351**, 535-541.

Horvitz, H. R. and Sulston, J. E. (1980). Isolation and genetic characterization of cell-lineage mutants of the nematode *Caenorhabditis elegans*. *Genetics* **96**, 435-454.

Hoskins, R., Hajnal, A. F., Harp, S. A. and Kim, S. K. (1996). The *C. elegans* vulval induction gene *lin-2* encodes a member of the MAGUK family of cell junction proteins. *Development* **122**, 97-111.

Huang, L. S., Tzou, P. and Sternberg, P. W. (1994). The *Caenorhabditis elegans lin-15* locus encodes two negative regulators of vulval development. *Molec. Biol. Cell* **5**, 395-412.

Irish, V. F. and Gelbart, W. M. (1987). The *decapentaplegic* gene is required for dorsal-ventral patterning of the *Drosophila* embryo. *Genes Dev.* **1**, 868-879.

Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V. E. and Hogan, B. L. M. (1992). DVR-4 (bone morphogenetic protein-4) as a posterior-ventralizing factor in *Xenopus* mesoderm induction. *Development 115*, 639-647.

Kadin, M. E., Cavaille-Coll., M. W., Gertz, R., Massugue, J., Cheifetz, S. and George, D. (1994). Loss of receptors for transforming growth factor β in human T-cell malignancies. *Proc. Natl. Acad. Sci. USA* **91**, 6002-6006.

Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tautigian, S. V., Stockert, E., Day III, R. S., Johnson, B. E. and Skolnick, M. H. (1994). A cell cycle regulator potentially involved in genesis of many tumor types. *Science* **264**, 436-440.

Katz, W. S., Hill, R. J., Clandinin, T. R. and Sternberg, P. W. (1995). Different levels of the *C. elegans* growth factor LIN-3 promote distinct vulval precusor fates. *Cell* **82**, 297-307.

Kim, S. K. and Horvitz, H. R. (1990). The *Caenorhabditis elegans* gene *lin-10* is broadly expressed while required specifically for the determination of vulval cell fates. *Genes Dev.* **4**, 357-371.

Kimble, J. (1981). Alterations in cell lineage following laser ablation of cells in the somatic gonad of *Caenorhabditis elegans*. *Dev. Biol.* **87**, 286-300.

Kimchi, A., Wang, X-F., Weinberg, R. A., Cheifetz, S. and Massugue, J. (1988). Absence of TGF-β receptors and growth inhibitory response in retinoblastoma cells. *Science* **240**, 196-199.

Koff, A., Ohtsuki, M., Polyak, K., Roberts, J. M. and Massugue, J. (1993). Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-β. *Science* **260**, 536-539.

Kornfeld, K., Guan, K-L. and Horvitz, H. R. (1995a). The *Caenorhabditis elegans* gene *mek-2* is required for vulval induction and encodes a protein similar to the protein kinase MEK. *Genes Dev.* **9**, 756-768.

Kornfeld, K., Hom, D. M. and Horvitz, H. R. (1995b). The *ksr-1* gene encodes a novel protein kinase involved in Ras-mediated signaling in *C. elegans*. *Cell* **83**, 903-913.

Koster, M., Plessow, S., Clement, J. H., Lorenz, A., Tiedemann, H. and Knuchel, W. (1991). Bone morphogenetic protein 4 (BMP-4), a member of the TGF-β family, in early embryos of Xenopus laevis: analysis of mesoderm inducing activity. *Mech. Dev.* **33**, 191-200.

Kretzschmar, D., Brunner, A., Wiersdorff, V., Pfugfelder, G. O., Heisenberg, M. and Schneuwly, S. (1992). *giant lens*, a gene involved in cell determination and axon guidance in the visceral system of *Drosophila melanogaster*. *EMBO J.* **11**, 2531-2539.

Lackner, M. R., Kornfeld, K., Miller, L. M., Horvitz, H. R. and Kim, S. K. (1994). A MAP kinase homologue, *mpk-1*, is involved in ras-mediated induction of vulval cell fates in *Caenorhabditis elegans*. *Genes Dev.* **8**, 160-173.

Laiho, M., DeCario, J. A., Ludlow, J. W., Livingston, D. M. and Massugue, J. (1990). Growth inhibition by TGF-β linked to expression of retinoblastoma protein phosphorylation. *Cell* **62**, 175-185.

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Econimides, A. N., Stahl, N., Yanaopoulous, G. D. and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Science* **262**, 713-718.

Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., Peters, G. and Bartek, J. (1995). Retinoblastoma protein-dependent cell cycle inhibition by the tumor suppressor p16. *Nature* **375**, 503-506.

Marsh, L., Nieman, A. M. and Herskowitz, I. (1991). Signal transduction during pheromone response in yeast. *Annu. Rev. Cell Biol.* **7**, 699-728.

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zbrorowska, E., Kinzer, K. W., Vogelstein, B., Brattain, M. and Willson, J. K. V. (1995). Inactivation of the type II TGF-β receptor in colon cancer cells with minisatellite instability. *Science* **268**, 1336-1338.

Masui, T., Wakefield, L. M., Lechner, J. F., LaVeck, M. A., Sporn, M. B. and Harris, C. C. (1986). Type β transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells. *Proc. Natl. Acad. Sci. USA* **83**, 2438-2442.

Matushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J. and Kato, J-Y. (1994). D-type cyclin-dependent kinase in mammalian cells. *Mol. Cell. Biol.* **14**, 2066-2076.

Medema, R. H., Herrera, R. E., Lam, F. and Weinberg, R. A. (1995). Growth suppression by $p16^{ink4}$ requires functional retinoblastoma. *Proc. Natl. Acad. Sci. USA* **92**, 6289-6293.

Moos Jr., M., Wang, S. and Krinks, M. (1995). Anti-dorsalizing morphogenetic protein is a novel TGF-β homolog expressed in the Spemann organizer. *Development* **121**, 4293-4301.

Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K. and Sugino, H. (1990). Activin-binding protein from rat ovary is follistatin. *Science* **247**, 836-838.

Nash, R., Tokiwa, G., Anand, S., Erikson, K. and Futcher, A. B. (1988). The *WHI1*$^+$ gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. *EMBO J.* **7**, 4335-4346.

Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-368.

Nieman, A. M. and Herskowitz, I. (1994). Reconstitution of a yeast protein kinase cascade *in vitro*: activation of the yeast MEK homologue *STE7* by *STE11*. *Proc. Natl. Acad. Sci. USA* **91**, 3398-3402.

Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K. and Carson, D. A. (1994). Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* **368**, 753-756.

Okano, H., Hayashi, S., Tanimura, T., Sawamoto, K., Yoshikawa, S., Watanabe, J., Iwasaki, M., Hirose, S., Mikoshiba, K. and Montell, C. (1992). Regulation of *Drosophila* neural development by a putative secreted protein. *Differentiation* 52, 1-11.

Padgett, R. W., St. Johnston, R. D. and Gelbart, W. M. (1987). A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-β family. *Nature* **325**, 81-84.

Padgett, R. W., Wozney, J. M. and Gelbart, W. M. (1993). Human BMP sequences can confer normal dorsal-ventral patterning in the *Drosophila* embryo. *Proc. Natl. Acad. Sci. USA* **90**, 2905-2909.

Pardee, A. B. (1989). G_1 events and regulation of cell proliferation. *Science* **246**, 603-608.

Park, K., Kim, S-J., Bang, Y-J., Park, J-G., Kim, N. K., Roberts, A. B. and Sporn, M. B. (1994). Genetic changes in the transforming growth factor β (TGF-β) type II receptor gene in human gastric cancer cells: correlation with sensitivity to growth inhibition by TGF-β. *Proc. Natl. Acad. Sci. USA* **91**, 8772-8776.

Peter, M., Gartner, A., Horecka, J., Ammerer, G. and Herskowitz, I. (1993). *FAR1* links the signal transduction pathway to the cell cycle machinery in yeast. *Cell* **73**, 747-760.

Peter, M. and Herskowitz, I. (1994). Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. *Science* **265**, 1228-1231.

Piccolo, S., Sasai, Y., Lu, B. and DeRobertis, E. M. (1996). Dorsovental patterning in *Xenopus*: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86**, 589-598.

Polyak, K., Kato, J-Y., Solomon, M. J., Sherr, C. J., Massugue, J., Roberts, J. M. and Koff, A. (1994a). $p27^{Kip1}$, a cyclin-Cdk inhibitor links transforming growth factor-β and contact inhibition to cell cycle arrest. *Genes Dev.* **8**, 9-22.

Polyak, K., Lee, M-H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P. and Massugue, J. (1994b). Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antagonistic signals. *Cell* **78,** 59-66.

Pringle, J. R. and Hartwell, L. H. (1981). The *Saccharomyces cerevisiae* cell cycle. In The molecular biology of the yeast *Saccharomyces*: life cycle and inheritance (eds. J. N.

Strathern, E. W. Jones and J. R. Broach), pp. 97-142. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Ray, R. P., Arora, K., Nusslein-Volhard, C. and Gelbart, W. M. (1991). The control of cell fate along the dorsal-ventral axis of the *Drosophila* embryo. *Development* **113**, 35-54.

Reed, S. I. (1980). The selection of *S. cerevisiae* mutants defective in the start event of cell division. *Genetics* **95**, 561-577.

Re'em-Kalma, Y., Lamb, T. and Frank, D. (1995). Competition between noggin and bone morphogenetic protein 4 activities may regulate dorsalization during *Xenopus* development. *Proc. Natl. Acad. Sci. USA* **92**, 12141-12145.

Reynisdottir, I., Polyak, K., Iavarone, A. and Massugue, J. (1995). Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-β. *Genes Dev.* **9**, 1831-1845.

Richardson, H. E., Wittenberg, C., Cross, F. and Reed, S. I. (1989). An essential G1 function for cyclin-like proteins in yeast. *Cell* **59**, 1127-1133.

Roberts, A. B., Anzano, M. A., Wakefield, L. M., Roche, N. S., Stern, D. F. and Sporn, M. B. (1985). Type β transforming growth factor: a bifunctional regulator of cellular growth. *Proc. Natl. Acad. Sci. USA* **82**, 119-123.

Roth, S., Stein, D. and Nusslein-Volhard, C. (1989). A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* **59**, 1189-1202.

Rutledge, B. J., Zhang, K., Bier, E., Jan, Y. N. and Perrimon, N. (1992). The *Drosophila spitz* gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis. *Genes Dev.* **6**, 1503-1517.

St. Johston, R. D. and Gelbart, W. M. (1987). Decapentaplegic transcripts are localized along the dorsal-ventral axis of the *Drosophila* embryo. *EMBO J.* **6**, 2785-2791.

St. Johnston, D. and Nusslein-Volhard, C. (1992). The origin of pattern and polarity in the *Drosophila* embryo. *Cell* **68**, 201-219.

Sasai, Y., Lu, B., Steinbessier, H., Geissert, D., Gont, L. K. and DeRobertis, E. M. (1994). *Xenopus chordin*: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.

Sasai, Y., Lu, B., Steinbessier, H. and DeRobertis, E. M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus* . *Nature* **376**, 333-336.

Schejter, E. D., Segal, D., Glazer, L. and Shilo, B-Z. (1986). Alternative 5' exons and tissue-specific expression of the *Drosophila* EGF receptor homolog transcripts. *Cell* **46**, 1091-1101.

Schmidt, J., Francois, V., Bier, E. and Kimmelman, D. (1995a). *Drosophila short gastrulation* induces an ectopic axis in *Xenopus*: evidence for conserved mechanisms of dorsal-ventral patterning. *Development* **121**, 4319-4328.

Schmidt, J. E., Suzuki, A., Ueno, N. and Kimmelman, D. (1995b). Localized BMP-4 mediates dorsal/ventral patterning in the early *Xenopus* embryo. *Dev. Biol.* **169**, 37-50.

Schreuder, H., Tardif, C., Trump-Kallmeyer, S., Soffientini, A., Sarubbi, E., Akeson, A., Bowlin, T., Yanofsky, S. and Barrett, R. W. (1997). A new cytokine-receptor binding mode revealed by the crystal structure of the IL-1 receptor with an antagonist. *Nature* **386**, 194-200.

Schweitzer, R., Howes, R., Smith, R., Shilo, B-Z. and Freeman, M. (1995a). Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos. *Nature.* **376**, 699-702.

Schweitzer, R., Shaharabany, M., Seger, R. and Shilo, B-Z. (1995b). Secreted spitz triggers the the DER signaling pathway and is a limiting component in embryonic ventral ectoderm determination. *Genes Dev.* **9**, 1518-1529.

Serrano, M., Hannon, G. J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366**, 704-707.

Shah, N. M., Groves, A. K. and Anderson, D. J. (1996). Alternative neural crest cell fates are instructively promoted by TGF-β superfamily members. *Cell* **85**, 331-343.

Sherr, C. J. (1993). Mammalian G_1 cyclins. *Cell* **73**, 1059-1065.

Shimell, M. J., Ferguson, E. L., Childs, S. R. and O'Connor, M. B. (1991). The *Drosophila* dorsal-ventral patterning gene *tolloid* is related to human bone morphogenetic protein 1. *Cell* **67**, 469-481.

Silberstein, G. B. and Daniel, C. W. (1987). Reversible inhibition of mammary gland growth by transforming growth factor-β. *Science* **237**, 291-293.

Simon, M. A. (1994). Signal transduction during the development of the *Drosophila* R7 photoreceptor. *Dev. Biol.* **166**, 431-442.

Simon, M. A., Bowtell, D. D. L., Dodson, G. S., Laverty, T. R. and Rubin, G. M. (1991). *Ras1* and a putative guanine nucleotide exchange factor perform critical steps in signaling by the *sevenless* protein tyosine kinase. *Cell* **67**, 701-716.

Simske, J. S. and Kim, S. K. (1995). Sequential signalling during *Caenorhabditis elegans* vulval induction. *Nature* **375**, 142-146.

Simske, J. S., Kaech, S. M., Harp, S. A. and Kim, S. K. (1996). LET-23 receptor localization by the cell junction protein LIN-7 during *C. elegans* vulval induction. *Cell* **85**, 195-204.

Slingerland, J. M., Hengst, L., Pan, C-H., Alexander, D., Stampfer, M. R. and Reed, S. I. (1994). A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor β-arrested epithelial cells. *Mol. Cell. Biol.* **14**, 3683-3694.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of *noggin*, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell* **70**, 829-840.

Smith, W. C., Knecht, A. K., Wu, M. and Harland, R. M. (1993). Secreted *noggin* protein mimics the Spemann organizer in dorsalizing *Xenopus* mesoderm. *Nature* **361**, 547-549.

Steinbeisser, H., Fainsod, A., Niehrs, C., Sasai, Y. and DeRobertis, E. M. (1995). The role of *gsc* and *BMP-4* in dorsal-ventral patterning of the marginal zone in *Xenopus*: a loss-of-function study using antisense RNA. *EMBO J.* **14**, 5230-5243.

Stern, M. J. and Horvitz, H. R. (1991). A normally attractive cell interaction is repulsive in two *C. elegans* mesodermal cell migration mutants. *Development* **113**, 797-803.

Sternberg, P. W. (1988). Lateral inhibition during vulval induction in *Caenorhabditis elegans*. *Nature* **335**, 551-554.

Sternberg, P. W. and Horvitz, H. R. (1986). Pattern formation during vulval development in *C. elegans*. *Cell* **44**, 761-772.

Sternberg, P. W. and Horvitz, H. R. (1989). The combined action of two intercellular signalling pathways specifies three cell fates during vulval induction in *C. elegans*. *Cell* **58**, 679-693.

Stevenson, B. J., Rhodes, N., Errede, B. and Sprague Jr., G. F. (1992). Constitutive mutants of the protein kinase *STE11* activate the yeast pheromone response pathway in the absence of the G protein. *Genes Dev.* **6**, 1293-1304.

Sulston, J. E. and Horvitz, H. R. (1977). Postembryonic cell lineages of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **56**, 110-156.

Sulston, J. E. and Horvitz, H. R. (1981). Abnormal cell lineages in mutants of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **82**, 41-55.

Sulston, J. E. and White, J. G. (1980). Regulation and cell autonomy during postembryonic development of *Caenorhabditis elegans*. *Dev. Biol.* **78**, 577-597.

Sundaram, M. and Han, M. (1996). Control and integration of cell signaling pathways during *C. elegans* vulval development. *Bioessays* **18**, 473-480.

Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K. and Ueno, N. (1994). A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early *Xenopus* embryo. *Proc. Natl. Acad. Sci. USA* **91**, 10255-10259.

Taira, M., Otani, H., Saint-Jeannet, J-P. and Dawid, I. G. (1994). Role of the LIM class homeodomain protein *Xlim-1* in neural and muscle induction by the Spemann organizer in *Xenopus*. *Nature* **372**, 677-679.

Thomas, J. H., Stern, M. J. and Horvitz, H. R. (1990). Cell interactions coordinate the development of the *Caenorhabditis elegans* egg-laying system. *Cell* **62**, 1041-1052.

Tio, M., Ma, C. and Moses, K. (1994). *spitz*, a *Drosophila* homolog of transforming growth factor-α is required in the founding photoreceptor cells of the compound eye facets. *Mech. Dev.* **48**, 13-23.

Tio, M. and Moses, K. (1997). The *Drosophila* TGFα homologue Spitz acts in photoreceptor recruitment in the developing retina. *Development* **124**, 343-351.

Tomlinson, A. (1988). Cellular interactions in the developing *Drosophila* eye. *Development* **104**, 183-193.

Toyoshima, H. and Hunter, T. (1994). p27, a novel inhibitor of G1-Cdk protein kinase activity, is related to p21. *Cell* **78**, 67-74.

Tyers, M., Tokiwa, G., Nash, R. and Futcher, B. (1992). The Cln3-Cdc28 kinase complex of *S. cerevisiae* is regulated by proteolysis and phosphorylation. *EMBO J.* **11**, 1773-1784.

Tyers, M. and Futcher, B. (1993). Far1 and Fus3 link the mating phromone signal transduction pathway to three G_1-phase Cdc28 kinase complexes. *Mol. Cell. Biol.* **13**, 5659-5669.

Tyers, M., Tokiwa, G. and Futcher, B. (1993). Comparison of the *Saccharomyces cerevisiae* cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. *EMBO J.* **12**, 1955-1968.

Valdivieso, M. H., Sugimoto, K., Jahng, K-Y., Fernandes, P. M. B. and Wittenberg, C. (1993). *FAR1* is required for postranscriptional regulation of *CLN2* gene expression in response to mating pheromone. *Mol. Cell. Biol.* **13**, 1013-1022.

Vigers, G. P. A., Anderson, L. J., Caffes, P. and Brandhuber, B. J. (1997). Crystal structure of the type-I interleukin-1 receptor complexed with interleukin-1β. *Nature* **386**, 190-194.

Weinberg, R. A. (1991). Tumor suppressor genes. *Science* **254**:1138-1146.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. *Cell* **81**, 323-330.

Wharton, K. A., Ray, R. P. and Gelbart, W. M. (1993). An activity gradient of *decapentaplegic* is necessary for the specification of dorsal pattern elements in the *Drosophila* embryo. *Development* **117**, 807-822.

Wilson, P. A. and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* **376**, 331-333.

Whittenberg, C., Sugimoto, K. and Reed, S. I. (1990). G1-specific cyclins of *S. cerevisiae*: cell cycle periodicity, regulation by mating pheromone, and association with the p34*CDC28* protein kinase. *Cell* **62**, 225-237.

Wolfel, T., Hauer, M., Schneider, Serrano, M., Wolfel, C., Klehmann-Hieb, E., De Plaen, E., Hankeln, T., Meyer zum Buschenfelde, K-H. and Beach, D. (1995). A p16^{INK4a}-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science* **269**, 1281-1284.

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M. and Wang, E. A. (1988). Novel regulation of bone formation: molecular clones and activities. *Science* **242**, 1528-1534.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F. and Massugue, J. (1994). Mechanism of activation of the TGF-β receptor. *Nature* **370**, 341-347.

Wu, Y. and Han, M. (1994). Suppressors of activated Let-60 Ras protein defines a role of *Caenorhabditis elegans* Sur-1 MAP kinase in vulval differentiation. *Genes Dev.* **8**, 147-159.

Wu, Y., Han, M. and Guan, K-L. (1995). MEK-2, a *Caenorhabditis elegans* MAP kinase kinase, functions in Ras-mediated vulval induction and other developmental events. *Genes Dev.* **9**, 742-758.

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. (1993). p21 is a universal inhibitor of cyclin kinases. *Nature* **366**, 701-704.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.

Zimmerman, L. B., De Jesus-Escobar, J. M. and Harland, R. M. (1996). The Spemann organizer signal *noggin* binds and inactivates bone morphogenetic protein 4. *Cell* **86**, 599-606.

Zusman, S. B., Sweeton, D. and Wieschaus, E. F. (1988). *short gastrulation*, a mutation causing delays in stage-specific cell shape changes during gastrulation in *Drosophila melanogaster*. *Dev. Biol.* **129**, 417-427.

Table 1. Examples cited of biological processes which involve antagonistic signaling systems are listed. Biological process, biological process regulated by antagonistic signals. Positive signal, signal responsible for producing a certain biological effect. Negative signal, signal that acts antagonistically to the positive signal so as to negatively regulate its effect. In several examples, positive and negative signal designations are arbitrary. Mechanism of negative regulation, mechanism, if known, by which the negative signal acts to antagonize, or negatively regulate, the positive signal or to inhibit the processes stimulated by the positive signal.

[a]The mechanism of action of the negative signal is unknown.

Table 1. Summary of reviewed antagonistic signaling systems and mechanisms

Biological Process	Positive Signal	Negative Signal	Mechanism of Negative Regulation
C. elegans vulval development	LIN-3	synMuv genes (class A, class B)	unknown[a]
S.cerevisiae growth	nutrients	mating pheromone	Cdc28-Cln inhibition by Far1
mammalian cell growth	mitogens (EGF, FGF, IGF-I, PDGF, insulin. etc.)	TGF-β	cdk inhibitor
Xenopus dorso-ventral polarity	BMP-4	Chordin, Noggin	titrate positive signal
Drosophila dorso-ventral polarity	Dpp	Sog	titrate positive signal
C. elegans larval development	EGL-15 (receptor)	CLR-1 (receptor?)	phosphatase
Drosophila ommatidial cell fate specification	Spitz	Argos	negative ligand of positive signal receptor
mammalian injury response	IL-1α, IL-1β	IL-1ra	negative ligand of positive signal receptor

Figure 1. Antagonistic signals controlling yeast cell division. (A) Nutrients act as a positive signal to activate a Cln-Cdc28 complex which allows cell division. (B) Mating pheromone acts through a distinct signal transduction cascade to activate Far1 via phosphorylation. Phosphorylated Far1 binds to the Cln-Cdc28 complex, inhibiting it and thereby inhibiting cell division.

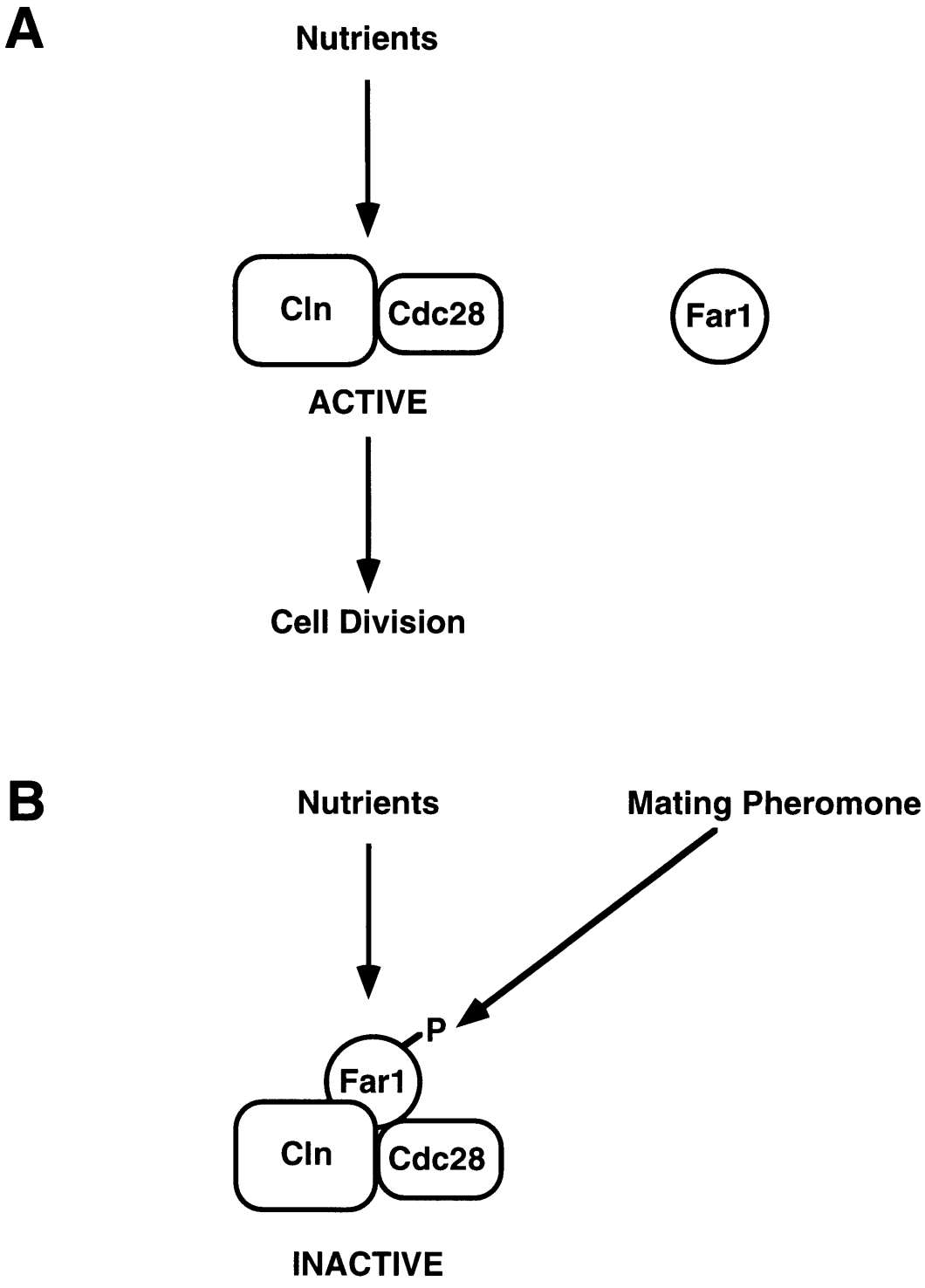

A
Nutrients
Cln
Cdc28
Far1
ACTIVE
Cell Division
B
Nutrients
Mating Pheromone
P
Far1
Cln
Cdc28
INACTIVE

Figure 2. Antagonistic signals controlling mammalian cell division. (A) Mitogen signaling activates Cyclin E-Cdk2 and Cyclin D-Cdk4 complexes which hyperphosphorylate Rb allowing cell division. (B) TGF-β-mediated inhibition in Mv1Lu cells. TGF-β signaling causes p27 to bind Cdk2 and inhibit cyclin-Cdk2 complex activity. *ink4* transcription is activated, leading to the production of p15 which associates with Cdk4 thereby inhibiting the cyclin D-Cdk4 complex. Rb is not hyperphosphorylated and cell division is prevented. (C) TGF-β-mediated inhibition in HaCaT cells. TGF-β signaling activates the transcription of *ink4b* and *cip1*. The protein products, p15 and p21, bind to Cdk-4 and Cdk2, respectively, inhibiting the cyclin-Cdk complexes. This prevents Rb hyperphosphorylation and cell division.

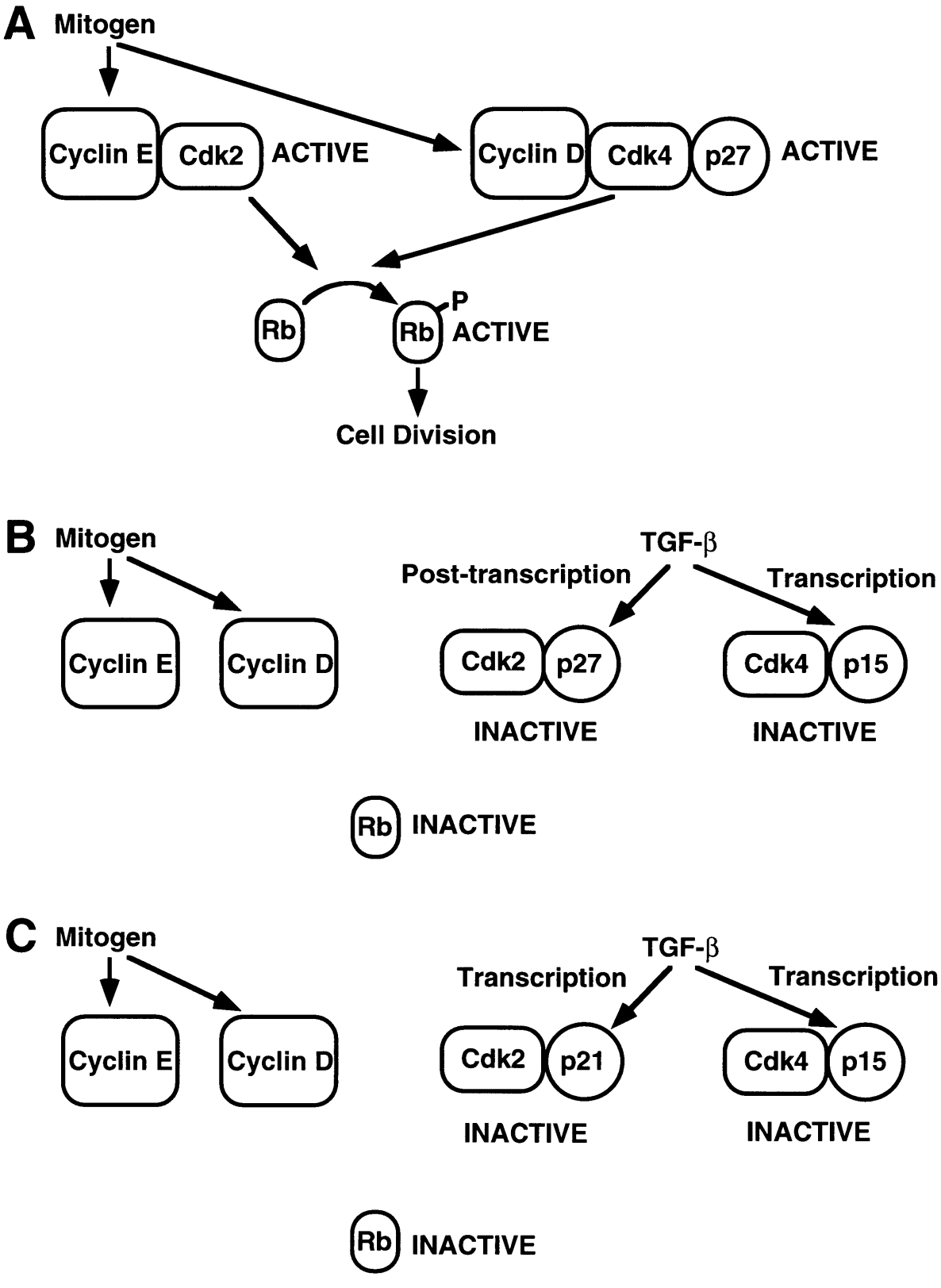
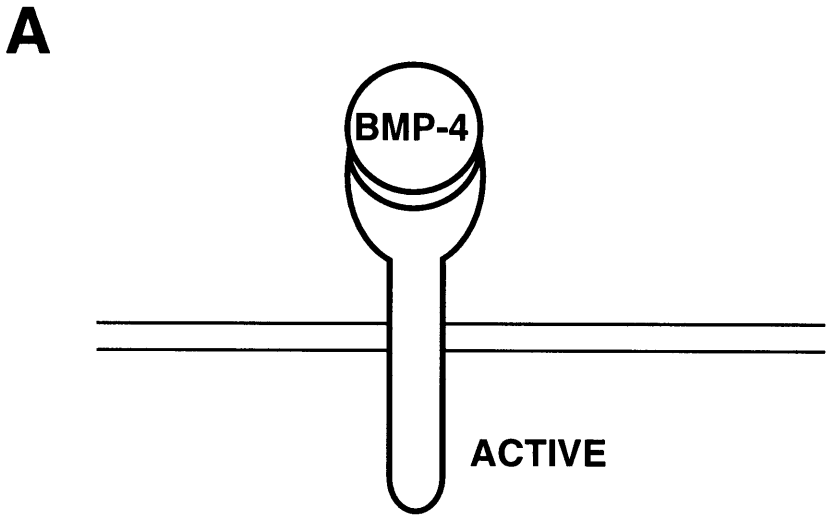

A
Mitogen
Cyclin E
Cdk2
ACTIVE
Cyclin D
Cdk4
p27
ACTIVE
Rb
P
Rb
ACTIVE
Cell Division
B
Mitogen
TGF-β
Post-transcription
Transcription
Cyclin E
Cyclin D
Cdk2
p27
Cdk4
p15
INACTIVE
INACTIVE
Rb
INACTIVE
C
Mitogen
TGF-β
Transcription
Transcription
Cyclin E
Cyclin D
Cdk2
p21
Cdk4
p15
INACTIVE
INACTIVE
Rb
INACTIVE

Figure 3. Negative regulation of a signal transduction system by a negative signal that titrates out the positive signal. *Xenopus* and *Drosophila* dorso-ventral polarity specification use this process. (A) In the absence of negative signal, the positive signal binds and activates the receptor. (B) In the presence of negative signal, the positive signal is bound by the negative signal molecule and is thereby prevented from binding and activating the receptor. Molecules depicted are from *Xenopus* dorso-ventral polarity specification. In *Drosophila*, BMP-4 is replaced by Dpp, and Noggin or Chd is replaced by Sog.


A
BMP-4
ACTIVE

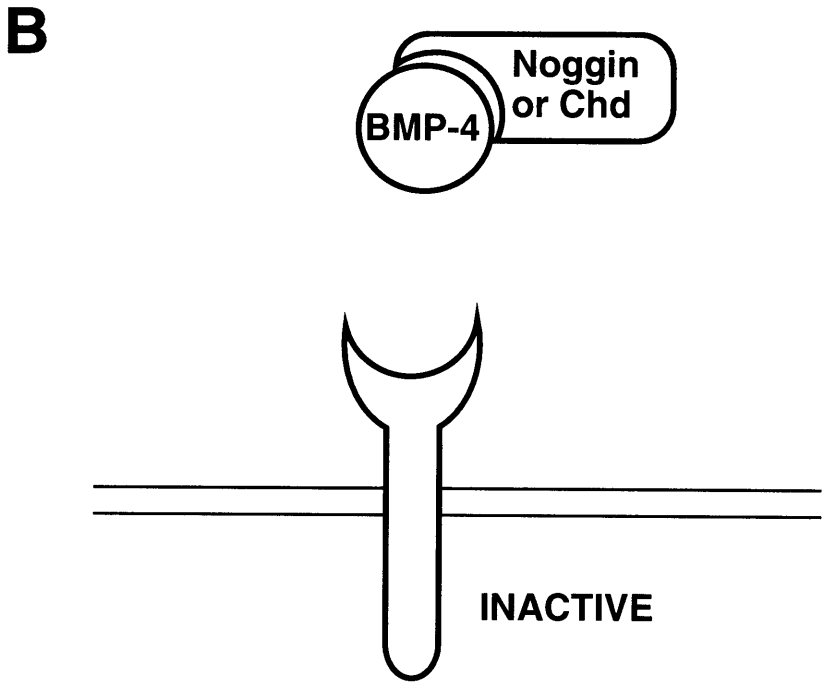

B
Noggin
or Chd
BMP-4
INACTIVE

Figure 4. *C. elegans* larval development regulatory system involving antagonistic signals mediated by the EGL-15 and CLR-1 proteins. (A) In the absence of a negative signal mediated by the CLR-1 receptor, the positive signal activates the EGL-15 receptor which leads to the phosphorylation of a molecule necessary for signal transduction. (B) In the presence of a CLR-1 ligand, CLR-1 dephosphorylates the signal transduction molecule phosphorylated in response to EGL-15 activity. This prevents positive signal transduction.

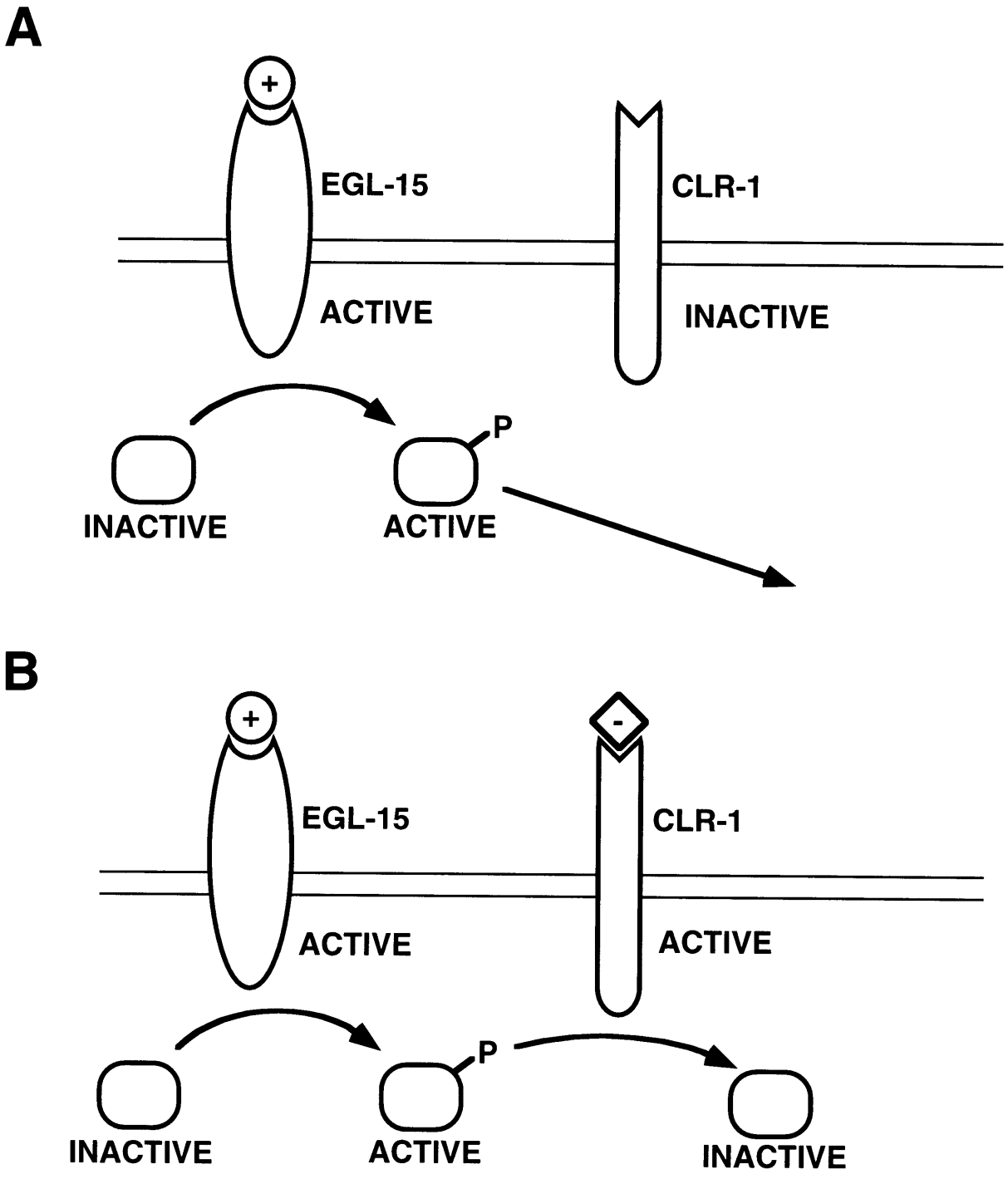
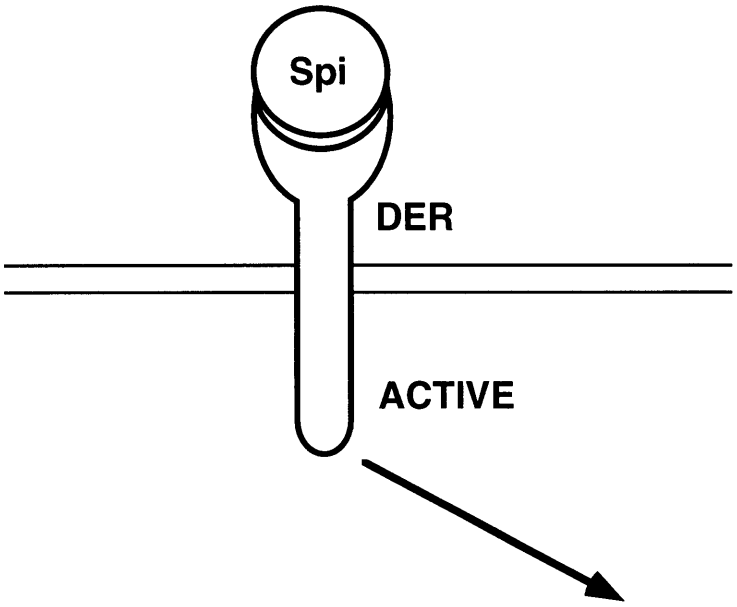
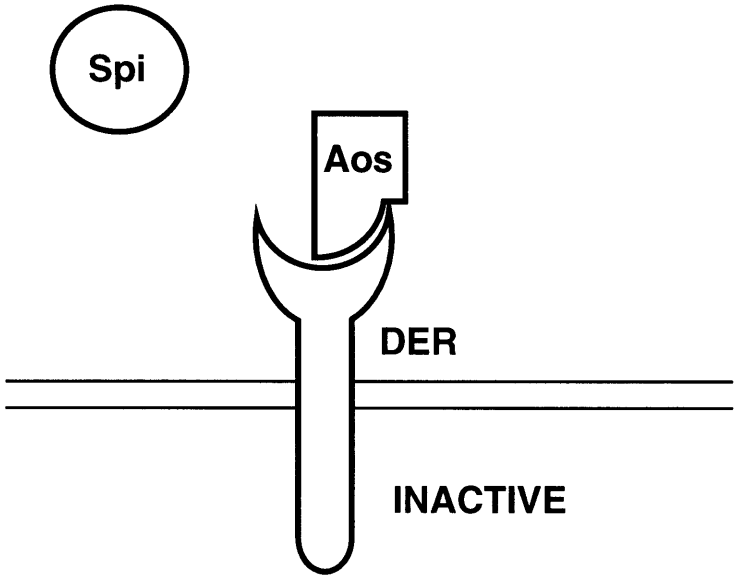

A
+
EGL-15
CLR-1
ACTIVE
INACTIVE
P
INACTIVE
ACTIVE
B
+
-
EGL-15
CLR-1
ACTIVE
ACTIVE
P
INACTIVE
ACTIVE
INACTIVE

Figure 5. Antagonistic signaling by means of a negative ligand for the receptor of the positive signal. (A) In the absence of negative signal, the ligand (positive signal) activates the receptor. In the specification of ommatidial cell fates in the *Drosophila* eye, Spitz is the ligand and DER is the receptor. In IL-1 signaling, IL-1 is the positive ligand that binds the IL-1 receptor. (B) Negative regulation is mediated by a negative ligand molecule which can bind the receptor without activating it and prevent postitive ligand binding. The negative ligand acts as a competitive inhibitor. In *Drosophila* ommatidial development, the negative ligand is Argos; in IL-1 signaling, it is IL-1ra.

A



B



Chapter 2

Isolation and Characterization of Mutants Defective in Two Functionally Redundant Pathways that Act as Negative Regulators of Vulval Development in *Caenorhabditis elegans*

Jeffrey H. Thomas and H. Robert Horvitz

Howard Hughes Medical Institute, Massachusetts Institute of Technology
Cambridge, MA 02139

This chapter will be submitted to *Genetics*

Summary

Previous studies have shown that a synthetic multivulva phenotype results from mutations in interacting genes that negatively regulate the *ras*-mediated intercellular signaling system of *Caenorhabditis elegans* vulval induction. The interaction of synthetic multivulva mutations in two different classes of genes, class A and class B, is required to produce the multivulva phenotype. The multivulva phenotype is produced by the formation of ectopic vulval tissue by vulval precursor cells which in wild type do not form vulval tissue. Mutations in either class alone do not cause a vulval phenotype. In this paper, we describe the isolation and characterization of 49 synthetic multivulva mutations and the identification of six new synthetic multivulva loci.

Introduction

A receptor tyrosine kinase and Ras-mediated signal transduction pathway is essential to the specification of vulval cell fates in the development of the hermaphrodite vulva of *Caenorhabditis elegans*. This signal transduction cascade has been implicated in the development and growth of other organisms and in oncogenesis. However, little is known about the negative regulation of this signal transduction pathway. In oncogenesis such negative regulators act as tumor suppressor genes. The synthetic Multivulva (synMuv) genes perform this role in *C. elegans* vulval development. Thus, these genes provide an opportunity to study the negative regulation of a receptor tyrosine kinase and Ras-mediated signal transduction pathway in a genetically tractable model system.

The hermaphrodite vulva of *Caenorhabditis elegans* is formed from the descendents of three hypodermal blast cells, P(5,6,7).p (Sulston and Horvitz, 1977). These cells are part of the vulval equivalence group, P(3-8).p, a set of six cells which have the potential to adopt either one of two vulval fates (1° or 2°) or a nonvulval fate (3°) (Sulston and White, 1980; Kimble, 1981; Sternberg and Horvitz, 1986). These cell fates are specified by several cell interactions (for review, see Horvitz and Sternberg, 1991; Eisenmann and Kim, 1994; Sundaram and Han, 1996). The gonadal anchor cell signals the nearest Pn.p cells to adopt vulval fates. A signal from the anchor cell is at least partly responsible for establishing the proper pattern of vulval fates (Kimble, 1981; Sternberg and Horvitz, 1986; Thomas et al., 1990; Simske and Kim, 1995; Katz et al., 1995). In addition to the influence of the gonadal anchor cell signal on the pattern of vulval cell fates, another signal is propagated amongst the induced Pn.p cells to prevent the formation of adjacent primary cells (Sternberg, 1988). Another signal, presumably emanating from the hypodermal syncytium, inhibits the adoption of vulval fates (Herman and Hedgecock, 1990). It is likely that the anchor cell signal overrides this inhibitory signal to induce the cells nearest to the uterus to adopt vulval fates.

Genetic dissection of vulval development has led to the identification and characterization of numerous genes involved in different aspects of this process (for review, see Horvitz and Sternberg, 1991; Eisenmann and Kim, 1994; Sundaram and Han, 1996). Many of these genes encode proteins implicated in signal transduction. Among those involved in the transduction of the inductive signal from the anchor cell are: *lin-3*, an EGF-like molecule (Hill and Sternberg, 1992), *let-23*, a receptor tyrosine kinase (Arioan et al., 1990), *sem-5*, an adaptor molecule (Clark et al., 1992), *let-60*, a *ras* GTPase (Han and Sternberg, 1990; Beitel et al., 1990), *lin-45*, a *raf* serine/threonine kinase (Han et al., 1993), *mek-2*, a mitogen-activated protein (MAP) kinase kinase

(Kornfeld *et al.*, 1995a; Wu et al., 1995), *mpk-1/sur-1*, a MAP kinase (Lackner et al., 1994, Wu and Han, 1994), and *ksr-1*, a protein kinase (Kornfeld et al., 1995b).

Many loss-of-function mutations in the genes described above result in a vulvaless (Vul) phenotype in which the vulva is not formed. Mutations in some other genes result in a multivulva (Muv) phenotype in which ectopic vulva tissue is produced. The multivulva (Muv) phenotype of certain mutant strains results from the interaction of two different mutations (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985; 1989). The mutations that interact to produce such a synthetic multivulva (synMuv) phenotype fall into two classes: A and B. Animals carrying both a class A and a class B mutation have a Muv phenotype. Animals that carry mutations in a single class have a wild-type vulval phenotype. Ferguson and Horvitz (1989) have proposed that the synMuv genes encode the components of two functionally redundant pathways that negatively regulate vulval development.

SynMuv mutants were discovered in the first screen for mutations that produced lineage defects. The mutant strain CB1322 had a Muv phenotype that was found to require the presence of two unlinked mutations, *lin-8(n111)* and *lin-9(n112)*. Neither of these mutations caused a vulval phenotype when alone (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981). Systematic mutagenesis of strains carrying either *lin-8(n111)* or *lin-9(n112)* and the mutagenesis of another strain carrying a previously undetected class A synMuv mutation in the background allowed the identification of additional mutations that behaved like *lin-8(n111)* (class A) or *lin-9(n112)* (class B). Several of these mutations failed to complement *lin-15* for either a class A or a class B activity (Ferguson and Horvitz, 1989). *lin-15* had been previously identified by alleles that produced a Muv phenotype (Ferguson and Horvitz, 1985). These genetic analyses suggested that *lin-15* is a complex locus that contained genetically separable class A and class B activities (Ferguson and Horvitz, 1989). Subsequent molecular analyses of *lin-15* revealed that *lin-15* encodes two nonoverlapping transcripts that encode the A and B activities (Clark et al., 1994; Huang et al., 1994). These genetic analyses resulted in the identification and characterization of three class A genes (*lin-8*, *lin-38* and *lin-15* A) and five class B genes (*lin-9*, *lin-35*, *lin-36*, *lin-37* and *lin-15* B). Three additional class B mutations were identified and found to complement mutations in the five class B genes and to complement each other, but were not further characterized (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1989).

SynMuv mutants in which the anchor cell has been ablated still display a Muv phenotype (Ferguson et al., 1987). This result suggests that in the absence of synMuv gene activity, the Pn.p cells do not require the anchor cell signal to adopt vulval cell fates.

Genetic mosaic analyses indicate that both *lin-15* AB and *lin-37* act cell non-autonomously and most likely in the hypodermal syncytium (Herman and Hedgecock, 1990; Hedgecock and Herman, 1995). These data suggest that the synMuv genes encode the components of two redundant signaling systems that inhibit the adoption of vulval fates by the Pn.p cells. Loss-of-function mutations in genes known to be involved inductive signal transduction, *let-23*, *sem-5*, *let-60 ras*, and *lin-45 raf* are epistatic to *lin-15* AB mutations (Ferguson et al., 1987; Beitel et al., 1990; Han et al., 1990, Clark et al., 1992; Han et al., 1993; Huang et al., 1994). Thus, the inductive signal transduction system, but not the anchor cell signal itself, is required for the adoption of vulval cell fates by Pn.p cells in the absence of inhibitory synMuv gene activity.

The molecular nature of the inhibitory signals encoded by the synMuv genes is unknown. Three genes, *lin-15* A, *lin-15* B and *lin-9* have been cloned and shown to encode novel proteins (Clark et al., 1994; Huang et al., 1994; G. Beitel and H. R. Horvitz, in preparation).

The synMuv genes provide an opportunity to study the negative regulation of receptor tyrosine kinase and Ras-mediated signal transduction pathways. Very little is known about the negative regulation of this signal transduction pathway. To provide the necessary reagents for an extensive study of the negative regulation of signal transduction in *C. elegans*, we conducted several screens to isolate more synMuv mutants. These mutants provide new alleles of previously identified synMuv genes which will facilitate the genetic and molecular analysis of these known genes. The screens also provided alleles that define new genes involved in the synMuv pathways.

In this paper, we identify and characterize 49 additional synMuv mutations. We define six new synMuv loci, including three which had been previously isolated by Ferguson and Horvitz (1989), but not completely characterized. These six loci include five class B loci and one class A locus which are demonstrated to be synMuv loci.

Materials and Methods

Strains and general techniques

Caenorhabditis elegans var. Bristol strain N2 was used as the wild-type strain and parent of all of the mutants used in this study. The alleles used in this study have been described by Hodgkin et al. (1988) unless otherwise noted.

LGI: *bli-3(e767)*; *sup-11(n403)*; *dpy-5(e61)*; *lin-35(n745)* (Ferguson and Horvitz, 1989); *unc-29(e1072)*; *dpy-14(e188)*; *unc-13(e1091)*; *lin-11(n566)*; *unc-75(e950)*; *unc-101(m1)*; *unc-54(e1092)* (Waterston et al., 1980).

LGII: *lin-8(n111)* (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985; 1989); *unc-85(e1414)*; *bli-2(e768)*; *dpy-10(e128)*; *rol-6(e187)*; *let-23(n1045, mn23, mn216)* (Herman, 1978; Sigurdson et al., 1984; Ferguson and Horvitz, 1985); *let-240(mn209)*; *unc-4(e120)*; *unc-53(e569)*; *rol-1(e91)*; *lin-38(n751)* (Ferguson and Horvitz, 1989); *unc-52(e444)*; *mnDf67* (Sigurdson et al., 1984); *mnDf85* (Sigurdson et al., 1984); *mnDf46* (Sigurdson et al., 1984); *mnDf71* (Sigurdson et al., 1984); *mnC1 dpy-10(e128) unc-52(e444)*.

LGIII: *dpy-1(e1)*; *daf-2(e1370)*; *unc-93(e1500)*; *dpy-27(y57)* (Plenefisch et al., 1989); *unc-79(e1068)*; *dpy-17(e164)*; *lon-1(e185)*; *sma-3(e491)*; *lin-37(n758)* (Ferguson and Horvitz, 1989); *egl-5(n945)*; *lin-36(n766)* (Ferguson and Horvitz, 1989); *unc-36(e251)*; *dpy-19(e1259)*; *lin-9(n112)* (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985; 1989); *unc-32(e189)*; *unc-16(e109)*; *unc-69(e587)*; *unc-25(e156)*; *unc-49(e382)*; *dpy-18(e364)*; *qC1* (Austin and Kimble, 1989; Graham and Kimble, 1993).

LGIV: *dpy-9(e12)*; *egl-18(n162)*; *unc-17(e245)*; *unc-5(e53)*; *dpy-20(e1282)*; *unc-22(e66)*; *unc-30(e191)*; *lev-1(x22)*; *ced-3(n717)*; *unc-26(e205)*; *dpy-4(e1166)*.

LGV: *unc-34(e566)*; *dpy-11(e224)*; *unc-51(e369)*.

LGX: *lon-2(e678)*; *unc-3(e151)*; *lin-15(n433, n744, n765, n767)* (Ferguson and Horvitz, 1985; 1989).

Methods for the culture and genetic manipulation of *C. elegans* have been described by Brenner (1974).

Genetic nomenclature used conforms to the standardized *C. elegans* nomenclature established by Horvitz et al. (1979). The assignment of *lin* (*lin*eage abnormal) gene names to new synthetic Multivulva genes and other nomenclature used to describe synthetic Multivulva strains has been established (Ferguson and Horvitz, 1989).

Mutagenesis of class A and class B mutants

Screens for new synthetic Multivulva strains were conducted essentially as described by Ferguson and Horvitz (1989). To isolate mutations in one class, L4 hermaphrodites carrying a single mutation of the other class were mutagenized with ethyl methanesulfonate (EMS). Two P_0 hermaphrodites were placed on a single plate and allowed to lay eggs. A total of 40 F_1 eggs was selected from each plate and picked, 20 per plate, to two plates. The F_2 generation was screened for the presence of Muv animals. These Muv animals were picked individually to plates and allowed to self-fertilize to generate a mutant strain. Only one Muv strain was selected from each pair of plates for subsequent analysis to ensure each mutation was independently derived. N2

males were crossed into the Muv strains and the F_2 progeny were scored for the Muv phenotype. Those that segregated 1/16 or less were selected as candidate synMuv strains. None of the Muv strains in which the original mutation was autosomal resulted from a synthetic interaction with a second mutation on the same linkage group. In mutagenesis experiments in which the original mutation was *lin-15*, strains that segregated 1/4 or less were also retained for further analysis. All candidate synMuv strains were backcrossed to their strain of origin two to five times.

To isolate class B mutations, a *lin-8(n111)* homozygous strain and a *lin-15(n433)* homozygous strain were mutagenized. Ferguson and Horvitz (1989) had previously mutagenized *lin-8(n111)* and *lin-15(n767)* to isolate class B mutations. In the mutagengenesis of *lin-8(n111)* described in this paper, approximately 6000 haploid genomes were screened and 15 synMuv strains were isolated. In the mutagenesis of *lin-15(n433)*, approximately 10,000 haploid genomes were screened and 15 synMuv strains were isolated. To isolate class A mutations, a *lin-36(n766)* homozygous strain and a *lin-15(n744)* homozygous strain were mutagenized. Ferguson and Horvitz (1989) had previously mutagenized *lin-9(n112)* to isolate class A mutations. In the mutagengenesis of *lin-36(n766)*, approximately 10,000 haploid genomes were screened and 5 synMuv strains were isolated. In the mutagenesis of *lin-15(n744)*, approximately 13,000 haploid genomes were screened and 15 synMuv strains were isolated.

Analysis of *lin-15* AB lesions

Genomic DNA was purified, essentially using standard methods, from *lin-15* AB strains isolated in the mutageneses of *lin-15(n744)* and *lin-15(n433)* (Sulston and Hodgkin, 1988). DNA was digested by *Eco*RI, separated by agarose gel electrophoresis, and probed with ^{32}P-labeled *lin-15* plasmid DNA (Sambrook et al., 1989; Clark et al., 1994). Some samples of genomic DNA which showed a lesion were digested with *Eco*R1 and either *Eag*I, *Sac*I, *Msc*I, *Bgl*I, *Mlu*I or *Nru*I, and probed with ^{32}P-labeled *lin-15* plasmid DNA.

Nomarski observation and Pn.p cell lineage analysis of *lin-54* animals

P(3-8).p cells and their descendents in *lin-8(n111); lin-54(n2231)* animals were observed using Nomarski optics at different times during vulval development. P(3-8).p cell lineages were observed using Nomarski optics as described by Sulston and Horvitz (1977). The nomenclature and criteria described by Sternberg and Horvitz (1986, 1989) were used to assign 1°, 2° and 3° cell fates.

Construction of strains homozygous for isolated new synMuv mutations

Chromosomes carrying the synMuv mutation to be isolated and homozygosed in the absence of another synMuv mutation were balanced by recessive markers that mapped very close to the synMuv mutation to be isolated. Males of genotype *r/+*, where *r* is one or more linked recessive markers, were crossed into synMuv strains of genotype *a; b*, where *a* is the mutation to be isolated and *b* is the original mutation. Progeny of genotype *a* +/+ *r; b/+* were isolated by picking L4 larvae that were non-Muv as adults and segregated R progeny, where R is the phenotype of strains of *r/r* genotype. Progeny of phenotype non-Muv non-R were picked individually to plates. The non-Muv progeny of animals that segregated 1/4 R and did not segregate Muv (genotype: *a* +/+ *r*, +/+) were picked individually to plates. Those that did not segregate R animals were of putative genotype *a/a*. This was confirmed by mating males of genotype *b/b* into the *a/a* candidates, picking at least 12 L4 larvae individually to plates and observing the segregation of Muv animals from each animal. In these experiments, *unc-79 dpy-27* balanced *lin-51(n770)*, *unc-69* balanced *lin-52(n771)*, *unc-29* balanced *lin-53(n833)*, *unc-22 ced-3 unc-26* balanced *lin-54(n2231)*, and *rol-6 unc-4* balanced both *lin-55(n2994)* and *lin-56(n2728)*.

Construction of unlinked synMuv double mutants

Class A; class B double mutants were constructed by crossing male *lin-8(n111)* and male *lin-15(n767)* animals into strains homozygous for a class B mutation (*lin-51(n770)*, *lin-52(n771)*, *lin-53(n833)*, *lin-54(n2231)*, and *lin-55(n2994)*) or male *lin-15(n744)* animals into a strain homozygous for a class A mutation (*lin-56(n2728)*). F_2 Muv progeny isolated were of genotype *a/a; b/b*.

To construct class A; class A or class B; class B double mutants carrying a new mutation and a *lin-15* mutation of the same class, N2 males were crossed into a strain of genotype *b1 r1*; *lin-15a* where *b1* is the new mutation (either class A or class B), *r1* is a tightly linked recessive marker in *cis*, and *lin-15a* is a *lin-15* mutation of the opposite class of *b1*. Male progeny of genotype *b1 r1/+ +*; *lin-15a/0* were crossed into animals of genotype *lin-15b* where *lin-15b* is a *lin-15* mutation of the same class as *b1*. Male progeny of this cross, half of which were of genotype *b1 r1/+ +; lin-15b/0* were crossed into animals of genotype *lin-15b*. Some progeny of this cross segregated R1 animals which were of putative genotype *b1 r1; lin-15b*. To ensure that a strain was isolated in which mutations were not lost by recombination, several independent lines were isolated for each strain.

To construct class A; class A or class B; class B double mutants carrying a new mutation and an autosomal mutation of the same class, N2 males were crossed into a strain of genotype *b1 r1; lin-15a*. Male progeny of genotype *b1 r1/+ +; lin-15a/0* were crossed into animals of genotype *b2 r2* where *b2* is an autosomal mutation of the same class as *b1*. Male progeny of this cross, half of which were of genotype *b1 r1/+ +; b2 r2/+ +; +/0* were crossed into animals of genotype *b2 r2*. Some R2 progeny of this cross segregated R1 R2 animals which were of putative genotype *b1 r1; b2 r2*. To ensure that a strain was isolated in which mutations were not lost by recombination, several independent lines were isolated for each strain.

In these constructions, *lin-15(n767)* and *lin-15(n744)* were used as the class A and class B *lin-15* alleles respectively. The autosomal class B mutation used was *lin-36(n766)*. This allele was marked in *cis* by *unc-32*. The following markers were linked in *cis* to the new mutations: *unc-32* to *lin-51(n770)*, *unc-32* to *lin-52(n771)*, *dpy-5* to *lin-53(n833)*, *dpy-20* to *lin-54(n2231)*, *rol-6* to *lin-55(n2994)*, and *rol-6* to *lin-56(n2728)*.

Construction of linked synMuv double mutants

The *lin-8 lin-55(n2994)* class A class B double mutant strain was isolated by crossing *lin-8* males to *lin-8 unc-85 dpy-10* hermaphrodites, picking male progeny and mating these males to animals of genotype *lin-55* and isolating heterozygotes of genotype *lin-8 unc-85 dpy-10 +/+ + + lin-55*. Unc non-Dpy recombinant progeny were picked and allowed to segregate 1/4 Unc Muv animals of genotype *lin-8 unc-85 lin-55*.

To construct a class B class B double between *lin-51(n770)* and *lin-36,* N2 males were crossed into an *egl-5 lin-36; lin-15* A strain. Progeny males were mated to hermaphrodites of genotype *lin-51 unc-32; lin-15* A, non-Unc progeny which segregated Egl animals were selected. These animals were of putative genotype *lin-51 + + unc-32/+ egl-5 +; lin-15* A. The frequency of recombination between *lin-51* and *egl-5* is greater than that between *lin-36* and *unc-32* and much greater than that between *egl-5* and *lin-36*. Muv non-Unc recombinant progeny of putative genotype *lin-51 egl-5 lin-36 +/lin-51 + + unc-32; lin-15* A were isolated. These recombinants segregated Muv, Muv Unc and Egl Muv animals. N2 males were crossed to the the Egl Muv animals of putative genotype *lin-51 egl-5 lin-36; lin-15* A and Egl non-Muv F_2 animals were isolated and picked to individual plates. Animals that did not segregate Muv progeny were of putative genotype *lin-51 egl-5 lin-36*. This genotype was confirmed by crossing N2 males into these animals and then crossing the male progeny into *lin-15* A. Half of the male progeny of this cross were of putative genotype *lin-51 egl-5 lin-36/+ + +; lin-15* A*/0*. These males

were used in complementation tests with *lin-51* and with *lin-36* to show that the strain contained both class B mutations.

A class B class B double between *lin-36* and *lin-52(n771)* was constructed in a manner similar to that for the construction of the double between *lin-51* and *lin-36*. Males of genotype *lin-36 unc-36/+ +; lin-15* A were mated to *sma-3 lin-52; lin-15* A hermaphrodites to generate heterozygotes of putative genotype + *lin-36 unc-36 +/sma-3 + + lin-52; lin-15* A. The frequency of recombination between *unc-36* and *lin-52* is greater than that between *sma-3* and *lin-36* and much greater than that between *lin-36* and *unc-36*. Muv non-Sma recombinant progeny of putative genotype + *lin-36 unc-36 lin-52/sma-3 + + lin-52; lin-15* A were isolated. These animals segregated Muv, Sma Muv and Unc Muv progeny which were of putative genotype *lin-36 unc-36 lin-52; lin-15* A. N2 males were mated to these animals and F_2 Unc non-Muv animals which did not segregate Muv animals were selected. These animals were of putative genotype *lin-36 unc-36 lin-52*. This genotype was confirmed by crossing male heterozygotes through *lin-15* A and using the male F_2 progeny in complementation tests with *lin-36* and with *lin-52*. These tests showed that the isolated strain was of genotype *lin-36 unc-36 lin-52*.

To construct a class A class A double between *lin-8* and *lin-56(n2728)*, *lin-8* males were mated to hermaphrodites of genotype *lin-8 unc-85 dpy-10; lin-9*, and non-Unc non-Dpy cross progeny of genotype *lin-8 + + +/lin-8 unc-85 dpy-10; lin-9/+* were isolated. Unc Dpy non-Muv animals which did not segregate Muv animals were of genotype *lin-8 unc-85 dpy-10*. Males of *lin-8* genotype were mated to these animals to generate males of genotype *lin-8 unc-85 dpy-10/lin-8 + +* which were crossed to hermaphrodites of genotype *rol-6 lin-56; lin-15* B. Cross progeny (non-Rol) which segregated Unc Dpy animals were of genotype *lin-8 unc-85 dpy-10 + +/+ + + rol-6 lin-56; lin-15* B/+. Unc non-Dpy non-Muv recombinant animals which did not segregate Muv progeny were isolated; these were of putative genotype *lin-8 unc-85 + rol-6 lin-56/lin-8 unc-85 dpy-10 + +*. Rol Unc segregants were of putative genotype *lin-8 unc-85 rol-6 lin-56*. To confirm the presence of *lin-8*, N2 males were mated to these animals and the progeny heterozygous males were mated to *lin-8 unc-85 dpy-10; lin-9* hermaphrodites. Unc non-Dpy progeny were allowed to self-fertilize and Unc Muv animals of putative genotype *lin-8 unc-85 + rol-6 lin-56/lin-8 unc-85 dpy-10 + +; lin-9* were picked individually to plates. This genotype was confirmed by the segregation of Unc Muv, Dpy Unc Muv and Unc Rol Muv progeny. The presence of *lin-56* was confirmed by crossing the heterozygous males to *rol-6 lin-56; lin-15* B hermaphrodites and allowing the Rol nonMuv progeny to self-fertilize. Rol Muv progeny, 2/3 of which were of putative genotype *lin-8 unc-85 rol-6 lin-56/+ + rol-6 lin-56; lin-15* B, were

picked individually to plates. This genotype was confirmed by the segregation of Rol Muv and Unc Rol Muv.

Results

Isolation of strains containing new class A and new class B mutations

We sought to identify new synthetic Multivulva mutations using the protocol of Ferguson and Horvitz (1989). To identify new class A mutations, we mutagenized *lin-36(n766)* and *lin-15(n744)* homozygotes, which display wild-type vulval development. Class A mutations interact with these class B mutations to produce a synthetic Multivulva phenotype (Ferguson and Horvitz, 1989). The morphology of synMuv strains can be readily distinguished from the morphology of Muv strains mutant in *lin-1* and *lin-31*. Muv strains that segregated as two unlinked loci when crossed with wild type males were candidates for being synMuv strains. Certain strains obtained in a *lin-15* background that did not fit this criteria were also possible candidates (see below). We obtained five synMuv strains from the mutagenesis of *lin-36(n766)* and 14 synMuv strains from the mutagenesis of *lin-15(n744)*. A total of 19 new class A mutations were identified in these screens (Table 1).

To identify new class B mutations, we mutagenized animals homozygous for the class A mutations *lin-8(n111)* and *lin-15(n433)*. Muv strains were tested for segregation of two loci as described for the isolation of class A mutations. We obtained 15 synMuv strains from the mutagenesis of *lin-8(n111)* and 15 synMuv strains from the mutagenesis of *lin-15(n433)*. A total of 30 new class B mutations were identified (Table 1).

Linkage and complementation

SynMuv mutations were expected to display linkage to two loci: the parental locus and the new locus (Ferguson and Horvitz, 1989). Mutations caused by most of the candidate synMuv strains displayed linkage to the parental mutation and to another linkage group. A few *lin-15* strains, discussed below, did not display linkage to a new location (Table 1).

The newly isolated synMuv strains were tested for complementation with the known synMuv genes: *lin-8*, *lin-9*, *lin-15* A, *lin-15* B, *lin-35*, *lin-36*, *lin-37*, and *lin-38* (Ferguson and Horvitz, 1989). Mutations that complemented all of these genes were tested against *lin(n770)*, *lin(n771)*, and *lin(n833)*, three previously identified but not extensively characterized mutations (Ferguson and Horvitz, 1989). Mutations that did

not fall into these complementation groups were tested against each other after strains carrying identical parental mutations of the opposite class were constructed.

Mutations were assigned to the same complementation group only if hermaphrodites of genotype *a; b1/b2*, where *a* is the background mutation required for the synthetic interaction and *b1* and *b2* are the two mutations being tested, were Muv and segregated only Muv progeny. As described by Ferguson and Horvitz (1989), this approach was necessary to distinguish intragenic noncomplementation from the intergenic noncomplementation observed in some doubly heterozygous synMuv strains. Several combinations of genotype *a; b1/+; b2/+* displayed intergenic noncomplementation; in most cases, the penetrance and expressivity of the Muv phenotype produced by intergenic noncomplementation was lower than that produced by homozygosity at either of the two loci which displayed intergenic noncomplementation. Thus, animals lacking the activity of one class of synMuv genes and having reduced doses of two genes of the other class as well as no maternal activity from one of these genes are occasionally Muv. This suggests that the synMuv genes are dose-sensitive and may not be completely redundant. *lin-53(n833)* was notable in that it showed very strong intergenic noncomplementation with other class B mutations. It is likely that lin-53 mutations act differnetly from the other class B synMuv mutations. *lin-53* activity may be very sensitive to dosage reduction or *lin-53* mutations may have an interfering activity that is enhanced in a background of reduced class B gene dosage.

A total of 38 mutations failed to complement defined synMuv genes. These included eight *lin-8* alleles, eight *lin-15* A alleles, 10 *lin-15* B alleles, six *lin-35* alleles, three *lin-36* alleles, one *lin-37* allele, and two *lin-38* alleles. Another six mutations failed to complement *lin-51(n770)* and *lin-53(n833)*, mutations that had previously been isolated but not extensively characterized. These include five *lin-51* alleles and one *lin-53* allele. Another four mutations defined three new complementation groups: *lin-54*, *lin-55*, and *lin-56*. There were two *lin-54* alleles, one *lin-55* allele, and one *lin-56* allele (Table 1).

Identification of *lin-15* AB double mutants

The mutations of several Muv strains isolated in a *lin-15* background did not segregate as two loci, yet displayed a Muv phenotype similar to that displayed by other synMuv strains. These included five strains isolated in a *lin-15(n433)* background and seven strains isolated in a *lin-15(n744)* background. These strains showed linkage only to *unc-3 X* which marked the parental *lin-15* mutation, and failed to complement *lin-*

15(n765), a *lin-15* allele defective in both class A and class B activities. Thus, the new strains are defective in both *lin-15* A and *lin-15* B activities.

lin-15 is a complex locus which contains genetically separable class A and class B activities (Ferguson and Horvitz 1989). This locus has been cloned and shown to encode two nonoverlapping transcription units under the control of the same promotor. One of these genes encodes the LIN-15 A protein and the other encodes the LIN-15 B protein (Clark et al., 1994; Huang *et al.*, 1994). All *lin-15* Muv mutants that have been analyzed, except *lin-15(n765)*, have gross mutations that disrupt both of the *lin-15* transcripts (Clark et al., 1994; Huang et al., 1994). *lin-15(n765)* is a deletion in the class B transcript and presumably a second point mutation in the A transcript. Several *lin-15* A and *lin-15* B mutations have been analyzed; none of these is a gross mutation that affect both transcription units (Clark et al., 1994).

To determine whether the Muv phenotype of the each of the *lin-15* AB mutants isolated in these screens is the result of only the newly induced mutation or the result of an interaction between the newly induced mutation and the parental mutation, we used Southern hybridization to analyze the *lin-15* locus in these strains. Four mutant strains show polymorphisms; three of these four are confined to only the A region or the B region. The *lin-15(n2993 n433)* strain has a polymorphism: the loss of an *Eco*RI site in the B region of *lin-15*. This is consistent with a second mutation affecting only the B locus; the Muv phenotype likely results from the interaction of this new mutation with the parental mutation. The *lin-15(n744 n2733)* strain has a small deletion of 0.3 kb in the A region of *lin-15*. The *lin-15(n744 n2735)* strain has a larger deletion of several kilobases in the A region. The Muv phenotype of these strains is also likely to result from the interaction of the new mutation, a deletion in only in one transcript region, with the parental mutation of the other class.

The *lin-15(n744 n2726)* strain has a deletion of about 0.9 kb in an *Eco*RI *Sac*I restriction fragment containing both A and B sequences. This region includes both the start of the class A transcript and the end of the class B transcript. It is likely that the deletion eliminates the 5' end of the class A transcriptional unit. It is possible that some of the 3' end of the class B transcriptional unit is also eliminated. This may be sufficient to give a class B defect. Thus the Muv phenotype in this strain may result entirely from the new mutation.

Polymorphisms were not detected in the other strains. Since the parental mutation of these strains is either a *lin-15* A or a *lin-15* B point mutation, and EMS produces predominantly point mutations, it is likely that the Muv phenotype of most, if not all,

these *lin-15* AB strains is the result of an interaction between class A and class B point mutations.

Phenotypes of newly isolated synMuv strains

Many of the newly isolated synMuv strains display a temperature-sensitive effect on vulval development such that the penetrance of the Muv defect increases at higher temperatures (Table 2). Similar observations were made by Ferguson and Horvitz (1989). The synMuv strains often show temperature-sensitive growth characteristics. Many strains show a reduced growth rate, or in some cases, lethality at 25° (Table 2). Similar observations were made on synMuv strains isolated by Ferguson and Horvitz (1989). Although there are some differences between strains in strain growth at 20°, the differences between these strains are much greater at 15° and 25°. Rare animals display a protruding excretory pore. Some animals rupture at the vulva, or rarely at a pseudovulval protrusion, as adults. Strains isolated in the screen in which *lin-15(n744)* was used as the parental mutation have a fairly high percentage of rupture, often exceeding 50% of adults.

Strains carrying *lin-54* mutations differ from other synMuv strains in that a greater proportion of these animals have a ventral protrusion that is more posterior to the vulva than is the case in most synMuv mutants. A number of these strains have two ventral protrusions posterior to the vulva, a rare occurrence in synMuv strains. This phenomenon is observed in both alleles of *lin-54*. In *lin-8(n111); lin-54(n2231)*, 13% of the animals have a relatively far posterior ventral protrusion and 11% of the animals have two posterior ventral protrusions (n=126). In *lin-54(n2231); lin-15(n767)*, 26% of animals have a far ventral protrusion and 19% of animals have two ventral protrusions (n=75). For *lin-54(n2990); lin-15(n433)* animals, 13% have a far ventral protrusion and 10% have two ventral protrusions (n=112).

Observation of the Pn.p cells and their descendents and lineage analysis of the Pn.p cells of *lin-8(n111); lin-54(n2231)* animals confirmed that the vulval equivalence group is composed of P(3-8).p, as in the wild type. Thus, the vulval equivalence group was not expanded to include P9.p as it is in certain mutants which enhance the *lin-15* Muv phenotype (Clark, 1992). Lineage analysis shows that the vulva is sometimes formed from P(4-6).p rather than P(5-7).p (Data not shown). This phenomenon has been observed in other synMuv strains, but is rare (Ferguson et al., 1987; Chapter 3). In these animals, both P7.p and P8.p are posterior to the developing vulva. These cells adopt vulval fates in the mutant animals and form either two posterior pseudovulvae or one posterior pseudovulva that has a greater relative distance from the misplaced vulva than does a pseudovulva formed from only P8.p relative to a properly positioned vulva. In one

case, the vulva was observed to form from P5.p, P6.p and part of P7.p. Some descendents of P7.p formed a pseudovulva with some P8.p descendents whereas other P8.p descendents formed a second posterior pseudovulva.

New complementation groups define synMuv genes

This study has resulted in the identification and characterization of six new synMuv genes: *lin-51*, *lin-52*, *lin-53*, *lin-54*, *lin-55* and *lin-56*. Mutations in three of these genes, *lin-51(n770)*, *lin-52(n771)* and *lin-53(n833)* were isolated previously (Ferguson and Horvitz 1989). The results from multiple-factor crosses and deficiency tests (Table 3) were used to position these six genes on their linkage groups (Figure 1).

lin-55 maps to the same interval as *let-23*, which encodes a receptor tyrosine kinase involved in inductive vulval signaling (Arioan et al. 1990). To determine if *lin-55(n2994)* is allelic to *let-23*, we performed complementation tests against *let-23(mn23)*, *let-23(mn216)* and *let-23(n1045)*. *lin-55(n2994)* complemented *let-23* for all phenotypes: Muv, Vul, hyperinduced (Hin) in which Pn.p cells adjacent to the developing vulva also adopt vulval fates, and lethal (Let). The allele used in these tests, *lin-55(n2994)*, is predicted to fail to complement a loss-of-function allele for the synMuv phenotype, since *lin-55(n2994)/mnDf67; lin-15* A animals are Muv (Table 3).

Two of the newly isolated synMuv genes, one class A gene and one class B gene, are represented by only one mutant allele. To test whether the phenotypes produced by these alleles are weaker than that expected from a null phenotype, these mutations were each tested in *trans* to a deficiency (Table 3). Animals of genotype *lin-56(n2728)/mnDf71; lin-15(n744)* had a Muv phenotype indistinguishable from that of animals of genotype *lin-56(n2728); lin-15(n744)*. The *trans*-deficiency heterozygotes were fertile and healthy. These results suggest that *lin-56(n2728)* is not a weak allele of a locus that has a stronger null phenotype. Animals of genotype *lin-55(n2994)/mnDf67; lin-15(n433)* had a Muv phenotype and an incidence of sterility similar to that of animals of genotype *lin-55(n2994); lin-15(n433)* when both are progeny of a mother of genotype *lin-55(n2994)/mnDf67; lin-15(n433)*. However, the fertile animals of genotype *lin-55(n2994)/mnDf67; lin-15(n433)* had a much greater incidence of maternal effect lethality than did in animals of genotype *lin-55(n2994); lin-15(n433)* when both are progeny of a mother of genotype *lin-55(n2994)/mnDf67; lin-15(n433)*. Animals of both of these genotypes have a stronger Muv phenotype and are less fertile than animals of genotype *lin-55(n2994); lin-15(n433)* when descended from animals of genotype *lin-55(n2994); lin-15(n433)*. These results suggest that *lin-55(n2994)* is a weak allele of a locus that has a stronger, possibly sterile, null phenotype.

To formally demonstrate that the newly defined synMuv genes are synMuv genes, we conducted tests similar to those used by Ferguson and Horvitz (1989). First, we isolated an allele of each gene from its parental mutation and showed that strains that carried only the isolated allele in homozygous condition displayed wild-type vulval development at the level of resolution of the dissecting microscope (Table 4). Double mutants were constructed between alleles of the new synMuv genes and alleles of previously defined synMuv genes. Double mutants carrying class A and class B mutations were Muv; double mutants carrying two class A mutations or two class B mutations were wild-type for vulval development (Table 4).

Maternal rescue of the synMuv phenotype depends on both class A and class B genes

Many of the new synMuv strains display maternal rescue of the Muv phenotype, such that animals of genotype *a; b* descended from animals of genotype *a/+; b/+* have lower penetrance and reduced expressivity compared to animals of genotype *a; b* descended from animals of *a; b* genotype (Table 5). Similar results have been shown for other synMuv strains (Ferguson and Horvitz, 1989). To determine whether this maternal rescue lay in only one of the two classes, we compared the Muv phenotype of animals of genotype *a; b* descended from animals of genotype *a/+; b/+* with the Muv phenotype of animals of genotype *a; b* descended from animals of genotype *a/a; b/+*, and with the Muv phenotype of animals of genotype *a; b* descended from animals of genotype *a/+; b/b*. Several different combinations of synMuv mutations were tested. The maternal rescue displayed in synMuv strains is the result of a synergistic interaction between genes of the two classes rather than the result of the maternal contribution of the genes of just one class (Table 5).

Discussion

In this paper, we describe the isolation of 49 synMuv mutants and their phenotypes at different temperatures and show linkage of the Muv phenotype to two loci. Six synMuv genes are mapped and described; these include five class B genes, *lin-51*, *lin-52*, *lin-53*, *lin-54*, *lin-55*, and one class A gene, *lin-56*. We showed that the maternal rescue of the synMuv phenotype is dependent on a synergistic interaction between the wild type alleles of both classes.

This study has identified additional constituents of the two proposed redundant signaling pathways controlled by the synMuv genes. The pathway defined by the class B

genes consists of at least ten genes, whereas that defined by the class A ganes consists of at least four. The synMuv genes may not encode the components of two duplicated pathways that perform the same biochemical function in the same manner. Instead, they likely regulate vulval development via two distinct mechanisms. The biochemical activities of the proteins encoded by the synMuv genes is, at present, unknown. The mechanism by which the synMuvs negatively regulate vulval induction is also uncertain.

The null phenotype of each of the synMuv genes is not known. It is likely that they do not all have the same null phenotypes. The class A mutation *lin-15(n767)* is a possible null allele by molecular criteria. The mutation is a deletion with a small insertion producing a frameshift in the class A transcript (Huang et al., 1994). *lin-15(n767)* mutants behave as synMuvs (wild-type vulval development alone, Muv in combination with a class B mutation), and are viable and fertile. Several *lin-15* AB mutations are double mutants for null alleles in both class A and class B transcripts. *lin-15(n309)* and *lin-15(e1763)* are deleted for most of the DNA of the locus (Clark et al., 1994; Huang et al., 1994). These mutants are Muv, viable and fertile. Thus, the *lin-15* A and *lin-15* B null phenotypes are not sterile. It is likely that the null phenotype of *lin-9* is sterile. A noncomplementation screen for *lin-9* mutants allowed the isolation of *lin-9* alleles which were sterile and behaved as synMuvs. The sterility was found to be independent of the presence of a class A mutation (Ferguson and Horvitz, 1989). The phenotype of animals carrying a strong *lin-9* mutation differs from that of animals carrying a null *lin-15* AB (and thus a null *lin-15* B) mutation.

The screens described in this paper were not designed to isolate synMuv alleles that had lethal or sterile pleiotropies. Thus, complete loss-of-function alleles of loci that have sterile or lethal null phenotypes were not isolated. Loci that are not readily mutated to a viable synMuv phenotype may not have been identified. For example, no *lin-9* alleles were isolated in this screen, even though a mutation similar to *lin-9(n112)* could have been isolated. Since 39,000 genomes were screened, it is possible that reduction-of-function mutations in loci with sterile or lethal complete loss-of-function phenotypes were isolated. Mutations in complementation groups with few alleles are candidates for such reduction-of-function mutations.

Two genes, the class A gene *lin-56* and the class B gene *lin-55*, are each represented by a single allele. To determine if these alleles are complete loss-of-function mutations, these alleles were each tested in *trans* to a deficiency that spanned the locus. Animals homozygous for *lin-56(n2728)* were indistinguishable from *trans*-heterozygotes for a deficiency of the locus, consistent with the hypothesis that *lin-56(n2728)* is a loss-of-function allele for the *lin-56* locus. *lin-55(n2994)* mutants have a stronger Muv

phenotype, are less fertile, and have a greater incidence of maternal effect lethality when they and their mothers are *trans*-heterozygotes for a deficiency of this locus. This suggests that *lin-55(n2994)* is probably a reduction-of-function mutation rather than a complete loss-of-function mutation.

With the exception of *lin-15* alleles, no class A and class B mutations mapped to the same site. This result, coupled with the result that *lin-15* encodes two nonoverlapping transcripts, one for each class, suggests that loci that are easily mutable to a synMuv A phenotype and a synMuv B phenotype are rare or nonexistent. It is possible that certain genes may be mutable to give a fertile synMuv phenotype in one class but not be mutable to a fertile or viable Muv phenotype, or fertile or viable synMuv phenotype of the other class. In such cases, these complex loci would not have been recognized.

The Muv phenotype of *lin-13* is similar to that of synMuv double mutants (Ferguson and Horvitz, 1985; 1989; Ferguson et al., 1987). This gene may be another locus that has genetically separable class A and class B activities; *lin-13(n387ts)* is Muv at 20° but acts as a class B synMuv at 15° (Ferguson and Horvitz, 1989). None of the mutations isolated in this screen mapped to the *lin-13* locus, so it is not clear whether it is possible to obtain mutations in this locus that act as distinctly class A or class B synMuv mutations.

Mutations in different complementation groups were isolated at different frequencies. Class A mutations fell into either a frequently isolated group, *lin-8* and *lin-15* A (eight alleles each), or an infrequently isolated group, *lin-38* and *lin-56* (one or two alleles each). *lin-38* was isolated at a higher frequency in previous screens (2 mutations in 8000 haploid genomes versus 2 mutations in 23,000 haploid genomes), although the sample size was much smaller (Ferguson and Horvitz, 1989). Class B mutations fell into one of three groups: a frequently isolated group consisting of *lin-15* B, *lin-35* and *lin-51* (five or more alleles each), an infrequently isolated group consisting of *lin-36*, *lin-37*, *lin-53*, *lin-54* and *lin-55* (three or fewer alleles each), or a group in which no alleles were isolated consisting of *lin-9* and *lin-52*. Given the number of haploid genomes screened and the expected frequency of mutation of the average *C. elegans* gene by EMS, 1/2000, we expected to isolate five to eleven alleles of each gene (Brenner, 1974; Greenwald and Horvitz, 1980).

There are several reasons why mutations may have been isolated at a lower frequency than expected. Some of the genes may have a sterile loss-of-function phenotype and only rare reduction-of-function mutations were isolated. This likely explains the failure to isolate any *lin-9* alleles. Since the only *lin-55* allele appears to be a reduction-of-function allele, *lin-55* may also fall into this catagory. Such genes should be

easily isolated in screens that allow the isolation of sterile and lethal mutants. Other genes may provide a small mutagenic target. Only one allele of *lin-56* was isolated, yet this allele appears to be a complete loss-of-function allele. Only one allele of *lin-37* was isolated in the screens described in this paper. This gene is physically small, and the allele isolated is consistent by molecular criteria with its being a loss-of-function allele (X. Lu, personal communication). We cannot rule out the possibility of bias due to the parental mutation used in the screens. The mutations *lin-8(n111)* and *lin-15(n433)* used to isolate class B mutations do not produce highly penetrant Muv phenotypes in conjunction with some class B mutations. This may have resulted in a lower isolation frequency of alleles of certain genes. Maternal rescue is unlikely to have played a significant role since the parental mutation was homozygous.

Several other examples of functional redundancy during development have been observed. In *Drosophila*, *achaete* and *scute* are functionally redundant for the specification of larval sense organs even though individually they are required for the specification of distinct groups of adult bristles (reviewed by Ghysen and Dambly-Chaudiere, 1991). In *C. elegans*, *lin-12* and *glp-1* are molecularly similar and functionally redundant for some aspects of development. Double mutants exhibit defects not found in either single mutant (Lambie and Kimble, 1991). Partial functional redundancy involving groups of genes or pathways is seen in the cell corpse engulfment genes of *C. elegans*. These mutants fall into two classes; animals carrying mutations in both classes have a more severe phenotype than animals carrying mutations in only one class (Ellis et al., 1991).

The synMuv genes differ from these previous examples in that alone they do not cause a vulval phenotype and they fall into two classes with multiple members. This indicates that they encode two pathways, each of which is unnecessary in the presence of the other for vulval development. Identifying other such redundant pathways by genetic means is difficult because mutations in the genes of such pathways do not cause a phenotype in the absence of a second site mutation. Because few examples are known, most redundant processes in development are relatively inaccessible. The synMuvs provide an excellent model for studying the role of redundant genetic systems in metazoan development because they have been identified and placed in the context of a well characterized developmental system.

The studies described in this paper have resulted in the identification of ten class B synMuv genes and four class A synMuv genes. The number of genes constitutuing each class is significantly different. This may indicate that genes of the two synMuv classes do not encode duplicate pathways. If such is the case, it is likely that class A and

class B genes encode pathways which control different biochemical activities. Functional redundancy at the genetic level implies that the two classes of genes exert the same ultimate effect, i. e. the negative regulation of vulval induction. Genetic redundancy does not imply that the two classes act via the same mechanism, only that only one is required for negative regulation. For example, class A synMuv genes may encode proteins necessary for the secretion and processing of a negative ligand that inhibits the LET-23 receptor tyrosine kinase which mediates vulval induction. This mechanism would require few genes. Note that since the two classes of synMuv genes are redundant, a *let-23* mutation only needs to be epistatic to one class of synMuv genes in order to produce a Vul phenotype. Thus, class B synMuv genes may be postulated to negatively regulate a component downstream of LET-23, thereby requiring a more elaborate signal transduction cascade within the Pn.p cells. In such an example, different biochemical mechanisms can be used to effect negative regulation; either is sufficient for negative regulation.

In addition to the opportunity provided to study the role of redundant genetic systems in development, the synMuv genes afford the opportunity to study the negative regulation of receptor tyrosine kinase and Ras-mediated signal transduction pathways. The synMuv genes encode the components of two signaling pathways involved in the negative regulation of a process regulated by a Ras signal transduction cascade. The further study of the synMuv genes may reveal how Ras signaling pathways are regulated and at what steps in the pathway hierarchy they are subject to negative regulation. It is likely that the synMuv genes are conserved in other organisms and may negatively regulate receptor tyrosine kinase and Ras signaling in mammals.

Acknowledgments

We thank current and former members of the Horvitz laboratory, especially Greg Beitel, Kerry Kornfeld and Shai Shaham, for helpful discussions during the course of this work. We thank Greg Beitel, Kerry Kornfeld and Xiaowei Lu for critically reading this manuscript. We thank the Caenorhabditis Genetics Center (supported by the National Institutes of Health National Center for Research Resources) for providing some of the strains used in this work. J.H.T. was a Predoctoral Fellow of the Howard Hughes Medical Institute. H.R.H. is an Investigator of the Howard Hughes Medical Institute.

References

Arioan, R. V., Koga, M., Mendel, J. E., Oshima, Y. and Sternberg, P. W. (1990). The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature* **348**, 693-699.

Austin, J., and Kimble, J. (1989). Transcript analysis of *glp-1* and *lin-12*, homologous genes required for cell interactions during development of *C. elegans*. *Cell* **58**, 565-571.

Beitel, G. J., Clark, S. G. and Horvitz, H. R. (1990). *Caenorhabditis elegans ras* gene *let-60* acts as a switch in the pathway of vulval induction. *Nature* **348**, 503-509.

Brenner, S., (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Clark, S. G., (1992). *Intercellular Signalling and Homeotic Genes Required During Vulval Development in* C. elegans. Ph. D. thesis, Massachusetts Institute of Technology.

Clark, S. G., Stern, M. J. and Horvitz, H. R. (1992). *Caenorhabditis elegans* cell-signalling gene *sem-5* encodes a protein with SH2 and SH3 domains. *Nature* **356**, 340-344.

Clark, S. G., Lu, X. and Horvitz, H. R. (1994). The *Caenorhabditis elegans* locus *lin-15*, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. *Genetics* **137**, 987-997.

Eisenmann, D. M., and Kim, S. K. (1994). Signal transduction and cell fate specification during *Caenorhabditis elegans* vulval development. *Curr. Opinion Genet. Develop.* **4**, 508-516.

Ellis, R., Jacobson, D. M. and Horvitz, H. R. (1991). Genes required for the engulfment of cell corpses during programmed cell death in *Caenorhabditis elegans*. *Genetics* **129**, 79-94.

Ferguson, E. L. and Horvitz, H. R. (1985). Identification and genetic characterization of 22 genes that affect the vulval cell lineages of the nematode *Caenorhabditis elegans*. *Genetics* **110**, 17-72.

Ferguson, E. L. and Horvitz, H. R. (1989). The Multivulva phenotype of certain *Caenorabditis elegans* mutants results from defects in two functionally redundant pathways. *Genetics* **123**, 109-121.

Ferguson, E. L. Sternberg, P. W. and Horvitz, H. R. (1987). A genetic pathway for the specification of the vulval cell lineages of *Caenorhabditis elegans*. *Nature* **326**, 259-267.

Ghysen, A., and Dambly-Chaudiere, C. (1988). From DNA to form: the *achaete-scute* complex. *Genes Dev.* **2**, 495-501.

Graham, P. L. and Kimble, J. (1993). The *mog-1* gene is required for the switch from spermatogeneis to oogenesis in *Caenorhabditis elegans*. *Genetics* **133**, 919-931.

Greenwald, I. S. and Horvitz, H. R. (1980). *unc-93(e1500)*: a behavioral mutant of *Caenorhabditis elegans* that defines a gene with a wild-type null phenotype. *Genetics* **96**, 147-164.

Han, M. and Sternberg, P. W. (1990). *let-60*, a gene that specifies cell fates during *C. elegans* vulval induction, encodes a ras protein. *Cell* **63**, 921-931.

Han, M., Golden, A., Han, Y. and Sternberg, P. W.(1993). *C. elegans lin-45 raf* gene participates in *let-60 ras*-stimulated vulval differentiation. *Nature* **363**, 133-140.

Hedgecock, E. M. and Herman, R. K. (1995). The *ncl-1* gene and genetic mosaics of *Caenorhabditis elegans*. *Genetics* **141**, 989-1006.

Herman, R. K. (1978). Crossover suppressors and balanced recessive lethals in *Caenorhabditis elegans*. *Genetics* **88**, 49-65.

Herman, R. K. and Hedgecock, E. M. (1990). Limitation of the size of the vulval primordium of *Caenorhabditis elegans* by *lin-15* expression in the surrounding hypodermis. *Nature* **348**, 169-171.

Hill, R. J., and Sternberg, P. W. (1992). The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans*. *Nature* **358**, 470-476.

Hodgkin, J., Edgley, M., Riddle, D. L. and Albertson, D. G. (1988). Appendix 4: Genetics, pp. 491-584 in *The Nematode* Caenorhabditis elegans, edited by W. B. Wood. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Horvitz, H. R. and Sternberg, P. W. (1991). Multiple intercellular signalling systems control the development of the *Caenorhabditis elegans* vulva. *Nature* **351**, 535-541.

Horvitz, H. R. and Sulston, J. E. (1980). Isolation and genetic characterization of cell-lineage mutants of the nematode *Caenorhabditis elegans*. *Genetics* **96**, 435-454.

Horvitz, H. R., Brenner, S., Hodgkin, J. and Herman, R. K. (1979). A uniform genetic nomenclature for the nematode *Caenorhabditis elegans*. *Molec. Gen. Genet.* **175**, 129-133.

Huang, L. S., Tzou, P. and Sternberg, P. W. (1994). The *Caenorhabditis elegans lin-15* locus encodes two negative regulators of vulval development. *Molec. Biol. Cell* **5**, 395-412.

Katz, W. S., Hill, R. J., Clandinin, T. R. and Sternberg, P. W. (1995). Different levels of the *C. elegans* growth factor LIN-3 promote distinct vulval precusor fates. *Cell* **82**, 297-307.

Kimble, J. (1981). Alterations in cell lineage following laser ablation of cells in the somatic gonad of *Caenorhabditis elegans*. *Dev. Biol.* **87**, 286-300.

Kornfeld, K., Guan, K-L. and Horvitz, H. R. (1995a). The *Caenorhabditis elegans* gene *mek-2* is required for vulval induction and encodes a protein similar to the protein kinase MEK. *Genes Dev.* **9**, 756-768.

Kornfeld, K., Hom, D. M. and Horvitz, H. R. (1995b). The *ksr-1* gene encodes a novel protein kinase involved in Ras-mediated signaling in *C. elegans*. *Cell* **83**, 903-913.

Lackner, M. R., Kornfeld, K., Miller, L. M., Horvitz, H. R. and Kim, S. K. (1994). A MAP kinase homologue, *mpk-1*, is involved in ras-mediated induction of vulval cell fates in *Caenorhabditis elegans*. *Genes Dev.* **8**, 160-173.

Lambie, E., and Kimble, J. (1991). Two homologous regulatory genes, *lin-12* and *glp-1*, have overlapping functions. *Development* **112**, 231-240.

Plenefisch, J. D., DeLong, L. and Meyer, B. J. (1989). Genes that implement the hermaphrodite mode of dosage compensation in *Caenorhabditis elegans*. *Genetics* **121**, 57-76.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Sigurdson, D. C., Spanier, G. J. and Herman, R. K. (1984). *Caenorhabditis elegans* deficiency mapping. *Genetics* **108**, 331-345.

Simske, J. S., and Kim, S. K. (1995). Sequential signalling during *Caenorhabditis elegans* vulval induction. *Nature* **375**, 142-146.

Sternberg, P. W. (1988). Lateral inhibition during vulval induction in *Caenorhabditis elegans*. *Nature* **335**, 551-554.

Sternberg, P. W. and Horvitz, H. R. (1986). Pattern formation during vulval development in *C. elegans*. *Cell* **44**, 761-772.

Sternberg, P. W., and Horvitz, H. R. (1989). The combined action of two intercellular signalling pathways specifies three cell fates during vulval induction in *C. elegans*. *Cell* **58**, 679-693.

Sulston, J. and Hodgkin, J. (1988). Methods, pp. 587-606 in *The Nematode* Caenorhabditis elegans, edited by W. B. Wood. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Sulston, J. E. and Horvitz, H. R. (1977). Postembryonic cell lineages of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **56**, 110-156.

Sulston, J. E. and Horvitz, H. R. (1981). Abnormal cell lineages in mutants of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **82**, 41-55.

Sulston, J. E. and White, J. G. (1980). Regulation and cell autonomy during postembryonic development of *Caenorhabditis elegans*. *Dev. Biol.* **78**, 577-597.

Sundaram, M. and Han, M. (1996). Control and integration of cell signaling pathways during *C. elegans* vulval development. *Bioessays* **18**, 473-480.

Thomas, J. H., Stern, M. J. and Horvitz, H. R. (1990). Cell interactions coordinate the development of the *Caenorhabditis elegans* egg-laying system. *Cell* **62**, 1041-1052.

Trent, C., Tsung, N. and Horvitz, H. R. (1983). Egg-laying defective mutants of the nematode *Caenorhabditis elegans*. *Genetics* **104**, 619-647.

Waterston, R. H., Thomson, J. N. and Brenner, S. (1980). Mutants with altered muscle structure in *Caenorhabditis elegans*. *Dev. Biol.* **77**, 271-301.

Wu, Y., and Han, M. (1994). Suppressors of activated Let-60 Ras protein defines a role of *Caenorhabditis elegans* Sur-1 MAP kinase in vulval differentiation. *Genes Dev.* **8**, 147-159.

Wu, Y., Han, M. and Guan, K-L. (1995). MEK-2, a *Caenorhabditis elegans* MAP kinase kinase, functions in Ras-mediated vulval induction and other developmental events. *Genes Dev.* **9**, 742-758.

Table 1: The mutations causing the Muv phenotype of candidate synMuv strains were mapped to linkage groups in a manner similar to that described previously (Ferguson and Horvitz, 1989). Males carrying both the original mutation and the new mutation were crossed to strains of genotypes *dpy-5 I; bli-2 II; unc-32 III*, *unc-5 IV; dpy-11 V; lon-2 X* (Trent et al., 1983), and *unc-3 X*. If the mutation did not display linkage to two of the markers, it was further tested against strains of the following genotypes: *bli-3 I unc-54 I*, *unc-85 II*, *mnC1 II*, *unc-52 II; unc-25 III; dpy-4 IV*, *dpy-1 III; unc-34 V*, *dpy-9 IV*, *egl-18 IV*, and *unc-51 V*. Muv animals were isolated from the F_2 progeny of these crosses and the frequency of marker segregation was scored. Markers linked to a mutation responsible for producing the Muv phenotype are expected to segregate at a frequency of 2p where p is the recombination frequency between the mutation being assayed and the linked marker. Unlinked markers are expected to segregate at a frequency of 2/3. The markers that displayed linkage are noted in the table.

[a] The mutations in these strains displayed linkage only to *unc-3 X*. Linkage of the new mutation to *unc-3 X* is assumed since the mutations in these strains segregated as single locus Muv mutations and failed to complement *lin-15(n765) X*.

[b] The mutation in this strain failed to display linkage to any marker listed other than *unc-3 X*. The mutations segregated as synMuv mutations and did not display complete linkage to the X chromosome.

TABLE 1

Origins and chromosomal linkages of new synMuv strains

Parental mutation	New mutation	Strain	Genotype of F2 Muv hermaphrodites with respect to the chromosomal marker linked to the: Parental mutation	New mutation
lin-8(n111) A				
	lin-15(n2230)	MT5833	5/16 *bli-2/+ II*	0/11 *unc-3/+ X*
	lin-15(n2233)	MT5835	4/13 *bli-2/+ II*	4/15 *lon-2/+ X*
	lin-15(n2241)	MT5841	4/21 *bli-2/+ II*	2/23 *unc-3/+ X*
	lin-15(n2244)	MT5844	6/19 *bli-2/+ II*	0/17 *unc-3/+ X*
	lin-15(n2245)	MT5845	3/10 *bli-2/+ II*	1/15 *unc-3/+ X*
	lin-35(n2232)	MT5834	4/24 *bli-2/+ II*	0/24 *dpy-5/+ I*
	lin-35(n2236)	MT5838	2/20 *bli-2/+ II*	0/20 *dpy-5/+ I*
	lin-35(n2239)	MT5839	7/28 *bli-2/+ II*	0/28 *dpy-5/+ I*
	lin-35(n2242)	MT5842	1/11 *bli-2/+ II*	0/11 *dpy-5/+ I*
	lin-36(n2235)	MT5837	4/14 *bli-2/+ II*	0/14 *unc-32/+ III*
	lin-36(n2240)	MT5840	11/36 *bli-2/+ II*	0/36 *unc-32/+ III*
	lin-36(n2243)	MT5843	3/16 *bli-2/+ II*	0/16 *unc-32/+ III*
	lin-37(n2234)	MT5836	6/24 *bli-2/+ II*	0/24 *unc-32/+ III*
	lin-51(n2238)	MT6031	14/48 *bli-2/+ II*	1/48 *unc-32/+ III*
	lin-54(n2231)	MT6101	10/35 *bli-2/+ II*	3/18 *unc-5/+ IV*
lin-15(n433) A				
	lin-15(n2980)	MT8087	2/20 *unc-3/+ X*	X^a
	lin-15(n2983)	MT8090	2/20 *unc-3/+ X*	X^a
	lin-15(n2989)	MT8096	0/20 *unc-3/+ X*	X^a
	lin-15(n2991)	MT8098	4/20 *unc-3/+ X*	X^a
	lin-15(n2993)	MT8100	0/20 *unc-3/+ X*	X^a
	lin-35(n2977)	MT8084	1/15 *unc-3/+ X*	1/15 *dpy-5/+ I*
	lin-35(n2996)	MT8103	1/20 *unc-3/+ X*	0/16 *dpy-5/+ I*
	lin-51(n2981)	MT8088	1/14 *unc-3/+ X*	1/16 *unc-32/+ III*
	lin-51(n2984)	MT8091	1/14 *unc-3/+ X*	0/16 *unc-32/+ III*
	lin-51(n2985)	MT8092	0/18 *unc-3/+ X*	0/16 *unc-32/+ III*
	lin-51(n2988)	MT8095	2/15 *unc-3/+ X*	0/14 *unc-32/+ III*
	lin-53(n2978)	MT8085	1/23 *unc-3/+ X*	1/12 *dpy-5/+ I*
	lin-54(n2990)	MT8097	3/18 *unc-3/+ X*	2/15 *unc-5/+ IV*
	lin-55(n2994)	MT8101	0/14 *unc-3/+ X*	0/9 *bli-2/+ II*
	lin(n2987)	MT8094	3/20 *unc-3/+ X*	N.D.[b]
lin-36(n766) B				
	lin-8(n2376)	MT6213	0/16 *unc-32/+ III*	2/16 *bli-2/+ II*
	lin-8(n2378)	MT6227	0/12 *unc-32/+ III*	0/12 *bli-2/+ II*
	lin-8(n2403)	MT6290	0/14 *unc-32/+ III*	4/14 *bli-2/+ II*
	lin-15(n2375)	MT6212	0/18 *unc-32/+ III*	3/22 *unc-3/+ X*
	lin-38(n2402)	MT6289	0/16 *unc-32/+ III*	0/10 *unc-52/+ II*
lin-15(n744) B				
	lin-8(n2724)	MT7606	0/19 *unc-3/+ X*	2/21 *bli-2/+ II*
	lin-8(n2731)	MT7611	2/20 *unc-3/+ X*	6/21 *bli-2/+ II*
	lin-8(n2738)	MT7616	0/19 *unc-3/+ X*	7/24 *bli-2/+ II*

lin-8(n2739)	MT7617	1/18 *unc-3/+ X*	6/24 *bli-2/+ II*
lin-8(n2741)	MT7618	2/18 *unc-3/+ X*	2/22 *bli-2/+ II*
lin-15(n2725)	MT7607	1/20 *unc-3/+ X*	X^a
lin-15(n2726)	MT7608	1/18 *unc-3/+ X*	X^a
lin-15(n2733)	MT7612	0/20 *unc-3/+ X*	X^a
lin-15(n2734)	MT7613	2/19 *unc-3/+ X*	X^a
lin-15(n2735)	MT7614	0/19 *unc-3/+ X*	X^a
lin-15(n2737)	MT7615	0/19 *unc-3/+ X*	X^a
lin-15(n2742)	MT7619	1/18 *unc-3/+ X*	X^a
lin-38(n2727)	MT7609	1/20 *unc-3/+ X*	0/17 *mnC1/+ II*
lin-56(n2728)	MT7610	0/24 *unc-3/+ X*	4/25 *bli-2/+ II*

Table 2: The penetrance of the Muv phenotype of each synMuv strain was determined at 15° and 20° after growth at the indicated temperature for two or more generations. Several stains displayed a temperature dependent reduction in viability. This reduction in viability was tested in a manner similar to that of Ferguson and Horvitz (1989) but differed in that exactly 10 eggs laid by hermaphrodites of the indicated genotype grown at 20° were placed on each of the four assay plates (each with a 2 cm diameter lawn of bacteria) used at each temperature. The plates were checked daily to determine when the bacterial lawn was consumed. The data were described according to the following criteria: at 15°: WT: 8.5-14 days, Slow: 14-24 days, Very slow: 24-28 days, Inviable: lethal or more than 28 days, at 20°: WT: 5.5-9 days, Slow: 9-17 days, Very slow: 17-28 days, Inviable: lethal or more than 28 days, at 25°: WT: 5-7.5 days, Slow: 7.5-15 days, Very slow: 15-28 days, Inviable: lethal or more than 28 days. The last value of the range described was included in that category. The data obtained from the wild type strain N2 was: 15°: 10 days, 20°: 6 days, 25°: 5 days.

TABLE 2

Phenotypes of new synthetic Multivulva strains

Genotype	Penetrance of Muv phenotype		Strain Growth		
	15°	20°	15°	20°	25°
lin-8(n111); lin-15(n2230)	20% (n=254)	93% (n=376)	Slow	WT	Slow
lin-8(n111); lin-15(n2233)	100% (n=235)	100% (n=385)	Slow	WT	Slow
lin-8(n111); lin-15(n2241)	100% (n=130)	100% (n=238)	Slow	WT	Inviable
lin-8(n111); lin-15(n2244)	91% (n=128)	99.6% (n=280)	WT	WT	Slow
lin-8(n111); lin-15(n2245)	6% (n=202)	98% (n=306)	WT	WT	WT
lin-35(n2232); lin-8(n111)	100% (n=200)	99.9% (n=942)	Inviable	WT	Inviable
lin-35(n2236); lin-8(n111)	99% (n=96)	100% (n=97)	Inviable	Slow	Inviable
lin-35(n2239); lin-8(n111)	100% (n=197)	100% (n=290)	Inviable	WT	Slow
lin-35(n2242); lin-8(n111)	N.D.	100% (n=278)	Inviable	Slow	Very slow
lin-8(n111); lin-36(n2235)	13% (n=279)	79% (n=313)	WT	WT	WT
lin-8(n111); lin-36(n2240)	7% (n=189)	79% (n=373)	WT	WT	WT
lin-8(n111); lin-36(n2243)	51% (n=115)	94% (n=593)	WT	WT	WT
lin-8(n111); lin-37(n2234)	96% (n=192)	100% (n=278)	WT	WT	Inviable
lin-8(n111); lin-51(n2238)	65% (n=391)	100% (n=74)	Very slow	Slow	Inviable
lin-8(n111); lin-54(n2231)	39% (n=334)	99% (n=164)	Very slow	WT	Slow
lin-15(n2980 n433)	2% (n=209)	99% (n=252)	WT	WT	Slow
lin-15(n2983 n433)	2% (n=232)	99% (n=227)	WT	WT	Slow
lin-15(n2989 n433)	0% (n=238)	100% (n=235)	WT	WT	WT
lin-15(n2991 n433)	2% (n=226)	100% (n=216)	WT	WT	WT
lin-15(n2993 n433)	0% (n=205)	79% (n=201)	WT	WT	WT
lin-35(n2977); lin-15(n433)	8% (n=257)	100% (n=234)	Slow	WT	Slow
lin-35(n2996); lin-15(n433)	18% (n=216)	100% (n=202)	Slow	Slow	Slow
lin-51(n2981); lin-15(n433)	0% (n=237)	84% (n=241)	WT	WT	Inviable
lin-51(n2984); lin-15(n433)	1% (n=217)	97% (n=239)	WT	WT	Inviable
lin-51(n2985); lin-15(n433)	2% (n=229)	94% (n=213)	WT	WT	Slow
lin-51(n2988); lin-15(n433)	33% (n=247)	97% (n=261)	Slow	Slow	Inviable
lin-53(n2978); lin-15(n433)	0% (n=211)	59% (n=203)	WT	WT	WT
lin-54(n2990); lin-15(n433)	4% (n=199)	95% (n=216)	WT	WT	Slow
lin-55(n2994); lin-15(n433)	4% (n=246)	78% (n=234)	WT	WT	Slow
lin(n2987); lin-15(n433)	0% (n=207)	91% (n=267)	WT	WT	WT
lin-8(n2376); lin-36(n766)	18% (n=382)	96% (n=189)	WT	WT	WT
lin-8(n2378); lin-36(n766)	19% (n=456)	100% (n=125)	WT	WT	WT
lin-8(n2403); lin-36(n766)	48% (n=402)	99% (n=549)	WT	WT	WT
lin-36(n766); lin-15(n2375)	0% (n=544)	82% (n=675)	WT	WT	Slow
lin-38(n2402); lin-36(n766)	28% (n=643)	99.7% (n=667)	WT	WT	WT
lin-8(n2724); lin-15(n744)	100% (n=211)	100% (n=126)	Slow	Slow	Slow
lin-8(n2731); lin-15(n744)	100% (n=217)	100% (n=167)	Slow	Slow	Slow
lin-8(n2738); lin-15(n744)	100% (n=128)	100% (n=140)	Slow	Slow	Very slow
lin-8(n2739); lin-15(n744)	100% (n=158)	100% (n=97)	Slow	Slow	Slow
lin-8(n2741); lin-15(n744)	100% (n=157)	100% (n=155)	WT	Slow	Slow
lin-15(n744 n2725)	100% (n=152)	100% (n=177)	Slow	Slow	Very slow
lin-15(n744 n2726)	100% (n=141)	100% (n=145)	Slow	Slow	Very slow
lin-15(n744 n2733)	100% (n=193)	100% (n=140)	Slow	Slow	Inviable
lin-15(n744 n2734)	100% (n=124)	100% (n=159)	Slow	Slow	Slow

lin-15(n744 n2735)	100% (n=132)	100% (n=176)	Slow	Slow	Very slow
lin-15(n744 n2737)	99% (n=199)	100% (n=121)	Slow	Slow	Inviable
lin-15(n744 n2742)	100% (n=121)	100% (n=173)	Slow	Slow	Very slow
lin-38(n2727); lin-15(n744)	100% (n=201)	100% (n=165)	Slow	Slow	Slow
lin-56(n2728); lin-15(n744)	100% (n=214)	100% (n=163)	Slow	Slow	Slow

Table 3: Two, three, and four factor crosses were performed as described previously (Brenner, 1974, Ferguson and Horvitz, 1989). A second mutation of the opposite class of the mutation being tested was present in the strain to allow the synMuv phenotype to be scored. From heterozygotes of genotype *r1 r2/b r3; a*, recombinant progeny of the phenotypes R1 non-R2, R2 non-R1, Muv non-R3, and R3 non-Muv were isolated. The progeny of each recombinant animal were scored for the presence of the the unselected markers. Deficiency heterozygotes were constructed and the vulval phenotype scored. The presence of the deficiency was confirmed in each animal scored by the segregation of 1/4 Lethal progeny.

TABLE 3

Two-factor crosses

Gene	Genotype of heterozygote	Segregants	Map distance (p)
lin-53	*lin-53 unc-75/+ +; lin-15(n767)*	406 wild type 124 Muv Unc 9 Muv 9 Unc	3.3%

Three- and four-factor crosses

Gene	Genotype of heterozygote	Phenotype of selected recombinants	Genotype of selected recombinants (with respect to unselected markers)
lin-51			
	lin-51 unc-32+ +/+ + unc-49 dpy-18; lin-15(n767)	Unc-32	0/2 *unc-49 dpy-18/+ +*
		Dpy	7/7 *lin-51 unc-32/+ +*
		Unc-49	0/1 *lin-51 unc-32/+ +*
	+ lin-51 + unc-32/daf-2 + dpy-17 +; lin-15(n767)	Muv	0/6 *daf-2/+*
			1/6 *dpy-17/+*
		Dpy	4/26 *lin-51/+*
		Unc	5/5 *daf-2 dpy-17/+ +*
	+ + lin-51/unc-93 dpy-27 +; lin-15(n767)	Unc	30/30 *lin-51/+*
	+ lin-51 +/dpy-27 + unc-79; lin-15(n767)	Unc	8/12 *lin-51/+*
lin-52			
	+ + unc-32 lin-52/lon-1 sma-3 + +; lin-15(n767)	Lon	1/1 *lin-52 unc-32/ + +*
		Sma	0/4 *lin-52/+*
		Muv	7/7 *lon-1/+*
	+ + lin-52/sma-3 unc-32; lin-15(n767)	Sma	3/3 *lin-52/+*
		Unc	0/13 *lin-52/+*
	+ lin-52 +/dpy-19 + unc-69; lin-15(n767)	Dpy	17/31 *lin-52/+*
		Unc	2/11 *lin-52/+*
	+ lin-52 +/unc-16 + unc-49; lin-15(n767)	Unc-16	0/2 *lin-52/+*
		Unc-49	13/16 *lin-52/+*
lin-53			
	dpy-5 lin-53 + +/+ + lin-11 unc-75; lin-15(n767)	Dpy	7/7 *lin-11 unc-75/+ +*
		Muv	0/12 *lin-11 unc-75/+ +*
		Vul	0/10 *dpy-5 lin-53/+ +*
		Unc	8/8 *dpy-5 lin-53/+ +*
	+ lin-53 +/unc-29 + lin-11; lin-15(n767)	Unc	1/3 *lin-53/+*
		Vul	11/19 *lin-53/+*
lin-54			
	lin-8; + + lin-54/unc-22 unc-30 +	Unc-22	19/19 *lin-54/+*
		Unc-30	0/15 *lin-54/+*
	lin-8; + lin-54 +/unc-30 + dpy-4	Unc	2/11 *lin-54/+*
		Dpy	21/22 *lin-54/+*
	lin-54 + +/+ lev-1 unc-26; lin-15(n433)	Lev	0/25 *lin-54/+*
lin-55			
	+ lin-55 +/dpy-10 + unc-53; lin-15(n433)	Dpy	6/9 *lin-55/+*
		Unc	6/11 *lin-55/+*
	+ lin-55 +/rol-6 + unc-4; lin-15(n433)	Rol	6/14 *lin-55/+*
		Unc	9/14 *lin-55/+*
	lin-55 + +/+ let-240 unc-4; lin-15(n433)	Unc	25/25 *lin-55/+*

lin-56			
	+ + lin-56/unc-85 dpy-10 +; lin-15(n744)	Unc	4/4 *lin-56/+*
		Dpy	0/6 *lin-56/+*
	+ lin-56 +/dpy-10 + unc-53; lin-15(n744)	Dpy	5/10 *lin-56/+*
		Unc	6/11 *lin-56/+*
	+ + lin-56/rol-6 unc-4 +; lin-15(n744)	Rol	16/16 *lin-56/+*
		Unc	0/9 *lin-56/+*

Deficiency heterozygotes

Gene	Genotype of heterozygote	Phenotype of heterozygote
lin-55		
	dpy-10 lin-55(n2994) +/+ mnDf46 unc-4; lin-15(n433)	wild type
	dpy-10 lin-55(n2994) +/+ mnDf85 unc-4; lin-15(n433)	wild type
	dpy-10 lin-55(n2994) +/+ mnDf67 unc-4; lin-15(n433)	Muv
lin-56		
	+ lin-56(n2728) unc-53/unc-4 mnDf71 +; lin-15(n744)	Muv

Table 4: Mutations in new genes were separated from the original mutation present in the synMuv strain as described in Materials and Methods. Double mutants carrying mutations in these new genes and mutations in class A and class B genes were constructed as described in Materials and Methods. *lin-51*, *lin-52* and *lin-54* display a weaker Muv phenotype in double mutants with *lin-8(n111)* than with *lin-15(n767)*. *lin-55* displays a weaker Muv phenotype in a *lin-15(n433)* background than in a *lin-15(n767)* background.

TABLE 4

Phenotypes of single and double mutants

			Double mutant with Class A		Double mutant with Class B	
	New Mutation	Single mutant	*lin-8(n111)*	*lin-15(n767)*	*lin-36(n766)*	*lin-15(n744)*
Class A						
	lin-56(n2728)	WT	WT	WT	Muv	Muv
Class B						
	lin-51(n770)	WT	Muv	Muv	WT	WT
	lin-52(n771)	WT	Muv	Muv	WT	WT
	lin-53(n833)	WT	Muv	Muv	WT	WT
	lin-54(n2231)	WT	Muv	Muv	WT	WT
	lin-55(n2994)	WT	Muv	Muv	WT	WT

Table 5: The contribution of class A and class B genes to the maternal rescue of the synMuv phenotype of doubly homozygous animals descended from either singly or doubly heterozygous mothers was determined by counting the number of Muv animals of phenotype R, where R is the phenotype produced by the *cis* marker *r*. Doubly heterozygous mothers were obtained by mating N2 males into marked doubly homozygous animals. Singly heterozygous mothers were obtained by mating males homozygous for one of the synMuv mutations into marked doubly homozygous animals. A strain homozygous for *lin-35* was obtained by the procedure used for the isolation of strains homozygous for the synMuv mutations described in this study. *dpy-14* was used to balance *lin-35*. *lin-8; lin-9* was marked with *lon-1*. *lin-35; lin-8* was marked with *unc-13*. *lin-8; lin-36* was marked with *unc-32*. *lin-8; lin-37* was marked with *lon-1*. *lin-38; lin-9* was marked with *unc-52*. *lin-51; lin-15(n767)* was marked with *unc-93* or *unc-32*. *lin-52; lin-15(n767)* was marked with *sma-3*. *lin-53; lin-15(n767)* was marked with *dpy-5* or *lin-11*. *lin-54; lin-15(n433)* was marked with *dpy-20*. *lin-55; lin-15(n433)* was marked with *rol-6*. *lin-56; lin-15(n744)* was marked with *rol-6*.

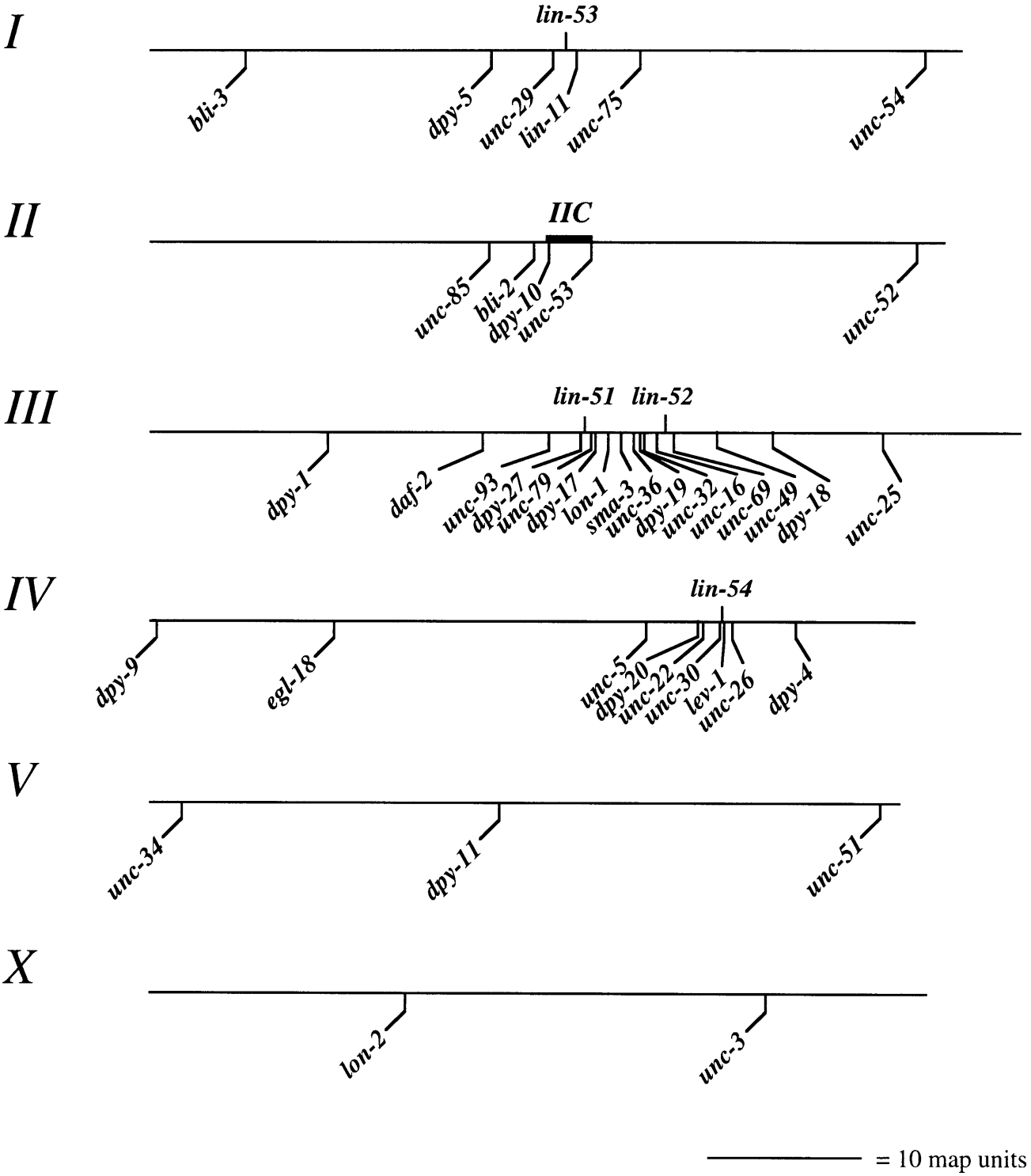
[a]This datum was obtained by estimating the penetrance and the number of doubly homozygous animals to be 1/4 of the R progeny.

TABLE 5

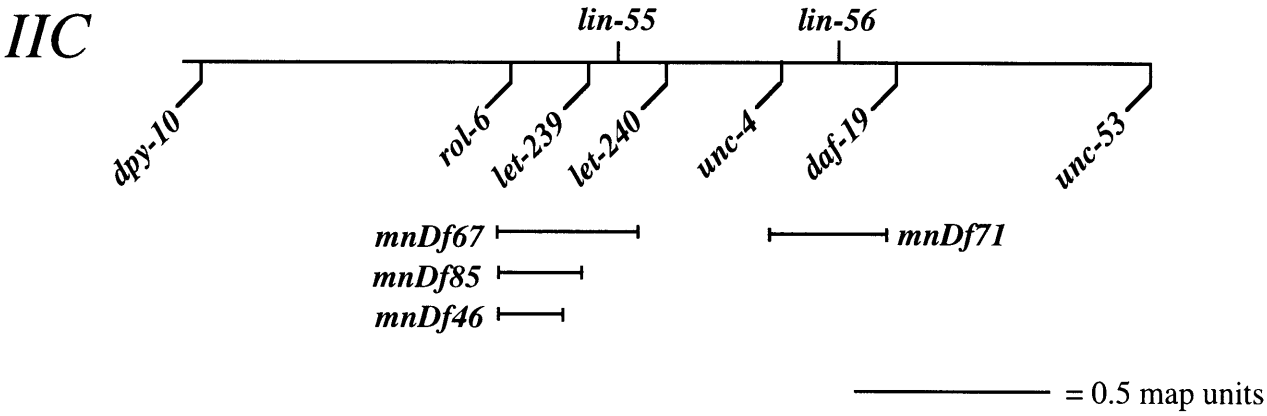
Maternal rescue of synMuv phenotype

		Penetrance of the Muv phenotype in animals of *a/a; b/b* genotype descended from animals of maternal genotype		
Class A mutation	Class B mutation	*a/+; b/+*	*a/+; b/b*	*a/a; b/+*
lin-8(n111)	*lin-9(n112)*	72% (n=57)[a]	83% (n=141)[a]	100% (n=183)
lin-8(n111)	*lin-35(n745)*	45% (n=65)[a]	N. D.	97% (n=149)
lin-8(n111)	*lin-36(n766)*	14% (n=93)[a]	23% (n=216)[a]	84% (n=300)
lin-8(n111)	*lin-37(n758)*	31% (n=69)[a]	98% (n=133)[a]	85% (n=188)
lin-15(n433)	*lin-54(n2231)*	9% (n=89)[a]	N. D.	21% (n=211)
lin-15(n767)	*lin-51(n770)*	58% (n=64)[a]	97% (n=238)[a]	90% (n=220)
lin-15(n767)	*lin-52(n771)*	83% (n=98)[a]	103% (n=225)[a]	90% (n=294)
lin-15(n767)	*lin-53(n833)*	103% (n=76)[a]	N. D.	100% (n=64)
lin-15(n433)	*lin-55(n2994)*	11% (n=95)[a]	75% (n=125)[a]	50% (n=298)
lin-38(n751)	*lin-9(n112)*	81% (n=65)[a]	100% (n=146)	99% (n=189)[a]
lin-56(n2728)	*lin-15(n744)*	93% (n=92)[a]	99% (n=200)	N. D.

Figure 1: a. Partial genetic map of *C. elegans* showing the locations of newly identified or characterized synMuv genes and the markers which were used to position these genes on the map. New synMuv genes are shown above the line representing the linkage group; marker genes are shown below the line. The cluster in linkage group II is depicted by a filled box and is shown in detail in b. b. Detailed view of the cluster of linkage group II extending roughly from *dpy-10* to *unc-53*. Deficiencies are drawn below the line and indicate which genes are deleted.


A
I
lin-53
bli-3
dpy-5
unc-29
lin-11
unc-75
unc-54
II
IIC
unc-85
bli-2
dpy-10
unc-53
unc-52
III
lin-51
lin-52
dpy-1
daf-2
unc-93
dpy-27
unc-79
dpy-17
lon-1
sma-3
unc-36
dpy-19
unc-32
unc-16
unc-69
unc-49
dpy-18
unc-25
IV
lin-54
dpy-9
egl-18
unc-5
dpy-20
unc-22
unc-30
lev-1
unc-26
dpy-4
V
unc-34
dpy-11
unc-51
X
lon-2
unc-3
= 10 map units

B


IIC
lin-55
lin-56
dpy-10
rol-6
let-239
let-240
unc-4
daf-19
unc-53
mnDf67
mnDf85
mnDf46
mnDf71
= 0.5 map units

Chapter 3

The *C. elegans* Gene *lin-36* Acts Cell Autonomously to Negatively Regulate Vulval Development

Jeffrey H. Thomas and H. Robert Horvitz

Howard Hughes Medical Institute, Massachusetts Institute of Technology
Cambridge, MA 02139

This chapter will be submitted to *Development*

Summary

Mutations in the *Caenorhabditis elegans* gene *lin-36* do not cause an abnormal phenotype alone but do cause a multivulva phenotype when combined with certain other mutations. Such interacting genes, known as synthetic multivulva genes, fall into two classes: A and B. Only a combination of a class A mutation and a class B mutation results in the multivulva phenotype. The synthetic multivulva genes act as negative regulators of the *ras*-mediated induction pathway of *C. elegans*. In this paper, we report that *lin-36* mutations do not cause vulval lineage defects alone, and that *lin-36* encodes a novel protein. We also report that *lin-36* is required cell autonomously for the regulation of vulval fate decisions and that a *lin-36::*GFP is expressed in the nuclei of the cells that decide whether to adopt vulval fates. We report that the synthetic multivulva genes act at the same hierachical level as, but independently of, the *lin-2*, *lin-7* and *lin-10*-mediated receptor localization pathway.

Introduction

Receptor tyrosine kinase and Ras-mediated signal transduction pathways have been implicated in the control of development in numerous organisms and in oncogenesis. Although the signal transduction pathway itself is relatively well understood, little is known about the negative regulation of the pathway *in vivo*. The receptor tyrosine kinase and Ras-mediated signal transduction pathway in *Caenorhabsditis elegans* provides a useful model for the analysis of this problem. A well-defined biological context and genetically defined negative regulators of the receptor tyrosine kinase signal transduction pathway are useful reagents for addressing the mechanism of the negative regulation of signal transduction.

Vulval induction, the biological context of C. elegans receptor tyrosine kinase and Ras-mediated signal transduction has been well defined. The hermaphrodite vulva of *C. elegans* is formed from the descendents of three hypodermal blast cells, P(5,6,7).p (Sulston and Horvitz, 1977). These cells are part of the vulval equivalence group, P(3-8).p, a set of six cells which have the potential to adopt either one of two vulval fates (1° or 2°) or a nonvulval fate (3°) (Sulston and White, 1980; Kimble, 1981; Sternberg and Horvitz, 1986). Cell fates are specified by several cell interactions (for review, see Horvitz and Sternberg, 1991; Eisenmann and Kim, 1994; Sundaram and Han, 1996). The gonadal anchor cell signals the nearest Pn.p cells to adopt vulval fates and is at least partly responsible for establishing the proper pattern of vulval fates (Kimble, 1981; Sternberg and Horvitz, 1986; Sternberg, 1988; Thomas et al., 1990; Simske and Kim, 1995; Katz et al., 1995). The receptor tyrosine kinase signal transduction pathway is activated by the anchor cell signal. Many of the components of this signaling system are known (Arioan et al., 1990; Beitel et al., 1990; Han and Sternberg, 1990; Horvitz and Sternberg, 1991; Clark et al., 1992; Hill and Sternberg, 1992; Han et al., 1993; Eisenmann and Kim, 1994; Lackner et al., 1994; Wu and Han, 1994; Kornfeld et al., 1995a, 1995b; Wu et al., 1995; Sundaram and Han, 1996).

A number of other genes have been identified that act as positive and negative regulators of the receptor tyrosine kinase and Ras-mediated signal transduction pathway. Mutations in the genes *lin-2*, *lin-7* and l*in-10* produce a vulvaless (Vul) phenotype similar to that produced by reduction-of-function mutations in components of the *let-60 ras* pathway. *lin-2*, *lin-7* and *lin-10* are not part of this signal transduction cascade, but rather act as positive regulators of the cascade. They have been implicated in the localization of the LET-23 receptor to the cell junction thereby upregulating the activity of the receptor

tyrosine kinase pathway (Ferguson et al., 1987; Kim and Horvitz, 1990; Hoskins et al., 1996; Simske et al., 1996).

Negative regulators of the receptor tyrosine kinase signaling pathway have also been identified. A group of genes known as the synthetic Multivulva (synMuv) genes have been shown to inhibit the adoption of vulval fates by Pn.p cells. The interaction of two different synMuv mutations produce a multivulva (Muv) phenotype (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985; 1989). The mutations that interact to produce such a synthetic multivulva (synMuv) phenotype fall into two classes: A and B. Animals carrying both a class A and a class B mutation have a Muv phenotype whereas animals which carry mutations in a single class have a wild-type vulval phenotype. Ferguson and Horvitz (1989) have proposed that the synMuv genes encode the components of two functionally redundant pathways which negatively regulate vulval development. Four class A genes (*lin-8*, *lin-15* A, *lin-38* and *lin-56*) and ten class B genes (*lin-9*, *lin-15* B, *lin-35*, *lin-36*, *lin-37*, *lin-51*, *lin-52*, *lin-53*, *lin-54*, *lin-55*) have been identified (Horvitz and Sulston, 1980; Ferguson and Horvitz, 1989; Chapter 2). *lin-15* is a complex locus with genetically separable class A and class B activities encoded by two nonoverlapping transcripts (Ferguson and Horvitz, 1989; Clark et al., 1994; Huang et al., 1994). Three of these genes, *lin-15* A, *lin-15* B and *lin-9*, have been cloned and shown to encode novel proteins (Clark et al., 1994; Huang et al., 1994; Beitel and Horvitz, in preparation).

SynMuv mutants in which the anchor cell has been ablated still display a Muv phenotype, suggesting that in the absence of synMuv gene activity, the Pn.p cells do not require the anchor cell signal to adopt vulval cell fates (Ferguson et al., 1987). However, loss-of-function mutations in genes known to be involved inductive signal transduction, *let-23*, *sem-5*, *let-60 ras*, and *lin-45 raf* are epistatic to *lin-15* AB mutations (Ferguson et al., 1987; Beitel et al., 1990; Han et al., 1990; Clark et al., 1992; Han et al., 1993; Huang et al., 1994). These results suggest that the inductive signal transduction system is required for the adoption of the Muv phenotype in the absence of inhibitory synMuv gene activity even though the anchor cell signal itself is not. Genetic mosaic analyses suggest that both *lin-15* AB and *lin-37* act cell non-autonomously and most likely in the hypodermal syncytium suggesting that the synMuv genes encode the components of two redundant signaling systems that negatively regulate vulval development (Herman and Hedgecock, 1990; Hedgecock and Herman, 1995).

lin-36, a class B gene, was selected for further genetic and molecular analysis on the basis of its map position and the observation that *lin-36* seems to act only in vulval development (Ferguson and Horvitz, 1989; Chapter 2). Here we describe the further

genetic characterization of *lin-36* mutants. The phenotype of *lin-36* is analyzed at the lineage level. We show that *lin-36* encodes a novel protein and acts cell autonomously. We show that *lin-36* is likely expressed in the nuclei of the Pn.p cells. This result suggests that negative regulation of vulval development by class B synMuv genes may be mediated in the nucleus of the responding cell even though the inhibitory signal emanates from another tissue. We also describe the genetic interaction between the synMuv genes which negatively regulate the *let-23* induction pathway and genes which positively regulate the *let-23* pathway.

Materials and Methods

General genetic methods and strains

Caenorhabditis elegans strains were maintained and genetically manipulated as described by Brenner (1974) and were grown at 20° unless otherwise stated. Genetic nomenclature used conforms to the standards established by Horvitz et al. (1979). N2 was used as the wild type strain and was the parent of all strains used in this study. Unless otherwise noted, the mutations used in this study are described in Hodgkin et al. (1988) and are listed below.

LGI: *lin-35(n745)* (Ferguson and Horvitz, 1989); *lin-10(n299)* (Ferguson and Horvitz, 1985); *lin-53(n833)* (Ferguson and Horvitz, 1989; Chapter 2).

LGII: *lin-8(n111)* (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985; Ferguson and Horvitz, 1989); *let-23(sy97)* (Aroian and Sternberg, 1991); *lin-55(n2994)* (Chapter 2); *rol-6(su1006)*; *lin-7(e1413)* (Ferguson and Horvitz, 1985; Simske et al., 1995); *mnC1 dpy-10(e128) unc-52(e444)*.

LGIII: *lin-51(n770)* (Ferguson and Horvitz, 1989; Chapter 2); *dpy-17(e164)*; *sma-2(e502)*; *sma-3(e491)*; *egl-5(n945)*; *egl-45(n999)*; *ncl-1(e1865)*; *lin-36(n747, n750, n766, n772)* (Ferguson and Horvitz, 1989) *(n2235, n2240, n2243)* (Chapter 2); *unc-36(e251)*; *lin-9(n112)* (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Ferguson and Horvitz, 1985; Ferguson and Horvitz, 1989); *unc-32(e189)*; *lin-52(n771)* (Ferguson and Horvitz, 1989; Chapter 2); *nDf20* (Finney et al., 1988); *sDp3(f)* (Rosenbluth et al., 1985).

LGX: *lon-2(e678)*; *xol-1(y70)* (Miller et al., 1988); *lin-2(n397)* (Ferguson and Horvitz, 1985; Hoskins et al., 1996); *lin-15(e1763, n767)* (Ferguson and Horvitz, 1985; Ferguson and Horvitz, 1989; Clark et al., 1994, Huang et al., 1994).

Noncomplementation screens for *lin-36* alleles

The 4,5',8-trimethylpsoralen (TMP) and ultraviolet radiation mutagenesis technique of Yandell et al. (1994) was used to conduct a noncomplementation screen for new *lin-36* alleles. L4 males of genotype *lin-8(n111)* were mutagenized with TMP and mated to hermaphrodites of genotype *lin-8(n111); sma-3(e491) lin-36(n766) unc-32(e189)* and the phenotypically non-Sma non-Unc F_1 cross-progeny were screened for Muv animals. A total of 23,666 haploid genomes were screened, and one Muv non-Sma non-Unc animal was isolated. This mutant was made homozygous and backcrossed three times with *lin-8(n111)*.

A second noncomplementation screen was conducted using ethyl methanesulfonate (EMS) as a mutagen. L4 males of genotype *lin-8(n111)* were mutagenized with EMS and mated to hermaphrodites of genotype *lin-8(n111); lin-36(n766) unc-32(e189); lon-2(e678) xol-1(y70)* and the phenotypically non-Unc non-Lon progeny were screened for Muv animals. A total of 21,003 haploid genomes were screened and five Muv non-Unc non-Lon animals were isolated. These mutants were made homozygous and then backcrossed three to four times by *lin-8(n111)*.

Pn.p cell lineage analysis

P(3-8).p cell lineages were observed using Nomarski optics as described by Sulston and Horvitz (1977). The nomenclature and criteria described by Sternberg and Horvitz (1986, 1989) were used to assign 1°, 2° and 3° cell fates.

General DNA manipulation

General DNA manipulations and analyses were essentially performed according to the protocols of Sambrook et al. (1989). Subcloning was conducted using the pBluescript SK and KS (+) and (-) vectors (Stratagene Cloning Systems, La Jolla CA) and the pIBI20 vector (VWR Scientific, Boston, MA). The RNA filter used for Northern blotting was a gift from L. Bloom.

Germline transformation

Germline transformation by microinjection was done according to Fire (1986) and Mello et al. (1991). DNA for injection was purified using Qiagen columns (Qiagen Inc., Chatsworth, CA). Cosmids and plasmids for rescue experiments were injected at concentrations of 20-80 µg/ml. The *lin-36::gfp* reporter was injected at 50 µg/ml. pRF4, a plasmid containing the *rol-6(su1006)* dominant allele was used as a coinjection marker in all rescue experiments (Mello et al., 1991). Animals were raised and injected at 20°.

F_1 Rol animals were placed individually on plates and scored for the Muv phenotype. Transmitting lines were established from these isolates and scored at 20°.

Isolation of cDNAs and sequence determination

We screened the Barstead mixed stage cDNA library (Barstead and Waterston, 1989) with the 5.0 kb minimum rescuing fragment cloned in pJHT16 and isolated one cDNA. Samples for sequencing were prepared from both strands using the limited *Exo III* digestion procedure. Single-stranded template was prepared using M13 phage according to Sambrook et al. (1989). The sequences of these templates were determined using the dideoxy chain termination method and the Sequenase enzyme (U. S. Biochemical, Cleveland, OH). A few regions required the use of the internal oligonucleotide primers listed below:

pssEx11: 5' CAAGAAACAAGAAGCAC
pssEx14: 5' GTCATTGATTCTATGG
pgapEx6: 5' ACTGAGTTGGATTCAG
pss-6B: 5' TTGAATTTTGAGGCAC
pss-27B: 5' GAGCTTGTGGAGACTG
pgap-27B: 5' TTAATCTCTGCTTCTC

One region in the 3' untranslated region required the use of *Taq* polymerase at 95° to sequence (Promega, Madison, WI). DNA sequences were assembled and analyzed using the *Geneworks* program (Intelligenetics, Inc., Mountain View, CA).

Molecular analysis of *lin-36* lesions

Genomic DNA was purified from N2 and strains carrying *lin-36*(*n747*, *n750*, *n766*, *n772*, *n2235*, *n2240*, *n2243*, *n3090*, *n3093*, *n3094*, *n3095*, *n3096*, *n3097*) mutations. *lin-36(n3090)* DNA was digested with *Sal*I and *Xba*I, *Nco*I, *Nco*I and *Xba*I, *Eco*RV, and *Hinf*I and analyzed by Southern blot (Sambrook et al., 1989) using the radiolabeled insert of pJHT16 as a probe. Four primer pairs were used to amplify the *lin-36* coding region and the regions of introns near the splice sites using PCR. These primer pairs are listed below:

P100: 5' ACTGTATTTTCTGTTTTTGTGCTC with:
R1100: 5' CGAGCTAACATATTCATTCACAAG
P200: 5' CTTGTGAATGAATATGTTAGCTCG with:
R1501A: 5' TTCACCAGATCCCATTGGAGCTTG
s9: 5' TCACCAACGAAAAAAGCCTACGTG with:
R1502: 5' ATCAATGACTTCTTCGACCATTTC

P400: 5' AGACCGGTAAGTTTAATATATTAG with:

R1503C: 5' GGATAACTTCTGTGGATATAAAAC)

The DNA fragments were gel-purified in low melting temperature agarose. The agarose was removed by digestion with ß-agarase, and the DNA purified according to standard protocols (Sambrook et al., 1989). The sequences of these PCR products were determined using an automated ABI 373A cycle sequencer (Applied Biosystems, Foster City, CA). In addition to seven of the eight primers (P100, R1100, P200, s9, R1501A, P400, and R1503C) used to generate the PCR fragments, the following primers were used to sequence the PCR products and are listed below:

s1: 5' AACCAGAAGACTCACACGTGACGG

s3: 5' TTTATTTTCAGATTCCCGTAATGC

s4: 5' TCCGTGCCAAGTTATTCCGGAGAC

s7: 5' TTTTTCACAGGTCCCAGAAGCTGG

P300: 5' CTGCGTAACGAAATTTAAAAAATC

s11: 5' GAGAACCAACAGTTTCACCAAATG

s13: 5' ATTCCTCTTTTACTTTGTAGCATG

s15: 5' TCGCGAGATCTTTTGTTGCTGGAG

s17: 5' ATCGTCTGAAGATGTTGCCACTTC

s19: 5' CAGGTGAGCCCATGGGAGGTCTAC

Genetic mosaic analysis

Genetic mosaic analysis of *lin-36* was conducted using the method of free duplication loss described by Herman (1984). We analyzed genetic mosaics using a strain of genotype *lin-8(n111); dpy-17(e164) ncl-1(e1865) lin-36(n766) unc-36(e251); sDp3(dpy-17(+) ncl-1(+) lin-36(+) unc-36(+))*. These worms are phenotypically wild-type. Most progeny are of two types: phenotypically wild-type progeny that carry the free duplication and progeny that are Dpy Ncl Unc that have lost the duplication in the maternal gametes or in the zygote. These Dpy Ncl Unc animals occur at a frequency of 31% (n=771). Rare progeny show a loss of the duplication mitotically, creating a mosaic of mutant cells in an otherwise wild-type animal. Distinct phenotypes result from the loss of the duplication in certain lineages in which the wild-type allele is required. *unc-36* has a focus in AB.p (Kenyon, 1986). *dpy-17* apparently has has a hypodermal focus in AB.a, AB.p and C (Yuan and Horvitz, 1990). *ncl-1* acts cell-autonomously and the enlarged nucleolus of the mutant can be scored in many cells (Hedgecock and Herman, 1995).

Two strategies were adopted to find mosaic animals. In the first, mosaic animals of the L3 and L4 larval stages (in addition to a few adults) were isolated on the basis of

gross morphology and then observed using Nomarski optics to further define the cell division at which duplication loss occurred. Unc non-Dpy animals result from duplication loss in AB or AB.p. Dpy non-Unc animals result from loss in P_1, P_2, or C. In these animals, a P_2 loss can be distinguished from a C loss by the segregation of all Dpy Ncl Unc progeny. Semi-Unc non-Dpy animals are the result of a loss in either AB.pl or AB.pr (Yuan and Horvitz, 1990). The second strategy was to examine seemingly wild-type L3 and L4 larvae for the Ncl phenotype using Nomarski optics. A total of 1515 animals were examined in this manner. This approach enabled us to identify AB.a losses and losses in the AB.p lineages later than AB.pl/r. The Ncl phenotype was scored primarily in neurons; it cannot be scored reliably in the Pn.p cells themselves. From the P_1 lineage, the pharyngeal neurons I4, I6, M1, M4 and M5, the tail neurons PVR and DVC, and occasionally hyp11 were scored for the Ncl phenotype. From AB.p, the HSNs and the AB.p-derived neurons of the head and tail were scored. The subset of AB.p-derived neurons with a Ncl phenotype and their lineage relationship (Sulston and Horvitz, 1977; Sulston et al., 1983; White et al., 1986) allowed us to determine the cell division at which duplication loss occurred. From AB.a, the BDUs, ALMs, the pharyngeal neurons I5, MI, the M2s and the M3s, and the AB.a-derived neurons of the head were scored.

To determine the maximum number of mosaic animals in which duplication loss occurred in lineages leading to the anatomical focus of *lin-36*, we counted the penetrance of the Muv phenotype in Dpy Ncl Unc animals. The penetrance of the Muv phenotype of Dpy Ncl Unc animals derived from duplication-bearing mothers was 69% (n=240). The penetrance of Muv non-Dpy non-Unc animals was 1.3% (n=531).

Construction of a *lin-36::* GFP reporter

A *lin-36::*GFP reporter was constructed by ligating the 4.4 kb *Sal*I *Sac*I fragment from pJHT16 to a linker fragment and *Sal*I *Xma*I double digested pPD95.79. pPD95.79 is a vector containing a green fluorescent protein coding region with a *C. elegans unc-54* 3' end and a fluorescence enhancing S65C mutation (Chalfie et al., 1994; Heim et al., 1994; 1995; A. Fire, personal communication). The linker fragment was constructed by using the primers pG5F (5' AGAGCAATGGAAGACGC) and pG3W (5' TCCCCCCGGGTTGTTGAGAATCCGATG) to amplify DNA from pJHT16. pG3W anneals to the end of the *lin-36* coding region including the last codon and adds an *Xma*I site followed by four additional nucleotides. This linker was digested with *Xma*I and an internal *Sac*I site.

Five lines were isolated from germline transformation experiments using a *lin-36(n766); lin-15(n767)* animal as a host and *rol-6(su1006)* as a coinjection marker. The reporter construct rescued the Muv phenotype of the strain in transformed lines. Animals from two of these lines were exposed to 4100 rads of γ-irradiation from a ^{60}Co source. The progeny of 894 F1 animals were screened for lines that segregated only Rol animals. Three such lines were isolated, but one had to be discarded because it had lost *lin-36* rescue ability and GFP staining. Line 2H1 expressed strongly, but the animals were sickly; line 3L26a was healthy and exhibited expression similar to the other transformants and was used in the characterization of *lin-36::*GFP reporter expression. Reporter expression in class B synMuv mutant backgrounds was analyzed in strains constructed by crossing the reporter construct into the strain bearing a class B mutation and isolating Muv Rol animals from the F2 generation.

Construction of strains for gene interaction studies

To constuct *lin-36*-containing triple mutants, N2 males were crossed into Vulvaless(Vul) strains mutant in *let-23*, *lin-2*, *lin-7*, or *lin-10*. Cross-progeny males were crossed into *lin-36; lin-15* hermaphrodites and cross progeny hermaphrodites were isolated. These hermaphrodites were allowed to self-fertilize and Muv and Vul animals were isolated. Progeny of the opposite phenotype (Vul segregants from Muv animals and Muv segregants from Vul animals) were isolated as the triple mutants. This scheme is essentially identical to that used previously (Ferguson et al., 1989). Double mutants containing mutations resulting in a Vul phenotype and the *lin-15(e1763)* Muv mutation were isolated in the same manner. In strains containing *lin-2*, a large number of animals were used in the crosses to insure that recombinants between the linked mutations in *lin-2* and *lin-15* would be isolated. For all genes tested, either null alleles or the strongest alleles known were used.

Laser microsurgery

Laser microsurgery was conducted essentially as described previously (Sulston and White, 1980; Avery and Horvitz, 1987; Thomas et al., 1990). 60 mM sodium azide was used as an anesthetic. Mock ablated animals were treated exactly as operated animals with the exception of laser microsurgery. Three mock ablated animals were used as an operation control in the *lin-36(n766); lin-15(n767)* experiment, and eight were used as a control in *lin-7(e1413); lin-36(n766); lin-15(n767)* experiment.

Results

Identification of new *lin-36* alleles

lin-36 mutations had previously been identified in screens for class B synMuv mutations (Ferguson and Horvitz, 1989; Chapter 2). To identify more mutations in this locus, two noncomplementation screens were conducted in a *lin-8(n111)* class A background. In one screen, 4,5',8-trimethylpsoralen was used as a mutagen. One mutation, *lin-36(n3090)* was isolated and shown to be associated with an *Eco*RV polymorphism by Southern hybridization. This RFLP was produced by a point mutation rather than a deletion. In the other screen, using EMS as a mutagen, five *lin-36* mutations (*n3093*, *n3094*, *n3095*, *n3096*, *n3097*) were isolated. This frequency of EMS-induced *lin-36* alleles, $2.4 \cdot 10^{-4}$ per haploid genome is close to that expected for the average EMS-induced loss-of-function mutation in *C. elegans*, $5 \cdot 10^{-4}$ (Brenner, 1974; Greenwald and Horvitz, 1980).

The penetrance of the Muv phenotype of each of the mutant strains was determined (Table 1). Three of the six strains, *lin-8(n111); lin-36(n3093)*, *lin-8(n111); lin-36(n3095)*, *lin-8(n111); lin-36(n3096)* are phenotypically similar to two strains carrying strong *lin-36* alleles, *lin-8(n111); lin-36(n747)*, *lin-8(n111); lin-36(n766)* (Ferguson and Horvitz, 1989). *lin-8(n111); lin-36(n3090)* is phenotypically similar to *lin-8(n111); lin-36(n2243)*; these mutations are missense mutations in adjacent residues (Table 3, and Chapter 2).

The phenotypes of the strongest mutant alleles (*n747*, *n766*, *n3093*, *n3095*, *n3096*) are essentially identical, suggesting that they may be complete loss-of-function alleles. The frequency of isolation in a noncomplementation screen is not inconsistent with the isolation of null alleles. We are not able to interpret the phenotype of animals carrying both *lin-36* alleles and deficiencies of the *lin-36* region since the phenotype of deficiency *trans*-heterozygotes is weaker than that of homozygotes. It is possible that a linked, haplo-insufficient suppressor of the synMuv phenotype is uncovered by the deficiencies or that the tested lin-36 alleles have some antimorphic character. The molecular natures of the strong lesions are consistent with complete loss-of-function alleles;four out of five are nonsense mutations (Table 3). Thus, much of the data is consistent with the identification of loss-of-function alleles, but these data are not compelling.

Vulval lineage defects of *lin-36*

The vulval defect in strains carrying *lin-36* mutations was analyzed at the lineage level. Each of the three fates adopted by the cells of the vulval equivalence in wild-type vulval development displays a characteristic and distinct cell division and morphology

pattern. We observed the lineages of the P(3-8).p descendents in various strains carrying synMuv mutations. Animals carrying only a strong *lin-36* mutation, *lin-36(n766)*, displayed wild-type Pn.p cell lineages, as did animals carrying only the strong class A mutation, *lin-15(n767)* (Table 2). Thus, animals homozygous for only a single, representative synMuv mutation of either class A or class B, are wild-type for vulval development at the lineage level. The members of the vulval equivalence group that are not induced in wild type, P(3,4,8).p, are induced in animals of genotype *lin-36(n766); lin-15(n767)*. In ten animals examined, 29/30 of these cells were completely induced and the remaining 1/30 showed partial induction. All but one of these cells that adopted 2° cell fates (n=7 cells), adopted the same polarity as the cell that in wild type adopts the 2° cell fate on the same side of the P6.p cell. 3/30 P(3,4,8).p cells adopted induced lineages which did not meet the criteria for assignment as either a 1° or a 2° cell fate. One of these included a P3.pap cell that adopted a hyp7-like nuclear morphology and fused with the syncytium; otherwise, the lineage was consistent with a 1° cell fate (Table 2). Ectopic vulval tissue from P3.p and P4.p formed invaginations consistent with the fates and polarities of the cells which composed this tissue. In cases where ectopic 2° cell lineages were adjacent to ectopic 1° lineages and their relative polarity was the same as that found in the wild-type vulva, a single invagination was observed. In cases in which the 2° lineage polarity was opposite that of the wild-type vulva, two invaginations were observed. In one case in which a P3.pap cell fused with the syncytium (described above), three invaginations were observed (Table 2).

Cloning of *lin-36*

Previous data placed *lin-36* between *lin-13* and *unc-36* on linkage group III (Ferguson and Horvitz, 1989). We mapped *lin-36* under *nDf20* and between *egl-5* and *unc-36* (Figure 1a) which had both been cloned and positioned on the physical map (Coulson et al., 1986; Coulson et al., 1991; Wang et al., 1993; L. Lobel, personal communication). We predicted the physical location of *lin-36* based on the relative map distances between *lin-36* and *egl-5* and *unc-36*, and selected cosmid pools from this region (Figure 1b). These pools of cosmids were injected into animals bearing a class A synMuv mutation and a *lin-36* mutation in germline transformation experiments. Cosmid E02E3 was shown to rescue the Muv phenotype of both *lin-8(n111); lin-36(n747)* and *lin-36(n766); lin-15(n767)* and was used to construct subclones. These experiments defined a 5.0 kb *Xba*I *Sal*I minimum rescuing fragment, pJHT16 (Figure 1c)

The *Xba*I *Sal*I insert of pJHT16 was used as a probe to a Northern blot containing poly-A+ RNA from mixed stage animals. This probe recognized a single band of

approximately 3.5 kb. The same fragment was used as a probe to screen the Barstead cDNA library (Barstead and Waterston, 1989) and isolate a single cDNA clone. The 3400 nucleotide cDNA included a poly-A tail and nine nucleotides of the SL1 trans-spliced leader, indicating that it was full length (Krause and Hirsh, 1987; Huang and Hirsh, 1989). The sequence of the cDNA was compared to genomic sequence from the region generated by the *C. elegans* genome sequencing project to define the exon and intron structure of the gene (Wilson et al., 1994).

lin-36 encodes a novel protein

The coding sequence of *lin-36* was determined from the cDNA clone (Figure 2). Conceptual translation yields a novel gene product of 962 amino acids with a predicted molecular weight of 108 kDa. The predicted protein product is highly hydrophilic and has no hydrophobic stretches of significant length. There are few significant protein motifs. Amino acids 428 to 532 are a potential nuclear localization sequence (for review, see Goldfarb, 1989) and a poly-glutamine stretch at the C-terminus. The region between residues 102 and 376 is cysteine- and histidine-rich; it contains 15 histidine and 14 cysteine residues. These residues are largely concentrated in the region between amino acids 151 and 300; 12 histidine residues and 12 cysteine residues lie in this region. Only two cysteine and two histidine residues fit zinc-finger spacing requirements (Rhodes and Klug, 1986; Tautz et al., 1987). Given the large number of Cys and His residues, it is unlikely that this region forms zinc fingers.

Molecular determination of *lin-36* lesions

The polymerase chain reaction was used to amplify *lin-36* DNA from mutants in order to determine the sequence changes in these strains. Six mutations are missense mutations, five are nonsense mutations, one is a splice site mutation, and one contains a missense mutation as well as a putative novel splice donor (Table 3). The nonsense mutations are expected to truncate the *lin-36* product. Three of the ochre mutations, *lin-36(n766)*, *lin-36(n3095)* and *lin-36(n3096)* delete 167, 394 and 521 amino acids from the 962 amino acid coding sequence respectively. All produce a strong phenotype. Another ochre mutation, *lin-36(n2235)* deletes 65 amino acids from the coding sequence and produces a weaker phenotype. The amber mutation, *lin-36(n3093)* deletes 384 amino acids from the protein. One mutant allele, *n750*, had two changes, one of which was identical to the *n2240* lesion. The other change is a silent glycine to glycine change but may generate a novel splice donor site. The mutation produces a sequence that closely conforms to the canonical *C.elegans* splice donor parameters (Fields, 1990). All of the

EMS induced mutations are the expected G:C to A:T transitions (Coulondre and Miller, 1977), except for *n766* which is a T:A to A:T transversion, a lesion rarely seen induced by EMS (Table 3).

lin-36 acts cell autonomously

To determine the site of action of *lin-36*, we conducted a genetic mosaic analysis. This analysis is commonly conducted in *C. elegans* by generating mutant clones in an otherwise wild-type animal through the loss of a mitotically unstable free duplication which complements the mutation of interest. By scoring the phenotypes of linked markers that have known sites of action and are complemented by the free duplication, and correlating these results with the known lineage of all *C. elegans* cells (Sulston and Horvitz, 1977; Sulston et al., 1983), the site of action of a gene can be determined (Herman, 1984).

A strain of genotype *lin-8(n111); dpy-17(e164) ncl-1(e1865) lin-36(n766) unc-36(e251); sDp3(f)* was used to generate genetic mosaics as described in Materials and Methods. Genetic mosaics were identified and characterized as L3 and L4 larvae and subsequently scored for the Muv phenotype upon reaching adulthood. Strains carrying *lin-36* exhibit maternal rescue of the Muv phenotype (Ferguson and Horvitz, 1989; Chapter 2). The penetrance of the Muv phenotype of Dpy Ncl Unc animals homozygous for *lin-36* descended from duplication bearing mothers was determined to be 69% (n=240). This number sets an upper limit on the fraction of Muv animals we expected from a mitotic loss of the duplication in the lineage from which the cellular focus of *lin-36* is derived.

Loss in AB, AB.p, AB.pl, or AB.pr often produced Muv animals. Loss in P_1 or in AB.a failed to result in Muv animals. These data are consistent with an anatomical focus in both the AB.pl and AB.pr. lineages; the Pn.p cells derive from both of these lineages. Note that P_1 makes the largest hyp7 contribution during embruogenesis. Note also that AB.a makes a larger contribution to hyp7 that either AB.pl or AB.pr, yet losses in AB.pl or AB.pr often result in a Muv phenotype whereas losses in AB.a or P_1 do not (Figure 3). Thus, the Pn.p cells, derived from AB.pl and AB.pr, are more likely candidates for the focus of *lin-36* activity than the hyp7 nuclei descended from thess lineages. As discussed below, synMuv genes with a postulated focus in hyp7 show very different clonal behavior. Three double losses were observed. One occurred in AB and MS/EMS and resulted in a Muv phenotype, presumably because of the AB loss. Another occurred in AB.pl and C resulting in a Muv phenotype, presumably because of the AB.pl loss. A third occurred in AB.prapp and AB.a resulting in a Muv phenotype, presumably because

of the AB.prapp loss. Thus, the phenotypes of mosaic animals with double losses could be explained on the basis of one of the two losses.

The simplest interpretation of these results is that *lin-36* acts cell autonomously in the Pn.p cells, although it is possible that *lin-36* is actually required in close relatives of the Pn.p cells, possibly hyp7 cells derived from V3 or V5, in both the AB.pl and AB.pr lineages.

These results are in marked contrast to the genetic mosaic analysis results of *lin-15* and *lin-37*. Loss of *lin-15* activity in AB or AB.p lineages results in a Muv phenotype, as does loss in AB.pl or AB.pr, albeit at a lower frequency. *lin-15* mosaics differ from *lin-36* mosaics in that loss of *lin-15* activity in P_1 results in a Muv phenotype. The P_1 lineage does not give rise to Pn.p cells, yet *lin-15* is required in this lineage as well as in the AB lineage. These findings suggest that *lin-15* is required in hyp7, which originates from both of these lineages (Herman and Hedgecock, 1990). The *lin-15* genetic mosaic analysis was conducted using a *lin-15* AB allele. Since *lin-15* A and *lin-15* B activities are redundant, both activities must be lost simultaneously in their respective anatomical foci in a mosaic analysis to produce a Muv phenotype. Since simultaneous loss of both *lin-15* A and *lin-15* B activities in P_1 is sufficient to produce a Muv phenotype, *lin-15* A and *lin-15* B are both required cell nonautonomously.

The *lin-36* mosaic results differ from the *lin-37* mosaic results. Only 3/23 animals in which *lin-37* was lost in AB had a Muv phenotype. Loss in AB.p was insufficient to produce a Muv phenotype (n=24). Loss in P_1 was also insufficient. Since loss in AB.p did not produce a Muv phenotype, these results have been interpretted to mean that *lin-37* activity is required cell nonautonomously. It has been proposed that the loss of *lin-37* activity in P_1 or AB.p lineages is insufficient to result in a Muv phenotype and that loss in AB is only occasionally sufficient. This is probably because the AB lineage produces almost all of the hyp7 nuclei in the animal. It is likely that *lin-37* is provided in greater excess than *lin-15* activity and that this accounts for the differences in the mosaic animals even though both are thought to act cell nonautonomously (Hedgecock and Herman, 1995). Thus, the requirements for *lin-36* activity differ substantially from those of *lin-15* and *lin-37*, and are consistent with a focus in the vulval precusor cells.

A *lin-36::*GFP reporter is expressed in the nuclei of Pn.p cells

To provide support for the evidence from the genetic mosaic analysis of *lin-36*, we determined the expression pattern of a *lin-36::*GFP reporter construct. This construct contains 1 kb of sequence 5' to the ATG start site and the entire *lin-36* open reading frame and all intervening sequences. GFP was fused, in frame, to the last amino acid residue of

lin-36. The *lin-36::*GFP reporter transgene rescued the Muv phenotype of a *lin-36; lin-15* A strain. This implies that expression in at least a subset of the cells in which the fusion protein is expressed is sufficient for normal *lin-36* function. *lin-36::*GFP is expressed in Pn.p cells and their descendents during vulval cell determination, division, and invagination (Figure 4). Similar observations have been made with an independently constructed *lin-36::*GFP reporter (Alex Hajnal, personal communication). This expression pattern is consistent with the data obtained in the genetic mosaic analysis. The Pn.p cell expression of *lin-36::*GFP is not altered by mutations in other class B genes. This was tested in *lin-9*, *lin-15*, *lin-35*, *lin-51*, *lin-52*, *lin-53*, and *lin-55* backgrounds (Ferguson and Horvitz, 1987; Chapter 2).

Besides the Pn.p cells, many other cells express GFP in strains bearing the reporter construct. Most notably, the neurons of the head, tail and ventral cord express *lin-36::*GFP throughout development. Some staining has also been observed in the gonad.

Interestingly, *lin-36::*GFP expression is observed in the nuclei of all cells in which it is expressed. No expression in the cytoplasm has been detected. The GFP reporter gene used in the construction of this reporter construct did not contain a nuclear localization sequence, but as mentioned earlier, the *lin-36* coding region contains a potential nuclear localization sequence. This sequence appears to be sufficient to allow localization of the LIN-36::GFP fusion protein to the nucleus. This data suggests that *lin-36* may act in the nucleus of the Pn.p cells to mediate its effect on vulval development.

The vulvaless phenotype of *lin-2*, *lin-7*, and *lin-10* is co-expressed with the multivulva phenotype in triple mutants with synMuv mutations

To determine where the synMuv genes act in the regulatory hierarchy controlling vulval induction, we constructed triple mutants using several vulvaless mutations and synMuv mutations (Table 4). The Vul phenotype of mutations in the *let-23* receptor tyrosine kinase gene is epistatic to the *lin-36; lin-15* A synMuv phenotype. Thus, *let-23* activity is required for the adoption of vulval fates by Pn.p cells in synMuv mutants. The *let-23* receptor tyrosine kinase acts downstream of, or in parallel to, the synMuvs. This is consistent with previous results using other synMuv mutations (Ferguson et al., 1987; Huang et al., 1994).

lin-2, *lin-7*, and *lin-10* positively regulate the *let-23*-mediated signal transduction cascade presumably by localizing the LET-23 receptor to the Pn.p cell junctions (Simske et al., 1995; Hoskins et al., 1996). In triple mutants with these mutations, the synMuv phenotype and the Vul phenotype are co-expressed (Table 4). Neither is epistatic to the

other. Animals are observed with either phenotype, a wild-type phenotype, or both phenotypes such that animals are either bloated with eggs or are bags of worms, and have ectopic ventral protrusions. This suggests that both groups of genes, the synMuv genes and the *lin-2*, *lin-7*, *lin-10* group, act at the same hierarchical level of regulation of the *let-23* pathway and that both sets of genes act independently of each other to regulate the *let-23* signal transduction pathway. The unusual distribution of phenotypes and the co-expression of phenotypes in the same animals suggests that, in the absence of both positive and negative regulation through these genes, the Pn.p cells are deregulated. Cells that in wild type normally adopt vulval fates often adopt nonvulval fates whereas cells that normally adopt nonvulval fates often adopt vulval fates in these animals (Table 2). It is worth noting that the anchor cell signal has not been eliminated in these animals.

The co-expression of Muv and Vul phenotypes has been reported for *lin-8; lin-9* synMuv strains with *lin-2*, *lin-7* and *lin-10*. However, double mutants between the synMuv *lin-15(n309)* mutation and *lin-2*, *lin-7* and *lin-10* have been reported to be Muv (Ferguson et al., 1987). We repeated this experiment using *lin-15(e1763)*. Both of these *lin-15* alleles are molecular null alleles (Clark et al., 1994). The *lin-15* double mutants with *lin-2*, *lin-7* and *lin-10* show co-expression of the Muv and Vul phenotypes (Table 4). These results are consistent with those of the *lin-36; lin-15* triples; however, unlike the *lin-36* triple mutants, most animals are either Muv or Muv Vul; few are wild type or Vul. Since almost all animals express the Muv phenotype and some express the Vul phenotype in addition to the Muv phenotype, it is likely that in previous studies, the Vul phenotype was not noticed.

To determine whether the anchor cell signal is responsible for the adoption of vulval fates by Pn.p cells in which receptor localization is disrupted and the negative regulatory pathways are eliminated, we removed the anchor cell by eliminating all of the somatic gonad. This was accomplished by laser ablating Z1 and Z4, the precursors of the somatic gonad (Kimble and Hirsh, 1979). These operations were performed at the early L1 larval stage. The ablation of Z1 and Z4 prevents germline proliferation and produces a sterile animal with no gonad (Kimble and White, 1981). This served as an internal control to confirm the ablation of the somatic gonad. As a further control, we ablated Z1 and Z4 in *lin-36; lin-15 A* animals; 11/11 of these animals produced ectopic vulval tissue. This is consistent with the laser ablation of the gonads of *lin-8; lin-9* and *lin-15* AB animals which also produce ectopic vulval tissue (Ferguson et al., 1987). We ablated Z1 and Z4 in *lin-7; lin-36; lin-15* A animals and observed that 14/14 operated animals had ectopic vulval tissue. Thus, in animals in which the gonadal signal, the receptor localization system, and the synMuv-mediated inhibitory pathways have been disrupted,

Pn.p cells can adopt vulval fates. These results, together with those described above, are consistent with the hypothesis that a ligand-independent and receptor localization-independent activity of the *let-23* signal transduction pathway can lead to the adoption of vulval cell fates. Presumably, it is this activity which the synMuvs normally inhibit.

Discussion

lin-36 encodes a novel protein and acts cell autonomously

Our results suggest that *lin-36* encodes a novel protein which is required in the Pn.p cells to negatively regulate vulval development. The *lin-36* mosaic data are in marked contrast to the mosaic data for *lin-15*. Herman and Hedgecock (1990) showed that *lin-15* acts cell non-autonomously in a genetic mosaic analysis experiment. Specifically, they found that loss of *lin-15(+)* in P_1, AB or AB.p, AB.pl, or AB.pr can result in a Muv phenotype. hyp7, which derives from these lineages, was postulated to be the anatomical focus of *lin-15* activity. The *lin-36* mosaic data are also different from the mosaic data for *lin-37*, which was shown by Hedgecock and Herman (1995) to act cell non-autonomously. Specifically, they found that loss of *lin-37(+)* in P_1, AB.p, AB.pl, or AB.pr did not cause a Muv phenotype; loss in AB infrequently resulted in a Muv phenotype. Loss in AB results in many hyp7 nuclei lacking *lin-37(+)*. Hedgecock and Herman (1995) have postulated that *lin-37* is also required in hyp7, but a relatively low dosage is sufficient for wild-type activity. Our results, which show a requirement for *lin-36(+)* only in those lineages leading to the Pn.p cells, suggest that *lin-36* acts in the Pn.p cells and not in hyp7. This is consistent with the *lin-36* expression data. Based upon these mosaic analysis results, we believe that the class B synMuvs encode components of a signaling system, some of which act in the hypodermis and some of which act in the Pn.p cells.

Redundancy in the specification of cell fates

lin-36 is a component of one of two redundant pathways involved in the specification of cell fates during *C. elegans* vulval development (Ferguson and Horvitz, 1989; Chapter 2). Several other examples of functional redundancy during development have been observed. The *Drosophila* genes *achaete* and *scute* are functionally redundant for the specification of larval sense organs but individually required for the specification of distinct groups of adult bristles (reviewed by Ghysen and Dambly-Chaudiere, 1991). Several additional examples of functional redundancy have been observed in *C. elegans*. The molecularly similar genes *lin-12* and *glp-1* act in a functionally redundant manner to

control several aspects of development even though they also have non-overlapping requirements in certain developmental decisions (Lambie and Kimble, 1991). These examples each involve functional redundancy between two molecularly similar genes. Partial functional redundancy between groups of genes or pathways is seen in the cell corpse engulfment genes of *C. elegans*. Cell corpse engulfment mutants fall into two classes; animals carrying mutations in both classes have a more severe phenotype than animals carrying mutations in only one class. However, an animal carrying one mutation exhibits a cell corpse engulfment phenotype (Ellis et al.,1991).

The synMuv genes differ from these previous examples in that alone they do not cause a vulval phenotype and they fall into two classes with multiple members. This indicates that they encode two pathways, each of which is unnecessary in the presence of the other for vulval development. Most redundant processes in development are relatively inaccessible because mutations in the genes of such pathways do not cause a phenotype in the absence of a second site mutation. The synMuv genes provide a model system for the analysis of the role of redundant systems in controlling metazoan development.

lin-36 acts as a component of a signaling pathway

The findings that some of the synMuv genes act outside of the Pn.p cells suggests that they encode the components of a signaling system that regulates the adoption of cell fates in the vulval equivalence group (Herman and Hedgecock, 1990, Hedgecock and Herman, 1995). The finding that *lin-36* acts in the Pn.p cells suggests that some of the class B genes encode the components of a negative regulatory signal transduction cascade within the vulval precursor cells. Thus the negative signal from the class B pathway is interpreted within the cytoplasm or nucleus of the Pn.p cell rather than on its surface. Sequence and expression data suggest that *lin-36* may be a nuclear protein. Thus *lin-36* may regulate transcription directly or indirectly. If this is indeed the case, then the class B synMuv pathway regulates vulval fate specification through an effect on transcription. Since *lin-36* likely acts in the nuclei of the Pn.p cells, it may encode a protein that acts near the end of the class B signal transduction pathway.

The negative signal produced by the components of the class A pathway may be interpreted on the surface of the Pn.p cell or in its interior (Figure 5). The number of components of the class A pathway is significantly smaller than that of the class B pathway (Ferguson and Horvitz, 1989; Chapter 2). This is not inconsistent with the class A signal acting at the Pn.p cell surface.

Negative regulation of inductive signaling pathways

lin-36 and the class B synMuvs may negatively regulate vulval development at any number of points. The class B synMuvs may directly negatively regulate the *let-23* receptor by preventing dimerization, by blocking access to downstream signaling molecules, or by phosphatase activity. Another protein in the inductive signal transduction cascade may be the target of negative regulation. For example, the synMuvs may inhibit *let-60 ras* through a *ras*-GTPase Activating Protein. They may inhibit positive regulators of the pathway that are required for the activity of the pathway, but are not a direct link in the transduction cascade. The class B synMuv genes may not directly affect the inductive signal transduction system, but instead inhibit the adoption of vulval cell fates at the level of transcription. The nuclear subcellular localization of the *lin-36::gfp* reporter suggests that this is the most likely possibility. However, some class B synMuv genes may regulate the transcription of a protein, possibly encoded by a class B synMuv gene, that negatively regulates the *let-23* pathway via one of the mechanisms described above.

The class A and class B genes need not have the same biochemical activities. Neither must they act at the same point in the inductive pathway. Epistasis experiments show that *let-23* is epistatic to the synMuvs. Since the two classes of synMuv genes are functionally redundant, only one class needs to act at the level of the *let-23* receptor. In the triple mutant, *let-23* bypasses at least one class; this suppression of one class masks the activity of the other since they are functionally redundant. The suppression of one class of synMuvs is genetically similar to having a mutation in only one class of synMuv genes; such animals display no vulval defects. Thus *let-23* need be epistatic to only one of the two classes of synMuv genes.

Epistasis experiments show that the synMuvs are epistatic to *lin-3* and that *let-23* is epistatic to the synMuv mutations (Ferguson et al., 1987; Huang et al., 1994). These results indicate that the inductive signal transduction system is required for the adoption of vulval fates in the absence of negative regulation by the synMuvs. These results, coupled with the finding that the synMuv mutants are Muv in gonad-ablated animals (Ferguson et al., 1987), also indicate that activation of the inductive signal transduction cascade by ligand is not necessary for the adoption of vulval fates in the absence of negative regulation by the synMuvs. These results are consistent with a model in which the synMuv genes inhibit (possibly via two distinct methods) the basal (ligand-independent) activity of the inductive pathway, whose activity is necessary for the adoption of vulval fates by the Pn.p cells. This inhibition is overpowered by the

activation of the inductive pathway by the binding of ligand to receptor in the subset of Pn.p cells nearest the anchor cell.

The synMuv phenotype is co-expressed with the Vul phenotype of *lin-2*, *lin-7* and *lin-10*, genes which positively regulate the inductive signal transduction cascade by localizing the LET-23 receptor to the Pn.p cell junctions (Ferguson et al., 1987; Kim and Horvitz, 1990; Hoskins et al., 1996; Simske et al., 1996). Thus, the synMuv genes act to negatively regulate the *let-23* pathway independently of the *lin-2*, *lin-7*, *lin-10* regulatory system. The synMuv genes do not act through the *lin-2*, *lin-7* and *lin-10* receptor localization process; they do not inhibit receptor localization. Likewise *lin-2*, *lin-7* and *lin-10* do not act by negatively regulating the synMuv genes. The absence of either of these modes of regulation can partially bypass the requirement for the other: they function at equivalent hierarchical steps. Neither takes precedence over the other. In the absence of both positive and negative regulation, the fate of the Pn.p cells becomes somewhat deregulated.

With the exception of pattern formation in the vulva, the fates seen in animals lacking positive regulation by *lin-2*, *lin-7*, *lin-10* genes and lacking negative regulation by the synMuv genes are similar to fates seen in animals lacking the stimulatory anchor cell signal as well as the positive and negative regulatory pathways. The expression of vulval fates in the absence of receptor localization is not due to the influence of the anchor cell signal; elimination of this signal still allows the adoption of vulval cell fates in synMuv mutants with a disabled receptor localization system. Not all Pn.p cells adopt vulval cell fates, however. In the absence of both inductive signal and receptor localization, the elimination of negative regulation by the synMuv genes is not sufficient to allow all Pn.p cells to adopt vulval cell fates. Thus, the activities of the synMuv pathways and the *lin-2*, *lin-7*, *lin-10* pathway are necessary to allow tight control over the adoption of vulval and nonvulval cell fates by the Pn.p cells.

The data described above suggest the following model. The six Pn.p cells are predisposed to adopt vulval fates by the unstimulated activity of the *let-23* signal transduction pathway. In the absence of this pathway, the Pn.p cells are incapable of adopting vulval fates. This predisposition to adopt vulval fates is inhibited through the action of the two functionally redundant signaling pathways encoded by the synMuv genes. The synMuv genes normally act to downregulate the unstimulated activity of the *let-23* signal transduction pathway. The action of the synMuv genes changes the inherent tendency of the Pn.p cells to adopt vulval cell fates to a tendency to adopt nonvulval cell fates. The inductive signal from the anchor cell stimulates the *let-23* pathway in the cells nearest it, instructing them to adopt a vulval cell fate. This stimulation overcomes the

effect of the two inhibitory pathways either by overriding or inhibiting negative regulation by the synMuv genes (Figure 5).

Multiple signaling pathways allow a more precise control over developmental events. It is likely that positive and negative signaling pathways that act together are common in the specification of cell fates in metazoa. Negative regulation of signaling pathways by other signaling pathways may be a common mode of regulation. Such systems are most readily studied *in vivo* where various tissue types are in contact; the homogeneous populations in tissue culture experiments are not likely to reveal such negative regulation.

Acknowledgments

We thank current and former members of the Horvitz laboratory, especially Greg Beitel, Kerry Kornfeld and Shai Shaham, for helpful discussions during the course of this work. We thank Bob Barstead for providing a cDNA library, Laird Bloom for providing RNA and Beth James for technical assistance. We thank Alex Hajnal and Stuart Kim for sharing unpublished data. We thank Stuart Kim and the Caenorhabditis Genetics Center (supported by the National Institutes of Health National Center for Research Resources) for providing some of the strains used in this work. J.H.T. was a Predoctoral Fellow of the Howard Hughes Medical Institute. H.R.H. is an Investigator of the Howard Hughes Medical Institute.

References

Arioan, R. V., Koga, M., Mendel, J. E., Oshima, Y. and Sternberg, P. W. (1990). The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature* **348**, 693-699.

Arioan, R. V. and Sternberg, P. W. (1991). Multiple functions of *let-23*, a *Caenorhabditis elegans* receptor tyrosine kinase gene required for vulval induction. *Genetics* **128**, 251-267.

Avery, L. and Horvitz, H. R. (1987). A cell that dies during wild-type *C. elegans* development can function as a neuron in a *ced-3* mutant. *Cell* **51**, 1071-1078.

Barstead, R. J. and Waterston, R. H. (1989). The basal component of the nematode dense-body is vinculin. *J. Biol. Chem.* **264**, 10177-10185.

Beitel, G. J., Clark, S. G. and Horvitz, H. R. (1990). *Caenorhabditis elegans ras* gene *let-60* acts as a switch in the pathway of vulval induction. *Nature* **348**, 503-509.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802-805.

Clark, S. G., Stern, M. J. and Horvitz, H. R. (1992). *Caenorhabditis elegans* cell-signalling gene *sem-5* encodes a protein with SH2 and SH3 domains. *Nature* **356**, 340-344.

Clark, S. G., Lu, X. and Horvitz, H. R. (1994). The *Caenorhabditis elegans* locus *lin-15*, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. *Genetics* **137**, 987-997.

Coulondre, C. and Miller, J. H. (1977). Genetic studies of the *lac* repressor IV: Mutagenic specificity of the *LacI* gene of *Escherichia coli*. *J. Mol. Biol.* **117**, 577-606.

Coulson, A., Sulston, J., Brenner, S. and Karn, J. (1986). Toward a physical map of the genome of the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **83**, 7821-7825.

Coulson, A., Kozono, Y., Lutterbach, B., Shownkeen, R., Sulston, J. and Waterston, R. (1991). YACs and the *C. elegans* genome. *Bioessays* **13**, 413-417.

Eisenmann, D. M. and Kim, S. K. (1994). Signal transduction and cell fate specification during *Caenorhabditis elegans* vulval development. *Curr. Opinion Genet. Develop.* **4**, 508-516.

Ellis, R., Jacobson, D. M. and Horvitz, H. R. (1991). Genes required for the engulfment of cell corpses during programmed cell death in *Caenorhabditis elegans*. *Genetics* **129**, 79-94.

Ferguson, E. L. and Horvitz, H. R. (1985). Identification and genetic characterization of 22 genes that affect the vulval cell lineages of the nematode *Caenorhabditis elegans*. *Genetics* **110**, 17-72.

Ferguson, E. L. and Horvitz, H. R. (1989). The Multivulva phenotype of certain *Caenorhabditis elegans* mutants results from defects in two functionally redundant pathways. *Genetics* **123**, 109-121.

Ferguson, E. L., Sternberg, P. W., and Horvitz, H. R. (1987). A genetic pathway for the specification of the vulval cell lineages of *Caenorhabditis elegans*. *Nature* **326**, 259-267.

Fields, C. (1990). Information content of *Caenorhabditis elegans* splice site sequences varies with intron length. *Nucl. Acids Res.* **18**, 1509-1512.

Finney, M., Ruvkun, G. and Horvitz, H. R. (1988). The *C. elegans* cell lineage and differentiation gene *unc-86* encodes a protein with a homeodomain and extended homology to transcription factors. *Cell* **55**, 757-769.

Fire, A. (1986). Integrative transformation of *Caenorhabditis elegans*. *EMBO J.* **5**, 2673-2680.

Ghysen, A. and Dambly-Chaudiere, C. (1988). From DNA to form: the *achaete-scute* complex. *Genes Dev.* **2**, 495-501.

Goldfarb, D. S. (1989). Nuclear transport. *Curr. Opinion Cell Biol.* **1**, 441-446.

Greenwald, I. S. and Horvitz, H. R. (1980). *unc-93(e1500)*: a behavioral mutant of *Caenorhabditis elegans* that defines a gene with a wild-type null phenotype. *Genetics* **96**, 147-164.

Han, M. and Sternberg, P. W. (1990). *let-60*, a gene that specifies cell fates during *C. elegans* vulval induction, encodes a ras protein. *Cell* **63**, 921-931.

Han, M., Golden, A., Han, Y. and Sternberg, P. W. (1993). *C. elegans lin-45 raf* gene participates in *let-60 ras*-stimulated vulval differentiation. *Nature* **363**, 133-140.

Hedgecock, E. M. and Herman, R. K. (1995). The *ncl-1* gene and genetic mosaics of *Caenorhabditis elegans*. *Genetics* **141**, 989-1006.

Heim, R., Prasher, D. C. and Tsien, R. Y. (1994). Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. USA* **91**, 12501-12504.

Heim, R., Cubitt, A. B. and Tsien, R. Y. (1995). Improved green flourescence. *Nature* **373**, 663-664.

Herman, R. K. (1984). Analysis of genetic mosaics of the nematode *C. elegans*. *Genetics* **108**, 165-180.

Herman, R. K. and Hedgecock, E. M. (1990). Limitation of the size of the vulval primordium of *Caenorhabditis elegans* by *lin-15* expression in the surrounding hypodermis. *Nature* **348**, 169-171.

Hill, R. J. and Sternberg, P. W. (1992). The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans*. *Nature* **358**, 470-476.

Hodgkin, J., Edgley, M., Riddle, D. L. and Albertson, D. G. (1988). Appendix 4: Genetics. In *The Nematode* Caenorhabditis elegans (ed. W. B. Wood), pp. 491-584. Cold Spring Harbor, New York: Cold Spring Harbor Press.

Horvitz, H. R. and Sternberg, P. W. (1991). Multiple intercellular signalling systems control the development of the *Caenorhabditis elegans* vulva. *Nature* **351**, 535-541.

Horvitz, H. R. and Sulston, J. E. (1980). Isolation and genetic characterization of cell-lineage mutants of the nematode *Caenorhabditis elegans*. *Genetics* **96**, 435-454.

Horvitz, H. R., Brenner, S., Hodgkin, J. and Herman, R. K. (1979). A uniform genetic nomenclature for the nematode *Caenorhabditis elegans*. *Molec. Gen. Genet.* **175**, 129-133.

Hoskins, R., Hajnal, A. F., Harp, S. A. and Kim, S. K. (1996). The *C. elegans* vulval induction gene *lin-2* encodes a member of the MAGUK family of cell junction proteins. *Development* **122**, 97-111.

Huang, L. S., Tzou, P. and Sternberg, P. W. (1994) The *Caenorhabditis elegans lin-15* locus encodes two negative regulators of vulval development. *Molec. Biol. Cell* **5**, 395-412.

Huang, X-Y. and Hirsh, D. (1989). A second *trans*-spliced RNA leader sequence in the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **86**, 8640-8644.

Katz, W. S., Hill, R. J., Clandinin, T. R. and Sternberg, P. W. (1995). Different levels of the *C. elegans* growth factor LIN-3 promote distinct vulval precusor fates. *Cell* **82**, 297-307.

Kenyon, C. (1986). A gene involved in the development of the posterior body region of *C. elegans*. *Cell* **46**, 477-487.

Kim, S. K. and Horvitz, H. R. (1990). The *Caenorhabditis elegans* gene *lin-10* is broadly expressed while required specifically for the determination of vulval cell fates. *Genes Dev.* **4**, 357-371.

Kimble, J. (1981). Alterations in cell lineage following laser ablation of cells in the somatic gonad of *Caenorhabditis elegans*. *Dev. Biol.* **87**, 286-300.

Kimble, J. and Hirsh, D. (1979). The postembryonic cell lineages of the hermaphrodite and male gonads in *Caenorhabditis elegans*. *Dev. Biol.* **70**, 396-417.

Kimble, J. E. and White, J. G. (1981). On the control of germ cell development in *Caenorhabditis elegans*. *Dev. Biol.* **81**, 208-219.

Kornfeld, K., Guan, K-L. and Horvitz, H. R. (1995a). The *Caenorhabditis elegans* gene *mek-2* is required for vulval induction and encodes a protein similar to the protein kinase MEK. *Genes Dev.* **9**, 756-768.

Kornfeld, K., Hom, D. M. and Horvitz, H. R. (1995b). The *ksr-1* gene encodes a novel protein kinase involved in Ras-mediated signaling in *C. elegans*. *Cell* **83**, 903-913.

Krause, M. and Hirsh, D. (1987). A *trans*-spliced leader sequence in actin mRNA in *C. elegans*. *Cell* **49**, 753-761.

Lackner, M. R., Kornfeld, K., Miller, L. M., Horvitz, H. R. and Kim, S. K. (1994). A MAP kinase homologue, *mpk-1*, is involved in ras-mediated induction of vulval cell fates in *Caenorhabditis elegans*. *Genes Dev.* **8**, 160-173.

Lambie, E. and Kimble, J. (1991). Two homologous regulatory genes, *lin-12* and *glp-1*, have overlapping functions. *Development* **112**, 231-240.

Mello, C. C., Kramer, J. M., Stinchcomb, D. and Ambros, V. (1991). Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**, 3959-3970.

Miller, L. M., Plenefisch, J. D., Casson, L. P. and Meyer, B. J. (1988). *xol-1*: a gene that controls the male modes of both sex determination and X-chromosome dosage compensation in *C. elegans*. *Cell* **55**, 167-183.

Rhodes, D. and Klug, A. (1986). An underlying repeat in some transcriptional control sequences corresponding to half a double helical turn of DNA. *Cell* **46**, 123-132.

Rosenbluth, R. E., Cuddeford, C. and Baillie, D. L. (1985). Mutagenesis in II: A spectrum of mutational events induced with 1500 r of γ-irradiation. *Genetics* **109**; 493-511.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Press.

Simske, J. S. and Kim, S. K. (1995). Sequential signalling during *Caenorhabditis elegans* vulval induction. *Nature* **375**, 142-146.

Simske, J. S., Kaech, S. M., Harp, S. A. and Kim, S. K. (1996). LET-23 receptor localization by the cell junction protein LIN-7 during *C. elegans* vulval induction. *Cell* **85**, 195-204.

Sternberg, P. W. (1988). Lateral inhibition during vulval induction in *Caenorhabditis elegans*. *Nature* **335**, 551-554.

Sternberg, P. W. and Horvitz, H. R. (1986). Pattern formation during vulval development in *C. elegans*. *Cell* **44**, 761-772.

Sternberg, P. W. and Horvitz, H. R. (1989). The combined action of two intercellular signalling pathways specifies three cell fates during vulval induction in *C. elegans*. *Cell* **58**, 679-693.

Sulston, J. E. and Horvitz, H. R. (1977). Postembryonic cell lineages of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **56**, 110-156.

Sulston, J. E. and Horvitz, H. R. (1981). Abnormal cell lineages in mutants of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **82**, 41-55.

Sulston, J. E. and White, J. G. (1980). Regulation and cell autonomy during postembryonic development of *Caenorhabditis elegans*. *Dev. Biol.* **78**, 577-597.

Sulston, J. E., Schierenberg, E., White, J. G. and Thomson, J. N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100**, 64-119.

Sundaram, M. and Han, M. (1996). Control and integration of cell signaling pathways during *C. elegans* vulval development. *Bioessays* **18**, 473-480.

Tautz, D., Lehmann. R., Schnürch, H., Schuh, R., Seifert, E., Kienlin, A., Jones, K. and Jäckle, H. (1987). Finger protein of novel structure encoded by *hunchback*, a second member of the gap class of *Drosophila* segmentation genes. *Nature* **327**, 383-389.

Thomas, J. H., Stern, M. J. and Horvitz, H. R. (1990). Cell interactions coordinate the development of the *Caenorhabditis elegans* egg-laying system. *Cell* **62**, 1041-1052.

Wang, B. B., Muller-Immerglück, M. M., Austin, J., Robinson, N. T., Chisholm, A. and Kenyon, C. (1993). A homeotic gene cluster patterns the anteroposterior body axis of *C. elegans*. *Cell* **74**, 29-42.

White, J. G., Southgate, E., Thomson, J. N. and Brenner, S. (1986). The structure of the nervous system of *Caenorhabditis elegans*. *Phil. trans. R. Soc. Lond. (B) Biol. Sci.* **314**, 1-340.

Wilson, R. et al. (1994). 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. *Nature* **368**, 32-38.

Wu, Y. and Han, M. (1994). Suppressors of activated Let-60 Ras protein defines a role of *Caenorhabditis elegans* Sur-1 MAP kinase in vulval differentiation. *Genes Dev.* **8**, 147-159.

Wu, Y. Han, M. and Guan, K-L. (1995). MEK-2, a *Caenorhabditis elegans* MAP kinase kinase, functions in Ras-mediated vulval induction and other developmental events. *Genes Dev.* **9**, 742-758.

Yandell, M. D. , Edgar, L. G. and Wood, W. B. (1994). Trimethylpsoralen induces small deletion mutations in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **91**, 1381-1385.

Yuan, J. and Horvitz, H. R. (1990). The *Caenorhabditis elegans* genes *ced-3* and *ced-4* act cell autonomously to cause programmed cell death. *Develop. Biol.* **138**, 33-41.

Table 1. The percentage of Muv animals of each genotype was determined at 20°. Complete genotypes are shown. Percent Muv, percent of animals having ventral protrusions. n, number of animals scored.

[a] data from Ferguson and Horvitz, 1989.

[b] data from Chapter 2.

Table 1. Penetrance of the Muv phenotype in strains carrying *lin-36* mutations

Genotype	Percent Muv	n
lin-8(n111); lin-36(n747)[a]	98	177
lin-8(n111); lin-36(n750)[a]	75	357
lin-8(n111); lin-36(n766)[a]	98	207
lin-36(n766); lin-15(n767)[a]	100	226
lin-36(n772); lin-15(n767)[a]	99	369
lin-8(n111); lin-36(n2235)[b]	79	313
lin-8(n111); lin-36(n2240)[b]	79	373
lin-8(n111); lin-36(n2243)[b]	94	593
lin-8(n111); lin-36(n3090)	81	265
lin-8(n111); lin-36(n3093)	96	320
lin-8(n111); lin-36(n3094)	53	278
lin-8(n111); lin-36(n3095)	94	420
lin-8(n111); lin-36(n3096)	97	456
lin-36(n3096); lin-15(n767)	99.7	317
lin-8(n111); lin-36(n3097)	57	334
lin-36(n3097); lin-15(n767)	99	309

Table 2. Pn.p cell lineages follow the nomenclature of Sternberg and Horvitz (1986; 1989). This nomenclature describes nuclear division and morphology: S, the nucleus adopted a characteristic morphology and fusion with the hypodermis; T, the nucleus divided transversely (left-right); L, the nucleus divided longitudinally (anterior-posterior); O, the nucleus divided obliquely; N, the nucleus did not divide and adopted a distinct morphology; ?, the nucleus had not divided four hours after the last divisions but did not adopt the distinct morphology of an N fate. Underlining and bold face type indicate that the daughter nuclei adhered to the cuticle. Gross phenotype is described in terms of pseudovulval invaginations in larvae and subsequent pseudovulval protrusions in adults. The number of pseudovulval invaginations anterior to the vulva is indicated by the number preceding the letter a; the number of pseudovulval invaginations posterior to the vulva is indicated by the number preceding the letter p. WT indicates that no pseudovulval invaginations formed. Vul, indicates that no functional vulva was formed; in the case described here, the vulval tissue protruded. Unless otherwise noted, in all animals, mutant and wild-type, a functional vulva formed. Pn.p cell lineages of *lin-7(e1413)* mutants show no vulval induction or partial vulval induction (Sternberg and Horvitz, 1989). 2° cell fates are defined by division to produce seven or eight great granddaughter cells that are polarized such that four cells in one half of the lineage adhere to the cuticle. Note that all but one of the Pn.p cells adopting ectopic 2° cell fates show polarity similar to that of the nearest Pn.p cell adopting a 2° cell fate and participating in the formation of the vulva.

Table 2. Pn.p cell lineages in *lin-36* mutants

Genotype	n	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	Gross Phenotype
N2	many	S	SS	LLTN	TTTT	NTLL	SS	WT
	many	SS	SS	LLTN	TTTT	NTLL	SS	WT
lin-15(n767)	5	S	SS	LLTN	TTTT	NTLL	SS	WT
	4	SS	SS	LLTN	TTTT	NTLL	SS	WT
	1	SS	SS	LLTN	TTTT	NOLL	SS	WT
lin-36(n766)	5	S	SS	LLTN	TTTT	NTLL	SS	WT
	5	SS	SS	LLTN	TTTT	NTLL	SS	WT
lin-36(n766); lin-15(n767)	1	LOLL	LLTT	LLON	TTTL	NOLL	OTOL	2a1p
	1	OTLO	LLOO	LLTN	TTTT	NTLL	LLTO	2a1p
	1	LSOO	LLTL	LLON	TTTT	NTLL	LTOT	3a1p
	1	TOOT	LLON	LLON	TTTT	NTLL	LTTT	2a1p
	1	LLTT	LLTN	LLON	TTTT	NTLL	OOLO	2a1p
	1	OOLO	LLTT	LLON	TTTT	NLLL	OOS	2a1p
	1	TTLL	LOTO	LLON	TTTT	NOLL	OOOO	2a1p
	1	LLON	TTTL	LLLN	TTTT	NTLL	LTOO	1a1p
	1	LLTN	TTOO	LLTN	TTTT	NLLL	OTTO	1a1p
	1	LLTO	TOOO	LLTN	TTTT	NLLL	TOTO	1a1p
lin-7(e1413); lin-36(n766); lin-15(n767)	1	LLTN	??OO	LLON	LOTO	NOLL	SOS	2a1p
	1	S	LLON	LLTT	TTOO	LOOL	LLTL	1a1p
	1	SOT	LLLN	LTTT	NTLL	SS	SS	1a
	1	LLLL	OOOT	LLTN	TTTT	NTLL	SSS	1a
	1	LTTN	OOON	SOT	LONO	SS	SS	3a, Vul

Table 3. Exon sequences are shown in capital letters and intron sequences are shown in lowercase letters. The *C. elegans* concensus splice donor and acceptor sites are RAG/gtaagttt and wwtttcag/NNN where R is A or G, W is A or T, and N is any nucleotide (Fields, 1990). Amino acid substitutions are shown as wild-type residue identity, residue number, predicted mutant residue. All mutations with the exception of *n3090* were generated with EMS. Twelve out of the thirteen changes observed in these the mutations generated with EMS are G:C to A:T transitions, consistent with the known specificity of EMS (Coulondre and Miller, 1977). The mutation in *n766*, generated by EMS, is a transversion (T:A to A:T). The remaining mutation, *n3090*, is also a transversion (T:A to A:T). This mutation was generated using TMP, which usually produces small deletions. In one mutant allele, *n747*, two lesions were observed. One produces a predicted missense mutation (identical to that found in *n2240*); the other produces a predicted novel splice donor site early in exon 3. Both the wild-type and mutant codons encode glycine.

Table 3. Sequences of *lin-36* mutations

Allele	Wild-Type Sequence	Mutant Sequence	Substitution or Splice Site Change
n747	GAA	AAA	E241K
n750	CCT	CTT	P246L
	ACGGCAAAGGC	ACG/gtaaaggc	Novel exon 3 donor
n766	TAT	TAA	Y796ochre
n772	CGC	CAC	R189Q
n2235	CAA	TAA	Q898ochre
n2240	CCT	CTT	P246L
n2243	GGA	GAA	G324E
n3090	TAT	AAT	Y325N
n3093	CAG	TAG	Q579amber
n3094	atttttag/GAT	atttttaa/GAT	Exon 2 acceptor
n3095	CAA	TAA	Q569ochre
n3096	CAA	TAA	Q442ochre
n3097	CAT	TAT	H284Y

Table 4. The vulval phenotype of strains of various genotypes is described. The complete genotype is shown. Triple mutants containing *lin-36(n766)* and double mutants containing *lin-15(e1763)* contain no markers; the construction of these strains is described in Materials and Methods. Phenotypic catagories were assigned as follows: Muv, animal had a Multivulva phenotype characterized by ectopic ventral protrusions; Vul, animal had a Vulvaless phenotype such that it was bloated with eggs or contained many internally hatched larvae (bag of worms); Muv Vul, animal co-expressed both a Multivulva and a Vulvaless phenotype, that is it was bloated with eggs or internally hatched larvae and had ectopic protrusions characteristic of a Muv phenotype; WT, animal had a functional vulva and was nonMuv. n, the number of animals scored. Several animals displayed a Hyperinduced (Hin) phenotype in addition to the phenotype described. These animals were assigned a phenotype based upon whether they had Multivulva-like ectopic vulval tissue (Muv), a nonfunctional vulva (Vul), both (Muv Vul), or no Multivulva-like ventral protrusions and a functional vulva (WT).

[a]datum from Ferguson and Horvitz (1989).

[b]These animals were not true Muv Vul animals, but were somewhat bloated with eggs and may have been scored as Muv Vul animals. This number therefore serves as a measure of the false positive noise in scoring Muv Vul animals in the *lin-15(e1763)* double mutants.

[c]datum from Hoskins et al. (1996).

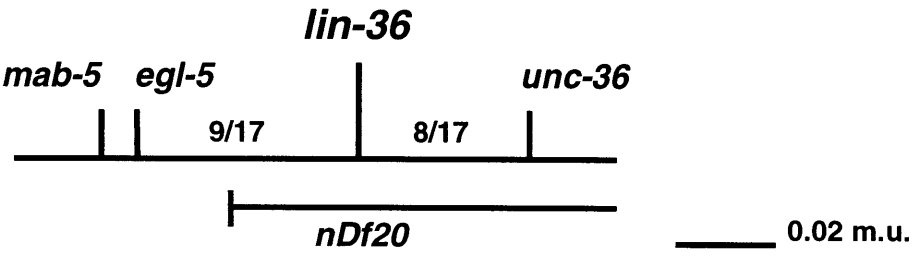
[d]data from Ferguson and Horvitz (1985).

Table 4. Gene Interactions

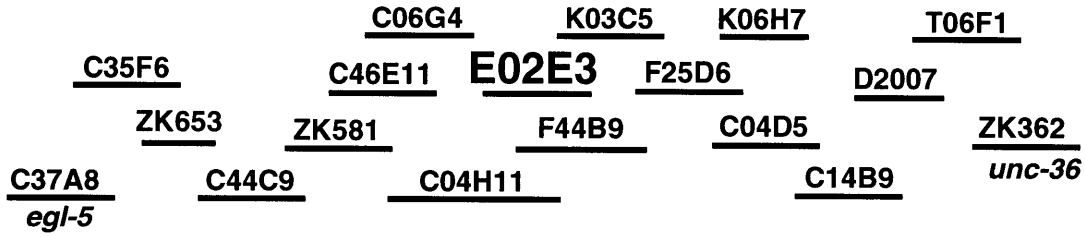
Genotype	Muv	Vul	Muv Vul	WT	n
lin-2(n397)[c]	0%	99%	0%	1%	280
lin-7(e1413)[d]	0%	98%	0%	2%	341
lin-10(n299)[d]	0%	97%	0%	3%	211
lin-36(n766); lin-15(n767)[a]	100%	0%	0%	0%	226
let-23(sy97); lin-36(n766); lin-15(n767)	0%	100%	0%	0%	203
lin-36(n766); lin-2(n397) lin-15(n767)	39%	14%	37%	9%	180
lin-7(e1413); lin-36(n766); lin-15(n767)	43%	9%	47%	2%	202
lin-10(n299); lin-36(n766); lin-15(n767)	29%	25%	42%	4%	224
lin-15(e1763)	97%	0%	3%[b]	0%	119
lin-2(n397) lin-15(e1763)	52%	0%	48%	0%	106
lin-7(e1413); lin-15(e1763)	63%	1%	35%	1%	140
lin-10(n299); lin-15(e1763)	56%	2%	42%	0%	113

Figure 1. Cloning of *lin-36*. (A) Part of the genetic map of linkage group III showing the region of *lin-36*. The deficiency *nDf20* failed to complement *lin-36* and three factor mapping positioned *lin-36* roughly halfway between *egl-5* and *unc-36*. (B) Physical map of cosmid clones in the *egl-5* to *unc-36* region. *egl-5* and *unc-36* are rescued by cosmids C37A8 and ZK362, respectively (Coulson et al., 1986; Wang et al., 1993; L. Lobel, personal communication). These clones formed the endpoints of the molecular region in which *lin-36* lies. The recombination distances shown in (A) suggested that *lin-36* lies in the middle of this region. Cosmids from this region were injected into *lin-8(n111); lin-36(n747)* at a concentration of 80 μg/ml. Cosmids C04H11, F44B9 and E02E3 were shown to rescue the Muv phenotype (2/2, 5/8 and 3/3, rescued lines respectively). (C) Subclones constructed from cosmid E02E3. E02E3 F2 rescue data is totaled in the figure; it consists of: 80 μg/ml into *lin-8(n111); lin-36(n747)*, 3/3; 20 μg/ml into *lin-8(n111); lin-36(n747)*, 4/4; 80 μg/ml into *lin-36(n766); lin-15(n767)*, 15/15; 20 μg/ml into *lin-36(n766); lin-15(n767)*, 17/17. Subcloned insert DNA is depicted by the line shown adjacent to each subclone plasmid name. Subclones were injected into *lin-36(n766); lin-15(n767)* and tested for the ability to rescue the Muv phenotype. A 5.0 kb minimum rescuing fragment, pJHT16, was defined on this basis. pJHT1, pJHT2, pJHT3 and pJHT4 were constructed by using *Mlu*I, *Nco*I, *Kpn*I, and *Eag*I, respectively, to delete DNA from the cosmid which was religated. pJHT1 and pJHT4 each had one of the restriction enzyme sites in the vector sequence. From left to right, the following restriction sites were used in the construction of the subclones: pJHT7: *Mlu*I and *Xba*I, pJHT8: *Mlu*I and *Sac*I, pJHT9: *Mlu*I and *Kpn*I, pJHT10: *Mlu*I and *Sal*I, pJHT11: *Xho*I and *Kpn*I, pJHT12: *Spe*I and *Kpn*I, pJHT13: *Sal*I and *Kpn*I, pJHT14: *Cla*I and *Kpn*I, pJHT15: *Sal*I and *Sac*I, pJHT16: *Sal*I and *Xba*I, pJHT17: *Cla*I and *Sac*I, pJHT18: *Cla*I and *Xba*I. The following concentrations of DNA were used: pJHT1, 50 μg/ml; pJHT2-4, 20 μg/ml; pJHT7-17, 30 μg/ml; pRF4, 80 μg/ml.

A Genetic Map



B Physical Map



C Subclones

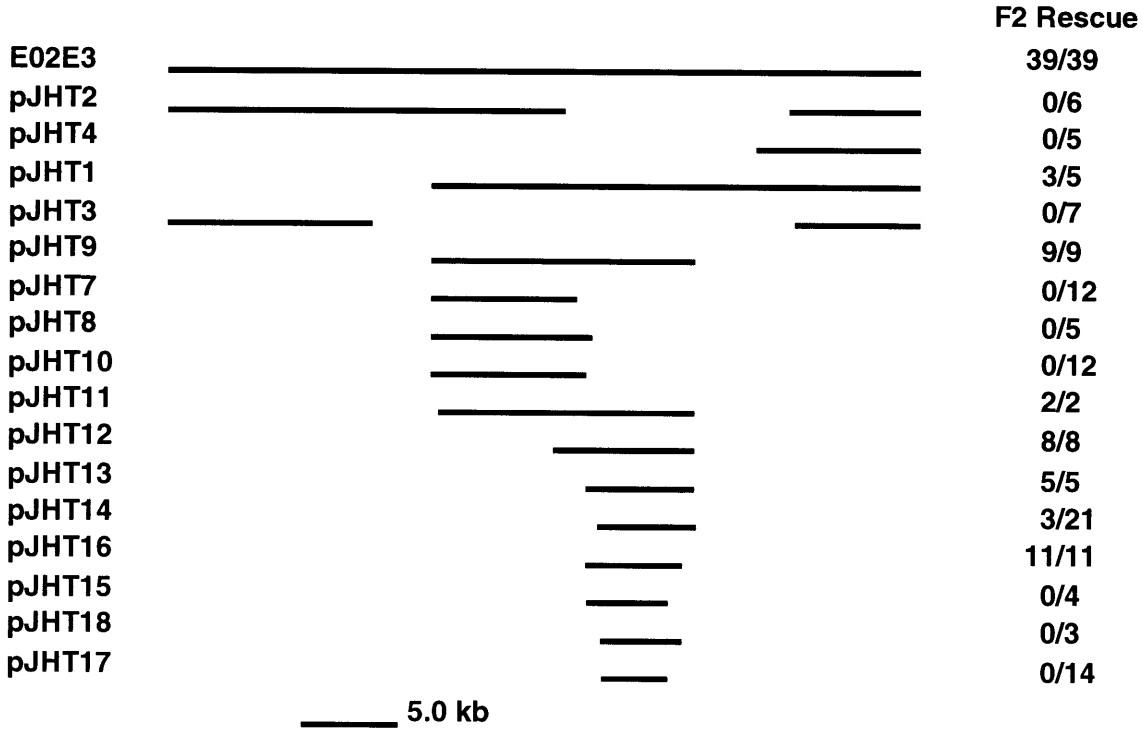


Figure 2. *lin-36* cDNA sequence. Nucleotide sequence of the cDNA and predicted amino acid sequence is shown. Nucleotides are numbered beginning at the first nucleotide after the *Eco*RI linker. These first nine nucleotides are the 3' end of the *trans*-spliced leader SL1 (Krause and Hirsh, 1987; Huang and Hirsh, 1989). Amino acids are numbered beginning at the presumptive ATG codon and ending at the residue just before the presumptive TAA stop codon. Arrowheads indicate the location of intervening sequences determined by comparison of cDNA sequences with genomic sequence determined by the *C. elegans* genome sequencing project (Wilson et al., 1994). The nine introns are 56, 75, 101, 75, 101, 123, 66, 48 and 215 nucleotides long, respectively.

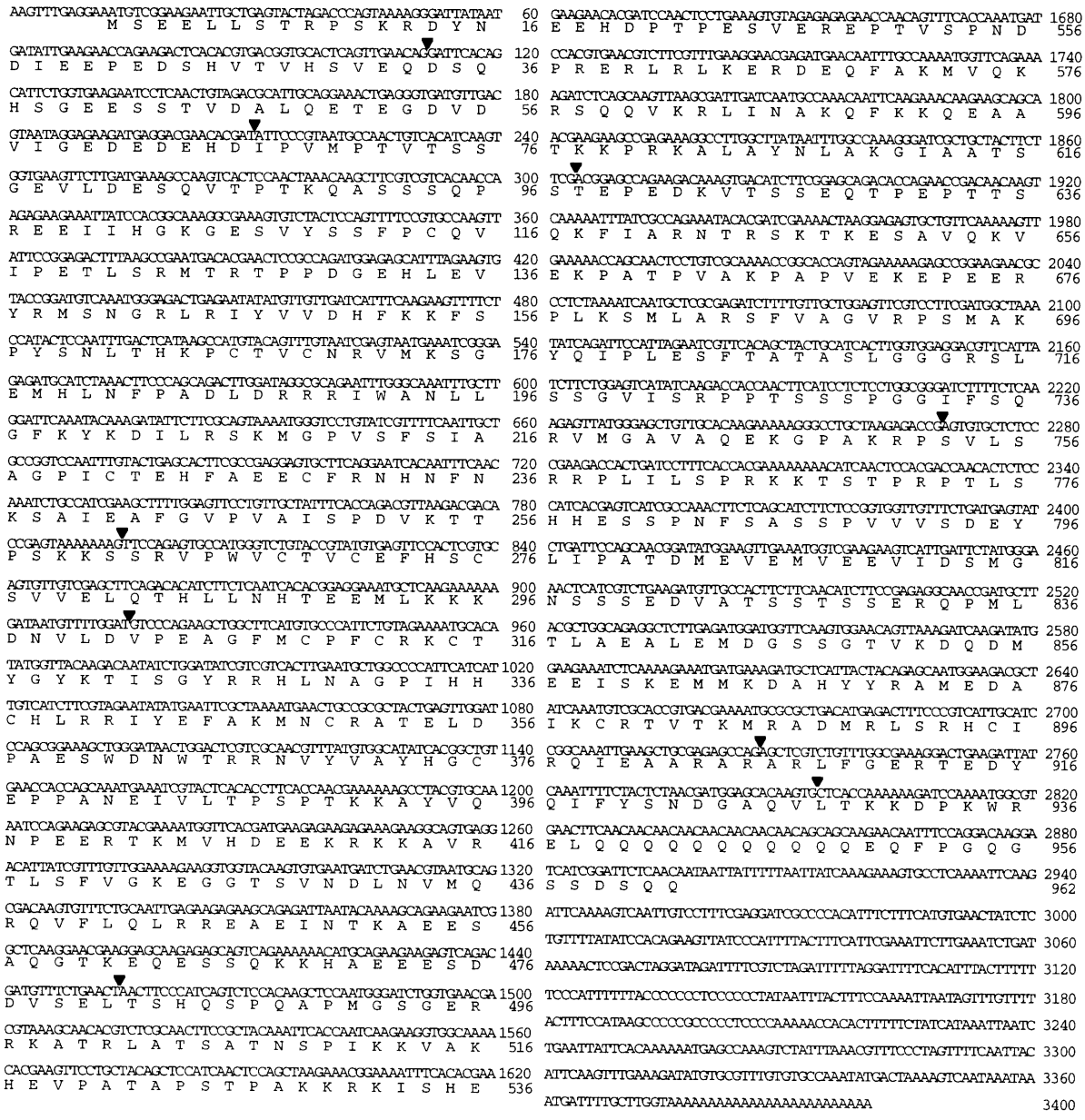


Figure 3. Mosaic analysis of *lin-36*. A partial embryonic lineage (Sulston et al., 1983) showing the origins of cells involved in vulva development. The gonadal anchor cell is derived from EMS; P(3-8).p are derived from both AB.plapp and AB.prapp; hyp7, the main body hypodermal syncytium, is derived from AB.a, AB.p, and C. The number of nuclei that each of these lineages contribute and the stage at which they are contributed to hyp7 is noted under the lineage. Only contributions from embryonic through larval stage 3, when Pn.p cells undergo vulval cell divisions, are noted (see Sulston and Horvitz, 1977; Sulston et al., 1983). E, embryonic; L1, larval stage 1; L2, larval stage 2; L3, larval stage 3. Contributions from P3.p, P4.p and P8.p 3° cell fates are noted in the L3 number even though it is not likely that these hyp7 nuclei play a role in vulval cell determination. These include two nuclei each from AB.pl and AB.pr and two additional nuclei from P8.p which descend either from AB.pl or AB.pr. In Muv animals, these cells often adopt vulval cell fates rather than hyp7 cell fates. Brackets below the lineage diagram show the lineages in which duplication loss occurred and the phenotype of the mosaic animal.

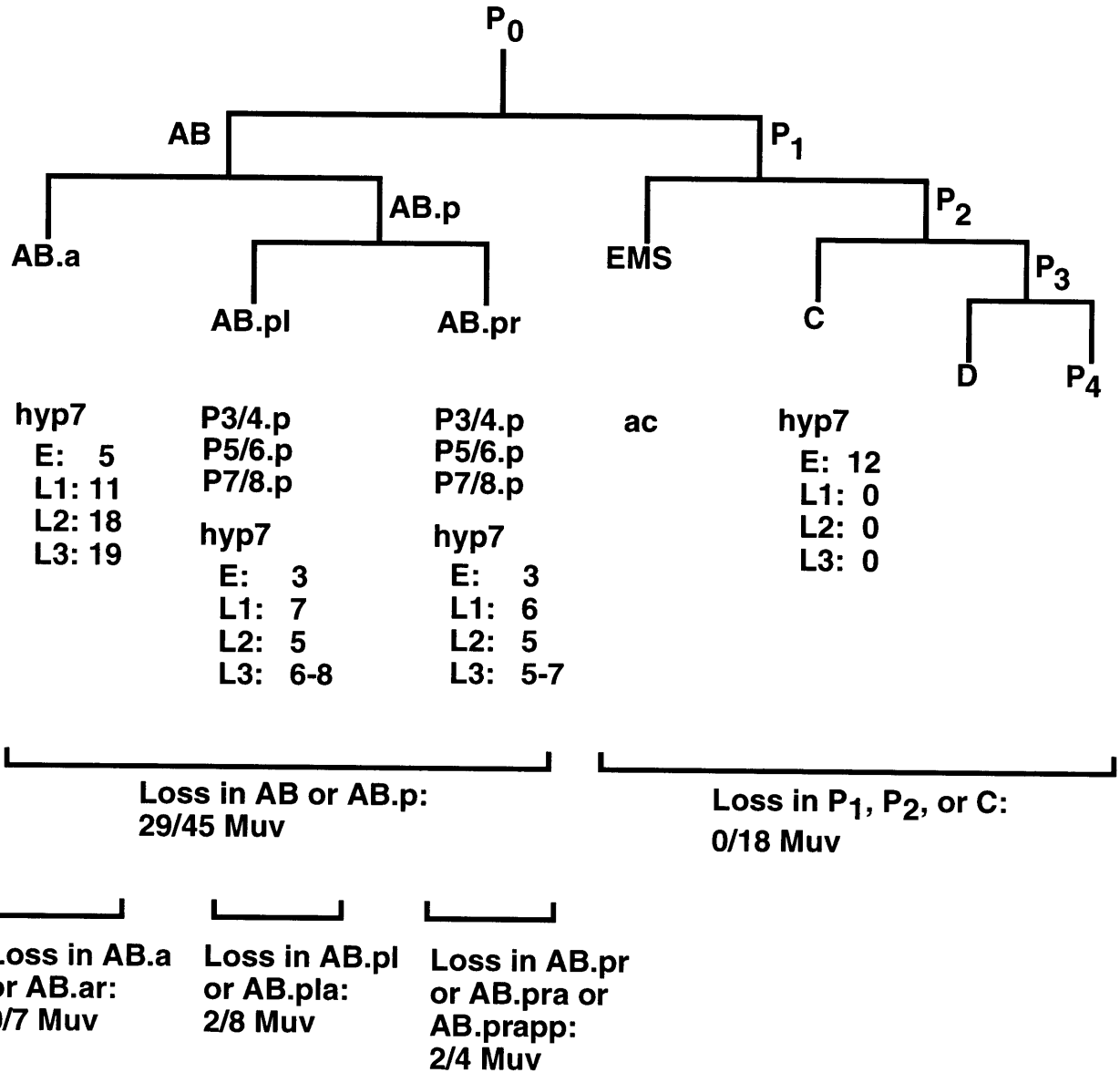

P0
AB
P1
AB.p
P2
AB.a
EMS
P3
AB.pl
AB.pr
C
D
P4
hyp7
E: 5
L1: 11
L2: 18
L3: 19
P3/4.p
P5/6.p
P7/8.p
hyp7
E: 3
L1: 7
L2: 5
L3: 6-8
P3/4.p
P5/6.p
P7/8.p
hyp7
E: 3
L1: 6
L2: 5
L3: 5-7
ac
hyp7
E: 12
L1: 0
L2: 0
L3: 0
Loss in AB or AB.p:
29/45 Muv
Loss in P1, P2, or C:
0/18 Muv
Loss in AB.a
or AB.ar:
0/7 Muv
Loss in AB.pl
or AB.pla:
2/8 Muv
Loss in AB.pr
or AB.pra or
AB.prapp:
2/4 Muv

Figure 4. *lin-36::*GFP reporter expression. Scale bar, 20 μm. Anterior is to the left. (A, C, E) photomicrographs taken under Nomarski optics. (B, D, F) fluorescent images of the animals seen in (A, C, E) observed using a LP-FITC filter. Animals are Rol. (A, B) L3 hermaphrodite showing reporter expression in the nuclei of Pn.p cells. P4.p, P5.p and P6.p are shown. P5.p and P6.p are expected to adopt vulval fates whereas P4.p is expected to adopt a nonvulval fate. The nuclei of ventral cord neurons adjacent to the Pn.p cells can also be observed expressing the reporter construct. (C, D) L3 hermaphrodites showing the nuclear expression of the reporter in the descendants of Pn.p cells during vulval cell division. The lineage relationships of these cells is shown by a lineage diagram superimposed on the images. The indicated nuclei expressing the reporter are the daughter and granddaughter cells of P5.p, P6.p and P7.p, the Pn.p cells which adopt vulval cell fates. (E, F) L4 hermaphrodites during early vulval invagination. *lin-36::*GFP expression persists after cell division. The arrowhead indicates the vulval invagination.

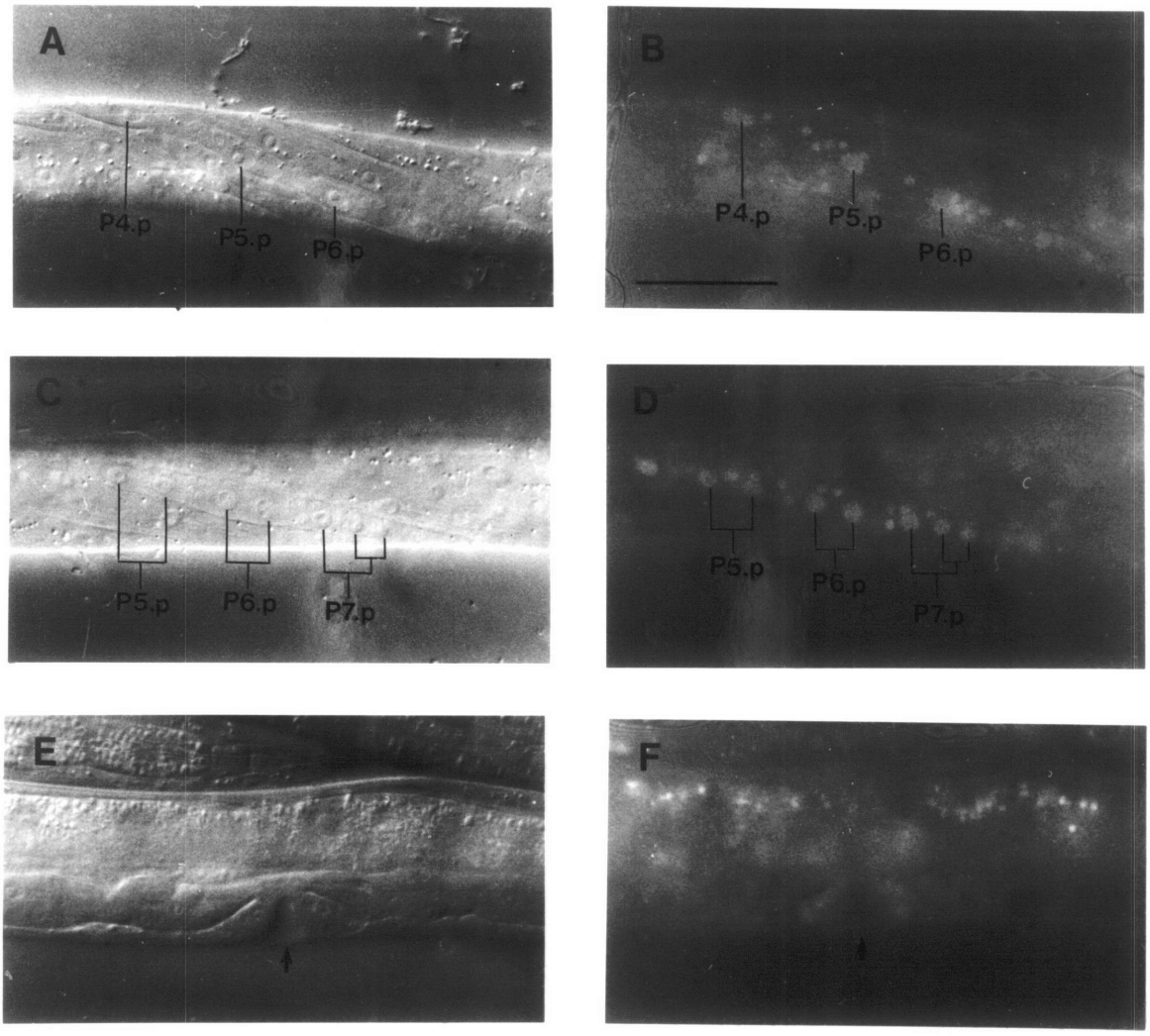
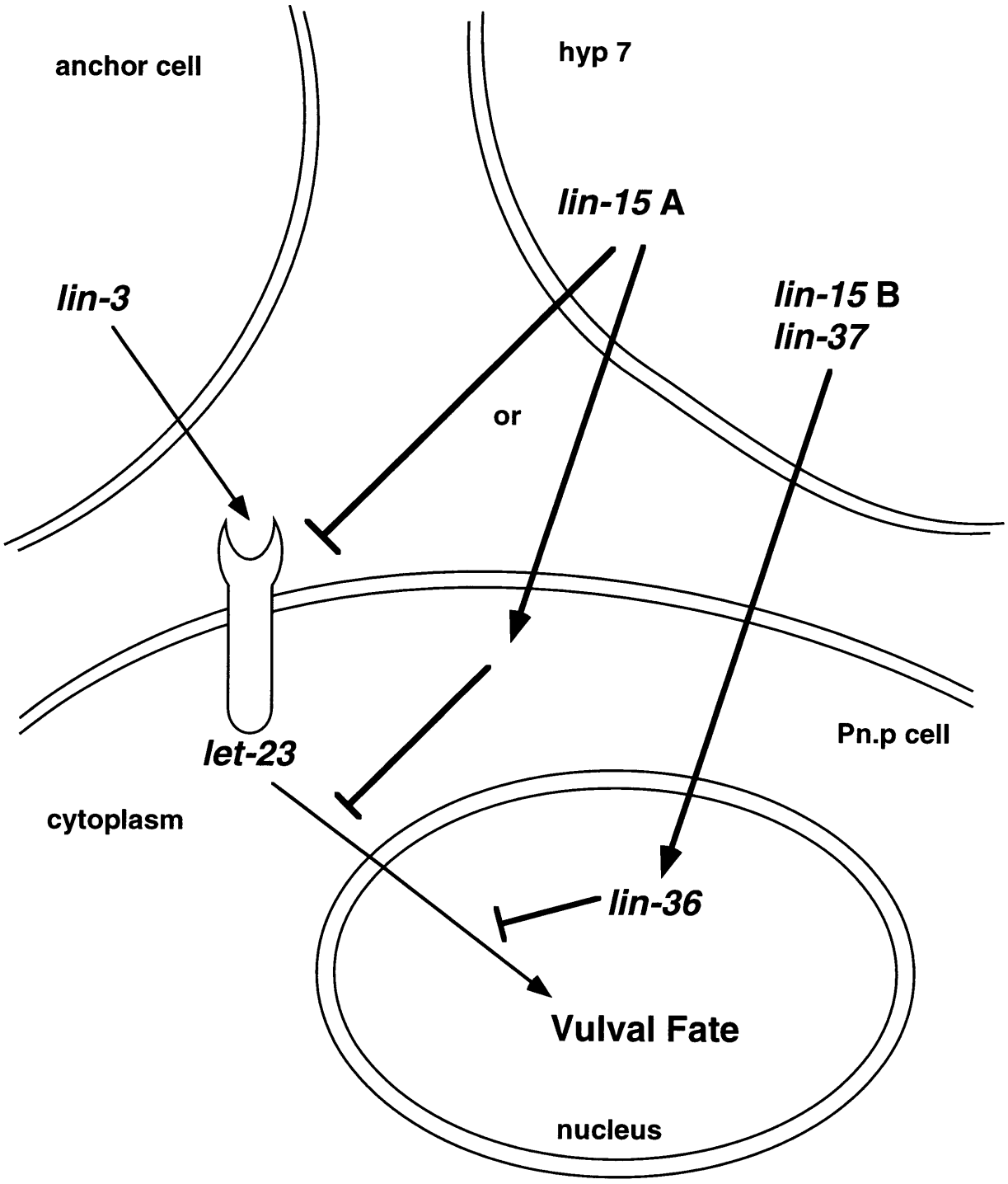

A
P4.p
P5.p
P6.p
B
P4.p
P5.p
P6.p
C
P5.p
P6.p
P7.p
D
P5.p
P6.p
P7.p
E
F

Figure 5. Model for the negative regulation of vulval development by the synMuv genes. The inductive signal encoded by *lin-3* binds the receptor tyrosine kinase encoded by *let-23*, activating the signal transduction pathway leading to adoption of a vulval fate by the Pn.p cell. The two functionally redundant synMuv pathways negatively regulate this process. The class B pathway has components in both hyp 7 (*lin-15* B, *lin-37* and possibly others) and the Pn.p cell (*lin-36* and possibly others). It likely that the class B synMuv pathway may not exert its negative regulation on the adoption of vulval cell fates until the level of transcription, as suggested by the nuclear subcellular localization of *lin-36*. The class A pathway has a component in hyp 7 (*lin-15* A) and may or may not have other components acting in the Pn.p cell. Thus it may act to negatively regulate the pathway by acting the extracellular surface of the *let-23*-encoded receptor, by inhibiting the *let-23*-encoded receptor tyrosine kinase cytoplasmically, by negatively regulating another component of the inductive signal transduction cascade (e.g. as a phosphatase, or a *ras*-GAP), or in a manner like that proposed for the class B pathway. The class A and class B pathways may act in biochemically distinct manners. Inductive signal transduction cascade activity is required for the adoption of vulval fates even in the absence of negative regulation by the synMuv genes; however, the inductive signal itself is not required. The ligand-independent activity of the *let-23* pathway is necessary for the adoption of vulval fates when the synMuv-mediated inhibition is eliminated.


anchor cell
hyp 7
lin-15 A
lin-3
lin-15 B
lin-37
or
let-23
Pn.p cell
cytoplasm
lin-36
Vulval Fate
nucleus

Appendix 1

Production of Polyclonal Antisera to LIN-36

Jeffrey H. Thomas and H. Robert Horvitz

Howard Hughes Medical Institute, Massachusetts Institute of Technology
Cambridge, MA 02139

Summary

Polyclonal antisera to LIN-36 were generated to facilitate the analysis of *lin-36*. Western blots identified a 130 kDa LIN-36 protein that was truncated in several nonsense mutants. LIN-36 did not display any identifiable protein modifications on Western blots in response to *lin-15*. Immunoflouresence staining of animals was unsuccessful, so the expression pattern and subcellular localization of LIN-36 could not be determined.

Introduction

To determine where *lin-36* is expressed, polyclonal antisera were produced. We planned to produce antisera that would allow us to determine which cells express LIN-36 protein and the subcellular localization of the protein in these cells. It was hoped that the determination of subcellular localization would provide some insight into the mode of action of the LIN-36 protein in vulval development.

In addition to providing information on the expression pattern of LIN-36, the antibodies would serve as a useful reagent for further experiments to elucidate the role of *lin-36*. Western blot analysis in synMuv mutant backgrounds may reveal whether LIN-36 is modified by other class B synMuvs. Modification by phosphorylation or dephosphorylation should be readily detectable. Detection of the modification of expression levels by other class B synMuvs through transcriptional or translational means may be possible. Changes to patterns of LIN-36 cellular expression and subcellular localization by synMuv mutants may be detectable using the same techniques used to determine the wild-type expression pattern. The methods described here to analyze modifications to the wild-type LIN-36 protein can be used to test whether other synMuv mutants or *lin-36* mutants regulate these characteristics. In the case of other synMuv mutants, these data may reveal regulatory hierarchies; in the case of *lin-36* mutations, these data may reveal important domains of LIN-36 and their function.

LIN-36 antibodies would also provide a useful reagent for further biochemical studies of synMuv function. Anti-LIN-36 antibodies could be used in immunoprecipitation experiments to identify interacting proteins. At present, the identities of any proteins with which LIN-36 interacts are unknown.

Materials and Methods

Production of antisera

Three peptide regions of the *lin-36* coding region were selected for antigenic potential based on hydrophilicity and charge. These included peptide 20, EERTKMVHDEEKRKKAVR; peptide 21, KEQESSQKKHAEEESDD; and peptide 22, DPRERLRLKERDEQFAK. These peptides were used to immunize rabbits. ELISA assays revealed that Ab20 (antisera generated against peptide 20) and Ab22 had high titers whereas Ab21 had a very low titer (Research Genetics, Huntsville, AL).

Affinity purification of antisera

The three peptides used for antisera production all map to a small region in the *Eco*RI *Stu*I fragment of the *lin-36* cDNA. A GST fusion protein was constructed by cloning this *Eco*RI *Stu*I fragment into the pGEX-5X-1 inducible bacterial expression vector (Pharmacia Biotech, Uppsala, Sweden). This fusion protein can be induced at very high levels in bacteria by IPTG as assayed by Coomassie staining.

To affinity purify anisera, fusion protein expression was induced, proteins separated by electrophoresis, and transferred to a nitrocellulose filter. 2 ml each of Ab20 and Ab22 were incubated with approximately 1 mg of fusion protein fixed to nitrocellulose. After washing, affinity purified antisera was eluted from the membrane.

Western blots

Western blots of bacterial expression of fusion protein were analyzed using goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (BioRad, Hercules, CA). Western blots of worm protein were analyzed using goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (BioRad, Hercules, CA). Chemiluminescence was developed using ECL detection agents (Amersham, Arlington Heights, IL).

Whole mount immunofluorescence

Animals were fixed and prepared for whole mount immunoflouresence essentially according to the protocol of Finney and Ruvkun (1990). Flourescein-conjugated secondary antibodies were used to immunolocalize the primary antibodies.

Results and Discussion

LIN-36 is present in worms as a 130 kDa protein that is not obviously modified by *lin-15*

Western blots were prepared from protein extracted from mixed stage worms. *lin-36* is expected to encode a protein of molecular weight 108 kDa (Chapter 3). Antibody Ab22 recognized a protein of 130 kDa from these extracts. That the 130 kDa protein was actually the LIN-36 protein was supported by the observation that Western blots of protein extracts from *lin-36(n766)* worms did not show a 130 kDa protein, but instead showed a 110 kDa protein. The *lin-36(n766)* mutation is an ochre stop codon at position 796 of the 962 residue protein (Chapter 3). Thus, the mutant protein encoded by *lin-36(n766)* is predicted to be a truncated protein. This is consistent with the supposition that the 130 kDa protein is LIN-36. Western blots of proteins isolated from *lin-36(n3093)* and *lin-36(n3095)* strains show even smaller proteins and no 130 kDa protein.

This is consistent with the sequences of these mutations: *lin-36(n3093)* has an amber codon at codon position 579 and *lin-36(n3095)* has an ochre codon at codon position 569) (Chapter 3).

Proteins extracted from a *lin-15(n309)* strain were also analyzed by Western blot. *lin-15(n309)* is a deletion of most of the *lin-15* AB locus and is expected to be a null allele (Clark et al., 1994). Thus activity of the *lin-15* B locus is completely eliminated. *lin-15* has been shown to act in the hypodermis (Herman and Hedgecock, 1990) whereas *lin-36* has been shown to act in the Pn.p cells (Chapter 3). Therefore, we think it is likely that *lin-15* acts upstream of *lin-36* in the class B signaling pathway. To test whether LIN-36 is modified by presumably upstream class B synMuv genes, we conducted a Western blot of LIN-36 in a *lin-15(n309)* mutant. No changes in protein size or expression levels were noted.

LIN-36 antisera has not been shown to usefully stain worms

Both mixed stage and L2 worms were fixed and permeabilized for antibody staining under various conditions within the parameters of the protocol of Finney and Ruvkun (1990). None of these various conditions produced any useful staining. Antisera generated to GOA-1 (M. Koelle, personal communication) were used a positive control for fixation and immunoflouresence staining. GOA-1 expression was observed in the expected cells (Mendel et al., 1995; Segalat et al., 1995; M. Koelle, personal communication). Under certain fixation and staining conditions, Ab22 stains seam cells whereas Ab20 stains intestinal nuclei. It is thought that this staining is produced by immunoreaction to contaminating antigens that were not completely removed in the affinity purified antisera.

Acknowledgments

We thank Michael Koelle for his expert advice on the experiments described in this Appendix. We also thank Alex Hajnal for providing additional advice on these experiments. J. H. T. was a predoctoral fellow of the Howard Hughe Medical Institute. H. R. H. is an Investigator of the Howard Hughes Medical Institute.

References

Finney, M. and Ruvkun, G. (1990). The *unc-86* gene product couples cell lineage and cell identity in *C. elegans*. *Cell* **63**, 895-905.

Herman, R. K. and Hedgecock, E. M. (1990). Limitation of the size of the vulval primordium of *Caenorhabditis elegans* by *lin-15* expression in the surrounding hypodermis. *Nature* **348**, 169-171.

Koelle, M. R. and Horvitz, H. R. (1996). EGL-10 regulates G protein signaling in the *C. elegans* nervous system and shares a conserved domain with many mammalian proteins. *Cell* **84**, 115-125.

Mendel, J. E., Korswagen, H. C., Liu, K. S., Hadju-Cronin, Y. M. Simon, M. I., Plasterk, R. H. A. and Trenberg, P. W. (1995). Participation of the protein G_0 in multiple aspects of behavior in *C. elegans*. *Science* **267**, 1652-1655.

Segalat, L., Elkes, D. A. and Kaplan, J. M. (1995). Modulation of seratonin-controlled behavior by G_0 in *C. elegans*. *Science* **267**, 1648-1651.

Appendix 2

Prospects and Perspectives

Jeffrey H. Thomas and H. Robert Horvitz

Howard Hughes Medical Institute, Massachusetts Institute of Technology
Cambridge, MA 02139

Summary

This appendix describes prospects for the continued study of the synMuv genes as well as some perspectives and speculations on a few issues raised by the study of the synMuv genes. The first section defines a strategy for the future analysis of synthetic Multivulva genes. The rest of this appendix presents several thoughts and speculations prompted by the study of redundant systems in development. The nature of redundant systems is discussed briefly. Evolutionary considerations raised by the existence of redundant systems are addressed and several models are proposed to account for the existence of such systems.

Strategy for the future analysis of the synthetic multivulva genes

In this section a strategy for the future analysis of the synthetic multivulva genes is outlined. The strategy focusses on experiments that extend our understanding of the signaling pathways modulated by the synMuv genes and how they regulate vulval development. Many of the experiments described involve the use of reagents developed over the course of the work described in this thesis.

Genetic analysis of the synMuv genes suggests that mutations in several class B genes may not have been isolated. There may be several unidentified class B genes that have not yet been identified because of pleiotropies that interfere with their isolation in the screens conducted to date. This is suggested by the observation that animals carrying strong *lin-9* alleles are sterile and by the observation that animals *trans*-heterozygous for *lin-55(n2994)* and a deficiency spanning the *lin-55* locus are sterile and maternal effect lethal (Ferguson and Horvitz, 1989; Chapter 2). Mutations with these pleiotropies were not expected to be isolated in the screens described in Chapter 2. These screens demanded that the synMuv mutations isolated produce a viable, fertile, Muv animals.

To isolate class B synMuv mutations that produce sterile or maternal effect lethal phenotypes, a screen must be conducted that allows the recovery of heterozygous siblings that are viable and fertile. Such a screen can be readily conducted by mutagenizing a class A strain and picking F1 progeny individually to single plates. F2 progeny are screened for Muv animals that are sterile or segregate only dead progeny. From plates that contain such animals, nonMuv animals would be isolated, 2/3 of which are expected to be heterozygous for the class B mutation. The new mutations would be maintained as balanced heterozygotes. The new mutations would then be tested to see if they are alleles of known genes or are alleles of hitherto undiscovered genes.

To extend the molecular characterization of the synMuv genes, additional genes should be cloned. This will provide molecular reagents for biochemical and expression studies. The expression studies will allow the synMuv genes to be partitioned into groups based on the cells in which they are expressed and on the subcellular localization within these cells. This data may suggest that certain synMuv gene products interact more closely with certain other synMuv gene products to effect negative regulation of vulval development.. Of particular interest are data on the similarity of synMuv genes to other genes with known biochemical functions. Strong similarity may reveal the mechanism by which a class of synMuv genes acts to regulate vulval development. Such data could lead to further experiments which are beyond the scope of this discussion.

Cloning many of the newly isolated synMuv genes described in Chapter 2 should be fairly easy. These genes have been well mapped, often to the point where they have

been positioned such that they have been locatalized to about a hundred kb of cloned DNA. The newly isolated class B genes that should be this easy to clone are *lin-55 II*, *lin-52 III*, and *lin-54 IV*. *lin-37 III* also falls into this category. Several other class B genes should be relatively straightforward to clone, these include *lin-53 I* and *lin-35 I*. The class A gene *lin-56 II* should be as easy to clone a member of the most accessible group of class B genes. These genes are thus readily amenable to molecular analysis and should be studied at the first opportunity. If these genes are cloned, nine out of the ten known class B genes and two out of the four known class A genes will have been cloned. This will provide an almost complete preliminary analysis of the synMuv genes.

Several strategies exist for placing the synMuv genes into well-ordered pathways within each class. Since mutations in the synMuv genes all have identical phenotypes, they cannot be ordered by conventional epistasis experiments. Therefore, non-genetic strategies must be used to order the pathways. Similar strategies may enable us to determine the identities of other molecules with which synMuv gene products interact. These molecules may be key elements involved in vulval development that are regulated by the synMuv genes. The methods that should be used for this analysis are yeast two-hybrid experiments and coimmunoprecipitation experiments.

Yeast two-hybrid experiments to order the synMuv genes into a pathway within each class can be conducted in two ways. The first is to test all pairwise combinations of cloned synMuv genes of the same class. If the genes noted above are cloned, this will allow most of the synMuv genes to be tested and ordered into a pathway. In the absence of any other data, it would only be possible to determine sequence of action without direction, i.e. without determining the beginning and end of the pathway. However, the the genetic mosaic results of *lin-15*, *lin-37* and *lin-36* should allow the direction of the pathway to be determined. This of course presupposes the most parsimonious pathway: signaling from hyp7 to the Pn.p cell. The second method is to use each of the cloned synMuv genes as bait to trap other genes in a two-hybrid screen. This method, although much slower than the first, has several advantages. These are that uncloned synMuv genes may be cloned, that new synMuv genes may be identified and cloned, and that genes that are not mutable to a synMuv phenotype, but are targets of synMuv gene regulation or are regulators of synMuv genes may be identified and cloned. In this latter case, it may be possible to determine if a class of synMuv genes acts to regulate a particular component of the vulval induction pathway, and if so, which one. Thus, the potential reward for inferring how synMuv genes work is enormous.

Coimmunoprecipitation experiments offer another approach to address these issues. Antibodies have been raised to LIN-36 (Appendix 1). These may be capable of

immunoprecipitating LIN-36 protein from worm extracts.. If so, other proteins may coimmunoprecipitate with LIN-36. These proteins could be microsequenced so that probes can be made and clones isolated to determine the identities of these proteins. In addition to determining which, if any, synMuv genes physically interact with LIN-36, it may be possible to isolate a target protein of LIN-36 that is not a synMuv if such a protein exists. A portnetially easier way to identify coprecipitating proteins is to immunoprecipitate LIN-36 from the extracts of worms carrying mutations in any of the various class B synMuv genes. The absence of a particular band in a particular extract may indicate a correspondence between mutant gene and missing protein. Together with the yeat two-hybrid experiments, these studies may define much of the logic of the synMuv pathways and posssibly even their targets.

Other biochemical experiments may provide insight into the mechanisms of synMuv gene regulation of vulval development. Unfortunately, by their nature, these experiments are not comprehensive searches for biochemical mechanisms but are instead merely tests of certain potential mechanisms amenable to experimental analysis with the reagents at hand. The MAP kinase active in vulval development has been cloned (Lackner et al., 1994; Wu and Han, 1994). This cloned gene can be used in a phosphorylation assay to see if the synMuv genes can negatively regulate MAP kinase activity either at the level of the MAP kinase or upstream. This would be done by comparing the relative activities of MAP kinase in the presence of extracts from wild-type and synMuv mutant worms. Kinase activity would be expected to be higher in synMuv mutant extracts if the synMuv genes encode negative regulators of kinase activity. Similar experiments can be conducted using GTPase assays and the cloned *let-60 ras* gene (Beitel et al., 1990; Han and Sternberg, 1990). MAPK::GFP nuclear transport could also be observed *in vivo* and tested in the presence of synMuv mutants to see if the process is inhibited by synMuv genes. Purified LIN-36 protein could be tested for DNA binding activity. Such activity may suggest that LIN-36 directly modulates transcription and may define a specific binding sequence for LIN-36. A *let-23*::GFP reporter (Hoskins et al., 1996; Simske et al., 1996) could be used to determine if synMuv genes partially control the transcription of *let-23*. However, since LET-23 must be present to receive the LIN-3 signal, the synMuv genes cannot be wholly responsible for *let-23* transcription but may downregulate its expression. Whereas these biochemical experiments are risky and can only specifically test a few mechanisms of synMuv activity, they may provide a useful insight into synMuv gene function.

The strategy for future analysis of the synMuv genes described here is relatively straightforward and requires no unusual effort. Many of these experiments are essentially

assured of success and would yield important findings. The plan outlined above stands a good chance of defining the biochemical mechanisms that the two classes of synMuv genes use to regulate vulval development. If not, these experiments may eliminate several models and provide a more detailed description of the genetics and molecular biology of the synMuv genes and vulval development.

Redundancy in development

Redundancy in development has long been an issue which has bedevilled developmental biologists. Experimentalists have always been concerned about the implications of redundancy for the analysis of development. Redundant genes and functions present a distinct dificulty for the analysis of development. The geneticist who wants to analyze a certain aspect of development through the isolation of mutants in which this process is perturbed must always bear in mind that some or all of the process may be controlled by redundant genes. In such a case, a mutation in such a gene is unlikely to be isolated because a redundant gene masks the mutation. The probability of finding a double mutant affecting the process is extremely low: the product of the frequencies of isolation of the two genes times the Poisson probability that two mutations will be induced in the same animal. Molecular biologists who are able to make a deletion mutation in a cloned gene of interest by homologous recombination in yeast or mice are often disappointed to isolate homozygous mutants with no phenotype. Often this is because of redundant gene is present in the organism. Similar examples are seen when a geneticist attempts to revert a dominant phenotype and isolates a loss-of-function mutation in the gene of interest that produces a wild-type phenotype. This may often be the result of redundant gene activities.

Redundant genes and their functions are usually identified in only a few ways. One method is the construction of double mutants between genes that control similar processes. The double mutant often has additional phenotypes not seen in the single mutants. Double mutants between genes that encode similar proteins are also often constructed because the genes are hypothesized to perform the overlapping function. These double mutants often have additional or more severe phenotypes than the single mutants. If neither single mutant has a phenotype, then a phenotype is often observed in the double mutant (for examples see Hadwiger et al., 1989; Richardson et al., 1989; Ghysen and Dambly-Chaudiere, 1991; Lambie and Kimble, 1991). In other cases, weak phenotypes are seen in single mutants and these phenotypes are enhanced in double mutants between weaker mutants with the same phenotype. Often this enhancement, in the case of null mutations, is the result of redundancy (for example, see Ellis et al., 1991).

The synMuv genes are distinct from these more common examples in that they have no vulval phenotype as single mutants. Some class B mutants have defects in viability and fecundity. It is notable that the synMuv genes were isolated by a fortuitous double mutant (Horvitz and Sulston, 1980).

Genetic redundancy can take two distinct forms: duplicated genes and functional redundancy. Redundant duplicated genes are of course functionally redundant, but they are functionally redundant by virtue of having essentially identical biochemical functions. Duplicated genes are genes that are molecularly similar and perform the same biochemical function. Often these sytems arose from the duplication of an ancestral gene or genes. In these cases, the control elemrnts of the gene as well as the structure and function of the gene are very similar. In other cases, the genes are more distantly related. Such genes may have the same basic biochemical function, such as kinase activity, but differ profoundly in control and structure. Despite these differences, the encoded proteins are able to perform the same biochemical role and thus act redundantly.

In the case of purely functional redundancy, the genes do not necessarily have to have similar biochemical functions, they only have to have similar biological functions. This biological function can be provided by different biochemical means. For example, two genes may act to increase the activity of a target gene above a certain threshold. One may be a transcriptional activator that leads to increased expression of the target protein, and the other may be a cofactor that increases the activity of the target protein. Either gene is sufficient to increase the activity of the target. Another example could involve the acquisition of a metabolite. One gene may act as a transporter of this metabolite from the environment whereas a functionally redundant gene may act to catalyze the production of this metabolite from another molecule in the cell. In this example, the redundant genes play the same biological role, but do so by very different biochemical means. These hypothetical examples serve to show how functional redundancy need not imply molecular or biochemical similarity.

Redundancy and evolution

The fundamental questions with regard to redundancy and evolution are how is redundancy evolutionarily feasible and why does it exist? Because evolution occurs by mutation and natural selection, these questions are inextricably linked. By answering the question why does redundancy exist, it is straightforward to determine how it is feasible. Knowing how redundancy is feasible may well indicate why it exists. One must bear in mind that organisms are not engineered, they are evolved. Redundancy is a common element in designed systems; such redundancy provides some insurance against failure.

For instance, the space shuttle is designed with many backup systems so that if the primary system fails, the backup system can take its place. This is a very safe design. However, such systems cannot be maintained throughout evolution where natural selection and mutation interact to determine the "design" of an organism. There is no selective advantage for a backup system. Since the system is redundant, there is no selective pressure to maintain two parallel systems. Random mutations that disable one or the other of the redundant systems are nondeleterious. In the absence o selective pressure to eliminate these mutations from the gene pool, it is likely that the redundant system will decay over time so that one redundant element is disabled in a brief time and the process is no longer redundant.

The argument cited above applies only to strictly redundant systems. In the case of incompletely redundant systems, selective pressure can maintain both components of such a redundant system. This is because both components are required; neither component is sufficient for complete function. However, the synMuv genes appear to be completely redundant for vulval development as seen by the absence of any defects in single mutants (Capter 3). I propose several models which may account for the presence of a redundant system in the control of *C. elegans* vulval development.

The first model is relatively trivial. It is that the two classes of synMuv genes are not truly redundant in the wild. These genes have been analyzed under laboratory conditions in which much of the selective pressure on nematodes has been eliminated. Thus, these genes may not be redundant for vulval development under narural conditions, or these genes may be required for functions necessary for survival in the wild that are not present in the laboratory and therefore not observable.

In the second model, the synMuv genes are redundant by virtue of accidental expression. The two classes of synMuv genes each play a distinct role in the biology of the organism. The reduced fecundity and viability of many class B synMuv mutants suggest a distinct biological role for these genes. The class A genes may be required for a process that has so far escaped notice or is not essential in the laboratory enviroment. Thus selective pressure maintains these two systems independently of each other. One of these processes controls vulval development and the other is expressed at the right time and place to do so as well. This expression may be the result of the pathway's requirement in another process at the same time and place or may be the result of the interaction of controls that express this system in several other locations at other times. In this model, one of the pathways just happens to be in the right place at the right time and can functionally substitute for the other pathway in terms of ultimate biological effect on vulval development. It is important to note that in this case there is no selective

pressure to maintain this overlap of expression and function except as an artifact of maintaining the proper expression and function of the two classes of genes for their primary functions.

The third model involves direct selective pressure acting to maintain a redundant system for the negative regulation of vulva development. In this model, the redundancy itself has some selective advantage over nonredundancy. Higher efficiency is a likely reason for such selective pressure. A slight growth advantage would be favored by natural selection. Over many generations, this slight advantage would be sufficient to favor animals which possessed it. Such a growth advantage would be predicted to be particularly advantageous to an organism such as *C. elegans* that has a short lifespan characterized by rapid growth. Note that this increased efficiency is not the result of a backup system in case something goes wrong, but an actual improvement in performance because of the operation of two systems. In such a case, it seem that there would have to be cross talk and back regulation between the synMuv genes and other components of the vulal development system in order to obtain the greater efficiency; there has to be a way for two pathways performing the same function to use less resources or take less time than having only one pathway perform this function. The situation postulated in this mode is one in which redundancy is actually favored by selection.

In this section I have attempted to address the vexing conundrum of how a redundant system is maintained during evolution. For this to be the case, there has to be some selective pressure, whether direct or indirect, to maintain such a system. Several possible models to explain how redundant systems are feasible and why they exist have been proposed and explored.

References

Beitel, G. J., Clark, S. G. and Horvitz, H. R. (1990). *Caenorhabditis elegans ras* gene *let-60* acts as a switch in the pathway of vulval induction. *Nature* **348**, 503-509.

Ellis, R., Jacobson, D. M. and Horvitz, H. R. (1991). Genes required for the engulfment of cell corpses during programmed cell death in *Caenorhabditis elegans*. *Genetics* **129**, 79-94.

Ferguson, E. L. and Horvitz, H. R. (1989). The Multivulva phenotype of certain *Caenorhabditis elegans* mutants results from defects in two functionally redundant pathways. *Genetics* **123**, 109-121.

Ghysen, A. and Dambly-Chaudiere, C. (1988). From DNA to form: the *achaete-scute* complex. *Genes Dev.* **2**, 495-501.

Hadwiger, J. A., Wittenberg, C., Richardson, H. E., De Barros Lopes, M. and Reed, S. I. (1989). A family of cyclin homologs that control the G_1 phase in yeast. *Proc. Natl. Acad. Sci. USA* **86**, 6255-6259.

Han, M. and Sternberg, P. W. (1990). *let-60*, a gene that specifies cell fates during *C. elegans* vulval induction, encodes a ras protein. *Cell* **63**, 921-931.

Horvitz, H. R. and Sulston, J. E. (1980). Isolation and genetic characterization of cell-lineage mutants of the nematode *Caenorhabditis elegans*. *Genetics* **96**, 435-454.

Hoskins, R., Hajnal, A. F., Harp, S. A. and Kim, S. K. (1996). The *C. elegans* vulval induction gene *lin-2* encodes a member of the MAGUK family of cell junction proteins. *Development* **122**, 97-111.

Lackner, M. R., Kornfeld, K., Miller, L. M., Horvitz, H. R. and Kim, S. K. (1994). A MAP kinase homologue, *mpk-1*, is involved in ras-mediated induction of vulval cell fates in *Caenorhabditis elegans*. *Genes Dev.* **8**, 160-173.

Lambie, E. and Kimble, J. (1991). Two homologous regulatory genes, *lin-12* and *glp-1*, have overlapping functions. *Development* **112**, 231-240.

Richardson, H. E., Wittenberg, C., Cross, F. and Reed, S. I. (1989). An essential G1 function for cyclin-like proteins in yeast. *Cell* **59**, 1127-1133.

Simske, J. S., Kaech, S. M., Harp, S. A. and Kim, S. K. (1996). LET-23 receptor localization by the cell junction protein LIN-7 during *C. elegans* vulval induction. *Cell* **85**, 195-204.

Wu, Y. and Han, M. (1994). Suppressors of activated Let-60 Ras protein defines a role of *Caenorhabditis elegans* Sur-1 MAP kinase in vulval differentiation. *Genes Dev.* **8**, 147-159.