IDENTIFYING ROLES FOR NON-ESSENTIAL GENES IN ESSENTIAL PROCESSES

by

MARC DAVID DORFMAN

A DISSERTATION

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

December 2008

University of Oregon Graduate School

Confirmation of Approval and Acceptance of Dissertation prepared by:

Marc Dorfman

Title:

"Identifying Roles for Non-Essential Genes in Essential Processes"

This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Biology by:

Christopher Doe, Chairperson, Biology
Bruce Bowerman, Advisor, Biology
Judith Eisen, Member, Biology
Karen Guillemin, Member, Biology
Tom Stevens, Outside Member, Chemistry

and Richard Linton, Vice President for Research and Graduate Studies/Dean of the Graduate School for the University of Oregon.

December 13, 2008

Original approval signatures are on file with the Graduate School and the University of Oregon Libraries.

An Abstract of the Dissertation of

Marc David Dorfman for the degree of Doctor of Philosophy

in the Department of Biology to be taken December 2008

Title: IDENTIFYING ROLES FOR NON-ESSENTIAL GENES IN ESSENTIAL PROCESSES

Approved: _____
Dr. Bruce Bowerman, Advisor

My dissertation has focused on identifying functions for non-essential genes in essential process, using the early *C. elegans* embryo as a model system. The fully sequenced *C. elegans* genome contains ~19,800 protein coding genes of which about half have identifiable homologs in humans. Classical forward genetic mutagenesis screens, and more recently, genome-wide RNA interference (RNAi) screens has led to the identification of most essential genes in the genome. Analysis of the phenotypic data from mutants and RNAi screens shows that roughly 15% of the genes are essential and an additional 15% produce some other easily identifiable knockdown phenotype. This leaves about 70% of genes that have no functional information. Genetic modifier screening allows for the identification of roles for genes that do not produce a loss of function phenotype on their own but are able to modify the phenotype of a specific mutant. In my first chapter, I introduce approaches to identifying new gene functions and the usefulness

of *C. elegans* as a model system in this pursuit. In Chapter II, I describe a type of high-throughput genetic modifier screen that combines the sensitized genetic background of temperature-sensitive (ts) embryonic lethal mutants, and RNAi, to identify genes that either enhance or suppress embryonic lethality seen in the mutant background. I also summarize results from screening four ts mutants using this method. The following two chapters describe the identification and characterization of genetic modifier genes for two different ts embryonic-lethal mutants. Chapter III describes modifiers of *rfl-1*, a conserved gene required for proper cytoskeletal regulation in the early *C. elegans* embryo. Chapter IV describes modifiers of *lit-1*, also a conserved gene, that is required for regulation of Wnt signaling and cell fate specification in *C. elegans*. These findings reveal novel genetic interactions and provide functional information about many conserved but non-essential genes that have had no previous characterization. Conclusions are also made about the effectiveness of ts mutant/RNAi screening in the pursuit of identifying new gene functions.

This dissertation contains co-authored material that has been previously published, and material that is currently in review, or is being prepared for publication.

CURRICULUM VITAE

NAME OF AUTHOR: Marc David Dorfman

PLACE OF BIRTH: Santa Barbara, California

DATE OF BIRTH: July 17, 1979

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene, Oregon
University of California at Santa Barbara, California

DEGREES AWARDED:

Doctor of Philosophy in Biology, 2008, University of Oregon
Bachelor of Science, 2002, University of California at Santa Barbara

AREAS OF SPECIAL INTEREST:

Cell Biology
Genetics
Developmental Biology

PROFESSIONAL EXPERIENCE:

Graduate Research Fellow, Department of Biology, University of Oregon, Eugene, Oregon, 2004-2008

Technology and Entrepreneurship Program Fellow, Lundquist College of Business, University of Oregon, 2008

Graduate Teaching Fellow, Department of Biology, University of Oregon, Eugene, Oregon, 2002-2003

Researcher, Dr. Joel Rothman Lab, University of California at Santa Barbara, 2002

Laboratory Technician, Qualtek Molecular Laboratories, Goleta, California, 1998 -2001

GRANTS, AWARDS AND HONORS:

NIH Developmental Biology Training Grant, University of Oregon, 2004-2007

Co-Host of 5th Annual Developmental Training Grant Symposium "(Re)Generating Stem Cells", University of Oregon, June 2007

President of Students in Biological Sciences (SIBS), University of Oregon, 2004-2005

PUBLICATIONS:

Dorfman, M.D., S.M. O'Rourke, J. Gomez and B. Bowerman, (2008) Using RNA interference to identify modifiers of a temperature-sensitive, embryonic-lethal mutation in the essential *C. elegans* gene *rfl-1*. Genetics (In Review).

O'Rourke, S. M., M. D. Dorfman, J. C. Carter and B. Bowerman, 2007 Dynein modifiers in *C. elegans*: light chains suppress conditional heavy chain mutants. PLoS Genet **3:** e128.

ACKNOWLEDGMENTS

I wish to thank my advisor, Dr. Bruce Bowerman, for always putting my best interests first and for providing excellent guidance and encouragement throughout my graduate school career. I also acknowledge all my lab members past and present for helping to create a positive work and learning environment. Specifically I would like to thank Dr. Sean O'Rourke. Sean is a brilliant scientist who was always generous in providing training, guidance and expert opinion. Working closely with him has greatly enhanced my scientific training. I would like thank Graduate Program Coordinator Donna Overall for always being vigilant about important deadlines and reminding me whenever I needed to complete something. Lastly, I thank Dr. Dan Graham for his help when the chips were down and a computer or a piece of equipment needed to be fixed in a hurry.

I co-dedicate this document to my parents and to my wife Jana, who have supported my endeavors, scientific and otherwise.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION TO FUNCTIONAL GENOMIC ANALYSIS USING THE MODEL SYSTEM *CAENORHABDITIS ELEGANS*

In recent years many whole genome sequencing projects have been completed, including that of humans, providing us with the DNA sequences that encode all of the heritable factors necessary for life. While the completion of these sequencing projects are heroic and important achievements, we are far from understanding the functions and requirements of most of the genes in any one of those genomes. Model organisms that are amenable to experimental genetic analysis are essential for identifying the functions of specific genes. With the availability of whole genome sequences, these functions can be extrapolated to understanding the roles of genes with similar sequences in humans.

One interesting finding from sequencing data and subsequent analysis, was the relatively small number of predicted gene coding sequences in humans and other multi-cellular organisms compared with previously sequenced genomes of single celled organisms. The relatively small number (25,000 for humans vs 6000 for the single celled yeast), did not seem to account for the vast complexity of regulatory processes required for the development and maintenance of multi-cellular organisms and complex phenotypes (LANDER *et al.* 2001). Alternative-RNA splicing (SCHELLENBERG *et al.* 2008), post-translational modification (MEINNEL and GIGLIONE 2008), and epigenetics (GUIL and ESTELLER 2008), have all been shown to play important roles in exploiting the

limited repertoire of heritable factors to allow for the development of higher organisms. Moreover, the identification of genetic interaction networks, how genes affect the regulation and function of other genes, will no doubt be essential in understanding fundamental biological processes and the development of complex traits. In addition, genetic interactions can provide important insight into the function of individual genes. Importantly, genetic interactions can reveal roles for genes that do not have an easily detectable loss-of-function phenotype of their own and therefore have avoided detection in previous genetic mutagenesis studies. The model system *C. elegans* has emerged as one of the most important model organisms in the pursuit of identifying such genetic interactions.

Genetic analysis in the model organism *Caenorhabdidtis elegans*: *Caenorhabditis elegans* is an ideal model organism for genetics study as it is complex enough to study development, behavior and specific disease models, but simple to maintain and amenable to various methods of forward and reversed genetics to identify gene function. The genome sequence of *C. elegans* released in 1998 (*C. elegans* Sequencing Consortium), was the first to be released of any multicellular organism. Analysis and comparison with the human genome sequence has predicted that roughly 50% of human genes have identifiable homologs in *C. elegans* (Wormbook 2005). Another import characteristic of *C. elegans* is that embryos and adults are transparent and therefore their invariant development to produce the adult animal consisting of 959 somatic cells, can be easily monitored with a light microscope. This allows for the identification of aberrant

phenotypes at a cellular level which can provide information about the specific function of genes when they are reduced in function.

It has been over three decades since Sydney Brenner published the first genetic screen in *C. elegans*. This paper describes the use of a chemical mutagen to produce random mutations throughout the genome (BRENNER 1974). Then, by screening through the F2 generation he identified 619 mutants with visible phenotypes which were then grouped and classified into distinct phenotype classes. This screen is an example of a "simple screen" where the experimenter's goal is to find mutations that cause any type of abnormal phenotype. This screen was critical for establishing phenotypic classes such as uncoordinated (*unc*), small body (*sma*), dumpy (*dpy*), long body (*lon*) and rolling locomotion (*rol*). Researchers later used these phenotype classes to identify mutations in different genes that caused the same or similar phenotype and thereby began to establish genetic pathways.

In the years since Syndey Brenners's landmark paper, researchers have designed and executed many different types of forward genetic screens, and this has led to the characterization of many thousands of genetic mutation alleles covering roughly 5,000 individual genes. Many screens have been designed to identify specific phenotypes such as maternal embryonic lethal phenotypes (*mel*). Maternal embryonic lethal mutants describe mutations in genes that are essential for embryonic development, but embryos homozygous for the mutation, but that have wildtype, maternally derived gene product (from heterozygous parent), are viable.

Selectable markers can be used to aid in the identification and/or the isolation of specific phenotypes such as *mel*. For example, *mel* screens have been done using an mutant allele of the gene *lin-2* that causes an egg laying defect (PRIESS *et al.* 1987). Embryos that have maternally deposited wildtype *mel* gene product are viable and will hatch inside of the adult hermaphrodite *lin-2* mutants, leading to the distinctive "bag of worms" phenotype, instead of being passed through the vulva. Embryos from homozygous *mel* mutant worms will fail to hatch but will remain inside the mother. While mutants obviously cannot be recovered from the worm containing dead eggs, heterozygotes containing the *mel* mutation can be isolated from siblings of the adult contained on the same plate. Thus selectable markers aid in reducing the amount of labor and time required to conduct large-scale screens.

Functional genomics using genome-wide RNA interference: Reducing gene function to identify phenotypes and therefore gene functions in *C. elegans* has benefitted greatly from the advent of RNA interference, the ability of double stranded RNA (dsRNA) to target and degrade endogenous mRNA, thereby thwarting protein production. First applied by microinjection (GUO and KEMPHUES 1995), RNAi can now be performed by feeding worms *E. coli* that expresses dsRNA corresponding to a particular gene (TIMMONS and FIRE 1998). This method has allowed for genome-wide RNAi screens to be performed routinely using various genetic backgrounds (KAMATH and AHRINGER 2003; MAEDA *et al.* 2001; SIMMER *et al.* 2003; SONNICHSEN *et al.* 2005). Data derived from these genome-wide RNAi screens and decades of forward mutagenesis screens have

led to the discovery of most if not all essential genes in the genome. Current estimates put the number of essential genes at roughly 15% of the genome while another 15% produce some other readily identifiable phenotype when depleted with RNAi. The remaining 70% of genes (about 14,000) have no known loss-of-function phenotype. Studies using deletion mutants in yeast *Saccharomyces cerevisiae* show a similar ratio of essential (19%) to non-essential genes. About one third of these 14,000 *C. elegans* genes have counterparts in mammalian organisms, and therefore it will be a worthwhile pursuit to elucidate functions for many of these genes. The challenge is finding the best way to identify such functions in an efficient manner. Various methods of genetic modifier screening will likely play a major role in such efforts.

The role of genetic modifier screening in determining gene functions: Genetic modifier screening is a powerful method to identify genes that function in or influence specific molecular pathways. By identifying second site mutations that either enhance (worsen) or suppress (alleviate) a particular phenotype, researchers can accumulate mutants, quantify their effect on the background phenotype, and then clone and characterize the mutated loci. Often, modifier screens can reveal functions for genes that do not have a loss of function phenotype of their own, because of genetic redundancy, genetic buffering or because the phenotype is too subtle to detect. But, when combined with a background mutation, certain mutants of non-essential genes display a synthetic phenotype. This is sometimes referred to as "sensitized screening" as it allows for detection of mutant phenotypes that would not be penetrant enough, or completely silent,

in an otherwise wildtype background. Reverse genetic screens using RNAi offers an attractive alternative to identifying second site modifiers, as it allows the researcher to perform targeted screens of suspected genetic modifiers, or large-scale, high-throughput screens to identify many candidate modifier genes, without the need for genetic mapping and sequencing to identify modifier loci as with forward genetics screens.

Temperature-sensitive mutations offer a unique and ideal background for genetic modifier screens as mutants of essential genes can be maintained at permissive temperature, and then raised at semi-permissive temperatures for the detection of enhancers and suppressors. The ability to control the function of ts mutants proteins by altering temperature also allows the researcher to identify requirements of the protein at different times during development. For example, a loss-of-function mutant of a gene that is essential for very early embryonic development likely would lead to a terminal phenotype before most of the embryonic cell divisions and morphogenesis has occurred. This would prevent the detection of functional requirements for the gene in later stages of development. With ts mutations, one can circumvent early requirements by maintaining the animals a permissive temperature and then "upshifting" to a non-permissive temperature later in development.

High-throughput genome-wide modifier screens necessitate a method of qualitative scoring that is relatively quick and accurate. Temperature-sensitive mutants are especially useful for high-throughput, qualitative screening as they can be raised at temperatures that both maximize genetic sensitivity and the ability to detect changes in embryonic lethality. For example, by growing a ts embryonic-lethal mutant at a

temperature at which only 2% of progeny hatch with control RNAi, makes a suppressor that increases viability by say 8%, a five fold increase in viability and therefore will likely be detected. Detection of slight changes in lethality may not be detectable at more intermediate temperatures. Furthermore, most ts mutants display higher degree of phenotype variability at intermediate temperatures compared with temperature near the points of 0% or100% penetrance.

In the following chapters, a novel type of high-throughput RNAi modifier screen is introduced and results of screening are presented. Chapter II contains published, co-authored material describing the screening procedure. Chapters III and IV present coauthored, in-depth analysis and characterization of genetic loci that modify the ts mutants *rfl-1(or189ts)* and *lit-1(or131ts)*. The gene *rfl-1* encodes a conserved gene required for proper cytoskeletal regulation in the early *C. elegans* embryo. The gene *lit-1* encodes a conserved gene that is required for regulation of Wnt signaling and cell fate specification in *C. elegans*. Together, a total of 59 reproducible genetic modifiers were identified for these two mutants. The majority of these genes have no previously identified function while about half have homologous counterparts in humans. The identification of novel roles for these genes and the preliminary work in characterizing some of the conserved but non-essential genes, will hopefully aid in the pursuit of understanding how essential biological pathways that control cell division and cell fate-patterning work. Moreover, non-essential genes that influence essential processes may prove to be valuable drug targets as altering their function may allow for modification of an essential process without causing deleterious side effects.

Bridge to Chapter II: In the preceding chapter, I introduced various approaches to identifying gene function, the usefulness of *C. elegans* in the study of functional genomics, and the attributes of temperature-sensitive mutants in genetic studies. In chapter II, I will present a novel screening approach using feeding RNAi and ts mutants to identify roles for non-essential genes in essential processes. I also present some results of screening four ts mutants, and discuss the overall effectiveness of the screening approach. The chapter contains some material from a previously published, coauthored publication.

CHAPTER II

A GENETIC MODIFIER SCREEN USING HIGH-THROUGHPUT RNAi AND TEMPERTURE-SENSITIVE, EMBRYONIC-LETHAL MUTANTS

The high-throughput screening procedure of ts mutants with feeding RNAi described in this chapter, was developed primarily be three lab members: Jose Gomez, Sean O'Rourke and myself. Jose Gomez conceived of the idea to use ts mutants to screen for both enhancers and suppressors of embryonic lethality. Sean O'Rourke played a major role in the development of the high-throughput procedure. Along with Sean O'Rourke I contributed to optimizing the screening procedure and also performed all screening and subsequent analysis of the mutants presented in this chapter.

INTRODUCTION

Functional genomic analysis of *Caenorhabditis elegans* has been accelerated in recent years by the ability to inhibit the expression of targeted genes using RNA interference (RNAi), the delivery of dsRNA into cells which causes a degradation of endogenous mRNA corresponding to the sequence of the dsRNA. In *C. elegans* RNAi can be performed by three different methods: soaking (MAEDA *et al.* 2001), injection (SONNICHSEN *et al.* 2005),or feeding (TIMMONS *et al.* 2001). With the availability of

whole genome sequence data released in 1998, researchers have already conducted genome-wide RNAi screens using each of the three methods of dsRNA (KAMATH and AHRINGER 2003; MAEDA *et al.* 2001; SIMMER *et al.* 2003; SONNICHSEN *et al.* 2005). In addition, genome-wide RNAi screens have been done in mutant backgrounds that have been shown to be hypersensitive to RNAi, presumable allowing for a more complete knockdown of genes that are relatively insensitive to the effects of RNAi (SIMMER *et al.* 2003). The data derived from these screens have lead to the identification of most if not all essential genes and genes that cause an easily identifiable visible phenotype (see Chapter 1 for more detailed discussion). About 15% of the 20,000 genes in the genome produce an embryonic lethal phenotype while another 15% produce some other readily identifiable abnormality. The remaining 70% of genes (roughly 14,000) have no known function, even though roughly one third of these genes have identifiable homologues in mammalian genomes. Identifying roles for these genes presents a challenge to researchers, but it seems to be a challenge worth pursuing as it is likely that many of the genes in this "silent majority" have important roles in major biological processes even if they lack a clear loss-of-function phenotype of their own.

Modifier screens using sensitized genetic backgrounds present the opportunity to identify roles for non-essential genes in essential processes. In this study, we have developed a novel method of high-throughput modifier screening using feeding RNAi with animals carrying temperature-sensitive (ts), embryonic-lethal mutations to identify genes that modify the degree of embryonic lethality normally present in the mutant background. The use of ts alleles allowed for the identification of both genetic enhancers

and suppressors by altering the incubation temperature to a semi-permissive (for identifying enhancers) or just non-permissive temperature (for identifying suppressers) during the screening process. We describe the systematic screening process which includes three screening steps (primary, secondary and tertiary), and present results of screening four different ts mutants; *spn-4 (or191ts)*; *zen-4(or153ts)*; *lit-1(or131ts)* and *rfl-1(or198ts)*, each defective in distinct processes essential for early embryonic development. We go on to discuss some of the candidate modifiers identified for *spn-4(or191ts)* in the discussion section; modifiers of *rfl-1(or198ts)* and *lit-1(or131ts)* are discussed and analyzed in chapters III and IV, respectively.

MATERIALS AND METHODS

Screening tools and basic procedure: To identify genes that, when reduced in function, can suppress or enhance conditional ts mutants, we developed a high-throughput RNAi based screen. To reduce gene function we used a library of 16,757 bacterial strains that each express dsRNA corresponding to an exon-rich gene sequence. After obtaining the *E. coli* RNAi library from the MRC Geneservice (Cambridge, UK), we rearrayed it into a 48-well microplate format using a liquid-handling Qiagen BioRobot 8000. *E. coli* strains were thawed from −80 °C storage and inoculated into 1 ml of LB + 100 mg/ml ampicillin-containing 96-well growth plates (Whatman) and covered with microporous sealing film (USA Scientific). Only 48 wells of the 96-well growth plates were filled with

media, corresponding to the rearrayed *E. coli* library. After overnight shaking incubation at 37 °C, 20 µl of the cultures were dispensed with a 24-channel electronic repeating pipette (Rainin) onto 48-well plates (Nunc) containing NGM agar, 100 µg/ml ampicillin, and 1 mM IPTG and allowed to dry and induce dsRNA at 37°C overnight. The 48-well agar plates were filled using a Wheaton Unispense peristaltic pump equipped with a custom-made adaptor (University of Oregon Technical Science Administration) that allowed simultaneous filling of eight wells with the agar solution. Approximately 15 hypochlorite-synchronized L1 mutant larvae were pipetted into each well of the 48-well plates with a multichannel pipette and allowed to produce broods. Screening for F1 viability was performed by visual examination with a stereomicroscope. Phenotypes were recorded on a Microsoft Excel spreadsheet.

Identifying candidate modifiers and testing for reproducibility: primary, secondary and tertiary Screens: During the primary screening process, a non-stringent scoring criterion for identifying putative enhancers and suppressors was used to reduce the possibility of failing to detect potentially interesting genetic interactions. To eliminate false-positive interactions, and to identify the most reproducible and penetrant genetic interactions worthy of further study, each positive interaction was systematically re-screened in both a "secondary" and "tertiary" screening process (Table 1). During the secondary screening process each interaction was retested using the same method as in the primary screening process. Only the genetic interactions that exhibited a reproducible effect were carried on to tertiary screening. During the tertiary screen each RNAi clone

that exhibited a reproducible genetic interaction was streaked out, and two colonies were selected for retesting. This procedure eliminated the possibility that two different RNAi clones, accidentally mixed in the library, were acting together to interact with the background mutant. Results from the tertiary screening process are also included in Table 1.

RESULTS

A sensitized genetic background: To identify semi-permissive temperatures that optimized our ability to identify enhancers and suppressors, we quantified embryonic viability for embryos derived from *spn-4(or191ts)* and *zen-4(or153ts)* animals grown at 5 different temperatures between 15°C and 25°C (see chapters III and IV for *rfl-1(or198ts)* and *lit-1(or131ts)* ts curves) (Figure 1). We chose to screen *spn-4(or191ts)* at 17°C for enhancement and 23°C for suppression. For the mutant *zen-4(or153ts)* we chose 15°C for enhancement and 18°C for suppression.

Candidate modifiers from qualitative screening: We screened four ts mutants using two separate conditions against a feeding RNAi library to identify enhancers and suppressors of embryonic lethality. The primary screen yielded 2101 total genetic modifiers which were consolidated and rescreened for reproducibility using the same procedure. This secondary screen reduced the number of candidate modifiers to 585. A final round of screening using bacterial cultures started from single colonies yielded 189

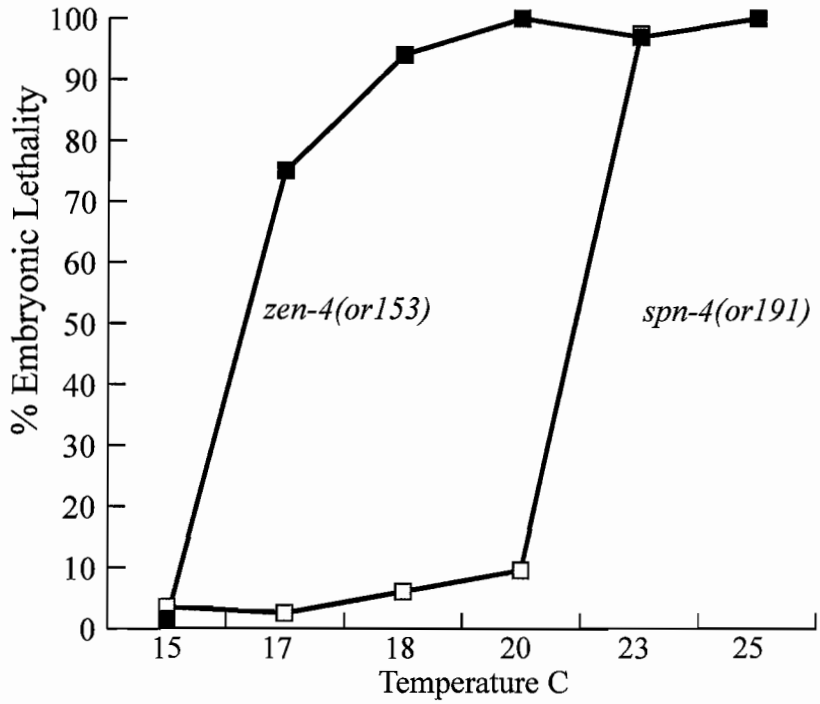

Figure 1.-Temperature versus embryonic viability for *spn-4(or191)* and *zen 4(or153)*. L1 larvae were raised to adulthood at the indicated temperatures on L4440 empty-vector control bacteria. Progeny were scored as hatched larvae or dead embryos (n>200 embryos for each temperature).

TABLE 1
Screening output

mutant	lit-1 enh	lit-1 supp	spn-4 enh	spn-4 supp	rfl-1 enh	rfl-1 supp	zen-4 enh	zen-4 supp
% library screened/ # of genes	77 12874	94 15716	54 9029	79 13208	49 8192	84 14045	56 9363	68 11370
Modifier candidates primary screen	328	239	271	180	248	388	420	27
Reproducible modifiers secondary screen	95	31	135	30	101	104	85	4
Tertiary screen positives	89	5	21	16	32	26	Not completed	0

modifiers [*zen-4(or153ts)* enhancer tertiary screening not completed]. Among these reproducible candidate modifiers are 142 enhancer genes which had no previously identified essential requirements in genome-wide RNAi screens. Thus, our screen was able to identify potential novel genetic interactions and functions for non-essential genes.

RNAi knock-down of the gene *oma-2* dramatically suppresses embryonic lethality in *spn-4(or191ts)* mutants: Enhancer and suppressor screening of *spn-4(or191ts)* yielded 21 candidate enhancers and 16 candidate suppressors. Among the enhancers are three genes that are highly conserved and have no RNAi or mutant phenotype in any of several previously published screens [in our screening process we only eliminated enhancer candidates that showed lethality in either a genome wide-feeding RNAi screen (KAMATH and AHRINGER 2003), or genome-wide soaking RNAi screen (MAEDA *et al.* 2001)]. These genes are H17B01.4, F12A10.7 and C45G9.1. H17B01.4 and F12A10.7 both encode uncharacterized proteins whereas C45G9.1 encodes a predicted casein kinase. Of the 16 suppressor candidates, one gene stood out during qualitative screening as a particularly penetrant suppressor. Sequence verification of the RNAi clone confirmed that it was the gene *oma-2*. Quantitative analysis of embryonic viability in *spn-4(or191ts)* mutants showed a dramatic amount of suppression, from 6% viability with negative controls, to 59% viability of animals fed *oma-2* RNAi. At an even more restrictive temperature of 24°C, at which only 0.5% of *spn-4(or191ts)* embryos hatch, viability was increased to 41% with *oma-2* RNAi. Importantly, *oma-2* was not detected as a suppressor

in any of the other three mutants screened in this report, supporting the possibility that *oma-2* has a direct role in *spn-4* related processes.

DISCUSSION

The goal of the work described here was to conduct a high-throughput RNAi modifier screen using multiple genetic backgrounds in parallel to detect reproducible enhancers and suppressors. Using the methods and tools presented in this chapter it is possible to conduct multiple genome-wide screens in parallel in as little as five weeks. The methods used have also proven to be a useful approach in identifying roles for non-essential genes in essential processes.

Screening procedure analysis: The work presented in this chapter took roughly two years to complete. Much of that time could have been eliminated had we not faced setbacks due to equipment problems, inactivation of IPTG, and recurrent bacterial and fungal contamination. Although equipment failure is not always preventable, extreme care should be taken to prevent contamination of plates and worm stocks during the procedure. Solutions containing synchronized L1 larvae should always be tested for contamination by aliquoting a small volume onto an NGM + 4xAMP plate and incubating overnight at 37°C. If any colony growth is observed, the solution should not be used, as even a very small amount of contamination will likely grow rapidly on the RNAi plates and negate results. All solutions containing IPTG should be immediately

frozen in single use aloquots. There is lack of documentation regarding IPTG shelf life and we found that activity of IPTG is very sensitive to freeze-thaw cycles or storage at temperatures above freezing. Furthermore, positive and negative controls should be performed during every RNAi assay to make sure the feeding RNAi is working. Lastly, it is important to allow bacterial cultures seeded onto agar to completely dry, as a liquid environment for the worms significantly alters hatching rates. Likewise, care should also be taken to prevent excessive drying of the agar as this can also affect hatching rates.

Candidate modifiers of *spn-4(or191ts)*: The gene *spn-4* encodes a protein containing an RNP-type RNA-binding domain and is required for several processes in the early *C. elegans* embryo including proper cell fate patterning and mitotic spindle orientation (GOMES *et al.* 2001; LABBE and GOLDSTEIN 2002). Loss of function of *spn-4* ultimately leads to reduced gut and pharyngeal tissue and excess body wall muscle and germline (GOMES *et al.* 2001). We identified one highly penetrant suppressor of *spn-4(or191ts)* mutants, that did not suppress lethality in any of the other three mutants in this study (qualitative analysis). RNAi knock-down of *oma-2* increased viability in *spn-4(or191ts)* mutants from 6% to 59% viability.

The gene *oma-2* is at least partially redundant with its paralog *oma-1* and has 63% amino acid similarity. Neither gene is essential, but double mutants have severe defects in oocyte maturation often leading to sterility (DETWILER *et al.* 2001). *oma* gene products encode CCCH-type zinc finger proteins that, like SPN-4, have been shown to bind to maternal germline mRNA targets and to regulate their expression. A recent study showed

that SPN-4 and OMA-1/2 have at least one mRNA target in common as both bind the 3'UTR of the maternal mRNA *nos-2* and suppress its translation (JADHAV *et al.* 2008). Requirement for *spn-4* has been identified in several studies examining the establishment of polarity in the early *C. elegans* embryo (GOMES *et al.* 2001; JADHAV *et al.* 2008; LABBE and GOLDSTEIN 2002), and it has become clear that both *spn-4* and *oma-1/2* have multiple different roles that influence these early events in development. The data presented here suggest that *oma-2* and *spn-4* may have some opposing role(s) which would explain why depleting *oma-2* would suppress lethality in a *spn-4* loss-of-function mutant. It is not known if RNAi to *oma-2* is specific, or if *oma-1* gene product is also partially depleted because of sequence homology. It would be interesting to cross *oma-1* and *oma-2* mutant alleles into the *spn-4(or191ts)* background and test whether lethality is enhanced or suppressed. A significant phenotype difference between these double mutants could indicate separate functions for *oma-1* and *oma-2*. Additionally, it would be interesting to identify all or most of the mRNA targets of these proteins. This information will likely explain the mechanism of the interaction identified in this RNAi screen.

Bridge to Chapter III: I identified many reproducible interactions using the screening approach outlined in the proceeding chapter. In chapter III, I present coauthored material currently in review (Genetics) describing a systematic analysis of enhancers and suppressors of the gene *rfl-1*. The analysis includes quantification of enhancement or suppression, a thorough specificity analysis of each modifier, protein localization data of several conserved and specific modifiers, and analysis of cellular phenotypes in enhanced

or suppressed *rfl-1* mutant embryos. Results of this work indicate that many of the genetic modifiers identified likely play novel and important roles in early embryonic cell division. Important conclusions are also made about assessing specificity of genetic interactions before formulating hypothesis about potential protein functions.

CHAPTER III

USING RNA INTERFERENCE TO IDENTIFY MODIFIERS OF A TEMPERATURE-SENSITIVE, EMBRYONIC-LETHAL MUTATION IN THE ESSENTIAL *C. ELEGANS* GENE *RFL-1*

Reproduced with permission from: Marc Dorfman, José-Eduardo Gomes*,
Sean O'Rourke* and Bruce Bowerman
*These authors contributed equally to this manuscript
Manuscript currently in review at Genetics

INTRODUCTION

Ubiquitin-mediated proteolysis regulates many biological processes (NANDI *et al.* 2006). These include, in the nematode *Caenorhabditis elegans*, the early developmental processes of oocyte maturation, cell cycle progression, cell polarization, and cell fate patterning, all of which require the timely destruction of maternally expressed proteins (BOWERMAN and KURZ 2006). One *C. elegans* protein targeted for proteolysis early in *C. elegans* embryogenesis is MEI-1, the AAA-ATPase subunit of the microtubule severing complex called katanin (DOW and MAINS 1998; KURZ *et al.* 2002; MAINS *et al.* 1990; PINTARD *et al.* 2003a; SRAYKO *et al.* 2000; XU *et al.* 2003). Katanin is a heterodimer of two subunits called p60 and p80 in vertebrates and MEI-1 and MEI-2 in *C. elegans*. Katanin in *C. elegans* is required for proper assembly and function of the small, barrel-

shaped meiotic spindles (ALBERTSON and THOMSON 1993; MCNALLY *et al.* 2006), but it must be degraded after meiotic divisions to permit assembly of the much larger first mitotic spindle in the one-cell zygote. In mutants that fail to degrade katanin after the completion of meiosis, the first mitotic spindle is fragmented and mis-oriented, cytokinesis is defective, and the embryos die without hatching (DOW and MAINS 1998; KURZ *et al.* 2002; SRAYKO *et al.* 2000).

The katanin subunit MEI-1 is targeted for poly-ubiquitylation and proteolytic destruction by a Cullin-based E3 ligase. This complex includes the Cullin scaffolding protein CUL-3 and a substrate specific adaptor called MEL-26 that binds to CUL-3 through a BTB domain and to MEI-1 through a MATH domain (PINTARD *et al.* 2003b). Cullin 3-based E3 ligases in mammals also utilize substrate-specific adaptor proteins that, like MEL-26, have both a Cullin-binding BTB/POZ domain and another protein/protein interaction domain that binds to the substrate (ANGERS *et al.* 2006; CULLINAN *et al.* 2004; GEYER *et al.* 2003). While MEI-1/Katanin down regulation by the CUL-3/MEL-26 E3 ligase is essential at most growth temperatures, a *mel-26* null mutation is viable at the low growth temperature of 15°C (LU and MAINS 2007). This bypass of *mel-26* at 15°C depends at least in part on the anaphase promoting complex and its targeting of MEI-1 for proteolytic degradation (LU and MAINS 2007). Phosphorylation by the kinase MBK-2 primes MEI-1 for proteolysis (QUINTIN *et al.* 2003; STITZEL *et al.* 2007), and also promotes the down-regulation of MEI-1 by the anaphase promoting complex (LU and MAINS 2007).

CUL-3 is the only *C. elegans* Cullin thus far identified that requires modification by the ubiquitin-like protein Nedd8 (BOWERMAN and KURZ 2006) In contrast, *C. elegans* CUL-2 is required for progression through meiosis, and for the localized degradation of cell fate determinants in one-cell stage embryos, but neddylation-defective mutants do not exhibit these early defects (BOWERMAN and KURZ 2006). The neddylation of CUL-3 is mediated by a Nedd8 protein conjugation pathway. It begins with a heterodimeric E1 activating enzyme consisting of ULA-1 and RFL-1 (Uba3p in budding yeast), and also includes the E2 conjugating enzyme UBC-12 (JONES and CANDIDO 2000; KURZ *et al.* 2002; SRAYKO *et al.* 2000) and the E3 ligase DCN-1 (KURZ *et al.* 2005).

The down-regulation of MEI-1/katanin by the CUL-3/MEL-26 E3 ligase requires a balance of both CUL-3 neddylation, mediated by the Nedd8 conjugation pathway, and de-neddylation, mediated by the highly conserved COP-9 Signalosome (PINTARD *et al.* 2003a). Other Cullin based E3 ubiquitin ligases also require a balance of neddylation and de-neddylation (BORNSTEIN *et al.* 2006; HETFELD *et al.* 2008; LYAPINA *et al.* 2001; SCHWECHHEIMER *et al.* 2001). De-neddylation may modulate activation of the E3 ligase and thereby prevent the premature degradation of substrate adaptor proteins that also can become poly-ubiquitylated and degraded as a result of E3 ligase function.

To identify additional factors that influence neddylation and the down regulation of MEI-1/katanin after the completion of meiosis in *C. elegans*, we report here our use of RNA interference (RNAi) to reduce gene functions in a temperature-sensitive neddylation-defective mutant, *rfl-1(or198ts).* The discovery of RNAi and its systemic properties in *C. elegans* have made it possible to systematically target *C. elegans* genes

for depletion by feeding worms bacterial strains that express double-strand RNAs corresponding to *C. elegans* gene sequences (BAUGH *et al.* 2005; FEINBERG and HUNTER 2003; FIRE *et al.* 1998; LEHNER *et al.* 2006; TIMMONS *et al.* 2001; VAN HAAFTEN *et al.* 2006). Furthermore, chemical mutagenesis screens have identified temperature-sensitive mutations in many essential *C. elegans* genes, providing opportunities to sensitize genetic backgrounds for synthetic screens by choosing intermediate growth temperatures that sensitize the genetic background and also optimize quantitative scoring of embryonic viability. Recently, genome-wide RNAi screens have been used to identify *C. elegans* genes that, when reduced in function, restore viability to temperature-sensitive embryonic-lethal mutants (LABBE *et al.* 2006; O'ROURKE *et al.* 2007). As a loss of suppressor function restores mutant viability, the suppressors may negatively regulate either the wild-type gene product or the process that requires the wild-type gene product.

Here we report our identification of 21 *C. elegans* genes that, when reduced in function by feeding RNAi, reproducibly suppressed *rfl-1(or198ts)* embryonic lethality and 32 genes that enhanced *rfl-1(or198ts)* lethality. Most of the suppressors were specific for *rfl-1(or198ts)*: reducing their function did not suppress embryonic lethality associated with temperature sensitive mutations in four other essential genes. In contrast, specific enhancement was less common. Finally, many of the *rfl-1*-specific suppressors and enhancers are conserved but appear to be non-essential.

MATERIALS AND METHODS

***C. elegans* strains and culture:** Strains were cultured according to standard procedures (BRENNER 1974). Temperature-sensitive mutants were maintained at 15°C, GFP-expressing strains were maintained at room temperature. Isolation of transgenic worms was performed with the microparticle bombardment method as previously described (O'ROURKE *et al.* 2007; PRAITIS 2006).

RNAi screening and quantification of embryonic viability: Methods used for RNAi screening and quantification of embryonic viability in this study as those described in detail in O'ROURKE et al. 2007, with the following modifications. For scoring enhancement, genes that previously had been identified as lethal when targeted by RNAi, as reported in large-scale screens, were not given candidate enhancer status (KAMATH and AHRINGER 2003; SIMMER *et al.* 2003) To quantify embryonic viability of embryos at 23.5°C, the broods of 7-10 gravid adult worms were analyzed. For all quantitative analysis, experiments were repeated at least four times and the average viability determined. For enhancement, we report the percentage of dead embryos (we calculated this percentage for each replicate and then took the average of the replicates). For suppression, we report the percentage of viable larvae, calculated again by taking the average of the replicates. The total number of progeny counted, percent viability or lethality and standard deviations used to generate Figure 3 and data for wildtype (N2 strain) embryonic viability are included in Supplemental Table 2.

Molecular biology: For all *pie-1* driven, N-terminal GFP constructs (specifically, the *rfl-1* suppressors), genes were amplified using Pfu Turbo polymerase (Stratagene) from a cDNA library (Invitrogen), with the exception of R10D12.14, which was amplified from N2 genomic DNA. PCR products were subsequently ligated into pGEM-T or pGEM-T-easy shuttle vectors (Promega). Inserted genes were sequenced in the University of Oregon sequencing facility prior to cleavage and ligation into pSO26 (pSO26 described in O'ROURKE *et al.* 2007).

To construct N-terminal GFP and tdTomato (SHANER *et al.* 2004) fusions for *sel-10*, C24D10.1 and *rfl-1*, standard recombineering methods were used as described in protocol #3, available at (http://recombineering.ncifcrf.gov/Protocol.asp) as previously described (WARMING *et al.* 2005). We used the following fosmid clones available from GeneService Limited (http://www.geneservice.co.uk): WRM0610aC12 (*sel-10*) WRM0633dC (C24D10.1) and WRM066dF09 (*rfl-1*). We used pSO26 as a template for amplifying GFP (O'ROURKE *et al.* 2007). For the gap repair step we used pPUB (SAROV *et al.* 2006), and designed primers to allow for inclusion of DNA sequence up to the next open reading frame (start or stop codon) of the gene of interest. To construct the N-terminal tdTomato::RFL-1 fusion we used the pAA64 Vector to amplify the fluorescent protein-encoding gene. The detailed recombineering protocol for generating *C. elegans* fusion proteins will be described in a subsequent publication; details are available upon request.

GFP imaging: Imaging of GFP and tdTomato fusion protein localization was done by mounting embryos or whole worms on M9 + 3% agarose pads on microscope slides and overlayed with a coverslip. Time-lapse videos were obtained on a spinning disk Leica DMI 4000B microscope using a Leica 63X/1.40-0.60 HCX Plan Apo oil objective, fitted with a Hamamatsu EM-CCD Digital Camera. Images in FIGURE 6 are projected stacks that include optical sections through the entire embryo or worm at spacing of 0.5μM for embryos and 1μM for worms. Images of GFP::C24D10.1 in FIGURE 6 D-F were taken with a Leica 40X/1.25-0.75 Plan Apo oil objective and all other images were taken with the Leica 63X objective described above. Data was recorded using Velocity software and videos and images were adjusted for contrast in ImageJ and adjusted for levels with Adobe Photoshop. ImageJ was used to obtain pixel intensities of GFP::RFL-1 in nuclei of unenhanced images (Supplemental FIGURE 1). Measurements were taken by selecting a circular area slightly smaller than the nuclei and obtaining average intensity values for each nucleus.

DIC imaging: Cellular phenotypic analysis for suppression was performed by incubating *rfl-1(or198ts)* L1 stage larvae at 20°C, followed by a shift to 23°C once the larvae reached the L4 stage (~60hrs at 20°C). The incubation of *rfl-1(or198ts)* mutants at 20°C through L4 stage allowed for slightly increased brood size and a decrease in the number of worms displaying a sterile phenotype. To image the embryos at a specific temperature we utilized a temperature controlled microscope stage, equipped with an HEC-400 Heat Exchanger (20/20 Technology) and a BC-110 Bionomic Controller (20/20 Technology).

To calibrate the apparatus we used an Omega HH12 temperature probe to acquire the temperature of a M9 + 3% agarose pad placed on a sapphire inset metallic microscope slide (20/20 Technology), while on the microscope stage with the light source turned on. We adjusted the Bionomic controller until a temperature of 23°C was achieved. Use of the temperature-controlled stage prevented us from achieving optimal focus of the condenser and therefore we were unable to obtain high contrast DIC images, although image quality was sufficient for analysis of phenotypes.

Following incubation at 23°C for 12 hrs embryos were mounted on the sapphire crystal inset/metal microscope slides and immediately transferred to the microscope stage Images were recorded every 5 s using a Dage MT1 VE1000 digital camera and Scion Image or ImageJ software. Contrast and levels were adjusted using Adobe Photoshop. Spindle angle measurements were done using the ImageJ angle tool. Spindle angles at cytokinesis were measured one minute (12 frames) following first appearance of a cytokinetic furrow. The same procedure was used to image embryos for enhancement of cellular phenotypes except incubation of mutant animals was done at a constant temperature throughout development as brood size was not significantly reduced at these less restrictive temperatures (17°C, 18°C or 20°C). The microscope stage temperature was calibrated to these lower temperatures for imaging as described above.

RESULTS

A sensitized genetic background: To identify semi-permissive temperatures that optimized our ability to identify enhancers and suppressors of *rfl-1(or198ts)* embryonic

lethality, we examined the viability of embryos produced by homozygous *rfl-1(or198ts)* hermaphrodite worms raised to adulthood at six different temperatures ranging from 15°C to 25°C (Figure1; see Materials and Methods). We chose 18°C (86.7% hatching) to screen for enhancers of embryonic lethality and 23.5°C (12.7% hatching) to screen for suppressors. These growth conditions optimized our ability to detect modifiers by initially scoring qualitatively for changes in embryonic lethality, using stereomicroscopes and 48-well agar plates with worm cultures (see Materials and Methods). At temperatures higher than 23.5°C (24°C and above), most *rfl-1(or198ts)* larvae matured into either sterile or fertile adults with ruptured vulvae and produced very few or no progeny (data not shown), precluding any use of higher temperatures to achieve a lower background level of embryonic hatching for suppressor screening.

Quantifying Embryonic Viability to Identify Reproducible Enhancers and Suppressors: To screen for modifiers, we used an RNAi feeding library of 16,757 bacterial strains, each capable of inducibly expressing double-stranded RNA corresponding to *C. elegans* gene sequences [some E. coli produce dsRNAs that correspond to two genes due to incorrect gene annotation] (KAMATH and AHRINGER 2003). We screened a total of 14,045 genes for suppression and 8,192 genes for enhancement of *rfl-1(or198ts)* embryonic lethality, after the mutant larvae matured into adults while feeding on the dsRNA-expressing bacterial strains at the semi-permissive temperatures. Our initial qualitative screen yielded 248 candidate enhancers and 388 candidate suppressors. We then consolidated and systematically re-screened each

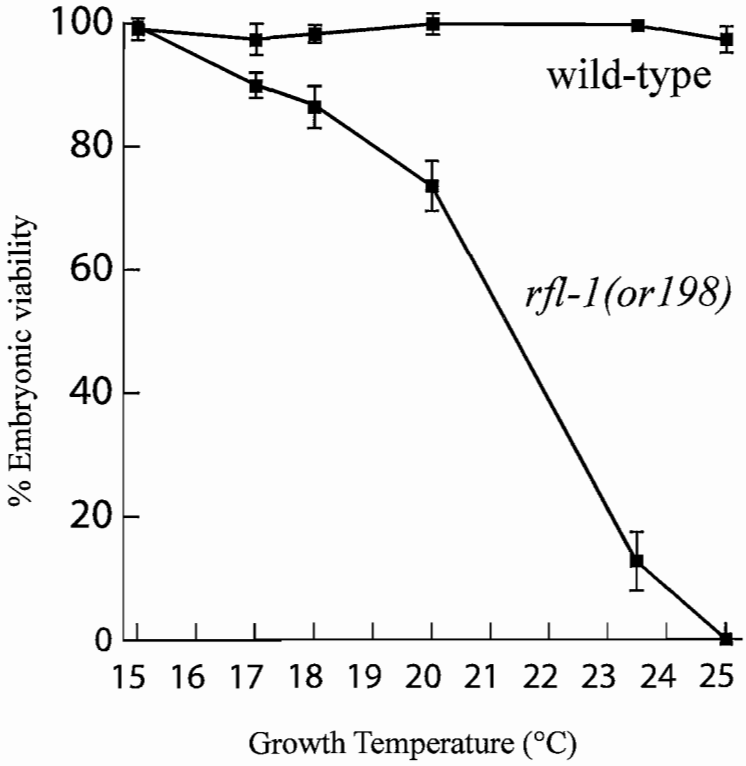

Figure 1.- Temperature versus embryonic viability for *rfl-1(or198ts)* and wildtype (N2). L1 larvae were raised to adulthood at the indicated temperatures on L4440 empty-vector control bacteria. Progeny were scored as hatched larvae or dead embryos (n>200 except for 25°C where n=7 due to sterile phenotypes at this temperature; see Methods).

candidate enhancer and suppressor dsRNA using the same qualitative scoring method, reducing the number of candidate enhancers and suppressors to 101 and 104, respectively (see O'Rourke et al 2007 for a description of the qualitative screening method).

To quantify the suppression and enhancement, we compared the embryonic viability of broods from enhanced or suppressed young adults, and from control young adults raised to adulthood on bacteria carrying the feeding RNAi vector without an insert (hereafter referred to as empty vector control broods). Thirty-two of the candidate enhancer genes, when reduced in function by RNAi, increased embryonic lethality in the broods of *rfl-1(or198ts)* mutants by at least 1.8 fold at 18°C, compared to empty vector control broods (Figure 2A). To verify that depletion of these enhancer loci themselves did not cause embryonic lethality, we depleted each of these 32 genes using feeding RNAi and wild-type worms to quantify embryonic viability. None of the enhancer genes were strongly required for embryonic viability under our conditions, with 5.7% embryonic lethality being the most penetrant essential requirement we observed. We also tested each of the 32 enhancers with *rfl-1(or198ts)* mutants raised at 17°C, at which temperature 90% (SD +/- 4%) of un-enhanced mutant embryos hatched. We found that fourteen of the 32 enhancers still increased embryonic lethality by two-fold or more at 17°C (Figure 2B). We limited further analysis to these fourteen enhancers that reproducibly acted at both semi-permissive temperatures (See Supplemental Table 1 Appendix A1 for modifier gene identities).

A similar quantitative brood analysis identified 21 suppressors that, when reduced in function by RNAi, consistently increased the viability of embryos from

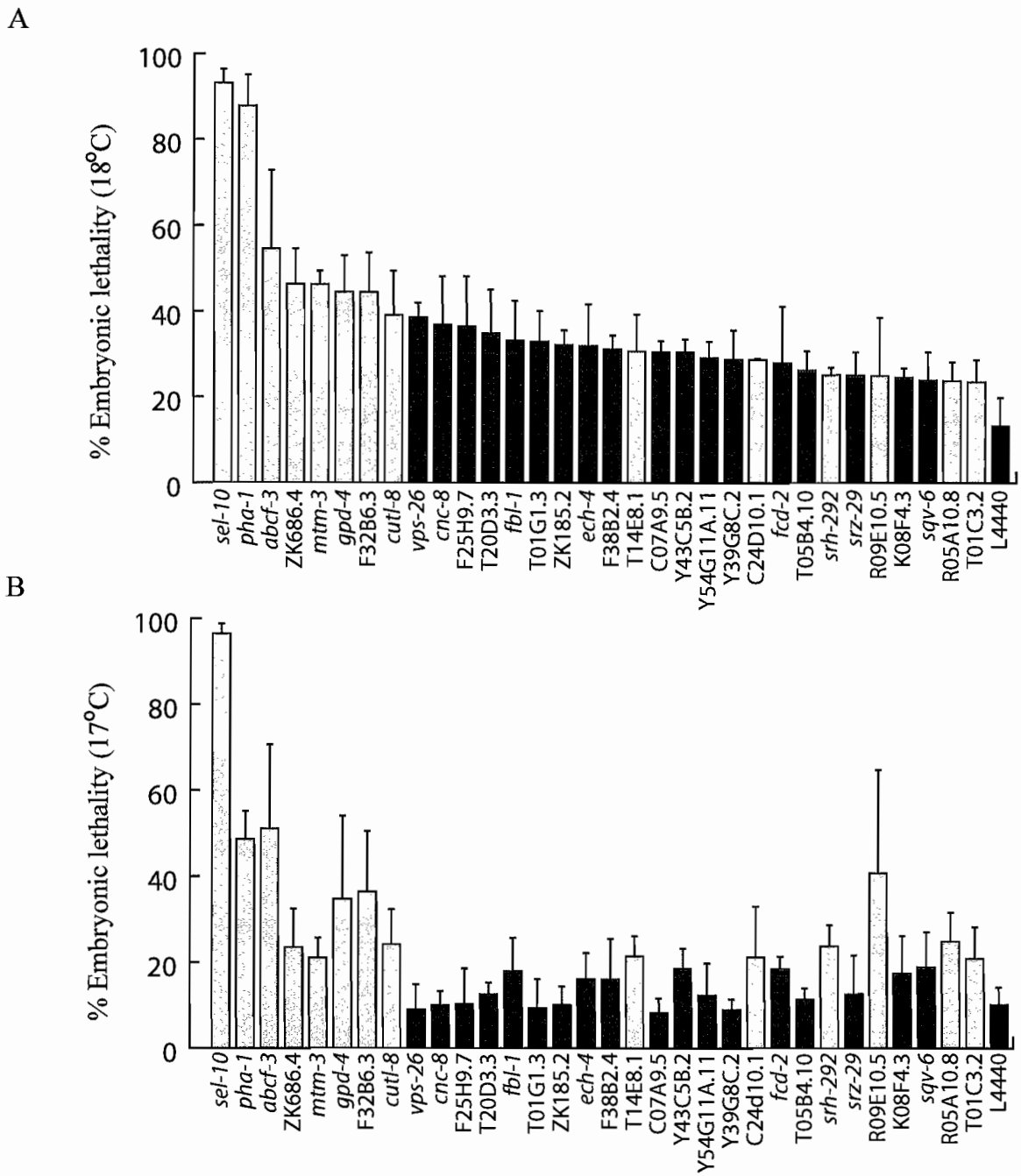

Figure 2.-Enhancement of *rfl-1(or198ts)* embryonic lethality. L1 larvae were raised to adulthood at (A) 18˚C and (B) 17˚C, with RNAi-mediated depletion of 32 candidate enhancer genes. L4440 empty-vector negative controls are at far right. Light bars indicate loci that enhance embryonic lethality by at least 2-fold at 17°C (these genes were selected for further analysis). Error bars indicate one standard deviation.

hermaphrodites raised at 23.5°C by 1.8-fold or more, compared to worms raised on the empty vector control *E. coli* (See Appendix for gene identities) . Depletion of thirteen of the suppressors restored viability to over 38% hatching (3-fold over the control); depletion of the strongest suppressor, *csn-5*, restored viability to 90% hatching.

Specificity of suppressors and enhancers: To test whether the modifiers we identified specifically influence *rfl-1* function, or if depleting them can non-specifically influence multiple conditionally mutant loci, we used four different temperature-sensitive embryonic-lethal mutants that to our knowledge are not defective in functions related to *rfl-1* or ubiquitin-mediated proteolysis: *lit-1(or131ts)*, *spn-4(or191ts)*, *dhc-1(or195ts)* and *spd-5(or213ts)*. The *lit-1* gene encodes a MAP Kinase that modulates Wnt signaling (MENEGHINI *et al.* 1999); *spn-4* encodes a protein with an RNA binding motif that regulates cell fate patterning in the early embryo (GOMES et al. 2001); *dhc-1* encodes the heavy chain of the minus-end-directed microtubule motor dynein (HAMILL et al. 2002); and *spd-5* encodes a coiled-coil protein required for centrosome maturation (HAMILL et al. 2002). For each of these mutants we used growth temperatures that gave nearly complete embryonic viability for testing enhancement, or nearly complete embryonic lethality for testing suppression (O'ROURKE *et al.* 2007).We quantified the effects of depleting modifier genes on embryonic viability with these four additional mutants and compared the results to those obtained with *rfl-1(or198ts)* (Figure 3).

We found that while enhancers were often non-specific, most of the suppressors were specific for *rfl-1*. Reducing the function of eight of the fourteen enhancers increased

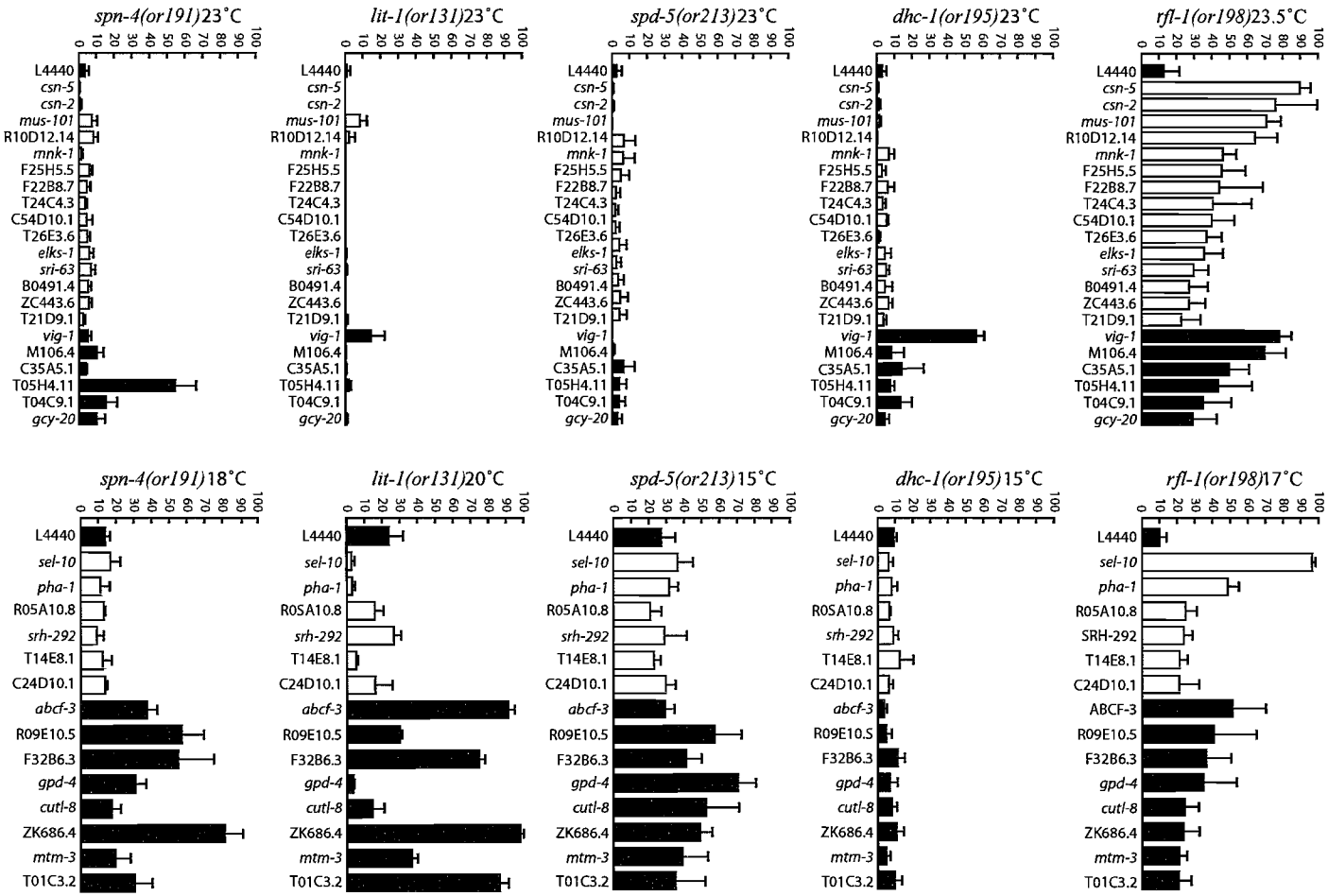

Figure. 3- Specificity of modifiers for *rfl 1(or198ts)* embryonic lethality. Percent embryonic viability (hatching) for suppression is shown on the left; percent embryonic lethality for enhancement is shown on the right. Identity of conditionally lethal mutations and growth temperatures are shown at the far left. Black bars indicate non-specific modifiers; white bars indicate specific modifiers far left bar (L4440) is neg-ctrl RNAi.

embryonic lethality by at least 2-fold in one or more of the four other conditionally mutant strains. Depletions of the remaining six enhancer loci specifically increased *rfl-1(or198ts)* embryonic lethality by between 2.1 and 9.6 fold (21 and 96.4% lethality), but reducing their function did not increase embryonic lethality by two-fold or more in any of the four other conditional mutants we tested (Figure 3). In contrast, of the 21 reproducible suppressor loci, 14 were specific for *rfl-1(or198ts)*: reducing their function failed to suppress embryonic lethality by more than 2-fold in at least three of the four of unrelated conditional mutants we tested (Figure 3). All of these 14 specific suppressors failed to raise viability to 10% or more in any of the four unrelated mutants, while background viabilities on empty vector control ranged from 1.2% for *lit-1(or131ts)* as the lowest of the four, to 3.7% for *spn-4(or191ts)* as the highest.

Two of the specific and most penetrant *rfl-1(or198ts)* suppressors were *csn-2* and *csn-5*, which encode components of the COP-9 signalosome and have been shown previously to suppress *rfl-1(or198ts)* embryonic lethality when reduced in function (PINTARD *et al.* 2003a). These were the only two signalosome components included in the 14,045 gene set that we tested for suppression, confirming our ability to identify functionally important suppressors.

Modifier Depletion and Cell Division Defects in *rfl-1(or198ts)* Mutant Embryos: We next used DIC time lapse videomicroscopy to examine in live embryos the first mitotic division in both suppressed and un-suppressed *rfl-1(or198ts)* mutants. In un-suppressed embryos from *rfl-1(or198ts)* mutants grown at the fully restrictive temperature of 25°C,

the failure to degrade MEI-1/katanin leads to defects in mitotic spindle orientation, ectopic membrane furrows, and a failure to complete cytokinesis (KURZ *et al.* 2002). In un-suppressed embryos, produced by *rfl-1(or198ts)* mutant worms raised at 23°C and fed bacteria carrying the empty vector, 54% of embryos failed to complete cytokinesis, 77% had one or more ectopic cleavage furrows following cytokinesis, and the first mitotic spindle was mis-oriented on the anterior-posterior axis in comparison to the wild type (Figure 4). Depletion of the strongest suppressor, *csn-5,* almost completely rescued the cytokinesis and ectopic furrowing defects: 0% and 10%, respectively, of the embryos exhibited these defects, and the spindle orientation defect also was also suppressed (Figure 4). Depletion of the specific suppressors *mnk-1*, *mus-101*, and R10D12.14 produced less complete but still substantial suppression of these defects in early stage mutant embryos (Figure 4). We conclude that these specific suppressors influence the same early embryonic processes that require *rfl-1*, and that the suppression of these cell division defects may account at least in part for the increased viability of the suppressed mutant embryos.

The synthetic embryonic lethality caused by depletion of specific enhancers in *rfl-1(or198ts)* mutants raised at a semi-permissive temperature could result from further compromising essential processes in the early embryo that require *rfl-1*, or from deleterious interactions that occur later in development. We therefore used DIC time-lapse videomicroscopy to examine the first mitotic division in enhanced and un-enhanced mutant embryos, focusing on the two most penetrant and specific enhancer genes, *sel-10* and *pha-1*. At 17°C on empty vector RNAi-expressing bacteria, 0/15 un-enhanced *rfl-*

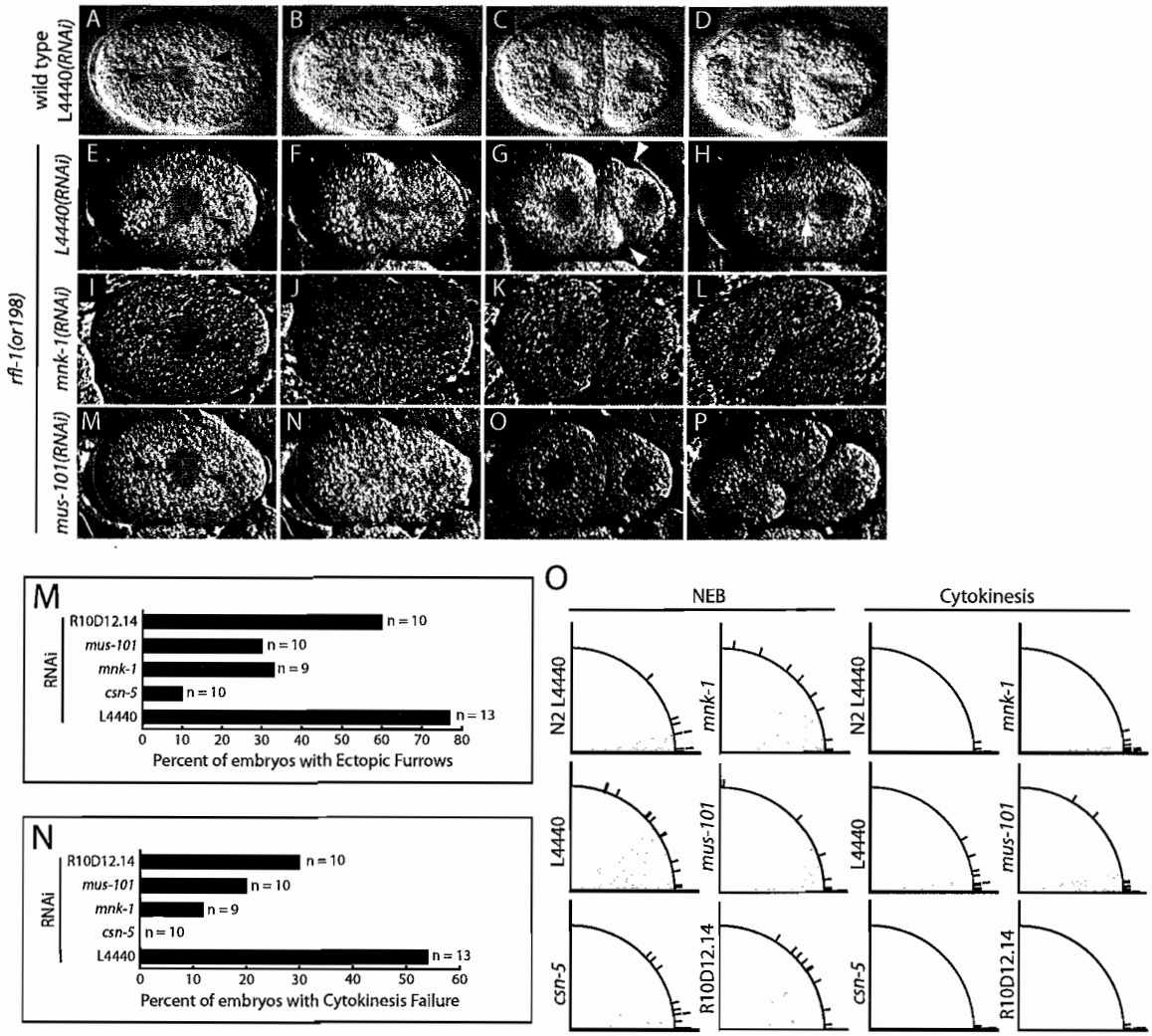

Figure 4.- Suppression of early embryonic cell division defects in *rfl-1(or198ts)* mutants raised at 20°C and then shifted to 23°C for 7-12 hours prior to imaging (see Materials and Methods). (A-L) show frames from time-lapse DIC videomicrographs at nuclear envelope breakdown (A, E, I), during cytokinesis one minute after first appearance of a cleavage furrow (B, F, J), and at the 2-cell stage after the completion of cytokinesis (C, G, K). Black arrowheads indicate centrosome position; white arrowheads indicate ectopic membrane furrows; white arrow indicates nuclei of AB cell and P1 cell advancing toward each other after cytokinesis failure; black arrow indicates regression of cytokinetic furrow. Temperature-controlled stage prevented use of optimal DIC optics (see Materials and Methods) (M) Quantification of ectopic cleavage furrows in suppressed and controls embryos. (N) Quantitation of cytokinesis failure in suppressor-RNAi treated and control RNAi treated embryos. Number of embryos scored are indicated for each condition in (M) and (N). (O) Angle of mitotic spindle at NEB and at cytokinesis (1 minute following appearance of cleavage furrow). Hash marks indicate individual data points and shading indicates mean angle of all data points.

1(or198ts) embryos exhibited cytokinesis failures, while 2/15 had ectopic cleavage furrows. At 18°C, 2/15 empty vector RNAi-fed embryos exhibited cytokinesis failure and 5/15 had ectopic furrows. While *sel-10(RNAi)* and *pha-1(RNAi)* both dramatically increase the rate of lethality at these temperatures, we observed no increase in cytokinesis failures, ectopic furrowing, or spindle orientation defects at either 17°C or 18°C (Table 1). This lack of enhancement of early cell division defects suggests that the synthetic lethal interaction(s) may occur at a later stage in embryonic development, or are not detected by our methods.

To ask whether *rfl-1* has functional requirements later in embryogenesis, beyond the first few cell divisions, we performed temperature up-shift experiments with *rfl-1(or198ts)* embryos, following the completion of early cell divisions at the permissive temperature of 15°C. After shifting 16-cell and later stage embryos to 26°C, we found that 18/42 embryos (48.2%) failed to hatch, while in a control experiment with no temperature up-shift only 3/51 embryos (5.8%) failed to hatch, indicating that *rfl-1* does have additional essential requirements later in embryonic development. We examined the terminal phenotypes of the un-hatched embryos using DIC microscopy and found that 16/18 embryos arrested after elongation to or beyond the 3 fold stage. We also used DIC microscopy to examine enhanced *rfl-1* mutant embryos produced at 18°C after *sel-10* depletion and found that 25/28 (89%) of these enhanced mutant embryos failed to hatch, and many of the unhatched embryos appeared to arrest after little if any elongation (data not shown), a more severe phenotype than we observed after up-shifts of un-enhanced *rfl-1(or198ts)* embryos to 26°C (see above). Other *rfl-1(or198ts)* embryos enhanced by *sel-*

TABLE 1

Modifier depletion does not enhancer early embryonic cell division defects in *rfl-1(or198ts)* mutants.

Genotype	# embryos with ectopic furrows	# embryos with cytokinesis failure
rfl-1(or198);empty vector RNAi 17°C	2/15	0/15
rfl-(or198); *sel-10(RNAi)* 17°C	2/11	0/11
rfl-1(or198); *pha-1(RNAi)* 17°C	2/11	0/11
rfl-1(or198); empty vector RNAi 18°C	5/15	2/15
rfl-(or198); *sel-10(RNAi)*18°C	5/13	0/13
rfl-1(or198); *pha-1(RNAi)* 18°C	3/13	1/13

10 depletion arrested after variable amount of elongation (data not shown). We conclude that the synthetic lethal interactions observed after enhancer depletion could result from interactions that occur after the early embryonic cell division processes known to require *rfl-1*. However, we have not determined precisely where or when in embryogenesis the synthetic lethal interactions occur.

Suppressor and enhancer protein localization overlap with RFL-1: To gain further insight into how the specific modifiers might influence *rfl-1* dependent processes, we first constructed N-terminal GFP fusions to *rfl-1* and to five of the specific suppressors, all driven by the maternal *pie-1* promoter, and isolated transgenic lines for all six fusions (see Methods). Previous immunocytochemistry studies of the neddylation pathway

components NED-8 and CSN-5 documented nuclear and cytoplasmic localization in fixed embryos, with a pronounced nuclear enrichment (KURZ *et al.* 2002; PINTARD *et al.* 2003a; PINTARD *et al.* 2003b). Consistent with these previous studies of Nedd8 pathway components, we observed a similar nuclear enrichment of the GFP::RFL-1 fusion protein in live embryos, using spinning disk confocal microscopy (Figure 5; see Methods). Fusion constructs for F25H5.5 (an ortholog of the human Protein CLASPIN), F22B8.7 (an uncharacterized conserved iron-sulfur domain-containing protein), T26E3.6 (related to Fibrillin), and MNK-1 (a conserved kinase) all displayed nuclear enrichment in early embryonic cells. GFP::R10D12.14 (a conserved protein of unknown function) localized more exclusively to the cytoplasm, appearing to be excluded from nuclear and mitotic spindle regions. In addition, GFP::R10D12.14 was cortically enriched near cytokinetic furrows, particularly during the division of the anterior-most two-cell stage blastomere, and at cell boundaries in oocytes (Figure 5 and data not shown). While these are very general localization patterns, most of the suppressor proteins exhibit localization patterns similar to those observed for RFL-1, NED-8 and CSN-5, consistent with the possibility that these suppressor proteins influence the neddylation pathway or the regulation of MEI-1/katanin in early embryonic cells.

We also examined the expression and localization of GFP fusions to the proteins encoded by the two most specific enhancers, SEL-10 (an F-box substrate adaptor for SCF-type E3 ligases) and C24D10.1 (an uncharacterized protein tyrosine phosphatase), using recombineering to construct GFP translational fusions regulated by native promoter and other non-coding sequences (see methods). We did not detect any expression of

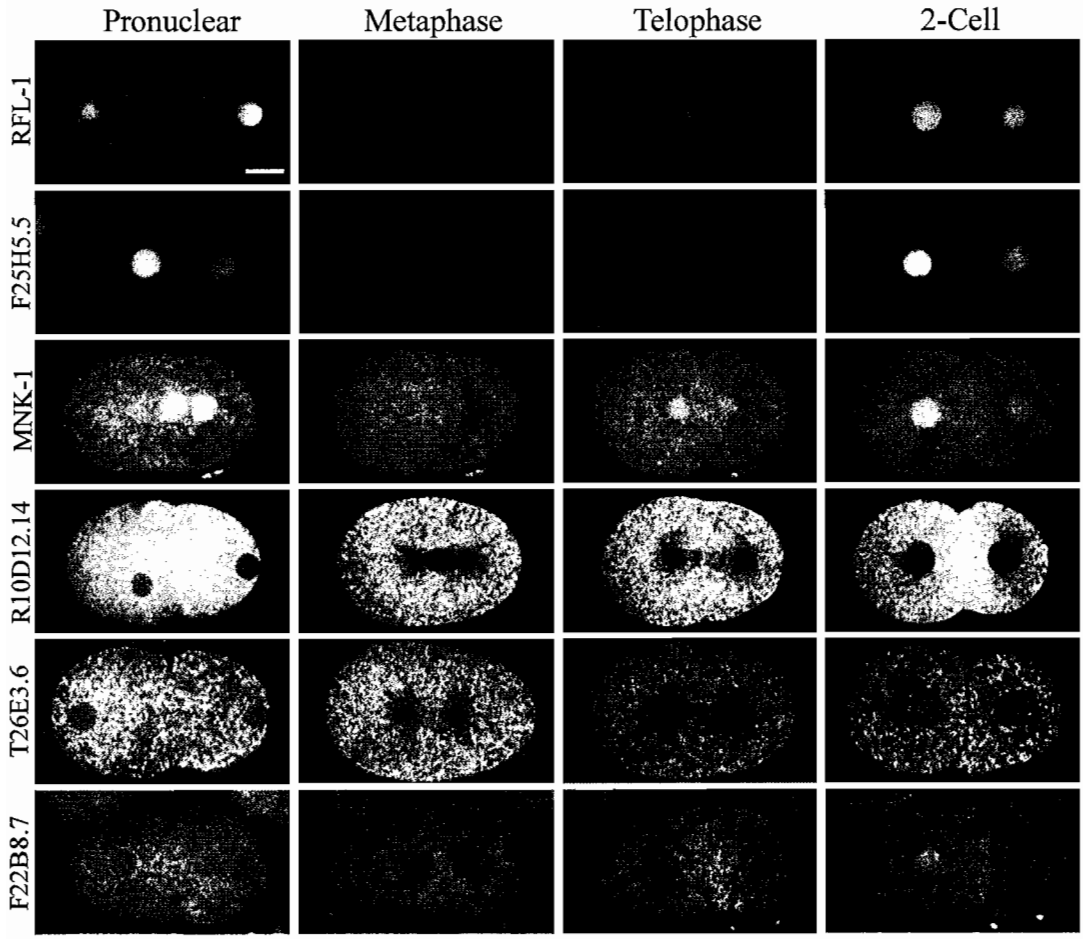

Figure 5.- Expression of GFP fusion to suppressor encoded proteins in early embryos from transgenic strains. Frames from single optical sections at indicated cell cycle stage from spinning-disk confocal time-lapse videomicrographs (see Materials and Methods). Scale bar equals 10μM.

GFP::SEL-10 in early embryos, but we did detect cytoplasmic expression in some cells beginning at about the 50-cell stage (Figure 6A). We detected higher levels of cytoplasmic expression beginning at about the bean stage (Figure 6B), after the completion of most embryonic cell divisions, in many cells throughout the embryo. In larvae and adults, we observed expression in head and tail neurons and in unidentified cells along the entire length of the body (Figure 6). For the fusion GFP::C24D10.1, we again did not detect any expression in early embryos but first detected expression beginning at approximately the bean stage of embryogenesis, predominantly in nuclei and in many cells throughout the embryo (Figure 6 D-E). In larvae and adults, we observed GFP::C24D10.1 in the nuclei of many cells throughout the head.

The lack of early embryonic expression for the two most penetrant enhancers could be due to transgene silencing in the maternal germline, a frequent outcome for maternally expressed genes in transgenic *C. elegans* strains (KELLY and FIRE 1998). However, we did detect presumably zygotic expression of the GFP fusions to these proteins in later stage embryos, and we wanted to compare these later expression patterns to RFL-1. We therefore used recombineering to produce an N-terminal-tagged tdTomato::RFL-1 fusion driven by the *rfl-1* promoter and generated a transgenic line that expresses this fusion (see Materials and Methods). We again did not observe any early embryonic expression, presumably due to germline silencing. However, we did detect strong cytoplasmic expression in larval stages and in adults, in both head and tail neurons, and in vulval epithelial cells and intestinal cells (Figure 6), consistent with previous studies of a *rfl-1::GFP* promoter fusion as a transcriptional reporter (HUNT-

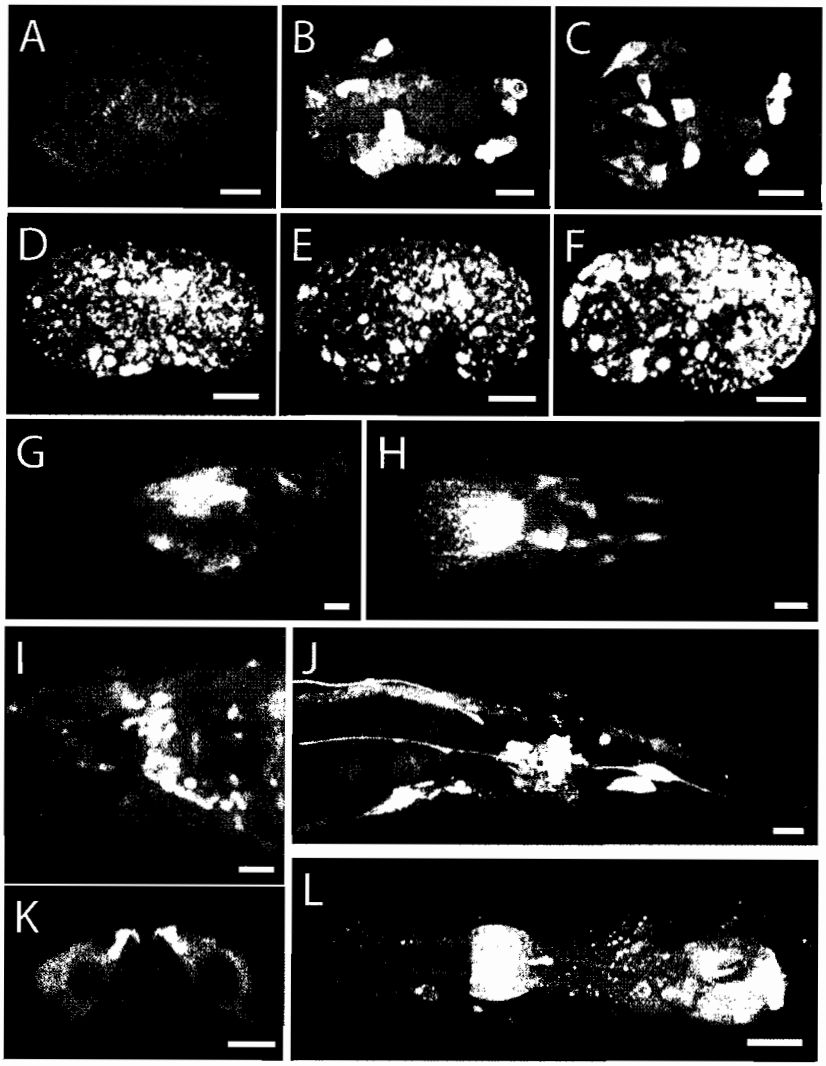

Figure 6.- Spinning disk confocal projected Z-stack images of GFP and tdTomato fusion proteins (see Materials and Methods). GFP::SEL-10 expression (A-C, G, H). (A) ~50-cell stage (B) bean stage (C) comma stage (G) expression in head of adult (H) Expression in tail neurons. GFP::C24D10.1 expression (D-F, I). Bean stage (D); comma stage (E); 1.5-fold stage (F); head of adult around pharynx (I). tdTomato::RFL-1 (J-L). (J) Tail neurons and intestine in adult stage; (K) Vulval VulB2 cells of young adult; (L) larval head. Anterior is to the left; scale bars equal 10μM.

NEWBURY *et al.* 2007). To summarize, although the two strongest specific *rfl-1(or198ts)* enhancers are expressed in later stage embryos, we did not detect any RFL-1 expression in later stage embryos. For this reason, and because we did not detect any maternal expression of the two enhancer GFP fusions, we do not know in which cells RFL-1 might interact with SEL-10 or C24D10.1 during embryogenesis, assuming such interactions are responsible for the enhanced embryonic lethality we observe. It is possible that such interactions do occur in later stage embryos but involve undetected low levels of RFL-1, or maternal expression of the two enhancers. Finally, while functional requirements for RFL-1 in larvae and adults remain uncharacterized, the expression of RFL-1 in vulval epithelial cells may relate to the frequent rupture at the vulva in young adult hermaphrodites raised at restrictive temperatures (data not shown).

To further investigate the relationship of the RFL-1 modifiers we have identified, we asked if depletion of the suppressor gene products can alter the localization of GFP::RFL-1, and if depletion of *rfl-1* or other neddylation pathway components can change the localization of the GFP suppressor protein fusions in early embryonic cells. We did not detect changes in localization for any of the suppressor fusions following *rfl-1*, *nedd-8* and *ula-1* depletions (data not shown). Similarly no change in GFP::RFL-1 localization was observed upon depletion of the suppressor genes *mus-101*, R10D12.14, *mnk-1*, F25H5.5, T26E3.6 or F22B8.7 (data not shown). We also did not observe any change in early embryonic GFP::RFL-1 localization upon depletion of *sel-10*, *pha-1*, or C24D10.1, a result consistent with our finding that *sel-10* and *pha-1* depletion do not enhance the cellular phenotypes in *rfl-1* mutants.

Finally, we also examined GFP::RFL-1 expression in transgenic embryos after depleting the neddylation pathway components *ula-1*, *nedd-8,* and *ubc-12* (Figure 7). We did not observe changes in RFL-1 localization after depletion either *nedd-8* or *ubc-12*, but we did observe a loss of GFP::RFL-1 nuclear enrichment following *ula-1* depletion. ULA-1 and RFL-1/UBA-3 form the E1 activating enzyme complex for the Nedd8 conjugation pathway, with RFL-1 being the catalytic AAA-ATPase subunit (JONES and CANDIDO 2000). A comparison of pixel intensities showed a nuclear/cytoplasmic ratio of 1.01 after *ula-1* depletion, compared to 1.49 in wild-type embryos (n = 6 embryos for each analysis and then averaged; see Material and Methods) Using western blots, we detected similar levels of GFP::RFL-1 in extracts from ULA-1 depleted worms and wild-type worms (data not shown), suggesting that ULA-1 is required for RFL-1 nuclear enrichment independent of any changes in protein levels. To our knowledge, such a requirement for ULA-1 orthologs has not been reported in other organisms.

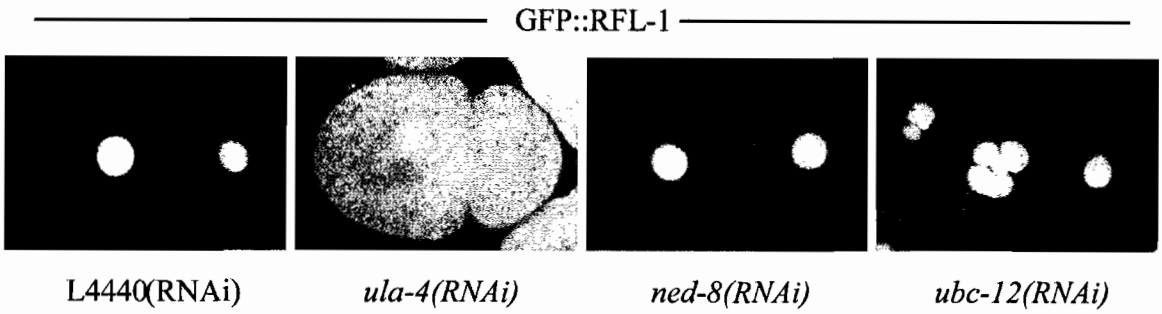

Figure 7.- Nuclear localization of GFP::RFL-1 is lost in *ula-1*(RNAi) but not in other neddylation pathway defective backgrounds.

DISCUSSION

To identify factors that influence the regulation of cell division in the early *C. elegans* embryo, we used a high throughput RNAi screen to identify suppressors and enhancers of the embryonic lethality associated with *or198ts*, a temperature-sensitive, embryonic-lethal mutation in the *C. elegans* gene *rfl-1*, which encodes a component of the ubiquitin-like Nedd8 protein conjugation pathway. Using ts mutations in other essential loci, we tested the modifiers to determine if they were specific for *rfl-1(or198ts)* embryonic lethality. We found that many of the enhancers and suppressors were non-specific, with specific suppressors being more common than specific enhancers. Depletion of the *rfl-1*-specific suppressors partially rescued the embryonic cell division defects associated with loss of *rfl-1* function, and similar sub-cellular distributions of the suppressor proteins and RFL-1 are consistent with the suppressors having RFL-1-related

roles in early embryonic cell division. We also identified two highly penetrant and specific enhancers of *rfl-1(or198ts)* embryonic lethality, but their depletion did not detectably enhance early embryonic cell division defects in mutant embryos. We speculate that *rfl-1* may have roles later in development that can explain the synthetic lethality that results when these enhancers are depleted in *rfl-1(or198ts)* mutants raised at a semi-permissive temperature.

Synthetic screening with RNAi and temperature-sensitive, embryonic-lethal *C. elegans* mutants--controlling for specificity: While some large-scale synthetic screens using RNAi and temperature-sensitive *C. elegans* mutants have addressed the issue of modifier specificity (FRASER 2004; LEHNER *et al.* 2006; O'ROURKE *et al.* 2007), relatively few such screens have been reported thus far. Moreover, this study represents the first time that a systematic and quantitative analysis of specificity has been reported for both enhancers and suppressors of a single embryonic-lethal *C. elegans* mutant.

In our analysis of the modifiers, we found that many of the enhancers were non-specific, while more suppressors were specific for *rfl-1*. Of the 21 suppressors 71% were specific: their depletion did not restore embryonic viability to other conditionally embryonic-lethal mutants grown at semi-permissive temperatures. In contrast, only 43% of enhancers were specific, and only 14% (2 of 14) strongly enhanced embryonic lethality. Enhancers we identified in modifier screens with other conditional mutants also exhibit, with very few exceptions, a high degree of non-specificity when tested with multiple conditionally lethal mutants (M.D. and S.O., unpublished data). We conclude

that when using RNAi to reduce gene function in temperature-sensitive, embryonic-lethal *C. elegans* mutant backgrounds, synthetic embryonic lethality most often results from additive and unrelated defects and probably does not result from the two genes influencing the same biological process or pathway. While genes that enhance embryonic lethality when depleted in more than one mutant background could represent functional links among the genes mutated in the different backgrounds, we suspect that most such synthetic-lethal interactions instead involve un-related defects.

Synthetic lethal screens in budding yeast have been powerful tools for the discovery of new gene functions (OOI *et al.* 2006; TONG *et al.* 2004), and it is interesting to compare the specificity observed in synthetic lethal screens done in *C. elegans* with the specificity observed in synthetic knockout screens done in budding yeast. For example, one yeast study analyzed 132 nonessential genes (TONG *et al.* 2004). Each of these 132 viable knockout mutants was mated with ~4700 additional viable knockout mutants to produce double mutants. A total of ~4000 synthetic lethal interactions were identified, with an average of 34 interactions per mutant. Of the interactions found, 27% were with pairs of genes known to be in the same or related genetic pathways. These findings suggest a high degree of specificity for synthetic lethal interactions in yeast, and that the interactions reflect participation in a common process. By contrast, one study in *C. elegans* found that over half of the genes on chromosome III, when reduced in function by feeding RNAi, significantly enhanced the embryonic lethality of a hypomorphic mutation in the transcription factor gene *dpl-1* (FRASER 2004). Similarly, we found that many of the strong enhancers, when depleted by RNAi, enhanced embryonic lethality

with multiple conditional mutants. This high degree of non-specificity compared to the synthetic lethal screens in budding yeast could reflect the added genetic complexity of the cellular and developmental processes in a multicellular organism. Alternatively, the different degrees of specificity may simply reflect the different kinds of mutant alleles used for screening. The yeast synthetic-lethal screens used double mutants made with viable deletion alleles. In contrast, the synthetic screens in *C. elegans* have used partial inactivation of essential genes, usually by growing temperature-sensitive mutants at intermediate temperatures to sensitize genetic backgrounds, and RNAi to deplete the expression of other genes that may or may not themselves be essential. Perhaps temperature-sensitive mutants grown at just-viable temperatures are particularly vulnerable to non-specific, synthetic lethality caused by the disruption of unrelated processes. It would be interesting to use genome-wide feeding RNAi screens to detect synthetic lethality in *C. elegans* strains that are homozygous for deletion mutations in non-essential genes. Conversely, it would be interesting to know if temperature-sensitive mutations in essential budding yeast genes are also prone to high levels of non-specific enhancement and suppression when other genes are reduced in function.

While non-specific enhancement of embryonic lethality is especially common, suppression of embryonic lethality by RNAi knockdown of other *C. elegans* loci also is frequently non-specific. In a genome-wide RNAi screen for suppressors of a temperature-sensitive dynein heavy chain mutant, *dhc-1(or195ts)*, 49 genes were identified that, when depleted by feeding RNA, restored *dhc-1(or195ts)* embryonic viability by 3-fold or more (O'ROURKE *et al.* 2007). However, depletion of 57% of the *dhc-1(or195ts)* suppressors

also significantly suppressed embryonic lethality for at least one of two other mutants with temperature-sensitive mutations in essential loci unrelated in function to *dhc-1*. As we report here, 29% of the *rfl-1(or198ts)* suppressors were similarly non-specific. For at least some of the non-specific *dhc-1(or195ts)* suppressor genes, their depletion has been reported by others to increase glycerol production, which may non-specifically stabilize temperature-sensitive proteins and thereby increase embryonic viability at semi-permissive temperatures (LAMITINA *et al.* 2006; O'ROURKE *et al.* 2007). Clearly, it is important to carefully assess the specificity of both enhancer and suppressor interactions that influence the degree of lethality associated with temperature-sensitive mutations in essential *C. elegans* genes. Many interactions are highly non-specific and presumably will not provide mechanistic insight into the conditionally defective biological process.

Known roles for specific modifier genes: One of the strongest specific suppressors, *mus-101*, encodes a widely conserved 1227 amino acid protein containing six copies of the BRCA1 carboxyl-terminal (BRCT) repeat (HOLWAY *et al.* 2005; YAMAMOTO *et al.* 2000). The *mus-101* gene is essential but feeding RNAi results in only partially penetrant embryonic lethality (MAEDA *et al.* 2001). BRCT domains are commonly found in proteins involved in DNA metabolism, and have been implicated in mediating protein-protein interactions between other BRCT domain-containing proteins (MANKE *et al.* 2003; YU *et al.* 2003). Some proteins with BRCT repeats, including BRCA1, have roles in mitosis as well as more established roles in DNA replication and repair. For example, BRCA1 binds to tubulin and localizes to centrosomes and spindle microtubules (Hsu and White, 1998), and high levels of BRCA1 are maintained throughout mitosis, while the

protein is ubiquitinated and degraded during G1 and S phase (Choudhury et al., 2004). Furthermore, in both mammalian and Xenopus cells, BRCA1 is required for spindle pole assembly and the centrosomal accumulation of TPX2 (JOUKOV *et al.* 2006). Perhaps the BRCA repeat protein encoded *mus-101* may also influence cell division functions such that it can restore viability when depleted in *rfl-1* mutants.

The other *rfl-1* suppressor with a possible link from other studies to cell division is R10D12.14. This gene is at least partially essential (PIANO *et al.* 2002), but its specific cellular requirements remain unknown. R10D12.14 encodes a protein with a GYF domain (poly-proline interaction motif) and was identified as a binding partner of Dynein Light Chain (DLC-1) in a large scale two-hybrid interaction screen of metazoan-specific genes from *C. elegans*; DLC-1 was the only binding partner of R10D12.14 identified in this study (LI *et al.* 2004). While the link to dynein might reflect roles in cell division, the GFP::R10D12.14 fusion protein we examined was largely excluded from nuclei and mitotic spindles and was mostly cytoplasmic, with some cortical enrichment at cytokinesis furrows and in oocytes.

The two strongest specific enhancer genes were *sel-10* and *pha-1*. The *sel-10* gene encodes an F-box protein and has been implicated as a substrate adaptor in E3 ligases that control the ubiquitin-mediated degradation of LIN-12/Notch, SEL-12/Presenilin, and the sex-determining proteins FEM-1 and FEM-3 (HUBBARD *et al.* 1997; JAGER *et al.* 2004; WU *et al.* 2001). There are no identified requirements for Cullin neddylation in the ubiquitin-mediated degradation of SEL-10 targets, but our finding of a strong and highly

specific enhancement of embryonic lethality raises this possibility. The *pha-1* gene encodes a novel protein that has been shown to function redundantly with class B SynMuv genes, such as lin-35/Rb and *efl-1*, to influence pharyngeal morphogenesis (FAY *et al.* 2004). Furthermore, reducing the function of both *pha-1* and any one of four ubiquitin ligases —*ubc-18*, C27A12.6, C27A12.7, *ari-1*—results in pharyngeal morphogenesis defects and partially penetrant early larval lethality (QIU and FAY 2006). ARI-1 has been shown to interact with CSN-5 by yeast two-hybrid screening, and inactivation of *csn-5* also enhances pharyngeal defects in some mutant backgrounds (D. Fay, personal communication). Our results suggest that neddylation may be important for proper proteolytic regulation of *pha-1* and/or *lin-35*/Rb dependent processes.

Identifying requirements for non-essential genes: While mutational studies and genome-wide RNAi screens in *C. elegans* have identified thousands of genes that are either essential or have visible requirements, roughly 70% of the known and predicted genes in the *C. elegans* still have no known requirements (KEMPHUES 2005) even though at least half have homologs in other animal phyla (LANDER *et al.* 2001). Understanding the function of conserved but non-essential genes is an important frontier of genetic research in model organisms, as many such genes are widely conserved and could be relevant to human disease processes. Moreover, non-essential genes that influence essential processes may prove to be valuable drug targets: altering their function may allow for modification of an essential process without causing deleterious side effects that might result from targeting a more pleiotropic and essential disease gene. In our screen for modifiers of the essential *C. elegans* gene *rfl-1*, 11 of the 16 specific

suppressors and all 6 of the specific enhancers we identified appear to be non-essential. Thus when appropriately controlled for specificity, modifier screens that use RNAi and conditional mutations in essential *C. elegans* genes may prove useful in identifying roles for non-essential but conserved genes.

CHAPTER IV

NOVEL REQUIREMENTS FOR THE GENES Y119C1A.1 AND *DRH-3* IN WNT/MAPK PATHWAYS AND ENDODERM SPECIFICATION IN *C. ELEGANS*

The screening procedure utilized to identify genetic modifiers of *lit-1(or131ts)* was developed primarily by Sean O'Rourke, Jose Gomez and myself. The identification of *lit-1(or131ts)* modifier genes and subsequent characterization analysis described in this chapter, was performed entirely myself.

INTRODUCTION

Wnt signal transduction pathways utilize highly conserved molecular components and mechanisms that play fundamental roles in embryonic development, tissue regeneration, cell polarity and cancer (CADIGAN and NUSSE 1997). In *C. elegans*, both a Wnt signaling pathway and a MAPK pathway are necessary for the posterior versus anterior sister cell-fate specification in developing embryos (MENEGHINI *et al.* 1999). These pathways are first utilized in the four-cell stage embryo where a MOM-2/Wnt signal from the P_2 blastomere signals anteriorly to the adjacent EMS cell, ultimately leading to its polarization and subsequent asymmetric cell division. The daughters of this cell division, MS and E, go on to become the mesoderm and endoderm, respectively (ROCHELEAU *et al.* 1997; ROCHELEAU *et al.* 1999; THORPE *et al.* 1997).

The Wnt and MAPK signaling pathways required for EMS cell polarization have been studied extensively and genetic and molecular characterization have identified many components of each pathway. One of the first components to be identified was the gene *pop-1*, a member of the TCF/LEF family of transcription factors. In *pop-1* mutants both daughters of the EMS cell adopt the MS cell fate. In wildtype embryos, lower levels of nuclear localized POP-1 are found in E (posterior cell) relative to MS. This relative difference does not appear to be due to POP-1 degradation in the E cell, but appears to be the result of a redistribution of nuclear POP-1 to cytoplasmic POP-1 in the E blastomere (MADURO *et al.* 2002). Together, these results support the model that unlike other TCF/LEF proteins in other Wnt pathways, that serve to activate transcription of target genes, POP-1 acts as a transcriptional repressor of MS cell fate genes. However, a recent study has revealed that remaining POP-1 protein in E cell nuclei acts as a transcriptional activator when bound to the β-catenin homolog SYS-1 (KIDD *et al.* 2005). Many other components of the Wnt and MAPK pathways were first identified as being required for the E cell adopting the endoderm fate, and were later shown to be required for the lower levels of nuclear POP-1 in the E cell. These genes were named for their shared mutant phenotypes: MOM (more-of-mesoderm) and LIT (loss-of-intestine).

Wnt signaling is induced by Wnt/MOM-2 (ROCHELEAU *et al.* 1997; THORPE *et al.* 1997) binding the receptor MOM-5/Frizzled (ROCHELEAU *et al.* 1997; THORPE *et al.* 1997) on the EMS cell which activates the downstream cytoplasmic components SGG-1/GSK-3 and APR-1/APC. In canonical Wnt signaling, GSK-3 and APC are components of the β-catenin destruction complex (XING *et al.* 2003) that are degraded in response to

the Wnt signal. The degradation of the β-catenin destruction complex allows for cytoplasmic accumulation of β-catenin and its subsequent translocation into the nucleus where it binds to and activates the TCF/LEF transcription factor. Curiously in *C. elegans*, EMS cell polarization requires the presence and positive activity of both SGG1 and APR-1 for WRM-1/ β-catenin mediated negative regulation of POP-1 in the E cell (YOST *et al.* 1996). It remains unclear what specific roles SGG-1 and APR-1 have in EMS cell polarity establishment.

The Wnt pathway converges with the MAPK pathway which includes the MAPKKK MOM-4 (SHIN *et al.* 1999), a homologue of vertebrate transforming-growth-factor TAK1, and the NEMO-like kinase LIT-1 (MENEGHINI *et al.* 1999). MOM-4 is required for LIT-1 kinase activity and functions upstream of LIT-1. LIT-1 forms a complex with β-catenin/WRM-1. *In vitro* experiments have shown that LIT-1 can phosphorylate POP-1, and WRM-1 mediated activation of LIT-1 is required for phosphorylation of POP-1 *in vivo* (ROCHELEAU *et al.* 1999). The WRM-1/LIT-1 complex translocates into the nucleus of the E cell and phosphorylates POP-1 leading to its export from the nucleus. Consistent with this function, LIT-1 is enriched in the posterior nuclei (LO *et al.* 2004). Recently, the conserved 14-3-3 protein PAR-5 was shown to be required for POP-1 asymmetry and likely has a direct role in promoting the export of phosphorylated POP-1 from the nucleus (LO *et al.* 2004). Proteins containing 14-3-3 domains previously had been shown to regulate the nuclear export of their binding partners (LOPEZ-GIRONA *et al.* 1999). One other factor that has been shown to be required for POP-1 nuclear export is ZK742.1 which is the homolog of the vertebrate exportin

CRM-1. RNAi mediated knock-down of *crm-1* inhibits nuclear export of POP-1 from the E cell nucleus and also causes a failure in endoderm development, in a strain containing an N-terminal, GFP tagged POP-1 (Lo *et al.* 2004). However, the loss of GFP::POP-1 nuclear asymmetry only occurs in 40% of AP sisters in the EMS lineage, indicating that RNAi does not cause complete knock-down of *crm-1*, or that other factors are sufficient for POP-1 nuclear export. It is not known what causes the asymmetry of LIT-1 although knockdown of both Wnt and MAPK signaling pathways causes a loss of LIT-1 enrichment in posterior nuclei. There are likely other factors required for recognition and/or transport of phosphorylated POP-1 and LIT-1 between the nucleus and cytoplasm.

To identify additional factors that influence POP-1 activity and localization and Wnt signaling dependent cell polarity in *C. elegans*, we have used RNA interference (RNAi) to reduce gene functions in the temperature-sensitive *lit-1* mutant *or131ts* (see chapter 2 and 3 for information of RNAi, screening methods, and discussion of sensitized genetic backgrounds). Here we report our identification of two genes that when reduced in function by RNAi, modify the amount of embryonic lethality in *lit-1(or131ts)* mutants. We go on to describe the initial characterization of these two modifiers that display both a very high degree of penetrance and genetic specificity for *lit-1(or131ts)* mutants. One of the modifiers, Y119C1A.1, is a protein containing two conserved domains, and when depleted with RNAi causes a 188.6-fold (0.5% with negative control and 94.3% with Y119C1A.1 RNAi at 17.5°C) increase in *lit-1(or131ts)* embryonic lethality. Remarkably, depletion of Y119C1A.1 has no loss-of-function phenotype of its own and fails to enhance lethality in five other ts mutants, each defective in other essential embryonic

processes. We also describe the identification and characterization of the conserved and essential gene *drh-3*, that produces strong and highly specific suppression of *lit-1(or131ts)* embryonic lethality when depleted with RNAi.

MATERIALS AND METHODS

Screening procedure and quantitative scoring: Techniques used for screening and scoring are identical to those described in Chapter III methods section.

Dark field microscopy: Visualization of intestinal granules was done using an Ziess Axioskop (Microscope) and a Plan-Apochromat 63X/1,4 oil immersion objective.

Molecular biology: To construct the *pei-1* driven N-terminal GFP fusion construct of Y119C1A.1 we first used Pfu Turbo polymerase (Stratagene) and a cDNA library (Invitrogen), and amplified the gene using the following primers containing spe-1 and asc-1 restriction sites (lowercase): 5'-ggtactagtATG CCT CAT CAA CCA GTG CT-3'; 5'- gtgtggcgcgccTTA GGC AGT GGA ATT AGC TGT G-3'. PCR products were subsequently ligated into pGEM-T or pGEM-T-easy shuttle vectors (Promega). Inserted genes were sequenced in the University of Oregon sequencing facility prior to cleavage and ligation into pSO26 [pSO26 described in (O'ROURKE *et al.* 2007)]. Transformation of constructs using particle bombardment is described in Chapter III methods.

Spinning Disk Confocal Microscopy: Equipment and techniques used for imaging GFP::Y119C1A.1 are the same as described in Chapter III methods section.

RESULTS

A Sensitized Genetic Background: To identify semi-permissive temperatures that optimize our ability to identify enhancers and suppressors of *lit-1(or131ts)* embryonic lethality, we examined the viability of embryos produced by homozygous *lit-1(or131ts)* hermaphrodite worms raised to adulthood at five different temperatures ranging from 15°C to 25°C. We chose 20°C (24% hatching N>200 progeny) and 23°C (1.04% hatching N>200 progeny) to screen enhancers and suppressors of embryonic lethality, respectively. These growth conditions optimized our ability to detect modifiers by initially scoring qualitatively for changes in embryonic lethality, using stereomicroscopes and 48-well agar plates with worm cultures. For later analysis and rescreening of *lit-1* enhancers we used a less restrictive temperature of 17.5°C (99.5% hatching) for assessing enhancement of embryonic lethality as this allowed for even greater sensitivity and reproducibility of results.

Identification of *lit-1(or131ts)* genetic modifiers: Using the RNAi feeding library we screened a total of 12,874 genes for enhancement and 15,716 genes for suppression of *lit-1(or131ts)* embryonic lethality. Our initial quantitative screen yielded 328 candidate enhancers and 239 candidate suppressors. We consolidated and systematically rescreened each candidate enhancer and suppressor using the same qualitative scoring method,

reducing the number of candidate enhancers and suppressors to 89 and 31, respectively (see Chapter 2 for a description of the qualitative screening method).

To quantify suppression, we compared the embryonic viability of broods from suppressed young adults, and from control RNAi treated young adults raised to adulthood on bacteria carrying the feeding RNAi vector without an insert (same method as in Chapter 3). From this analysis, five of the candidate suppressors, increased embryonic viability in the broods of *lit-1(or131ts)* mutants at 23°C by at least 6-fold over background (quantitative data shown in Figure 1).

The relatively large number of genetic enhancer candidates remaining after qualitative screening, and the high degree of non-specificity generally observed with enhancers of ts mutants, led us to perform an additional round of screening to aid in identifying the most penetrant and specific candidate modifiers before proceeding with quantitative analysis (see methods). Following this additional screening procedure we identified 12 genes that when reduced in function by RNAi enhanced lethality in *lit-1(or131ts)* mutants that by qualitative analysis did not enhance two unrelated ts mutants *dhc-1* and *spd-5* (data not shown). Only one of these RNAi clones, Y119C1A.1 a gene containing a RanBP-Type 2 domain and a DNA methylase domain, produced a dramatic increase in lethality observed in all rounds of qualitative screening, while the other 11 clones produced only mild enhancement (less than ~2-fold increase). Following sequencing the vectors to verify the identity of the RNAi clone, we quantified the enhancement caused by RNAi depletion of Y119C1A.1 in *lit-1(or131ts)* mutants and found that at 17.5°C lethality is increased to 94.3% (n=539) from 0.5% (n=985) on

empty-vector RNAi. At temperatures ≥20°C adult *lit-1(or131ts)* worms become sterile when depleted of Y119C1A.1.

Specificity analysis yields two highly specific *lit-1(or131ts)* modifiers: Modifiers of embryonic lethality in ts mutant backgrounds often function in a non-specific manner, as they can enhance or suppress multiple unrelated mutant backgrounds (See Chapter III and (O'ROURKE *et al.* 2007). We performed RNAi treatment of each of the five suppressors and the *lit-1(or131ts)* enhancer Y119C1A.1 with five other ts mutants: *spn-4(or191ts)*, *dhc-1(or195ts), spd-5(or213ts)*, *rfl-1(or198ts)* and *mel-26(or543ts)*. We found that the enhancer Y119C1A.1 and a suppressor *drh-3*, a gene containing a dead-box helicase, and has been shown to regulate RNAi induced gene silencing in the germline, are both completely specific modifiers as they fail to significantly modify lethality in any of the other five mutants tested (Figure 2). This very high genetic specificity and penetrance, suggests that *drh-3* and Y119C1A.1 have roles that directly influence Wnt signaling and/or cell polarity in the *C. elegans* embryo.

Allele specificity and genetic interactions with *mom-4*: Genetic modifiers of *lit-1(or131ts)* may function to influence Wnt or MAPK signaling, or they may act in a way that compensates for the specific molecular defect caused by the *or131ts* mutation. We therefore, used two additional mutant alleles of *lit-1* to evaluate the effect of both *drh-3* and Y119C1A.1 depletion on embryonic lethality. We observed strong enhancement of both *lit-1(ne1991ts)* and *lit-1(or393ts)* embryonic lethality with RNAi depletion of

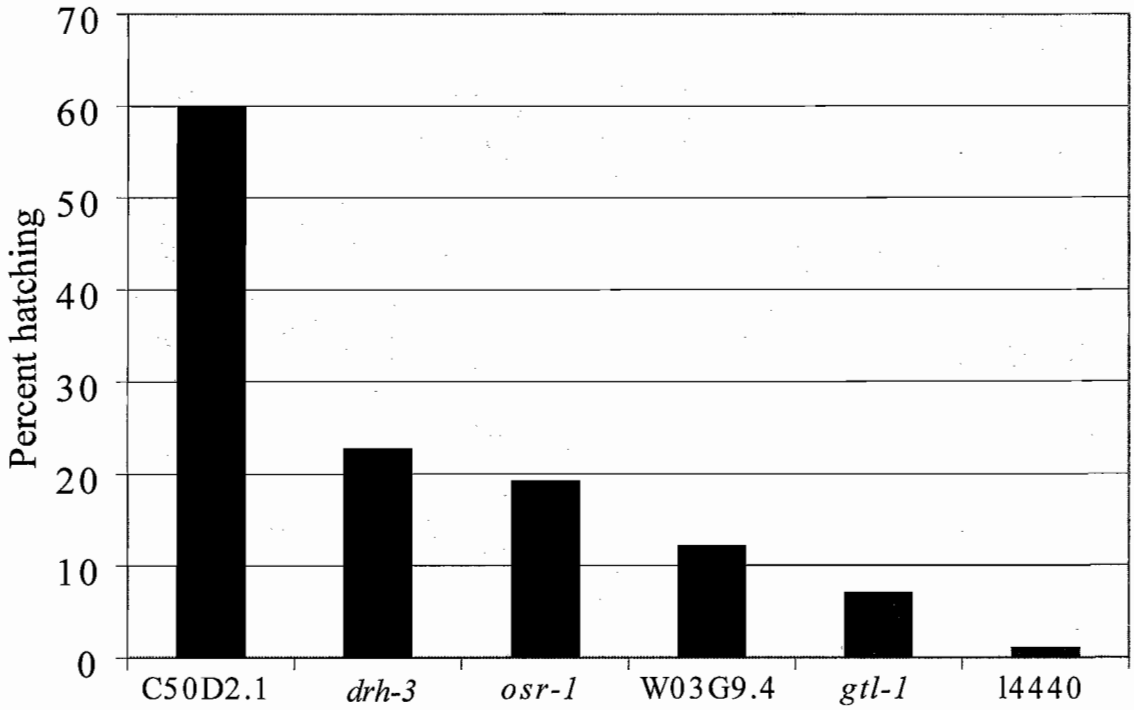

Figure 1.- Suppression of *lit-1(or131ts)* lethality by RNAi.

Y119C1A.1 (Figure 2). We also observed significant suppression of embryonic lethality with RNAi depletion of *drh-3* (Figure 2). These results are consistent with both modifiers somehow influencing *lit-1* related processes, and not simply compensating for an unstable or miss-folded LIT-1 protein.

Y119C1A.1 knock-down produces strong additive effect on embryonic lethality of a mutant of *mom-4*, a gene upstream of *lit-1*: The identification of penetrant genetic modifiers that are specific, and affect multiple different alleles of *lit-1* suggests that both modifiers influence *lit-1* dependent processes, but does not provide information about where or what the genes are directly influencing. The gene *mom-4* encodes a MAPKKK protein that has been shown to function upstream of LIT-1 (SHIN *et al.* 1999). If Y119C1A.1 functions in the same or related genetic pathway influencing cell polarity and cell fate specification, we would expect a significant additive effect on embryonic lethality when both loci are compromised. We used a ts mutant allele of the gene *mom-4* to test for this additive effect. With empty vector RNAi at 20°C, 8% of *mom-4(ne1539ts)* embryos fail to hatch. At this temperature, depletion of Y119C1A.1 with RNAi increases lethality to 56.6% (Figure 3). This result is consistent with Y119C1A.1 having a function that contributes to *lit-1* dependent processes.

***drh-3* Knock-Down Enhances Embryonic Lethality In *mom-4* Mutant:** The penetrant and highly specific suppression of embryonic lethality in three *lit-1* mutants after RNAi depletion of *drh-3* suggests some type of functional negative regulation on MAPK or

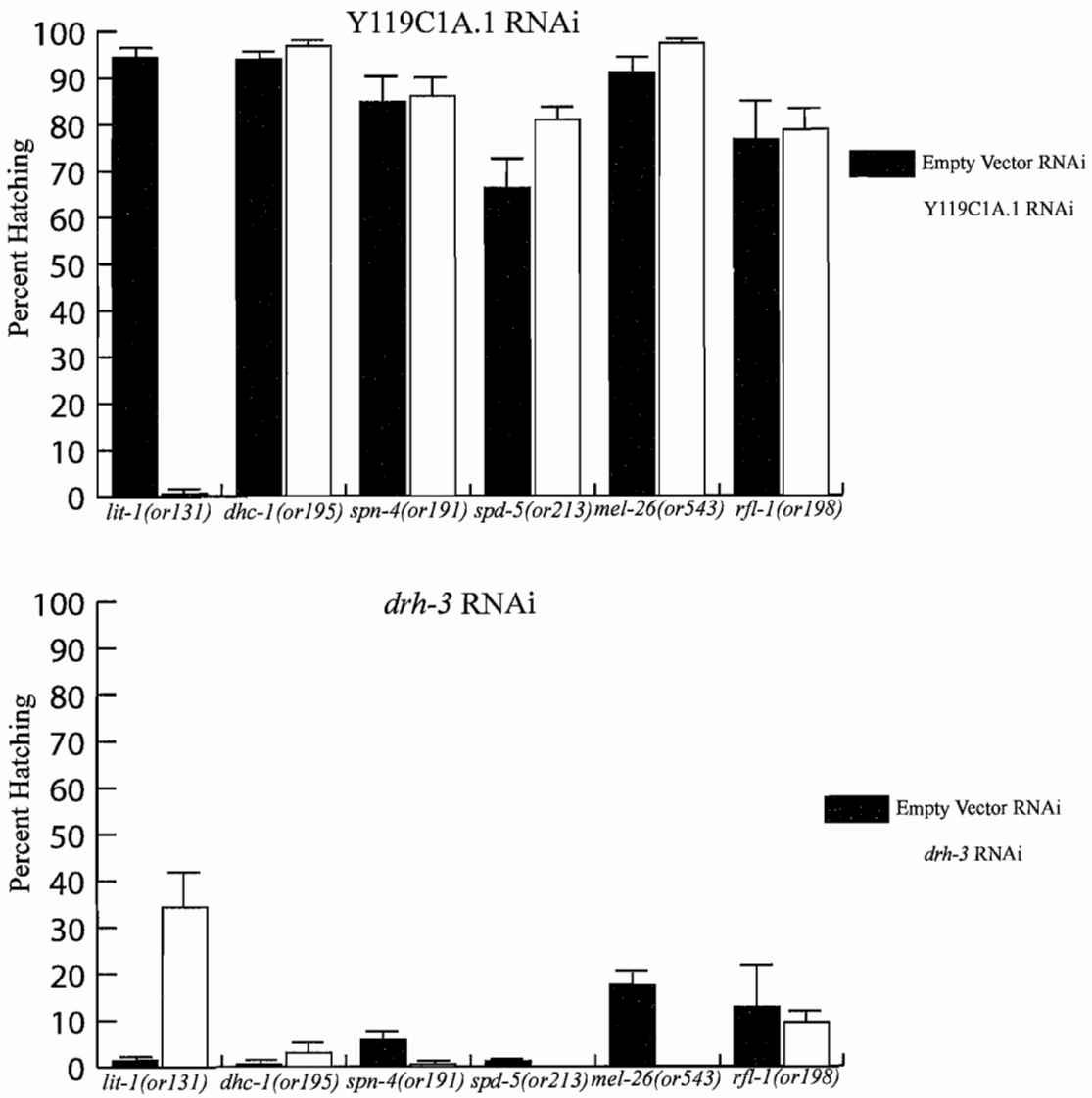

Figure 2.- Specificity analysis of genetic interactions caused by Y119C1A.1 (enhancer of *lit-1*) or *drh-3* (suppressor of *lit-1*) RNAi. Data points used to generate the graphs are averaged data from at least four replicate experiments. Error bars equal one standard deviation.

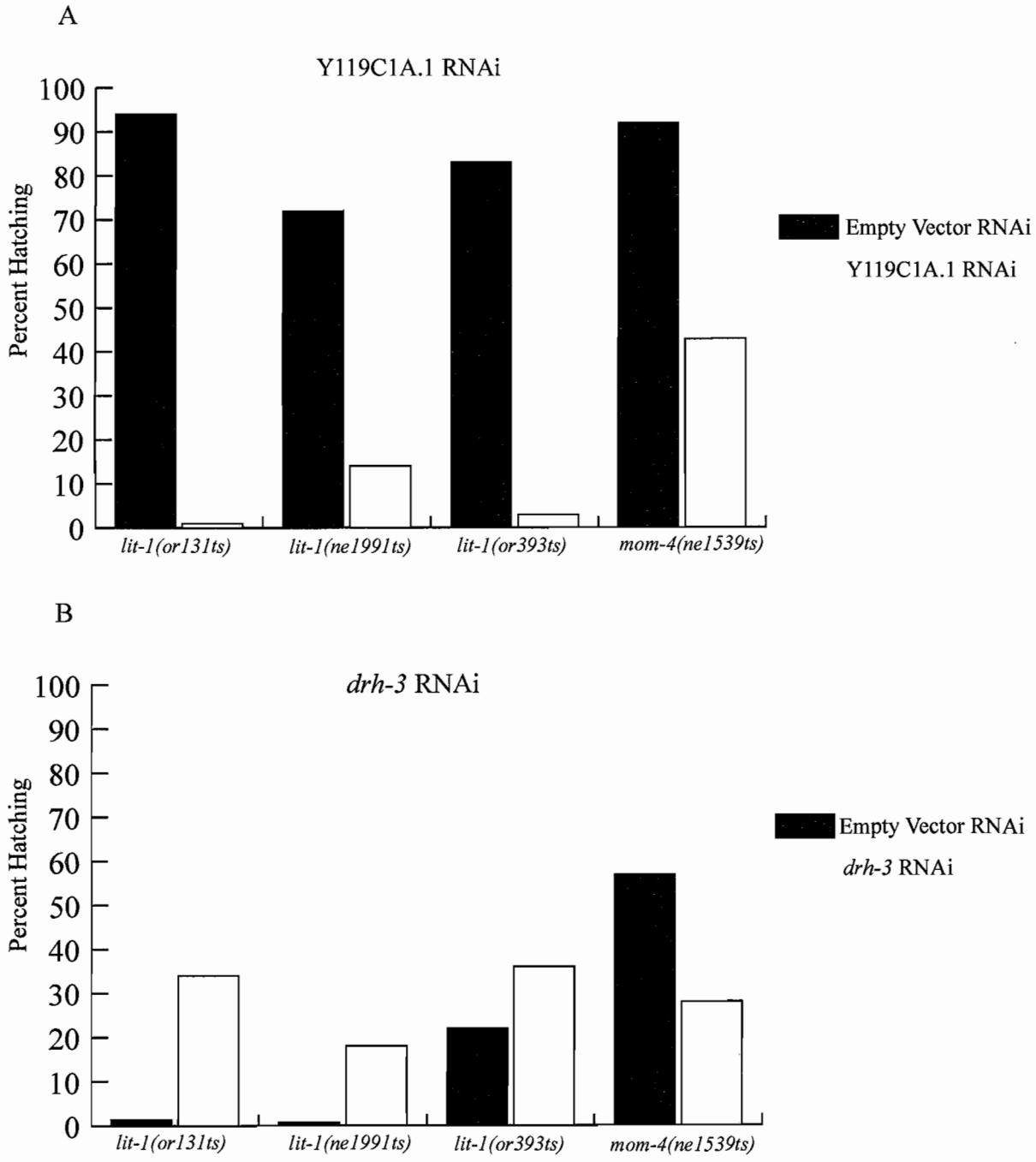

Figure 3.- RNAi knockdown of Y119C1A.1 (A) and *drh-3* (B) effect on embryonic viability in three different ts alleles of *lit-1* and one ts allele of *mom-4*. N>200 embryos for all data points shown.

Wnt signaling. To test this further we asked whether RNAi depletion of *drh-3* also suppresses embryonic lethality in *mom-4(ne1539ts)* mutants. We found that depleting *drh-3* gene product with RNAi increases lethality in this mutant background (Figure 3) suggesting a more complex relationship with LIT-1/MOM-4 signaling (see discussion). Since *drh-3* is itself required for embryonic viability, enhancement of *mom-4(ne1539ts)* could indicate that it may be functioning to negatively regulate Wnt or MAPK signaling downstream of *mom-4* but upstream of *lit-1*. Additional genetic and molecular analysis will be needed to determine the epistatic relationship between these genes.

Y119C1A.1 enhances loss of gut and *drh-3* suppresses loss of gut: *lit-1* loss-of-function mutants fail to produce intestine during embryogenesis. If Y119C1A.1 specifically influences endoderm specificaton induced by the Wnt/MAPK pathways, then depletion of Y119C1A.1 should enhance the loss of intestine phenotype. Indeed we found a striking enhancement of this phenotype indicating that Y119C1A.1 does influence LIT-1 in a way that affects endoderm specification, presumably through the regulation of POP-1 activity or localization (Figure 4 and Table 1). We also tested if depletion of *drh-3* in *lit-1(or131ts)* mutants suppresses the loss of intestine seen at a highly restrictive temperature. We observed significant suppression of the loss-of-intestine phenotype with the depletion of *drh-3* (Figure 4 and Table 1).

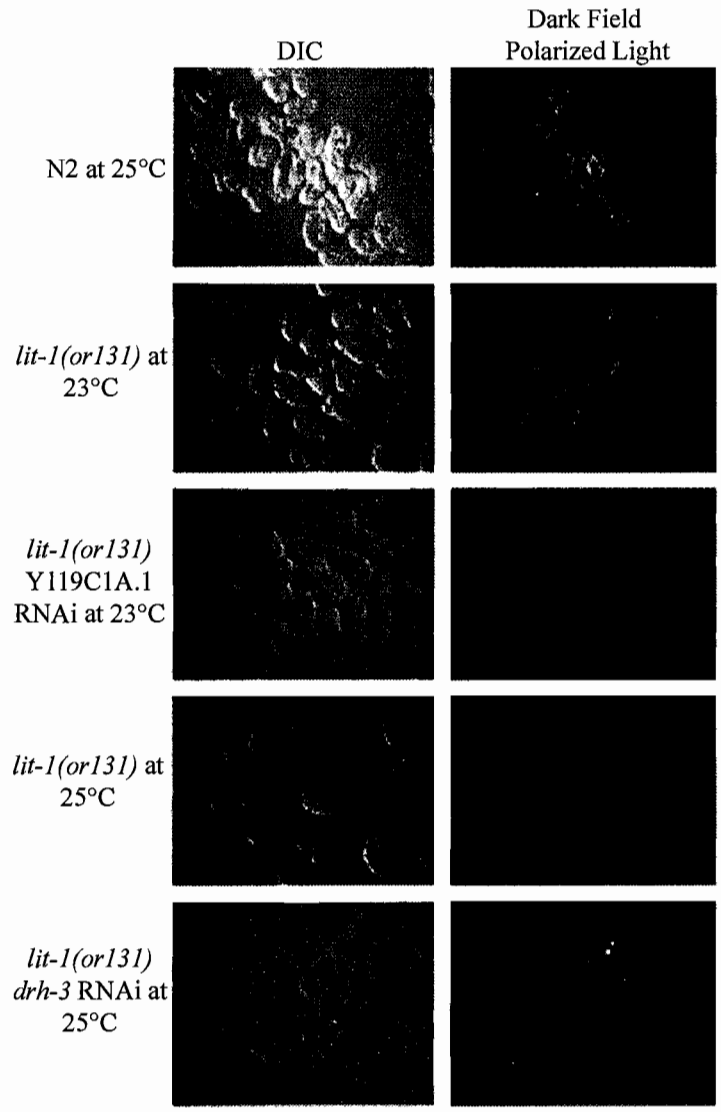

Figure 4.- Gut granules serve as a marker for endodermal development

TABLE 1
Percent embryos with gut granules

	Control RNAi	**Y119C1A.1 RNAi**
lit-1(or131ts) at 23°C	49% N=269	8% N=369
	Control RNAi	***drh-3* RNAi**
lit-1(or131ts) at 25°C	9.5% N=429	51% N=305

GFP::Y119C1A.1 localizes to nuclear membranes in the early embryo: To gain further insight into how Y119C1A.1 may influence WNT/MAPK signaling we constructed an N-terminal GFP fusion construct of Y119C1A.1. We isolated transformed GPP expressing lines and analyzed the localization patterns during early cell divisions. We observed cytoplasmic and nuclear localization of GFP::Y119C1A.1 in all early cells. During mitosis and early interphase we observed diffuse cytoplasmic localization. During late interphase and nuclear envelope breakdown, the fusion protein localized to the nuclei of all cells. Initially, signal is seen surrounding the nuclei similar to nuclear pore proteins, but then becomes more nuclear shortly before NEB (Figure 5). Localization to the nucleus is supportive of Y119C1A.1 somehow functioning to regulate nuclear-cytoplasmic transport of Wnt and/or MAPK components.

DISCUSSION

We have used feeding RNAi to conduct a large-scale screen for enhancers and suppressors of *lit-1(or131ts)* embryonic lethality. Quantitative analysis eliminated all but two genes that modify embryonic lethality in *lit-1(or131ts)* mutants, but not in other unrelated ts embryonic-lethal mutants. Here we report the identification of two genes that both have a strong and specific interaction with mutants of *lit-1* and *mom-4*, and appear to play a role in endoderm specification in *C. elegans*.

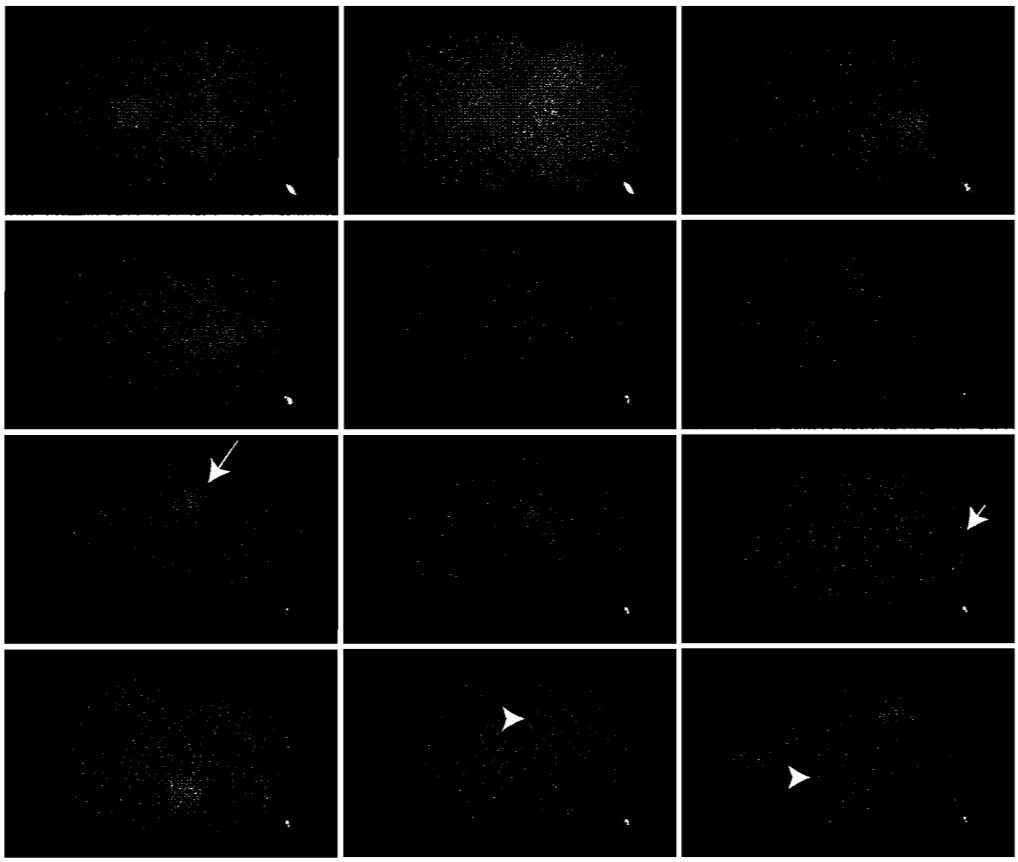

Figure 5.- Expression of GFP fusion to Y119C1A.1 encoded protein in early embryos from transgenic strain. All images are from the same embryo at various (unspecified) time-points during early embryonic cell divisions. Frames from six optical sections were projected from spinning-disk confocal time-lapse videomicrographs. Anterior is to the left. Long arrow indicates nuclear signal. White arrowhead indicates nuclear envelope signal. Short arrow indicates P granules.

Y119C1A.1: A strong candidate for regulating LIT-1 nucleo-cytoplasmic transport: While Y119C1A.1 has no previously known function or knockdown phenotype, we show that this gene is essential for both viability and endoderm formation in three different ts *lit-1* mutant strains grown at semi-permissive temperatures. RNAi mediated knock-down also strongly enhances mutants of *mom-4*, a protein known to function upstream of LIT-1 (SHIN *et al.* 1999). The gene Y119C1A.1 encodes a novel protein containing a RanBP-Type 2 Zinc Finger domain near the C-terminus. This domain is found in five different worm genes including the nuclear pore proteins *npp-7* and *npp-9*/NUP358/RanBP2. Mammalian RanBP2/Nup358 is a protein that is comprised largely of a repetitive series of RanBP2 zinc finger domains, and is one of three known nuclear pore components that reside on the cytoplasmic face of the nuclear pore complex (DELPHIN *et al.* 1997). Nup358 is thought to be the major, if not the only, component of the cytoplasmic filaments that extend from the nuclear pore out into the cytoplasm. The zinc finger domains of Nup358 have been shown to associate with the nuclear export factor CRM1 and RNAi mediated knockdown of Nup358 in mouse blastocysts cause a loss of CRM-1 localization at the cytoplasmic side of the nuclear pore (BERNAD *et al.* 2004). Furthermore, RNAi mediated knock-down of Nup358 in MCF-7 cells causes a partial, but significant reduction in nuclear export of a reporter construct (BERNAD *et al.* 2004). Importantly, the authors state that in experiments in which RNAi was less efficient (<90% knock-down of protein expression), little or no loss of nuclear export was observed. This finding indicates that only a small of amount of Nup358 is sufficient for nuclear export. If Y119C1A.1 has a similar role in mediating export of nuclear factors,

the lack of lethality by RNAi in a wildtype background may be simply because of insufficient knock-down of the gene product. Likewise it would make sense that a mutant of a nuclear export substrate (potentially LIT-1) would produce an additive negative effect and result in lethality.

Additional support for Y119C1A.1 acting to regulate nuclear localization of LIT-1, comes from a large scale Y2H screen of metazoan specific *C. elegans* genes (Li *et al.* 2004), which showed Y119C1A.1 is a reciprocal binding partner of LIT-1. This apparent physical interaction combined with the very pentrant and specific enhancement of both *lit-1* and *mom-4* mutants strongly suggests that Y119C1A.1 has a direct role in Wnt/MAPK signaling, and likely has some direct interaction with LIT-1. Importantly, the Y119C1A.1 bait construct also interacted with nine additional proteins, six of which are nuclear associated proteins. Five of these nuclear associated proteins (RPY-1, NHR-111, EYA-1, TAF-9, VAB-3) are transcription factors while the other is a component of the nuclear transport machinery: IMA-3 (Importin Alpha nuclear transport factor). Finally, we showed that GFP::Y119C1A.1 localizes to the nuclear envelope consistent with it functioning at the nuclear pore complex. Additional experiments will be required to confirm this hypothesis.

Potential role for siRNA mediated regulation of wnt signaling: We show that depletion of *drh-3*, suppresses lethality in *lit-1* mutants, yet curiously enhances lethality in a mutant of *mom-4*. While we cannot yet predict a specific role for this essential and conserved gene, our data suggests a direct and novel function for this gene in Wnt/MAPK

signaling and/or endoderm specification in *C. elegans*. *drh-3* encodes a Dicer-related helicase that interacts with Dicer, is required for RNAi in the germline, and for the production of endogenous small interfereing RNAs (siRNA) (DUCHAINE *et al.* 2006). *drh-3* is essential for embryonic viability as both RNAi and deletion mutants are lethal (RNAi produces partially penetrant lethality). Phenotype analysis of *drh-3* mutants show multinucleate germ cells often resulting in an overall sterile phenotype, as well as lagging mitotic chromosome and anaphase bridges in mitosis (DUCHAINE *et al.* 2006). A recent report showed that *drh-3* may be required for an siRNA pathway that regulates Ras signaling in developing larvae. It is possible that some small RNA species generated by a DRH-3 RNAi complex could regulate the expression of Wnt or MAPK signaling components. Additional genetic epistasis analysis, localization analysis and identification of additional DRH-3 generated siRNAs will be needed to pursue this possibility. It would be interesting to evaluate protein levels of several Wnt or MAPK signaling components in a background deficient of DRH-3. Identifying a significant change in protein levels of any of these components (most likely an increase), could indicate siRNA mediated regulation of their expression. Alternatively, DRH-3 may have an as yet undiscovered function, independent of its siRNA generating activity.

Concluding remarks: Using a mutant of the MAPK *lit-1* we have identified two genes that function to modify *lit-1* mutant induced embryonic lethality in a way that also affects endoderm specification. The enhancement of lethality caused by depleting Y119C1A.1 in *lit-1* mutants, but the absence of any phenotype in a wildtype background, is a striking

example of the power of modifier screening in the identifying gene function. We do not yet have defined roles for either *drh-3* or Y119C1A.1, but the evidence presented here clearly shows that both genes have functions that influence Wnt signaling in *C. elegans* embryos.

CHAPTER V

CONCLUSIONS

The completion of genome sequencing projects has presented researchers with the comprehensive genetic code of many different organisms including that of humans. These projects represent an incredible achievement in the pursuit of understanding the heritable factors required for life. An even greater challenge will be to identify the functions for each gene contained in the vast sequences of DNA we now have access to. This includes identifying how genes interact with each other to coordinate complex processes that require the proper function and interplay of many proteins. Model organisms such as *C. elegans* provide us with a valuable resource to study the function of genes and genetic interactions, that can then be used to gain insight into the biology and disease mechanisms of more complex organisms including humans.

Conditional mutant modifier screening: The goal of my dissertation was to identify novel genetic requirements for essential processes that control embryogenesis in *C. elegans*. To do this I used temperature-sensitive mutants defective in specific processes that occur in the early embryo and screened them with a genomic RNAi library for genes that, when depleted with RNAi, modify the amount of lethality caused by the mutation. We identified ~70 reproducible genetic interactions from screening three ts mutants. For modifiers of the Wnt signaling defective mutant *lit-1(or131ts)*, and the neddylation

defective mutant *rfl-1(or198ts)*, we showed that quantitatively assessing the specificity of genetic interactions is crucial step in beginning the processes of characterizing the nature of genetic interactions. After identifying the most specific and penetrant modifiers we proceeded to characterize the modifier loci using various techniques.

We show that specific suppressors of *rfl-1* mutants encode proteins that have localization patterns similar to many of the components of the neddylation pathway. We also show that knocking down expression of the strongest suppressors of *rfl-1(or198ts)* significantly reduces the cellular defects caused by the mutation. We identified two highly penetrant enhancers of lethality in *rfl-1* mutants that produce little or no lethality in a wildtype background or in ts mutants of unrelated genes. These enhancers failed to increase early embryonic defects associated with *rfl-1* mutants. Further analysis showed the enhancement was likely due to an as yet undiscovered function for *rfl-1* later in development that was only detectable when both *rfl-1*, and the enhancer gene were reduced in function. In sum, we identified novel roles for 17 conserved genes, validating the usefulness and efficiency of ts mutant modifier screening.

In chapter IV we presented data describing the interaction between mutants of *lit-1* and depletion of *drh-3* and Y119C1A.1 gene products. We show that *lit-1* ts mutant lethality, at largely permissive temperature, is dramatically enhanced by RNAi mediated depletion of Y119C1A.1. This enhancement is completely specific for mutants of *lit-1* and the related gene *mom-4*, as they fail to enhance any of the five additional ts embryonic lethal mutants of unrelated genes that we tested. Sequence analysis indicated the presence of a conserved zinc finger motif, found in specific proteins that function in

the nuclear pore complex (NPC). While Y119C1A.1 is uncharacterized, it was identified as a reciprocal binding partner of LIT-1 in a large-scale 2-hybrid screen. Additional targets identified in this 2-hybrid screen are nuclear factors including one component of the nuclear transport machinery, supporting a possible role in regulating nucleo-cytoplasmic transport. We show a localization pattern consistent with this possibility as GFP::Y119C1A.1 localizes to the nuclear membrane. Finally we show a striking enhancement of the loss-of-intestine phenotype characteristic of *lit-1* mutants and other Wnt signaling components that act upstream of *pop-1*. Together these findings show that Y119C1A.1 has an important role in Wnt/MAPK signaling. This role was completely illusive in previous RNAi studies and may indicate that RNAi mediated knockdown of Y119C1A.1 is insufficient for causing a phenotype, or that Y119C1A.1 is simply not essential for proper development in an otherwise wildtype background. Both of these scenarios support the importance of modifier screening in determining gene functions.

The gene *drh-3* has been previously characterized as essential for development and is required for RNAi in the germline (DUCHAINE *et al.* 2006). We show here that knockdown of *drh-3* causes strong and specific suppression of *lit-1* mutant induced embryonic lethality. The suppression of lethality also correlates with suppression of the loss-of-intestine phenotype characteristic of *lit-1* mutants. Recent functional characterization of *drh-3* indicates important roles in the production of siRNAs and siRNA mediated regulation of gene expression. The identification of *drh-3* as a suppressor of *lit-1* mutants shows that the screening method used here can also detect new roles for essential genes. While we cannot easily predict any specific mechanisms

that explain the suppression of *lit-1* mutants, a role for siRNA pathways in regulating Wnt signaling would be a major discovery in the study of these important molecular pathways.

APPENDIX

Summary of *rfl-1(or198ts)* modifiers

Gene	Encoded Protein	Interaction	Essential	Specific	Human Homolog	Blast P value	% length
csn-5	COP9 Signalosome subunit	suppressor	yes	yes	COPS5	5.2e-100	84.8
csn-2	COP9 Sinalosome subunit	suppressor	yes	yes	COPS2	6.81e-151	83.8
mus-101	Nucleotide Excision Repair Factor	suppressor	yes	yes	TOPBP1	3e-50	61.7
R10D12.14	Unknown Function	suppressor	yes	yes	TNRC15	6.4e-06	25.7
mnk-1	Kinase	suppressor	no	yes	Q9BUB5-2	2.1e-31	
F25H5.5	Claspin (kinase)	suppressor	yes	yes	CLSPN	1.4e-25	91.3
F22B8.7	Fe-S Protein	suppressor	no	yes	MOSC1	8.6e-43	85.5
T24C4.3	Novel	suppressor	no	yes	-	-	-
cdr-2	glutathione S-transferase-like	suppressor	no	yes	C6orf168	7.3e-22	82.7
T26E3.6	Fibrillin related protein	suppressor	no	yes	SORL1	0.0001	84.5
elks-1	Rab6 GTPase-interacting protein	suppressor	no	yes	ERC2	6.4e-36	77.3
sri-63	7TM chemoreceptor	suppressor	no	yes	CCR1	0.011	87.7
gcy-20	guanylate cyclase	suppressor	no	yes	NPR1	1.4e-133	89
lgc-20	Acetylcholine receptor	suppressor	no	yes	CHRNB1	1.8e-07	71.6
ugt-16	UDP-glucuronosyl transferase	suppressor	no	yes	UGT2B7	1.6e-43	95.5

col-164	Collagen protein	suppressor	no	yes	Q59HB5	6.4e-43	73
vig-1	RNA Binding Protein	suppressor	yes	no	Q8NC51-4	2.1e-31	97.9
M106.4	GMP Synthase	suppressor	no	no	GMPS	2.7e-151	74.7
aqp-5	Aquaporin	suppressor	no	no	AQP8	6.4e-26	73.8
T05H4.11	Novel	suppressor	no	no	-	-	-
T04C9.1	Rho GTPase-activating protein	suppressor	no	no	ARHGAP10	3.1e-144	79
sel-10	F-Box,WD40 Protein	enhancer	no	yes	FBXW7	1.9e-126	76.7
pha-1	novel	enhancer	yes/no	yes	Q96Q89-3	0.018	71.1
R05A10.8	novel	enhancer	no	yes	-	-	-
srh-292	7TM chemoreceptor	enhancer	no	yes	LMBR1L	.071	75.8
T14E8.1	Protein tyrosine kinase	enhancer	no	yes	MET	1.8e-48	50.8
C24D10.1	Protein tyrosine phosphatase	enhancer	no	yes	Q12923-2	1.2e-06	50.6
abcf-3	ABC transporter	enhancer	no	no	ABCF3	1.3e-183	98
F32B6.9	RNA splicing factor	enhancer	yes/no	no	PRPF18	6e-58	96.6
gpd-4	glyceraldehyde-3-phosphate dehydrogenases	enhancer	yes	no	GAPDH	3.2e-132	99.4
cutl-8	Cuticulin precursor	enhancer	no	no	MAST1	.066	50.9
ZK686.4	Zinc finger protein	enhancer	no	no	ZMAT2	3.4e-50	96.8
mtm-3	Myotubularin-related protein	enhancer	no	no	Q13615-2	1.5e-74	64.1
T01C3.2	Chromatin modifying protein	enhancer	no	no	MIT:ENY2-001	0.0014	92.9

REFERENCES

ALBERTSON, D. G., and J. N. THOMSON, 1993 Segregation of holocentric chromosomes at meiosis in the nematode, *Caenorhabditis elegans*. Chromosome Res. **1:** 15-26.

ANGERS, S., C. J. THORPE, T. L. BIECHELE, S. J. GOLDENBERG, N. ZHENG *et al.*, 2006 The KLHL12-Cullin-3 ubiquitin ligase negatively regulates the Wnt-beta-catenin pathway by targeting Dishevelled for degradation. Nat. Cell Biol. **8:** 348-357.

BAUGH, L. R., J. C. WEN, A. A. HILL, D. K. SLONIM, E. L. BROWN *et al.*, 2005 Synthetic lethal analysis of *Caenorhabditis elegans* posterior embryonic patterning genes identifies conserved genetic interactions. Genome Biol. **6:** R45.

BERNAD, R., H. VAN DER VELDE, M. FORNEROD and H. PICKERSGILL, 2004 Nup358/RanBP2 attaches to the nuclear pore complex via association with Nup88 and Nup214/CAN and plays a supporting role in CRM1-mediated nuclear protein export. Mol. Cell Biol. **24:** 2373-2384.

BORNSTEIN, G., D. GANOTH and A. HERSHKO, 2006 Regulation of neddylation and deneddylation of cullin1 in SCFSkp2 ubiquitin ligase by F-box protein and substrate. Proc. Natl. Acad. Sci. U S A **103:** 11515-11520.

BOWERMAN, B., and T. KURZ, 2006 Degrade to create: developmental requirements for ubiquitin-mediated proteolysis during early *C. elegans* embryogenesis. Development **133:** 773-784.

BRENNER, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics **77:** 71-94.

CADIGAN, K. M., and R. NUSSE, 1997 Wnt signaling: a common theme in animal development. Genes Dev. **11:** 3286-3305.

CULLINAN, S. B., J. D. GORDAN, J. JIN, J. W. HARPER and J. A. DIEHL, 2004 The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. Mol. Cell. Biol. **24:** 8477-8486.

DELPHIN, C., T. GUAN, F. MELCHIOR and L. GERACE, 1997 RanGTP targets p97 to RanBP2, a filamentous protein localized at the cytoplasmic periphery of the nuclear pore complex. Mol. Biol. Cell **8:** 2379-2390.

DETWILER, M. R., M. REUBEN, X. LI, E. ROGERS and R. LIN, 2001 Two zinc finger proteins, OMA-1 and OMA-2, are redundantly required for oocyte maturation in *C. elegans*. Dev. Cell **1:** 187-199.

DOW, M. R., and P. E. MAINS, 1998 Genetic and molecular characterization of the *Caenorhabditis elegans* gene, *mel-26*, a postmeiotic negative regulator of *mei-1*, a meiotic-specific spindle component. Genetics **150:** 119-128.

DUCHAINE, T. F., J. A. WOHLSCHLEGEL, S. KENNEDY, Y. BEI, D. CONTE, JR. *et al.*, 2006 Functional proteomics reveals the biochemical niche of *C. elegans* DCR-1 in multiple small-RNA-mediated pathways. Cell **124:** 343-354.

FAY, D. S., X. QIU, E. LARGE, C. P. SMITH, S. MANGO *et al.*, 2004 The coordinate regulation of pharyngeal development in *C. elegans* by *lin-35/Rb*, *pha-1*, and *ubc-18*. Dev. Biol. **271:** 11-25.

FEINBERG, E. H., and C. P. HUNTER, 2003 Transport of dsRNA into cells by the transmembrane protein SID-1. Science **301:** 1545-1547.

FIRE, A., S. XU, M. K. MONTGOMERY, S. A. KOSTAS, S. E. DRIVER *et al.*, 1998 Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391:** 806-811.

FRASER, A., 2004 Towards full employment: using RNAi to find roles for the redundant. Oncogene **23:** 8346-8352.

GEYER, R., S. WEE, S. ANDERSON, J. YATES and D. A. WOLF, 2003 BTB/POZ domain proteins are putative substrate adaptors for cullin 3 ubiquitin ligases. Mol. Cell **12:** 783-790.

GOMES, J. E., S. E. ENCALADA, K. A. SWAN, C. A. SHELTON, J. C. CARTER *et al.*, 2001 The maternal gene *spn-4* encodes a predicted RRM protein required for mitotic spindle orientation and cell fate patterning in early *C. elegans* embryos. Development **128:** 4301-4314.

GUIL, S., and M. ESTELLER, 2008 DNA methylomes, histone codes and miRNAs: Tying it all together. Int. J. Biochem. Cell Biol.

GUO, S., and K. J. KEMPHUES, 1995 *par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell **81:** 611-620.

HAMILL, D. R., A. F. SEVERSON, J. C. CARTER and B. BOWERMAN, 2002 Centrosome maturation and mitotic spindle assembly in *C. elegans* require SPD-5, a protein with multiple coiled-coil domains. Dev. Cell **3:** 673-684.

HETFELD, B. K., A. PETH, X. M. SUN, P. HENKLEIN, G. M. COHEN *et al.*, 2008 The COP9 signalosome-mediated deneddylation is stimulated by caspases during apoptosis. Apoptosis **13:** 187-195.

HOLWAY, A. H., C. HUNG and W. M. MICHAEL, 2005 Systematic, RNA-interference-mediated identification of *mus-101* modifier genes in *Caenorhabditis elegans*. Genetics **169:** 1451-1460.

HUBBARD, E. J., G. WU, J. KITAJEWSKI and I. GREENWALD, 1997 *sel-10*, a negative regulator of *lin-12* activity in *Caenorhabditis elegans*, encodes a member of the CDC4 family of proteins. Genes Dev. **11:** 3182-3193.

HUNT-NEWBURY, R., R. VIVEIROS, R. JOHNSEN, A. MAH, D. ANASTAS *et al.*, 2007 High-throughput in vivo analysis of gene expression in *Caenorhabditis elegans*. PLoS Biol. **5:** e237.

JADHAV, S., M. RANA and K. SUBRAMANIAM, 2008 Multiple maternal proteins coordinate to restrict the translation of *C. elegans* nanos-2 to primordial germ cells. Development **135:** 1803-1812.

JAGER, S., H. T. SCHWARTZ, H. R. HORVITZ and B. CONRADT, 2004 The *Caenorhabditis elegans* F-box protein SEL-10 promotes female development and may target FEM-1 and FEM-3 for degradation by the proteasome. Proc. Natl. Acad. Sci. U S A **101:** 12549-12554.

JONES, D., and E. P. CANDIDO, 2000 The NED-8 conjugating system in *Caenorhabditis elegans* is required for embryogenesis and terminal differentiation of the hypodermis. Dev. Biol. **226:** 152-165.

JOUKOV, V., A. C. GROEN, T. PROKHOROVA, R. GERSON, E. WHITE *et al.*, 2006 The BRCA1/BARD1 heterodimer modulates ran-dependent mitotic spindle assembly. Cell **127:** 539-552.

KAMATH, R. S., and J. AHRINGER, 2003 Genome-wide RNAi screening in *Caenorhabditis elegans*. Methods **30:** 313-321.

KELLY, W. G., and A. FIRE, 1998 Chromatin silencing and the maintenance of a functional germline in *Caenorhabditis elegans*. Development **125:** 2451-2456.

KIDD, A. R., 3RD, J. A. MISKOWSKI, K. R. SIEGFRIED, H. SAWA and J. KIMBLE, 2005 A beta-catenin identified by functional rather than sequence criteria and its role in Wnt/MAPK signaling. Cell **121:** 761-772.

KURZ, T., N. OZLU, F. RUDOLF, S. M. O'ROURKE, B. LUKE *et al.*, 2005 The conserved protein DCN-1/Dcn1p is required for cullin neddylation in *C. elegans* and *S. cerevisiae*. Nature **435:** 1257-1261.

KURZ, T., L. PINTARD, J. H. WILLIS, D. R. HAMILL, P. GONCZY *et al.*, 2002 Cytoskeletal regulation by the Nedd8 ubiquitin-like protein modification pathway. Science **295:** 1294-1298.

LABBE, J. C., and B. GOLDSTEIN, 2002 Embryonic development: a new SPN on cell fate specification. Curr. Biol. **12:** R396-398.

LABBE, J. C., A. PACQUELET, T. MARTY and M. GOTTA, 2006 A genomewide screen for suppressors of *par-2* uncovers potential regulators of PAR protein-dependent cell polarity in *Caenorhabditis elegans*. Genetics **174:** 285-295.

LAMITINA, T., C. G. HUANG and K. STRANGE, 2006 Genome-wide RNAi screening identifies protein damage as a regulator of osmoprotective gene expression. Proc. Natl. Acad. Sci. U S A **103:** 12173-12178.

LANDER, E. S., L. M. LINTON, B. BIRREN, C. NUSBAUM, M. C. ZODY *et al.*, 2001 Initial sequencing and analysis of the human genome. Nature **409:** 860-921.

LEHNER, B., C. CROMBIE, J. TISCHLER, A. FORTUNATO and A. G. FRASER, 2006 Systematic mapping of genetic interactions in *Caenorhabditis elegans* identifies common modifiers of diverse signaling pathways. Nat. Genet. **38:** 896-903.

LI, S., C. M. ARMSTRONG, N. BERTIN, H. GE, S. MILSTEIN *et al.*, 2004 A map of the interactome network of the metazoan *C. elegans*. Science **303:** 540-543.

LO, M. C., F. GAY, R. ODOM, Y. SHI and R. LIN, 2004 Phosphorylation by the beta-catenin/MAPK complex promotes 14-3-3-mediated nuclear export of TCF/POP-1 in signal-responsive cells in *C. elegans*. Cell **117:** 95-106.

LOPEZ-GIRONA, A., B. FURNARI, O. MONDESERT and P. RUSSELL, 1999 Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. Nature **397:** 172-175.

LU, C., and P. E. MAINS, 2007 The *C. elegans* anaphase promoting complex and MBK-2/DYRK kinase act redundantly with CUL-3/MEL-26 ubiquitin ligase to degrade MEI-1 microtubule-severing activity after meiosis. Dev. Biol. **302:** 438-447.

LYAPINA, S., G. COPE, A. SHEVCHENKO, G. SERINO, T. TSUGE *et al.*, 2001 Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. Science **292:** 1382-1385.

MADURO, M. F., R. LIN and J. H. ROTHMAN, 2002 Dynamics of a developmental switch: recursive intracellular and intranuclear redistribution of *Caenorhabditis elegans* POP-1 parallels Wnt-inhibited transcriptional repression. Dev. Biol. **248:** 128-142.

MAEDA, I., Y. KOHARA, M. YAMAMOTO and A. SUGIMOTO, 2001 Large-scale analysis of gene function in *Caenorhabditis elegans* by high-throughput RNAi. Curr. Biol. **11:** 171-176.

MAINS, P. E., K. J. KEMPHUES, S. A. SPRUNGER, I. A. SULSTON and W. B. WOOD, 1990 Mutations affecting the meiotic and mitotic divisions of the early *Caenorhabditis elegans* embryo. Genetics **126:** 593-605.

MANKE, I. A., D. M. LOWERY, A. NGUYEN and M. B. YAFFE, 2003 BRCT repeats as phosphopeptide-binding modules involved in protein targeting. Science **302:** 636-639.

MCNALLY, K., A. AUDHYA, K. OEGEMA and F. J. MCNALLY, 2006 Katanin controls mitotic and meiotic spindle length. J. Cell Biol. **175:** 881-891.

MEINNEL, T., and C. GIGLIONE, 2008 Protein lipidation meets proteomics. Front. Biosci. **13:** 6326-6340.

MENEGHINI, M. D., T. ISHITANI, J. C. CARTER, N. HISAMOTO, J. NINOMIYA-TSUJI *et al.*, 1999 MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in *Caenorhabditis elegans*. Nature **399:** 793-797.

NANDI, D., P. TAHILIANI, A. KUMAR and D. CHANDU, 2006 The ubiquitin-proteasome system. J. Biosci. **31:** 137-155.

O'ROURKE, S. M., M. D. DORFMAN, J. C. CARTER and B. BOWERMAN, 2007 Dynein modifiers in *C. elegans*: light chains suppress conditional heavy chain mutants. PLoS Genet. **3:** e128.

OOI, S. L., X. PAN, B. D. PEYSER, P. YE, P. B. MELUH *et al.*, 2006 Global synthetic-lethality analysis and yeast functional profiling. Trends Genet. **22:** 56-63.

PIANO, F., A. J. SCHETTER, D. G. MORTON, K. C. GUNSALUS, V. REINKE *et al.*, 2002 Gene clustering based on RNAi phenotypes of ovary-enriched genes in *C. elegans*. Curr. Biol. **12:** 1959-1964.

PINTARD, L., T. KURZ, S. GLASER, J. H. WILLIS, M. PETER *et al.*, 2003a Neddylation and deneddylation of CUL-3 is required to target MEI-1/Katanin for degradation at the meiosis-to-mitosis transition in *C. elegans*. Curr. Biol. **13:** 911-921.

PINTARD, L., J. H. WILLIS, A. WILLEMS, J. L. JOHNSON, M. SRAYKO *et al.*, 2003b The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase. Nature **425:** 311-316.

PRAITIS, V., 2006 Creation of transgenic lines using microparticle bombardment methods. Methods Mol. Biol. **351:** 93-107.

PRIESS, J. R., H. SCHNABEL and R. SCHNABEL, 1987 The *glp-1* locus and cellular interactions in early *C. elegans* embryos. Cell **51:** 601-611.

QIU, X., and D. S. FAY, 2006 ARI-1, an RBR family ubiquitin-ligase, functions with UBC-18 to regulate pharyngeal development in *C. elegans*. Dev. Biol. **291:** 239-252.

QUINTIN, S., P. E. MAINS, A. ZINKE and A. A. HYMAN, 2003 The *mbk-2* kinase is required for inactivation of MEI-1/katanin in the one-cell *Caenorhabditis elegans* embryo. EMBO Rep. **4:** 1175-1181.

ROCHELEAU, C. E., W. D. DOWNS, R. LIN, C. WITTMANN, Y. BEI *et al.*, 1997 Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. Cell **90:** 707-716.

ROCHELEAU, C. E., J. YASUDA, T. H. SHIN, R. LIN, H. SAWA *et al.*, 1999 WRM-1 activates the LIT-1 protein kinase to transduce anterior/posterior polarity signals in *C. elegans*. Cell **97:** 717-726.

SAROV, M., S. SCHNEIDER, A. POZNIAKOVSKI, A. ROGUEV, S. ERNST *et al.*, 2006 A recombineering pipeline for functional genomics applied to *Caenorhabditis elegans*. Nat. Methods **3:** 839-844.

SCHELLENBERG, M. J., D. B. RITCHIE and A. M. MACMILLAN, 2008 Pre-mRNA splicing: a complex picture in higher definition. Trends Biochem. Sci. **33:** 243-246.

SCHWECHHEIMER, C., G. SERINO, J. CALLIS, W. L. CROSBY, S. LYAPINA *et al.*, 2001 Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIRI in mediating auxin response. Science **292:** 1379-1382.

SHANER, N. C., R. E. CAMPBELL, P. A. STEINBACH, B. N. GIEPMANS, A. E. PALMER *et al.*, 2004 Improved monomeric red, orange and yellow fluorescent proteins

derived from *Discosoma sp.* red fluorescent protein. Nat. Biotechnol. **22:** 1567-1572.

SHIN, T. H., J. YASUDA, C. E. ROCHELEAU, R. LIN, M. SOTO *et al.*, 1999 MOM-4, a MAP kinase kinase kinase-related protein, activates WRM-1/LIT-1 kinase to transduce anterior/posterior polarity signals in *C. elegans*. Mol. Cell **4:** 275-280.

SIMMER, F., C. MOORMAN, A. M. VAN DER LINDEN, E. KUIJK, P. V. VAN DEN BERGHE *et al.*, 2003 Genome-wide RNAi of *C. elegans* using the hypersensitive *rrf-3* strain reveals novel gene functions. PLoS Biol. **1:** E12.

SONNICHSEN, B., L. B. KOSKI, A. WALSH, P. MARSCHALL, B. NEUMANN *et al.*, 2005 Full-genome RNAi profiling of early embryogenesis in *Caenorhabditis elegans*. Nature **434:** 462-469.

SRAYKO, M., D. W. BUSTER, O. A. BAZIRGAN, F. J. MCNALLY and P. E. MAINS, 2000 MEI-1/MEI-2 katanin-like microtubule severing activity is required for *Caenorhabditis elegans* meiosis. Genes Dev. **14:** 1072-1084.

STITZEL, M. L., K. C. CHENG and G. SEYDOUX, 2007 Regulation of MBK-2/Dyrk kinase by dynamic cortical anchoring during the oocyte-to-zygote transition. Curr. Biol. **17:** 1545-1554.

THORPE, C. J., A. SCHLESINGER, J. C. CARTER and B. BOWERMAN, 1997 Wnt signaling polarizes an early *C. elegans* blastomere to distinguish endoderm from mesoderm. Cell **90:** 695-705.

TIMMONS, L., D. L. COURT and A. FIRE, 2001 Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene **263:** 103-112.

TIMMONS, L., and A. FIRE, 1998 Specific interference by ingested dsRNA. Nature **395:** 854.

TONG, A. H., G. LESAGE, G. D. BADER, H. DING, H. XU *et al.*, 2004 Global mapping of the yeast genetic interaction network. Science **303:** 808-813.

VAN HAAFTEN, G., R. ROMEIJN, J. POTHOF, W. KOOLE, L. H. MULLENDERS *et al.*, 2006 Identification of conserved pathways of DNA-damage response and radiation protection by genome-wide RNAi. Curr.Biol. **16:** 1344-1350.

WARMING, S., N. COSTANTINO, D. L. COURT, N. A. JENKINS and N. G. COPELAND, 2005 Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res. **33:** e36.

WU, G., S. LYAPINA, I. DAS, J. LI, M. GURNEY *et al.*, 2001 SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. Mol. Cell Biol. **21:** 7403-7415.

XING, Y., W. K. CLEMENTS, D. KIMELMAN and W. XU, 2003 Crystal structure of a beta-catenin/axin complex suggests a mechanism for the beta-catenin destruction complex. Genes Dev. **17:** 2753-2764.

XU, L., Y. WEI, J. REBOUL, P. VAGLIO, T. H. SHIN *et al.*, 2003 BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. Nature **425:** 316-321.

YAMAMOTO, R. R., J. M. AXTON, Y. YAMAMOTO, R. D. SAUNDERS, D. M. GLOVER *et al.*, 2000 The Drosophila mus101 gene, which links DNA repair, replication and condensation of heterochromatin in mitosis, encodes a protein with seven BRCA1 C-terminus domains. Genetics **156:** 711-721.

YOST, C., M. TORRES, J. R. MILLER, E. HUANG, D. KIMELMAN *et al.*, 1996 The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. Genes Dev. **10:** 1443-1454.

YU, X., C. C. CHINI, M. HE, G. MER and J. CHEN, 2003 The BRCT domain is a phospho-protein binding domain. Science **302:** 639-642.