Analysis of Cell Polarity Signaling in C. elegans: A Dissertation

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ANALYSIS OF CELL POLARITY SIGNALING IN C. elegans

A Dissertation Presented

By

Christian Ernest Rocheleau

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

DECEMBER, 3 1999

CELL BIOLOGY
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The chapters of this dissertation have appeared in separate publications:


*These authors contributed equally.
ANALYSIS OF CELL POLARITY SIGNALING IN C. elegans

A Dissertation Presented
By
Christian Ernest Rocheleau

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ABBREVIATIONS

A-P  anterior-posterior
APC  adenomatous polyposis coli
Apr  APC-related
Arm  armadillo
Bli  blistered cuticle
Df  deficiency
Dpy  dumpy
Egl  egg laying-defective
Fz  frizzled
Gsk  glycogen synthase kinase
HMG  High mobility group
LEF  lymphoid enhancer factor
Lin  lineage abnormal
Lit  loss of intestine
MAPK  mitogen activated protein kinase
MAPKK  MAPK kinase
MAPKKK  MAPKK kinase
Mex  muscle in excess
Mom  more mesoderm
Nlk  Nemo-Like kinase
Par  embryonic partitioning abnormal
Pie  pharynx, intestine in excess
Pop  posterior pharynx-defective
Porc  porcupine
RNAi  RNA interference
Ser  serine
Sgg  shaggy
Skn  skin (hypodermis) in excess
TAK  TGF-β Activated Kinase
TCF  T-cell factor
TGF  transforming growth factor
Thr  threonine
Unc  uncoordinated
Wnt/wg  Wnt/Wingless
Wrm  worm arm-motif protein or “wormadillo”
ABSTRACT

During embryonic development of the nematode *Caenorhabditis elegans*, cell fates are specified by asymmetric segregation of cell fate determinants and via cell-cell signaling events. Specification of the eight-cell stage blastomere E, the endoderm progenitor cell, requires both cell signaling and asymmetric cell division. At the four-cell stage, a polarity-inducing signal from the P_2_ cell is required for the EMS cell to divide asymmetrically to produce an anterior daughter MS, and posterior daughter E. In the absence of signal, the EMS cell divides symmetrically to produce two daughters that adopt the MS fate. This thesis describes the identification and analyses of seven genes required to transduce this polarity-inducing signal and specify endoderm formation. The *mom-1, mom-2, mom-5, apr-1*, and *wrm-1* genes are homologous to components of the Wnt/Wingless signal transduction pathway, and the *mom-4*, and *lit-1* genes are related to components of the mitogen-activated protein kinase pathway. Biochemical analysis of these signaling molecules reveal a novel convergence of these pathways at the level of the LIT-1 and WRM-1 proteins, which appear to function as a kinase complex and are required for the downregulation of POP-1. Together these genes constitute components of a complex genetic pathway required for specification of the E cell fate.
GENERAL INTRODUCTION

BACKGROUND

Metazoans, multicellular organisms such as ourselves, can be composed of different tissues, each of which can be composed of many different cell types, all of which are born from the zygote, a single cell fertilized embryo. During embryonic development cells must become different from each other to produce all the different cell types and tissues that exist in an adult organism. An important area of research in developmental biology is to understand how cell fates become specified. How do two sister cells become different from each other?

Introduction to C. elegans

The free-living nematode Caenorhabditis elegans is an ideal model organism for the study of cell fate specification. The adult hermaphrodite is composed 959 somatic cells that make up the pharynx (muscular organ in the head used for feeding), intestine, neurons, muscles, the somatic gonad, and the hypodermis (epidermis). C. elegans are clear allowing for easy view of the internal organs. The cell lineage is invariant and has been mapped out from zygote to adult allowing for identification of cell fate changes by virtue of changes in the amounts of specific tissues produced and by cell lineage analysis. Additionally C. elegans are well suited for genetic manipulation. C. elegans exists as self-fertilizing hermaphrodites and males allowing for easy maintenance of stocks and for crossing of different strains. The generation time is three days and a hermaphrodite has
300-350 progeny making for rapid genetics with no shortage of progeny for analysis. More information about *C. elegans* can be found at the *C. elegans* website (http://elegans.swmed.edu/) and Wood (1988) and Riddle et al. (1997).

**RNA-mediated interference**

*C. elegans* is the first metazoan with a nearly complete genome sequence (*C. elegans* Sequencing Consortium, 1998) making it an ideal organism for reverse genetic analysis in addition to forward genetics. PCR-based deletion screening and transposon insertion reverse genetic techniques are being utilized to identify gene function in *C. elegans*. Another technique, RNA-mediated interference (RNAi), can be used to rapidly identify biological roles for genes of interest (Rocheleau et al., 1997; Fire et al., 1998; for review see Tabara et al., 1998). RNA corresponding to a specific gene injected into a hermaphrodite can result in progeny that phenocopy the loss-of-function phenotype of that gene, and in some cases the injected animal itself is affected. Phenotypes can be seen as soon as twelve hours post-injection. The mechanism by which RNAi works is not yet known. RNAi is different from anti-sense, in that it is more potent and both anti-sense and sense strands are effective. Recently it has been shown that double-stranded RNA (dsRNA) works more efficiently than either single strand alone (Fire et al., 1998). Although RNAi for many genes phenocopies the known mutant phenotype, there are some genes for which RNAi does not work. Some genes appear to be more sensitive than others, for example maternal genes are generally more sensitive than zygotic genes. Because the mechanism of action is not known, RNAs that due not exhibit a phenotype...
can not be assumed to not have a function. It is currently believed that RNAi functions post-transcriptionally (for review, see Tabara et al., 1998). Mutations have been identified that block the effects of RNAi (Tabara et al., 1999). The cloning of two of these mutations has revealed that rde-1 (RNAi-deficient) encodes a protein with some homology to rabbit eIF-2A, a protein implicated in translation initiation (Tabara et al., 1999) and mut-7 (mutator) which shares homology with the exonuclease domain of ribonuclease D (Ketting et al., 1999). Thus, how these genes function in RNAi remains a mystery. Perhaps the cloning of the additional RNAi-deficient mutants identified by Tabara et al. (1999) will provide more incite into the mechanism of RNAi. RNAi is no longer a C. elegans specific technique, and has been extended to Drosophila (Kennerdell and Carthew, 1998) suggesting the mechanism is conserved and may eventually be applicable in other model organisms such as the mouse.

C. elegans embryonic development

During C. elegans development a series of determined cell cleavages produce six cells that generate distinct sets of tissues, the primordial germ cell P₄, and the five somatic founder cells AB, MS, E, C, and D (Figure I-1, p.5 and Sulston et al., 1983). These and other early cell fates are specified by cell-cell interactions and by asymmetric divisions, either induced or under intrinsic control. Genetic screens have identified a number of genes required for the fates of these early blastomeres (Schnabel and Priess, 1997).
Initial anterior-posterior (A-P) polarity is established during fertilization with the point of sperm entry defining the posterior of the embryo (Goldstein and Hird, 1996). The fertilized embryo (P₀) and the subsequent divisions of P₁-P₃ occur asymmetrically.

Figure I-1. Early embryonic cell lineage. A) Lineage tree depicting the cell divisions that result in the formation of the five somatic cells (AB, MS, E, C, and D) and the germ cell (P₄) and the tissues produced. Cell divisions are depicted by horizontal lines. Anterior is to the left and posterior is right. (Adapted from Sulston et al., 1983; Kemphues and Strome, 1997). B) Diagram of embryos at the different stages of development corresponding to the birth of the somatic cells in A. Each founder cell and its lineage are differentially shaded.
across the A-P axis (Sulston et al., 1983). These cells appear to have an intrinsic polarity that is not present in other cells of the embryo (Kemphues and Strome, 1997). Mutations in pie-1 result in the P2 cell adopting the EMS somatic cell fate, though this mutation results in a cell fate change, it does not appear to disrupt the intrinsic polarity of this lineage (Mello et al., 1992; Goldstein, 1995a). Mutations in the par genes disrupt A-P polarity in the early embryo resulting in a first cleavage that is symmetric. This loss of asymmetry results in equal segregation of maternal cell fate determinants and as a consequence the loss and duplication of many early cell fates (reviewed in Kemphues and Strome, 1997; Bowerman et al., 1997). Thus the par genes are likely required to maintain this intrinsic A-P polarity in the embryo after it has been established during fertilization.

Cell-cell interactions have been shown to be required for the specification of some cell fates in the AB lineage (reviewed in Schnabel and Priess, 1997). The division of the AB cell along the dorsal-ventral axis is symmetric (Priess and Thomson, 1987). Due to the constraints of the egg shell, only the ABp daughter contacts the P2 cell (Figure 1-1B, p.5), this interaction specifies it to adopt a cell fate different from its sister ABa (Bowerman et al., 1992b; Hutter and Schnabel, 1994; Mango et al., 1994; Mello et al., 1994; Moskowitz et al., 1994). Mutations in either glp-1, or apx-1 result in the ABp cell adopting the fate of its sister ABa (Priess et al., 1987; Mango et al., 1994; Mello et al., 1994). GLP-1 encodes a transmembrane receptor homologous to Drosophila Notch and C. elegans LIN-12 (Priess et al., 1987; Yochem and Greenwald, 1989) and is present on both ABa and ABp (Evans et al., 1994). APX-1 encodes a transmembrane protein related
to the *Drosophila* Notch ligands, Delta and Serrate (Mello et al., 1994), and is located on the surface of P$_2$ (Mickey et al., 1996). Thus a Notch signaling pathway transduces a signal from the intrinsically polarized P$_2$ cell to specify the ABp cell fate.

Another signal from the P$_2$ cell has been identified that is required for the specification of the E cell fate. EMS divides asymmetrically to produce an anterior daughter MS, that gives rise to primarily mesodermal tissues, and a posterior daughter, E, that forms all the endoderm (intestine) (Figure I-1, p.5 and Sulston et al., 1983). When EMS is cultured in isolation it fails to form intestinal tissue, and only MS-like tissues are produced (Goldstein, 1992; 1995a). The formation of intestinal tissue from the isolated EMS cell can be restored when EMS is placed back in contact with P$_2$, but not when it is placed in contact with the ABa or ABp cells (Goldstein, 1992). In addition, the restored E cell is always born from the side of EMS in contact with P$_2$ (Goldstein, 1995a). This indicates an inductive signal from P$_2$ cell is required to induce polarity in the EMS cell such that it divides asymmetrically to specify the E cell fate.

This thesis describes the identification and analysis of genes that encode and transduce this polarity-inducing signal. These genes are related to components of two well studied signal transduction pathways, Wnt/Wingless (Wnt/Wg) and Mitogen activated protein kinase (MAPK) signaling.

**Signal transduction pathways**

Signal transduction pathways consist of a group of proteins that function together to relay messages from outside the cell. Often these signals are secreted proteins that
bind to transmembrane receptors at the cell surface. Ligand binding triggers a cascade of biochemical events often requiring multiple proteins in the cytosol. This signaling cascade can result in both transcriptional and non-transcriptional outputs. Many signal transduction pathways have been identified and are well studied. Early analysis of these pathways had identified genes that appear to function in linear cascades of events required for the transduction of these signals. Over the years, it has become clear is that signaling is not so simple (Arias et al., 1999; Peifer, 1999). Often signaling downstream of the receptors is branched, having multiple outputs. Cross talk between signaling pathways can occur as a result of cells responding to and integrating multiple signals. Overall it appears that signal transduction is a complex process in which these pathways can form networks to mediate cellular responses to extracellular stimuli.

Wnt/Wg signaling

Wnt/Wg signaling and many of its components were identified as genes required for proper segment polarity during Drosophila embryonic development and since have been found to be a commonly utilized signaling mechanism in animal development (for review see Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). Figure I-2, p.9, is a simplistic model of the Wnt/Wg signaling pathway. Armadillo/β-catenin (Arm/β-catenin) plays a central role (Willert and Nusse, 1998). In the absence of signal, cytoplasmic levels of Arm/b-catenin are repressed, and TCF (T cell factor)/LEF (lymphoid enhancer factor) proteins repress Wnt/Wg responsive gene transcription. Upon Wnt/Wg signaling, repression of Arm/β-catenin is lifted and cytoplasmic levels of
Figure I-2. Simple model of Wnt/Wg signaling pathway. The diagram depicts the status of Wnt/Wg signaling components in two cells, without Wnt/Wg signal (left) and with Wnt/Wg signal (right). This model was adapted from the Roel Nusse’s Wnt Gene Homepage (www.stanford.edu/~rnusse/wntwindow.html ).
ArB-catenin can increase allowing for the interaction of ArB-catenin with TCF/LEF where in the nucleus it functions as a transcriptional activator of Wnt/Wg responsive genes (Bienz, 1998; Eastman and Grosschedl, 1999; Nusse, 1999).

The Wnt/Wg signal is a secreted glycoprotein whose family is partly defined by 23 or 24 conserved cysteine residues in nearly parallel positions throughout the length of the protein (Nusse and Varmus, 1992). Secretion of Wnt/Wg requires Porcupine (Porc), a multiple transmembrane protein that localizes to the ER of the signaling cell and appears to function in the processing of Wnt/Wg (Kadowaki et al., 1996). porc mutants can result in the retention of Wnt/Wg in the signaling cell (van den Heuval et al., 1993).

Wnt/Wg is a ligand for the Frizzled (Fz) family of serpentine or seven transmembrane receptors (Ingham, 1996; Perrimon, 1996). Fz was originally identified as being required for planar polarity (Gubb and Garcia-Bellido, 1982). Later Fz2 was found to function as a Wnt receptor (Bhanot et al., 1996) and more recently it has been shown that Fz and Fz2 function redundantly during Drosophila development (Bhat, 1998; Kennerdell and Carthew, 1998; Bhanot et al., 1999; Chen and Struhl, 1999; Müller et al., 1999). Recently Dally, a proteoglycan, has been proposed to facilitate Wnt/Wg interaction with Fz possibly by functioning as a coreceptor (Lin and Perrimon, 1999; Tsuda et al., 1999).

Disheveled (Dsh/Dvl) functions downstream of Wnt/Wg and upstream of Zeste white 3/GSK-3β (Zw3/GSK3) (Klingensmith et al., 1994; Noordermeer et al., 1994; Siegfried et al., 1994; Theisen et al., 1994). Dsh/Dvl is a cytoplasmic protein that upon signaling is believed to be phosphorylated and localized to the membrane (Yanagawa et
Dsh/Dvl contains three conserved domains found in other signaling molecules, none that would be predicted to be catalytic, and has been suggested that it may function as an adaptor (Boutros and Mlodzik, 1999). As yet, no direct interactions between Dsh/Dvl and Fz have been detected, and the mechanism by which Dsh/Dvl relays the signal from Fz to downstream components of the pathway remains unknown. Though casein kinase I (CKI) has recently been shown to phosphorylate Dsh/Dvl, and to be required for Wnt/Wg signaling in *Xenopus* axis duplication assays and in *C. elegans* for specification of endoderm (Peters et al., 1999; discussed in the conclusion).

Activation of Dsh/Dvl results in the downregulation of Zw3/GSK3, a serine/threonine kinase (Siegfried et al., 1994), possibly via protein kinase C, PKC (Goode et al., 1992; Cook et al., 1996). Zw3/GSK3 functions as part of a complex required to maintain low cytoplasmic levels of Arm/β-catenin. This complex consists of Zw3/GSK3 as well as Axin and APC (adenomatous polyposis coli) (reviewed in Wodarz and Nusse, 1998). Exactly how this complex functions is currently a major area of study. Both APC and Axin can bind Zw3/GSK3 and Arm/β-catenin, as well as interact with each other (Rubinfeld et al., 1993, 1996; Su et al., 1993; Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Sakanaka et al., 1998). Zw3/GSK3 can phosphorylate β-catenin and APC. Zw3/GSK3 phosphorylation of APC is thought to promote interaction between Arm/β-catenin and APC (Rubinfeld et al., 1996). Zw3/GSK3 phosphorylation of the N-terminus of β-catenin as well as binding of Arm/β-catenin and APC that are thought to be required for the targeted degradation of cytoplasmic Arm/β-catenin (reviewed in Wodarz...
Axin appears to promote Zw3/GSK3 phosphorylation of β-catenin (Ikeda et al., 1998; Sakanaka et al., 1998). Upon the reception of the Wnt/Wg signal, Zw3/GSK3 is down-regulated, and cytoplasmic levels of Arm/β-catenin are allowed to increase (Figure I-2, p.9). It has recently been reported that Axin can bind Dsh/Dvl, suggesting a link between Dsh/Dvl and Zw3/GSK3 (Smalley et al., 1999).

TCF/LEF proteins are HMG (high mobility group) box DNA binding proteins that require interactions with other proteins to regulate transcription. When coupled with Groucho they can function as a transcriptional repressor. When cytoplasmic levels of Arm/β-catenin are increased as a result of Wnt/Wg signaling, TCF/LEF proteins can form a complex with Arm/β-catenin and function as a transcriptional activator of Wnt/Wg responsive genes (Nusse, 1999; Roose and Clevers, 1999).

**MAPK signaling**

Mitogen-activated protein kinases (MAPK) and their upstream activators are important intermediates for the relay of extracellular signals from the cell surface to the nucleus (Davis, 1993; 1994). MAPK signaling cascades are highly conserved from yeast to mammals and is involved in an array of different physiological processes (Davis, 1993; 1994; Herskowitz, 1995).

MAPK signaling has also been found to function multiple times in the development of flies and worms (Duffy and Perrimon, 1996; Sundaram and Han, 1996; Ip and Davis, 1998; Sternberg and Han, 1998; Tan and Kim, 1999). In *C. elegans* MAPK signaling functions downstream of *let-23 EGFR* and *let-60 ras* for the specification of the
vulval precursor cell fates, male tail development, and meiotic cell cycle progression as well as functioning downstream of an unidentified receptor to regulate the migrations of the sex myoblast cells (Han et al., 1993; Lackner et al., 1994; Wu and Han, 1994; Church et al., 1995; Kornfeld et al., 1995; Wu et al., 1995; Sundaram et al., 1996).

MAPK signaling consists of a cascade of kinases that can transduce a variety of signals from both RTKs (receptor tyrosine kinases) and seven transmembrane receptors (Herskowitz, 1995). MAPK is a ser/thr kinase that is activated by phosphorylation of a tyr and thr residues within its activation loop. This phosphorylation is carried out by a dual specificity MAPK kinase (MAPKK), which in turn is activated via phosphorylation by a MAPKK kinase (MAPKKK) (Davis, 1993). MAPKKK can be activated in a number of ways including by another upstream kinase or via small GTPases such as ras (Fanger et al., 1997). The targets of MAPK activity are also numerous including other kinases, transcriptional regulators and the cytoskeleton (Davis, 1993).

Different MAPKs can be activated by more than one MAPKK, and likewise MAPKKs is can be the target of multiple MAPKKKs. This crosstalk between members of different kinase modules raises the question of how of signal specificity can be obtained (Schaeffer and Weber, 1999). Scaffold proteins such as Ste5p, JIP1, and JIP2 can bind specific sets of MAPKs, MAPKKs, and MAPKKKs, thus localizing them and facilitating activation of the correct downstream kinases (Garrington and Johnson, 1999; Yasuda et al., 1999).
OVERVIEW

Maternal effect lethal screens have identified mutations that result in the duplication of the MS cell fate at the expense of the E fate, and vice versa. (Lin et al., 1995; Rocheleau et al., 1997; Thorpe et al., 1997; Kaletta et al., 1997). Mutations in pop-

I, a HMG box protein of the TCF/LEF family, result in the duplication of the E cell fate at the expense of the MS fate (Lin et al., 1995). POP-1 staining is nuclear and is present in high levels in the MS cell and low levels in the E cell. Thus, POP-1 is required for the MS cell fate, and at low levels, or in the absence of POP-1, MS adopts the E cell fate.

Mutations at five loci were identified that result in a loss of the E cell fate and a duplication of the MS fate (Rocheleau et al., 1997; Thorpe et al., 1997). These five mom (more mesoderm) genes were candidates for encoding the polarity-inducing signal from P2 that specifies the E cell fate. Cloning of mom-1, mom-2, and mom-5 revealed that they encode genes homologous to porc, wnt/wg, and fz, respectively, components of a highly conserved Wnt/Wg signal transduction pathway (Rocheleau et al., 1997; Thorpe et al., 1997). In addition to mom-1, mom-2, and mom-5, reverse genetics utilizing RNAi identified wrm-1 and apr-1 genes related to Wnt/Wg signaling components Arm/β-
catenin and APC respectively as being required for specification of the E cell fate (Rocheleau et al., 1997). Genetic analysis of mom-1, mom-2, mom-5, apr-1, wrm-1 and pop-1 mutations and RNAi phenotypes suggested they might function in a branched genetic pathway (Figure 1-6, p.50). In this pathway two parallel inputs, one encoded by mom-1, mom-2 and mom-5, and the other encoded by apr-1 converge at or upstream of wrm-1. This pathway functions to repress pop-1. In wrm-1(RNAi) embryos POP-1
staining is equal in both MS and E suggesting that the signaling pathway functions to repress the levels of POP-1 in the E cell (Rocheleau et al., 1997).

Throughout C. elegans development a majority of the embryonic divisions occur along the A-P axis, and like the EMS blastomere, result in A-P daughters with different cell fates (Sulston et al., 1983). Mutations in pie-1 and mex-1 result in ectopic expression of the MS cell fate (Mello et al., 1992). Despite their abnormal location within the embryo, these ectopic MS cells appear to execute an MS-like cell lineage with the proper A-P polarity, suggesting the existence of a global A-P polarity cue that cells can recognize regardless of location within the embryo (Mello et al., 1992).

Mutations in lit-1 result in the E cell adopting the MS fate, as seen in mom mutants, resulting in embryos that fail to produce intestine (Kaletta et al., 1997). Lineage analysis of a conditional lit-1 allele revealed that not only is the E cell fate lost and the MS fate duplicated, but that in all daughters of A-P divisions observed, the posterior fates are lost and the anterior fates duplicated. Thus, lit-1 appears to function as a binary switch required for specification of posterior cell fates after an A-P division (Kaletta et al., 1997). Genetic analysis of pop-1, which results in MS adopting an E fate (Lin et al., 1995), found pop-1 is genetically downstream of lit-1 (Kaletta et al., 1997). Thus, lit-1 may function to suppress pop-1 function in the E cell.

Monoclonal antisera raised against POP-1 can detect POP-1 in the nuclei of most embryonic cells as well as in many post-embryonic cells during development (Lin et al., 1998). Not only is POP-1 staining asymmetric in the EMS daughters MS and E, but POP-1 staining is asymmetric in most or all daughters resulting from an A-P division.
POP-1 staining is brighter in the nucleus of the anterior daughter, and POP-1 staining is equal in the daughters of a transverse cleavage. Lineage analysis of pop-1 mutants indicated that it is also required for cell fates of cells of the AB lineage and POP-1 asymmetric staining requires mom-2, wrm-1, and apr-1 (Lin et al., 1998). Like lit-1, pop-1 appears to be required in the specification of cell fates resulting from an A-P cell division, suggesting that LIT-1 and POP-1 are functioning together to determine cell fates as a consequence of an A-P polarity cue. The requirement of mom-2, wrm-1, and apr-1 for the asymmetry of POP-1 in the AB lineage suggests that genes required for the polarity of the EMS blastomere are also required for polarity of other cells in the embryo that divide along the A-P axis.

Cloning of lit-1 revealed that it is homologous to the *Drosophila* polarity gene nemo, a serine (ser)/threonine (thr) kinase related to the MAPK family (Meneghini et al., 1999; Rocheleau et al., 1999). nemo is required for the polarity of the ommatidia in the *Drosophila* eye. The ommatidia of nemo mutants fail to complete their 90° rotation during morphogenesis (Choi and Benzer, 1994). lit-1 mutants and RNAi phenotypes resemble wrm-1 RNAi embryos, and both appear to function genetically upstream of pop-1 (Kaletta et al., 1997; Rocheleau et al., 1997), suggesting that LIT-1 and WRM-1 may function together to regulate POP-1. Consistent with that hypothesis, POP-1 staining is symmetric in lit-1 mutant embryos (Meneghini et al., 1999; Rocheleau et al., 1999). Binding studies found that WRM-1 and LIT-1 can bind each other, and that this interaction is required for LIT-1 kinase activity. The active WRM-1/LIT-1 kinase can phosphorylate LIT-1, WRM-1 and POP-1, suggesting that WRM-1/LIT-1 might regulate
POP-1 via phosphorylation. Expression studies in vertebrate cells show that POP-1 alone is localized predominantly in the nucleus, though addition of WRM-1 and LIT-1 results in a redistribution of POP-1 staining from nucleus to cytoplasm (Rocheleau et al., 1999). Thus, phosphorylation of POP-1 by the WRM-1/LIT-1 kinase complex may be necessary for maintaining low levels of POP-1 in the E cell.

The homology between LIT-1 and members of the MAPK family of ser/thr kinases suggests that LIT-1 might be activated by upstream kinases. The cloning of mom-4 revealed that it encodes a gene related to the vertebrate TGF-β activated kinase, TAK1 (Meneghini et al., 1999; Shin et al., 1999). TAK1 was found in an assay to identify mammalian MAPKKK activity that can substitute for the yeast MAPKKK, Ste11p, as can activated forms of Raf and MEKK1 (Yamaguchi et al., 1995). TAK1 can phosphorylate the MAPKK, XMEK2 (SEK1) in vitro, and has been shown to activate JNK and NF-kB pathways in response to IL-1 signaling (Yamaguchi et al., 1995; Moriguchi et al., 1996; Shirakabe et al., 1997; Ninomiya-Tsuji et al., 1999). Mutations in mom-4 genetically enhance the mom phenotypes of the Wnt/Wg signaling components mom-2, mom-5, and apr-1, as do weak mutations in lit-1, suggesting that MOM-4 functions in a pathway separate from that of the Wnt/Wg pathway and may be an activator of LIT-1 (Shin et al., 1999). Coexpression of MOM-4 with LIT-1/WRM-1 kinase in vertebrate cells can activate LIT-1 kinase activity, and this activation requires the LIT-1 activation loop (Shin et al., 1999). This data suggests that MOM-4 does function as an upstream activator of LIT-1 and that MOM-4 and LIT-1 are part of a MAPK-like cascade that converges with a Wnt pathway to specify the E cell fate.
Chapter I of this thesis describes the identification of components of a Wnt/Wg signaling pathway required for the specification of the E cell fate from the EMS blastomere. Both forward and reverse genetics identified the genes *mom-1, mom-2, mom-5, wrm-1,* and *apr-1* that are homologous to the Wnt/Wg pathway components *porc, wnt/wg, fz, arm/β-catenin,* and APC respectively. Genetic analysis indicates that these genes can encode parallel inputs, one encoded by *mom-1, mom-2,* and *mom-5,* and the other by *apr-1,* and that these inputs likely converge upstream or upon *wrm-1.* In addition these genes are genetically upstream of *pop-1,* which exhibits the opposite phenotype. Staining of POP-1 which has previously been shown to be asymmetrically distributed between the MS and E cells indicates that it is distributed symmetrically in mutant backgrounds consistent with corresponding cell fate change. These genes are also shown to be required for the specification of the E cell fate from the P_2 cell in a *pie-1* mutant background in which the P_2 cell adopts the fate of its sister EMS. In addition it was found that the *mom-1, mom-2,* and *mom-5* genes, but not *wrm-1, apr-1* or *pop-1* are required for the spindle orientation of the ABar blastomere in the 8-cell stage embryo, indicating that Wnt/Wg signaling is involved in controlling spindle orientation using different downstream signaling components. This work presents data that represents a departure from genetic data of Wnt/Wg signaling in other systems. First, *apr-1* is functioning positively with *wrm-1* for specification of the E cell fate. In other systems APC is functioning to downregulate Arm/β-catenin, thus it would be expected that *apr-1* should have the opposite phenotype of *wrm-1* if APR-1 was functioning like APC. Second, in other systems Arm/β-catenin in response to Wnt/Wg signal binds to TCF/LEF
and activates transcription of Wnt responsive genes. This study shows that POP-1 and WRM-1 have opposite phenotypes and WRM-1 appears to repress POP-1. The relationship between these genes appears to be different in these different systems.

Though, it has been shown that TCF/LEF proteins can function as both activators and repressors of Wnt/Wg responsive genes (discussed in the general discussion).

Chapter II describes the identification of a C. elegans homolog of the Drosophila tissue polarity gene nemo as being required for the specification of the E cell fate. We show that CeNemo is lit-1. POP-1 staining is symmetric in the lit-1 mutant background. LIT-1 is a ser/thr kinase related to MAPK family and requires WRM-1 as a coactivator. LIT-1/WRM-1 can form a complex both in vitro and in vivo. The LIT-1/WRM-1 complex can phosphorylate POP-1 and change its subcellular localization in vertebrate cells, suggesting a mechanism for downregulation of POP-1 in the embryo. lit-1 is required for multiple Wnt/Wg signaling pathways in C. elegans, functioning downstream of mom-2 and mom-5 in the embryo, and downstream of lin-44 (Wnt/wg) and lin-17 (fz) in T cell polarity. This work is the first report of a MAPK-related protein functioning as a necessary component of a Wnt signaling pathway and the only report of a β-catenin related molecule binding and functioning as an activator for a kinase analogous to a cyclin for a cdk (cyclin dependent kinase).

Chapter III describes the cloning and genetic analysis of mom-4. Genetic analysis suggests mom-4 functions in a separate pathway from the other mom genes that encode components of the Wnt signaling pathway. MOM-4 is related to the MAPKKK, TAK1. Like lit-1 and the Wnt/Mom pathway, mom-4 is required for the asymmetric POP-1
staining between A-P daughters. MOM-4 can activate the LIT-1/WRM-1 kinase complex, and this activation requires the LIT-1 activation loop. This data suggests that MOM-4 and LIT-1 encode components of a MAPK-related cascade. More interesting though is that it appears that this cascade converges with a Wnt/Wg pathway at the level of LIT-1 and WRM-1, and that these MAPK and Wnt/Wg pathways function together to repress POP-1.

Together these studies describe the molecular identification of a polarity-inducing signaling pathway that is required to polarization the EMS blastomere and specify the posterior cell fate, the E blastomere. This pathway is composed of a Wnt signaling pathway and a MAPK-related pathway that converge at the point of an Arm/β-catenin related molecule, WRM-1, and LIT-1, a MAPK-related protein homologous to Drosophila Nemo and mouse Nlk. Together these pathways function to repress the POP-1, TCF/LEF transcription factor related protein, in the posterior daughter of an A-P cell division, E. This pathway appears to transduce a polarity cue that specifies the cell fates of cells born of an A-P cell division during C. elegans embryogenesis.
CHAPTER I: WNT SIGNALING AND AN APC-RELATED GENE SPECIFY ENDODERM IN EARLY C. elegans EMBRYOS

SUMMARY

In a 4-cell stage C. elegans embryo, signaling by the P2 blastomere induces anterior-posterior polarity in the adjacent EMS blastomere, leading to endoderm formation. We have taken genetic and reverse genetic approaches toward understanding the molecular basis for this induction. These studies have identified a set of genes with sequence similarity to genes that have been shown to be, or are implicated in, Wnt/Wingless signaling pathways in other systems. The C. elegans genes described here are related to wnt/wingless, porcupine, frizzled, β-catenin/armadillo and the human adenomatous polyposis coli gene, APC. We present evidence that there may be partially redundant inputs into endoderm specification, and that a subset of these genes appear to also function in determining cytoskeletal polarity in certain early blastomeres.
INTRODUCTION

In *C. elegans* embryogenesis, many of the divisions of early cells, called blastomeres, result in anterior and posterior sisters with different fates. Part of the anterior-posterior (A-P) polarity of the early embryo appears to be determined by the site of sperm entry (Goldstein and Hird 1996). A second source of A-P polarity requires a cell-cell interaction at the 4-cell stage of embryogenesis between blastomeres called EMS and P2 (Goldstein 1992, 1993). In normal development, the EMS blastomere divides into an anterior daughter called MS that produces mesoderm, and a posterior daughter called E that produces only endoderm. The ability of EMS to produce daughters with different fates appears to require signaling by the P2 blastomere. If P2 is prevented from contacting EMS, neither EMS daughter produces endoderm, and both daughters adopt MS-like fates. P2 normally contacts the posterior surface of EMS, however if P2 is repositioned to contact the anterior surface, the fates of the EMS daughters are interchanged. Thus P2 induces both endoderm formation and the A-P polarity of the EMS blastomeres; we refer to this induction as P2-EMS signaling.

Previous studies suggested that P2-EMS signaling may induce the E fate by lowering the amount, or activity, of POP-1 protein in the E blastomere (Lin et al., 1995). In a wild-type embryo, POP-1 appears to be present at a high level in the MS nucleus and at a lower level in the E nucleus. In a mutant lacking detectable POP-1 in both MS and E, both blastomeres adopt E-like fates and produce endoderm. POP-1 is an HMG-domain protein similar to the vertebrate TCF-1 and LEF-1 proteins, and to the recently described Pangolin/dTCF protein of *Drosophila* (Brunner et al., 1997; van de Wetering et
Genetic and/or biochemical studies suggest that Pangolin/dTCF and TCF/LEF family members can function as downstream components in a conserved Wnt/Wingless (Wnt/Wg) signaling pathway (see Nusse and Varmus, 1992; Klingensmith and Nusse, 1994; and Moon et al., 1997 for reviews). Thus P2-EMS signaling might involve POP-1 and a Wnt/Wg-like pathway in *C. elegans*. However loss of Wg signaling and mutations in *pangolin* cause similar phenotypes (Brunner et al., 1997), while loss of P2-EMS signaling and mutations in *pop-1* cause opposite phenotypes (Lin et al., 1995).

To determine the molecular basis for P2-EMS signaling, we performed genetic screens to look for mutants that lacked endoderm (the E fate) and that overproduced mesoderm (the MS fate). As a second approach, we searched the *C. elegans* Genome Sequence database for homologs of genes implicated in Wnt/Wg signaling, and tested by a reverse genetic assay whether these genes were required for P2-EMS signaling. In this report, we show that three new genes identified in our genetic screens encode products that are similar to components of the Wnt/Wg pathway. Two additional genes identified by our reverse genetic assay also appear to be required for P2-EMS signaling; one can encode a protein related to β-catenin, and the other is similar to the human adenomatous polyposis coli (APC) gene (Groden et al., 1991). Our results provide strong evidence that P2-EMS signaling in *C. elegans* involves a Wnt/Wg-like pathway, although downstream components in this pathway may have novel roles. Finally, our studies provide evidence for partial redundancy within this pathway involving the APC-related gene. These observations indicate that the E blastomere, which has one of the simplest developmental
patterns of any cell in *C. elegans*, may be specified by a surprisingly complex mechanism.
RESULTS

Molecular identification of genes required for endoderm formation

Previous genetic screens by several laboratories have identified genes required for endoderm development. For example, mutations in the \( \text{skn-1} \) gene cause a high percentage of embryos to lack endoderm (Bowerman et al., 1992a). However none of the mutations reported thus far cause the specific transformations predicted from a defect in P2-EMS signaling. When P2-EMS signaling is prevented in wild-type embryos, the E blastomere develops like an MS blastomere (Goldstein 1992, 1993). First, E does not produce endoderm, and instead produces pharyngeal tissue and body-wall muscles, which are mesodermal tissues normally produced by MS. Second, the E blastomere adopts an accelerated cleavage rate similar to MS; for example, the E daughters divide prematurely at about the same time as the MS daughters (Figure 1-1, p.26).

We used a reverse genetic assay to test whether 12 genes in the \( \textit{C. elegans} \) database that have homology to known, or potential components, of the Wnt/Wg pathway function in P2-EMS signaling (see Experimental Procedures). For this assay, RNA from the relevant cDNA or PCR-amplified gene fragment was injected into the gonads of wild-type adult hermaphrodites (see Experimental Procedures). We and others have found that this RNA procedure precisely reproduces phenotypes that are known to result from strong or null mutations in almost all of the maternally-expressed genes tested (Guo and Kemphues, 1995;1996; Lin et al., 1995; Mello et al., 1996; Powell-Coffman et al, 1996; Guedes and Priess, 1997). For each of the genes examined thus far, this procedure has been shown to result in a lack of protein expression (Lin et al., 1995; Powell-Coffman et
Figure 1-1. MS and E development. A schematic diagram of a 4-cell stage embryo is shown with abbreviated lineages of the sister blastomeres MS and E. These lineages show the different division times of the MS and E daughters in wild-type embryogenesis, and the unequal divisions (>) and cell death that normally occur in one branch of the MS lineage. The shaded boxes refer to abnormalities observed in mutants described in the text.
The mechanism underlying the RNA-induced defect appears to be distinct from that of conventional antisense RNA because both the sense and antisense RNA strands cause similar defects (see above references, S. Driver and C. Mello unpublished observations). Because the mechanism is not known, we will refer to this technique as RNAi, for RNA mediated interference; embryos thus treated will be indicated by listing the gene name followed by RNAi.

We identified a gene related to Drosophila armadillo (arm) and vertebrate β-catenin, and call this gene wrm-1 (for worm arm motif gene; Figure 1-2, p.28). The predicted WRM-1 protein contains the same number of the repeated "arm" motif that is found in Arm/β-catenin (Riggleman, 1989) but is considerably diverged in overall amino acid sequence identity (Figure 1-2, p.28 and data not shown). We found that wrm-1(RNAi) embryos have phenotypic defects that are very similar to those caused by defects in P2-EMS signaling; all of these embryos fail to produce endoderm and instead produce abnormally large quantities of pharyngeal tissue (Figure 1-3, p.30 and Table 1-1, p.32). We examined the fate of the E blastomere in wrm-1(RNAi) embryos by killing all other blastomeres with a laser microbeam after the period that P2-EMS signaling normally occurs (see Experimental Procedures). After E was allowed to develop for several hours, the resulting partial embryos were examined, then fixed and stained with tissue-type specific antibodies. The E blastomere failed to produce endoderm and instead produced pharyngeal tissue in 8/8 laser operated embryos. We followed the early cleavages patterns in 11 wrm-1(RNAi) embryos, and found that these patterns are normal except for
Figure 1-2. Schematic representations of the predicted WRM-1, APR-1, MOM-1, MOM-2, and MOM-5 proteins. The predicted proteins shown are based on cDNA sequences and/or genomic sequences available from the C. elegans Genome Sequencing Project; molecular lesions in some of the mom mutants are indicated. Proteins are not drawn to scale; the predicted sizes in amino acids are indicated at the C-termini (right). Potential signal peptides and transmembrane domains are indicated by black and gray
boxes, respectively. WRM-1 has 12 arm motifs (stippled boxes) that are 23% identical to human β-catenin (Hülsken et al., 1994) and 22% identical to Drosophila Armadillo (Riggleman 1989); no significant homologies are observed outside of these domains. APR-1 is much smaller than the 2843 amino acid APC protein in humans (Groden et al., 1991), however both proteins contain 7 arm motifs, and APR-1 and APC are 31% identical in these regions. APC and APR-1 contain 3 and 2 copies, respectively, of the sequence [I/L-X-E/R-C/S-I-X-S-A/E-M-P-T/K]; in APC this motif occurs at residue 1574, 1723 and 2038 within a region believed to be important in interactions with β-catenin. MOM-1 has 29% identity to Drosophila Porcupine (Kadowaki et al., 1996), including five, similarly positioned possible transmembrane domains. MOM-2 has 34% identity to Drosophila Wingless (Rijsewijk et al, 1987) and 35% identity to human Wnt2 (Wainwright et al., 1988). Conserved cysteine residues found in all Wnt proteins are indicated by vertical lines. MOM-5 has 37% identity to Drosophila Frizzled (Adler et al., 1990) and 33% identity to Frizzled2 (Bhanot et al, 1996). The shaded box at the N-terminus corresponds to the CRD (cysteine rich extracellular domain) thought to mediate interactions between Frizzled2 and Wg (Bhanot et al., 1996); conserved cysteine residues are indicated by vertical lines.
Figure 1-3
Figure 1-3. The *Mom* phenotype. The E blastomere in *wrm-1(RNAi)* embryos, *apr-1(RNAi)* embryos, and *mom* mutant embryos can produce mesoderm instead of endoderm. This defect results in the phenotype illustrated by the *mom-1(se2)* mutant shown in the right column; the ABar cleavage defect unique to the *mom* mutants is shown in (h). (a and b) Light micrographs of living embryos. The wild-type embryo has initiated morphogenesis and will ultimately elongate fourfold. The terminal stage *mom* mutant embryo has not elongated. (c and d) Immunofluorescence micrographs of terminal stage embryos stained with mABICB4; this antibody recognizes the intestine (endoderm), non-endodermal valve cells (pair of long arrows, Bowerman et al., 1992b) and a few neurons (pair of short arrows). (e and f) Immunofluorescence micrographs of embryos after staining with mAB3NB12; this antibody stains a subset of muscles in the pharynx, many of which are derived from the MS blastomere. (g and h) Early cell divisions. In the wild-type embryo the ABar spindle (left arrow) is orthogonal to other AB descendants such as ABpr (right double arrow). In the *mom* mutant ABar (left double arrow) and ABpr (right double arrow) have parallel spindles.
Embryos lacking MS/E cleavage timing

<table>
<thead>
<tr>
<th>Embryo type</th>
<th>Embryos lacking Endoderm (n)</th>
<th>MS/E cleavage timing*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-5min</td>
</tr>
<tr>
<td>wild-type</td>
<td>0% (350)</td>
<td>0</td>
</tr>
<tr>
<td>pop-1 (zu189)</td>
<td>0% (200)</td>
<td>n.d.</td>
</tr>
<tr>
<td>(RNAi)</td>
<td></td>
<td>7**</td>
</tr>
<tr>
<td>wrm-1 (RNAi)</td>
<td>100% (179)</td>
<td>14</td>
</tr>
<tr>
<td>apr-1 (RNAi)</td>
<td>26% (383)</td>
<td>5</td>
</tr>
<tr>
<td>mom-1 (se2)</td>
<td>62% (144)</td>
<td>2</td>
</tr>
<tr>
<td>(ne117)</td>
<td>62% (100)</td>
<td>n.d.</td>
</tr>
<tr>
<td>(zu188)</td>
<td>56% (296)</td>
<td>n.d.</td>
</tr>
<tr>
<td>(zu204)</td>
<td>41% (397)</td>
<td>n.d.</td>
</tr>
<tr>
<td>(zu237)</td>
<td>41% (100)</td>
<td>n.d.</td>
</tr>
<tr>
<td>mom-2 (ne141)</td>
<td>39% (879)</td>
<td>4</td>
</tr>
<tr>
<td>(RNAi)</td>
<td>14% (269)</td>
<td>0</td>
</tr>
<tr>
<td>(ne141)/ mDf3</td>
<td>40% (322)</td>
<td>n.d.</td>
</tr>
<tr>
<td>(ne141) (RNAi)</td>
<td>67% (196)</td>
<td>n.d.</td>
</tr>
<tr>
<td>mom-5 (ne12)</td>
<td>5% (63)</td>
<td>0</td>
</tr>
<tr>
<td>(zu193)</td>
<td>5% (126)</td>
<td>0</td>
</tr>
<tr>
<td>(RNAi)</td>
<td>2% (186)</td>
<td>0</td>
</tr>
<tr>
<td>(zu193)/(RNAi)</td>
<td>8% (229)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table 1-1. Development of the E blastomere. *Wild-type E daughters divide 20 to 25 minutes after MS daughters (see Figure 1-1, p.26). The time between MS and E divisions has been broken into three intervals. The number of embryos that exhibited E division within each interval is indicated. For example in all 14 wrm-1 (RNAi) lineages examined the E daughters divided within a five minute interval following the division of the MS daughters. **In pop-1 (RNAi) embryos, MS daughters adopt an E-like cleavage, and divide later than normal. n.d. = not determined.
the E daughters, which divide prematurely (Figure 1-1, p.26 and Table 1-1, p.32). The close similarity between these defects and those caused by defects in P2-EMS signaling suggests that the wrm-1 gene plays an essential role in P2-EMS signaling in wild-type embryogenesis.

To test if wrm-1 was required for the different apparent levels of POP-1 protein observed in the MS and E nuclei of wild-type embryos (Figure 1-4a, p.34), we stained 8-cell stage wrm-1(RNAi) embryos with an antibody that recognizes POP-1. We found that the E nucleus appears to contain an abnormally high level of POP-1 that is similar to the level in the MS nucleus (Figure 1-4b, p.34). To determine whether pop-1(+) activity prevents E from producing endoderm in the wrm-1(RNAi) embryos, we constructed and analyzed pop-1(zu189);wrm-1(RNAi) embryos. In contrast to wrm-1(RNAi) embryos, which produce no endoderm (Table 1-1, p.32), all of the pop-1(zu189);wrm-1(RNAi) embryos contain endoderm, as do pop-1(RNAi) wrm-1(RNAi) embryos (Table 1-2, p.44, see also Experimental Procedures). To determine if a loss of pop-1(+) activity allows E to produce endoderm irrespective of P2 signaling, we removed the P2 blastomere from 4-cell stage pop-1(zu189) embryos following the procedures of Goldstein (1992), and found that both daughters of EMS produced endoderm in 7/7 experiments (data not shown). These results together are consistent with a model that P2-EMS signaling decreases or inhibits pop-1(+) activity in E (Lin et al., 1995), and suggest that wrm-1(+) activity plays a role in this process.

Several recent studies have implicated the human colon cancer associated gene, APC, as a possible regulator of β-catenin (Morin et al., 1997; Korinek et al., 1997;
wild type    wrm-1

POP-1 localization

Figure 1-4
Figure 1-4. POP-1 localization. Immunofluorescence micrographs of a wild-type and a *wrn-1(RNAi)* embryo at the 8-cell stage stained with mABRL2, an antibody that recognizes the POP-1 protein (a and b) and with DAPI to visualize nuclei (bottom panels). Each doubleheaded arrow points to an MS nucleus (c and d) and an E nucleus (right end).
Rubinfeld et al., 1997). We identified from the database a gene related to APC that we call apr-1, for APC-related gene (Figure 1-2, p.28). We found that about 26% of apr-1(RNAi) embryos lack endoderm, overproduce pharyngeal tissue, and have a premature division of the E daughters (Table 1-1, p.32); these embryos have partial body morphogenesis, in contrast to wrm-1(RNAi) embryos that have no body morphogenesis (Figure 1-3, p.30 and data not shown). Laser ablation experiments similar to those described above confirmed that the E blastomere in 2/9 apr-1(RNAi) embryos adopts an MS-like fate; E produces pharyngeal tissue and the E daughters divide prematurely. Thus apr-1 and wrm-1 both appear to play a role in endoderm specification, while wrm-1 may have additional functions required for proper morphogenesis.

We tested eleven other genes that showed sequence similarity to components of the Wnt/Wg signaling pathway for their effects on endoderm development (see Experimental Procedures). None of our experiments on two disheveled-related genes resulted in endoderm defects. However experiments on one of five wingless-related genes, and one of three frizzled-related genes, caused endoderm defects. Because these same two genes were identified independently in our genetic screens, they are described below.

The mom genes and P2-EMS signaling

In genetic screens we identified 13 independent mutants that produce embryos that lack endoderm and that have abnormally large amounts of pharyngeal tissue; we call these mom mutants for more mesoderm. These mutants identified five genes: mom-1 (five
alleles), mom-2 (one allele), mom-3 (one allele), mom-4 (four alleles), and mom-5 (two alleles). In this paper, we describe the mom-1, mom-2, and mom-5 genes, which we have cloned. All of the mutations in the mom genes cause maternal-effect lethality (see Experimental Procedures). One hundred percent of the embryos produced by homozygous mom mutant mothers fail to hatch and exhibit severe abnormalities in morphogenesis (Figure 1-3, p.30).

The percentage of embryos lacking endoderm varies in the different mom mutants, and also varies between broods from individuals in each strain. For example, a total of 39% of the embryos from mom-2(nei41) mutants lack endoderm (Table 1-1, p.32; see also Figure 1-5a, p.38), but this percentage varies between 14% to 76% in individual broods; embryos lacking endoderm invariably have large amounts of pharyngeal tissue. Laser ablation experiments as described above showed that in mom-1 and mom-2 embryos the MS blastomere produces pharyngeal tissue, as in wild-type embryos, however the E blastomere also adopts an MS-like fate; E produces pharyngeal cells, and the E daughters divide prematurely. The percentage of isolated E blastomeres with this pattern of development correlated with the expected frequencies of embryos lacking endoderm; E produced pharyngeal cells instead of endoderm in 10/16 mom-1(se2) mutants, 4/10 mom-2(nei41) mutants, and 2/15 mom-5(zui93) mutants. We stained 8-cell stage mom-1(se2) mutants for POP-1 and found that about 65% (n=30) had higher than normal levels of staining in the E nucleus (data not shown). These results suggest that the mom genes are involved in endoderm specification, and that they are likely to have a role in P2-EMS signaling.
Figure 1-5

a mom-2(ne141)
b mom-2(ne141);mom-5(RNAi)
c apr-1(RNAi);mom-2(RNAi)
d apr-1(RNAi);mom-2(ne141);pop-1(RNAi)
Figure 1-5. Endoderm differentiation. (a)-(d) each show a field of embryos viewed with polarization microscopy; endoderm-specific granules are birefringent and appear white.
In contrast to the variable penetrance of the endoderm defect, all of the *mom-1*, *mom-2*, and *mom-5* embryos examined had defects in the cleavage of one of the early blastomeres, a defect that was not observed in *wrm-1(RNAi)* or *apr-1(RNAi)* embryos, or in *pop-1* mutants or *pop-1(RNAi)* embryos. In a wild-type, 8-cell stage embryo there are four descendants of the AB blastomere. Three of these descendants have mitotic spindles that are oriented approximately in parallel, however the spindle of the third descendant, ABar, is oriented perpendicular to the others (Figure 1-3g, p.30). We found that the orientation of the ABar spindle is abnormal in *mom* mutants, such that it is parallel to that of the other AB descendants (Figure 1-3h, p.30). The ABar spindle was also abnormal in 4/4 *mom-2(ne141);pop-1(RNAi)* embryos. Because *pop-1* mutants and *pop-1(RNAi)* embryos have normal ABar spindles, these observations suggest that *mom-2(+)* acts through a *pop-1* independent mechanism to control spindle orientation (see Discussion).

To determine if mutations in the *mom* genes cause cleavage defects in other lineages, we examined the development of the MS blastomere in *mom-1(se2)* mutants. We found that certain MS descendants that have unequal cleavages in wild-type embryos instead have equal, or reversed cleavages in *mom-1* mutants; defects were observed in 5/5 embryos examined, although the nature of the polarity defect was variable (Figure 1-1, p.26). One of these polarity defects correlated with a defect in cell differentiation. In wild-type embryos an MS descendant called MSpaapp is born in an unequal cleavage and soon afterward undergoes programmed cell death. In lineaged *mom-1* mutant embryos, we found that MSpaapp was born in a cleavage that was either equal or reversed, and did
not undergo programmed cell death (Figure 1-1, p.26). Thus in addition to their role in the P2-EMS interaction, the *mom* genes have a role in determining the orientation of the ABar spindle, and at least *mom-1* has a role in determining the proper polarities of later MS divisions.

**Molecular analysis of *mom-1*, *mom-2*, and *mom-5***

Mutations in *mom-1* and *mom-5* were identified in screens using a genetic background in which the transposon Tc1 is mobilized (Mello et al., 1994). We found novel Tc1 insertions that mapped to the physical-genetic locations of the *mom-1* and *mom-5* mutations, and used standard techniques to recover the flanking sequences and clone the genes (See Experimental Procedures). We found that *mom-1* encodes a gene related to the *Drosophila* gene *porcupine* (Kadowaki et al., 1996) and that *mom-5* encodes a member of the *frizzled* gene family (Vinson et al., 1989).

The *mom-2* gene was identified by correlating the genetic position of *mom-2(ne141)* to cloned genes present on the *C. elegans* Genome Sequence. Within this interval we found a gene with homology to *wnt/wg* that gave a *mom-2*-like phenotype when tested by the RNAi assay. We confirmed that this gene was *mom-2* by showing that the *mom-2(ne141)* mutation is a lesion in this gene (Figure 1-2, p.28). Thus the predicted MOM-2 protein is homologous to Wnt/Wg, and the predicted MOM-1 and MOM-5 proteins are homologous to proteins implicated in the secretion and reception of the Wnt/Wg signal, respectively.
Because our mutations in the *mom* genes cause incomplete penetrance of the endoderm defect, we initially expected to find that these mutations would create only minor changes in the predicted protein products. However many of the mutations in the *mom-1* gene would be predicted to create a severely truncated protein (Figure 1-2, p.28). The predicted MOM-1 protein is 442 amino acids in length, with five potential transmembrane domains; the *mom-1(zu188)* mutation would be expected to truncate the MOM-1 protein before the first transmembrane domain at only 47 amino acids. The *mom-2(nei41)* mutation is expected to replace a glycine that is highly conserved in Wnt/Wg proteins with an arginine; a glycine to aspartic acid mutation at this same site in the *Drosophila wg* IN67 mutation appears to block Wg secretion and results in a strong, embryonic lethal phenotype resembling the presumptive null phenotype (van den Heuvel et al., 1993). Similarly, the *mom-5(nei2)* mutation would be expected to truncate the MOM-5 protein before the first transmembrane domain.

**Genetic analysis of endoderm specification**

To further address whether the variability in the endoderm defect of the *mom* mutants could be attributed to partial activity of the *mom* gene products, we compared the phenotypes of *mom-2* and *mom-5* mutants with *mom-2(RNAi)* and *mom-5(RNAi)* embryos. We found that *mom-2(RNAi)* and *mom-5(RNAi)* embryos lack endoderm at frequencies that are comparable to the *mom-2* or *mom-5* mutants (Table 1-1, p.32). In another test, we constructed and examined *mom-2(nei41);mom-5(zu193)* double mutants. We expected to find the endoderm defect in this double mutant to be at least as severe as
in the \textit{mom-2(ne141)} mutant. However, only 8\% of these double mutant embryos lack endoderm, compared to 39\% of the \textit{mom-2(ne141)} single mutants (Table 1-2, p.44).

Essentially identical results were observed in \textit{mom-2(ne141);mom-5(RNAi)} embryos (Table 1-2, p.44 and Figure 1-5b, p.38) and in \textit{mom-2(RNAi);mom-5(RNAi)} embryos (Table 1-2, p.44). Finally, we tested the \textit{mom-2(or42)} allele, which contains a partial deletion of the gene (Thorpe et al., 1997) in combination with \textit{mom-5(RNAi)}, and found that only about 9\% of the resulting embryos lack endoderm (data not shown). The observation that more than 90\% of the embryos with mutations in both \textit{mom-2} and \textit{mom-5} are nevertheless able to produce endoderm suggests that neither of these genes are essential for endoderm development (see Discussion).

Because 26\% of the \textit{apr-1(RNAi)} embryos lack endoderm, we asked whether mutations in the \textit{mom} genes would suppress, or enhance, this defect. We found very strong enhancement in all combinations tested (Table 1-2, p.44). For example, 100\% of \textit{apr-1(RNAi); mom-5(zu193)} embryos lack endoderm and produce excess mesoderm, as do 99\% of both \textit{apr-1(RNAi); mom-2(ne141)} embryos and \textit{apr-1(RNAi);mom-2(RNAi)} embryos (Table 1-2, p.44 and Figure 1-5c, p38). Furthermore we found that 22/22 of the latter embryos showed premature division of the E daughters. To determine if \textit{pop-1(+)} activity is responsible for the lack of endoderm in \textit{apr-1(RNAi);mom-2(ne141)} embryos, we constructed \textit{apr-1(RNAi);mom-2(ne141);pop-1(RNAi)} embryos and found that all such embryos have endoderm (Table 1-2, p.44 and Figure 1-5d, p.38).
Table 1-2. Genetic analysis of endoderm development

<table>
<thead>
<tr>
<th>Embryo type</th>
<th>Embryos lacking Endoderm (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pop-1(zu189);wrm-1(RNAi)</td>
<td>0% (86)</td>
</tr>
<tr>
<td>pop-1(zu189);apr-1(RNAi)</td>
<td>0% (173)</td>
</tr>
<tr>
<td>pop-1(RNAi);wrm-1(RNAi)</td>
<td>0% (69)</td>
</tr>
<tr>
<td>pop-1(RNAi);apr-1(RNAi)</td>
<td>0% (121)</td>
</tr>
<tr>
<td>pop-1(RNAi);mom-2(ne141)</td>
<td>0% (88)</td>
</tr>
<tr>
<td>pop-1(RNAi);mom-5(zu193)</td>
<td>0% (47)</td>
</tr>
<tr>
<td>wrm-1(RNAi);apr-1(RNAi)</td>
<td>100% (134)</td>
</tr>
<tr>
<td>wrm-1(RNAi);mom-2(ne141)</td>
<td>98% (256)</td>
</tr>
<tr>
<td>wrm-1(RNAi);mom-5(RNAi)</td>
<td>100% (79)</td>
</tr>
<tr>
<td>mom-2(ne141)</td>
<td>39% (879)</td>
</tr>
<tr>
<td>mom-5(zu193)</td>
<td>5% (126)</td>
</tr>
<tr>
<td>mom-2(ne141);mom-5(zu193)</td>
<td>8% (200)</td>
</tr>
<tr>
<td>mom-2(ne141);mom-5(RNAi)</td>
<td>8% (607)</td>
</tr>
<tr>
<td>mom-2(RNAi);mom-5(RNAi)</td>
<td>7% (259)</td>
</tr>
<tr>
<td>apr-1(RNAi);</td>
<td>26% (383)</td>
</tr>
<tr>
<td>apr-1(RNAi);mom-2(ne141)</td>
<td>99% (145)</td>
</tr>
<tr>
<td>apr-1(RNAi);mom-2(RNAi)</td>
<td>98% (326)</td>
</tr>
<tr>
<td>apr-1(RNAi);mom-5(zu193)</td>
<td>100% (98)</td>
</tr>
<tr>
<td>apr-1(RNAi);mom-5(RNAi)</td>
<td>99% (237)</td>
</tr>
<tr>
<td>apr-1(RNAi);mom-2(ne141);pop-1(RNAi)</td>
<td>0% (58)</td>
</tr>
<tr>
<td>apr-1(RNAi);mom-2(ne141);mom-5(RNAi)</td>
<td>100% (160)</td>
</tr>
</tbody>
</table>
Autonomous endoderm in *pie-1* mutants may involve autocrine signaling

In addition to signaling the EMS blastomere to produce endoderm, the P2 blastomere appears to have a latent ability to produce endoderm itself. In wild-type embryos, P2 is prevented from producing endoderm by the maternal gene *pie-1* (Mello et al., 1992, 1996), which appears to function in a general repression of transcription in P2 (Seydoux et al., 1996). Although an isolated wild-type, or *pie-1* mutant, EMS blastomere cannot produce endoderm without P2 signaling, an isolated *pie-1* mutant P2 blastomere is able to produce endoderm (Goldstein, 1995a). Therefore we were interested in determining whether the genes described in this thesis also are required for the "autonomous" endoderm from P2 in *pie-1* mutants. We found that in all combinations tested (Table 1-3, p.46), the endoderm produced by the P2 and EMS blastomeres in *pie-1* mutant embryos shows the same dependence on these genes as does the endoderm produced by the EMS blastomere in *pie-1*(+) embryos. For example, many *pie-1(zui27);mom-5(RNAi)* embryos and *pie-1(zui27);apr-1(RNAi)* have endoderm, while almost no *pie-1(zui27);apr-1(RNAi);mom-5(RNAi)* embryos have endoderm. The observation that the P2 blastomere requires *wrm-1*, *apr-1*, and the *mom* genes in order to produce endoderm suggests that P2 may undergo autocrine signaling in *pie-1* mutant embryos.
Table 1-3. Endoderm development in *pie-1* mutant embryos

<table>
<thead>
<tr>
<th>Embryo type</th>
<th>Embryos lacking Endoderm (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pie-1</em>(zu127)</td>
<td>0 % (400)</td>
</tr>
<tr>
<td><em>pie-1</em>(zu127);<em>wrm-1</em>(RNAi)</td>
<td>99 % (443)</td>
</tr>
<tr>
<td><em>pie-1</em>(zu127);<em>wrm-1</em>(RNAi);<em>pop-1</em>(RNAi)</td>
<td>0 % (173)</td>
</tr>
<tr>
<td><em>pie-1</em>(zu127);<em>apr-1</em>(RNAi)</td>
<td>54 % (147)</td>
</tr>
<tr>
<td><em>pie-1</em>(zu127);<em>mom-2</em>(ne141)</td>
<td>9 % (200)</td>
</tr>
<tr>
<td><em>pie-1</em>(zu127);<em>apr-1</em>(RNAi);<em>mom-2</em>(RNAi)</td>
<td>93 % (659)</td>
</tr>
<tr>
<td><em>pie-1</em>(zu127);<em>apr-1</em>(RNAi);<em>mom-2</em>(RNAi); <em>pop-1</em>(RNAi)</td>
<td>0 % (48)</td>
</tr>
<tr>
<td><em>pie-1</em>(zu127);<em>mom-5</em>(zu193)</td>
<td>3 % (104)</td>
</tr>
<tr>
<td><em>pie-1</em>(zu127);<em>apr-1</em>(RNAi);<em>mom-5</em>(RNAi)</td>
<td>99 % (673)</td>
</tr>
<tr>
<td><em>pie-1</em>(zu127);<em>apr-1</em>(RNAi);<em>mom-5</em>(RNAi); <em>pop-1</em>(RNAi)</td>
<td>0 % (91)</td>
</tr>
</tbody>
</table>

Table 1-3. Endoderm formation in *pie-1* mutants
DISCUSSION

The mom genes

In an effort to understand how interactions between blastomeres contribute to the numerous A-P differences observed in the early C. elegans embryo, we have begun a genetic analysis of P2-EMS signaling. In this chapter we described the results of genetic screens for mom mutants that lack endoderm and that have abnormally large quantities of mesoderm, the terminal phenotype predicted for a mutant defective in P2-EMS signaling. Our analysis suggests that when the E blastomere fails to produce endoderm in the mom mutants, it produces pharyngeal tissues that normally are made by MS. Thus the mom genes appear to play a role in the P2-EMS signaling pathway as defined by the blastomere isolation and recombination experiments of Goldstein (1992,1993). Our molecular cloning of the mom genes showed that each could encode a protein similar to one of the known components of the Wnt/Wg pathway; MOM-2 is related to the signaling protein Wnt/Wg, and MOM-1 is related to Porc, a protein involved in Wg secretion. These molecular results are consistent with the results of Thorpe et al. (1997) showing that in chimeric embryos mom-2(+) and mom-1(+) functions are required in the signaling blastomere, P2.

The predicted MOM-5 protein has nearly equal similarity to the Frizzled2 (Fz2) and Frizzled (Fz) proteins of Drosophila. Fz2 and Fz are closely related, serpentine receptor-like transmembrane proteins. Fz2 has been proposed to function as a receptor in Wg-mediated signaling events such as cell fate determination (for reviews see Klingensmith and Nusse 1994, Orsulic and Peifer 1996; Perrimon, 1996). Fz appears to
function in a signaling pathway that coordinates the cytoskeletal polarities of epidermal cells (see Adler 1992 for a review). Relatively little is known about the molecular components of the Fz pathway, however the only component known to be shared with the Wg pathway is Disheveled (Gubb, 1993; Theisen et al., 1994). A recent study has implicated a RhoA homolog in the Fz pathway, and suggested possible similarities with G protein mediated, pheromone signaling in yeast (Stutt et al., 1997). Thus Fz and Fz2 may function in distinct pathways, either or both of which may be relevant to understanding mom-5 function in C. elegans. Because the mom mutants are defective in both cytoskeletal polarity and cell fate determination, we discuss these defects separately below.

The role of the mom genes in spindle orientation

We have shown that mom-1, mom-2, and mom-5 mutants have fully penetrant defects in the mitotic spindle orientation of an early AB descendant called ABar, and lineage analysis of mom-1 embryos revealed additional, later defects in the cleavage polarities of other blastomeres. Although the effect of P2 removal on the ABar spindle has not been reported, laser ablation of the P2 blastomere markedly alters the development of ABar while having no, or minor, effects on the development of the other early AB descendants (Schnabel, 1995; Hutter and Schnabel, 1995). There appears to be complex, and distinct, mechanisms that control the normally invariant spindle orientations of early blastomeres in C. elegans (see Goldstein et al., 1993 for review). G protein signaling is implicated in these controls; mutations in gbp-1, a gene encoding the Gβ subunit, cause the early
mitotic spindles to be randomly oriented, and the GBP-1 protein localizes to the spindle asters of dividing blastomeres (Zwaal et al., 1996). Thus the mom genes play a role in spindle orientation (Figure 1-6, p.50), and it is possible this role involves G proteins.

As discussed below, pop-1 and wrm-1 may function downstream of the mom genes to regulate transcriptional events required for endoderm specification, however we have not detected a function for these genes in controlling spindle orientations. Thus the role of the mom genes in spindle orientation may either not involve transcription, or may involve transcriptional regulators other than pop-1. Although most models for Wnt/Wg signaling emphasize transcriptional or chromatin targets of this pathway, early Xenopus embryos that are transcriptionally silent have been shown to respond to Wnt1 class members by increasing gap junctional permeability (Olson et al., 1991). These and similar results have suggested that chromatin cannot be the sole target of Wnt signaling (for review see Moon et al., 1997). In experiments to address which features of early C. elegans development require transcription, Powell-Coffman et al., (1996) inhibited transcription by injecting anti-sense RNA from the ama-1 gene into the gonads of wild-type adults and analyzed the resulting embryos; the ama-1 gene encodes the large subunit of RNA polymerase. They found that all blastomeres had normal cleavages until the 26-cell stage of embryogenesis, consistent with earlier observations that alpha-amanatin treated embryos appeared to have normal early cleavages (Edgar et al., 1994). We have repeated similar ama-1(RNAi) experiments to examine the orientation of the ABar spindle specifically, and found that it was normal (unpublished observations). The
Figure 1-6. Genetic model for the role of the *mom* genes in spindle orientation and in endoderm development. *wrm-1, pop-1*, and *apr-1* appear to have no role in determining the early spindle orientations, and so are shown on a separate branch leading to endoderm formation. In the absence of signaling (as in the *mom-1* and *mom-2* mutants), negative interactions may exist between *mom-5* and *apr-1* (not shown).
observation that spindle orientations in early wild-type blastomeres do not appear to be
determined by transcriptional events suggests that mom mutants disrupt spindle
orientations by non-transcriptional effects, or through inappropriate transcription.

The role of wrm-1, apr-1, and the mom genes in determining the E fate

In wild-type embryogenesis, one of the first visible consequences of P2-EMS
signaling is the delayed division of the E daughters relative to the division of the MS
daughters. This delay does not occur in transcription inhibited embryos (Powell-Coffman
et al., 1996), in wrm-1(RNAi) embryos, apr-1(RNAi) embryos, or in mom mutant
embryos. Thus we propose that each of these genes, at some level, is involved in an early
transcription-mediated event that makes E different from MS, and ultimately leads to E
producing endoderm rather than mesoderm. We have shown that wrm-1 can encode a
protein related to β-catenins such as the Armadillo protein. In Drosophila, Armadillo has
been shown to have functions in cell signaling through the Wg pathway, in addition to
functioning in cell adhesion (see Peifer 1995 for review). We see no evidence of defects
in cell adhesion between the early blastomeres in wrm-1(RNAi) embryos, and recent
studies have shown that a second β-catenin-like protein, HMP-2, is expressed in these
blastomeres; hmp-2 mutants and hmp-2(RNAi) embryos show no defects in endoderm
development (M. Costa and J. Priess, unpublished results). Thus the early C. elegans
embryo contains at least two β-catenin-like proteins that may have different functions.

Mutations in the maternal gene pop-1 and pop-1(RNAi), allow endoderm to form
in wrm-1(RNAi) embryos, and allow the E blastomere to produce endoderm independent
of P2-EMS signaling (Lin et. al., 1995; this thesis). Thus we propose that the E fate in normal development requires low, or no, pop-1(+) activity, and that the wrm-1 gene product is required to repress pop-1(+) activity (Figure 1-6, p.50). In wild-type embryos, an antiserum against the POP-1 protein shows a relatively low level of staining in the E nucleus compared to the MS nucleus; we have shown that MS and E in wrm-1(RNAi) have comparable, high levels of POP-1 staining, as do wild-type MS and E blastomeres that are not exposed to P2-EMS signaling. Thus it is possible that wrm-1(+) activity decreases the absolute level of POP-1 protein in the E nucleus, or that the mechanism by which wrm-1(+) represses pop-1(+) activity alters the ability of POP-1 to be recognized in immunostaining experiments. POP-1 is an HMG domain protein related to the vertebrate transcription factors LEF-1 or TCF-1 and Drosophila Pan. Therefore we propose that wrm-1 plays a role in transcriptional regulation through pop-1. In current models of Wnt/Wg signaling pathways, β-catenin has been proposed to form a complex with a TCF/LEF-related protein to activate transcription. Therefore mutations in the genes that encode LEF-1 and β-catenin would be predicted to result in similar phenotypes, while our results show that pop-1 mutants and wrm-1(RNAi) embryos have opposite phenotypes. Thus pop-1 and/or wrm-1 may have novel roles in the Wnt/Wg-like pathway in C. elegans.

The APC protein has been shown to interact with β-catenin and glycogen synthase kinase 3 (GSK-3), both of which are components of the Wnt/Wg signaling pathway (for a review see Peifer, 1996). Recent studies have shown that APC and β-catenin appear to have similar properties in axis-inducing assays in Xenopus (Vleminckx et al., 1997). We
have analyzed the *C. elegans* APC-related gene *apr-1*, and have shown that *apr-1(RNAi)* embryos and *wrm-1(RNAi)* embryos have similar defects in the development of the E blastomere and do not affect the early spindle orientations, suggesting that *apr-1* might function with *wrm-1* in the P2-EMS signaling pathway leading to E specification.

**E specification may involve multiple or branched pathways**

In contrast to *wrm-1(RNAi)* embryos, all of which lack endoderm, the *mom* mutants produce many embryos that contain endoderm. For example, endoderm is present in about 40% and 90% of the *mom-2* and *mom-5* mutant embryos, respectively. The incomplete penetrance of the endoderm defect contrasts with the completely penetrant defects in spindle orientation caused by every *mom-1*, *mom-2* and *mom-5* allele we identified. Incompletely penetrant defects could simply result from non-null alleles. However our molecular analysis suggests that many of the mutations in the *mom* genes should result in truncated or severely defective proteins. For example, the *mom-5(ne12)* mutation is a stop codon before the first of the seven transmembrane spanning domains, making it difficult to imagine how this mutant protein would retain partial function in signal transduction. We also do not consider it likely that the *mom-5(ne12)* mutation creates a partial protein with novel patterns of interaction with other components of the signaling pathway, because *mom-5(RNAi)* embryos exhibit phenotypes identical to our mutant alleles.

How is endoderm specified in the absence of wild-type *mom* gene products? We have shown that although only 26% of *apr-1(RNAi)* embryos lack endoderm when *mom-*
2(+) and mom-5(+) activities are both present, apr-1(+) function becomes essential for endoderm formation when either mom-2(+) or mom-5(+) activities are not present. A simple interpretation of these results is that there are partially redundant inputs into the specification of the E fate (and thus endoderm formation). The first input is the product of a linear pathway containing the mom-1, mom-2, and mom-5 genes, and the second input involves apr-1. Both inputs might then converge to alter wrm-1(+) activity, and consequently pop-1(+) activity (Figure 1-6, p.50).

If the mom genes provide only a single, linear input into endoderm formation, null alleles in all mom genes might be expected to cause similar percentages of embryos with endoderm. However we have shown that 90% of mom-5 mutants have endoderm, compared to much lower percentages in mom-1 or mom-2 embryos. Because removing mom-5(+) activity in a mom-2 mutant background, greatly increases the number of embryos that have endoderm, this result suggests that mom-5(+) activity has a negative influence on endoderm formation when mom-2(+) activity is not present. Several models are consistent with this result; for example, as an extension of the model presented above, it is possible that when the mom-2 signal is not present mom-5(+) activity has a negative effect on the apr-1 input to endoderm formation. Genetic interactions between Wg and Fz2 have not yet been analyzed in Drosophila, however complex genetic interactions analogous to those reported here have also been observed in postembryonic development in C. elegans between a second Wnt/wg-like gene, lin-44, and a fz-like gene, lin-17 (Sawa et al., 1996).
In summary, our results show that the MOM proteins are related to components of the Wnt/Wg signaling pathway and are required for proper cytoskeletal polarity as well as cell fate determination in the early *C. elegans* embryo. However the *mom* pathway appears to involve some complexities compared to models for the Wnt/Wg pathway. First, our results suggest that the effect of the *mom* genes on endoderm formation is mediated by *wrm-1* and *pop-1*, but that the effect of the *mom* genes on spindle orientation is not mediated by either *wrm-1* or *pop-1*. Second, WRM-1 and/or POP-1 may have different roles than *Drosophila* Arm and Pan/dTcf, respectively, since *pop-1* mutants and *wrm-1*(RNAi) embryos have opposite phenotypes. Finally, our results provide evidence that the specification of the E fate may involve parallel inputs provided by the *mom* genes and *apr-1*. The ability to isolate and recombine the blastomeres involved in these interactions, plus the ability to rapidly test by RNAi experiments whether specific genes are involved, suggest that a detailed molecular understanding of these interactions in the early *C. elegans* embryo should be possible.
EXPERIMENTAL PROCEDURES

Strains and Alleles

The Bristol strain N2 was used as the standard wild-type strain. The marker mutations, deficiencies and balancer chromosomes used are listed by chromosome as follows: LGI: pop-1(zi89), dpy-5(e61), unc-13(e1091), nDf9, nDf24, qDf6, mnDf111, lin-11(n566), hT1(I;IV), hT2(I;III); LGII: bli-2(e768); LGIII: unc-32(ei89), dpy-18(e364), eT1(III;V); LGIV: unc-5(e53), DnT1(IV;V); LGV: dpy-11(e224), unc-42(e270), sDf35, mDf3, him-5(ei409); LGX: lin-2(e1309), lon-2(e678). C. elegans culture, mutagenesis, and genetics were as described in Brenner (1974).

Genetic analysis

Mutant alleles were isolated as described previously (Mello et al., 1992; 1994). Standard genetic crosses were used to map mom-1(se2) to the dpy-8, unc-6 interval on LGX, mom-2(ne141) to the dpy-11, unc-42 interval of LGV, and mom-5(zi193) and mom-5(ne12) to the unc-13, lin-11 interval on LGI. Data from these crosses are available from the C. elegans database, ACEDB. Self progeny from mom-1(se2), mom-2(ne141) or mom-5(zi193) adult hermaphrodites appeared identical in all respects to cross progeny by wild-type males, indicating that these gene activities are required maternally.

Complementation tests were performed as follows: mom-1(se2) against all other mom-1 alleles, mom-2(ne141) against mom-2(or42), mom-2(ne141) against the chromosomal deficiency mDf3, mom-5(zi193) against mom-5(ne12). In all cases, heterozygotes grew to adults that produced dead embryos resembling those produced by
the test strain (first listed). All mom-1 mutant strains have a non-complementing, incompletely penetrant vulva defect as adults.

**RNAi reverse genetics**

Templates for RNA synthesis were produced from PCR directly on cDNA phage lysates using T7 and T3 primers. For genomic clones, PCR primers were chosen to span exons and generate fragments between 0.5-2Kb in size. A nested pair of PCR primers, one containing the T7 promoter (5'TAATACGACTCACTATAGGGAGACCAC3'), were used for a second round of PCR. RNAs were synthesized using T7 polymerase and commercially available synthesis kits. Unmodified RNA was resuspended for injection at 1-5 mg/ml in DEPC treated water. Microinjection into both arms of hermaphrodite gonads were performed as described in Mello et al., (1991). Preliminary studies indicate that at cosuppression of different, but related genes requires at least 70% to 80% nucleotide sequence identity (C. Mello, unpublished observations).

Coinjection of two RNA's appears to result in the phenotype equivalent to that observed in doubles constructed with actual mutant alleles for the corresponding genes (C. Mello unpublished; this study). Because RNA injections might fail for technical reasons, additional controls were performed where epistasis was expected. For example wrm-1(RNAi);pop-1(RNAi) coinjections yield a phenotype identical to pop-1(RNAi) singles as do injections of wrm-1(RNAi) into pop-1(zu189) mutant mothers. To insure that wrm-1 activity was blocked we injected wild-type animals with wrm-1(RNAi) first, followed 12 hours later by a second injection of pop-1(RNAi). These sequential assays
allowed us to first confirm conversion to the *wrm-1*, no-endoderm phenotype, followed by conversion to the *pop-1*(RNAi) extra-endoderm phenotype. Performing the injections in the opposite order resulted in the same final result, embryos with extra-endoderm.

RNAs from the following clones or genes were tested but failed to induce embryonic phenotypes: Wnt related; *lin-44*(yk120c7), *cwn-1*, *cwn-2*, and W08D2.1. Frizzled related; *Y34D9.00718*(yk107h2), and *F27E11*(yk117b4). Disheveled related; C34F11.9a/(yk10b11), yk46b11. We do not know whether these genes have no function in the early embryo, or whether the RNA procedure did not inhibit the function of these genes.

**Molecular Analysis**

The genes described in this study correspond to completely sequenced genomic clones. Coding sequences were determined by sequencing cDNA clones or by sequencing RT-PCR products amplified using primers predicted from genomic sequence. Our results for *mom-5*T23D8.1 and *apr-1*K04G2.9 were consistent with Genefinder predictions. Our results for *mom-1*T07H6.2, *mom-2*F38E1.7 and *wrm-1*B0336.1 did not agree with Genefinder predictions; these differences are detailed in Genbank. Mutant alleles were sequenced using standard protocols from PCR-amplified genomic DNA. The accession numbers for the sequences described are as follows: *mom-1*, AF013489; *apr-1*, AF013950; *wrm-1*, AF013951; *mom-2*, AF013952; and *mom-5*, AF013953.
Microscopy

Light and immunofluorescence microscopy and laser microsurgery were as described in Bowerman et al. (1992a and 1993). The identity of differentiated cells were assigned based on morphological criteria in the light microscope, followed, in most cases by fixation and staining with tissue-specific probes. Criteria for assigning cell fates, and antibodies were as described in Bowerman et al. (1992a) and Mello et al. (1992); the mABRL2 antibody has staining properties similar to the 94I antiserum (Lin et al., 1995; 1998).
CHAPTER II: WRM-1 ACTIVATES THE LIT-1 PROTEIN KINASE TO TRANSDUCE ANTERIOR-POSTERIOR POLARITY SIGNALS IN *C. elegans*

**SUMMARY**

During *C. elegans* development, Wnt/Wg signaling is required for differences in cell fate between sister cells born from anterior-posterior divisions. A β-catenin-related gene, *wrm-1*, and the *lit-1* gene are effectors of this signaling pathway and appear to downregulate the activity of POP-1, a TCF/LEF-related protein, in posterior sister cells. We show here that *lit-1* encodes a serine/threonine protein kinase homolog, related to the *Drosophila* tissue polarity protein Nemo. We demonstrate that the WRM-1 protein binds to LIT-1 in vivo and that WRM-1 can activate the LIT-1 protein kinase when coexpressed in vertebrate tissue culture cells. This activation leads to phosphorylation of POP-1 and to apparent changes in its subcellular localization. Our findings provide evidence for novel regulatory avenues for an evolutionarily conserved Wnt/Wg signaling pathway.
INTRODUCTION

The early blastomeres of the *C. elegans* embryo initiate region-specific patterns of development through several mechanisms including position-dependent cell-cell interactions and the asymmetric expression of maternally-provided transcription factors (for review, see Schnabel and Priess, 1997). Despite the various mechanisms that lead to region-specific development, several experimental and genetic studies have suggested that cells throughout the embryo share a common mechanism for linking cell division to cell fate (Mello et al., 1992; 1994; Kaletta et al., 1997; Lin et al., 1998). Part of this process appears to involve POP-1, a protein related to vertebrate TCF (T-cell factor)/LEF (lymphoid enhancer factor) transcription factors (Lin et al., 1995; 1998). Most of the cell divisions in all regions of the early embryo are oriented along the anterior-posterior (A-P) axis, and essentially all of these divisions result in A-P sister cells with different fates (Sulston et al., 1983). Antibodies specific for POP-1 show a higher level of nuclear staining in the anterior daughters of A-P divisions than in the posterior daughters, and genetic studies have shown that POP-1 function is required for several of the early A-P differences in cell fate (Lin et al., 1995; 1998).

POP-1 activity and localization is regulated by a group of genes that are called *mom* genes. Cloning of several *mom* genes revealed that each of these genes encodes a protein clearly related to known components of the Wnt/Wingless (Wnt/Wg) signaling pathway defined in vertebrates, *Drosophila*, and *C. elegans*. These proteins include MOM-1(Porcupine), MOM-2(Wnt/Wg), and MOM-5(Frizzled) (Rocheleau et al., 1997; Thorpe et al., 1997). Reverse genetic studies showed that the loss of function of a *C.
The *Caenorhabditis elegans* homolog of β-catenin/Armadillo (called WRM-1) and of APR-1, an Adenomatous polyposis coli (APC) homolog, also resulted in defects resembling *mom* mutants (Rocheleau et al., 1997). In most models for the Wnt/Wg signaling pathway, β-catenin is a principal effector of signaling, and genetic studies of the *mom* genes and *wrm-1* (β-catenin) in the *C. elegans* embryo are consistent with this view (Rocheleau et al., 1997; for review see Cadigan and Nusse, 1997; Han, 1997). However, there is one important difference in the roles of WRM-1 and β-catenin. In the Wnt/Wg model, β-catenin enters the nucleus in response to signaling and stimulates transcriptional activator function of TCF/LEF proteins. Thus, the loss of β-catenin and loss of TCF/LEF would result in similar phenotypes (for review see Cavallo et al., 1997; Kuhl and Wedlich, 1997; Willert and Nusse, 1998). However, studies on the *C. elegans* embryo have shown that the loss of WRM-1 (β-catenin) has the opposite consequences in numerous cell fate decisions to the loss of POP-1 (TCF/LEF); loss of WRM-1 causes both A-P sisters to adopt anterior fates (Rocheleau et al., 1997), while loss of POP-1 causes duplication of posterior fates (Lin et al., 1995; 1998). Thus, a critical question for understanding how the Wnt/Wg pathway functions in *C. elegans* is to understand the role of WRM-1 in relation to the rest of the signaling components.

In the present study, we show that the Wnt/Wg pathway in *C. elegans* involves the *C. elegans* polarity gene *lit-1* (Kaletta et al., 1997) and that *lit-1* encodes a protein related to the *Drosophila* tissue polarity protein Nemo (Choi and Benzer, 1994). We show that WRM-1 can bind LIT-1 and can activate a LIT-1-dependent kinase activity. Finally, we show that the WRM-1/LIT-1 kinase complex precipitated from vertebrate
cells can phosphorylate POP-1 in vitro.
RESULTS

The *lit-1* gene encodes a putative protein kinase implicated in Mom(Wnt/Wg) signaling

We have used an RNA-mediated reverse genetic approach (RNAi) to ask whether any of the polarity genes identified in other systems had Mom-related functions in *C. elegans* (Rocheleau et al., 1997 and our unpublished data). We found a completely penetrant *mom* phenotype resulted from RNAi targeted to a *C. elegans* homolog of the *Drosophila* polarity gene *nemo* (Table 2-1, p.70 and Experimental Procedures). We noted that the physical map position of the *nemo* homolog was consistent with the genetic position of *lit-1*. Previous studies (Kaletta et al., 1997) had shown that mutations in the *lit-1* gene cause at least one defect that is identical to that of *mom* mutants, i.e. the transformation of intestinal precursors into mesodermal precursors. We found that a Yeast Artificial Chromosome with *C. elegans* DNA containing the *nemo* homolog completely rescued *lit-1(tl512)* (see Experimental Procedures). These observations suggested that the *nemo* homolog might be the *lit-1* gene. We sequenced the *nemo* homolog in the *lit-1* mutants *lit-1(tl512)* and *lit-1(tl534)*, and found a mutation in each (Figure 2-1, p.65). Taken together, the proximity of the respective physical and genetic map positions of the *nemo* and *lit-1* genes, the similarity of their RNAi and mutant phenotypes, and the existence of point mutations predicted to alter conserved residues of the Nemo-like protein in each *lit-1* mutant lead us to conclude that *lit-1* is the *C. elegans* *nemo* gene.
Figure 2-1
Figure 2-1. LIT-1 encodes a homolog of *Drosophila* Nemo and mouse Nlk.

(A) Alignment of LIT-1 amino acid sequence (LIT-1a, see below) with *Drosophila* Nemo form II and mouse Nlk (Nemo-like kinase). Identical residues are shaded in gray. The conserved residues altered in *lit-I(t1512)* (Leucine 177 to Serine) and in *lit-I(t1534)* (Glutamate 222 to Lysine) are bold in the LIT-1 sequence.

(B) Schematic representation of *lit-I* genomic structure. Two SL1 trans-spliced isoforms are shown. The longer transcript *lit-Ia* is predicted to contain three non-coding exons (white boxes) in front of the predicted start codon. The second transcript *lit-Ib* starts at an alternatively spliced exon (gray box) and contains two in frame methionine codons 15 and 24 nucleotides upstream of the start site for *lit-Ia*. Black boxes indicate predicted coding exons from which the amino acid sequence in (A) was deduced. The exons containing the kinase domain are also indicated. Positions of the *lit-I(t1512)* and *lit-I(t1534)* lesions are shown.
LIT-1 is highly homologous to *Drosophila* Nemo (Choi and Benzer, 1994) and mouse Nlk (Nemo-like kinase) (Brott et al., 1998) within its predicted kinase domain, and also contains a conserved C-terminal region. The N-terminal region is the most diverged between LIT-1, Nemo and Nlk (Figure 2-1a, p.65). *lit-1* can encode at least two different 5’ splice variants (Figure 2-1b, p.65). The *lit-1(t1512)* mutation changes a conserved Leucine in the predicted kinase domain, and the *lit-1(t1534)* mutation changes a Glutamate to a Lysine in a regulatory motif believed to activate related kinases (THE to THK; Figure 2-1, p.65) (Payne et al., 1991).

Previous genetic tests had shown that mutations in *pop-1* are epistatic to mutations in *lit-1* (Kaletta et al., 1997); however, whether POP-1 localization and/or levels are regulated by *lit-1(+) activity had not been determined. We immunostained *lit-1(t1512)* mutant embryos for POP-1 and found that POP-1 protein levels appear equal in the nuclei of sister cells resulting from A-P divisions (Figure 2-2, p.68). Thus, a reduction or loss of *lit-1(+) activity appears to have the same effect on POP-1 asymmetry as previously described mutations in the *mom* genes (Rocheleau et al., 1997; Thorpe et al., 1997; Lin et al., 1998).

To determine if *lit-1* had genetic interactions with the *mom* genes, we examined the phenotypes of *lit-1* mutant embryos in which *mom* gene activity was removed by mutation or by RNAi. We found that *lit-1* mutants strongly enhance the polarity defects associated with *mom-2(Wnt/wg), mom-5(frizzled)*, and of *apr-1(APC-related)* (Table 2-1, p.70). For example, double mutants with the temperature-sensitive *lit-1(t1512)* allele and a partially penetrant *mom-2(ne141)* allele produced a fully penetrant *mom* phenotype.
wild type  \textit{lit-1(t1512)}

POP-1 localization

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Figure 2-2}
\end{figure}
Figure 2-2. POP-1 is localized symmetrically in *lit-I(t1512)*.

Top panels show immunofluorescence staining of POP-1 in either wild-type (A) or *lit-I(t1512)* mutant embryos (B). The anterior of the embryo is to the left, and the posterior to the right. Double-headed arrows indicate pairs of anterior/posterior sisters. In wild-type embryos, the anterior cells show higher levels of POP-1 protein than their posterior sisters. *lit-I(t1512)* embryos show equal staining in the nuclei of both anterior and posterior sisters. The embryos as well as their mothers were kept at restrictive temperature for *lit-I(t1512)*. The lower panels show corresponding DAPI staining of the nuclei in either wild-type (C) or *lit-1(t1512)* (D).
Table 2-1. Genetic Analysis of Endoderm Specification in lit-1.

<table>
<thead>
<tr>
<th>Embryo Type</th>
<th>% Embryos Lacking Endoderm (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lit-1(RNAi)</td>
<td>100(544)</td>
</tr>
<tr>
<td>lit-1(t1534)</td>
<td>2(195)</td>
</tr>
<tr>
<td>lit-1(t1512)</td>
<td>0(216) 15°C</td>
</tr>
<tr>
<td></td>
<td>96(473) 25°C</td>
</tr>
<tr>
<td>mom-2(ne141)</td>
<td>53(304)</td>
</tr>
<tr>
<td>mom-2(ne141);lit-1(t1534)</td>
<td>100(225)</td>
</tr>
<tr>
<td>mom-2(ne141);lit-1(t1512)</td>
<td>100(235) 15°C</td>
</tr>
<tr>
<td></td>
<td>100(220) 25°C</td>
</tr>
<tr>
<td>mom-5(RNAi)</td>
<td>2(511)</td>
</tr>
<tr>
<td>mom-5(RNAi);lit-1(t1534)</td>
<td>97(458)</td>
</tr>
<tr>
<td>mom-5(RNAi);lit-1(t1512)</td>
<td>60(105) 15°C</td>
</tr>
<tr>
<td></td>
<td>98(259) 25°C</td>
</tr>
<tr>
<td>apr-1(RNAi)</td>
<td>49(603)</td>
</tr>
<tr>
<td>apr-1(RNAi);lit-1(t1534)</td>
<td>98(219)</td>
</tr>
<tr>
<td>pop-1(RNAi)</td>
<td>0(123)</td>
</tr>
<tr>
<td>pop-1(RNAi);lit-1(RNAi)</td>
<td>0(62)</td>
</tr>
<tr>
<td>pop-1(RNAi);lit-1(t1512)</td>
<td>0(75) 15°C</td>
</tr>
<tr>
<td></td>
<td>0(235) 25°C</td>
</tr>
</tbody>
</table>

Table 2-1. Genetic analysis of endoderm specification.

Strains containing the temperature sensitive mutant, lit-1(t1512), were cultured at 25°C, the restrictive temperature, and 15°C, the permissive temperature, as indicated at right.

All other strains were cultured at room temperature (~22°C).
even at permissive temperature for lit-1(t1512) (Table 2-1, p.70). Consistent with previous analysis of the pop-1(zu189) mutant (Kaletta et al., 1997), we found that pop-1(RNAi) was fully epistatic to lit-1(RNAi) and to lit-1(t1512) (Table 2-1, p.70). In summary, we conclude that Wnt/Wg signaling in the early embryonic divisions requires lit-1(+) to downregulate pop-1(+) activity.

**LIT-1 functions in multiple Wnt/Wg-related signaling events in C. elegans**

We found that a transgene expressing a fusion protein consisting of LIT-1 and green fluorescent protein (GFP) fully rescues the lit-1(t1534) mutant and partially rescues the more severe lit-1(t1512) mutant (see Experimental Procedures). Although GFP fluorescence could not be detected in early embryos, fluorescence was detected in the nuclei of most embryonic cells beginning around the 100 cell stage. Faint GFP was also visible in the cytoplasm of some embryonic and larval cells (data not shown). In larvae, GFP::LIT-1 was detected in numerous cells, some of which had been shown previously to contain POP-1 (Lin et al., 1998). These results suggest a possible role for LIT-1 in larval cell fate decisions involving POP-1.

We found GFP::LIT-1 expression in a larval cell called the T cell and in its descendants; these cells were of interest because proper T cell development has been shown to involve a Wnt/Wg-like signaling pathway (Herman et al. 1995; Sawa et al. 1996). To ask whether lit-1(+) activity was required in this pathway, we examined the development of the T cell in temperature-sensitive lit-1(t1512) mutants shifted to restrictive temperature during late embryogenesis. We observed several defects in T cell
development that were similar to those caused by mutations in the *lin-17* gene (Sternberg and Horvitz, 1988), which encodes a protein related to *Drosophila* Frizzled (Table 2-2, p.73; Sawa et al. 1996). For example, *lit-1* animals exhibit a 33% frequency of symmetric T-cell division, a frequency similar to that seen in weak *lin-17* mutants. As with *lin-17* mutations, the symmetric division phenotype of *lit-1* appears to suppress the reversed polarity phenotype of *lin-44* (*Wnt/wg*) mutants (see Table 2-2, p.73). These results suggest that *lit-1* is involved in the T cell *lin-17/lin-44* signaling system and appears to function downstream of *lin-44* (*Wnt/wg*).

**LIT-1-dependent kinase activity is activated by WRM-1 and phosphorylates POP-1.**

Mouse Nlk has been shown to have an apparent autophosphorylation activity when expressed in mammalian cell culture (Brott et al., 1998). We failed to detect LIT-1 kinase activity when an epitope tagged LIT-1 was expressed and immunoprecipitated from vertebrate cells. However, if WRM-1 was coexpressed with LIT-1, two proteins present in the LIT-1 immunoprecipitation complex could be phosphorylated; these proteins were identified as LIT-1 itself and WRM-1 (Figure 2-3A, p.74). This kinase activity requires the putative ATP-binding site in the LIT-1 kinase domain, as a point mutation in this position (K89G) abolished the phosphorylation of both LIT-1 and WRM-1. Thus, WRM-1 is both a substrate for and an activator of LIT-1-dependent kinase activity. We next asked if POP-1 could be phosphorylated by the activated LIT-1 kinase. We found a bacterially expressed GST-POP-1 protein can be phosphorylated by the LIT-1 immunoprecipitation complex and that phosphorylation required WRM-1 and an intact
Table 2-2. Genetic Analysis of T cell Polarity in *lit-1*.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>% Normal</th>
<th>% Symmetric</th>
<th>% Reversed</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>lin-44(n1792)</em></td>
<td>85</td>
<td>6</td>
<td>14</td>
<td>80</td>
</tr>
<tr>
<td><em>lin-17(mn589)</em></td>
<td>62</td>
<td>56</td>
<td>31</td>
<td>13</td>
</tr>
<tr>
<td><em>lin-17(n3091)</em></td>
<td>61</td>
<td>8</td>
<td>82</td>
<td>10</td>
</tr>
<tr>
<td><em>lit-1(t1512)</em></td>
<td>91</td>
<td>67</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td><em>lin-44(n1792); lit-1(t1512)</em></td>
<td>66</td>
<td>3</td>
<td>85</td>
<td>12</td>
</tr>
<tr>
<td><em>lin-17(mn589); lit-1(t1512)</em></td>
<td>64</td>
<td>2</td>
<td>98</td>
<td>0</td>
</tr>
<tr>
<td><em>lin-17(n3091); lit-1(t1512)</em></td>
<td>81</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
A

Immunoprecipitation with α-FLAG

In vitro kinase assay

+ SDS/sample buffer

SDS/PAGE

Dilution and immunoprecipitation with α-FLAG or α-HA

HA-WRM-1   −   −   +   +   −   +

FLAG-LIT-1  −   +   −   +   +   +

120

81

46

IP: αFLAG

αFLAG

B

Immunoprecipitation with α-FLAG

+ GST-POP-1

In vitro kinase assay

Precipitation with glutathione beads

SDS/PAGE

120

81

HA-WRM-1   −   −   +

FLAG-LIT-1  −   +   +   +

Figure 2-3
Figure 2-3. LIT-1 immunoprecipitates can phosphorylate LIT-1, WRM-1 and GST-POP-1.

(A) LIT-1-dependent phosphorylation of LIT-1 and WRM-1 requires coexpressed WRM-1. COS-7 cells were transfected as indicated at the bottom of the gel, and in vitro kinase assays were performed on FLAG-LIT-1. The anti-FLAG immunoblot (lower panel) shows that FLAG-LIT-1 and FLAG-LIT-1 K89G were expressed at comparable levels. Two radioactive bands were identified as WRM-1 and LIT-1 by immunoprecipitation using either the anti-FLAG or anti-HA antibody.

(B) In vitro phosphorylation of GST-POP-1 by the LIT-1 immunoprecipitation complex. Cell lysates from COS-7 cells transfected as indicated beneath the gel were subjected to immunoprecipitation with the anti-FLAG antibody followed by in vitro kinase assays with bacterially expressed GST-POP-1 protein as a substrate. GST-POP-1 was precipitated from the kinase reaction with glutathione-Sepharose. This precipitate contained a phosphorylated protein with the electrophoretic mobility expected for GST-POP-1. A faint comigrating band corresponding to a small amount of nonspecifically phosphorylated GST-POP-1 was precipitated with the glutathione-Sepharose (first two lanes and data not shown). GST alone, without the fusion to POP-1, was not a substrate for phosphorylation by the LIT-1 immunoprecipitates (data not shown).
LIT-1 kinase domain (Figure 2-3B, p.74 and not shown).

**WRM-1 and LIT-1 form a stable complex**

The experiments described above indicate that the LIT-1 immunoprecipitate contained sufficient amounts of WRM-1 to be detected after the in vitro phosphorylation reaction using radiolabeled ATP. Although this may represent a transient interaction between the kinase and the substrate, apparent activation of LIT-1 kinase by WRM-1 raises the possibility that the two proteins may form a stable complex.

To examine how WRM-1 might activate LIT-1, we asked if WRM-1 could bind LIT-1 directly. In the yeast two-hybrid assay, we found that the full-length WRM-1 could interact with LIT-1 (Figure 2-4, p.77). We mapped the minimal interaction domain in WRM-1 to a small N-terminal region (Figure 2-4, p.77). Similarly, we found that a bacterially expressed N-terminal WRM-1 protein, containing the first 214 amino acids, was sufficient to bind LIT-1 that had been translated in vitro (data not shown). The binding domain in LIT-1 has not been mapped in detail; however, neither the first 378 amino acids nor the last 74 amino acids were sufficient to bind WRM-1 (data not shown). Interaction between WRM-1 and LIT-1 were also observed in transfected vertebrate cells by reciprocal coimmunoprecipitation experiments (Figure 2-5A, p.78). This interaction was apparently unaffected by the presence of POP-1 (Figure 2-5A, p.78).

To ask if WRM-1 can associate with LIT-1 in the *C. elegans* embryo, we prepared embryo extracts from transgenic GFP::LIT-1 strains and used a monoclonal antibody against GFP to precipitate GFP::LIT-1. The immunoprecipitate was analyzed for the
Full length WRM-1 and a series of truncation proteins (schematically diagrammed) were cloned into the GAL4 activation domain vector, pACT2, and were tested for interactions with POP-1 and LIT-1 in the GAL4 DNA-binding domain vector pAS1. LIT-1 interacts with full-length WRM-1. An N-terminal 148 amino acid region in WRM-1 is sufficient to bind LIT-1. POP-1 interacts weakly with full-length WRM-1 but not with any of the truncated proteins we tested. (+/-) indicates growth on 5mM but not on 10mM 3-AT medium. (+) indicates growth on 10mM 3-AT. The shaded boxes represent the twelve conserved Armadillo (Arm) motifs. A lightly shaded box represents a region with weak homology to the consensus Arm motif and may represent a thirteenth repeat.
Figure 2-5
Figure 2-5. LIT-1 and WRM-1 form a stable complex.

(A) FLAG-LIT-1 and HA-WRM-1 coimmunoprecipitate from vertebrate cells. COS-7 cells were transfected with a combination of plasmids expressing FLAG-LIT-1, HA-WRM-1 and Myc-POP-1, as indicated at the bottom of the gel. HA-WRM-1 or FLAG-LIT-1 was immunoprecipitated with monoclonal antibodies against the respective epitope tags, and the presence of coprecipitated FLAG-LIT-1 or HA-WRM-1 was determined by immunoblotting using the anti-FLAG or anti-HA antibody, respectively. Similar experiments failed to detect co-precipitation of Myc-POP-1 with either FLAG-LIT-1 or HA-WRM-1 (data not shown). (B) Immunodetection of WRM-1 from embryo extracts. Immunoblotting with a monoclonal antibody, P3C8, raised against bacterially expressed WRM-1 protein (see Experimental Procedures), revealed a single band of approximately 110-120kD in *C. elegans* embryo extracts. An independent monoclonal antibody P5D6, as well as an affinity purified polyclonal antiserum, all raised against WRM-1 expressed in *E. coli*, recognized a band which apparently comigrated with the 110-120kD band reactive to P3C8 (data not shown). (C) GFP-LIT-1 associates with WRM-1 in vivo. Protein extracts were prepared from two independent transgenic worm strains homozygous for the *lit-I(t1534)* mutation and rescued by GFP::LIT-1 (see Experimental Procedures). GFP-LIT-1 was immunoprecipitated using a monoclonal anti-GFP antibody (3E6) from embryo extracts and visualized by immunoblotting using a second monoclonal anti-GFP antibody (7.1/13.1) (top panel). Coprecipitation of WRM-1 was determined by immunoblotting using the anti-WRM-1 monoclonal antibody P3C8 (bottom panel). P3C8 detected a single major band with the mobility expected for endogenous WRM-1 protein. Immunoprecipitation using extracts prepared from non-transgenic wild-type strain (N2) did not yield coprecipitated WRM-1. Neither GFP-LIT-1 nor WRM-1 was precipitated in the absence of the anti-GFP antibody.
presence of co-precipitated WRM-1 by immunoblotting. A monoclonal antibody raised against bacterially expressed WRM-1 (Experimental Procedures) detected a single band in the GFP::LIT-1 immunoprecipitate that has approximately the correct electrophoretic mobility to be WRM-1 (Figure 2-5B and 2-5C, p78). A second monoclonal antibody against WRM-1 recognized an apparently identical band in GFP::LIT-1 (data not shown). We conclude that WRM-1 forms a stable complex with GFP-LIT-1 in vivo.

In vertebrates and Drosophila, proteins related to C. elegans WRM-1 and POP-1 form a stable complex (see discussion). In the yeast two-hybrid assay, we detected only weak interactions between POP-1 and full-length WRM-1 and no interactions with any of the truncated forms of WRM-1. Similarly, we were unable to detect POP-1 in immunoprecipitates of WRM-1 or LIT-1 from vertebrate cells expressing these proteins (data not shown). These findings suggest that WRM-1 does not form a stable complex with POP-1.

**LIT-1 and WRM-1 may regulate subcellular localization of POP-1**

We have shown that WRM-1 can exist in a complex with LIT-1 in vivo and can also activate a LIT-1-dependent kinase that phosphorylates GST-POP-1 in vitro. These findings raise the possibility that LIT-1 and WRM-1 may form an active kinase complex that regulates POP-1 activity or localization. We therefore decided to address the consequences of WRM-1/LIT-1 regulation of POP-1 upon expression in vertebrate cells. We found that in transfected vertebrate cells, POP-1 became hyperphosphorylated, as represented by a change in electrophoretic mobility, when LIT-1 and WRM-1 were
coexpressed but not when the kinase-inactive form of LIT-1 was substituted for LIT-1 (Figure 2-6A, p.82). When expressed alone in vertebrate cells, POP-1 is primarily nuclear, as it is in anterior daughters of A-P divisions in *C. elegans* embryos (Figure 2-6B and 2-6C, p.82; see Figure 2-2, p.68). However, when POP-1 is coexpressed in vertebrate cells with both LIT-1 and WRM-1, it becomes prominent in the cytoplasm (Figure 2-6B and 2-6C, p.82). This redistribution of POP-1 required both an intact kinase domain in LIT-1 and the coexpression of LIT-1 and WRM-1, suggesting that it is mediated by the kinase activity of the LIT-1/WRM-1 complex.
Figure 2-6
Figure 2-6. Regulation of POP-1 by LIT-1/WRM-1 in vertebrate cells.

(A) POP-1 phosphorylation depends on WRM-1 and kinase active LIT-1. COS-7 cells were transfected with Myc-POP-1 and combinations of HA-WRM-1, FLAG-LIT-1, and FLAG-LIT-1 K89G. Two forms of Myc-POP-1 were detected, and the appearance of the slower migrating form was dependent on coexpression of HA-WRM-1 and kinase active FLAG-LIT-1. Treatment of the sample with phosphatase converted the slow migrating band to the faster form, and this conversion was blocked by adding phosphatase inhibitors (last two lanes), indicating that the slower mobility reflects phosphorylation of POP-1. (B and C) Cytoplasmic levels of Myc-POP-1 are increased by WRM-1/LIT-1. COS-7 cells transfected with POP-1 alone (left panels) or with POP-1, WRM-1 and LIT-1 (right panels), were fixed and stained for Myc-POP-1 by immunofluorescence (B, and B0) and for nuclei with DAPI (Bc and Bd). The arrows indicate individual transfected cells in each field of view (B, and B0) and the corresponding nuclei (Bc and Bd). (C) Graphic representation showing the percentages of transfected cells with predominantly cytoplasmic Myc-POP-1. Sets of three bars represent three independent transfection experiments. The total numbers of cells scored are: 2127 for Myc-POP-1, 1563 for Myc-POP-1/HA-WRM-1, 1367 for Myc-POP-1/FLAG-LIT-1, 1744 for Myc-POP-1/FLAG-LIT-1/HA-WRM-1 and 1454 for Myc-POP-1/FLAG-LIT-1 K89G/HA-WRM-1. In parallel experiments, the localization of an endogenous nuclear protein, MKK7, was monitored by immunofluorescence microscopy. The pattern of MKK7 localization was not affected in these transfected populations (data not shown), indicating that the effects on POP-1 are not due to alterations in protein nuclear localization in general.
DISCUSSION

Activation of the LIT-1 protein kinase

In *C. elegans*, embryonic cells that divide A-P have high levels of nuclear POP-1 in anterior daughters and relatively low levels of nuclear POP-1 in posterior daughters (Lin et al., 1995; 1998). The MOM proteins, WRM-1, and APR-1 (Rocheleau et al., 1997; Thorpe et al., 1997; Lin et al. 1998) and LIT-1 (Rocheleau et al., 1999) are all required for the low levels of nuclear POP-1 in posterior daughters. We have shown here that *lit-1* encodes a protein related to the *Drosophila* Nemo kinase (Choi and Benzer, 1994) and mouse Nlk (Brott et al., 1998). The observation that mutations present in *lit-1(t1512)* and *lit-1(t1534)* alleles alter conserved residues in the predicted kinase domain suggests that the kinase activity of LIT-1 is essential for POP-1 asymmetry. Consistent with this idea, we have shown that LIT-1 kinase activity causes phosphorylation of POP-1. This kinase activity also promotes phosphorylation of both LIT-1 and WRM-1 and is dependent on WRM-1(β-catenin).

There are several phenotypic similarities in embryos depleted of LIT-1 and WRM-1. Nearly all such embryos fail to differentiate intestine and lack POP-1 asymmetry (Kaletta et al., 1997; Rocheleau et al., 1997; 1999). In contrast, at least 20% of the embryos produced by all other *mom* mutants differentiate intestinal cells and retain POP-1 asymmetry (Rocheleau et al., 1997; Thorpe et al., 1997; Lin et al., 1998). Furthermore, almost all *mom* mutants have defects in mitotic spindle orientation that are not observed in embryos lacking either *wrn-1(+) or lit-1(+) (Rocheleau et al., 1997; Thorpe et al., 1997). These observations support the view that LIT-1 and WRM-1 might
function downstream of the other MOM proteins and in a closely related event that generates POP-1 asymmetry.

WRM-1 can be coimmunoprecipitated with LIT-1 from *C. elegans* extracts, providing evidence that WRM-1 and LIT-1 may form a complex in vivo. Using the yeast two-hybrid system, we have shown that the N-terminal region of WRM-1 is sufficient to bind LIT-1. This region is outside of the canonical 12 "Armadillo" (ARM) repeats that are known to be involved in protein-protein interactions in WRM-1 related proteins (for reviews, see Cavallo et al., 1997; Kuhl and Wedlich, 1997; Wilert and Nusse, 1998). If LIT-1 binds in vivo to only the N-terminal region of WRM-1, it may leave the ARM repeat region available for interactions with other proteins involved in signaling. The phosphorylation of WRM-1 observed when bound to LIT-1 may in turn alter the binding properties of the complex to promote binding or release of factors involved in POP-1 regulation.

**WRM-1, LIT-1, and POP-1**

When POP-1 is coexpressed with both LIT-1 and WRM-1 in vertebrate cells, POP-1 becomes phosphorylated; this phosphorylation requires the putative ATP-binding site in LIT-1. Thus, an attractive possibility is that activated LIT-1 phosphorylates POP-1 directly. Consistent with this idea, the LIT-1 complex immunoprecipitated from transfected cells can phosphorylate GST-POP-1 in vitro. In *C. elegans* embryos, WRM-1 and LIT-1 appear to downregulate *pop-I* (+) activity in posterior daughters of A-P divisions and cause a reduction in the apparent nuclear level of POP-1 in these daughters.
It is not yet known whether the difference in POP-1 observed after immunostaining results primarily from changes in protein levels, subcellular distribution, or modifications of the immunoepitope (see Lin et al., 1998). The findings described here suggest a possible model to explain POP-1 regulation. We propose that in *C. elegans*, the primary function of WRM-1 may be to activate LIT-1, which then results in phosphorylation of POP-1. As in other systems, Wnt/Wg signaling might serve to increase levels of WRM-1 (β-catenin). Phosphorylation of POP-1 by LIT-1 might directly inactivate POP-1 in the nucleus or stimulate the nuclear export or cytoplasmic retention of POP-1. The observation that POP-1 protein, which is nuclear in mammalian cells when expressed alone, accumulates in the cytoplasm when coexpressed with WRM-1 and LIT-1 would be consistent with this later possibility.

**Wnt/Wg signaling and tissue polarity.**

The *lit-1* homolog *nemo* was identified in *Drosophila* by a mutant with altered patterns of rotation in the ommatidia of the compound eye (Choi and Benzer, 1994) and is considered to be part of the “tissue-polarity” pathway (Zheng et al., 1995). This pathway involves the Frizzled protein (Vinson and Adler, 1987, Vinson et al., 1989) which together with Frizzled2 (Bhanot et al., 1996) has recently been implicated as a receptor for *Drosophila* Wingless (Bhat, 1998; Kennerdell and Carthew, 1998; Müller et al., 1999). However, there are no known requirements for a WRM-1-related or POP-1-related proteins in the tissue-polarity pathway.
Specification of A-P differences in the blastomeres of early *C. elegans* embryos might involve the chance convergence of a tissue-polarity pathway involving LIT-1 and a largely separate Wnt/Wg pathway. Perhaps consistent with this view, the genetics of the *mom* genes is complex and suggests a pathway involving multiple branches (see Rocheleau et al., 1997). However, we have shown here that LIT-1 functions in at least one additional developmental regulation: the T cell fate decision. The observation that LIT-1/Nemo/Nlk appears to be an integral part at least two Wnt/Wg mediated cell fate decisions in *C. elegans* raises the interesting possibility that members of this highly conserved protein kinase family may also have roles in Wnt/Wg signaling in other systems.

**Comparison with Wnt/Wg signaling in other organisms**

Current models for Wnt/Wg signaling in vertebrates and *Drosophila* suggest that this signaling stabilizes the β-catenin protein, making it available for binding to TCF/LEF-related transcription factors. The β-catenin-TCF/LEF complex in turn directly activates Wnt/Wg target genes in the nucleus (for review see Cavallo et al., 1997; Kuhl and Wedlich, 1997; Willert and Nusse, 1998). WRM-1 (β-catenin) and POP-1 (TCF/LEF), rather than working as cofactors, appear to have opposing functions (for review, see Cadigan and Nusse, 1997; Han, 1997); WRM-1 negatively, rather than positively, regulates POP-1 (TCF/LEF).

Findings reported here point to possible explanations for the reversed genetic relationship between WRM-1 and POP-1. We have shown that rather than binding stably
with POP-1, WRM-1 is required for POP-1 phosphorylation. It is easy to imagine several possible molecular models based on these observations. For example, the LIT-1-related kinases Nemo and Nlk may phosphorylate the corresponding POP-1 homologs in vertebrates and *Drosophila*. In the context of a stable association with β-catenin/Armadillo, this phosphorylation could lead to positive rather than negative regulation.

**Multiple upstream branches for polarity signaling in *C. elegans***.

Understanding how upstream signals control WRM-1 and LIT-1 will require much more genetic and biochemical investigation. β-catenin and its related proteins, Plakoglobin, Armadillo, and *C. elegans* HMP-1 and BAR-1 (Costa et al., 1998; Eisenmann et al., 1998), are all reported to associate with cell junctions. The interaction of β-catenin and plakoglobin with cell adhesion molecules such as E-cadherin appears to influence their signaling properties (reviewed by Klymkowsky and Parr, 1995; Gumbiner, 1997; and Bullions and Levine, 1998), and a recent study suggests that integrin linked kinase (Novak et al., 1998) regulates both β-catenin levels and LEF-1 transcriptional activity. The apparent complexity of A-P polarity signaling in *C. elegans* embryos (Rocheleau et al., 1997) could reflect the existence of multiple signals that converge into WRM-1. The identification of LIT-1 as a co-effecter in polarity signaling increases the possibilities and raises the question of whether upstream activators may directly target LIT-1 kinase activation.
In summary, the current study increases the repertoire of activities associated with β-catenin related proteins. In addition, these findings suggest a possible new target via regulation of LIT-1/Nemo/Nlk kinase activity for controlling the transcriptional activity of TCF/LEF related transcription factors. It will now be interesting to follow the pathway upwards from WRM-1 and LIT-1 to identify how MOM-5/LIN-17/Frizzled and potentially other cell surface receptors activate signaling.
EXPERIMENTAL PROCEDURES

Strains and alleles

The Bristol strain N2 was used as the standard wild-type strain. The marker mutations, deficiencies, and balancer chromosomes used are listed by chromosome as follows: LG III: unc-32(e189), lit-1(t1512), lit-1(t1534), eTi(III; V), qC1; LG IV: him-3(e1147); LG V: dpy-11(e224), mom-2(ne141). *C. elegans* culture and genetics were as described in Brenner (1974).

Microinjection

RNAi was performed as described in Fire et al. (1998) and Rocheleau et al. (1997). The *lit-l* cDNA clone yk457d2 was used to prepare dsRNA.

Transformation rescue of *lit-l(t1512)* and *lit-l(t1534)* was performed using YAC Y26C10, which contains a large segment of *C. elegans* genomic DNA, including the *nemo* homolog. The GFP gene was inserted into Y26C10 by using homologous recombination in yeast. First, a GFP::sup4° cassette was engineered to contain the yeast ochre suppressor tRNA, sup4°, embedded within a synthetic *C. elegans* intron. This GFP::sup4° cassette was then used to create vectors, in which GFP is flanked by short 5' and 3'-flanking sequences of the *nemo* homolog. This configuration allowed homologous recombination in yeast to drive the formation of an in-frame N- or C-terminal insertion of GFP in the *nemo* homolog in Y26C10. Recombinant yeast strains were selected by virtue of suppression of the ochre mutation ade2-1 present in the host yeast strain, AB1380 (*MATa ade2-1 can1-100 lys2-1 trpl ura3 his5*). The N-terminal
GFP insertion resulted in full rescue of *lit-I(t1534)* and partial rescue of *lit-I(t1512)*. Rescue of *lit-I(t1534)* by GFP::LIT-1 was abolished by RNAi targeting the GFP tag, suggesting the tagged gene is responsible for *lit-I* rescue in this strain. The YAC DNA was prepared for injection by making total yeast genomic DNA from the YAC-bearing strain and purifying the DNA over a Qiagen column (Qiagen). In each experiment, 200 µg/ml of total yeast DNA was mixed with 100 µg/ml of the dominant *rol-6* marker plasmid pRF4 (Mello et al., 1991; Mello and Fire, 1995). Approximately 50 separate transgenic strains were made with each construct, and 1 in 5 were found to have incorporated the coinjected YAC sequences.

**Molecular analysis and plasmids**

Coding sequences in *lit-I* and mutant alleles were determined by sequencing RT-PCR products as described in Rocheleau et al. (1997). The *lit-I* cDNA differ slightly from the genome center’s GeneFinder predictions for the corresponding open reading frame W06F12.1 and are detailed in the GenBank accessions AF143243 and AF143244.

For expression in COS-7 cells, we cloned the full-length WRM-1 (Rocheleau et al., 1997), LIT-1, and POP-1 (Lin et al., 1995) were tagged at the N-terminus with HA, FLAG, and Myc epitopes, respectively, and cloned in vector pCDNA3 (Invitrogen). Point mutations and truncations of the full-length genes were constructed using protocols described in Ausubel et al. (1997). The vectors pACT2 and pAS1 were used for the two-hybrid assays (Clontech).
Cell culture, transfection and immunoprecipitation-kinase assay.

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Transient transfections were performed using lipofectamine (Life Technologies). Cells were harvested 48 hrs after transfection following serum starvation for 12 hrs by lysis in Triton X-100 lysis buffer (20 mM Tris-HCl [pH 7.4], 137 mM NaCl, 10% glycerol, 1% Triton X-100, 25 mM β-glycerophosphate, 2 mM pyrophosphate, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM PMSF and 10 μg/ml leupeptin). The immunoprecipitation-kinase assay was performed as described previously (Whitmarsh et al., 1997), with or without added purified GST-POP-1 (1 μg).

Immunoprecipitation and phosphatase treatment.

Immunoprecipitation of tagged proteins from COS-7 cells was performed using a mouse anti-FLAG antibody M2, a rat anti-HA antibody 3F10 (Roche Molecular Biochemicals), or a mouse anti-Myc antibody 9E10. The phosphatase treatment of immunoprecipitated Myc-POP-1 was performed as described previously (Papavassiliou and Bohmann, 1992). For immunoprecipitation from the *C. elegans* extracts, ~2.5x10⁶ embryos were homogenized in buffer (25 mM HEPES-NaOH [pH7.4], 140 mM NaCl, 1 mM DTT, 10% glycerol, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM PMSF, 5 μg/ml aprotinin, 4 μg/ml E-64, 2 μg/ml pepstatin and 5 μg/ml leupeptin) using a stainless steel homogenizer and lysed with 1% NP-40. Approximately 1 mg of protein extract was used for immunoprecipitation with a mouse monoclonal anti-GFP
antibody, 3E6 (Quantum Biotechnologies). Following the immunoprecipitation, WRM-1 was visualized by immunoblotting using a monoclonal antibody, P3C8 (1/10 dilution). We estimate that 1%-2% of the total WRM-1 protein present in the extract immunoprecipitated with GFP::LIT-1 under the conditions described here. This number may reflect a significant underestimate, as the conditions for immunoprecipitating GFP and for maintaining the WRM-1/LIT-1 complex were not optimized.

Microscopy

Cellular differentiation in mutant and lit-1(RNAi) embryos was analyzed as described previously (Mello et al., 1992; Rocheleau et al., 1997). For example, lit-1(RNAi) embryos were analyzed for endoderm differentiation using both cell lineage analysis and light microscopy. Briefly, the E cell lineage was examined in six embryos, and in each case Ea and Ep were found to divide prematurely, with timing similar to the mesodermal precursors MSa and MSP. The descendents of E fail to gastrulate, and after 6 hours, when E descendents in wild-type would normally begin to exhibit gut birefringence, instead exhibited evidence of pharyngeal differentiation (see Mello et al., 1992). In all respects, the terminal phenotype of lit-1(RNAi) embryos is indistinguishable from lit-1(t1512) embryos. The POP-1 mABRL2 antibody and the staining procedure were described by Lin et al. (1998). Analysis of postembryonic phenotype of lit-1 was done as follows. Homozygous lit-1(t1512) animals grown at 15°C were allowed to lay eggs at 15°C for 3 hours. Then, the adults were removed and eggs were incubated at 25°C for 14
to 16 hours. The T cell polarity was scored in late L1 larvae as described by Herman and Horvitz (1994).

Immunofluorescence microscopy for COS-7 cells were performed essentially as described (Tournier et al., 1999) except for the following modifications. Cells were fixed in 1x PBS (pH 7.2), 3% paraformaldehyde for 10 min at room temperature, followed by permeabilization in methanol for 10 min at -20°C. Myc-POP-1 was detected by a monoclonal antibody, 9E10. Endogenous M KK7 was visualized as described by Tournier et al. (1999).

**Two hybrid assay.**

A yeast strain, HF7c (MATa, his3-200, trp1-901, leu2-3112, gal4-542, gal80-538, LYS2::GALI_UAS-GALI_TATA-HIS3, URA3::GAL4_17-mer x3-CYC1_TATA-lacZ) was transfected with one of the pACT2 WRM-1 plasmids or pACT2 together with either pAS1 LIT-1, pAS1 POP-1, or pAS1. Two hybrid interactions were determined by colony formation on -Trp/-Leu/-His medium containing 5 to 25mM 3-AT (3-amino-1,2,4-triazole, Sigma).

**Antibody production.**

Monoclonal antibodies were generated against a full-length, His-tagged WRM-1 fusion protein. RBF/Dn mice (The Jackson Laboratory) were injected subcutaneously with 100μg of fusion protein and boosted at 2 week intervals according to published protocols.
(Wayner and Carter, 1987) in the Hybridoma Production Facility at the Fred Hutchinson Cancer Research Center.
CHAPTER III: MOM-4, A MAP KINASE KINASE KINASE-RELATED PROTEIN, ACTIVATES WRM-1/LIT-1 KINASE TO TRANSDUCE ANTERIOR-POSTERIOR POLARITY SIGNALS IN C. elegans

SUMMARY

In C. elegans, a Wnt/Wg-like signaling pathway downregulates the TCF/LEF related protein, POP-1, to specify posterior cell fates. Effectors of this signaling pathway include a β-catenin homolog, WRM-1, and a conserved protein kinase, LIT-1. WRM-1 and LIT-1 form a kinase complex that can directly phosphorylate POP-1, but how signaling activates WRM-1/LIT-1 kinase is not yet known. Here we show that mom-4, a genetically defined effector of polarity signaling, encodes a MAP kinase kinase kinase-related protein that stimulates the WRM-1/LIT-1 dependent phosphorylation of POP-1. LIT-1 kinase activity requires a conserved residue analogous to an activating phosphorylation site in other kinases including MAP kinases. These findings suggest that anterior-posterior polarity signaling in C. elegans may involve a MAP kinase-like signaling mechanism.
INTRODUCTION

During *C. elegans* embryonic development many of the cell divisions in all regions of the embryo are oriented along the anterior-posterior (A-P) axis, and essentially all of these divisions result in A-P daughter cells with different fates (Sulston et al., 1983). Genetic studies of this polarity signaling process have defined a mechanism that involves several proteins with similarities to known Wnt/Wg signaling components (Rocheleau et al., 1997; Thorpe et al., 1997). For example, MOM-2 is related to the secreted protein Wnt/Wg and MOM-5 is related to the membrane protein Frizzled, a candidate Wnt/Wg receptor. As in other Wnt/Wg systems, these upstream factors appear to act through a β-catenin related protein, WRM-1 (Rocheleau et al., 1997; for review, see Cadigan and Nusse, 1997; Han, 1997).

A key difference between polarity signaling in *C. elegans* and Wnt/Wg signaling in vertebrates and *Drosophila* concerns the relationship of WRM-1 and a protein related to vertebrate TCF (T-cell factor)/LEF (lymphoid enhancer factor) transcription factors, POP-1 (Lin et al., 1995). In vertebrates and *Drosophila* the WRM-1 related proteins, β-catenin and Armadillo, are thought to enter the nucleus in response to signaling where they bind to and activate TCF/LEF related factors (for review see Cavallo et al., 1997; Kuhl and Wedlich, 1997; Willert and Nusse, 1998). In *C. elegans*, although WRM-1 is an effector of signaling, WRM-1 appears to have the opposite downstream activity, downregulating rather than activating POP-1 (Rocheleau et al., 1997). In studies on wild-type embryos POP-1 exhibits a lower level of nuclear immunofluorescence staining in the posterior daughters of many A-P divisions than in the anterior daughters (Lin et al., 1995,
1998). Genetic studies have shown that WRM-1 and other signaling components are required for this POP-1 asymmetry between A-P sister cells (Lin et al., 1998; Rocheleau et al., 1997, 1999; Thorpe et al., 1997). Thus in *C. elegans* Wnt/Wg signaling through WRM-1 leads to downregulation of POP-1.

Possible insights into POP-1 regulation by WRM-1 have come from analysis of the gene *lit-1*. Mutations in *lit-1* result in a loss of A-P cell fate asymmetries (Kaletta et al., 1997). Phenotypic and genetic analysis of *lit-1* place this gene in both the MOM-2 and LIN-44 Wnt/Wg signaling systems in *C. elegans*. In the embryo, LIT-1 appears to function along with WRM-1 in a process that reduces POP-1 levels or activity in posterior daughters of A-P divisions (Rocheleau et al., 1999). The LIT-1 protein is related to serine/threonine protein kinases and is most similar to the *Drosophila* tissue polarity protein, Nemo (Choi and Benzer, 1994), and to the mouse protein Nlk (Brott et al., 1998). WRM-1 and LIT-1 appear to form a stable protein complex in vivo in *C. elegans* and in transfected vertebrate cells (Rocheleau et al., 1999). In vertebrate cells WRM-1 activates the LIT-1 protein kinase leading to phosphorylation of WRM-1, LIT-1 and POP-1. These observations support a model in which signaling activates the WRM-1/LIT-1 kinase complex. This complex then directly phosphorylates POP-1 leading to its functional inhibition in posterior daughters of A-P divisions.

How upstream signaling events lead to activation of the WRM-1/LIT-1 kinase is not understood. LIT-1/Nemo/Nlk kinases make up a small subfamily of protein serine/threonine kinases distinct from, but closely related to, MAP kinases (MAPK) (Choi and Benzer, 1994; Brott et al., 1998; Rocheleau et al., 1999). MAPK signaling
pathways, which involve sequential activation of protein kinases called MAPK kinase kinases and MAPK kinases, are highly conserved from yeast to metazoans and regulate many developmental decisions in *C. elegans, Drosophila* and vertebrates (for review see Schaeffer and Weber, 1999; Tan and Kim, 1999; Ip and Davis, 1998; Madhani and Fink, 1998). In the present study, we report the cloning of a new A-P polarity gene, *mom-4*. The *mom-4* locus was previously identified by a set of maternal mutations that cause defects in polarity signaling in the early embryo (Rocheleau et al., 1997; Thorpe et al., 1997). We show here that *mom-4* activity is required for POP-1 asymmetries between anterior and posterior daughters of A-P divisions. *mom-4* encodes a *C. elegans* homolog of mammalian TAK1 (TGF-β activated kinase), which is thought to function as a MAPK kinase kinase (Yamaguchi et al., 1995; Shirakabe et al., 1997; Wang et al., 1997; Fanger et al., 1997). When expressed in cultured mammalian cells, both MOM-4 and TAK1 are able to stimulate WRM-1/LIT-1 kinase activity leading to the increased phosphorylation of POP-1. Finally, we show that this activation is dependent on the putative kinase activation loop of LIT-1 that serves as a target for activating phosphorylation in related kinases. The structural similarities of LIT-1 to MAPK and of MOM-4 to MAPK kinase kinase raise the possibility that a MAPK-like kinase cascade contributes to A-P polarity signaling in *C. elegans*. 
RESULTS

*mom-4* interacts genetically with Wnt/Wg components to regulate POP-1

In an effort to understand the mechanism of A-P polarity signaling in *C. elegans* we sought to identify additional genes that function in this process. Two previous studies reported the identification of the *mom-4* locus as one of a set of *mom* genes whose maternal activities are required for a decision between mesodermal versus endodermal cell fates for daughters of the four-cell stage blastomere called EMS (Rocheleau et al., 1997; Thorpe et al., 1997). The division of EMS is oriented along the A-P axis, and the previously described *mom* genes control cell fate differences between the A-P daughters of this division (Rocheleau et al., 1997; Thorpe et al., 1997). As with the other *mom* mutants, some *mom-4* embryos (9-43%, Table 3-1, p.101) lack endoderm and appear to contain excess pharyngeal mesoderm, a phenotype consistent with a posterior to anterior transformation for the EMS daughter, E. To further examine this possibility we used laser ablation to prevent all blastomeres except E from differentiating in *mom-4(ne19)* mutant embryos. In 13 of 22 such embryos examined we found that E failed to produce endoderm and instead produced mesodermal tissues similar to those normally produced by its anterior sister, MS (Experimental Procedures and data not shown). Furthermore, in lineaged embryos this transformation in cell fate was correlated with accelerated division timing in the E lineage (data not shown). In both of these respects *mom-4* mutant embryos are similar to the previously characterized *mom* mutants (Rocheleau et al., 1997; Thorpe et al., 1997).

It has been reported that *mom-4* mutations strongly enhance EMS polarity defects
Table 3-1. Genetic Analysis of Endoderm Specification in mom-4.

<table>
<thead>
<tr>
<th>Embryo Type</th>
<th>% Embryos Lacking Endoderm (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mom-4(ne4)</td>
<td>9 (214)</td>
</tr>
<tr>
<td>mom-4(ne135)</td>
<td>11 (252)</td>
</tr>
<tr>
<td>mom-4(ne82)</td>
<td>17 (382)</td>
</tr>
<tr>
<td>mom-4(ne19)</td>
<td>43 (1134)</td>
</tr>
<tr>
<td>mom-4(ne19)/qDf9</td>
<td>36 (341)</td>
</tr>
<tr>
<td>mom-4(ne19);mom-4(RNAi)</td>
<td>51 (285)</td>
</tr>
<tr>
<td>mom-2(ne141)</td>
<td>53 (304)</td>
</tr>
<tr>
<td>mom-2(ne141);mom-4(ne19)</td>
<td>100 (98)</td>
</tr>
<tr>
<td>mom-5(RNAi)</td>
<td>2 (511)</td>
</tr>
<tr>
<td>mom-5(RNAi);mom-4(ne19)</td>
<td>100 (251)</td>
</tr>
<tr>
<td>mom-5(RNAi);mom-4(ne135)</td>
<td>100 (54)</td>
</tr>
<tr>
<td>mom-5(zu193);mom-4(RNAi)</td>
<td>56 (304)</td>
</tr>
<tr>
<td>lit-1(RNAi)</td>
<td>96 (604)</td>
</tr>
<tr>
<td>lit-1(RNAi);mom-4(ne19)</td>
<td>95 (193)</td>
</tr>
<tr>
<td>apr-1(RNAi)</td>
<td>42 (234)</td>
</tr>
<tr>
<td>apr-1(RNAi);mom-4(ne19)</td>
<td>100 (329)</td>
</tr>
<tr>
<td>pop-1(RNAi)</td>
<td>0 (117)</td>
</tr>
<tr>
<td>pop-1(RNAi);mom-4(ne19)</td>
<td>0 (116)</td>
</tr>
</tbody>
</table>
associated with \textit{mom-2} mutations and that \textit{mom-4} lies genetically upstream of \textit{pop-1} (Thorpe et al., 1997). However, previous studies indicated that not all \textit{mom} mutants synergize with each other suggesting possible branched pathways that contribute to polarity signaling (Rocheleau et al., 1997). We therefore examined genetic interactions between \textit{mom-4} and the entire set of previously described polarity genes. We found that \textit{mom-4} exhibits strong synergy in genetic tests with \textit{mom-2}, \textit{mom-5}, and \textit{apr-1}, and like these other genes \textit{mom-4} appears to lie upstream of \textit{pop-1} (Table 3-1, p.101). These genetic findings, together with the previous observation that \textit{mom-4(+) }activity is required in the responding cell for A-P polarity signaling (Thorpe et al., 1997), are consistent with a place for \textit{mom-4} either after a convergence between Wnt/Wg and other polarity signals or in a pathway parallel to Wnt/Wg signaling (See Discussion).

In order to ask if \textit{mom-4} is required for POP-1 asymmetry, we stained \textit{mom-4} mutant embryos with an antibody specific for POP-1. We found that POP-1 staining levels were equal and high in the nuclei of sister cells resulting from A-P divisions (Figure 3-1, p.103, and data not shown). Taken together, the genetic relationship of \textit{mom-4} with Wnt/Wg signaling and its strong effect on POP-1 asymmetry suggests that \textit{mom-4} is an integral part of the polarity signaling mechanisms that downregulate the activity of POP-1.
**wild type**  **mom-4(ne19)**

POP-1 localization

Figure 3-1
Figure 3-1. POP-1 is localized symmetrically in *mom-4(ne19)*. 

Top panels show immunofluorescence staining of POP-1 in either wild-type (A) or *mom-4(ne19)* mutant embryos (B). The anterior of the embryo is to the left, and the posterior to the right. Bars connect nuclei of anterior/posterior sister cells MS and E. In wild-type embryos (A), the anterior cell nucleus shows higher levels of POP-1 immunostaining than the posterior sister. *mom-4(ne19)* embryos (B) show equal staining in the nuclei of both anterior and posterior sisters. The lower panels show corresponding DAPI staining of the nuclei in either wild-type (C) or *mom-4(ne19)* (D). Each embryo is approximately 50 microns in length.
mom-4 encodes a homolog of vertebrate TAK1

To clone the mom-4 gene, we mapped it to a small interval on genetic and physical maps of LGI. We then used RNA interference (RNAi) to determine whether the loss of function of any of the predicted genes within this interval could result in a phenotype similar to that of mom-4 (Rocheleau et al., 1997; see Experimental Procedures). One of the genes we tested in this RNAi assay (F52F12.3) gave a low frequency of mom-4-like dead embryos (data not shown). We further analyzed this gene by conducting the RNAi assay in the genetic background of the mom-5 mutation and found that the Mom phenotype associated with mom-5 was strongly enhanced (Table 3-1, p. 101). These observations suggested that F52F12.3 may encode the mom-4 gene. We therefore sequenced the F52F12.3 gene in five mom-4 mutant alleles and found that each contains a mutation in the predicted exons (Figure 3-2A, p. 106). We conclude that F52F12.3 is mom-4. The predicted MOM-4 protein shows an overall similarity to vertebrate TAK1 (Figure 3-2B, p. 106; Yamaguchi et al., 1995) and resembles MAPK kinase kinases in its predicted kinase domain. MOM-4 and TAK1 share additional similarities outside of the respective kinase domain including a serine-rich N-terminal region (Figure 3-2B, p. 106).

The five mom-4 mutants appeared to be strong hypomorphs, possibly including null alleles (Figure 3-2A, p. 106; see also Experimental Procedures). For example, the mom-4(nei9) allele is predicted to cause an early frame shift after codon 46 and a premature translation termination as a consequence. We also found that mom-4(nei9) behaves like a deficiency in genetic complementation tests (Table 3-1, p. 101). Nevertheless, none of the mom-4 mutants produced a fully penetrant maternal effect
Figure 3-2
Figure 3-2. MOM-4 encodes a TAK1 homolog.

(A) Schematic representation of mom-4 genomic structure. mom-4 is trans-spliced to SL1. Black boxes indicate coding exons from which the amino acid sequence in (B) was deduced. The exons containing the kinase domain are indicated with a bracket. Positions of the mom-4 lesions ne4, ne19, ne82, ne135, and ne227 are shown (See Experimental Procedures).

(B) Alignment of MOM-4 amino acid sequence with a C. elegans MOM-4 homolog on Y105C5, and vertebrate TAK1. Identical residues are shaded in gray. The MOM-4 related sequence on clone Y105C5 was predicted based on homology with MOM-4 and on nucleotide homology with known splice acceptor and donor sequences.
Mom phenotype suggesting that the *mom-4* function is not absolutely required for A-P polarity control in the EMS cell. This could reflect a role for MOM-4 in one of multiple parallel pathways for activating the WRM-1/LIT-1 kinase (see Discussion). One possible source of A-P polarity signaling in the absence of *mom-4(+)* activity is a highly similar *mom-4* homolog present in clone Y105C5 in the *C. elegans* genome sequence (Figure 3-2B, p.106). However, we found that RNAi targeting this Y105C5 homolog failed to enhance the phenotype of *mom-4(nei9)* (data not shown). Further genetic tests will be needed to determine if this *mom-4* homolog contributes to A-P polarity signaling.

**MOM-4 activates the WRM-1/LIT-1 kinase**

The identification of MOM-4 as a MAPK kinase kinase homolog prompted us to test whether MOM-4 can activate the MAPK-related kinase LIT-1. Previous work on vertebrate TAK1 had suggested that the serine-rich N-terminal region negatively regulates TAK1 and had shown that an N-terminal truncation that removes this region activates TAK1 (Yamaguchi et al., 1995). We therefore constructed an analogous N-terminal truncation of MOM-4, MOM-4Δ(1-21), that removes the first 21 amino acids of MOM-4. As previously reported (Rocheleau et al, 1999), LIT-1, expressed in and immunoprecipitated from cultured mammalian cells, can phosphorylate itself, WRM-1 and POP-1 in a manner strictly dependent on WRM-1 (Figure 3-3A, lane 3, p.109). Coexpression of MOM-4Δ(1-21) resulted in a three to four-fold increase in the phosphorylation of all three substrates by LIT-1 immunoprecipitates (Figure 3-3A, lane 4, p.109). The increased LIT-1, WRM-1 and POP-1 phosphorylation was completely
Figure 3-3
Figure 3-3. WRM-1/LIT-1 kinase is activated by MOM-4 and is dependent on the LIT-1 activation loop-like motif.

(A) MOM-4 stimulates phosphorylation of POP-1, LIT-1 and WRM-1 by LIT-1 immunoprecipitation complex. COS-7 cells were transfected with expression plasmids encoding epitope tagged LIT-1, WRM-1 and/or N-terminally truncated MOM-4 as indicated at the top of the gel. FLAG-LIT-1 was either wild-type or mutant K89G, which is predicted to be defective in the ATP-binding site and therefore to be kinase inactive. In vitro kinase assays were performed on FLAG-LIT-1 immunoprecipitates with bacterially produced GST-POP-1 as a substrate. FLAG-LIT-1 coimmunoprecipitated with, and subsequently phosphorylated WRM-1. Quantitative comparisons of the radioactive bands reveals that phosphorylation of POP-1, WRM-1 and LIT-1 are increased by 3.5-, 3.3- and 3.8-fold (average of three experiments), respectively, when FLAG-LIT-1 complex was precipitated from MOM-4Δ(1-21) expressing cells (lane 4) compared to FLAG-LIT-1 complex from non-expressing cells (lane 3). The anti-FLAG immunoblot (lower panel) shows comparable amounts of FLAG-LIT-1 present in each immunoprecipitate.

(B) Alignment of the activation loop regions of human CDK2, LIT-1 and human p38 MAPK. Thr160 of human CDK2, Thr180 and Tyr182 of human p38 MAPK are the sites of activating phosphorylation.

(C) The activation loop of LIT-1 is required for its kinase activity. Lysates from COS-7 cells transfected with epitope tagged LIT-1 and WRM-1 were subjected to an immunoprecipitation/kinase assay. FLAG-LIT-1 was either wild-type or contained the
following amino acid substitutions within the putative activation loop: Glu$^{222}$Lys (THK; lane 2), Thr$^{220}$Ala (AHE; lane 3) and double Glu$^{222}$Lys Thr$^{220}$Ala (AHK; lane 4). The genetically identified lit-$1(t1534)$ (Kaletta et al., 1997) is predicted to encode a Glu$^{222}$Lys (THK) mutant protein, with diminished kinase activity (lane 2).
dependent on LIT-1 kinase activity since a point mutation in the predicted ATP-binding site in LIT-1, K89G, abolished all phosphorylation (Figure 3-3A, lane 2, p.109). Full-length MOM-4 expressed at comparable levels to MOM-4Δ(1-21) failed to stimulate LIT-1 kinase activity (data not shown), suggesting that as in TAK1 the N-terminal region of MOM-4 may be a site of negative regulation in mammalian cells. The MOM-4Δ(1-21)-dependent increase in LIT-1 kinase activity required WRM-1 (Figure 3-3A, lane 1, p.109), suggesting that MOM-4 activation does not simply bypass the WRM-1-dependent mechanism for LIT-1 activation. Furthermore, when MOM-4Δ(1-21) was coexpressed we did not observe an increase in the amount of WRM-1 protein present in cell extracts or in coimmunoprecipitates with LIT-1 (data not shown). This finding suggests that the stimulation in LIT-1 kinase activity does not reflect an increase in WRM-1 protein levels or in WRM-1 affinity for LIT-1.

In the MAPK signaling cascade, MAPK kinase kinase induces the activation of MAPK through the phosphorylation/activation of MAPK kinase. MAPK kinase in turn phosphorylates MAPK on threonine and tyrosine residues in its activation loop (Payne et al., 1991) that correspond to Thr180 and Tyr182 in human p38 MAPK (see Figure 3-3B, p.109). LIT-1 contains a putative activation loop within kinase subdomains VII and VIII (Rocheleau et al., 1999) and Thr220 in LIT-1 is analogous to Thr180 in p38 MAPK (Figure 3-3B, p.109). LIT-1 does not contain a Tyr182 equivalent but instead contains a negatively charged Glu222 at this position. We refer to these residues using the single letter amino acid designations in Figure 3-3B and C. We examined the potential importance of the putative activation motif in LIT-1 by altering Thr220 and Glu222...
separately or in combination. The hypomorphic allele, *lit-1(t1534)*, is reported to contain a single Glu222 to Lys mutation (“THK” instead of “THE” in Figure 3-3C, p.109). The majority of *lit-1(t1534)* mutant embryos are severely defective in morphogenesis and yet are able to form the endoderm (Kaletta et al., 1997; Rocheleau et al., 1999). Consistent with these observations, we found that the THK mutant protein could phosphorylate POP-1, LIT-1 and WRM-1 but only weakly (Figure 3-3C, compare lane 1 and lane 2, p.109). The single mutant protein Thr220Ala (AHE, in Figure 3-3C, p.109) and the double mutant protein AHK showed no detectable kinase activity either in the absence (Figure 3-3C, lanes 3 and 4, p.109), or in the presence of MOM-4Δ(1-21) (data not shown). These observations support the view that the activation loop-like motif in LIT-1 is important for LIT-1 kinase activity.
DISCUSSION

In C. elegans, the relative A-P positions of sister cells at birth is coupled to cell fate decisions by a polarity signaling mechanism that involves Wnt/Wg like components (Lin et al., 1995, 1998; Rocheleau et al., 1997; Thorpe et al., 1997). A key target of this signaling pathway is the POP-1 protein, which appears to be downregulated by signaling (Lin et al., 1998; Rocheleau et al., 1997; Thorpe et al., 1997). Two proteins WRM-1, a β-catenin related protein, and LIT-1, a conserved serine/threonine protein kinase, appear to be essential for POP-1 downregulation (Rocheleau et al., 1999). In cell culture assays WRM-1 and LIT-1 form an active kinase complex that can directly phosphorylate the POP-1 protein, suggesting that the WRM-1/LIT-1 kinase may directly downregulate POP-1 in vivo. Genetic studies suggest that the signaling pathway is branched upstream of WRM-1 and LIT-1 and may have polarity inputs from sources other than the MOM-2/Wnt related protein (Rocheleau et al., 1997). For example a large percentage of embryos from mutant strains carrying apparent null alleles of both mom-2(Wnt) and mom-5(Frizzled), nevertheless exhibit proper specification of posterior cell fates, strongly suggesting that alternative polarity signals must be able to activate the WRM-1/LIT-1 kinase.

In the present study we have described a new polarity signaling protein, MOM-4, homologous to the vertebrate protein TAK1. Mutations in mom-4 strongly synergize with mutations in mom-2 and mom-5 raising the possibility that mom-4(+) activity is required for Wnt/Wg independent polarity signaling. TAK1 is thought to be a MAPK kinase kinase and can phosphorylate and activate MAPK kinases both in vitro and in
transfection assays (Yamaguchi et al., 1995; Wang et al., 1997; Fanger et al., 1997 for review, Ninomiya-Tsuji et al., 1999). The LIT-1/Nemo/Nlk kinases belong to a small subfamily of serine/threonine kinases that are distinct from but closely related to MAPK (Choi and Benzer, 1994; Brott et al., 1998; Rocheleau et al., 1999). Within its kinase domain, LIT-1 is approximately 45% identical (132 out of 292 residues) to human p38 MAP kinase and 43% identical (126 out of 292 residues) to human ERK1, respectively. Furthermore, we have shown here that, LIT-1 activation appears to require a conserved motif analogous to a site required for activating phosphorylation by MAPK kinases. Thus we have shown that MOM-4 is similar to MAPK kinase kinase and that LIT-1 which is similar to MAPK in amino acid sequence, is also similar to MAPK in its activation. These observations together with the genetic synergy between mom-4 mutants and Wnt/Wg pathway mutants suggest that a MAP kinase-like cascade may function in parallel with Wnt/Wg signaling to specify A-P cell fate differences during C. elegans development. In the future, understanding how MOM-4 is activated and how MOM-4 in turn activates LIT-1 is likely to shed light on how Wnt/Wg signals interact with other signaling pathways to control cell polarity and cell fate.
EXPERIMENTAL PROCEDURES

Strains and Alleles

The Bristol strain N2 was used as the standard wild-type strain. The marker mutations, deficiencies and balancer chromosomes used are listed by chromosome as follows: LGI: dpy-5(e61), unc-13(e1091), hDf9, nDf25, nDf24, nDf29, qDf9, qDf10, mnDf111, lin-11(n566), hT1(I;IV), hT2(I;III); LGII: bli-2(e768); LGIII: unc-32(e189), dpy-18(e364), eT1(III;V), qCl; LGIV: unc-5(e53), nT1(IV;V); LGV: dpy-11(e224), him-5(e1409); LGX: lin-2(e1309), lon-2(e678). C. elegans culture, mutagenesis, and genetics were as described in Brenner (1974).

Genetic analysis

Mutant alleles were isolated as described previously (Mello et al., 1992; 1994). Standard genetic crosses were used to map mom-4(nei35) to the unc-13 lin-11 interval on LGI and deficiency mapping was used to map mom-4(nei9) between cloned genes mec-8 and mom-5. Data from these crosses are available from the C. elegans database, ACEDB. Self progeny from mom-4(nei9) adult hermaphrodites appeared identical in all respects to cross progeny by wild-type males, indicating that these gene activities are required maternally. Complementation tests were performed as follows: mom-4(nei9) against all other mom-4 alleles, mom-4(nei9) against the chromosomal deficiency qDf9. In all cases, heterozygotes grew to adults that produced dead embryos resembling those produced by mom-4(nei9).
**RNAi reverse genetics**

RNAi was performed for 29 *mom-4* candidate genes in the *mec-8 lin-11* genetic interval (data not shown). If available, cDNA clones from Yuji Kohara’s collection were used as template for RNA synthesis, if no cDNA clone was available template was made by PCR using primers tagged with the T7 promoter (Rocheleau et al. 1997). Synthesis and injection of RNAi was done as described in Fire et al. (1998) and Rocheleau et al. (1997).

**Molecular analysis and plasmids**

Coding sequences in *mom-4* were determined by sequencing RT-PCR products amplified using primers for the SL1 trans-splice leader sequence and the gene specific sequence. The *mom-4* coding sequences we determined show some inconsistencies with the GeneFinder predictions for the corresponding open reading frame F52F12.3, these differences are detailed in the GenBank accession AF143242. Mutant alleles were sequenced using standard protocols from PCR-amplified genomic DNA. *ne19* encodes a single nucleotide deletion in codon 46 that results in a frameshift followed by a subsequent stop codon. *ne82*, *ne4* and *ne135* respectively encode for Trp156, Trp482 and Arg370 to stop. *ne227* changes a splice site at the end of exon 10 to a stop.

For expression in COS-7 cells, we cloned the full-length MOM-4 (2523nt) and N-terminal truncated MOM-4 (lacking the first 21 amino acids), tagged at the N-terminus with the T7 gene 10 protein epitope, into pCDNA3 (Invitrogen). The full-length cDNA for the LIT-1 isoform LIT-1a (1365nt) was tagged at the N-terminus with the FLAG
epitope and cloned into pCDNA3. We used a PCR-based site-directed mutagenesis method to create amino acid changes in the putative T-loop within the LIT-1 kinase domain. The HA-WRM-1 and the GST-POP-1 plasmids were described previously (Rocheleau et al., 1999).

**Cell culture, transfection and immunoprecipitation-kinase assay**

Cell culture and transfection procedures and the immunoprecipitation-kinase assays were performed as previously described (Rocheleau et al., 1999). For quantitative analyses of LIT-1 kinase activity, the amounts of FLAG-LIT-1 and HA-WRM-1 were normalized for each immunoprecipitation. Following the kinase reaction, radioactive bands corresponding to FLAG-LIT-1, HA-WRM-1 and GST-POP-1 were quantitated using a phosphoimager (Molecular Dynamics).

**Microscopy**

Light and immunofluorescence microscopy and laser microsurgery were as described previously (Bowerman et al., 1992a; Lin et al., 1998; Rocheleau et al., 1997). The identities of differentiated cells were assigned based on morphological criteria in the light microscope, followed, in most cases by fixation and staining with tissue-specific probes. Criteria for assigning cell fates, and antibodies were as described in Bowerman et al. (1992a) and Mello et al. (1992). The POP-1 mABRL2 antibody and the staining procedure were described previously (Lin et al. 1998).
GENERAL DISCUSSION

REVIEW OF WNT/WG SIGNALING IN C. elegans

Wnt/Wg signaling has been shown to function multiple times during the development of Drosophila, Xenopus, and mouse. Similarly in C. elegans Wnt/Wg signaling functions multiple times during development in numerous processes. There are five Wnt genes in the C. elegans genome. The egl-20 and lin-44 wnt genes have been implicated in many post-embryonic polarity signaling events while mom-2 is required for polarity signaling during early embryonic development (Herman and Horvitz, 1994; Herman et al., 1995; Rocheleau et al., 1997; Thorpe et al., 1997; Maloof et al., 1999). All three wnt genes appear to play a similar role in providing the A-P polarity cues necessary for asymmetric cells divisions, cell migrations and cell fate specification. cwn-1, and cwn-2 were identified by homology (Kamb et al., 1989; Waterston et al., 1992).

Northern analysis indicated both cwn-1 and cwn-2 are expressed and are most abundant during embryogenesis (Shackleford et al., 1993), but no biological role for cwn-1 and cwn-2 has been identified. Reviewed below are C. elegans developmental events that have been shown to require Wnt/Wg signaling or at least downstream components of the pathway. A comparison of C. elegans Wnt/Wg signaling pathways with each other and Wnt/Wg signaling pathways from other organisms may shed light on the mechanism of Wnt/Wg signal transduction and the evolution of the Wnt/Wg pathway.
The MS/E decision

As described in this thesis, the MS/E decision is encoded by multiple inputs (Rocheleau et al., 1997). One encoded by *mom-2* (*Wnt/wg*), *mom-1* (*porc*), and *mom-5* (*fz*) the second encoded by *apr-1* (APC) (Rocheleau et al., 1997; Thorpe et al., 1997). These inputs likely converge at or upon the *arm/B*-catenin related gene *wrm-1* (Rocheleau et al., 1997). This *Wnt/Wg* pathway also requires *lit-1*, a kinase related to *Drosophila nemo* and an upstream activating kinase, *mom-4*, related to vertebrate TAK1 (Meneghini et al., 1999; Rocheleau et al., 1999; Shin et al., 1999). Thus, LIT-1 and MOM-4 encode a novel kinase cascade that converges with the *Wnt/Wg* pathway (Figure C-1B, p.133).

Two-hybrid screening and database searches have identified a *C. elegans* gene related to TAB1 (Meneghini et al., 1999; C.E.R. and C.C. Mello). TAB1 (TAK1 binding protein), can bind and activate TAK1 kinase (Shibuya et al., 1996). Although RNAi for *tap-1* (TAB1-like protein) does not have an embryonic lethal phenotype (C.E.R. and C.C. Mello), *tap-1*(RNAi) into a weak *mom-4* mutation can enhance the phenotype (Meneghini et al., 1999). Like TAB1, TAP-1 can bind and activate the MOM-4 kinase, and likely functions upstream of *mom-4* in the kinase cascade (Meneghini et al., 1999; Figure C-1B, p.133). Recently TAB1 and TAK1 have been shown to function downstream of TRAF6 in IL-1 signaling (Ninomiya-Tsuji et al., 1999). It will be interesting to determine if TRAF6 or any of the upstream components of the IL-1 pathway have homologs in *C. elegans*, and if they have a role in endoderm specification.

The *mom-3* gene remains uncloned, though blastomere isolation experiments show that it along with *mom-1* and *mom-2* are required in the signaling cell, P2 (Thorpe et
al., 1997). Mutations in porc and wg can lead to retention of Wg in the signaling cell suggesting that proper modification and folding of Wg is likely required for Wg secretion (van den Heuvel et al., 1993). Porc can lead to increased N-linked glycosylation of Wg when they are coexpressed in cell culture (Kadowaki et al., 1996). Thus, mom-3 may encode a protein also involved in the glycosylation/modification of MOM-2.

We recently found that two C. elegans dsh homologs C27A2.6 and mig-5 function redundantly to specify endoderm. RNAi for both dsh homologs results in an embryonic lethal mom phenotype with embryos that fail to produce intestine at a frequency similar to that seen with mom-5 mutations. Like mom-5, mig-5/C27A2.6(RNAi) displays an enhanced mom phenotype in the mom-4 mutant background. The mig-5/C27C2.6(RNAi) embryos also share the same ABar spindle orientation defects as mom-2 and mom-5 mutant embryos (discussed below). Thus the mig-5/C27A2.6 dsh genes function in the mom-2/mom-5 branch of the pathway, consistent with it being downstream of Fz (Figure C-1B, p.133; C.E.R., H. Sawa, and C. Mello, unpublished results).

Recently it has been found that a C. elegans casein kinase I (CKI) homolog, kin-19, displays a mom phenotype (Peters et al., 1999; C.E.R., T. Shin, and C.C. Mello, unpublished data). Xenopus CKI has been shown to bind and phosphorylate Dsh, though epistasis experiments indicates that CKI functions downstream of Dsh and upstream of GSK-3 (Peters et al., 1999). Thus, it would be predicted that kin-19 may function with or downstream of C27A2.6 and mig-5. It has not yet been determined whether kin-19 has a defect in the spindle orientation of ABar cell as might be predicted.
A *C. elegans* zw3/GSK3 homolog, *sgg-1*, has also been found to function in the Wnt/Mom pathway. Interestingly *sgg-1* is required for specification of endoderm from the E cell and inhibiting the formation of endoderm from the C lineage, as *sgg-1(RNAi)* embryos frequently fail to form E derived intestine and produce ectopic intestine from the C lineage (Y. Bei and C. Mello, unpublished data; Schlesinger et al., 1999). Thus, *sgg-1* is functioning positively with the *mom* genes to specify the E fate. Genetic placement of *sgg-1* into the Wnt/Mom pathway is complicated as it enhances the *mom* phenotypes of genes from all branches of the pathway (Y. Bei and C. Mello). In vertebrate systems APC functions with Axin and GSK3 to suppress β-catenin in the absence of signal (Figure C-1A, p.133). Interestingly both *sgg-1* and *apr-1* function positively in the MOM pathway in the same direction as wtm-1/β-catenin (Figure C-1B, p.133; Rocheleau et al., 1997; Schlesinger et al., 1999; Y. Bei and C. Mello; discussed below).

Polarity signaling during embryonic development appears to be complex (Figure C-1B, p.133). Genetically the Mom pathway appears to be different from the caniconical Wnt/Wg pathway as determined in other systems (Figure C-1A and B, p.133). The differences between the Mom and other Wnt/Wg pathways are discussed in more detail in general discussion. Identification of more components and biochemical analysis of interactions between components will be necessary for sorting out their functions in this pathway.
**Male tail development**

During post-embryonic development, Wnt signaling is required for the proper A-P polarity of the B and T blast cell lineages in the male tail. The T cells are required for the generation of hypodermal cells that join the hypodermal syncytium, a group of neurons, and a hypodermal blast cell that divides in a sex specific manner producing three sensory rays during male tail development. The B cell is required to generate neurons and structural cells of the tail (Sulston and Horvitz, 1977; Sulston et al., 1980).

LIN-44 encodes a Wnt/Wg protein (Herman et al., 1995). Mutations in lin-44 often result in a polarity reversal of asymmetric A-P cell divisions of the B and T cell lineages (Herman and Horvitz, 1994). LIN-17 encodes a seven transmembrane receptor related to Fz family of receptors (Sawa et al., 1996). Mutations in lin-17 cause the B and T cells lineages to lose their asymmetry and divide to produce daughters cells of the same fate (Sternberg and Horvitz, 1988). lin-44; lin-17 double mutants have B and T cell lineage defects identical to that of the lin-17 single mutant in which the B and T cells divide symmetrically (Herman and Horvitz, 1994). Thus, lin-17 is downstream of lin-44, the first genetic evidence placing a Fz receptor downstream of Wnt/Wg (Figure C-1C and D, p.133). LIN-44 has been found to be expressed in the tail hypodermal cells posterior to the B and T cells during late embryogenesis and larval development (Herman et al., 1995). The difference in phenotypes, polarity reversal versus loss of polarity, suggests that in the absence of the posteriorly secreted LIN-44 Wnt/Wg signal, LIN-17 is able to respond to an anteriorly localized signal, thus reversing the polarity of the cell division, and in the absence of LIN-17, the cell is unable to respond to either signal and is not
polarized and divides symmetrically (Sawa et al., 1996). A analogous situation may exist with MOM-5 and MOM-2. Greater than 90% of mom-5 embryos produce intestine, while only about 50% of mom-2 embryos produce intestine, suggesting that MOM-5 can transduce an inhibitory signal in the absence of MOM-2 (Rocheleau et al., 1997; see general discussion). MOM-5 may either possess an intrinsic signaling capability or respond to a second ligand in the absence of MOM-2. In the absence of MOM-5, neither positive (MOM-2), or negative polarity signals can be transduced, but the EMS cell can be correctly polarized 90% of the time due to other parallel polarity signals likely encoded by apr-1 and/or mom-4.

Recently mutations in lit-1 and egl-27 have been shown to affect the polarity of the T cell lineages and mutations in son-1 are required for certain asymmetries of the B cell lineage (Herman et al., 1999; Jiang and Sternberg, 1999; Rocheleau et al., 1999). In lit-1 animals, the T cells divide symmetrically with the same frequency as observed with lin-17, and like lin-17 functions downstream of lin-44 (Rocheleau et al., 1999; Figure C-1C, p.133). Thus, lit-1 is functioning in both embryonic and post-embryonic Wnt/Wg pathways.

Mutations in the egl-27 gene result in T cell polarity defects similar to lin-17 (Herman et al., 1999). EGL-27 encodes a protein similar to Mta1, a chromatin regulatory factor overexpressed in metastatic cells (Ch'ng and Kenyon, 1999; Herman et al., 1999; Solari et al., 1999). EGL-27 displays defects in Q cell and HSN migrations as do mutations in other Wnt/Wg signaling components (see below) suggesting that EGL-27
may be a common component of the Wnt/Wg pathway in *C. elegans* (Herman et al., 1999). EGL-27 functions cell autonomously, and is expressed in the nucleus. It has not been positioned in the pathway, but has been suggested to function at the bottom of, or in parallel to the Wnt/Wg pathway (Herman et al., 1999; Figure C-1C, p.133).

In *lin-17* mutants in which the early B lineage is wild-type, late lineage defects, such as a sheath to neuron cell fate transformation can occur. Mutations in *son-1* result in the same defect as these *lin-17* mutants, in addition, *son-1* mutations exhibit other defects seen in *lin-17* mutants such as vulval lineage defects and single arm gonads (Ferguson and Horvitz, 1985; Ferguson et al., 1987; Sternberg and Horvitz, 1988; Jiang and Sternberg, 1999). SON-1 is an HMG1/2 protein distinct from the TCF/LEF class of HMG proteins (Jiang and Sternberg, 1999). Overexpression of POP-1 results in the same phenotype of mutations in *lin-17*, or *son-1*, thus POP-1 or a POP-1-related activity can function to negatively regulate the specification of the sheath cell fate. Genetic analysis favors a model in which SON-1 may function after inhibition of POP-1 to activate gene transcription (Jiang and Sternberg, 1999; Figure C-1D, p.133). Thus *lin-44* and *lin-17* function in the control of polarity of the T and B cells, and in addition *lit-1* and *egl-27* function downstream in the T cell, and *son-1* and maybe *pop-1* function in control of SPD sheath cell fate in the B cell lineage (Figure C-1C and D, p.133).
Q cell migrations

The Q neuroblasts, QL and QR are born in the same A-P position on opposite sides of the embryo. The QL and QR neuroblasts undergo an identical sequence of divisions, though they and their descendents migrate in opposite direction along the A-P axis to populate regions of the body with sensory neurons (Sulston and Horvitz, 1977; Chalfie and Sulston, 1981). The posterior migration of the QL descendents (QL.d) depends on the expression of the HOX gene, mab-5. In mab-5 loss of function mutations both Q neuroblasts migrate anteriorly, and mab-5 gain of function mutations result in both Q neuroblasts to migrate posteriorly (Kenyon, 1986; Salser and Kenyon, 1992; Antebi et al., 1997).

A number of genes have been identified as being required for the expression of MAB-5 and/or the proper posterior migration of the QL.d cells. Mutations in bar-1, egl-20, egl-27, lin-17, mig-1, mig-5, and mig-14, result in QL.d to migrate anteriorly like QR.d (Hedgecock et al., 1987; Way et al., 1992; Guo, 1995; Harris et al., 1996; Herman et al., 1999; Maloof et al., 1999). Four of these genes are homologs of the Wnt/Wg signaling genes, egl-20 (wnt/wg), lin-17 (fz), mig-5 (dsh), and bar-1 (arm/β-catenin) (Guo, 1995; Sawa et al., 1996; Eisenmann et al., 1998; Maloof et al., 1999; Figure C-1E, p.133). The egl-27 gene is related to Mta1 (discussed above; Herman et al., 1999), and mig-1 and mig-14 remain uncloned. Mutations in pry-1 result in the opposite phenotype, the QR.d cells express MAB-5 and migrate posteriorly (Maloof et al., 1999). Genetic analysis of pry-1 indicates that it functions downstream of egl-20, but upstream of bar-1, and functions to repress bar-1 in the QR neuroblast to prevent MAB-5 expression.
(Maloof et al., 1999; Figure C-1E, p.133). The molecular identification of pry-1 has not been determined, but could possibly encode any one of number of negative regulators of Arm/β-catenin such has Zw3/GSK3, Axin, or APC (Peifer et al., 1994; Siegfried et al., 1994; Munemitsu et al., 1995; Rubinfeld et al., 1996; Polakis, 1997; Zeng et al., 1997). as well as components of the SCF (Skp1p, Cdc53/Cul1, F-box protein) complex required for the targeted degradation of β-catenin (for review see Maniatis, 1999). Genetic epistasis between pry-1, mig-1 and mig-14 indicate that mig-1, and mig-14 function upstream of pry-1 (Maloof et al., 1999; Figure C-1E, p.133). It will be interesting to see if mig-1 and mig-14 encode known or novel components of the Wnt/Wg signaling pathway.

**P12 cell fate specification**

The P12 cell is the most posterior of the row of P1-P12 neuroectoblasts. The anterior daughters of P11 and P12, P11.a and P12.a undergo identical lineages to produce ventral cord neurons. The P11.p cell fuses with the large hypodermal syncytium, hyp7, though P12.p divides again to produce a cell that undergoes a program cell death and to produce P12.pa, a unique hypodermal cell, hyp12 (Sulston and Horvitz, 1977). lin-44 is also been found to be required for the specification of the P12 cell fate (Herman and Horvitz, 1994). In a percentage of lin-44 worms, P12 adopts a P11 cell fate.

Additionally, mutations in components of the RTK/Ras/MAPK pathway, encoded by let-23/let-60/mpk-1, result in a P12 to P11 cell fate change (Fixsen et al., 1985; Aroian and Sternberg, 1991; Clark et al., 1992). Double mutant analysis of lin-44 or lin-17 with components of the RTK/Ras/MAPK pathway synergize and result in a higher penetrance
of P12 to P11 cell fate changes suggesting that Wnt/Wg signaling and RTK/Ras/MAPK signaling are functioning in parallel (Jiang and Sternberg, 1999). Mutations in egl-5 Hox gene result in a fully penetrant P12 to P11 defect (Kenyon, 1986; Chisholm, 1991) and egl-5 appears to be downstream of both these signals (Jiang and Sternberg, 1999). The time requirement for Wnt/Wg signal is earlier than the time requirement for LIN-3 (EGF), suggesting that these pathways specify the P12 cell fate at different times.

### Vulva Development

A number of studies have found evidence for the function of Wnt/Wg signaling during multiple steps of vulval development (see below). During L1 development, a single row of cells form along the ventral midline, P1-12. Each Pn cell divides to generate a Pn.a neuroblast and a Pn.p hypodermal cell. Five Pn.p cells (P1.p, P2.p, P9.p, P10.p, P11.p) adopt the F (fused) fate, in which the cell fuses with the hypodermal syncytium and the P3.p-P8.p generate the vulval precursor cells (VPCs) (Sulston and Horvitz, 1977). The P3.p cell 50% of the time adopts the F fate during wild-type development (Sulston and White, 1980; Sternberg and Horvitz, 1986). Well characterized signaling events are required to further induce the VPCs to adopt the 1°, 2°, or 3° vulval cell fates during the L2/early L3 stage (reviewed in Kornfeld, 1997). The anchor cell (AC) sends an inductive signal that causes the P6.p cell to adopt a 1° cell fate. This signal from the AC is a RTK/Ras/MAPK signaling pathway encoded by LET-23, LET-60, and MPK-1/SUR-1 respectively. Lateral signaling mediated by the LIN-12 Notch pathway, and possibly from the RTK/Ras/MAPK pathway induces P5.p and P7.p
to adopt the 2° cell fate. The remaining cells P3.p, P4.p and P8.p do not receive either signal and adopt the 3° fate. The 1° and 2° cells will divide to produce the vulva, the 3° cells divide once and fuse with the hypodermal syncytium. Mutations in bar-1 result in the VPCs adopting the F cell fate, a phenotype seen in partial reduction-of-function mutations in the Hox gene lin-39 (Eisenmann et al., 1998). lin-39 appears to function first for the specification of the VPC fate over the F fate (Clark et al., 1993; Wang et al., 1993), and then again is required for the specification of the 1° and 2° fates versus the 3° fate (Clandinin et al., 1997; Maloof and Kenyon, 1998). Again mutations in bar-1 resemble those of lin-39, in cells that do become a VPC, and normally adopt either a 1° or 2° cell fate sometimes adopt the 3° fate (Eisenmann et al., 1998). As mentioned above BAR-1 encodes a protein related to Arm/β-catenin. Mosaic analysis shows bar-1 functions cell autonomously in the VPCs and BAR-1::GFP is expressed in the VPCs. Analysis of LIN-39 expression in bar-1 mutants revealed that P3.p and P4.p lose LIN-39 staining during the L2 stage, and P5.p-P8.p exhibit less penetrant defects in LIN-39 expression, coincident with the fact that mutations in bar-1 have a greater affect on the fates of the P3.p and P4.p cells, than seen on P5.p-P8.p (Eisenmann et al., 1998). The LET-23/LET-60/MPK-1 pathway has been shown to regulate LIN-39 expression in P5.p-P7.p (Maloof and Kenyon, 1998). In let-23; bar-1 double mutants, fewer animals showed LIN-39 staining in P5.p-P7.p, and the frequency in which P5.p-P7.p adopts the F fate increases. This suggests that bar-1 and let-23 have partially redundant roles to regulate LIN-39 expression in P5.p-P7.p (Eisenmann et al., 1998). Genetic epistasis analysis of bar-1 with mutations or transgenic constructs that result in a multi-vulva
(Muv) phenotype indicate that bar-1 functions downstream of let-60 (ras), and mpk-I (MAPK), and upstream or in parallel to lin-1 and lin-31 transcription factors in specifying the 1° and 2° fates over the 3°. Thus, bar-1 is required twice during specification of the vulva, once in specifying the fates of the VPCs and again during specification of the 1°, 2°, and 3° cell fates, in both cases it appears to be functioning to turn on expression of the lin-39 Hox gene (Eisenmann et al., 1998). The genetic data suggest bar-1 is functioning with a MAPK pathway to regulate lin-39, as it is in the specification of the P12 cell fate. Interestingly other Wnt/Wg pathway genes appear to have diverse functions during vulval development, but none appear to have the same phenotype as bar-1.

Mutations in lin-17 (fz) result in a multi-vulva (Muv) phenotype and generate a supernumerary vulva-like structure immediately posterior to the vulva (Ferguson and Horvitz, 1985). In lin-17 mutants the P7.p cell lineage either undergoes a symmetric division or divides asymmetrically with a reversed polarity (Ferguson et al., 1987). Mutations in lin-18 also specifically affect the lineage of the P7.p cell in a similar manner suggesting that a may encode a gene that might function with lin-17 in this process (Ferguson et al., 1987). Mutations in lin-44 result in animals with egg-laying defects and can have protruding vulva (Herman and Horvitz, 1994), though the basis for these defects has not been determined.

Genes that function during embryonic development might also have functions in the vulva. Mutations in mom-1 display partially penetrant vulval defects, and both a GFP:LIT-1 and POP-1 staining is detected in cells of the developing vulva (Rocheleau et
al., 1997; Lin et al., 1998; C.E.R. and C.C. Mello, unpublished data). Further investigation will be required to determine if these genes might function in the as part of a Wnt/Wg pathway. Thus, it appears that Wnt/Wg signaling, in addition to RTK/Ras/MAPK and Notch signaling is required at multiple steps of vulval development.

Additionally various genes related to Wnt/Wg signaling components have been found to be required for proper development of the somatic gonad and the ventral hypodermis as well as other cell migrations. Less is known about their functions in these developmental events and will not be discussed further.

There appears to be an overall theme to Wnt/Wg signaling in C. elegans for the transduction of A-P polarity. In the absence of signal, cells appear to recognize the A-P axis, but fail to distinguish which end is anterior and which is posterior. For example, in the absence of signal, cells still divide or migrate along the A-P axis, but divide symmetrically or with a reverse polarity or migrate in the opposite direction. Thus, Wnt/Wg signaling is likely only one of the polarity cues that informs the cell of its location within the organism with respect to axis. Cell adhesion molecules and other signaling pathways are likely to also be required.

Not enough is known about each of these postembryonic Wnt/Wg pathways to make an in depth comparison between them and the Mom/Wnt pathway. The Mom pathway differs from the canonical Wnt/Wg pathway as below. For example, WRM-1 appears to negatively regulate POP-1, where Arm/β-catenin functions as a transcriptional activator for TCF/LEF. APR-1 and SGG-1 appear to function as positive effectors of
signaling, while their counterparts in other systems function as negative regulators of Wnt/Wg signaling. Whether these differences hold for all *C. elegans* Wnt/Wg pathways or just the Mom pathway will require further investigation. Does POP-1 function similarly in other Wnt/Wg signaling events, or are there other proteins that might function as positive effector of signaling? Can LIT-1 be activated by BAR-1? Are there roles for *sgg-1, wrm-1* and *apr-1* during postembryonic development? It is possible there are, but RNAi phenotypes might fail to reveal these later requirements. Identification of more components of these pathways will help to provide insight into the mechanism by which the Wnt/Wg signal is transduced in *C. elegans* as well as in other systems.
Figure C-1
Figure C-1. *C. elegans* Wnt/Wg signaling pathways. A). Model of the Wnt/Wg signaling components from other systems.

(B). Model of Wnt/Wg signaling for specification of endoderm. *mom-3* is uncloned and its position relative to *mom-2* and *mom-1* is unknown.

(C). Model of Wnt/Wg signaling for specification of A-P cell fates of the T cell lineages.

(D). Model of Wnt/Wg signaling for the B cell lineage. All genes are required for specification of the SPD sheath cell fate, though no role for *pop-1* or *son-1* for specification of early lineage fates has been determined.

(E). Model for Wnt/Wg signaling for control of *mab-5* expression in the QL lineage. *pry-1*, *mig-1*, and *mig-14* have not been cloned. *pry-1* functions between *egl-20* and *bar-1*, and both *mig-1* and *mig-14* are upstream of *pry-1*. 
COMPARISON OF MOM/WNT SIGNALING WITH WNT/WG SIGNALING IN OTHER ORGANISMS

The genetics of the Mom/Wnt signaling pathway of *C. elegans* suggest that this pathway differs in some fundamental ways from the canonical Wnt/Wg signaling pathway as determined in other model organisms. As mentioned above, it is premature to suggest that all *C. elegans* Wnt/Wg pathways share the differences seen with the Mom/Wnt signaling pathway. Although genetically the Mom/Wnt pathway differs from that of other organisms in a number of ways. It is not clear whether all of these differences are a result of different biochemical functions. Discussed below are the major differences seen between these pathways and possible explanations for these differences. It is likely that further study of these differences will shed light on the function of these signaling components in both *C. elegans* and in other organisms, and may find that some of these differences are not so different once more is known about the mechanisms of these signal transduction pathways.

*wrm-1, bar-1, hmp-2 and cell adhesion*

β-catenin plays dual roles in the cell. β-catenin was originally identified as protein that interacts with E-cadherin and is part of a cell adhesion complex (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; McCrea and Gumbinger, 1991). Armadillo was originally identified as mutations that affect segment polarity in the *Drosophila* embryo (Klingensmith et al., 1989; Peifer et al., 1991). Subsequently, Arm was found to be an integral part of the Wnt/Wg signaling pathway (Noordermeer et al., 1994; Siegfried et al.,
1994) and that β-catenin is homologous to Armadillo (Butz et al., 1992). Arm/β-catenin the cell adhesion protein, and Arm/β-catenin the Wnt/Wg signaling protein appear to be separable functions, but suggests a link between the regulation of cell adhesion and signal transduction during development (Gumbinger, 1996).

The C. elegans Arm/β-catenin homologues WRM-1 and BAR-1 function to transduce Wnt/Wg signals (Rocheleau et al., 1997; Eisenmann et al., 1998). Though no cell adhesion defects have been seen or reported for either wrm-1 or bar-1, a third C. elegans Arm/β-catenin gene hmp-2 functions with an α-catenin homolog, hmp-1, and an E-cadherin homolog, hmr-1, to regulate cell adhesion during morphogenesis (Costa et al., 1998). Mutations in hmp-2 do not appear to display any defects consistent with a role in Wnt/Wg signaling. Thus C. elegans Arm/β-catenin molecules may have diverged such that separate proteins fulfill the cell adhesion and Wnt/Wg signaling roles.

APR-1 and APC

APC functions to negatively regulate Arm/β-catenin, preventing interaction with TCF/LEF proteins and activation of Wnt/Wg responsive genes (reviewed in Peifer, 1996). In C. elegans apr-1 functions positively with respect to wrm-1 to specify endoderm formation (Rocheleau et al., 1997). Thus apr-1 function with respect to wrm-1 is opposite that for APC and Arm/β-catenin.

Both apr-1 and wrm-1 are quite diverged from APC and Arm/β-catenin molecules from higher organisms. APR-1 is roughly one-third the size of mammalian APC, containing conserved domains over the entire length of the protein, likewise, WRM-1
exhibits low amino acid identity within the arm motifs and little or no homology in either the N-terminal or C-terminal domains (Rocheleau et al., 1997). It is not inconceivable that both proteins have evolved differently in the nematode such that they have a different biochemical function from their \textit{Drosophila} and vertebrate counterparts. Although it is easy to speculate that this part of the pathway has diverged in \textit{C. elegans}, data in \textit{Xenopus} suggest that vertebrate APC can function positively in Wnt signaling. In \textit{Xenopus} embryos, overexpression of Wnt1, or \( \beta \)-catenin, results in a duplication of the embryonic axis (Moon et al., 1997). Interestingly, overexpression of APC resulted in an embryonic axis duplication phenotype similar to that seen with Wnt1 or \( \beta \)-catenin, and this duplication activity required the presence of \( \beta \)-catenin (Vleminckx et al., 1997). This suggested that APC and \( \beta \)-catenin have a signaling function different than would be predicted if APC was negatively regulating \( \beta \)-catenin. Though there are caveats to such overexpression studies and duplication of the embryonic axis could reflect an artificial result.

\textbf{APC contains multiple functional domains, and has been reported to interact with many different proteins and even DNA} (Rubinfeld et al, 1993; 1996; Su et al., 1993; 1995; Matsumine et al., 1996; Deka et al., 1999). APC has also been reported to co-localize with the actin and microtubule cytoskeletons (Munemitsu et al., 1994; Smith et al., 1994; McMartney et al., 1999; Yu and Bienz, 1999). It is likely that APC has multiple roles outside of Wnt/Wg signaling, and it is not hard to imagine that APC in different cellular contexts could have both positive and negative roles in the Wnt/Wg pathway. It is also possible that there is no true APC homolog in \textit{C. elegans}, and that
*apr-1*, although related to APC, is functionally more similar to an as yet unidentified positive regulator of Wnt/Wg signaling in *Drosophila* and vertebrates. Recently APC homologs (APC2) have been identified in human, mouse and *Drosophila* (McMartney et al., 1999; van Es et al., 1999). APR-1 may be less related to APC2 than APC (C.E.R. unpublished observation). Recent findings suggest that APC2 functions like APC in the Wnt/Wg pathway as a negative regulator of Arm/β-catenin (McMartney et al., 1999; van Es et al., 1999), and thus it too does not appear to function like APR-1.

APC functions as part of a complex with Axin, Zw3/GSK3, and Arm/β-catenin (reviewed in Wodarz and Nusse, 1998). Determining how APR-1 functions will require testing for interactions with known signaling components such as WRM-1 and SGG-1 as well as screening for APR-1 interacting proteins. If APR-1 is functioning to negatively regulate a protein in *C. elegans* as APC does in other systems, perhaps it is POP-1. POP-1 is the target of negative regulation by the Wnt/Wg pathway in *C. elegans* (Rocheleau et al., 1997; Thorpe et al., 1997). POP-1 expressed in vertebrate cells is redistributed from the nucleus to the cytoplasm when coexpressed with WRM-1 and LIT-1 (Rocheleau et al., 1999). In the embryo, no cytoplasmic accumulation of POP-1 protein is seen in the E cell that exhibits low nuclear staining in response to Wnt/Wg signal (Lin et al., 1995; 1998). An attractive model would be that APR-1 function is to lower the levels of POP-1, as does APC with Arm/β-catenin, in response to phosphorylation by the WRM-1/LIT-1 kinase complex.

Genetic data indicate that *apr-1* functions in parallel to the *mom-1, mom-2, mom-5*, and C27A2.6/mig-5 genes for the specification of the E cell fate (Rocheleau et al.,
1997; C.E.R. and C. Mello, unpublished). Genetic data also indicates that \textit{mom-4} functions in parallel to the \textit{mom-1}, \textit{mom-2}, \textit{mom-5}, and C27A2.6/mig-5 genes (Shin et al., 1999; C.E.R. and C.C. Mello, unpublished). Finally, \textit{mom-4} and \textit{apr-1} also appear to function in parallel (Shin et al., 1999). Thus, at least three parallel inputs function to specify the E cell fate, and removal of any two results in a fully penetrant \textit{mom} phenotype. A model, such as the one above, in which APR-1 functions downstream or with the WRM-1/LIT-1 kinase complex, but is not absolutely required for repressing POP-1 could explain the genetics. Removal of \textit{apr-1} or either of the upstream inputs from \textit{mom-2} or \textit{mom-4} would result in a decrease in the efficiency of POP-1 downregulation. Removal of \textit{apr-1} and either of the \textit{mom-2} or \textit{mom-4} polarity inputs would damage the signaling process to the extent that a total loss of POP-1 regulation would occur. Alternatively, \textit{apr-1} could be absolutely required and function directly with \textit{wrm-1} and \textit{lit-1}, in which case it would be predicted that \textit{apr-1(RNAi)} is not fully penetrant and does not represent a null phenotype. The \textit{apr-1} embryonic lethal phenotype does differ from the \textit{mom} mutations in that hypodermal enclosure often occurs (Rocheleau et al., 1997; C.E.R. and C.C. Mello). Another alternative is that APR-1 functions similar to a scaffold protein that can recruit downstream components of the pathway, making signaling more efficient. Identification of APR-1 binding partners and determining if APR-1 might be a substrate for kinases in the pathway such as SGG-1 (GSK-3), LIT-1(Nemo/Nlk), MOM-4 (TAK1), or KIN-19 (CKI) will be required to determine how APR-1 is functioning.
Where is Axin?

Studies in *Drosophila* and vertebrates have implicated Axin as a central player in the negative regulation of Arm/β-catenin. Axin has been shown to be part of the APC and Zw3/GSK3 complex that downregulates Arm/β-catenin in the absence of signal. Upon Wnt/Wg signaling, it believed that Zw3/GSK3 kinase activity is downregulated, relieving the inhibition of Arm/β-catenin. Axin has been shown to interact with APC, Zw3/GSK3, Arm/β-catenin and most recently Dsh (reviewed in Wodarz and Nusse, 1998; also see Wnt/Wg signaling in background). The ability of Axin to bind all these proteins suggest that Axin may have a key role in the complex. Given the importance of Axin in Wnt/WG signaling it is interesting to note that no *C. elegans* Axin homolog appears to be present in the nearly fully sequenced *C. elegans* genome (Ruvkun and Hobert, 1998; C.E.R. and C.C. Mello, unpublished observation). If the *C. elegans* Wnt/Wg pathway components, SGG-1, APR-1 and WRM-1, are functioning in a similar manner to their counterparts in others systems, then a worm Axin would presumably be required. It is possible that a protein structurally/functionally conserved, but not conserved in primary sequence is functioning in the pathway in a manner similar to Axin. If a functionally related protein is required for this pathway then it will have to be identified by traditional forward genetic screening and/or screening for APR-1 interactors. APR-1 contains two SAMP or 12 amino acid motifs (Rocheleau et al., 1997) required for interactions between APC and Axin (Behrens et al., 1998; van Es et al., 1999), suggesting that this interaction would be conserved.
Alternatively, an Axin homolog may not be required for Wnt/Wg signaling in *C. elegans*. Since *sgg-1* and *apr-1* are functioning positively in the pathway (Rocheleau et al., 1997; Schlesinger et al., 1999; Y. Bei and C.C. Mello, unpublished), it possible that this part of the pathway has adapted differentially since *C. elegans* split from a common ancestor shared by *Drosophila* and vertebrates.

**WRM-1 and POP-1**

In response to Wnt/Wg signaling, Arm/β-catenin complexes with TCF/LEF DNA binding proteins to activate transcription of Wnt/Wg responsive genes such as Siamois in *Xenopus*, and *Ubx* in *Drosophila* (reviewed in, Eastman and Grosschedl, 1999; Nusse, 1999; Roose and Clevers, 1999). In addition to activating Wnt/Wg responsive genes TCF/LEF proteins also function to repress Wnt/Wg responsive genes (Nusse, 1999; Roose and Clevers, 1999). An interaction of TCF/LEF proteins with members of the Groucho/TLE family of proteins converts TCF/LEF into a transcriptional repressor. CBP (Creb-binding protein), an acetylase, has been shown to act as a transcriptional activator presumably by acetylating histones and reconfiguring chromatin making DNA more accessible to transcriptional activators (Tsukiyama and Wu, 1997). CBP has been shown act as a repressor of TCF/LEF transcriptional activity. CBP can acetylate a lysine residue in the Arm/β-catenin binding site of TCF/LEF, thus affecting the ability of Arm/β-catenin to bind and activate transcription (Waltzer and Bienz, 1998).
Mutations in the *C. elegans* TCF/LEF related gene *pop-1* result in a phenotype opposite that of the *mom* genes (Lin et al., 1995), suggesting that the Mom/Wnt pathway is repressing POP-1 activity (Rocheleau et al., 1997; Thorpe et al., 1997). Thus, POP-1 is unlikely to have dual functions in the presence or absence of Wnt/Wg signaling. In the absence of Wnt/Wg signal, POP-1 may function to repress E cell specific genes while simultaneously activating MS cell specific genes. POP-1 likely has no role in specifying the E cell fate, as mutations in *pop-1* result in both MS and E adopting the E cell fate (Lin et al., 1995).

Arm/β-catenin interacts with TCF/LEF proteins, but no significant interaction between WRM-1 and POP-1 has been observed (C.E.R., J. Yasuda, T. Shin, R. Davis and C. Mello, unpublished data). WRM-1 together with the LIT-1 kinase appears to regulate POP-1 by a different mechanism (Rocheleau et al., 1999). It is possible that other factors may exist that can be activated by WRM-1 for specification of the E cell fate, either directly or by virtue of POP-1 repression. For example, *son-1*, a *C. elegans* HMG1/2 protein, (described above) appears to function downstream of *lin-44* (Wnt) and *lin-17* (fz) to specify cell fates in the B cell lineage (Jiang and Sternberg, 1999). Overexpression of POP-1 results in the same phenotype as seen in the *son-1* and *lin-17* mutations. The authors propose a model in which SON-1 functions to positively regulate transcription after POP-1, or a POP-1 related activity is repressed by the Wnt/WG signaling pathway.

Interestingly RNAi of *C. elegans* CBP homolog, *cbp-1*, results in embryonic lethality (Shi and Mello, 1998). *cbp-1(RNAi)* embryos fail to produce intestine as well as
many other tissues and produce excess neuronal cell fates. Although cbp-1(-) embryos do not look like mom embryos, they are similar in that they overproduce anterior specific cell fates (neuronal) at the expense of more posteriorly derived cell fates. It is possible that cbp-1 may function downstream of the Mom/Wnt pathway for the activation of posterior cell fate genes, in addition to other functions. If so it would suggest that cbp-1 and pop-1 have opposite roles in specifying cell fate. Thus CBP-1 could function as a transcriptional activator that is recruited to Wnt/Wg responsive genes after POP-1 is repressed. If so, then perhaps CBP-1, SON-1, and EGL-27 function together in a similar manner.

LIT-1 and vertebrate Nlk

In C. elegans, the LIT-1 and WRM-1 proteins form a complex (Rocheleau et al., 1999) that can be activated by MOM-4 MAPKKK-like protein (Shin et al., 1999). Loss of activity of either wrm-1, or lit-1, result in a 100% penetrant defect in the specification of the E cell fate (Kaletta et al., 1997; Rocheleau et al., 1997). WRM-1 is required for LIT-1 kinase activity (Rocheleau et al., 1999), suggesting that binding of WRM-1 to LIT-1 may cause a conformational change necessary for kinase activity. This kinase complex can phosphorylate POP-1, and are required for downregulation of POP-1 (Rocheleau et al., 1999).

In vertebrates, the MOM-4 homolog, TAK1, can stimulate the kinase activity of the LIT-1 homolog, Nlk (Ishitani et al., 1999). TAK1 and Nlk function as negative regulators of Wnt/Wg signaling. Injection of Nlk mRNA into Xenopus embryos can
strongly suppress β-catenin induced axis duplication, but not that induced by Wnt/Wg

target genes Siamois and Twin. Additionally, TAK1 and Nlk can inhibit β-catenin-TCF-4
induced transcription, suggesting that Nlk is blocking Wnt/Wg signaling at the level of
TCF-4. Nlk can cause β-catenin-TCF-4 DNA complexes to disassociate, but has no
effect on TCF-4 alone bound to DNA, suggesting that Nlk can regulate TCF/LEF
proteins in a β-catenin dependent manner. Nlk can associate with and phosphorylate
TCF/LEF proteins in vertebrate cells (Ishitani et al., 1999). Thus Nlk and LIT-1 appear
to be function in a similar manner, both can phosphorylate and downregulate TCF/LEF
proteins (Ishitani et al., 1999; Rocheleau et al., 1999).

In C. elegans LIT-1 requires coexpression of WRM-1 for kinase activity
(Rocheleau et al., 1999), and no binding has been detected between LIT-1 and POP-1
(C.E.R., J. Yasuda, T. Shin, R.J. Davis, and C.C. Mello). In vertebrates Nlk does not
appear to require β-catenin for kinase activity, although it is possible that endogenous β-
catenin in the vertebrate cell line is interacting with Nlk to coactivate Nlk function.
Another possibility is that interactions between Nlk and TCF/LEF proteins may be
indirect. Nlk can interact with both β-catenin and TCF-4 in tumor cells, but fails to
interact with TCF/LEF proteins in two-hybrid assays (Ishitani et al., 1999). That data and
the dependence upon β-catenin for Nlk induced disassociation of TCF-4 from DNA
suggest that it is possible that Nlk and LIT-1 share a common mechanism to repress
TCF/LEF proteins that requires WRM-1/β-catenin. It will be interesting to determine if
Nlk binds β-catenin, and if this interaction is required for Nlk kinase activity.
The function of Nlk in mouse has not been determined. *Drosophila* Nemo is required for planar polarity in the ommatidia (Choi and Benzer, 1994). Nemo is a planar polarity gene that may function downstream of Fz (Zheng et al., 1995). No role for Arm or dTCF in the planar polarity pathway has been identified (Axelrod et al., 1998; Boutros et al., 1998). Likewise, no role for Nemo in Wnt/WG signaling has been reported. Whether *Drosophila nemo* and arm function together as seen in *C. elegans*, or presumed to function in vertebrates has yet to be determined. Further investigation of Nemo will be required to determine if its role in *C. elegans* Wnt/Wg signaling is conserved in *Drosophila*.

**Planer polarity and spindle orientation**

*Drosophila frizzled* was identified as a gene required for tissue or planar polarity (Vinson et al., 1989). Mutations in *frizzled* result in a disruption of the polarity of the bristles of the legs, wing, and thorax, as well as the polarity of ommatidia in the eye (reviewed in Shulman et al., 1998). More recently, it was found that Wnt/Wg proteins can bind Dfrizzled2 (Dfz2) (Bhanot et al., 1996) and now it has been shown that Fz and Dfz2 function redundantly as a receptor for Wg (Bhat, 1998; Kennerdell and Carthew, 1998; Bhanot et al., 1999; Chen and Struhl, 1999; Müller et al., 1999). Like Fz, Dsh is also a component of both the Wnt/Wg and planar polarity pathways (reviewed in Boutros and Mlodzik, 1999). Other components of the Wnt/Wg pathway do not have a roles in planar polarity, and other genes required for planar polarity do not appear to function in the Wnt/Wg pathway. Although both pathways utilize Fz and Dsh they appear to be
separate signaling pathways. No ligand has been identified for the tissue polarity pathway, though it has been speculated that an unidentified Wnt/Wg gene may be involved (Shulman et al., 1998).

Interestingly in *C. elegans* there appears to be a differential requirement for Wnt/Wg signaling components for the spindle orientation of the ABar and EMS blastomeres (Rocheleau et al., 1997; Thorpe et al., 1997; Schlesinger et al., 1999). Blastomere isolation experiments that identified the signal from P₂ required to specify the E cell fate from EMS, also found that a signal was required for the spindle orientation of the EMS blastomere. These signals were shown to have overlapping, but different temporal windows of action (Goldstein, 1995b). The *mom-1, mom-2, mom-3, mom-5*, but not *mom-4, wrm-1, apr-1, lit-1 or pop-1* genes are required for the spindle orientation of the ABar and EMS blastomeres (*mom-2* does not appear to be required of the spindle orientation in EMS, and has been speculated that a different or redundant signal functions for EMS) (Rocheleau et al., 1997; Thorpe et al., 1997; Schlesinger et al., 1999; C.E.R. and C.C. Mello, unpublished data). Thus, the spindle orientation pathway in *C. elegans* may be analogous to the *Drosophila* planar polarity pathway. Consistent with that hypothesis the *C. elegans* dsh homologs, C27A2.6 and *mig-5* that function redundantly in endoderm specification are also required for the spindle orientation of the ABar blastomere (C.E.R. and C.C. Mello unpublished results).

Interestingly, the *C. elegans* Zw3/GSK3 homolog, *sgg-1*, is also required for the proper spindle orientation of the ABar and EMS blastomeres (Y. Bei and C. Mello,
unpublished; Schlesinger et al., 1999). In *Drosophila* Zw3 does not appear to function in the planar polarity pathway (Boutros and Mlodzik, 1999). Additionally, *lit-1*, functions in the Wnt/Wg signaling pathway in *C. elegans*, and *nemo* functions in planar polarity in *Drosophila* (Choi and Benzer, 1994; Zheng et al., 1995). These differences suggest that planar polarity and spindle orientation may not be analogous pathways between *C. elegans* and *Drosophila*. Though if they were, the requirement of *mom-2* for in spindle orientation would indicate that a Wnt/Wg molecule does in fact function as a ligand for Fz in the planar polarity pathway.

In *C. elegans* it is believed that this spindle orientation pathway may act directly on the cytoskeleton. RNAi of the *ama-1* gene, which encodes the large subunit of RNA polymerase II, results in embryos that lack differentiation due to a block in zygotic transcription (Powell-Coffman et al., 1996). The ABar and EMS blastomeres divide in the correct orientation in *ama-1*(RNAi) embryos suggesting that transcription is not required for spindle orientation (Schlesinger et al., 1999; Y. Bei and C.C. Mello, unpublished). *ama-1*(RNAi) into *mom-2* fails to rescue the defective ABar cleavage axis indicating the spindle orientation defect is not due to inappropriate transcription (Y. Bei and C.C. Mello, unpublished data).

In addition to the Wnt/Wg pathway genes, RNAi for two *C. elegans* Skp1 related genes F46A9.4 and F46A9.5 result in embryonic lethality. The skp1-related genes are required for the ABar spindle orientation, but not for the specification of the E cell fate as all embryos produce intestinal tissue (C.E.R. and C.C. Mello). Skp1p is a component of
the SCF complex that targets specific proteins for proteosome degradation by ubiquitin conjugation pathway (Elledge and Harper, 1998). Ubiquitin-dependent proteolysis is required for numerous biological processes including cell cycle progression, transcription and signal transduction (Peters et al., 1998). Consistent with skp1’s role in regulating the cell cycle, F46A9.4/5 RNAi embryos make about double the number of gut cells that are about half the normal size suggesting that they entered an additional round of cleavage (C.E.R. and C.C. Mello). The SCF has also been shown to target Cubitus interruptus (Ci), NF-κB and β-catenin for degradation in the absence of signal (Maniatis, 1999). Thus it is likely that protein degradation is a requirement for the spindle orientation of the ABar blastomere.

MOM-5 signaling is complex

Signaling via the MOM-5 receptor does not appear to be a simple linear event. MOM-5 functions to transduce the MOM-2 (Wnt/Wg) signal, that results in two outcomes via different downstream signaling components. MOM-5 as mentioned above is required for the spindle orientation of the ABar blastomere. MOM-5 is also required to specify the E cell fate via repressing the function of POP-1. Both MOM-5 and MOM-2 appear to be 100% required for the specifying the spindle orientation of the ABar blastomere, though they are only partially required for the specification of the E cell fate (Rocheleau et al., 1997; Thorpe et al., 1997). Approximately 40% of mom-2 mutant and RNAi embryos fail to produce intestine from the E cell, while approximately 5% of mom-5 mutant and RNAi embryos fail to produce intestine from the E cell. Embryos born of a
mom-2;mom-5 double homozygote display the weaker 5% no intestine phenotype identical to the mom-5 single mutant (Rocheleau et al., 1997). There appears to be differential requirements for the mom-2 and mom-5 genes for endoderm specification, suggesting that mom-2 and mom-5 do not simply function in a linear pathway. The simplest genetic explanation for this difference in phenotypes would be that in the absence of MOM-2, MOM-5 has a negative input on endoderm specification. A similar genetic relationship exists between lin-44 (wnt/wg) and lin-17(fz). Mutations in lin-44 results in a reversal in the polarity of the T and B cell divisions (Herman and Horvitz, 1994), while mutations in lin-17 result in a loss of this polarity such that the T and B cells divide to produce identical daughters (Sternberg and Horvitz, 1988). The lin-44;lin-17 double mutants are phenotypically identical to the lin-17 single mutant (Herman and Horvitz, 1994). It is hypothesized that the difference in phenotype may be due to an as yet identified anteriorly secreted Wnt/Wg molecule (Sawa et al., 1996). Thus, in the absence of LIN-44, LIN-17 is able to receive a weaker signal from a different Wnt/Wg molecule causing the polarity of the division to be reversed, and in the absence of LIN-17, the cell can not receive either signal resulting in a loss of the polarity. Similarly, in the absence of MOM-2, it is possible that MOM-5 may be capable of receiving an anteriorly localized signal that disrupts the polarity of the EMS cell, such that almost half of the time EMS divides with no polarity and fails to specify endoderm formation.

Though in the absence of MOM-5, EMS can not receive either signal, and other polarity inputs encoded by APR-1 and/or MOM-4 are sufficient to polarize EMS ~95% of the time.
In *Drosophila*, mutations in *fz* or *Dfz2* do not resemble the phenotypes of *wg* mutants, but have been found to function redundantly in multiple *wg* signaling events (Bhat, 1998; Kennerdell and Carthew, 1998; Bhanot et al., 1999; Chen and Struhl, 1999; Müller et al., 1999). The difference in penetrance between *mom-2* and *mom-5* could be explained by the existence of a second receptor capable of transducing the MOM-2 signal. Thus, in the absence of *mom-5* and this hypothetically redundant receptor embryos should have a phenotype similar to that of *mom-2*. If such a receptor exists, it would have to have a partial redundancy with MOM-5. This second receptor would have to be specific for PEMS signaling, as MOM-5 is 100% required for the spindle orientation of the ABar blastomere and 100% of *mom-5* embryos die and lack morphogenesis (Rocheleau et al., 1997; Thorpe et al., 1997). The existence of a redundant receptor does not fit with genetic data showing that the *mom-2;mom-5* double mutants displays the same low penetrant *mom* phenotype as *mom-5* single mutants (Rocheleau et al., 1997). If a redundant receptor exists, then it would be expected that the *mom-2;mom-5* double mutant phenotype would have the same penetrance as the *mom-2* single mutant.

Feedback loops have been found to exist in *Drosophila Wg* signaling. *Wg* signaling results in the transcriptional downregulation of *Dfz2* (Cadigan et al., 1998) and the transcriptional upregulation of the newly identified *Dfz3* (Sato et al., 1999). Such feedback loops can result in complicated genetic analysis of signaling pathways. Though the existence of a feedback loop regulating the transcription of *mom-5* or another Fz-related receptor would fail to explain the genetics between *mom-2* and *mom-5*. It is more
likely that an additional Wnt/Wg molecule exists that can perturb the normal polarity of EMS in the absence of MOM-2.

CONCLUSION

This work describes the identification and analysis of genes required as a polarity inducing signal that species the endoderm from the EMS blastomere. This signal is encoded by a Wnt/Wg signaling pathway and a MAPK-related pathway (Rocheleau et al., 1997; 1999; Thorpe et al., 1997; Meneghini et al., 1999; Shin et al., 1999). Analysis of these genes has found that some aspects of these signaling systems do not conform to the paradigm of Wnt/Wg signaling as determined in other organisms. APR-1 functions positively in C. elegans, whereas APC functions as a negative regulator of Wnt/Wg signaling in other systems. WRM-1 functions to repress POP-1, as where Arm/β-catenin and TCF/LEF function together as a transcriptional activator (Rocheleau et al., 1997). Most intriguing are the interactions of WRM-1 and the LIT-1 kinase. WRM-1 interacts with LIT-1, a MAPK-related protein, and this interaction is required for LIT-1 kinase activity. Together they regulate POP-1, likely by phosphorylation (Rocheleau et al., 1999). MOM-4, a MAPKKK related protein, can further activate LIT-1 when expressed with WRM-1 (Shin et al., 1999). Thus a Wnt/Wg and a MAPK-related pathway converge at the level of Arm/β-catenin, and MAPK. Understanding these differences in C. elegans will lead to a better understanding of the function of their counterparts in Drosophila, and vertebrates and their roles in carcinogenesis.
In the future it will be interesting to determine how all the components of this pathway function biochemically with respect to each other. How do APR-1 and SGG-1 fit into the pathway? Are they positive regulators of WRM-1 and/or LIT-1 activity, or do they retain their role in marking a protein for degradation (normally Arm/β-catenin) but instead target a different protein such as POP-1? Is there a MAPKK-related protein that functions between MOM-4 and LIT-1? What activates MOM-4, is there an additional receptor that functions to transduce addition polarity-inducing signals? The ease of genetic manipulation of C. elegans should allow for the answering of these questions in addition to long standing questions in the field such as what are the factors that link Fz receptors to downstream signaling components?
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