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Analysis of Polarity Signaling in Both Early Embryogenesis and Germline Development in C. Elegans: A Dissertation

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ANALYSIS OF POLARITY SIGNALING IN BOTH EARLY EMBRYOGENESIS AND GERMLINE DEVELOPMENT IN *C. elegans*

A DISSERTATION PRESENTED

BY

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APPROVAL PAGE

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ABBREVIATIONS

A/P	anterior-posterior
APC	adenomatous polyposis coli protein
APR	APC related
BAR	β-catenin/Armadillo related protein
Ceh	*C. elegans* homeobox
CK	casein kinase
CRD	cystein rich domain
Csk	C-terminal Src kinase
D/V	dorsal/ventral
Dsh	dishevelled
DTC	distal tip cell
EGF	epidermal growth factor
Egl	egg-laying defective
ER	endoplasmic reticulum
Fem	feminization of XX and XO animals
Frat	frequently rearranged in T-cell lymphomas
Fz	frizzled
GBP	GSK-3 binding protein
GLP	germ line proliferation defective
GSK	glycogen synthase kinase
Hh	hedgehog
HMP	humpback
L/R	left-right
LEF/TCF	lymphoid enhancer factor/T-cell factor
Lin	lineage defective
Lip	Lateral-signal-Induced Phosphatase
Lit	loss of intestine
LRP	low-density-lipoprotein (LDL) receptor-related protein
MAPK	mitogen activated protein kinase
Mes	maternal effect sterile
Mig	migration defective
Mom	more mesoderm
NLK	Nemo like kinase
PAL	posterior alae in males
Pan	pangolin
PCP	planar cell polarity
PIE	pharynx and intestine excess

PKB	protein kinase B
Pop	posterior pharynx defective
Por	Porcupine
Pry	poly ray
RNAi	RNA interference
ROK	Rho-associated kinase
Rrf	RNA-dependent RNA polymerase Family
RTK	receptor tyrosine kinases
RYK	related to tyrosine kinase
SFK	Src family kinase
SKN	skinhead
SS	sheath and spermatheca precursor
TAK	transforming growth factor β–activated protein kinase
Unc	uncoordinated
Wnt/wg	Wnt/Wingless
Wrm	worm armadillo

ABSTRACT

In a 4-cell *C. elegans* embryo the ventral blastomere EMS requires polarity signaling from its posterior sister cell, P2. This signaling event enables EMS to orient its division spindle along the anterior-posterior (A/P) axis and to specify the endoderm fate of its posterior daughter cell, E.

Wnt pathway components have been implicated in mediating P2/EMS signaling. However, no single mutants or various mutant combinations of the Wnt pathway components disrupt EMS polarity completely. Here we describe the identification of a pathway that is defined by two tyrosine kinase related proteins, SRC-1 and MES-1, which function in parallel with Wnt signaling to specify endoderm and to orient the division axis of EMS. We show that SRC-1, a *C. elegans* homolog of c-Src, functions downstream of MES-1 to specifically enhance phosphotyrosine accumulation at the P2/EMS junction in order to control cell fate and mitotic spindle orientation in both the P2 and EMS cells.

In the canonical Wnt pathway, GSK-3 is conserved across species and acts as a negative regulator. However, in *C. elegans* we find that GSK-3 functions in a positive manner and in parallel with other components in the Wnt pathway to specify endoderm during embryogenesis. In addition, we also show that GSK-3 regulates *C. elegans* germline development, a function of GSK-3 that is not associated with Wnt signaling. It is required for the differentiation of somatic gonadal cells as well as the regulation of meiotic cell cycle in germ cells. Our results indicate that GSK-3 modulates multiple signaling pathways to regulate both embryogenesis and germline development in *C. elegans*.

CHAPTER I

GENERAL INTRODUCTION

Overview

Multicellular organisms, no matter how complicated, all begin as a single cell, the zygote. How does the zygote generate cell diversity to form different tissues and eventually develop into a functional organism? The simple answer to this question is asymmetric cell division, in which a cell divides into two cells with different developmental potentials. Asymmetric cell divisions may involve intrinsic or extrinsic factors. With intrinsic factors, unequal amounts of cell-fate determinants are partitioned into the two daughter cells. With extrinsic factors, daughter cells are initially equivalent but adopt different fates as the result of the interactions of the daughter cells with each other or with their environment, referred to as cell-cell signaling events.

Signaling events in *C. elegans* occur as early as the 4-cell stage. One of the earliest signaling events occurs between P2 and ABp which involves the conserved Delta/Notch like signaling pathway, APX-1/GLP-1 signaling. This P2-ABp signaling event renders ABp different from its sister cell, ABa, that has the same development potential (Mello et al., 1994). The other signaling event is P2/EMS signaling, which enables EMS divide asymmetrically along the anterior-posterior (A/P) axis to give rise to two daughters with different cell fates. The anterior daughter, MS, is a mesodermal precursor and develops into body wall muscle and pharyngeal muscle. The posterior daughter, E, is the only source of intestine (endoderm).

P2/EMS signaling was best demonstrated by culturing isolated blastomeres from wild-type embryos *in vitro*. EMS isolated early in its cell cycle and cultured alone produces two MS-like daughters that make only mesodermal tissue consisting of body wall muscle

and pharyngeal muscle. When P2, and P2 only, is placed in contact with EMS, EMS makes endoderm (E fate) in addition to mesoderm (MS fate) (Goldstein, 1992). Moreover, when EMS is cultured alone, its mitotic spindle elongates along the axis established by centrosome migration without any rotation. However, when EMS is put in contact with P2 early in its cell cycle, it rotates its centrosomes following centrosome migration and reorients its division axis in accordance to its contact site with P2. Therefore, P2 signaling polarizes EMS in such a way that a posterior daughter (E) is born next to P2 and produces endoderm (Goldstein, 1995a; Goldstein, 1995b). EMS remains competent to E cell fate specification potential in response to P2 signaling for a longer time than it remains competent to orient its mitotic spindle (Goldstein, 1995a; Goldstein, 1995b), suggesting that the signals or their responses may be different.

Genetic and molecular insights into the nature of P2/EMS signaling have come, in part, from the identification of five *mom* (**mo**re **m**esoderm) genes required for endoderm specification. Homozygous mothers for any of the *mom* genes produce dead embryos that are nicely differentiated but lack gut and have extra pharyngeal tissue. This occurs because both EMS daughters adopt an MS like fate (Rocheleau et al., 1997; Thorpe et al., 1997). Another maternal effect lethal mutant, *pop-1 (**p**osterior **p**harynx defective)*, has the opposite phenotype to the mom mutants. *pop-1* mutant mothers lay dead embryos with extra gut at the expense of pharynx (Lin et al., 1995) Molecular cloning of the *mom* and *pop-1* genes has identified them as components of the conserved Wnt signaling pathway. *mom-1* encodes a homolog of *porcupine* (*porc*) and *mom-2* and *mom-5* are similar to Wnt/*wingless* (*wg*) and *frizzled* (*fz*) respectively. *pop-1*is related to *Drosophila pangolin/dTCF* (*pan*) and mammalian TCF/LEF family of transcription factors that are regulated by Wnt signaling pathway. Reverse genetics using RNAi (RNA interference) also identified other conserved components involved in Wnt signaling including dishevelled homologs *dsh-2* (**Dish**evelled) and *mig-5* (**mig**ration defective), *apr-1* (**APC r**elated) and

wrm-1 (**w**orm **arm**adillo, also known as β-catenin), as genes required for E cell fate specification (Rocheleau, 1999; Rocheleau et al., 1997). Therefore, the widely conserved Wnt signal transduction pathway is required for endoderm induction during embryogenesis in *C. elegans*. Genetic studies also indicate that other signaling mechanisms contribute to endoderm specification. These mechanisms include components related to MAPK (**m**itogen **a**ctivated **p**rotein **k**inase) signaling factors. *mom-4*, is a TAK1 (**t**ransforming growth factor β--**a**ctivated **k**inase-1) MAP Kinase, kinase kinase homolog, and *lit-1 (loss of intestine)* encodes a Nemo, MAP Kinase related protein (Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999; Shin et al., 1999). Genetic data suggest that several parallel signaling inputs converging on the WRM-1/LIT-1 protein complex to downregulate POP-1 activity in the E cell to specify E cell fate (Rocheleau et al., 1997; Rocheleau et al., 1999; Shin et al., 1999).

Several of the genetically defined signaling components for E cell fate specification also exhibit a skewed A/P orientation of the EMS division axis. However, within the intact embryo, no single or multiple mutant combinations among the above mentioned mutants have a complete loss of EMS centrosome rotation resulting in a default left-right (L/R) division axis (Schlesinger et al., 1999). These findings indicate that an as-yet-unidentified factor(s) must direct EMS spindle orientation and possibly determine E cell fate specification during P2/EMS signaling. Figure I-1 summarizes the genetic pathways involved in P2/EMS signaling.

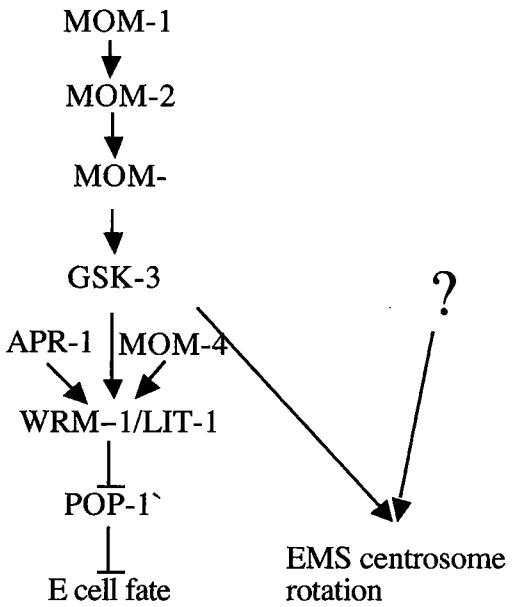

Figure I-1. Genetic pathways for P2/EMS signaling. At least three parallel pathways function upstream of WRM-1/LIT-1, which in turn negatively regulates POP-1 to specify E cell fate. In addition to E cell fate specification, MOM-1, MOM-2, MOM-5 and GSK-3 are also required for EMS centrosome rotation, possibly functioning redundantly with some unknown pathway(s) (?) to direct the EMS cell division axis.

The studies described in this thesis focus on the further examination of the polarity P2/EMS signaling in *C. elegans* as outlined in figure I-1.

Chapter II describes the discovery of a pathway that is represented by the "?"in figure I-1. Genetic data presented in Chapter II suggest that a MES-1/SRC-1 pathway functions in parallel with Wnt signaling to direct the EMS cell to divide along the A/P axis as well as to specify E cell fate. MES-1 protein, localized at the P2/EMS cell junction in a 4-cell stage embryo, is an atypical receptor tyrosine kinase that is not believed to have a functional kinase domain (Berkowitz and Strome, 2000). *C. elegans* SRC-1 is homologous to the proto-oncogene product, c-Src. SRC-1 functions downstream of MES-1 to form an enhanced phospho-tyrosine accumulation at the P2/EMS junction which directs EMS spindle orientation. Therefore, the two pathways that were originally identified and are often connected with cancers, Wnt signaling and Src-1 (cellular form of v-Src) mediated signaling, genetically cooperate with each other to regulate an experimentally approachable

biological event, the asymmetrical division EMS cell during early *C. elegans* embryogenesis.

GSK-3β (Glycogen synthase kinase-3β) is one of the major components of the Wnt signaling pathway. In addition, it is also involved in other signaling pathways, which include the insulin response and Hedgehog (Hh) signaling, and regulates multiple biological events (Jope and Johnson, 2004). Chapter III studies the functions of GSK-3 during *C. elegans* development focusing on its role during early embryogenesis as a component of the Wnt signaling pathway and its role during germline development. Studies in this chapter as well as reports published by Schlesinger et al. (1999) put GSK-3 as a positive influence on the asymmetrical cell division of EMS (account for both E cell fate specification and EMS spindle orientation). However, Chapter III presents genetic data placing GSK-3 in a pathway parallel to the MOM-2 (Wnt)-MOM-5 (Fz) mediated signaling instead of a straight downstream effector. Zygotic functions of GSK-3 on germline development are also described in this chapter.

In summary, work presented in this thesis provides a better understanding of Wnt signaling both in its cooperation with other signaling events (SRC-1 mediated signaling) as well as the genetic interactions between the known components already implicated in the pathway.

Introduction to *C. elegans* early embryogenesis

The transparent body of *C. elegans* allows live study of development at the cellular level through the use of light microscopy. This convenience of manipulation together with the relatively simple and almost invariant cell lineage makes *C. elegans* a useful experimental organism for the study of embryogenesis.

The polarity of the *C. elegans* embryo is determined as early as fertilization. The sperm pronucleus and its associated centrosomal asters provide a cue that establishes the anterior-posterior (A/P) body axis. The sperm entry site marks the future posterior pole of the embryo (Figure I-2A). The first division of the *C. elegans* zygote is asymmetric. The one-cell *C. elegans* embryo, called P_0 (Figure I-2B), divides along its A/P axis to produce a large anterior blastomere, AB, and a smaller posterior blastomere, P1 (Fig. I-2C). AB divides symmetrically, giving rise to two daughter cells with equal potential, ABa and ABp. Following AB division, P1 undergoes an asymmetric division to produce a larger anterior daughter, EMS, and a smaller posterior daughter cell called P2. After this second round of cell division, the dorsal-ventral (D/V) axis is established in the 4-cell stage embryo, which places ABp dorsal to EMS, leaving ABa at the anterior and P2 at the posterior pole of the embryo (Fig. I-2D). In the third round of cell division, the AB daughters continue to divide symmetrically, while both EMS and P2 undergo asymmetric cell division. EMS cell divides along the A/P axis, producing an anterior daughter MS cell that produces future mesodermal tissue (body wall muscle and pharyngeal muscle) and a posterior daughter E is the only source of intestine (endoderm). P_2 also divides along the A/P axis resulting in two sister cells, P3 and C. P_3 divides as previous P lineage cells eventually producing P4 (germ cell precursor) and D (body wall muscle) while C develops into hypodermis and body wall muscle. Due to the constraint of the eggshell, P_3 and C blastomeres end up dorsal-ventral to each other and not on the same focal plane (Fig. I-2E). After the fourth round of cell

division, a 16-cell embryo is established containing all of the founder cells. These founder cells are AB, MS, E, C, P4 and D. The descendants of each founder cells divide in an invariant pattern and 558 cells are generated at hatching. Figure I-3 shows the lineage of these founder cells.

Several maternally expressed proteins are required for the early blastomere cell fates. SKN-1 (**skin**head) is a composite bZIP/homeodomain trascription factor required for EMS fate (Blackwell et al., 1994; Bowerman et al., 1992). *skn-1* homozygous mutant mothers produce dead embryos that produce excessive hypodermal (skin) and body wall muscle tissue (C fate) at the expense of intestine (E) and pharynx (MS) (Bowerman et al., 1992). PAL-1 (**p**osterior **al**ae in males) is a Caudal-like transcription factor that is required in both C and D blastomeres (Hunter and Kenyon, 1996). Mutant *pal* worms lacking the germline activity of pal-1 lay dead embryos with both C and D cells producing unfimilar cells (Hunter and Kenyon, 1996). Although PAL-1 is also localized in EMS cell, its activity is inhibited by SKN-1. Therefore, in *skn-1* mutants, EMS adopts C like fate. PIE-1 (**p**harynx and **i**ntestine **e**xcess) is a zinc protein that is maternally required in the germline blastomere (P lineage) to inhibit somatic differentiation. Mutations of *pie-1* results in germ line blastomeres adopting somatic fates, with the P2 cell behaving similarly to an EMS blastomere (Mello et al., 1992).

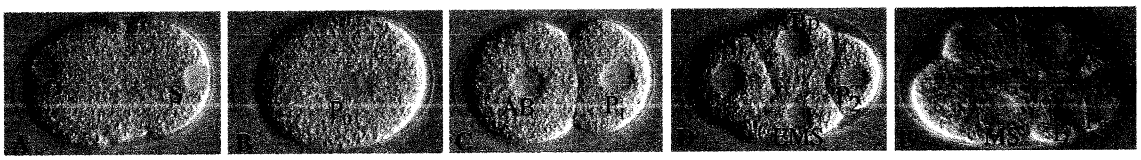

Figure I-2. Nomarski photomicrograph of a *C. elegans* embryo at successive stages between fertilization and the third cleavage. Embryos are oriented with posterior pole to the right and the dorsal axis on the top. (A) Pronuclear-stage embryo with oocyte (o) pronucleus at anterior and sperm (s) pronucleus at the posterior. (B) One-cell stage embryo, P_0. (C) Two-cell stage embryo. Anterior cell, AB, is bigger than its posterior sister cell, P_1. (D) Four-cell stage embryo. Dorsal-ventral axis of the embryo is established with EMS on the ventral side. Signaling events occur at this stage (see text). (E) Eight-cell stage embryo. The nucleus of C blastomere is not visible in this focal plane. Some nuclei of the AB descendants are also out of the focal plane.

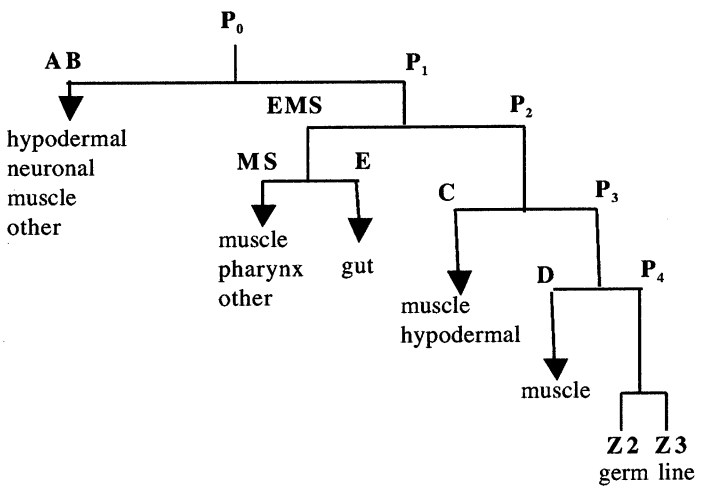

Figure I-3. Lineage pattern of early cleavages in the *C. elegans* embryo, showing derivation of the six founder cells AB, MS E, C, D and P4. Future cell types are indicated under each founder cell. (Adapted from The Nematode *Caenorhabditis elegans*, Cold Spring Harbor Laboratory Press).

Introduction to Wnt signaling

Signaling by the Wnt family of secreted growth factors have been found in all animal species. The first Wnt gene, Wnt1 (originally known as int-1), was discovered as an oncogene 22 years ago in the mouse mammary tumors (Nusse and Varmus, 1982). Since then there have been more than 2000 papers published on Wnt signaling. Wnt signaling not only is involved in tumorigenesis, but also regulates a large number of developmental processes, such as segmentation, axis formation and central nervous system (CNS) patterning (reviewed in Logan and Nusse, 2004; Wodarz and Nusse, 1998). The Wnt signaling pathway was initially elucidated as genetic epistasis hierarchy in *Drosophila*. In combination with biochemical analysis, many key Wnt signaling components have been identified and a canonical pathway has been delineated.

The canonical Wnt pathway (also known as the β-catenin pathway) leads to activation of target genes in the nucleus (Figure I-4). In the absence of Wnt receptor activation, the modular protein Axin/Conductin provides a scaffold for the binding of GSK-3β (**g**lycogen **s**ynthase **k**inase-3β), APC (**a**denomatous **p**olyposis **c**oli protein) and β-catenin. This protein complex facilitates β-catenin phosphorylation by GSK-3β resulting in proteasome-mediated degradation of β-catenin (Figure I-4A). In the presence of signaling, the Wnt proteins bind to the co-receptors, Frizzled (Fz) and LRPs/Arrow, at the cell surface. Through some yet-not-well-understood mechanism, the Axin-GSK-3-APC- β-catenin complex is then disrupted in a Dishevelled (Dsh) dependent manner and, thus, the β-catenin protein is no longer phosphorylated and targeted for degradation. The accumulated cytoplasmic β-catenin is, therefore, translocated into the nuclei where it interacts with members of the lymphoid enhancer factor (LEF)/T-cell factor (TCF) classes of transcription factors to regulate the expression of target genes (Figure I-4B). Major components of the canonical Wnt signaling pathway will be described.

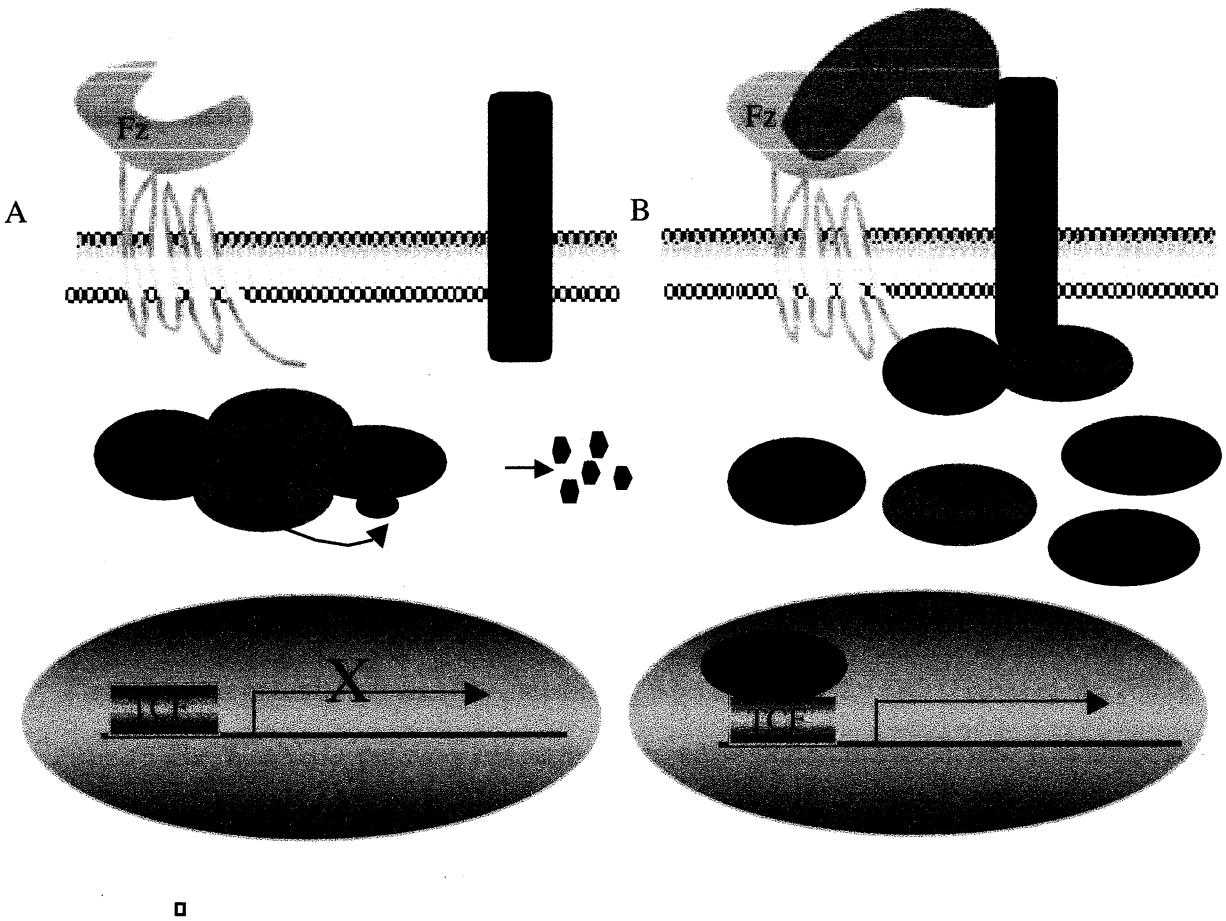

Figure I-4. A working model for the canonical Wnt signaling pathway. (A) In the absence of Wnt signal, β-catenin is phosphorylated by GSK-3 within the destruction complex consisting of Axin, APC, GSK-3 and β-catenin itself and the phosphorylated β-catenin is subsequently degraded. Wnt-responsive gene is suppressed by TCF. (B) Wnt simulation results in a Dsh-dependent inhibition of the Axin complex and, thus, β-catenin is no longer phosphorylated and degraded. Therefore, β-catenin accumulates and translocates into the nucleus where it associates with TCF and activates Wnt responsive genes (adapted from He et al., 2004).

Diversification of the Wnt signaling

Recent evidence suggests that at least three different branches are being distinguished within the Wnt pathway. These include the canonical Wnt pathway mentioned above, the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway.

The planar cell polarity (PCP) pathway, also known as tissue polarity, controls the orientation of hairs, bristles and ommatidia in *Drosophila* and it controls gastrulation movements in vertebrates (reviewed in Fanto and McNeill, 2004). PCP signaling diverges away from canonical Wnt signaling at the level of Dishevelled and directs cytoskeletal rearrangements without the involvement of gene transcription. Small GTPase Rho has been shown to act down stream of Dsh to mediate PCP (Strutt et al., 1997)with Rho-associated kinase (ROK) acting as an effector (Winter et al., 2001). The formin homology protein Daam1 binds to both Dsh and Rho, mediating Rho activation by Dsh (Habas et al., 2001).

The Wnt/Ca2+ pathway has so far been only reported in vertebrates. It is implicated in *Xenopus* ventralization and in the regulation of convergent extension movements (Kuhl et al., 2001; Wallingford et al., 2001). The Wnt/ Ca^{2+} pathway is stimulated by certain Wnt ligands and Fz receptors. It involves the mobilization of intracellular Ca^{2+} and activation of phospholipase C, protein kinase C (PKC) and calmodulin-dependent kinase II (CamKII) (reviewed in Kuhl et al., 2000). The calcium responsive transcription factor NF-AT has been suggested as a potential target (Murphy and Hughes, 2002; Saneyoshi et al., 2002). Recently, Dsh has been shown to be involved in Wnt/Ca^{2+} signaling (Sheldahl et al., 2003), making Dsh instead of Fz the branch point between Wnt/Ca^{2+} and canonical signaling. This also suggests a potential overlap between PCP and Wnt/ Ca^{2+} pathways.

Key components of the canonical Wnt signaling pathway

The signal: Wnt proteins

The Wnt genes encode a large family of secreted growth factors, approximately 350-400 amino acids in length. About 100 genes have been isolated from numerous species ranging from human to hydra. All of these genes encode proteins with a hydrophobic signal sequence, a conserved asparagine-linked oligosaccharide consensus sequence and a nearly invariant pattern of 23-24 cytein residues.

The Porcupine (Porc) protein, which is a multi-transmembrane protein predominantly found in the endoplasmic reticulum (ER), may be involved in the secretion or ER transport of Wnt proteins, as *Wingless* (Wnt) is retained in the ER in *Drosophila porcupine (por)* mutant embryos (Kadowaki et al., 1996). Recent studies have shown that Wnt proteins are highly hydrophobic and are post-translationally modified by palmitoylation on a highly conserved cystein residue. Porcupine might be involved in this modification event (Willert et al., 2003).

The receptor: Fz/LRP complex

Wnt ligands signal via seven transmembrane spanning receptors of the Frizzled family(Bhanot et al., 1996) together with LRP5 and LRP6 co-receptors, which are members of the low-density-lipoprotein (LDL) receptor-related protein family (LRP), identified as *Arrow* in *Drosophila* (Bhanot et al., 1996; Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000).

Frizzled proteins share common structural motifs: a putative N-terminal signal sequence, a conserved cysteine-rich domain (CRD), a hydrophilic and highly divergent linker region, seven transmembrane segments and a variable intracellular C-terminal domain. The CRD is required for binding with the Wnt proteins (Bhanot et al., 1996). The

conserved Lys-Thr-X-X-X-Trp motif in the cytoplasmic domain is required for Fz interaction with Dsh upon Wnt binding (Umbhauer et al., 2000; Wong et al., 2003). However, how Fz activates Dsh remains largely an unknown.

LRPs/Arrow were identified as Wnt co-receptors a few years after Fzs were shown to be receptors for Wnt molecules (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). LRPs are single transmembrane proteins. They contain, in their extracellular domain, four epidermal growth factor (EGF)-like repeats and three LDL-receptor type repeats followed by a transmembrane domain and a conserved cytoplasmic domain that contains several PPPSP motifs. There are reports that Wnts bind to LRPs directly (Cong et al., 2004; Tamai et al., 2000) although this is not the case in *Drosophila* (Wu and Nusse, 2002). Nonetheless, recent results support a model that Wnt stimulation induces a Wnt-Fz-LRP/Arrow ternary complex, enabling LRPs to interact with Axin, leading to Wnt target gene expression (Cong et al., 2004; Mao et al., 2001; Tolwinski et al., 2003). The interaction between LRPs and Axin is mediated through the conserved PPPSP motifs that are phosphorylated upon Wnt stimulation (Tamai et al., 2004).

Recently RYKs (**r**elated to **tyrosine** **k**inases) (Hovens et al., 1992), a subfamily of atypical receptor tyrosine kinases (RTKs), have emerged as Wnt receptors. They include vertebrate RYK (Lu et al., 2004), *Drosophila* Derailed (Yoshikawa et al., 2003), and *C. elegans* LIN-18 (Inoue et al., 2004). The extracellular domain of each RYK family member contains a Wnt inhibitory factor (WIF) domain (Patthy, 2000). Other secreted proteins containing a WIF domain have been shown to bind and inhibit Wnts proteins (Hsieh et al., 1999). The intracellular kinase domain of RYK family members appears to lack catalytic activity (Katso et al., 1999)suggesting a possibility that this family of receptors transduce Wnt signals through interaction with another catalytically active kinase (Yoshikawa et al., 2001).

Dishevelled

Dishevelled (Dsh) is the cytoplasmic protein that acts directly downstream of the Fz/LRPs receptor complex. Dsh is the component at which the canonical Wnt signaling pathway and the noncanonical Wnt signaling pathway (see above) diverge (Axelrod et al., 1998; Boutros et al., 1998; Li et al., 1999b; Sheldahl et al., 2003). Dsh proteins have three distinct domains: an N-terminal DIX (**Dis**hevelled, **A**xin) domain, a central PDZ (**P**SD-95/SAP90, **D**iscs-large, **Z**O-1) domain, which represents a globular protein-protein interaction domain contained in many adaptor molecules found in cellular junction complexes, and a C-terminal DEP (**D**ishevelled, **E**GL-10, **P**leckstrin) domain. Of these three, the PDZ domain, which is required for direct binding with Fz (Wong et al., 2003), appears to play an important role in both the canonical and noncanonical Wnt pathways (Moon and Shah, 2002). The DIX domain of Dsh is required for the direct interaction between Dsh and Axin (Kishida et al., 1999; Smalley et al., 1999)and proper canonical Wnt signaling (Axelrod et al., 1998; Boutros et al., 1998; Penton et al., 2002; Rothbacher et al., 2000). The DEP domain is only required for planar polarity and is dispensable for canonical Wnt signaling (Boutros et al., 1998; Penton et al., 2002)

While the mechanism of action of Dsh is not well defined in canonical Wnt signaling, it has been shown that Dsh interacts with Axin and relocates Axin to the plasma membrane upon Wnt stimulation, where it interacts with the cytoplasmic domain of LRPs/Arrow (Cliffe et al., 2003; Mao et al., 2001; Tolwinski et al., 2003)

The multiprotein β-catenin degradation complex

The multiprotein β–catenin degradation complex contains several major proteins including Axin/Conductin, APC, GSK3β and β–catenin protein itself. Within the complex GSK3β phosphorylates β–catenin, leading to β–catenin degradation. Wnt proteins signal to inhibit β–catenin degradation, resulting in β–catenin accumulation and association with

TCF/LEF transcription factors within the nucleus to activate Wnt responsive gene transcription.

Axin/Conductin

Axin/Conductin, also called Axil, was originally identified as the gene mutated in the *fused* mutant mouse characterized by axial duplications reminiscent of the phenotype seen in *Xenopus* after activation of the Wnt signal (Zeng et al., 1997). Axin/Conductin functions as a scaffold protein by approximating all components needed for phosphorylation of β-catenin, i.e., GSK3, APC, and β-catenin itself. Axin/Conductin consists of an N-terminal RGS (regulator of G-protein signaling) domain that binds to APC (Behrens et al., 1998; Hart et al., 1998; Kishida et al., 1998), centrally located separate binding domains for GSK3β and β-catenin (Behrens et al., 1998; Ikeda et al., 1998), and a C-terminal DIX domain that mediates dimerization of Axin (Ikeda et al., 1998; Zeng et al., 1997)or interaction with Dsh (Kishida et al., 1999; Smalley et al., 1999). The domain involved in LRPs/Arrow binding is poorly defined. The DIX domain is required but not sufficient, whereas the N-terminal RGS domain is inhibitory to the binding (Mao et al., 2001; Tolwinski et al., 2003). Despite the observation that GSK-3β phosphorylation of β-catenin is stimulated 20,2000-fold in the presence of Axin (Dajani et al., 2003), exactly how Axin facilitates β-catenin phosphorylation by GSK-3β is not well defined. Recent data suggest that Axin functions beyond this role. It can regulate Wnt signaling (β-catenin pathway) independent of GSK-3β (Tolwinski et al., 2003) most likely by anchoring β-catenin within the cytoplasm (Tolwinski and Wieschaus, 2001)or by promoting β-catenin nuclear export (Cong and Varmus, 2004).

APC

The APC (adenomatous polyposis coli) gene is mutated in **f**amilial **a**denomatous **p**olyposis (FAP), a hereditary form of colon cancer, and is as well truncated in most sporadic colonic tumors (Kinzler and Vogelstein, 1996; Laken et al., 1999). Its involvement in Wnt signaling was first discovered because of interaction with β-catenin and GSK-3 protein (Rubinfeld et al., 1996; Rubinfeld et al., 1993).

APC is an enormous protein approximately 2800 amino acids in length. The N-terminus of APC contains a coiled-coil domain involved in dimerization of the protein (Day and Alber, 2000; Joslyn et al., 1993). The coiled-coiled domain is followed by Armadillo (Arm) repeats that bind to APC-stimulated **g**uanine nucleotide **e**xchange **f**actor (GEF) for Rho family proteins (Asef) (Kawasaki et al., 2000), and Kap3 (**K**inesin **a**ssociate **p**rotein) (Jimbo et al., 2002). The N-terminal region also contains two functional nuclear exit signals (NES) that are required for shuttling APC between the nucleus and cytoplasm (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000). The central domain of APC consists of three 15-amino-acid repeats followed by seven 20-amino-acid motifs. These repeats bind to β-catenin and GSK-3β respectively (Rubinfeld et al., 1996; Su et al., 1993). Dispersed in between the 20-amino-acid repeats are three Ser-Ala-Met-Pro (SAMP) motifs that mediate interaction between APC and Axin (Behrens et al., 1998; Hart et al., 1998; Nakamura et al., 1998). The C-terminal region of APC is the least conserved between species and has been shown to interact with a number of structural proteins, such as EB1, a microtubule associated protein (Berrueta et al., 1999; Su et al., 1995).

Cancer cells carrying mutations in the APC gene have high levels of cytoplasmic β-catenin suggesting that APC plays a role in β-catenin degradation (reviewed in Peifer and Polakis, 2000). The molecular mechanism by which APC promotes destruction of β-catenin is not clear. It appears that activity of APC depends on its interaction with Axin/Conductin because mutations in the SAMP repeats lead to loss of APC activity in β-catenin

degradation (von Kries et al., 2000). It has also been shown that APC shuttles β-catenin out of the nucleus (Henderson, 2000), suggesting that APC controls the nuclear accumulation of β-catenin through nuclear export and cytoplasmic degradation.

GSK-3β

GSK-3β (Glycogen synthase kinase 3β) is a serine/threonine kinase that was originally identified as a regulator of glycogen synthase (Embi et al., 1980; Woodgett and Cohen, 1984). It has since been shown to be involved in several signaling pathways including Wnt signaling. In this section, I will focus on its involvement in the Wnt pathway only.

GSK-3β was first shown to be a negative regulator of Wnt signaling in *Drosophila*, in which it is known as *Shaggy* (Sgg), *Zeste-white 3* (Zw3) (Siegfried et al., 1992). It phosphorylates β-catenin leading to the subsequent degradation of this molecule (Aberle et al., 1997; Orford et al., 1997; Yost et al., 1998). In addition to β-catenin, GSK-3β also phosphorylates APC and Axin (Rubinfeld et al., 1996; Yamamoto et al., 1999). Phosphorylation of Axin increases its stability and its ability to bind to β–catenin (Ikeda et al., 1998; Itoh et al., 1998; Yamamoto et al., 1999) while phosphorylation of APC increases its ability to bind to and to downregulate β–catenin (Rubinfeld et al., 1997; Rubinfeld et al., 2001).

The optimal consensus site for GSK-3 phosphorylation is S/T-X-X-X-S/T^P; where S/T corresponds to serine or threonine, X to any other residue and S/T^P to phosphorylated serine or threonine, which is also known as "priming". Priming phosphorylation occurs at position P_{+4} and GSK-3 transfers a phosphase to position P_0, where it can create an adjacent GSK-3 site. In Wnt signaling, Casein Kinase Iα (CK1α) has been identified as the priming kinase for β–catenin phosphorylation by GSK-3β (Liu et al., 2002), while Ha et al. (2004)

have shown that APC phosphorylation requires mutual priming of CK1ε and GSK-3β sites.

In vertebrate, GBP (**G**SK-3 **b**inding **p**rotein) in *Xenopus* or Frat-1 (**fr**equently **a**rranged in **T**-cell lymphomas) in mice has been identified as a GSK-3 binding protein and acts as a negative regulator of GSK-3β activity (Yost et al., 1998). Frat also interacts with Dsh (Li et al., 1999a). It has been proposed that Dsh recruits Frat to the β-catenin destruction complex to inhibit GSK-3 activity (Li et al., 1999a; Salic et al., 2000). However, whether GBP/Frat plays a key role in Wnt signaling is not quite clear as a Dsh mutant protein lacking the Frat binding domain still activates β-catenin signaling (Hino et al., 2003). In addition, no Frat/GBP homolog has been identified in invertebrates.

β-catenin

β-catenin, also known as Armadillo (Arm) in *Drosophila*, was originally discovered as a patterning mutation with a phenotype similar to loss-of-function of Wg/Wnt mutations (Riggleman et al., 1989; Wieschaus and Riggleman, 1987). It was originally thought to be a cell-cell adhesion protein because of its association with cadherins (McCrea and Gumbiner, 1991). Later work defined a specific role for β-catenin as a transcriptional activator activating gene transcription in conjunction with the TCF/LEF family of transcription factors (Behrens et al., 1996; Molenaar et al., 1996). At the N-terminus, β-catenin is phosphorylated at S33, S37, T41, and S45, which leads to β-TrCP/SLMB mediated protein degradation of β-catenin (Aberle et al., 1997; Yost et al., 1996). Casein kinase Iα (CK1α) has been identified as the priming kinase which phosphorylates S45, leading to phosphorylation at S33, S37 and T41 by GSK-3β (Liu et al., 2002). This regulatory sequence in β-catenin is biologically significant since it is mutated in a wide variety of human cancers as well as in chemically and genetically induced animal tumors (Polakis, 1999). The core domain of β-catenin consists of twelve arm repeats, which are also present

in APC. The arm repeats bind to various partners such as cadherins, TCF/LEF, APC, and Axin/Conductin (Hulsken et al., 1994; von Kries et al., 2000). Within the C-terminal region of β-catenin is the transactivation domain (van de Wetering et al., 1997). It is presently unclear how the transactivation domain of β-catenin activates transcription, however, β-catenin has been shown to indirectly associate with the TATA-binding protein (TBP) (Bauer et al., 2000). In addition, β-catenin has also been shown to recruit Brg-1, a component of the SWI/SNF complex, to TCF target gene promoters, facilitating chromatin remodeling for transcriptional activation (Barker et al., 2001).

The TCF/LEF family of transcription factors

TCF-1 (**T** cell **f**actors)/LEF-1 (**L**ymphoid **E**nhancer **F**actor) was originally identified in screens for T cell specific transcription factors (Oosterwegel et al., 1991). Only one TCF exists In *Drosophila* and *C. elegans*, referred to as dTCF/pangolin (Brunner et al., 1997; van de Wetering et al., 1997) and POP-1 respectively (Lin et al., 1995). Proteins of the TCF/LEF family contain an 80-amino-acid high mobility group (HMG) box. HMG boxes bind DNA as monomers and can do so in a sequence-specific manner, which induces a dramatic bend in the DNA structure (reviewed in Grosschedl et al., 1994) enabling the binding of various transcription factors (reviewed in Bianchi and Beltrame, 1998). The first identified binding partner of TCF was β-catenin which via its arm-repeats binds to a conserved N-terminal region within TCF/LEF (Behrens et al., Molenaar et al., 1996). The transactivation domain of β-catenin is thought to recruit transcriptional machinery components such as TBP and Brg-1, to Wnt target genes (Barker et al., 2001; Hecht et al., 2000; Takemaru and Moon, 2000). TCF/LEF acts as a transcriptional repressor in the absence of Wnt signaling by forming a complex with one of several co-repressors. For example, Groucho (Gro) binds to TCFs and acts as a co-repressor (Cavallo et al., 1998). The repressing effect of Groucho is mediated by interactions with histone deacetylases

(HDAC), which are thought to make DNA refractive to transcriptional activation (Chen et al., 1999).

The major components in the Canonical Wnt signaling pathways and protein-protein interactions are diagramed in Figure I-5. The approximate binding sites and distinctive protein domains are also presented.

In summary, most studies on canonical Wnt signaling support a textbook model in which Wnt stimulation functions to inhibit β-catenin phosphorylation and phosphorylation dependent degradation. This leads to the accumulation of β-catenin in the cytoplasm and its subsequent translocation into the nucleus and its association with TCF/LEF, resulting in activation of Wnt-responsive gene transcriptions. A recent study in *Drosophila* by Tolwinski et al. (2003), however, suggested that Wnt/β-catenin signaling can occur independent of GSK-3. In this study, the authors found that a weak armadillo/β-catenin loss-of-function allele is regulated in a Wnt-dependent manner in *gsk-3* homozygous mutant embryos but not embryos mutant for *axin*. This implies a Wnt pathway that inhibits Axin and is independent of β-catenin phosphorylation by GSK-3. This GSK-3 independent activity of Axin maybe explained by its ability to anchor β-catenin (Tolwinski and Wieschaus, 2001) within the cytoplasm or by its ability to export β-catenin out of the nucleus (Cong and Varmus, 2004) or a combination of both. APC has also been shown to negatively regulate β-catenin activity by promoting the nuclear export of β-catenin (Giannini et al., 2004; Henderson, 2000). Taken together, the canonical Wnt signaling pathway maybe is not as linear as it is widely accepted but rather branched and complex.

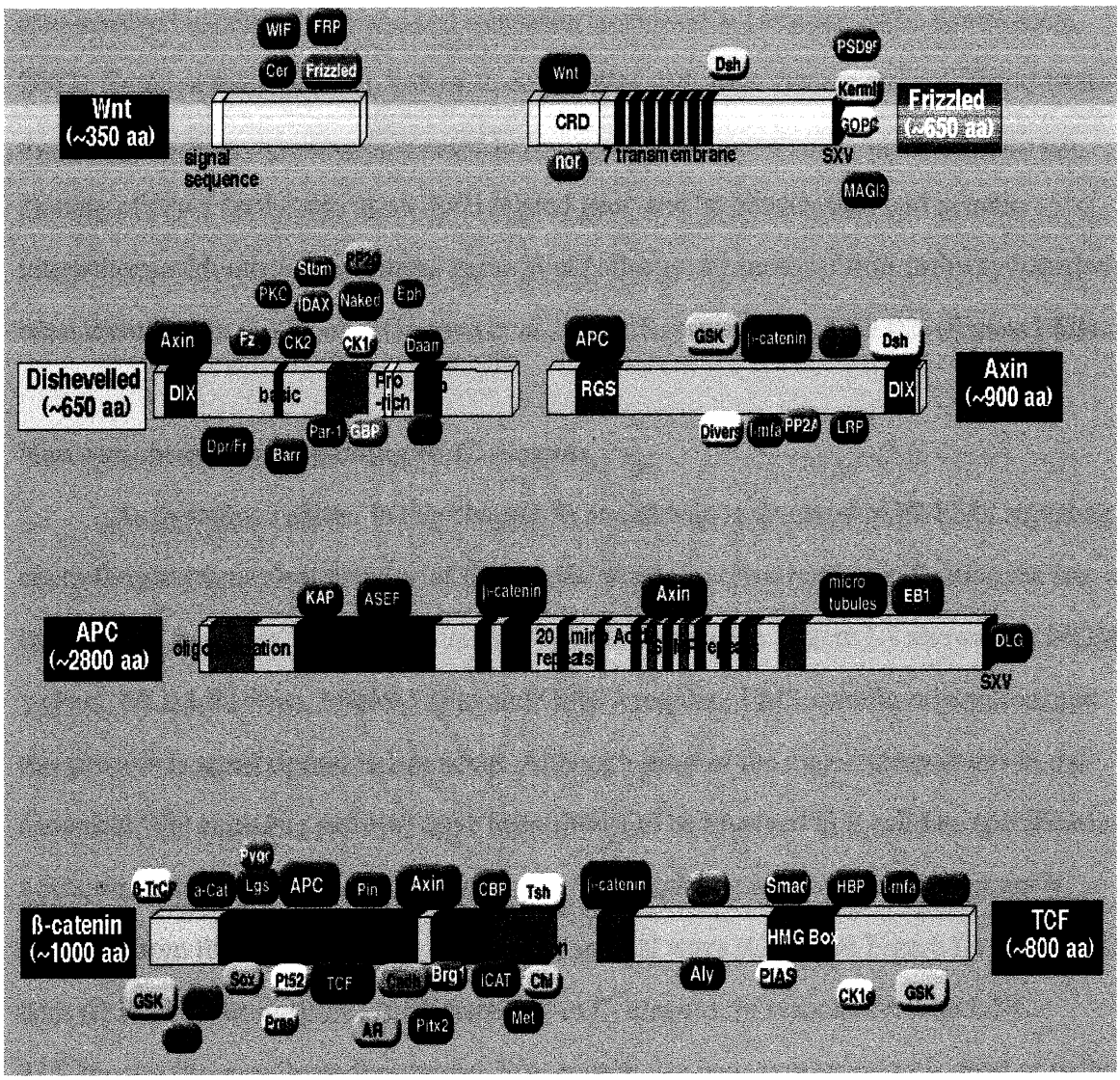

Figure I-5. Components of the Wnt pathway and protein-protein interactions. (Adapted from Wnt gene homepage: http://www.stanford.edu/~rnusse/pathways/binding.html)

Wnt signaling in *C. elegans* development

Wnt signaling also plays an important role during *C. elegans* development. Besides the role in EMS asymmetrical cell division mentioned earlier in the chapter, Wnt signaling also involved in cell polarity and cell migration (reviewed in Herman, 2002; Korswagen, 2002). Some of the processes, however, define Wnt pathways that are quite different from the canonical Wnt signaling delineated in *Drosophila* and vertebrates. Recently with the cloning of *pry-1* (**p**oly **r**a**y**) as an Axin related gene and its biochemical and genetic interactions with other components in the Wnt pathway, a canonical Wnt pathway has also been identified in *C. elegans* (Gleason et al., 2002; Korswagen et al., 2002) (Figure I-6B)

Non-canonical Wnt signaling in *C. elegans*

As mentioned earlier in this chapter, Wnt pathway is involved in P2/EMS signaling controlling asymmetrical division of EMS cells, which involves both specification the fate of its posterior daughter, E, and EMS mitotic spindle orientation. Figure I-1 shows that MOM-1 (Por), MOM-2 (Wnt), MOM-5 (Fz) and GSK-3 regulate EMS spindle orientation, and this process is transcription independent. Although most of the major components in the canonical Wnt signaling pathway have been shown to be involved in E cell fate specification, a process that involves gene transcription, the Wnt signaling involved in this process is different from the canonical Wnt signaling in several ways (Compare Figure I-1 to Figure I-6A): (i) POP-1, the only TCF/LEF homolog in *C. elegans*, in association with UNC-37/Groucho (**unc**oordinated) represses Wnt-responsive gene transcription instead of being a Wnt-responsive transcription activator. Thus, the MOM/Wnt signal inhibits POP-1 repressive function in E and this is achieved by reducing POP-1 protein level in the nucleus (Lin et al., 1995; Rocheleau et al., 1997; Thorpe et al., 1997b). (ii) Instead of associating with TCF/LEF and activating Wnt-responsive gene transcription, WRM-1, one of the three β-catenin/Armadillo homologs in *C. elegans*, functions to export POP-1 out of the nucleus

in E (Rocheleau et al., 1999) (iii) MAPK pathway consisting MOM-4/TAK1 and LIT-1 is involved in POP-1 downregulation. LIT-1, a MAPK related protein in *C. elegans*, forms a protein complex with WRM-1, leading to POP-1 phosphorylation by LIT-1 and subsequent nuclear export via a 14-3-3 protein, PAR-5 (Lo et al., 2004; Rocheleau et al., 1999). (iv) Both GSK-3 (this thesis and Schlesinger et al., 1999) and APR-1, the APC related protein in *C. elegans*, are positive regulators (Rocheleau et al., 1997) as opposed to negative regulators promoting β-catenin degradation in the canonical Wnt pathway. (v) Instead of being a linear pathway proposed in other systems, several pathways converge on WRM-1/LIT-1 to downregulate POP-1 activity. Any single mutation or mutations within the same pathway upstream of *wrm-1/lit-1* results in incomplete loss of E cell fate, while combinations of mutations between two different pathways cause a fully penetrant *mom* phenotype (Rocheleau et al., 1997, Shin et al., 1999). Based on the differences mentioned above, the Wnt signal pathway that controls asymmetrical EMS cell division is often referred as a non-canonical Wnt pathway. In *C. elegans*, other non-canonical Wnt pathways that are yet very well defined are also involved in the polarity of asymmetric cell divisions during *C. elegans* post-embryonic development, including somatic gonadal cell precursor cells Z1 and Z4 (Siegfried and Kimble, 2002)(Figure I-6C), vuval precursor cell P7p patterning (Inoue et al., 2004) (Figure I-6D) and T cell polarity in the tail (Herman, 2001).

Canonical Wnt signaling in *C. elegans*

The canonical Wnt signaling is best exemplified in the process of Q cell migration in *C. elegans*. The Q cells are similar to neural crest cells in that they delaminate from an epidermal epithelium and go on to populate regions of the body with sensory neurons (Sulston and Horvitz, 1977). The Q cells and their descendants migrate to highly reproducible positions in a relatively uniform region of the body. QL and QR are born in the same A-P position on opposite side of the animal. Although they undergo an identical

sequence of cell divisions, the two Q cells and their descendants migrate in opposite directions: QL and its descendants migrate towards the posterior, whereas QR and its descendants migrate towards the anterior (Sulston and Horvitz, 1997). A canonical Wnt pathway that includes EGL-20/Wnt, LIN-17/Fz and MIG/Fz and MIG-5/Dsh, GSK-3, PRY-1/Axin, BAR-1/β-catenin, and POP-1/TCF/LEF control the expression of the Antennapedia-like homeobox gene *mab-5*, which enables QL cells to migrate toward posterior (Harris, 1996; Herman, 2001; Korswagen et al., 2002; Maloof et al., 1999; Salser and Kenyon, 1992). In this pathway, destruction complex consisting of PRY-1/Axin, GSK-3, and APR-1 negatively regulates BAR-1/β-catenin (Korswagen et al., 2002) (Figure I-6B).

Canonical Wnt signaling is also involved in VPC (**v**ulva **p**recursor **c**ell) cell fate and P12 cell fate specification (Gleason et al., 2002; Jiang and Sternberg, 1998)In these two cases, Wnt signaling functions with a Ras pathway by controlling the expression of a Hox gene: *lin-39* (**lin**eage defective) for VPC cell fate and *egl-5* (**egg l**aying defective) for P12 cell fate. The Wnt signal renders the cells competent to respond to Ras signaling for the cell fate specification (Gleason et al., 2002; Jiang and Sternberg, 1998)

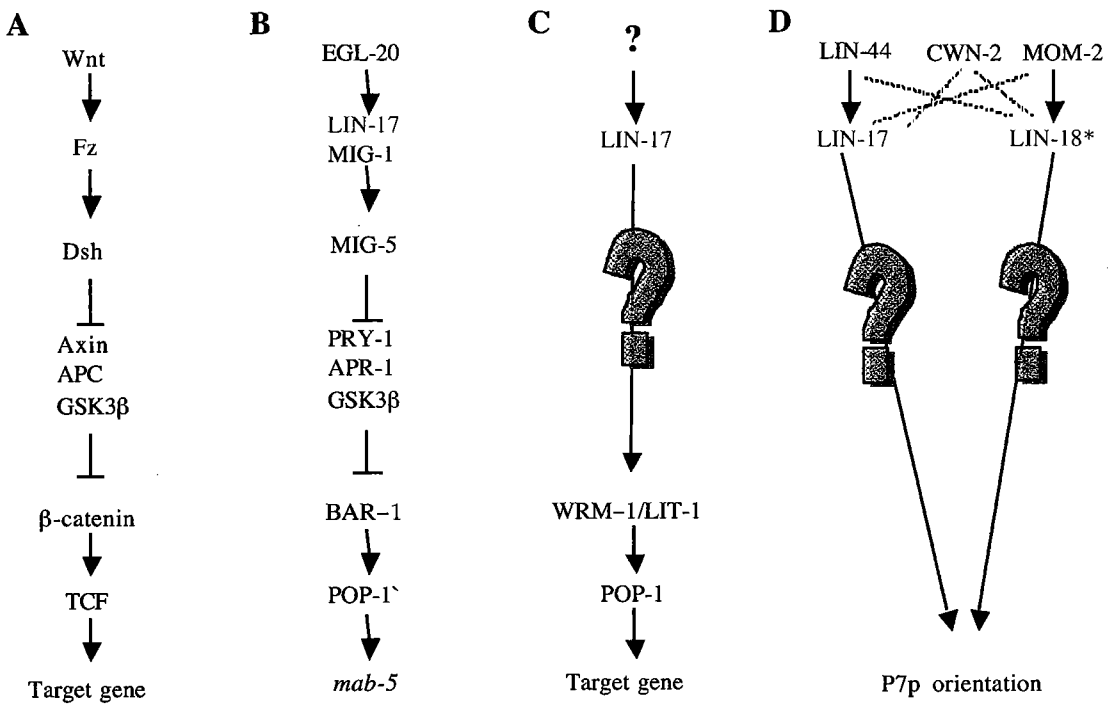

Figure I-6. Representative Wnt signaling pathways in *C. elegans* compared to canonical Wnt signaling pathway from other organisms (A). (B) A representative of canonical Wnt signaling during Q cell migration in *C. elegans*. (C) Z1/Z4 polarity during gonadogenesis (D) During P7.p patterning, RYK ortholog, LIN-18, functions in parallel with LIN-17/Fz as multiple receptors for multiple Wnt signals. Dotted line indicates minor signal/receptor interactions while solid arrow line indicates preferential signal/receptor interactions.
* indicates that although LIN-18 functions at the receptor level it is not a Fz homolog. "?" represents some unknown components function at that point in a pathway.

Wnt pathway components and their functions in *C. elegans*

Forward genetic screenings and the subsequent cloning of the mutants together with the homology searches of the complete genome sequence have identified a fairly complete list of Wnt pathway components in *C. elegans*. Conversed and novel Wnt signaling components identified in *C. elegans* and their involvements in different developmental processes are listed in Table I-1.

Among them two proteins, MOM-1 and MOM-3 (also known as MIG-14), are required within the signaling cell probably by regulating the maturation of signal proteins, Wnt proteins (Thorpe et al., 1997). MOM-1 is the *Drosophila* Porcupine homolog, a multi-transmembrane protein predominantly localized to the endoplasmic reticulum (ER). *Porcupine* mutants fail to secrete wingless (Wnt) to the cell surface (Kadowaki et al., 1996). *mom-3/mig-14* is yet to be cloned (Rocheleau et al., 1997; Thorpe et al., 1997). Its involvement in multiple pathways that utilize different Wnt signals suggest that it is involved in the production of the majority if not all of the Wnt signal proteins.

There are five Wnt genes in *C. elegans* genome, *lin-44, mom-2, egl-20, cwn-1, 2* (***C. elegans* Wnt** family) (Herman et al., 1995; Maloof et al., 1999; Rocheleau et al., 1997; Shackleford et al., 1993; Thorpe et al., 1997). Four *frizzled* related genes have been identified in *C. elegans* genome, *mom-5, lin-17, mig-1* and *cfz-2* (***C. elegans* Frizzled** family) (Rocheleau et al., 1997; Sawa et al., 1996) and there are three Dsh homologs in *C. elegans*, MIG-5, DSH-1 and DSH-2 (Rocheleau, 1999 and this thesis). Some of the developmental processes are less well defined with several components along the pathway unidentified (Figure I-6 and Table I-1). Recent data suggest that functional redundancy among the same family of proteins being one of the reasons. For example, during VCP cell fate specification, none of the single mutation of any of the five *Wnt* related genes mutations displays an apparent vulvaless phenotype while *cwn-1 (ok546); egl-20 (n585)* double mutant strain exhibits an almost fully penetrant vulvaless phenotype (Szyleyko and

Eisenmann, personal communication). Similarly, during P7p cell patterning, while mutation or RNAi of *lin-44, mom-2*, or *cwn-2* each does not affect vulval development, simultaneous disruption of two or three of these *wnt* genes results in a bivulva phenotype, suggesting functional redundancy among these Wnt family proteins (Inoue et al., 2004). The functional redundancy also occurs within the Frizzled like proteins as well as the Dsh like proteins (Table I-1).

The importance of LRP/Arrow as a co-receptor at the cell surface to transduce Wnt signals has been well documented in both vertebrates and *Drosophila* (reviewed in He et al., 2004). Three LRP related genes in *C. elegans*: *lrp-1* (F29D11.1), *lrp-2* (T21E3.3) and *rme-2* (**r**eceptor-**m**ediated **e**ndocytosis) have been identified. These genes do not encode typical LRP5, 6/Arrow like proteins and do not contain the PPPSP motif that is essential to transduce Wnt signal in the cytoplasmic domain (Tamai et al., 2004). There is no evidence that they are involved in Wnt signaling in *C. elegans* (Grant and Hirsh, 1999; Kamikura and Cooper, 2003; Yochem et al., 1999).

In *Drosophila* and vertebrates, β-catenin is not only a central player as a transcriptional coactivator with TCF/LEF for Wnt-responsive gene transcription, but also a component of cell-cell adhesion, linking cadherins to the actin cytoskeleton network through its association with both E-cadherin and α-catenin (Nelson and Nusse, 2004). Interestingly, three β-catenin related proteins have been identified in *C. elegans*, WRM-1, BAR-1 (**β**-catenin/**A**rmadillo **r**elated protein) and HMP-2 (**Hump**back) (Costa et al., 1998; Eisenmann et al., 1998; Rocheleau et al., 1997). Both phenotype analysis of mutations of the three different β-catenin like genes and protein-protein interactions *in vitro* indicate that Wnt signaling and adhesion functions of β-catenin are mediated by separate β-catenins in *C. elegans (Korswagen et al., 2000)*. BAR-1 and WRM-1, function specifically in Wnt signaling and HMP-2 mainly functions as a structural protein at the cell-cell adherens junctions. BAR-1 is mostly involved in the canonical Wnt signaling in which it interacts

with POP-1 (TCF/LEF) and activates gene transcription (Gleason et al., 2002, Korswagen et al., 2002). WRM-1 in association with LIT-1 phosphorylates POP-1 resulting in nuclear export of POP-1 in Wnt responsive cells (Lo et al., 2004; Rocheleau et al., 1999). HMP-2, interacts with HMP-1 (α-catenin like) and HMR-1 (**ham**mer, E-cadherin like) and these three proteins co-localize in adherens junctions. Although *C. elegans* utilizes different β-catenins for Wnt signaling and cellular adhesion, over-expression of HMP-2 or WRM-1 can partially rescue the phenotype of *bar-1* in vulva development suggesting some functional redundancy among them (Natarajan et al., 2001).

Table I-1. Wnt pathway components and their functions in *C. elegans*

gene	*C.elegans* homolog	*C. elegans* development processes								
		P2-EMS signaling		Vulva development		Q cell migration	P12 fate	V5 fate	Z1/Z4 polarity	T cell polarity
		E fate	EMS spindle	VPC fate	P7p polarity					
Porcupine	*mom-1*	+	+	+					+	
	mom-2	+	+		(+)					
	lin-44				(+)		+			+
Wnt	*egl-20*			(+)[1]		+		+		
	cwn-1			(+)[1]						
	cwn-2				(+)					
	mom-5	+	+							
Fz	*lin-17*				+[2]	+	+	+	+	+
	mig-1					+				
	cfz-2									
	mig-5	(+)	(+)	+		+	+			
Dsh	*dsh-1*	--	--							
	dsh-2	(+)	(+)							
GSK-3β	*gsk-3*	+	+			+ (n)				
Axin	*pry-1*	--	--	+ (n)		+ (n)	+ (n)	+ (n)		
APC	*apr-1*	+	--	+ (n)						--
	wrm-1	+	--						+	+
β-catenin	*bar-1*			+		+	+[3]	+		
	hmp-2	--	--							
TCF	*pop-1*	+ (n)	--	+		+	+[3]	+	+	+
NLK	*lit-1*	+	--	--					+	+
TAK	*mom-4*	+	--						--	
?	*mom-3/mig-14*	+	+	+	+	+	+	+		--

+, involved; --, not involved; (+), required redundantly with other homologs, no phenotype alone; + (n), involved but is a negative regulator

[1]Elizabeth Szyleyko, Julie E. Gleason, David M. Eisenmann

[2] LIN-17/Fz functions in parallel with a RYK ortholog, LIN-18, for P7p patterning, see Figure I-5 also.

[3]Rashmi Deshpande, Xin Li, Qi-Ming Li, Russell J Hill

Introduction to Src kinase

The first molecularly defined proto-oncogene, c-Src, is the cellular form of v-Src, which encodes the transforming gene product of the avian tumor virus, Rous sarcoma virus (reviewed in Yeatman, 2004). c-Src belongs to a family of non-receptor kinases, known as Src family kinases (SFKs). Vertebrate SFKs include nine members, some of which (Src, Fyn, Yes) are ubiquitously expressed and while others display tissue specific expression (reviewed in Brown and Cooper, 1996). In *C. elegans*, two SFK members have been identified, *src-1*, which will be discussed in this thesis, and *kin-22* (F49B2.5) (This thesis and Hirose et al., 2003).

SFK proteins have common structures (Figure I-7A). The N-terminal SH4 domain contains a glycine-2 residue for myristylation. This myristylation is required for SFK association with the cell membrane. The SH3 and SH2 domains are involved in protein-protein interactions. The SH3 domain binds to a PPXP motif while the SH2 domain binds to a phosphorylated tyrosine. The inactive state of Src is phosphorylated at the C-proximal tyrosine-527 residue by the tyrosine kinase Csk (**C**-terminal **S**rc **k**inase) (Nada et al., 1991; Okada et al., 1991). Once phosphorylated this tyrosine then binds to the SH2 domain resulting in a "closed" or inactive conformation (Xu et al., 1997; Yamaguchi and Hendrickson, 1996). v-Src protein lacks tyrosine 527, thus rendering it constitutively active. Activation of Src can be achieved by several means, several of which include dephosphorylation of tyrosine-527 by a tyrosine phosphatase, deletion or mutation of tyrosine-527, binding to a phosphorylated Tyrosine residue with higher affinity to the SH2 domain and displace the C-tail by a competition, or phosphorylation of tyrosine-416 within the kinase domain (Figure I-7B) (reviewed in Frame, 2002).

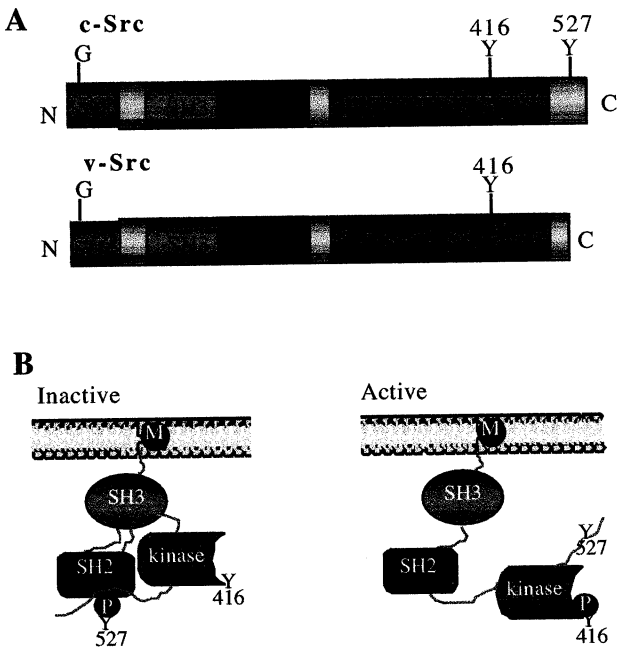

Figure I-7. Structure and activation of Src protein (A) Domain of cellular (c-) and viral (v-) Src. The Src molecule is composed of an N-terminal SH4 sequence with glycine at position 2 for myristylation, SH3 and SH2 protein interaction domains, a kinase domain (SH1) and a C-terminal regulatory domain that contains tyrosine-527. v-Src protein lacks the c-terminal regulatory domain and is constitutively active. (B) Inactivation of c-Src occurs when its C-terminal Tyrosine 527 is phosphorylated and it binds back to the SH2 domain intramolecularly. This interaction and an interaction between the SH3 domain and the kinase domain result in a closed structure. Conversely, when tyrosine 527 is dephosphorylated, the SH2 domain no longer binds to the kinase domain, resulting in an open confirmation, which allows the kinase domain to gain access to its substrate. Full activation of c-Src involves phosphorylation of tyrosine 419 within the kinase domain. G, glycine. Y, tyrosine. M, myristylation. P, phosphorylation.

Src kinases are involved in signal transduction pathways that control cell proliferation, differentiation, survival and cellular architecture (reviewed in Thomas and Brugge, 1997). Src kinases can be activated by variety of cell surface receptors. They include receptor tyrosine kinases, such as epidermal growth factor receptor (EGF-R) (reviewed in Bromann et al., 2004), steroid hormone receptors (Boonyaratanakornkit et al., 2001)and many surface receptors that contain relative short cytoplasmic domains or those that lack cytoplasmic cytoplasmic domains altogether (Davy et al., 1999; Howe and Weiss, 1995).

Src phosphorylates numerous proteins, notably among them are regulators of Rho family GTPases including, p190 RhoGAP, a GTPase-activating protein for Rho (Arthur et al., 2000; Chang et al., 1995), FRG (**F**GD1(faciogenital dysplasia gene product)-**r**elated Cdc42-**G**EF), GEFs (**g**uanine-nucleotide **e**xchange **f**actors) for Cdc42 (Fukuhara et al., 2004), Vav2 , and Rac (Kawakatsu et al., 2004; Servitja et al., 2003). Rho family GTPases are key regulators not only of actin filament organization but also microtubule polarization. They might direct microtubule organizing center (MTOC) movement through their effectors (Fukata et al., 2003). Thus Src may be involved in cell polarization by regulating the activity of Rho family GTPases. Src also phosphorylates cell adhesion molecules, including focal adhesion kinase (FAK), p120 and β-catenin (reviewed in Brown and Cooper 1996). Src phosphorylation of cell adherens junction components leads to disassembly of cell-cell adhesion in epithelial cells (Owens et al., 2000). Since β-catenin is also a component of cell adherens junctions, disassembly of cell-cell adhesion could result in an increased cytoplasmic pool of β-catenin, which indicates potential cross talk between Wnt signaling and Src mediated signaling.

More on GSK-3

GSK-3 is a highly conserved serine/threonine kinase that was originally identified over 20 years ago as a kinase that phosphorylates glycogen synthase (Embi et al., 1980; Woodgett and Cohen, 1984). Despite the specificity suggested by its name, GSK-3 has ever since been shown to act as an important regulator in several signaling pathways, including the Wnt pathway that has been described earlier in this chapter and the insulin pathway, and regulates a wide range of cellular processes, ranging from glycogen metabolism to cell cycle regulation and proliferation (reviewed in Jope and Johnson).

GSK-3 is different from other protein kinases in that it has a high basal activity and is generally considered constitutively active in resting cells. Therefore, GSK-3 activity is down regulated upon signal stimulation. Unlike Wnt signaling, in which GSK-3 activity is down regulated by protein complex (consisting of Axin, APC GSK-3 and its substrate β-catenin) formation, insulin stimulation results in phosphorylation of GSK-3 on a serine residue at the N-terminus of the protein by PKB (**p**rotein **k**inase **B**) and inhibits GSK-3 activity (Cross et al., 1995).

Although GSK-3 plays an important role in the Wnt pathway as well as the insulin pathway, it is selectively coupled to each agonist such that the consequences of it's regulation are specific to the agonist. For example, insulin signaling does not impact components of the Wnt signaling pathway. Treatment of cells with insulin does not lead to accumulation of β–catenin. Conversely, Wnt signaling does not affect insulin signaling (Ding et al., 2001; Yuan et al., 1999). How the cross-talk is prevented between different signaling pathways that involve GSK-3 is not yet clearly understood

GSK-3 substrates

GSK-3 phosphorylates a plethora of substrates to regulate multiple cellular processes, including transcription, cell cycle progression, cytoskeletal regulation and apoptosis. A few examples will be listed below.

One of the many functions of GSK-3 is to phosphorylate transcription factors such as Jun (de Groot et al., 1993), NFAT (**N**uclear **f**actor of **a**ctivated **T**-cells) (Beals et al., 1997). Phosphorylation of these transcriptions factors could directly increase or decrease their ability to bind to DNA. It can also affect their stability or nuclear import or export (reviewed in Frame and Cohen, 2001). GSK-3 phosphorylates cyclin D1 and cyclin E, leading to their degradation and in the case of cyclin D, GSK-3 phosphorylation also leads to its nuclear export (Diehl et al., 1998; Welcker et al., 2003). Microtubule-associated proteins (MAPs) play a central role in regulating microtubule function. Several MAPs, such as tau and MAP1B are phosphorylated by GSK-3, which regulates their binding to microtubules, thus the microtubule dynamics (Goold et al., 1999; Lovestone et al., 1996; Sanchez et al., 2000). GSK-3 also plays a role in mitotic spindle dynamics probably by regulating the activity of kinesin (Morfini et al., 2002; Wakefield et al., 2003).

Biological functions of GSK-3

GSK-3 is highly conserved and has been identified in every eukaryote from yeast to humans. In *S. cerevisiae* and *S. pombe*, GSK-3 related protein has been shown to be required in meiosis (Puziss et al., 1994; Plyte et al., 1996) and in *Dictyostelium* GSK-3 is required for cell fate specification (Harwood et al., 1995). During animal development, GSK-3 function has mostly been limited to embryogenesis because of the embryonic lethality in gsk-3 homozygous flies or mice (Bourouis et al., 1989; Hoeflich et al., 2000; Shannon et al., 1972). Mosaic studies in flies suggest that GSK-3 also is required for

several developmental processes in the larvae and adult (Simpson et al., 1988; Siegfried et al., 1990, 1992). Interestingly, in their methods and material session, Siegried et al., (1992) described that mosaic flies lacking gsk-3 activity in the germline lay abnormal oocytes, suggesting a role of gsk-3 in germline development. More recently, GSK-3 has been shown to be expressed in both testis sections as well as oocytes in mice (Guo et al., 2003; Wang et al., 2003). These results indicate a possible conserved role of GSK-3 for germline development in animals.

Introduction to *C. elegans* germline

Anatomy of C. elegans adult gonad

The *C. elegans* hermaphrodite reproductive system consists two mirror-image U-shaped gonadal arms, each of which at the proximal end is connected to a spermatheca, where sperms are stored and fertilization occurs. The distal arm of the gonad contains syncytial germline nuclei without complete cell membrane. A specialized somatic cell, the distal tip cell (DTC), signals the germ cells in the distal-most region to proliferate through mitotic divisions. As the germ cells progress further away from DTC, they exit mitosis and enter the pachytene stage of meiosis. Within the proximal gonad the germ cells undergo complete cellularization and initiate gametogenesis. During L4 stage they differentiate as spermatocytes which complete meiosis to form sperms and store in the spermatheca (reviewed in L'Hernault and Roberts, 1995). In the adult stage the germ cells differentiate into oocytes. These oocytes fill the proximal gonad arm in a single line and are arrested at diakinesis stage of meiotic prophase I. In the presence of the sperms, they undergo maturation and are ovulated into the spermatheca in an assemble line fashion (McCarter et al., 1999; Miller et al., 2001). The fertilized oocytes are then transferred from the

spermatheca to the uterus where meiotic maturation divisions of the oocytes are completed and embryogenesis begins.

In addition to the germ cells, the gonad also contains somatic cells that all originate from two precursor cells, Z1 and Z4. Each gonad arm contains one distal tip cell (DTC), 5 pairs of specialized myo-epithelial cells, sheath cells, which encircle most of the germ cells, spermathecal cells and a shared uterus by the two gonadal arms (reviewed in Hubbard and Greenstein, 2000).

Soma-germ cell interactions

The somatic gonad has been shown to be important for both germline development and the physiological functions of the sexually mature gonad in several biological systems. In *C. elegans*, the best-characterized somatic soma-germline interaction is the distal tip cell (DTC)-germ cell interactions. Germ cells originate from two precursor cells Z2 and Z3. The distal tip cells (DTC) regulate germ cell proliferation and entry into meiosis. Laser ablation of the distal tip cells during gonadal development leads to arrest of mitosis and initiation of meiosis in all descendants of Z2 and Z3 (Kimble and White, 1981). The DTC-germ cell interaction is mediated by the GLP-1 (**g**erm **l**ine **p**roliferation defective)/Notch signaling to prevent germ cells to enter meiosis (Austin and Kimble, 1987; Henderson et al., 1994; Tax et al., 1994). In addition to controlling germ cell proliferation, DTC cells also influence the spatial organization of the germ line tissue. An alteration in the position of the DTCs results in a corresponding shift in the axial polarity of the gonad (Kimble and White, 1981).

Another example of the importance of somatic gonadal cell during germline development came from laser ablation of sheath and spermathecal cells (SS cells) although the molecular mechanism of this SS-germ cell interaction is not defined yet. Laser ablation of SS studies indicate that somatic sheath cells and spermathecal cells play critical roles in germ cell proliferation, gamete differentiation, oocyte cell cycle control (McCarter et al.,

1997) The importance of the somatic sheath cells and spermathecal cells are also manifested by mutations that affect the differentiation/function of the somatic sheath cells or the spermathecal cells (Aono et al., 2004; Clandinin et al., 1998; Rose et al., 1997). These mutations not only exhibit defects in the physiological functions of the gonad, namely ovulation and fertilization, but also result in loss of oocyte cell cycle control.

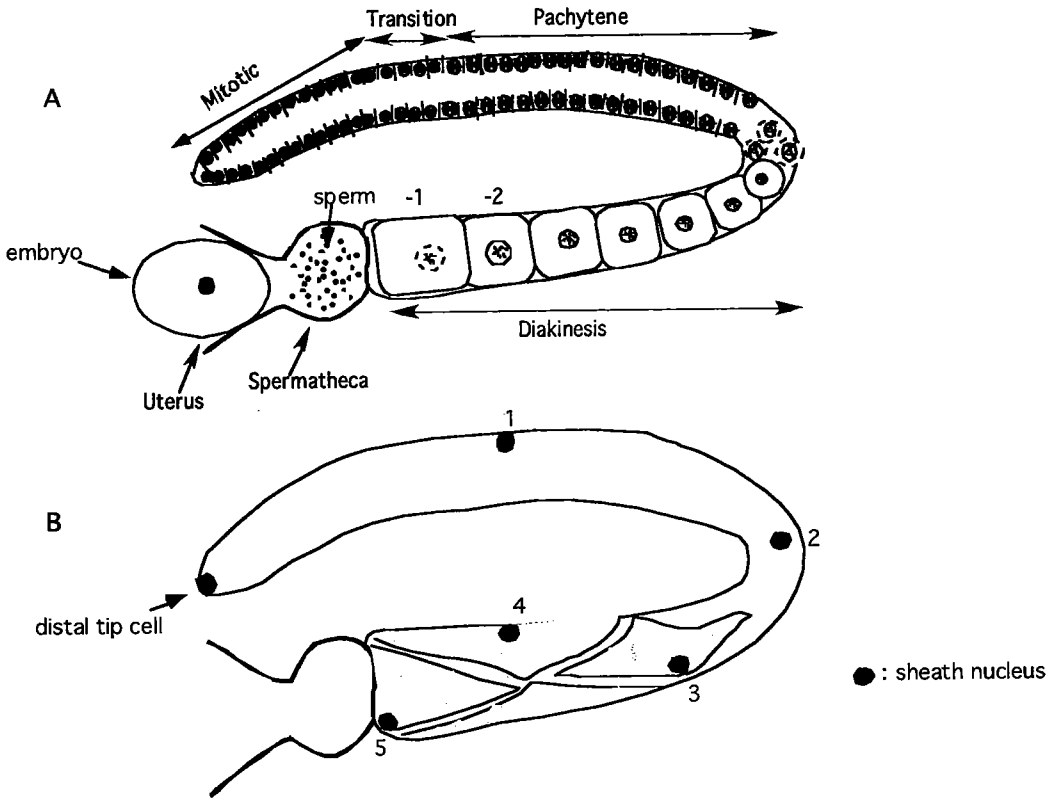

Figure I-8. Representation of one of the two gonad arms of an adult hermaphrodite. (A) The germ line. Mitotic cells are located in the distal tip of the arm. After a brief transition zone, meiotic germ cell nuclei in the pachytene stage extend to the loop region. Oocytes cellularize in the proximal arm and are arrested at diakinesis stage of meiosis I. Fertilized embryo is released into the uterus from the spermatheca and embryogenesis begins. (B) Position of sheath cells and the distal tip cells. One of each pair of sheath nuclei is indicated. Sheath cells 3-5 in the proximal arm are visibly contractile and contain thick and thin filaments. (Adapted from Rose et al., 1997)

CHAPTER II

SRC-1 SIGNALING AND WNT SIGNALING ACT TOGETHER TO SPECIFY ENDODERM AND TO CONTROL CLEAVAGE ORIENTATION IN EARLY *C. elegans* EMBRYOS

Contributors to the work presented in Chapter II:

Jennifer Hogan from John Collins' lab at University of New Hampshire identified the *src-1* gene and isolated the *src-1 (cj293)* allele

Laura A. Berkowitz of University of Tulsa provided the MES-1 antibody

Christian E. Rocheleau of Mello lab identified MIG-5 and DSH-2 as redundant Dishevelled homologs required for E cell fate specification

Ka Ming Pang helped with the co-focal microscopic photograph presented in Figure II-3

SUMMARY

In early *C. elegans* embryos signaling between a posterior blastomere, P2, and a ventral blastomere, EMS, specifies endoderm and orients the division axis of the EMS cell. Although Wnt signaling contributes to this polarizing interaction, no mutants identified to date abolish P2/EMS signaling. Here we show that two tyrosine kinase related genes, *src-1* and *mes-1*, are required for the accumulation of phosphotyrosine between P2 and EMS. Moreover, *src-1* and *mes-1* mutants strongly enhance endoderm and EMS spindle-rotation defects associated with Wnt pathway mutants. SRC-1 and MES-1 signal bi-directionally to control cell fate and division orientation in both EMS and P2. Our findings suggest that Wnt and Src signaling function in parallel to control developmental outcomes within a single responding cell.

INTRODUCTION

During development, cells often orient their division axes with respect to internal asymmetries or with respect to the tissue or body axis. In several well-studied cases of oriented cell division the mitotic apparatus appears to recognize cell-intrinsic cues that direct the spindle to orient along a predetermined axis of the cell. Examples include recognition of the budding axis by the mitotic spindle in yeast, and recognition of the anterior-posterior (A/P) axis by the mitotic spindle in early germline blastomeres of *C. elegans* embryos. In both cases astral microtubules emanating from one of the two centrosomes capture and become anchored at a corticle site and then shorten, rotating the mitotic apparatus into the correct position prior to division (Hyman, 1989 and reviewed in Schuyler and Pellman, 2001; Segal and Bloom, 2001). In these examples division axes appear to be determined by intrinsic asymmetries within the dividing cells. However, in other cases cell-cell contacts appear to orient or influence the cell division axis. For example, a recent study in *Drosophila* suggests that cell contact between epithelial cells can mask polarity cues that would otherwise direct the spindle complex to orient along the intrinsically polarized basal/apical axis of the epithelium (Lu et al., 2001). Epithelial cells can also respond to tissue polarity signals that orient the development of clusters of epithelial cells with respect to a common tissue axis. Tissue polarity signaling is best characterized at present in *Drosophila,* where Wnt/Wg related components including the seven membrane spanning receptor Frizzled, and a PDZ domain protein Disheveled have been implicated in controlling planar polarity within the epithelium (reviewed in Adler and Lee, 2001; Mlodzik, 1999; Mlodzik, 2000).

C. elegans endoderm induction provides one of the best-studied examples of a cell division that is oriented in response to extrinsic signals. Endoderm induction occurs at the 4-cell stage when the posterior-most cell, called P2, induces its anterior sister cell, called

EMS, to divide A/P and to produce a posterior descendant that gives rise to the entire endoderm of the animal. The nature of P2/EMS signaling has been the subject of classical embryological studies involving the isolation of blastomeres and their re-assembly into chimeric embryos. In intact embryos, after EMS is born its centrosomes migrate to occupy positions on the L/R axis of the embryo and then rotate with the mitotic apparatus to align on the A/P axis. EMS then divides to produce an anterior descendant called MS that produces primarily mesoderm, and a posterior descendant called E that produces endoderm. When cultured in isolation, EMS fails to rotate its mitotic apparatus and divides symmetrically to produce two daughters that resemble the anterior mesodermal precursor, MS. If the P2 cell is placed back in contact with the isolated EMS cell, then EMS can orient its spindle toward this contact site and can divide asymmetrically to produce an E-like daughter at the P2 proximal side of the EMS cell (Goldstein, 1995a; Goldstein, 1995b). Thus P2/EMS signaling induces not only the endoderm fate but can also direct the cleavage orientation of the EMS blastomere.

Genetic studies have identified genes involved P2/EMS signaling. Several of these genes define components of the conserved Wnt/Wg signaling pathway, including *mom-1* (Porcupine), *mom-2* (Wnt/Wg), *mom-5* (Frizzled), *wrm-1* (ß-catenin/Armadillo), *apr-1* (adenomatous polyposis coli, APC) and *pop-1* (TCF/LEF) (Lin et al., 1995; Rocheleau et al., 1997; Thorpe et al., 1997). Genetic studies also indicate that other signaling mechanisms contribute to endoderm specification. These mechanisms include components related to MAP Kinase signaling factors, *mom-4* (TAK1, MAP Kinase, kinase kinase related) and *lit-1* (Nemo, MAP Kinase related) (Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999; Shin et al., 1999; Thorpe et al., 1997). A key target of P2/EMS signaling is the down-regulation of POP-1 protein in the posterior daughter of EMS, called E. Certain single mutants as well as certain double mutant combinations among the P2/EMS signaling factors cause POP-1 levels to remain high in the E blastomere, and

consequently induce E to adopt the fate of its sister blastomere MS (Rocheleau et al., 1997; Thorpe et al., 1997). Several of the genetically defined P2/EMS signaling components exhibit alterations in cleavage axes in the early embryo and exhibit skewed A/P orientation of the EMS division. However, within the intact embryo no single or multiple mutant combinations completely prevent the A/P orientation of the EMS division axis (Rocheleau et al., 1997; Schlesinger et al., 1999a; Thorpe et al., 1997). These findings indicate that as-yet-unidentified factors must direct endoderm specification and EMS spindle orientation during P2/EMS signaling.

The *mes-1* gene encodes a probable trans-membrane protein with overall structural similarity to receptor tyrosine kinase and was previously described as a factor required for proper asymmetry and cell-fate specification in embryonic germlineage (Berkowitz and Strome, 2000). Null mutations in *mes-1* cause a maternal-effect sterile phenotype in which the progeny of homozygous mothers are viable but mature without germcells. Most cell-types are specified properly in *mes-1* sterile animals, but the germline cell named P4 adopts the fate of its sister cell, a muscle precursor, called D, and produces ectopic muscle at the expense of the germline (Strome et al., 1995). Interestingly, MES-1 protein is localized intensely at the contact site between the germline blastomere and intestinal precursor at each early developmental stage, starting from the 4-cell stage where MES-1 is localized at the contact site between P2 and EMS (Berkowitz and Strome, 2000).

Here we show that an intense phosphotyrosine signal that depends on *mes-1* (+) activity is correlated with MES-1 protein localization. We show that MES-1 is required in both P2 and EMS and appears to act through a second gene, *src-1*, a homolog of the vertebrate proto-oncogene c-Src^{pp60} (Takeya and Hanafusa, 1983). We describe a probable null mutant of *src-1* that exhibits a fully penetrant maternal-effect embryonic lethal phenotype. The *src-1* and *mes-1* mutants exhibit similar germline defects and have a nearly identical set of genetic interactions with Wnt/Wg pathway components. We show that

double mutants between *mes-1* or *src-1* and each of several Wnt/Wg signaling components exhibit a complete loss of P2/EMS signaling, including a loss of the A/P division orientation in the EMS cell. Finally we show that *mes-1* functions in both EMS and P2 to direct MES-1 protein localization at EMS/P2 junction and to specify A/P cleavage orientation in the EMS cell, while *src-1* is required cell-autonomously in EMS for the induction of the EMS A/P division axis. Our findings suggest that a homotypic interaction between MES-1-expressing cells, P2 and EMS, induces a SRC-1-mediated phosphotyrosine signaling pathway that functions in parallel with Wnt/Wg signaling to specify endoderm and to orient the division axis of EMS in early *C. elegans* embryos.

RESULTS

SRC-1 is required for embryonic body morphogenesis and germline asymmetries

We identified a *C. elegans* homolog of the vertebrate proto-oncogene c-SRC^{pp60} (reviewed in Schwartzberg, 1998), designated *src-1*. The *src-1* gene is one of two *C. elegans* genes that contain all of the hallmarks of Src family tyrosine kinases (Figure II-1, see Experimental Procedures). The second gene F49B2.5, has not been studied previously but has no phenotype by RNAi (Figure II-1B and data not shown). We isolated a deletion allele of *src-1*, *cj293*. This deletion removes 4.5 kilobases (kb) of genomic sequence including sequences encoding the SH2 domain as well as the catalytic site of the kinase, resulting in a frame-shifted and truncated protein of 137 amino acids (Figure II-1A and B). This probable null allele of *src-1* causes a recessive maternal effect embryonic lethal phenotype. Consistent with the idea that this embryonic lethal phenotype represents a loss of function phenotype for *src-1*, we found that RNAi targeting *src-1* induced an identical embryonic lethal phenotype (see below). Homozygous *src-1 (cj293)* hermaphrodites are themselves viable but produce inviable embryos. These *src-1 (cj293)* arrested embryos contain well-differentiated tissues including hypodermis, muscle, pharynx and intestine but these embryos fail to undergo body morphogenesis (Figure II-2A-F, see Experimental Procedures).

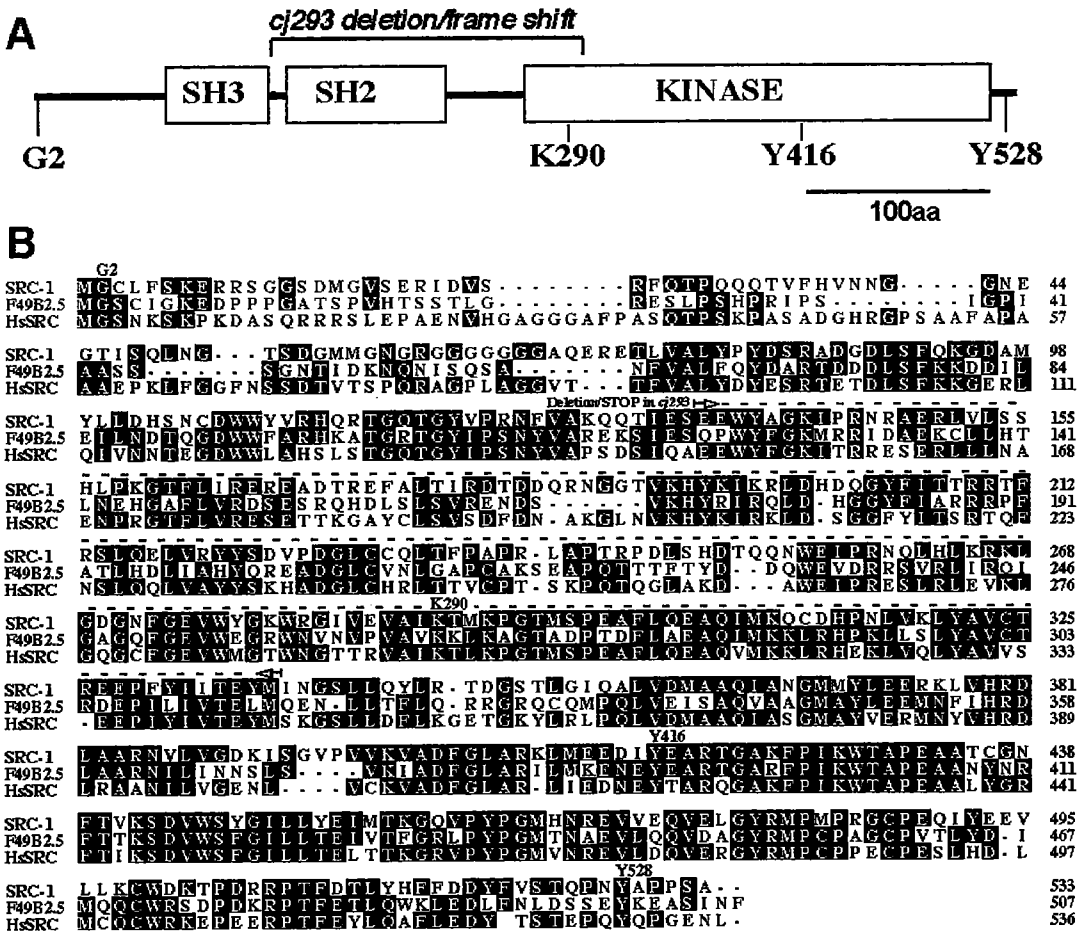

Figure II-1. SRC-1 is a Src-family kinase (A) Schematic diagram of protein features and domains conserved in SRC-1. Conserved domains and key residues are indicated including; the potential myristylation site, glycine 2 (G2), the Src Homology 2 (SH2) and SH3 domains, the kinase domain along with the catalytic active site, lysine 290 (K290), and conserved regulatory tyrosine residues, Y416 and Y528. The region indicated beneath the brackets corresponds to the region deleted in the *cj293* mutant. (B) Alignment of SRC-1 with a second *C. elegans* Src-family kinase (F49B2.5) and human c-Src (HsSRC). The conserved residues indicated in (A) and the position of the *cj293* deletion is indicated above the aligned sequences.

Table II-1. Genetics of P2/EMS signaling

Embryo type	% embryos with L-R EMS daughters (n)	% embryos lacking intestine (n)
src-1(cj293)	**15 (59)**	**0 (n>500)**
src-1(RNAi)	**10 (42)**	**0 (n>500)**
mes-1(bn74)	0 (28)	0 (n>500)
mes-1(RNAi)	0 (17)	0 (n>500)
src-1(RNAi);mes-1(bn74)	**13 (52)**	**0 (356)**
mom-1(or10)	**0 (12)**	**48 (208)**
mom-1(or10),mes-1(RNAi)	**100 (11)**	**100 (97)**
mom-1(or10); src-1(RNAi)	**100 (9)**	**100 (104)**
mom-2(ne141)	**0 (21)**	**66 (212)**
mom-2(ne141);mes-1(bn74)	**90 (31)**	**100 (534)**
pop-1(RNAi)	**0 (12)**	**0 (336)**
mom-2(ne141);mes-1(bn74);pop-1(RNAi)	**91 (22)**	**0 (389)**
mom-2(ne141);src-1(RNAi)	**94 (17)**	**99 (461)**
followed by pop-1(RNAi)	**n.d.**	**0 (49)**
mom-3(zu21)	**0 (14)**	**56 (182)**
mom-3(zu21); mes-1(bn74)	**100 (17)**	**100 (374)**
mom-3(zu21);src-1(RNAi)	**100 (9)**	**99 (103)**
mom-5(zu193)	**0 (11)**	**4 (137)**
mom-5(RNAi)	**0 (12)**	**3 (184)**
mom-5(RNAi);mom-1(or10)	**0 (12)**	**18 (81)**
mom-5(zu193);mes-1(bn74)	**100 (19)**	**100 (489)**
mom-5(zu193);mes-1(bn74);pop-1(RNAi)	**100 (16)**	**0 (197)**
mom-5(zu193), src-1(RNAi)	**97 (30)**	**100 (247)**
followed by pop-1(RNAi)	**n.d.**	**0 (79)**
dsh-2(RNAi);mig-5(RNAi)	**0 (27)**	**4 (348)**
dsh-2(RNAi);mig-5(RNAi);mom-2(ne141)	**0 (9)**	**7 (415)**
dsh-2(RNAi);mig-5(RNAi);mom-5(zu193)	**0 (11)**	**8 (351)**
dsh-2(RNAi);mig-5(RNAi);mes-1(bn74)	**97 (27)**	**99 (353)**
dsh-2(RNAi);mig-5(RNAi);src-1(RNAi)	**94 (17)**	**100 (573)**
dsh-2(RNAi);mig-5(RNAi);src-1(cj293)	**100 (12)**	**100 (254)**
gsk-3(RNAi)	**0 (18)**	**11(491), E to MS fate 56(22)***
gsk-3(RNAi);mes-1(bn74)	**97 (35)**	**11(255), E to MS fate100(15)***
gsk-3(RNAi);src-1(RNAi)	**95 (22)**	**11(228), E to MS fate 100(12)***
mom-4(ne19)	**0 (15)**	**46 (384)**
mom-4(ne19);dsh-2(RNAi);mig-5(RNAi)	**0 (14)**	**100 (357)**
mom-4(ne19;mes-1(bn74)	**0 (12)**	**57 (702)**
mom-4(ne19),src-1(RNAi)	**12 (34)**	**98 (273)**
apr-1(RNAi)	**0 (17)**	**31 (396)**
apr-1(RNAi);dsh-2(RNAi);mig-5(RNAi)	**0 (10)**	**100 (228)**
apr-1(RNAi);mes-1(bn74)	**0 (19)**	**33 (218)**
apr-1(RNAi),src-1(cj293)	**19 (26)**	**99 (215)**
lit-1(RNAi)	**0 (18)**	**100 (237)**
lit-1(RNAi);mes-1(bn74)	**0 (19)**	**100 (389)**
lit-1(RNAi);src-1(cj293)	**16 (19)**	**100 (187)**
wrm-1(RNAi)	**0 (15)**	**100 (372)**
wrm-1(RNAi);mes-1(bn74)	**0 (24)**	**100 (447)**
wrm-1(RNAi);src-1(cj293)	**17 (24)**	**100 (235)**
pop-1(zu189)	**0 (14)**	**0 (n>500)**
pop-1(RNAi);mes-1(bn74)	0 (11)	0 (247)
pop-1(zu189),src-1(RNAi)	**9 (22)**	**0 (364)**

n.d.= not determined.

*In approximately 90% of *gsk-3(RNAi)* embryos the C blastomere makes intestine (Y.B. unpublished results; Schlesinger et al., 1999). A similar frequency of intestinal differntiation was observed in the C lineage of double mutants *gsk-3;src-1* and *gsk-3;mes-1*.

During early development *src-1* embryos exhibit normal initial asymmetries in blastomere size and division timing and exhibit a wild-type pattern of cell-division axes until the 6-cell stage (data not shown). All of the *src-1* mutant embryos examined specified endoderm, and laser ablation studies indicated that endoderm was specified correctly in posterior descendant of the EMS blastomere, the E blastomere (Table II-1, and data not shown). Approximately 15% of *src-1 (cj293)* embryos exhibited a L/R rather than A/P EMS division resulting in L/R daughters that both contact the P2 cell (Table II-1). Approximately 60% of *src-1* embryos exhibited a skewed A/P-L/R division (Figure II-2H). In these skewed EMS divisions, although the spindle began to elongate at a L/R angle, it ultimately aligned with the A/P axis to produce daughters that occupy A/P positions that were within the norm for wild-type (data not shown). This skewed division-axis phenotype was similar to a defect observed in many Wnt-pathway mutants (Schlesinger et al., 1999).

In addition to the defects in the EMS division axis, *src-1* mutant embryos exhibit alterations in the divisions of the germline blastomeres P2, P3 and P4. For example, the P3 cell which normally is smaller than, and divides after, its sister cell, named, C, was instead equal to C in size in 5 of 37 embryos examined, and divided at the same time as C in 1 of 8 embryos examined. Similarly, P4 was equal in size to its somatic sister cell, D, in 19 of 37 embryos examined and 5 out of 8 embryos exhibited a precocious P4 division. Consistent with transformation from a P4 to a D-cell fate, we observed muscle differentiation in 18 out 20 P4 cells isolated by laser ablation (data not shown, and see Experimental Procedures). We stained *src-1* embryos with the antibody K76, which recognizes the normally germline specific P-granules. In wild type embryos K76 only stains the P lineage cells, P0, P1, P2, P3, P4 and at hatching stains only the daughters of P4, Z2 and Z3 (Strome and Wood, 1982). In *src-1* mutant embryos, we found that the P-granules were segregated properly to P1 and P2 but were frequently mis-segregated to both daughters of P2 and P3 as well as to

their descendants (Figure II-2I-L, Table II-2). Taken together these findings suggest that SRC-1 is required in the early embryo for proper orientation of the EMS cell division and for proper asymmetry in the P2 and P3 divisions.

We wondered if the morphogenesis defect of *src-1* embryos was caused by the mis-positioning of blastomeres due to the early defects in asymmetric divisions, or instead might reflect a second independent role for *src-1* in morphogenesis. We reasoned that the early role for *src-1 (+)* in asymmetric division was likely to require maternal *src-1 (+)* activity, while the later function in morphogenesis might be provided either maternally or zygotically. We therefore mated homozygous *src-1(cj293)* mothers with wild-type males and analyzed the phenotypes of the resulting heterozygous cross-progeny embryos. We found that these *src-1*/+ progeny of *src-1* homozygous mothers were partially rescued for their morphogenesis defects, and approximately 10% (n=2160) hatched, while 40% (n=77) underwent extensive body morphogenesis but failed to hatch. The zygotically rescued *src-1*/+ progeny exhibited a spectrum of post-embryonic phenotypes including larval lethality and sterility, however, rare fertile adults were also observed. In contrast, the early phenotypes including EMS division orientation defects and the P-granule localization defects were not rescued in the *src-1*/+ embryos. For example, we found that 16% (n=18) of EMS blastomeres examined in *src-1*/+ embryos divided L/R, and 78% (n=63) of terminally arrested embryos exhibited mislocalized P-granules. These findings indicate that SRC-1 functions in two distinct developmental events. First maternally provided SRC-1 functions in the early embryo to control proper specification of EMS and P-lineage division axes; second, later in embryogenesis, zygotic and maternal products function together in body morphogenesis.

Table 2. *src-1* is required for P-granule localization

Stage (# of cells)	Cells with p- granules	% of cells with P-granule (n)
2-3	P1	100 (n>50)
4-7	P2	100 (n>50)
8-15	P3	79 (24)
	P3+C	21 (24)
16-55	P4	21 (51)
	P4+D	69 (51)
	P4+D+C	10 (51)
terminal	Z2+Z3	20 (113)
	4-6 cells	30 (113)
	>10 cells	49 (113)

Embryos were scored at 25°C.

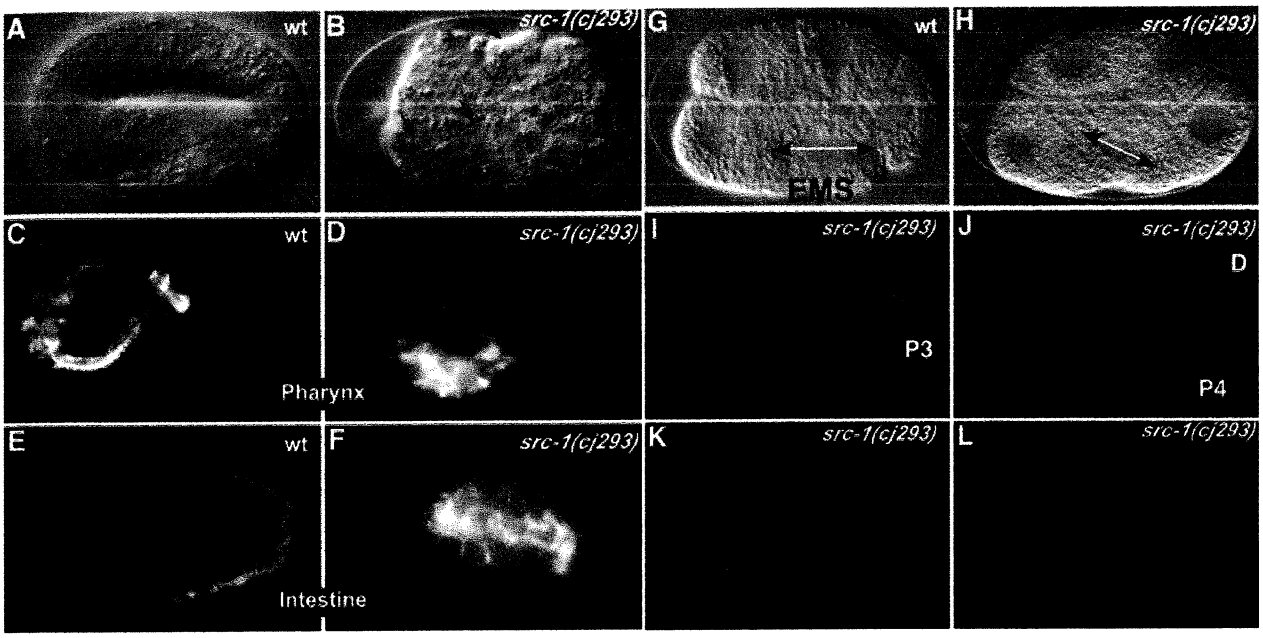

Figure II-2. *src-1* is required for morphogenesis and cell polarity (A-F) *src-1* is required for morphogenesis. (A-B) Light micrographs showing morphogenesis in (A) wild-type and (B) *src-1 (cj293)* embryos (anterior is to the left and dorsal is up in all micrographs shown). The wild-type embryo (A) has elongated and is ready to hatch. The *src-1* embryo (B) contains a wild-type number of well-differentiated cells, but has an amorphous appearance with hypodermal cells located on the dorsal side (arrowhead) and normally internal organs including the pharynx (arrows) located outside on the ventral surface of the embryo. (C-G) Immunofluorescence micrographs showing pharyngeal differentiation (C and D) and intestinal differentiation (E and F) in wild-type and *src-1* embryos. In *src-1* embryos (D and F) both the pharynx and intestine are correctly specified but are disorganized, fail to elongate and fail to define clear basal-apical polarity. (G-J) *scr-1* is required for proper cell division polarity. (G-H) Light micrographs showing a wild-type EMS cell division (G) and a skewed *src-1* mutant EMS cell division (H). (I-L) Immunofluorescence images showing progressively later staged *src-1* early embryos stained with the P-granule monoclonal antibody K76 (red) and nuclear stain DAPI (blue). The embryo (I) is shown with the germline cell P3 in telophase, P-granules are distributed

uniformly on both sides of the dividing cell. A slightly older 28-cell stage embryo (J) is shown after the P-granules have been partitioned into both descendants of P3 (named P4 and D). After one more division (K) and in terminally arrested embryos (L), P-granules are observed in 4 and 16 descendants (respectively) of P3.

src-1 and the receptor tyrosine kinase related gene _mes-1_ function together to direct phosphotyrosine accumulation between EMS and P2

The germline phenotypes observed in *src-1* mutant embryos appeared similar to phenotypes associated with loss of function alleles of a previously described gene, *mes-1*. Like *src-1* mutants, mutations in *mes-1* lead to defects in the asymmetric divisions that give rise to the germline blastomere P4. In *mes-1* mutants, P2, and P3 exhibit a partial loss of polarity and the P-granules are mislocalized to both descendants of these divisions (Strome et al., 1995). Also as observed in *src-1* mutants, we found that 63% (n=27) of *mes-1* mutant embryos exhibited an initially skewed alignment of the EMS spindle. However, whereas 15% of *src-1* mutant embryos exhibited a fully L/R EMS division axis, we did not observe L/R EMS divisions in the *mes-1* mutant embryos examined (Table II-1). Furthermore, unlike *src-1* mutants, the majority (>85%) of *mes-1* mutants properly execute morphogenesis and develop into sterile adults (Strome et al., 1995).

Since both SRC-1 and MES-1 are related to protein tyrosine kinases we asked if phosphotyrosine, pTyr, staining in the early embryo was correlated with their activities. We found that the pTyr-specific monoclonal antibody, pY99, stained cell-cell contact sites both in early embryo and throughout development (Figure II-3A and data not shown). Interestingly, at the 4-cell stage in wild-type embryos, the junctional pTyr staining between P2 and EMS was enhanced relative to other contact sites (Figure II-3A, C, E and F). This enhanced pTyr staining overlapped with MES-1 protein localization throughout the stages

when MES-1 protein is detectable, including at the P2/EMS contact site (Figure II-3B, C and D) as well as the P3/E and P4/Ep contact sites (data not shown).

We next asked if this enhanced pTyr staining required *mes-1 (+)* and *src-1 (+)* activities. We found that throughout the 4-cell stage *mes-1* mutants exhibited normal levels of pTyr staining at other cell junctions but exhibited a reduced level of staining at the P2/EMS junction (compare Figures II-3E and F to Figures II-3G and H). Likewise, pTyr staining was reduced at the P3/E, and P4/Ep contact sites (data not shown). In contrast, *src-1* mutants exhibited greatly reduced, uniformly low levels of pTyr staining at all cell contact sites, both throughout the 4-cell stage (Figure II-3 I and J), and until at least the 44-cell stage in development (data not shown). Thus at the 4-cell stage, *mes-1* is required for enhanced pTyr staining at the P2/EMS contact site while *src-1* is required for most of the visible pTyr staining.

The wild-type MES-1 protein is not predicted to encode an active kinase domain (Berkowitz and Strome, 2000), therefore these findings suggest that MES-1 localized at the P2/EMS contact site activates SRC-1 to direct tyrosine phosphorylation of junctional proteins. Consistent with this idea we found that MES-1 protein localization did not depend on SRC-1 activity (data not shown). Furthermore, we found that double mutants between *mes-1 (bn74)* and *src-1 (cj293)* did not exhibit enhanced or novel phenotypes and instead appeared identical to *src-1* single mutants (Table II-1). These findings are consistent with the idea that MES-1 functions upstream of SRC-1 in a cell-contact-mediated signaling process that controls P-lineage asymmetries after the 4-cell stage, and may also contribute to the specification of the EMS division axis.

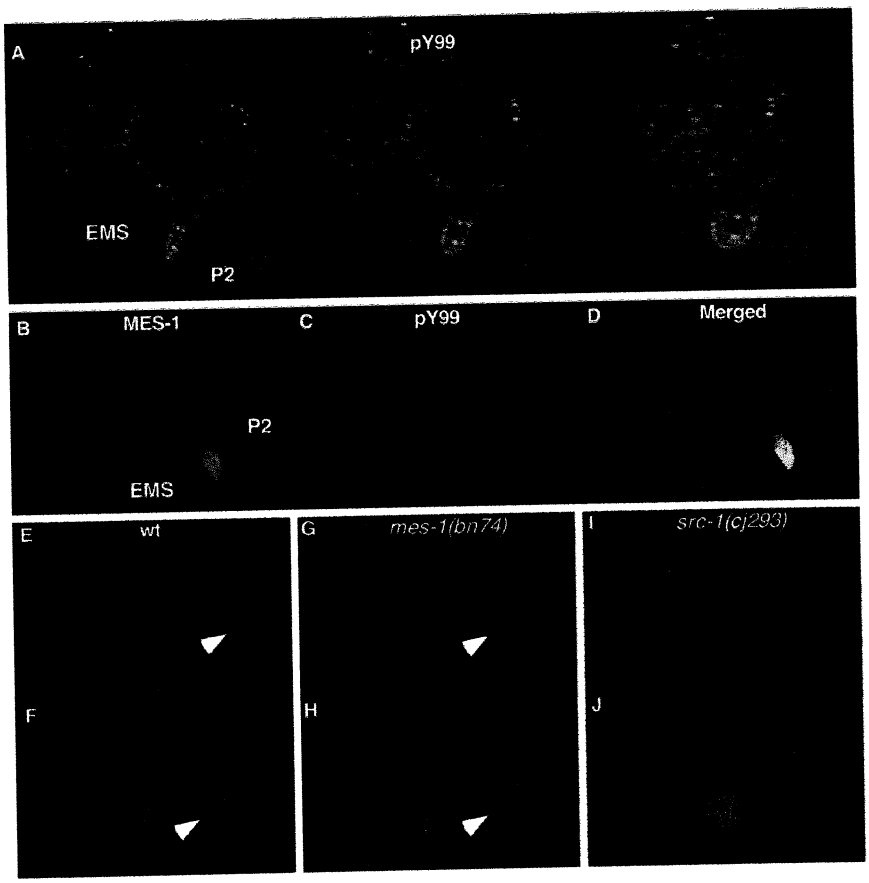

Figure II-3. MES-1 and SRC-1 are required for asymmetric Phosphotyrosine staining in 4-cell stage embryos (A) Three-dimensional reconstruction of phosphotyrosine junctional staining in a wild-type embryo. Each succesive image is rotated by 5° to reveal the phosphotyrosine signal that lies along the plane of each cell contact .
(B-D) A single embryo is shown stained with MES-1 specific antibody (B) and with pY99 antibody (C). The merged image (D) reveals extensive overlap (yellow) at the junction between EMS and P2. (E-J) Genetic analysis of phosphotyrosine localization during the 4-cell stage in wild-type embryos (E and F), in *mes-1* mutant embryos (G and H) and in *src-1* mutant embryos (I and J). Confocal microscopy was used to image phosphotyrosine, pY99, staining (red), and interphase nuclei (green). Nuclei were stained using an antibody that recognizes an epitope absent in mitotic cells (anterior and dorsal two cells in panels F,H and J).

src-1 and *mes-1* interact genetically with Wnt signaling components

The defects in the EMS division orientation of many *src-1* and *mes-1* mutant embryos resemble a partially penetrant phenotype observed in mutants of several Wnt-pathway related genes required for the P2/EMS cell-cell interaction (Schlesinger et al., 1999). We therefore decided to ask if *src-1* and *mes-1* interact genetically with previously described genes implicated in P2/EMS signaling. To do this we constructed double mutants between *mes-1* and *src-1* and each of the known components of the P2/EMS signaling pathway (Table II-1). We found that several of these double mutants exhibited strong synergistic interactions consistent with a complete loss of P2/EMS signaling. This syngergy included an endoderm to mesoderm transformation in the E-cell lineage (as assayed by laser ablation, see Experimental Procedures) and a fully L/R division axis in EMS (Table II-1). The L/R division in EMS appeared to result from a failure of the nascent mitotic apparatus to rotate onto the A/P axis prior to division. As in wild-type embryos, we observed that the newly duplicated centrosomes in EMS migrated properly around the nuclear envelope ending up positioned correctly on the L/R axis, orthagonal to the previous A/P division axis. In wild-type embryos astral microtubules at one pole appear to capture a cortical site and rotate the spindle complex onto the A/P axis. This rotation was not observed in the double mutants resulting in a uniform L/R division axis (Table II-1, and data not shown).

Synergy was observed between *mes-1* or *src-1* mutants and each of the following previously described mutants: *mom-1* (Porcupine), *mom-2* (Wnt/Wg), *mom-5* (Frizzled), *sgg-1* (GSK-3*)* and *mom-3* (uncloned). In addition, we observed identical synergies in the phenotypes of embryos produced by *mes-1* or *src-1* homozygotes after injection with a mixture of two double stranded RNAs targeting the *C. elegans* Disheveled homologues *dsh-2* (C27A2.6) and *mig-5* (T05C12.6). RNAi targeting these Disheveled homologues

individually did not induce visible defects in P2/EMS signaling (Table II-1 and data not shown).

SRC-1 signaling and Wnt signaling appear to act in parallel during P2/EMS signaling

Previous studies have shown that double mutants between Wnt pathway components fail to exhibit enhanced defects in P2/EMS signaling (Rocheleau et al., 1997; Thorpe et al., 1997). For example in double mutants between *mom-2/Wnt* and its presumptive receptor, *mom-5/Frizzled*, greater than 90% of embryos correctly specified intestine from the posterior daughter of EMS and 100% of the EMS blastomeres lineaged produced A/P daughters. Thus the less penetrant gutless phenotype of *mom-5* mutants partially suppresses the more penetrant gutless phenotype of *mom-2* mutants (See Table II-1 and Rocheleau et al. 1997). This finding suggests that aberrant signaling via MOM-5 can interfere with gut specification when the MOM-2 ligand is absent. The *mom-1* gene appears to be the only *C. elegans* homolog of *Porcupine,* a gene required for the proper secretion of Wnt/Wg in *Drosophila* (Kadowaki et al., 1996). Presumptive null alleles of *mom-1* result in egg-laying defects as well as maternal effect embryonic lethality, phenotypes consistent with defects in both embryonic and post-embryonic Wnt signaling (Rocheleau et al., 1997; Thorpe et al., 1997). Consistent with the idea that MOM-1 may be required for MOM-2 activity in the early embryo, *mom-1* mutants produce gutless embryos at a frequency of 48%, (Table II-1), a frequency similar to that observed for *mom-2* mutants (66%, Table II-1). We therefore asked if *mom-5* mutants can partially suppress the gutless phenotype of *mom-1,* and found that, *mom-5(RNAi)* in the *mom-1(or10)* mutant background resulted in a decrease in the number of gutless embryos to 18% (Table II-1). As expected, we found that *dsh-2/mig-5(RNAi)* failed to enhance defects in P2/EMS signaling in doubles with *mom-5(zu193),* and like *mom-5* mutants, *dsh-2/mig-5(RNAi)* partially suppressed the *mom-2*

gutless phenotype (Table II-1). Taken together these findings suggest that MOM-5 and DSH-2/MIG-5 function together downstream of MOM-1 and MOM-2, and that in the absence of MOM-2 activity, the activities of MOM-5 and DSH-2/MIG-5 can interfere with a parallel mechanism for gut induction.

The finding that all of the existing embryonic lethal mutants in the canonical Wnt pathway components including presumptive null alleles at various steps in the pathway, nevertheless, appear to properly execute P2/EMS signaling suggests that redundant pathways must function in parallel with these activities (Rocheleau et al., 1997). This conclusion is supported by the finding that double mutant combinations between mutants that affect distinct steps in the canonical Wnt pathway do not exhibit enhanced P2/EMS signaling defects and can instead result in reduced penetrance of P2/EMS signaling defects (Rocheleau et al., 1997 and Table II-1). These finding are in contrast to expectations for a combination of weak alleles wherein double mutants are expected to exhibit the phenotype consistent with the stronger allele or may show an enhanced phenotype. Finally, the finding that double mutant combinations between canonical Wnt pathway mutants and *src-1* or *mes-1* each exhibit very similar synergistic phenotypes, including a nearly total loss of P2/EMS signaling, suggests first of all, that the Wnt components do indeed function together in a pathway, and second, that this pathway functions in parallel to *src-1*, *mes-1* signaling.

SRC-1 signaling and Wnt signaling converge to regulate POP-1

Endoderm induction is correlated with a downregulation of the POP-1 protein in the E blastomere (Rocheleau et al., 1997; Thorpe et al., 1997). Therefore, we examined whether inhibition of *pop-1* could restore endoderm specification in double mutants. Consistent with this idea we found that inhibiting *pop-1* by RNAi resulted in restored differentiation of the endoderm but did not restore the A/P division orientation of the EMS cell (Table II-1).

Finally, we examined POP-1 levels in *mes-1 (bn74)*; *mom-2 (ne141)* and *src-1 (RNAi); mom-2 (ne141)* double mutants, and found that POP-1 protein which is normally restricted to MS as a result of P2/EMS signaling was present at equal, high levels in both daughters of EMS (data not shown). These findings indicate that MES-1/SRC-1 signaling collaborates with Wnt-signaling factors to downregulate POP-1and to specify the E cell fate.

Several genes previously implicated in P2/EMS signaling behaved as though they function in parallel with both Wnt and Src signaling, or lie below the convergence in signaling. These included *mom-4* (TAK1) and *apr-1* (adenomatous polyposis coli, APC), as well as *wrm-1* (ß-catenin) and *lit-1* (Nemo/NLK) (Table II-1). Consistent with previous studies, none of these genes appeared to have a role in controlling the division orientation of EMS (Rocheleau et al., 1997; Schlesinger et al., 1999; Thorpe et al., 1997), and mutants in these genes failed to exhibit synergy for EMS division defects in doubles with either Wnt or Src components (Table II-1, and data not shown). For example, just as *mom-4* and *apr-1* mutants enhanced endoderm defects observed in canonical Wnt components (Rocheleau et al., 1997), we found that these mutants also exhibited enhanced endoderm defects in doubles with *src-1* mutants (Table II-1). This finding suggests that *mom-4* and *apr-1* function in parallel with both pathways or function downstream of the convergence between Wnt and Src signaling. *mom-4* and *apr-1* exhibited little or no genetic interaction with *mes-1* (Table II-1), supporting the idea that *src-1* is more critical than *mes-1* for P2/EMS signaling (see discussion).

The finding that *wrm-1* and *lit-1* do not exhibit synergy for the control of EMS division orientation (Table II-1) is consistent with previous data that place WRM-1 and LIT-1 as direct regulators of POP-1 (Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999). Taken together these findings are consistent with a model in which MES-1 and SRC-1 function in parallel with certain upstream Wnt signaling factors and converge with

this parallel pathway to downregulate POP-1 and to specify the A/P division axis of EMS. Finally, consistent with this model, we found that, MES-1 protein localization and phosphotyrosine accumulation were not affected in the Mom mutants examined including, *mom-2*, *mom-3*, *mom-4*, *mom-5* and *wrm-1* (Berkowitz and Strome, 2000 and data not shown).

Autonomy and non-autonomy in P2/EMS signaling

Previous studies on P2/EMS signaling described methods that permit isolated blastomeres and chimeric embryos to be cultured *in vitro* (Goldstein, 1992). We used these techniques to examine the genetic requirements for EMS spindle orientation in 4-cell stage embryos. As shown previously (Goldstein, 1995b), isolated wild-type EMS cells placed back in contact with P2, (but not two EMS cells placed together) correctly orient their mitotic spindles in accordance with the P2 contact site (Figure II-4A and B). We next examined chimeric embryos assembled using a combination of wild-type and *src-1* mutant blastomeres. We found that chimeric embryos lacking *src-1* function in P2 exhibited a wild-type orientation of the EMS division axis (Figure II-4C), while embryos lacking *src-1* function in EMS, always failed to do so (Figure II-4D). An identical set of experiments were performed on *mes-1* mutant embryos. Interestingly, we found that *mes-1 (+)* activity was required in both EMS and P2 to orient the mitotic spindle of EMS (Figure II-4E and F). Thus SRC-1 functions cell-autonomously in EMS to control the EMS division axis, while MES-1 functions in both cells.

We next wanted to examine the genetic requirements for MES-1 protein localization. To do this we first removed P2 from a wild-type 4-cell embryo and then immediately placed it back in a new orientation. We found that when P2 was placed back in such a way that it made contact with EMS, MES-1 protein localization was restored at the newly established P2/EMS contact site (arrow in Figure II-4G). MES-1 protein localization

was never observed at other P2 cell-contacts. For example when placed away from EMS in contact only with the anterior blastomeres no MES-1 protein was observed at P2 cell contact sites (Figure II-4H). As described previously (Berkowitz and Strome, 2000), a remnant of the previous P2/EMS junctional staining was almost always retained in the P2 cell (see arrowheads in Figure II-4G, H and K). Consistent with the idea that MES-1 is required in both cells, the junctional MES-1 staining was not restored if MES-1 was absent in either P2 or EMS (Figure II-4I and J). As expected from previous staining of intact *src-1* mutant embryos, *src-1* (+) activity was not required in either P2 or EMS for MES-1 protein localization (data not shown). Furthermore, as in intact embryos, we found that intense pTyr staining at junctions was correlated with MES-1 protein localization at those junctions (data not shown). Thus just as proper control of EMS division orientation requires MES-1 in both cells, MES-1 protein localization at the P2/EMS contact site also requires MES-1 protein in both cells.

We noticed that although junctional staining was only observed at the P2/EMS contact site, all blastomeres including the AB blastomeres contain abundant MES-1 protein. This non-junctional MES-1 protein often appeared to localize as small punctae, perhaps indicating the existence of MES-1-containing vesicles in the cytoplasm (compare *mes-1 (-)* and *mes-1 (+)* blastomeres in Figure II-4I and J). This punctate appearance was often especially intense near the former EMS contact site within P2 or EMS cells (Figure II-4G, H, Kand data not shown). These findings suggest that there is some property unique to the P2/EMS junction that permits MES-1 to re-localize from the cytoplasm to the cell-cell junction. In order to ask if this ability to recruit MES-1 to the junction required factors unique to P2 or EMS, we placed wild-type P2 in contact with wild-type P2 (Figure II-4K), and wild-type EMS in contact with wild-type EMS (Figure II-4L). We found that in both types of experiment the junctional MES-1 staining was restored (Figure II-4K and L). Thus P2/EMS, EMS/EMS and P2/P2 cell contacts can all induce cortical localization of

MES-1 protein, while cell contacts with or between other blastomeres fail to do so. Taken together, these studies indicate that P2 and EMS undergo a dynamic interaction that involves signaling in both directions.

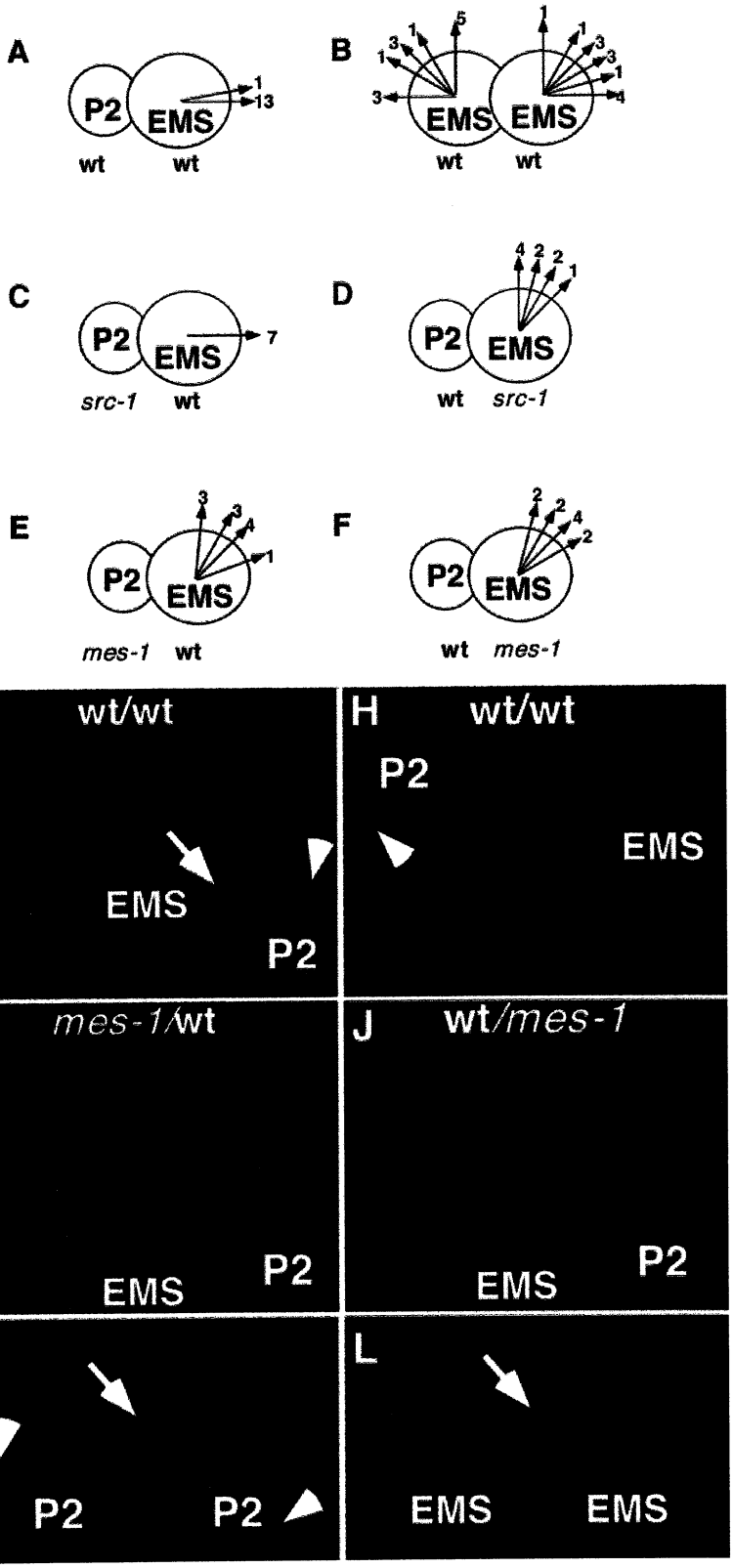
A
P2
EMS
1
13
wt wt
B
EMS EMS
wt wt
C
P2
EMS
7
src-1 wt
D
P2
EMS
wt src-1
E
P2
EMS
mes-1 wt
F
P2
EMS
wt mes-1
G wt/wt
EMS
P2
H wt/wt
P2
EMS
I mes-1/wt
EMS
P2
J wt/mes-1
EMS
P2
K
P2
P2
L
EMS
EMS

Figure II-4. Autonomy and Non-autonomy in SRC-1/MES-1 signaling

(A-F) Diagrams representing experiments in which early blastomeres from one or more embryos were first isolated and then placed in contact with each other as shown. The arrows indicate the direction of the EMS cell division and the number in front of the arrow indicates the number of experiments in which that orientation of division was observed. The origins of each blastomere, *wt* (wild-type), *mes-1* or *src-1* are indicated beneath each diagram. The division axis of the P2 cell was not determined.

(G-L) P2/EMS, EMS/EMS or P2/P2 contacts can direct MES-1 protein localization. Immunofuorescence micrographs showing MES-1 protein localization (red), and nuclei stained with DAPI (blue). Embryos were assembled from wild-type or mutant blastomeres as indicated. (G,H) Wild-type chimeras assembled by first removing P2 and then placing it back in contact either in its correct, posterior position, next to EMS (G) or at the anterior, away from EMS (H). (I,J) Similar experiments in which either EMS (I) or P2 (J) was derived from a *mes-1* mutant embryo. (K,L) Chimeras assembled by placing two wild-type P2 cells (K) or two wild-type EMS cells (L) in contact with each other. The arrows in (G, K and L) indicate restored MES-1 staining. A remnant of cortical MES-1 associated with the previous P2/EMS contact is indicated with an arrowhead (G, H and K).

Wnt components and SRC-1 function in parallel in *C. briggase*

C. briggsae is a soil nematode estimated to have diverged from *C. elegans* approximately 50 million years ago. We then identified homologs of SRC-1, MOM-2 and MOM-5 respectively in C. briggsae and asked if similar pathways is conserved between these two species that are 50 millions year apart through evolution using RNAi. As shown in Table II-3, the C. briggsae EMS cell divides in a-p axis in wild type and this a-p division requires both Wnt components as well as SRC-1. Similarly, endoderm induction also depends on both Wnt signaling as well as signaling mediated by SRC-1.

Table II-3. P2/EMS signaling in *C. briggsae*

RNAs used*	%embryos with L-R EMS daughters(n)	% embryos lacking intestine(n)
none	0 (15)	0(n>500)
src-1	0 (17)	0 (279)
mom-2	0 (14)	22 (192)
mom-2;src-1	92 (12)	95 (261)
mom-5	0 (18)	6 (406)
mom-5;src-1	92 (12)	95 (405)

* RNAs used in the experiments are *C. briggsae* homologs of *C. elegans*

DISCUSSION

Convergence between SRC and Wnt signaling

The present study provides evidence for a genetic *in vivo* link between the Wnt and Src pathways during P2/EMS signaling. We have shown that mutations in six different genes, that are implicated in a canonical Wnt-like signaling pathway, including *mom-1* (Porcupine), *mom-2* (Wnt/Wg), *mom-3*(uncloned), and *mom-5* (Frizzled), as well as two Disheveled homologs *dsh-2* and *mig-5* all exhibit identical synergistic interactions with a *C. elegans* Src family tyrosine kinase, *src-1*. Furthermore, we have shown that SRC-1 functions together with a second, novel protein, MES-1, related to receptor type tyrosine kinases. Together, SRC-1 and MES-1 are required for the enhanced accumulation of phosphotyrosine at the junction between EMS and P2, and function along with Wnt signaling components to specify endoderm and the A/P division orientation of the EMS cell.

Our findings indicate that the activities of the Wnt and Src pathways independently target a common set of downstream factors that control the developmental outcomes of P2/EMS signaling. The alleles analyzed include presumptive null alleles in both Wnt signaling and Src signaling components, and more importantly, synergy occurs only between components of distinct pathways and not between components of the same pathway. For example double mutants between *mom-2*(Wnt) and its presumptive receptor *mom-5*(Frizzled) fail to exhibit synergy and exhibit wild-type P2/EMS signaling in greater than 90% of the embryos analyzed, a frequency similar to that observed in *mom-5* single mutants (Rocheleau et al., 1997). In contrast double mutants between either *mom-2* or *mom-5* and *src-1* exhibit striking synergy and lack endoderm in nearly 100% of the embryos analyzed. The lack of synergy between mutants that affect different steps within the same pathway suggests that the alleles used strongly inhibit signaling through the

pathway. Thus it is not plausible, for example, that *src-1* mutants enhance P2/EMS signaling defects by reducing signaling through the Wnt pathway. Rather these findings indicate that the Wnt and Src pathways are redundant and converge to regulate a common set of downstream factors that control cell-fate as well as the orientation of cell division (see Model, Figure II-5).

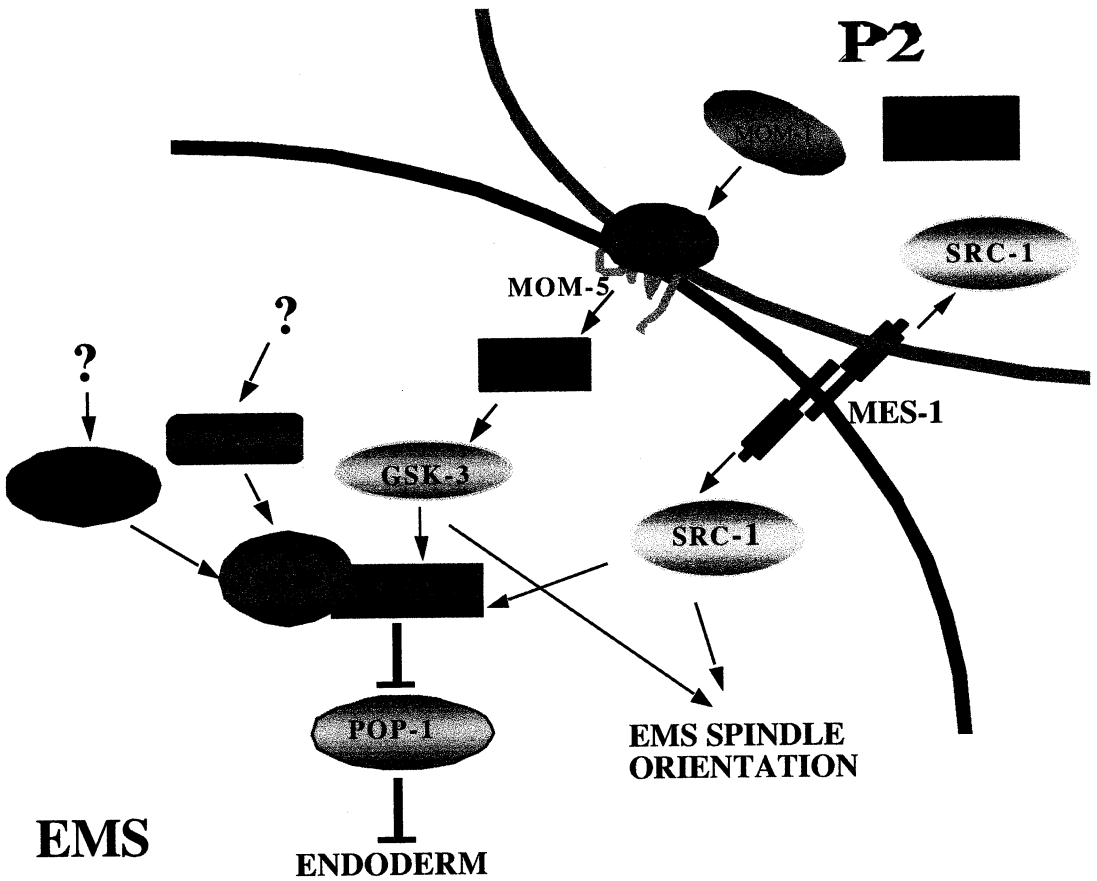

Figure II-5. Model for P2/EMS signaling

MES-1/SRC-1 signaling

Our findings suggest that the P2 and EMS cells signal in both directions via MES-1 and SRC-1. In EMS cell, MES-1/SRC-1signaling functions in parallel with Wnt signaling, as discussed above, while in P2 it functions to control germline asymmetries including the localization of the P-granules. MES-1 is predicted to be a single-pass transmembrane protein with a 471 amino acid extracellular domain and a 500 amino acid cytoplasmic domain with similarity to receptor-type protein tyrosine kinases (Berkowitz and Strome, 2000). MES-1 may therefore function directly as both ligand and receptor in a homotypic interaction between P2 and EMS. Although all early blastomeres contain MES-1, the junctional localization of MES-1 is observed only at the P2/EMS contact site. The localization of MES-1 protein to the cell junction requires MES-1 protein in both EMS and P2, but can also occur when two EMS or two P2 cells are placed in contact. The P2/EMS interaction that leads to MES-1 protein localization does not require SRC-1 or any of the Wnt signaling factors examined in this study. Thus MES-1 protein and other as yet unidentified factors unique to P2 and EMS are required for MES-1 to become localized to the cell junction.

Interestingly, despite the fact that both EMS and P2 can direct MES-1 protein localization to the cortex, cortical remnants containing MES-1 protein are often found on P2 but rarely on EMS. Thus MES-1 is either more firmly anchored at the cortex of P2, or P2 differs from EMS in having a slower recycling process for removing cortical MES-1. These findings suggest a possible explanation for previous observations that P-lineage cells, including P2, can retain their polarity when isolated (Goldstein, 1995b; Schierenberg, 1988). The retention of cortical MES-1 in P2 may reflect a greater potential for P-lineage cells (in general) to retain localized cortical factors. Retention of localized cortical factors from one cell division to the next could in turn serve to provide an intrinsic source of polarity in P-lineage cells.

Signaling downstream of MES-1 requires SRC-1 activity in the EMS cell. However, it is clear that SRC-1 has additional activities that do not depend on MES-1 activity. SRC-1 is required for phosphotyrosine staining that outlines each cell-cell junction in the early embryo until at least the 44-cell stage, and *src-1* mutants arrest embryogenesis without completing body morphogenesis. Furthermore, SRC-1 directs basal levels of tyrosine phosphorylation at the P2/EMS junction even in the absence of MES-1. Perhaps consistent with this finding, SRC-1 is more critical than MES-1 in controlling EMS division orientation and endoderm induction (see Table II-1). For example, *src-1* mutants exhibited a L/R EMS divisions in 15% of embryos examined, while *mes-1* single mutants were never observed to undergo L/R EMS divisions. Similarly, src-1 mutants but not mes-1 mutants enhanced endoderm defects of *mom-4* and *apr-1*. These findings suggest that SRC-1 signals from the P2/EMS cell junction even when MES-1 is absent. Alternatively it is possible that Wnt signaling or other signaling pathways contribute to SRC-1 activation.

A network of signaling downstream of cell contacts

The Wnt and Src signaling pathways converge to control diverse cellular and genetic targets (Figure II-5). For example both pathways control EMS division orientation and both contribute to asymmetries in the level of the TCF/LEF-related protein POP-1. POP-1 is downregulated in response to phosphorylation by a protein kinase complex that consists of WRM-1/ß-catenin and LIT-1/Nemo/NLK (Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999; Shin et al., 1999). Thus Wnt and Src signaling could downregulate POP-1 by activating WRM-1 or LIT-1. The GSK-3 protein functions positively along with MOM-2 (Wnt/Wg) in P2/EMS signaling, and may negatively regulate POP-1. Although we have indicated potential activation of GSK-3 by both pathways (Figure II-5), the genetics of GSK-3 suggests that it has multiple functions and may function in a constitutive rather

than activated manner (Figure II-5, Schlesinger et al., 1999; and our unpublished observations).

The literature on Src and Wnt signaling suggests numerous potential convergence points for these signaling pathways (dashed lines in Figure II-5). These include potential convergence directly on WRM-1 (ß-catenin) (Hinck et al., 1994; Papkoff, 1997), on GSK-3 (Hughes et al., 1993; Kim et al., 1999a; Kim et al., 1999b; Peifer and Polakis, 2000; Wang et al., 1994), or on G-proteins of the Rho family (Arthur et al., 2000; Billuart et al., 2001; Crespo et al., 1997; Habas et al., 2001; Winter et al., 2001). We have shown that two *C. elegans* Disheveled related genes, *dsh-2* and *mig-5*, contribute to P2/EMS signaling and exhibit a phenotype very similar to that of *mom-5* (Frizzled). In other systems Disheveled is thought to act downstream of Frizzled and behaves like a branch point in signaling via ß-catenin/Armadillo and via Rho and MAP kinase (Boutros et al., 1998; Strutt et al., 1997). In *C. elegans* P2/EMS signaling was previously shown to involve both canonical Wnt-like signaling via WRM-1/ß-catenin and via proteins similar to MAP-kinase/LIT-1 and MAP-kinase kinase kinase/MOM-4 (Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999; Shin et al., 1999). Perhaps P2/EMS signaling via both Wnt and Src pathways activates an as yet unidentified Rho-like G-protein that in turn directs spindle orientation through its effects on the actin cytoskeleton and activates endoderm induction via a MAP kinase-like signaling cascade that targets LIT-1 (Figure II-5).

Recent work on dorsal closure in *Drosophila* has identified a possible convergence between Src and Wnt signaling at the level of regulation of the Jun N-terminal kinase (JNK). Dorsal closure is the process in which epithelial sheets spread over and enclose the dorsal region of the *Drosophila* embryo during morphogenesis. JNK signaling is essential for dorsal closure and mutants lacking JNK exhibit a dorsal-open phenotype and also exhibit loss of expression of a TGF-ß homolog *decapentaplegic (dpp)* in the epithelial cells that lead the closure process (Hou et al., 1997; Riesgo-Escovar and Hafen, 1997). Recent

genetic studies have implicated both Src-like kinases (Tateno et al., 2000) and Wnt signaling components (McEwen et al., 2000) in the dorsal closure process and in regulating the expression of *dpp*. Mutations in Src and Wnt signaling factors produce dorsal closure phenotypes similar to JNK mutants and activation of JNK signaling can partially suppress defects caused by these mutants. These findings suggest that Wnt and Src may converge to regulate JNK activity and dorsal closure in *Drosophila* and thus provide evidence from another system for interactions between these pathways in a developmental process.

Our studies in *C. elegans* indicate that Wnt and SRC signaling function redundantly in a relatively simple developmental interaction between two early embryonic cells. The genetic tools developed here should facilitate the identification of additional components of both pathways. In the future it will be interesting to learn whether convergence between these two important signaling pathways occurs on a single key intermediate, or instead as seems more likely, occurs on multiple levels to coordinately control both cell fate and cell division axis.

MATERIAL AND METHODS

Strains and alleles

The Bristol strain N2 was used as the standard wild-type strain. Marker mutations, deficiencies and balancer chromosomes used are listed by chromosome as follows: *LGI: unc-13(e51), mom-4(ne19), mom-5(zu193), pop-1(zu189), src-1(cj293), szT1(I; X); LGII: unc-4(e120), mom-3(zu21), mnC1; LGIV: him-3(e1147); LGV: dpy-11(e224), mom-2(ne141); X: mes-1(bn74), unc-6(n102), mom-1(or10).* *C. elegans* culture and genetics were as described in Brenner (1974).

Isolation of the *src-1* gene and deletion allele

The *src-1* gene was identified by screening a mixed stage *C. elegans* cDNA library with a *v-src* probe. The *src-1* cDNA sequence differs from the GeneFinder predictions for Y92H12A.1 and is detailed in the GenBank accession listed in this paper. To identify a mutation in *src-1*, approximately 30,000 haploid genomes were screened by PCR according to protocols of (Zwaal et al., 1993).

Microinjection and Molecular Biology

RNAi was performed as described in Fire et al, (1998) and Rocheleau et al, (1997). *src-1* dsRNA was prepared from a full-length *src-1* cDNA. yk365b3, yk46b11 and yk55h11 were used to prepare dsRNA for *mes-1*, *mig-5* and *dsh-2* respectively.

Microscopy

Light and immunofluorescence microscopy and laser microsurgery were described previously (Bowerman et al., 1992; Rocheleau et al., 1997). Tissue differentiation of *src-1* embryos was analyzed using morphological criteria at the light microscope and with tissue-

specific antibodies. Antibodies used include: monoclonal antibodies 9.2.1 and J126 to visualize pharyngeal muscle and intestinal valve cells respectively. The P4 cell fate was determined by isolateing the P4 blastomere using laser-microsurgery to kill other blastomeres. POP-1 staining was done as described (Lin et al., 1995). pY99 and MES-1 staining were done using the POP-1 immunostaining protocol with one modification; after freeze cracking and methanol fixation the slides were incubated for 5 minutes in ice-cold acetone and then air-dried. pY99 antibody was obtained from (Santa Cruz Biotechnology) and used at 1:300 dilution.

Blastomere isolation:

Blastomeres were isolated as described by Edgar and Wood (1993), Goldstein (1995b) with modifications from Caroline Goutte (personal communication). Briefly, after chitinase treatment, embryos were transferred into SGM medium with 35% calf serum. The blastomeres were separated by mouth-pipetting them through pre-drawn (35um in diameter) glass capillary (FHC). For the EMS cleavage axis experiments, P1 was isolated from two-cell stage embryo. EMS and P2 were then separated right after P1 divided. Blastomeres were immediately put in contact with each other and EMS division axis was examined under light microscope. Reconstituted blastomeres were cultured for 5 minutes or more, fixed with 4% paraformalhyde (5 minutes), washed with Tris-Tween (5 minutes) followed by antibody incubation.

Genbank Accession Number

The accession number for *src*-1 sequence reported in this paper is AF475094.

CHAPTER III

GSK-3 MODULATES MULTIPLE SIGNALING PATHWAYS TO REGULATE GERMLINE DEVELOPMENT AND ENDODERM SPECIFICATION IN *C. elegans*

Contributors to the work presented in Chapter III:

Nemapharm Incorporated (Formerly of Cambridge, MA) isolated *gsk-3 (nr2047)* deletion allele.

Kaming Pang took the co-focal images of GSK-3::GFP localization in the embryos.

SUMMARY

GSK-3β has been shown to be involved in several distinct signal transduction pathways and regulate multiple cellular functions. However, genetic analysis on its biological function during animal development has been limited mostly to embryogenesis due to the embryonic lethality caused by gsk-3β mutations. In this paper we show that in *C. elegans*, a probable null allele of *gsk-3* homozygous worms are viable with germline defects and are maternal effect lethal, which allows us to study its role during not only embryogenesis but also germline development. GSK-3 protein is localized to *C. elegans* somatic gonad cells and is required for the proper differentiation and the functions of these cells. In addition, GSK-3 is also localized to the pachytene stage germ cells. It functions redundantly with dual-specificity phosphatase, LIP-1, to down regulate MAPK activity to control G2/M transition during oocyte development. During embryogenesis, GSK-3 is required for proper cell fate specification in both E and C blastomeres. Instead of being a negative regulator of the Wnt/Wg signaling pathway shown in other systems, GSK-3 functions positively and in parallel with Wnt/Wg signal for intestine induction during *C. elegans* embryogenesis. Our data indicate that GSK-3 functions redundantly with other factors or pathways to regulate *C. elegans* development.

INTRODUCTION

Glycogen synthase kinase 3ß (GSK-3β) is a conserved serine-threonine protein kinase in all eukaryotes. It was first identified as an inhibitor of glycogen synthase (Embi et al., 1980; Rylatt et al., 1980) but has since been shown to be a key regulator in a number of signaling pathways that are implicated in multiple biological events, including embryonic development, cell proliferation and cell death (reviewed in Doble and Woodgett, 2003; Frame and Cohen, 2001; Grimes and Jope, 2001). For example, studies in *Drosophila* and vertebrate cells have identified gsk-3β as a key regulator of the highly conserved Wnt/Wg signaling pathway (Siegfried et al., 1992). In the absence of Wnt signaling, GSK-3β phosphorylates β-catenin, the primary downstream effector of the signaling pathway, promoting its ubiquitin-mediated proteolysis. The Wnt signal functions to inactivate GSK-3β, permitting stabilized β-catenin to enter the nucleus, and activate target gene expression (reviewed in Jones and Bejsovec, 2003; Peifer and Polakis, 2000a).

Disruption of gsk-3β gene function causes embryonic lethality in mice and *Drosophila* (Bourouis et al., 1989; Hoeflich et al., 2000; Shannon et al., 1972), which masks its role for later development. Mosaic studies in *Drosophila* have shown that GSK-3ß (also known as Shaggy, *sgg*, and Zest White 3, *zw3*) is not only required for embryogenesis but also for several developmental processes in the larvae and adult (Siegfried et al., 1992; Siegfried et al., 1990; Simpson and Carteret, 1989). There are lines of evidence suggesting that GSK-3β also plays a role in animal reproduction. In *Drosophila*, females with germlines that are homozygous mutant for *zeste-white 3 (zw3)* probably have defects in oogenesis (Siegfried et al., 1992). In *Xenopus*, in addition to controlling specification of the dorsoventral axis during embryogenesis, GSK-3β also mediates cell cycle arrest in oocytes (Dominguez et al., 1995; Fisher et al., 1999; He et al.,

1995; Sarkissian et al., 2004) In rodents, GSK-3β is expressed in cells entering meiosis in testis sections, suggesting a role of GSK-3β in mammalian meiosis and spermatogenesis (Guo et al., 2003).

Communication between the somatic gonad and germ cells is required to produce a functional reproductive system in animals. Soma-germ cell interactions influence germ cell migration, proliferation and differentiation (reviewed in Saffman and Lasko, 1999). *C. elegans* provides an excellent model system to study germ line development and the role of soma-germcell interactions. In *C. elegans*, a specialized somatic gonadal cell, the distal tip cell (DTC) utilizes a Notch-related signaling pathway to maintain a mitotic population of germ cells in the distal-most region of the gonad (Austin and Kimble, 1987; Henderson et al., 1994; Kimble and White, 1981; Tax et al., 1994). A POU-domain homeobox gene, *ceh-18*, is required for the proper differentiation specialized myo-epithelial cells, gonadal sheath cells, appears to affect cell cycle control during oogenesis (Greenstein et al., 1994; Rose et al., 1997). Ablation of the sheath and spermatheca precursor (SS) cells reduces germline proliferation, meiotic progression and gametogenesis (McCarter et al., 1997).

Here we describe a deletion and probable null allele of *gsk-3* in *C. elegans*. Homozygous mutants are viable but have defects in the germ line and produce exclusively inviable embryos with extensive defects in cell-fate specification. We show that GSK-3 protein is localized to the somatic gonadal cells including DTC, sheath cells as well as spermatheca. GSK-3 protein is also localized to the germ cells in the pachytene zone. In embryos, GSK-3 is localized in all cells where it is present in the cytoplasm and exhibits cell-cycle dependent localization to the mitotic spindle, centrosome and nuclei. During germ line development, GSK-3 activity is required both in the somatic gonad and in the germ cells. It is required for the differentiation of somatic gonad cells. In germ cells it functions in parallel with the dual specificity phosphatase LIP-1 to inhibit MAP-kinase activation and the meiotic G2 to M transition. During embryogenesis, GSK-3 functions in parallel with

Wnt signaling to promote endoderm specification. Finally, we show that *gsk-3* mutant embryos have delayed degradation of the maternal transcription factor SKN-1, causing the C blastomere to adopt a fate similar to the endoderm precursor EMS. These findings indicate that GSK-3 has important roles in modulating signaling in multiple pathways during germline development and embryogenesis.

RESULTS

GSK-3 is required for germline development and embryogenesis

The *gsk-3 (nr2047)* allele was isolated by Nemapharm incorporated (Formerly of Cambridge, MA) using a PCR-directed screening method (Liu et al., 1999). This deletion removes about 1.5 kilobases (kb) of *gsk-3* genomic sequence including sequence encoding the majority of the kinase domain, resulting in a frameshifted and truncated open reading frame endcoding at most 127 amino acids (Figure III-1). Animals homozygous for this probable null allele of *gsk-3* are viable but are either completely sterile (40%, n=47) or have a brood size of only 14±8, (n=17), dramatically reduced compared to the wild-type brood size of 284±22, (n=16). All of the embryos produced by homozygous mothers (n>1000) fail to hatch and arrest development with no body morphogenesis and with abnormal patterns of cell-fate specification. Homozygous adults also display other defects, including: 1) a slightly protruding vulva with mild egg laying defects; and 2) uncoordinated body movement (*unc*) with backward-movement difficulty. Very similar sets of phenotypes were observed after RNAi targeting *gsk-3,* suggesting that all of these phenotypes stem from a loss of *gsk-3* function. Furthermore, all of these phenotypes are rescued by a GFP::GSK-3 transgene constructed in the yeast artificial chromosome (YAC) Y18D10A (See Experimental Procedures), and the pattern of GFP expression in this transgenic strain is consistent with the spectrum of phenotypes observed (see below).

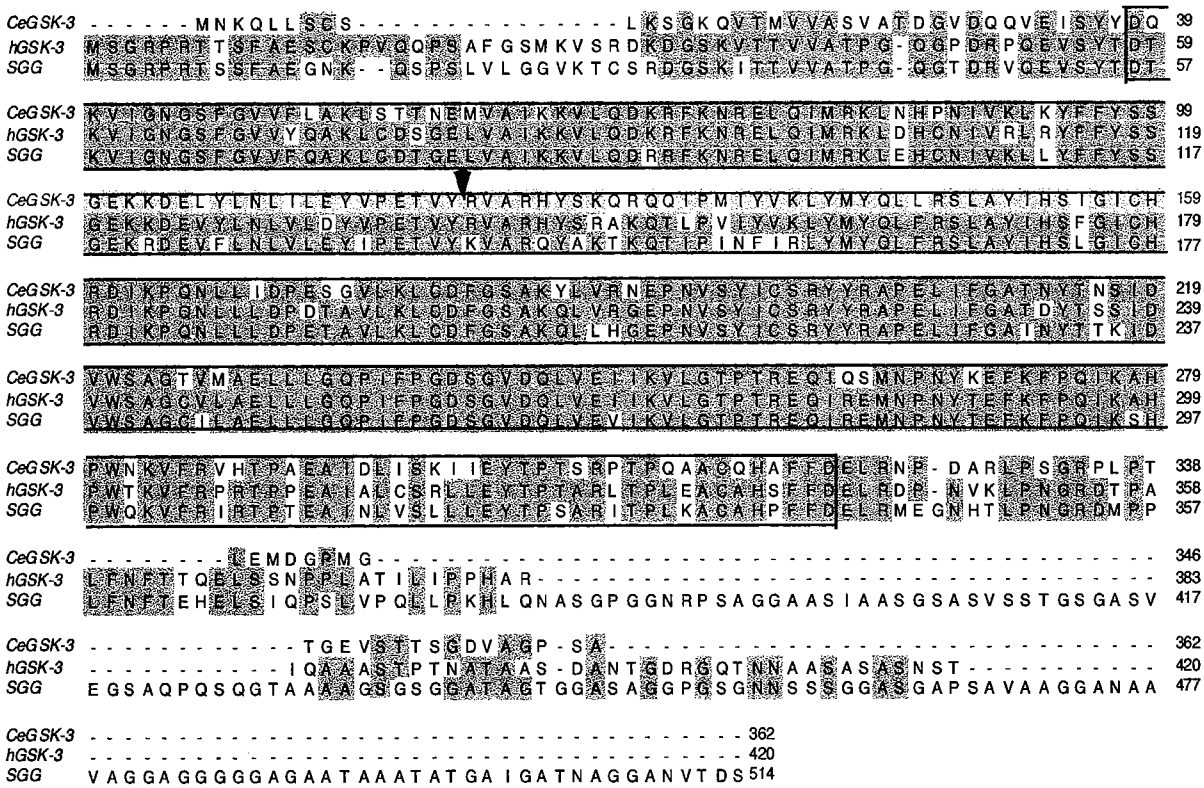

Figure III-1. Alignment of *C. elegans* GSK-3 (CeGSK-3) with human GSK-3β (hGSK–3) and *Drosophila* GSK-3 homolog, SGG (*Shaggy*). Identical residues are highlighted in gray backgrounds. The kinase domain is indicated with red lines. The deletion *nr2047* allele results in a framedshifted and truncated protein. The premature stop codon is located 5 amino acids (aa) downstream of the tyrosine (Y) residue marked by the black arrowhead.

gsk-3 (nr2047) worms exhibit multiple incompletely penetrant germline defects (Table III-1). *gsk-3 (nr2047)* gonad arms are shorter, narrower and have only approximately 1/4th the number of germ cells present in similarly staged wild-type gonads (Figure III-2A, B). Staining with the DNA dye, DAPI, revealed a distribution of germ-cell populations similar to those observed in wild-type, including a distal mitotic zone of proliferating germ-cells, followed by a transition to meiotic-pachytene morphologies (Figure III-2A, B). However, the pachytene germ cells sometimes extends well into the proximal arm and the most-proximal region was swollen with multiple rows of oocytes compared to a single row of oocytes in the whole proximal arm in wild type worms (Table III-1, Figure III-2D, E). Furthermore, 11% of *gsk-3 (nr2047)* gonad arms contained polyploidy (endomitotic) oocytes resulting from multiple rounds of DNA replication without cell division (Table III-1, Figure III-2B.). This endomitotic (Emo) phenotype was not suppressed by *fem-1 (hc17),* a mutation that prevents sperm development (9%, n=46), suggesting that these Emo oocytes do not result from excess sperm to oocyte signaling (McCarter et al., 1999; Miller et al., 2001).

Finally, sperms were often reduced in number or absent in *gsk-3 (nr2047)* hermaphrodites (Table III-1, Figure III-2C). However, the sterility (40%, n=47) or the reduced brood size of *gsk-3 (nr2047)* was not rescued by mating with wild type males (16±12, n=24), indicating that the sterility and small brood size phenotypes of *gsk-3 (nr2047)* hermaphrodites are not caused solely by a lack of functional sperms. *gsk-3 (nr2047)* males have much fewer sperms than wild-type males, and although they attempt to mate, they fail to produce cross progeny when crossed with unc-13 (n=14) hermaphrodites. These findings suggest that *gsk-3 (nr2047)* males may have sperm defects, however, we could not rule out the possibility that *gsk-3 (nr2047)* males fail to mate was also due to mobility defects (unc) or to a defective mating apparatus (data not shown).

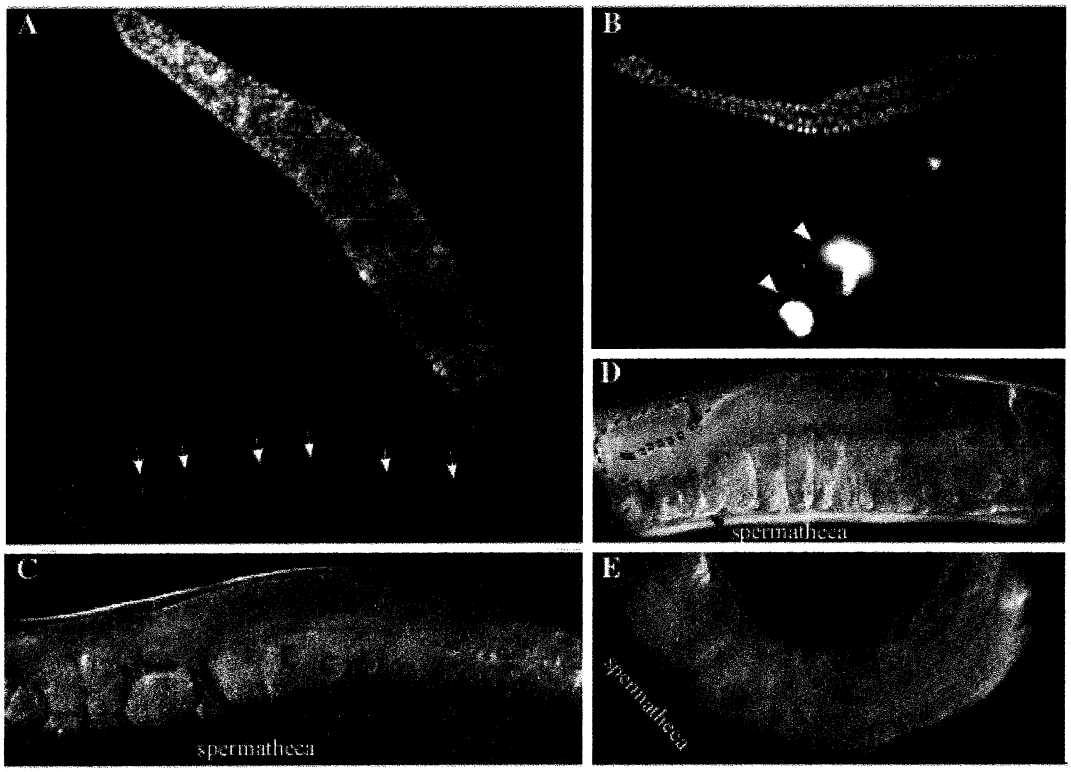

Figure III-2. *gsk-3 (nr2047)* worms have multiple germline defects. Dissected wild type and *gsk-3 (nr2047)* gonads stained with DAPI (A and B). (A) Wild type hermaphrodite gonad arm. White arrows indicate condensed bivalent chromosomes in diakinesis stage oocytes of the proximal arm. (B) *gsk-3 (nr2047)* adult gonad. White arrowheads indicate polyploidy oocyte nuclei with many condensed chromosomes (emo). Note that *gsk-3 (nr2047)* gonad has far fewer germ cells than the wild type gonad. Nomarski images of wild type (C) and *gsk-3 (nr2047)* (D&E) gonads. (D) shows path-finding defects outlined in red in *gsk-3 (nr2047)*. Also note that there is no sperm stored in the spermatheca in (D), compare to spermatheca in wild type worm shown in (C). (E) shows the pachytene exit defects in *gsk-3 (nr2047)*. Black arrowheads indicate the pachytene stage germ cells in the proximal arm, which is normally filled with cellularized oocytes arrested at diakinesis stage of meiosis prophase I (C).

Table III-1. Germline defects of *gsk-3 (nr2047)* worms

Phenotype	Penetrance (%)	Number of gonad scored
Germline hypoproliferation	77	43
Defective pachytene exit	53	43
Feminization of germline	23	53
Abnormal gonad migration	21	43
Endomitotic oocytes	11	53

GSK-3 is required for proper morphogenesis of the somatic gonad

The spectrum of germ line defects described above are very similar to those observed after ablation of gonadal and spermathecal precursor (SS) cells (McCarter et al., 1997). We therefore examined somatic gonad differentiation in *gsk-3 (nr2047)* worms. Staining with an anti-CEH-18 antibody (Greenstein et al., 1994), revealed that each *gsk-3 (nr2047)* gonad arm contained a single DTC and 5 pairs of sheath cells expressing CEH-18, as do wild type gonad arms. However, 24% (n=42) of the *gsk-3 (nr2047)* gonad arms had mispositioned DTCs, localized at the periphery of the arm about 5-8 cell diameters away from the tip (Figure III-3 B, C). These mispositioned DTC are apparently still able to signal germ cells to proliferate as revealed by the DAPI staining. However, minor gonad migration defects observed in 21% (n=43) of the worms (Table III-1, Figure III-2C) might result from improper pathfinding due to the mispositioned DTC (Kimble and White, 1981). We examined the differentiation of the sheath cells by staining the dissected gonad with FITC-phalliodin to label the actin filaments (Figure III-3D, E). We found that the proximal sheath cells pairs 3-5 in *gsk-3 (nr2047)* mutants are disorganized. The gaps between the

phalliodin staining of adjacent sheath cells are larger than normal and the sheath cells are often irregular in shape and size. Thus GSK-3 appears to be required for the proper differentiation of gonadal sheath cells. Similarly, although *gsk-3* mutant worms have the same number of spermathecal cells as wild type worms do, the organization of these cells as visualized by junctional staining with the ajm-1::GFP was less organized in *gsk-3(nr2047)* worms. Furthermore, the mutant spermatheca often fails to contain sperms, which instead accumulate outside on both sides of spermatheca (data not shown).

Perhaps consistent with the morphological defects described above, the gonadal sheath cells exhibit slower and less extensive contractions than observed in wild type. They contract at a peak rate of 7.9 (n=12) times per minute compared to 15.9 (n=10) times per minute in wild type. The oocyte maturation rate is also dramatically reduced (1 in 149± 23 minutes, n=11) compared to wild type (1 in 49±10 minutes n=9). Ovulation was only observed in one-day-old adults, in older worms, the oocytes fail to ovulate and tend to become endomitotic. And during ovulation, oocytes often failed to fully enter or exit the spermatheca and were pinched off resulting in the accumulation of cellular debris within the spermatheca (Figure III-3 F, G).

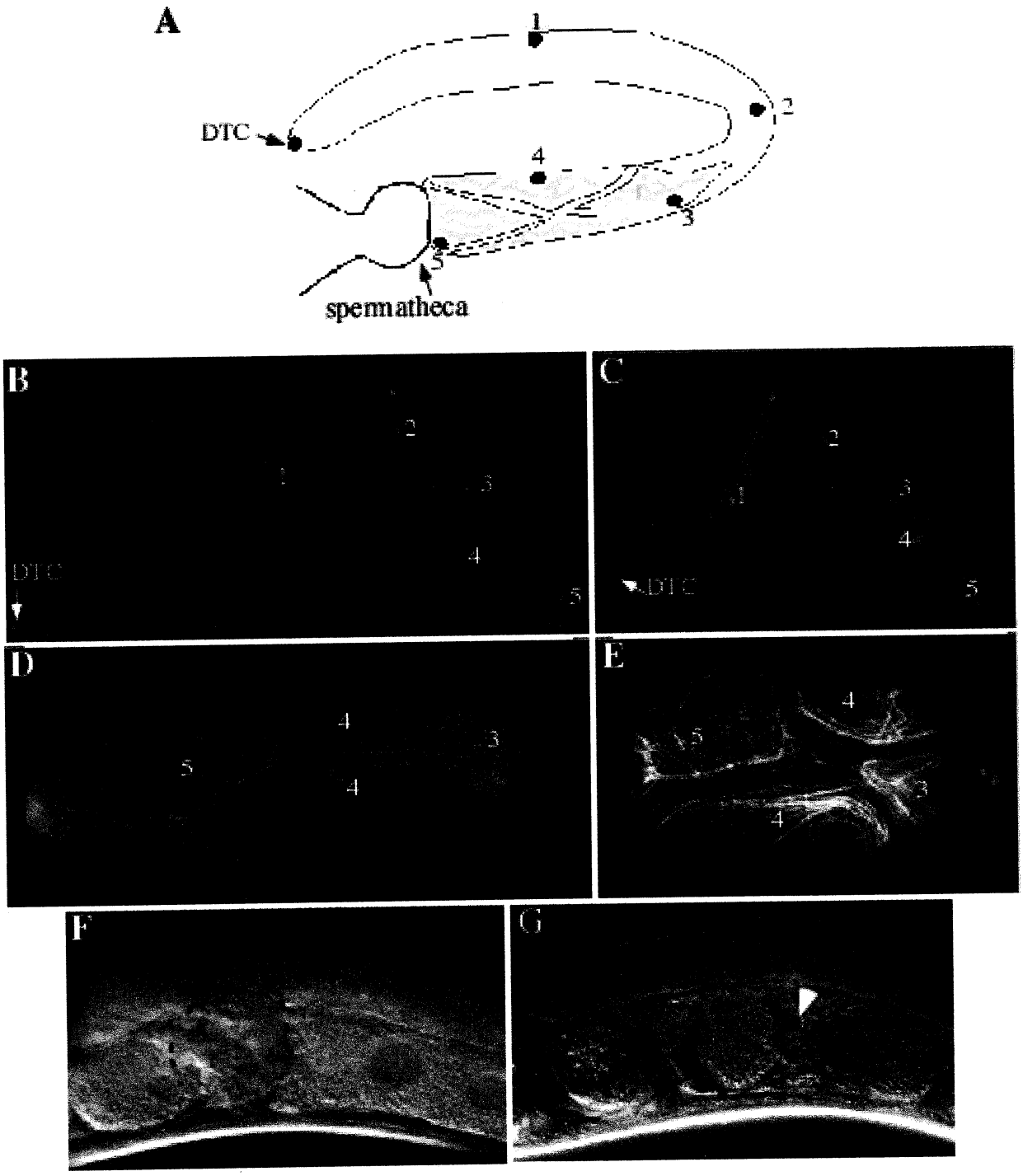
A
1
2
DTC
4
3
5
spermatheca
B
2
1
3
4
DTC
5
C
2
1
3
4
DTC
5
D
4
3
5
4
E
4
5
3
4
F
G

Figure III-3. Somatic gonadal cell disorganization and dysfunction in *gsk-3 (nr2047)* (A) Schematic diagram of the organization of DTC (distal tip cell) and sheath cells. One nucleus of each of the 5 pairs of sheath cells is indicated as 1-5. Sheath cells pairs 3-5 in the proximal gonad are contractile. Immunofluorescence staining of dissected gonads with anti-CEH-18 antibodies (B), wild type and (C), *gsk-3* (nr2047). *gsk-3 (nr2047)* mutants have mispositioned DTC. FITC-labeled phalloidin staining of dissected gonads from wild type (D) and *gsk-3 (nr2047)* (E). Note that the gaps between the sheath cells in *gsk-3 (nr2047)* are much bigger than those of the wild type. Normarski images of wild type (F) and *gsk-3 (nr2047)* (G) adult hermaphrodites showing the spermathecal outlined with dotted lines. Wild type spermatheca is filled with sperms and the *gsk-3 (nr2047)* spermatheca is filled with cytoplasmic debris (indicated by the black arrow), which is a result of "pinch off" during ovulation (indicated by the white arrowhead).

GSK-3 localizes to nuclei and microtubules in a cell-cycle dependent manner

To study the *in vivo* localization pattern of GSK-3, we used the GSK-3::GFP rescuing transgene described above (see Material and Methods). GSK-3::GFP is widely expressed throughout *C. elegans* development, notably in the embryos throughout embryogenesis, in the pharynx, the neurons, the vulva, the adult germ line and the male tale (Figure III-4, 5 and data not shown).

Consistent with the idea that GSK-3 functions in the somatic gonad, GSK-3 is expressed in the nuclei of DTC, all 5 pairs of sheath cells and the cytoplasm of the spermathecal cells (Figure III-4 A-D). GSK-3 also shows a perinuclear pattern in germ cells in the pachytene zone in the distal arm and in some germ cells in the loop region. GSK-3 is consistently expressed in only the distal portion of the pachytene zone, which account for 1/3 to 1/2 of the total population of the pachytene germ cells (Figure III-4A, B). GSK-3 is also expressed in the sperms in both hermaphrodites and males (Figure III-4C, D and data not shown). The punctate perinuclear localization pattern of GSK-3 resembles that of P-granules in the germ cells. P-granules, also known as germ granules in other organisms, are large cytoplasmic particles composed of proteins and RNAs. They are segregated to the germline precursors during embryogenesis and remain in germ cells at all stages of development. We stained the dissected gonad from GSK-3::GFP transgenic worms with antibody K-76 that recognizes P-granules (Strome and Wood, 1982a). Figures III-4 E-G show that in the germ cells where GSK-3 is expressed, the GSK-3 punctates and the P-granules punctates are usually adjacent to each other with some overlapping, but they do not exactly co-localize although their overall localization patterns are very similar.

In the embryos, GSK-3 is localized to the nuclei in a cell cycle dependent manner (Figure III-5). GSK-3::GFP is not detectable in the nuclei during interphase of the cell cycle. It starts to accumulate in the nuclei during prophase and is most abundant during metaphase, when it is localized to both the mitotic spindle and the centrosomes. The

centrosome localization persists till after telophase while the mitotic spindle localization decreases during anaphase and is undetectable in telophase.

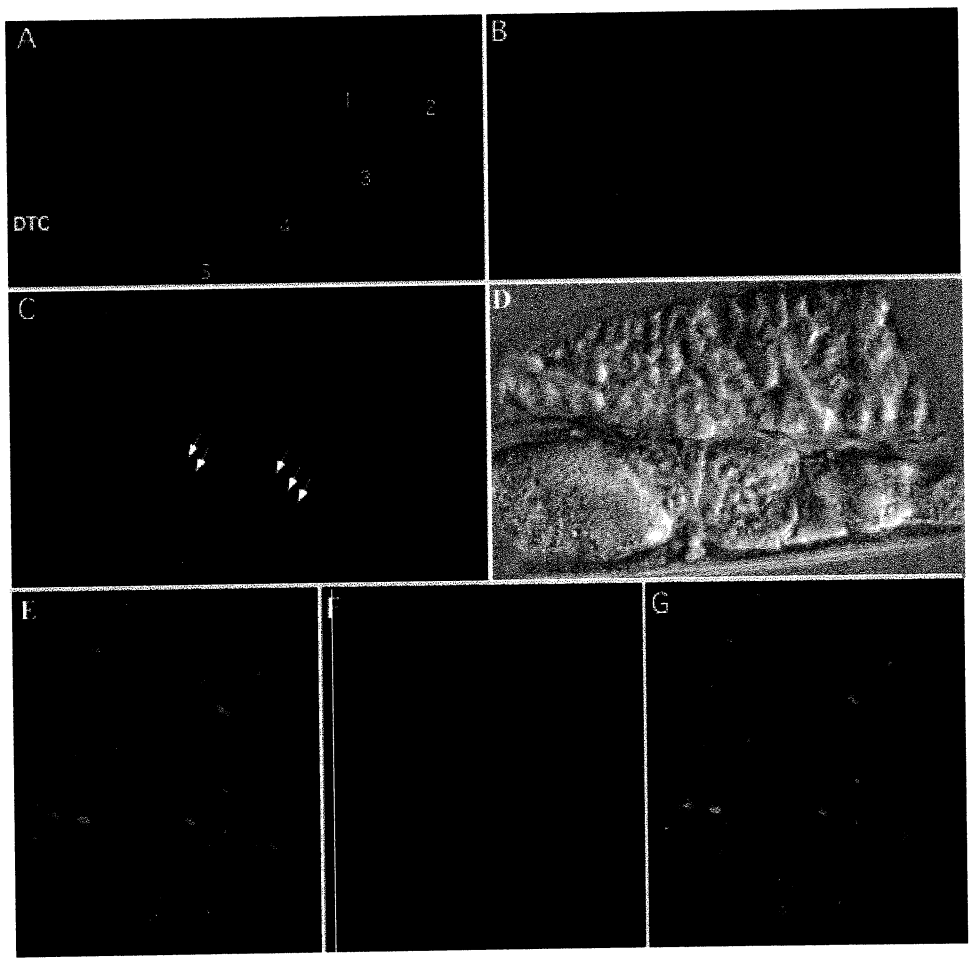

Figure III-4. *GSK-3*::GFP localization in the gonad. Dissected gonad from GSK-3::GFP transgene rescued *gsk-3 (nr2047)*. (A) GSK-3::GFP is localized to the nuclei of DTC (distal tip cell) and the sheath cells (indicated with number 1-5). It is also localized to the germ cells in the posterior region of the pachytene zone in a perinuclear pattern. The same gonad stained with DAPI. GSK-3::GFP is localized to the spermatheca and the sperms

(indicated by the white arrow) in (C). (D), Normarski image of the same worm in (C). (E-G) Higher magnification view of pachytene germ cells. (E) *GSK-3*::GFP signal. (F) Same cells stained with k76 antibody to label P-granules. (G) Merged image of (F) and (G), showing that GSK-3 does not exactly co-localize with P-granules.

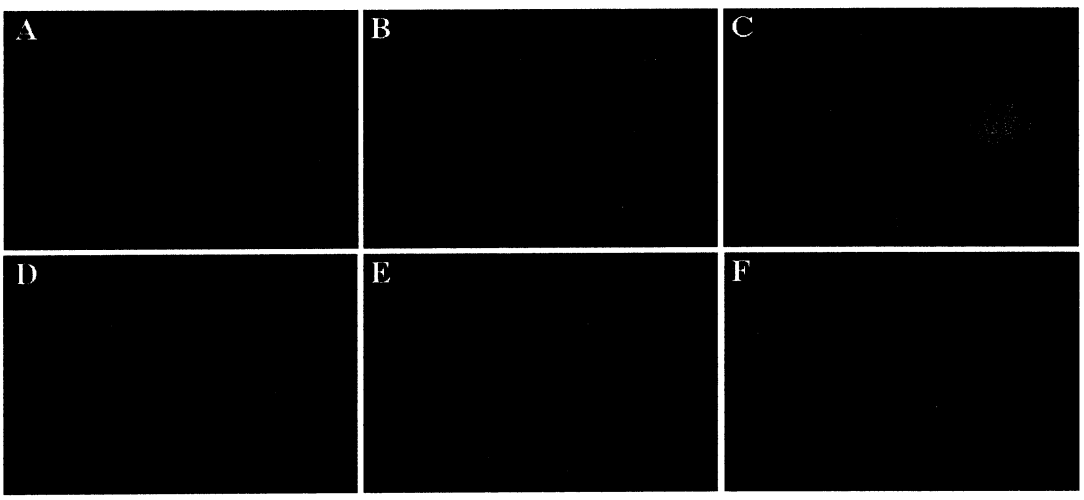

Figure III-5. GSK-3 is localized to the nuclei of each blastomere cells during early embryogenesis in a cell cycle dependent manner. (A) 1-cell embryo in anaphase. (B) 1-cell embryo in telophase. (C) 2-cell stage embryo, AB is in later anaphase and P1 is in metaphase. (D) 2-cell stage embryo, AB is in telophase and P1 is in early anaphase. (E) 4-cell stage embryo, ABa and ABp are in prophase and EMS and P2 are in interphase. (F) 6-cell stage embryo, ABa and ABp daughter are in interphase and EMS is in metaphpase and P1 is in late prophase.

GSK-3 function is required in both the germ cells and somatic gonadal cells

In order to distinguish the functions of GSK-3 in the soma and the germline we utilized *rrf-1 (pk1417)* mutant worms, which are resistant to RNAi in the soma but sensitive to RNAi in the germ line (Sijen et al., 2001). When gravid wild type or *rrf-1* (pk1417) mutant worms are exposed to RNAi targeting *gsk-3*, they produce live progeny first before laying dead embryos. Some of these live progeny are carriers of RNAi that inherited sufficient GSK-3 protein for embryogenesis but lack further expression of GSK-3 (revealed by GSK-3::GFP signal) during post-embryonic development. We refer these worms as *gsk-3 (RNAi)* carriers. In wild-type animals with a functional somatic RNAi pathway, *gsk-3 (RNAi)* carriers mature to become either sterile adults (11%, n=28) or produce dramatically reduced brood sizes (17±5, n=25), phenotypes very similar to those of *gsk-3 (nr2047)* mutants (see above). *gsk-3 (RNAi)* carriers lacking somatic RNAi in *rrf-1 (pk1417),* as evidenced by GSK-3::GFP signal persisting in the somatic gonadal cells and disappearing in the germ cells (data not shown), produced dead embryos with phenotypes identical to those of *gsk-3(nr2047)* homozygous hermaphrodites (See Below). However, unlike gsk-3 (RNAi) carriers in wild type, these animals exhibit rather normal looking germ line (Figure III-6 C, D), have normal rates of ovulation and produce nearly normal numbers of embryos (Table III-2). These findings suggest that GSK-3 is required in the somatic gonad for proper gonad function and fertility.

Although the brood size was restored to near normal levels, the *rrf-1 (pk1417); gsk-3(RNAi)* carriers still exhibited abnormalities in the proximal arm of the gonad, where there were increased numbers of smaller than normal oocytes (Figure III-6 C, D and Table III-2). These oocytes show some signs of accelerated development, including premature disappearance of the nucleolus (McCarter et al., 1999; Page et al., 2001) (Table III-2). Taken together, these results suggest that GSK-3 has functions in both the soma and the

germ cells. In the somatic gonadal cells it is required for DTC, sheath and spermathecal cell positioning and differentiation, and the lack of GSK-3 activity in the somatic gonadal cells is likely responsible for the most dramatic germline abnormalities listed in Table III-1. In the germ cells GSK-3 is required for proper oocyte maturation and for the subsequent embryogenesis.

Table III-2. GSK function in the germ cells

Genotype	Avg. # of oocytes	Position of nucleolar breakdown (%)		Ovulation rate Min/ovulation	Brood size
rrf-1	9.0±0.9	-2 (48) -3 (44) -4 (7)	n=27	44±13 (n=11)	282±22 (n=19)
rrf-1; gsk-3 (RNAi) arriers	15.7±2.3	-4 (44) -5 (32) -6 or more (20)	n=25	40±9 (n=13)	300±40 (n=24)
Wild type	7.9±1.1	-2 (30) -3 (70)	n=30	49±10 (n=9)	284±22 (n=16)

GSK-3 negatively regulates MAP kinase activation in the Germ Cells

Previous studies have shown that in wild type hermaphrodites, MAPK is activated in pachytene germ cells, which is required for the pachytene exit, it is also activated in one to three oocytes most proximal to the spermetheca (Church et al., 1995; Miller et al., 2003; Page et al., 2001). We asked if the accelerated oocyte development in rrf-1 (pk1417); gsk-3 (RNAi) carrier resulted from ectopic MAPK activation by staining dissected gonads from the *rrf-1(pk1417); gsk-3 (RNAi)* carrier animals, with anti-phospho-MAPK-specific antibody (Page et al., 2001). Strikingly, we found that both zones of MAPK activation were dramatically expanded, and often merged into a single zone (Figure III-6 K).

In the most proximal oocytes, MAPK activation is mediated by signaling from the sperms (Miller et al., 2003; Page et al., 2001). Consequently, *fem-1 (hc17)* animals, which lack sperm, fail to exhibit phospho-MAPK staining in proximal oocytes. We found that *fem-1 (hc17)* animals lacking germ-line expression of *gsk-3* also failed to exhibit phospho-MAPK staining in proximal oocytes, indicating that sperm-to-oocyte signaling occurs in these mutants (data not shown). Finally, the expanded zone of phospho-MAPK including proximal staining in diakinetic oocytes was still present in the *fem-1 (hc17); rrf-1 (pk1417); gsk-3 (RNAi)* carriers. These results suggest down-regulation of MAPK activation during or after pachytene exit also requires GSK-3.

GSK-3 and G2/M phase arrest in oocytes

It has been previously reported that MAPK phosphatase LIP-1 is localized to the pachytene stage germ cells and is required to down-regulate MAPK activity as germ cells exit pachytene stage. *lip-1* mutants produce increased number of oocytes that show signs of accelerated development in the proximal gonad arm (Hajnal and Berset, 2002). As described above, GSK-3 is also localized to the pachytene germ cells and is required to down-regulate

MAPK. We therefore decided to ask if GSK-3 and LIP-1function in the same or different pathways. Consistent with the latter model, double mutant *gsk-3(nr2047); lip-1(zh15)* animals are completely sterile and exhibit a dramatically enhanced phenotype relative to either single mutant (Table III-3). As shown in Figure III-6 G&H, all the germ cells in the proximal gonad arm become endomitotic (emo) due to a failure to arrest in G2 of the cell cycle. Cellularized germ cells extend well into the distal arm, which is normally filled with syncytial germline nuclei without complete cell membranes.

Since *gsk-3 (nr2047)* and *lip-1 (zh15)* are large deletions and hence presumptive null alleles of the respective genes, we conclude that the synergy observed is likely to reflect a role for GSK-3 and LIP-1 in parallel pathways that function to arrest the oocytes at G2 phase. Previous studies have suggested that failed ovulation resulting from defective sheath and/ or spermathecal functions can lead to a similar endomitotic phenotype (Aono et al., 2004; Bender et al., 2004; Clandinin et al., 1998; Iwasaki et al., 1996; McCarter et al., 1997; Myers et al., 1996; Rose et al., 1997). However, the *gsk-3 (nr2047); lip-1 (zh15)* double-mutant phenotype is unlikely a result from defective ovulation. First of all, in *gsk-3 (nr2047); lip-1 (zh15)* hermaphrodites, the endomitotic oocytes not only fill the proximal gonad arms but also the uterus (Figure III-6 G&H) suggesting that ovulation in these worms does occur although the oocytes are endomitotic before entering the spermatheca. Secondly, previous work has shown that LIP-1 is not required for ovulation but rather functions in germ cells to promote oocyte maturation (Hajnal and Berset, 2002). And thirdly, we observed the same strong synergistic defects in G2 arrest *rrf-1 (pk1417); lip-1 (zh15); gsk-3 (RNAi) carriers* that lack GSK-3 activity in the germ cells and maintain its activity in the somatic cells (Table III-3).

LET-60/RAS appears to act upstream of MAPK to direct its activation. Gain-of-function allele, *let-60 (nr2046),* in some case can stimulate the MAPK pathway in the absence of an upstream signal (Wu and Han, 1994). Although *let-60 (nr2046)* has no

germline phenotype alone, *gsk-3 (nr2047); let-60 (nr2046)* double mutant worms are 100% sterile and exhibit a G2-arrest defect identical to that observed in *gsk-3;lip-1* double mutants (Table III-3). These results suggest that GSK-3 and LIP-1 function in parallel in germ cells to reduce RAS-MAPK activation. Together, the concerted activities of these factors prevent cell cycle progression until after ovulation.

Table III- 3. GSK-3 and LIP-1 function in parallel to control oocyte cell cycle

Genotype	% of Emo	n
Wild type	0	54
gsk-3(nr2047)	11	53
lip-1 (0)	6	35
gsk-3 (nr2047); lip-1(0)	100	78
let-60 (nr1046gf)	0	22
gsk-3 (nr2047); let-60 (nr1046gf)	100	34
rrf-1 (pk1417)	0	64
gsk-3 (RNAi) carries	15	52
rrf-1 (pk1417);gsk-3(RNAi) carriers	4	25
lip-1(0); rrf-1 (pk1417);gsk-3(RNAi) carriers	100	56

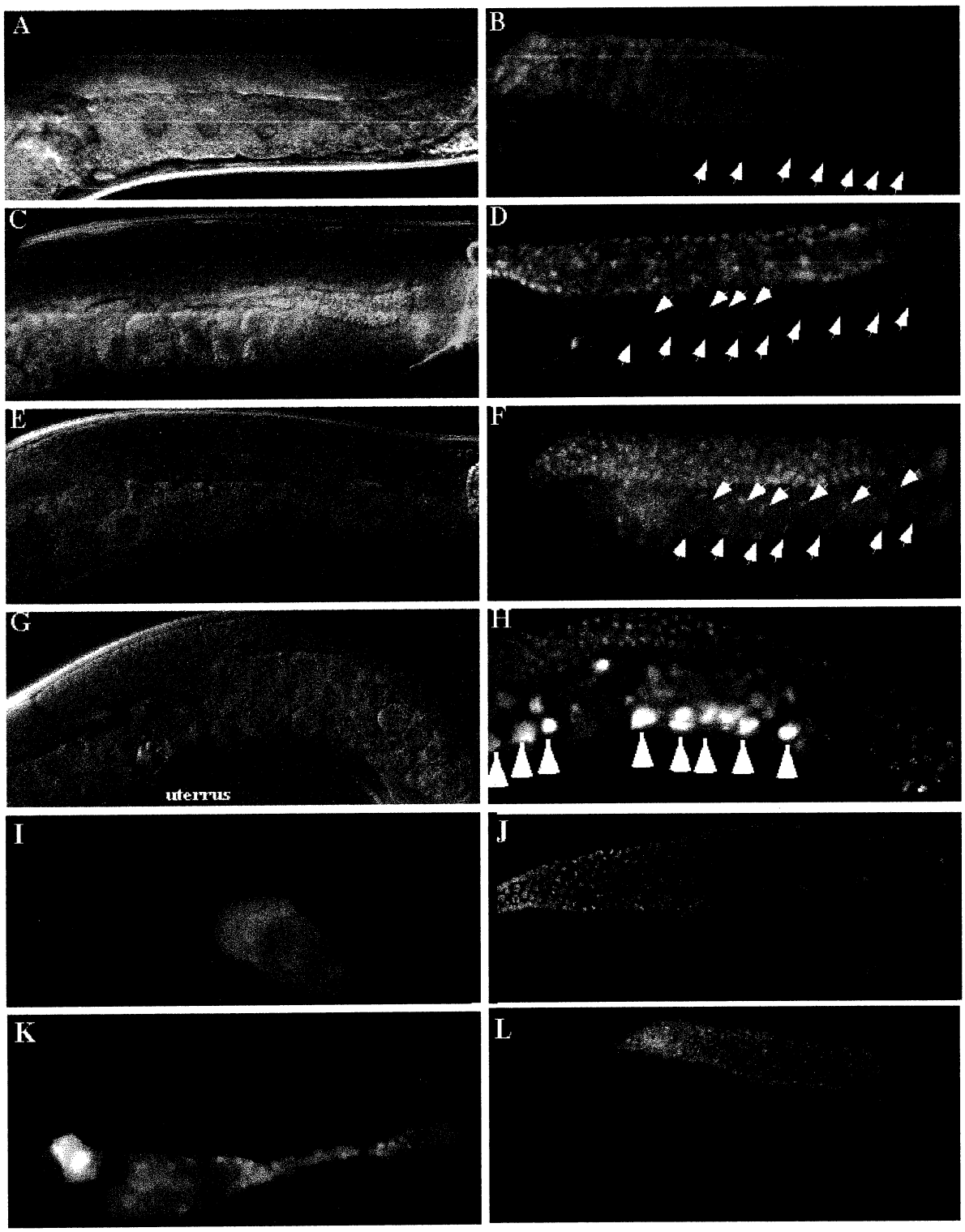
A
B
C
D
E
F
G
uterrus
H
I
J
K
L

Figure III-6. Accelerated oocyte development in worms lacking GSK-3 activity in the germ cells. Nomarski images of (A) *rrf-1 (pk1417)*, (C) *rrf-1(pk1417);gsk-3(RNAi)* carrier, (E) *lip-1 (zh15)*, (G) *rrf-1 (pk1417); lip-1 (zh15); gsk-3 (RNAi)* carrier. DAPI stained whole worm of (B) wild type, (D) *rrf-1(pk1417);gsk-3(RNAi)* carrier, (F) *lip-1 (zh15)*, (H) *rrf-1 (pk1417); lip-1 (zh15); gsk-3 (RNAi)* carrier. The arrows in (B), (D) and (F) point at oocytes in diakinesis in the proximal gonad arm. Arrowheads show the polyploidy nuclei. Note that in (C-F) there are increased numbers of smaller oocytes that are aligned in different focal planes in the proximal arm. (I-L) Dissected adult gonad from *rrf-1 (pk1417)* in (I-J) and *rrf-1 (pk1417); gsk-3 (RNAi)* carrier stained with activated MAPK antibodies in (I&K) and DAPI in (J&L). Note that in *rrf-1 (pk1417); gsk-3* (RNAi) carrier, the activated MAPK persists after pachytene stage and extends into oocytes in the proximal arm.

GSK-3 is required for proper cell-fate specification during embryogenesis

As mentioned above, *gsk-3 (nr2047)* worms are maternal effect lethal. This means that GSK-3 mRNA and protein produced by heterozygous mothers is sufficient to rescue her homozygous progeny which then mature to produce exclusively inviable embryos. These dead embryos contain well-differentiated tissues but fail to undergo body morphogenesis (Figure III-7 A, B). Because of the sterility and small brood size associated with *gsk-3 (nr2047)* mutants, we studied the function of GSK-3 during embryogenesis. mostly by using RNAi, which allowed us to bypass the requirement of GSK-3 function for germline development before reach adulthood (see above). The resulting *gsk-3 (RNAi)* dead embryos showed phenotypes identical to those of *gsk-3 (nr2047)* mothers, with the exception that 2% of the embryos produced by *gsk-3 (nr2047)* worms were undifferentiated and multinucleated apparently due to defective ovulation (see above).

As reported previously (Schlesinger et al., 1999a), *gsk-3* mutant embryos have cell fate changes during early embryogenesis. These include a partial loss of endoderm production by the E cell, (the normal and sole source of endoderm in wild-type embryos), and an ectopic production of endoderm from another early embryonic cell, called C (Schlesinger et al., 1999; Table III-4). We found that among the terminally arrested embryos, ~40% produce extra intestine, while ~10% completely lack intestine. By using laser ablation to prevent the differentiation of specific early blastomeres we found that; 1) more than 80% of partial embryos lacking E still produce intestine (Table III-4), 2) the C cell is the source of this excess endoderm and that more specifically, only the posterior daughter of C, Cp produces endoderm (see Materials and methods). These findings suggest that C adopts an EMS-like fate producing a posterior, E-like daughter. Similar results were also reported by Schlesinger et al. (1999).

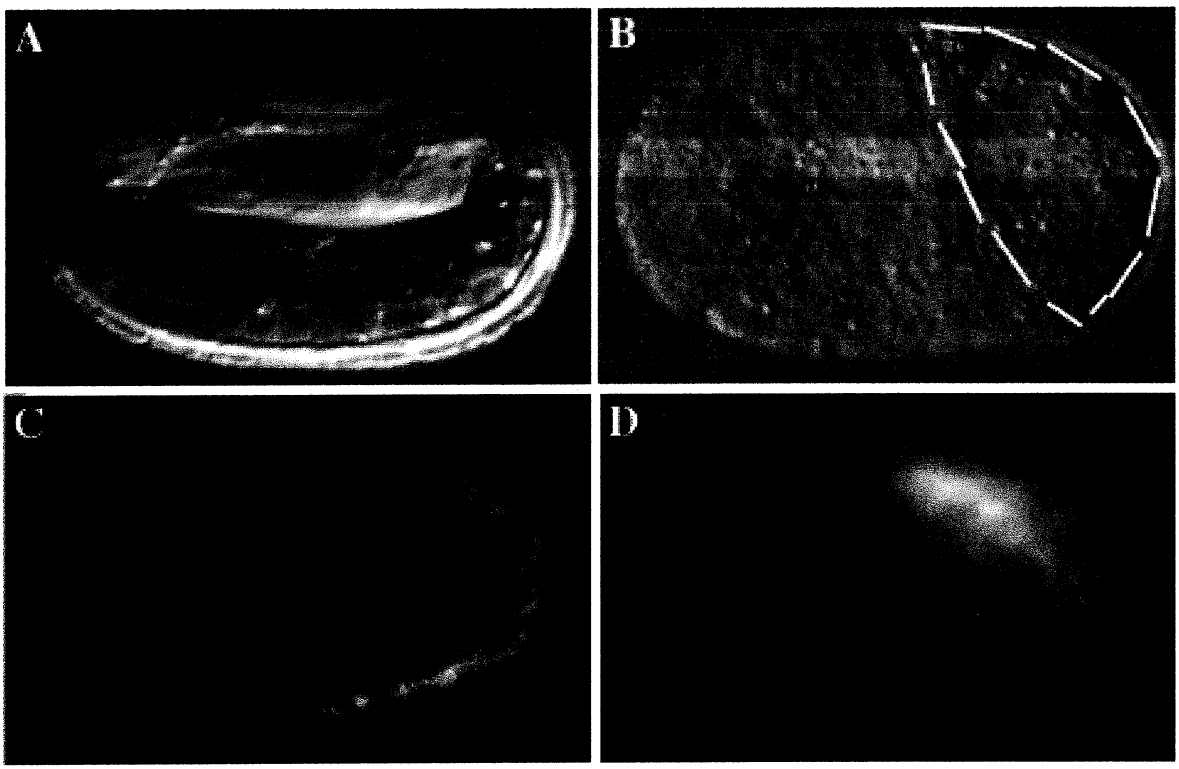

Figure III-7. GSK-3 is required for cell fate specification and morphogenesis in the embryos. (A-B) Nomarski images showing morphogenesis in (A) wild type and (B) *gsk-3* embryos. The wild-type embryo (A) has elongated and is ready to hatch. The gsk-3 (nr2047) embryo (B) contains well-differentiated cells but has no morphogenesis with gut exposed outside in the posterior (outlined by the dotted lines). (C-D) Immunofluorescence micorgraphs showing gut differentiation in wild type (C) and *gsk-3* (D) embryos. *gsk-3* embryo (D) makes extra gut cells that are well differentiated but fail to elongate and lack the basal-apical polarity.

GSK-3 functions in parallel with Wnt-signaling to specify endoderm

We first studied the role of GSK-3 in E cell-fate specification. Previous studies have shown that signaling from the posterior cell, P2, is required for EMS to orient its division axis and to divide asymmetrically producing intestine from its posterior daughter, E. In the absence of signaling, EMS divides Left/Right (L/R) instead of Anterior/Posterior (A/P) and gives rise to two MS-like daughters at the expense of intestine (Goldstein, 1992; Goldstein, 1995b). Genetic studies have identified several genes involved in P2/EMS signaling, including components of the conserved Wnt/Wg signaling pathway. These include *mom-1* (Porcupine), *mom-2* (Wnt/Wg), *mom-5* (Frizzled), *dsh-2* and *mig-5* (disheveled homologs), *wrm-1* (ß-catenin/Armadillo), *apr-1* (adenomatous polyposis coli, APC), and *pop-1* (TCF/LEF) (Bei et al., 2002; Lin et al., 1995; Rocheleau et al., 1997; Thorpe et al., 1997a). GSK-3ß is a conserved component of the Wnt/Wg signaling pathway and its role in the pathway has been well studied in other systems. In the absence of Wnt/Wg signal, GSK-3β phosphorylates β-catenin/Armadillo and promotes it to ubiquitin-mediated protein degradation. Signaling down-regulates GSK-3ß, stabilizing ß-catenin and permitting it to translocate into the nucleus to activate target gene expression (Jones and Bejsovec, 2003; Peifer and Polakis, 2000a).

However, as described above, GSK-3 appears to function positively, along with Wnt-signaling to promote endoderm specification during *C. elegans* embryogenesis, and based on these findings Schlesinger et al. (1999) proposed that GSK-3 functions downstream of *mom-2* (Wnt/Wg) and *mom-5* (Frizzled) as a positive regulator within the pathway.

Genetic studies indicate that there are several signaling inputs that function in parallel to specify endoderm. Consequently, even presumptive null alleles of nearly all genes involved in endoderm induction upstream of WRM-1/LIT-1 (β-catenin and NLK like

MAP kinase respectively) exhibit at most a partial loss-of-endoderm phenotype. The upstream components of the Wnt-signaling pathway including MOM-1, MOM-2, MOM-5 and DSH-2/MIG-5 comprise one of these inputs. Double mutants between any of these Wnt-signaling genes fail to exhibit enhanced endoderm defects, while double mutants between any of these genes and factors required in parallel pathways exhibit a dramatically increased loss of endoderm (Bei et al., 2002; Rocheleau et al., 1997).

To study how GSK-3 functions in P2/EMS signaling, we constructed double mutants between *gsk-3* and other components of the P2/EMS signaling. We found that all the double mutants we tested exhibited synergistic interactions, resulting in a fully penetrant endoderm (E cell fate) to mesoderm (MS cell fate) transformation (Table III- 4). For example, *gsk-3 (RNAi)*; *mom-2 (ne141)* (Wnt/Wg) double mutant embryos completely lack intestine. Similarly, 100% of *gsk-3 (RNAi)*; *mom-5 (RNAi)* double-mutant embryos fail to produce the E-derived endoderm (Table III-4). These synergistic interactions suggest that GSK-3 functions positively and in parallel with Wnt/Wg signaling for E cell fate specification.

GSK-3 down-regulates SKN-1 to prevent inappropriate gut induction

We next examined the genetic requirements for gut induction in the C lineage. Interestingly, we found that gut specification in Cp in *gsk-3* embryos is also dependent on Wnt-signaling. For example, the frequency of Cp-derived gut was dramatically reduced in *gsk-3* mutant embryos lacking *mom-2 (Wnt/Wg)* or *apr-1 (APC)* (Table III-4). Similarly, gsk-3 mutant embryos lacking, *mom-5* (Frizzled) or *mom-4* (TAK1) also exhibit a marked reduction in Cp-derived gut. Finally, as expected, when the primary down-stream effecter of P2/EMS signaling, WRM-1, is removed in gsk-3 embryos, neither E nor C makes gut

(Table III-4). These results suggest that like EMS, the C blastomere in *gsk-3* embryos requires signaling for its posterior daughter to produce intestine.

What renders C blastomere susceptible to P2/EMS like signaling in *gsk-3* embryos? One candidate is SKN-1, a transcription factor that is required for EMS cell-fate specification (Bowerman et al., 1993). In wild type embryos, SKN-1 protein is detected in EMS and P2 cells in 4-cell stage embryos and in 8-cell stage embryos, it is expressed in MS, E, P3 and C, daughters of EMS and P2 respectively, but by the 12-cell stage SKN-1 protein is not detectable in any blastomere (Bowerman et al., 1993). In gsk-3 embryos, we consistently detect SKN-1 protein in P3 and C blastomeres in 12 and 14-cell stage embryos (Figure III-7 I-L) suggesting that GSK-3 is required for the timely removal of SKN-1 protein in P3 and C blastomeres in wild type embryos. We find that eliminating the activity of *lin-23*, an F-box/WD-repeat protein orthologous to βTRCP/SLMB in SCF ubiquitin-ligase complex (Kipreos et al., 2000), by RNAi also results in embryos with ectopic gut formation out of C blastomere (Data not shown). Taken together, these results suggest that the delayed SKN-1 protein degradation in *gsk-3* mutants may enable C to produce endoderm in response to EMS-P2-like signaling.

Table 4. GSK-3 functions in parallel with Wnt-signaling to specify endoderm

Embryo Type	% embryos lacking gut (n)	% E to MS fate (n)[a]	% C making gut[b] (n)
gsk-3 (nr2047)	12 (170)[c]	53 (15)	89 (17)
gsk-3(RNAi)	10 (234)	56 (34)	85 (13)
mom-2 (ne141)	39 (263)	n.d.	n.d.
gsk-3(RNAi);mom-2(ne141)	97 (180)	n.d.	n.d.
apr-1(RNAi)	21(142)	n.d.	n.d.
gsk-3(RNAi);apr-1(RNAi)	93(177)	n.d.	n.d.
mom-4(ne82)	12(185)	n.d.	n.d.
gsk-3(RNAi);mom-4(ne82)	45(372)	100(18)	n.d.
mom-5(zu193)	5(197)	n.d.	n.d.
gsk-3(RNAi);mom-5(zu193)	68(123)	100(21)	n.d.
wrm-1(RNAi)	100(389)	n.d.	n.d.
gsk-3(RNAi);wrm-1(RNAi)	99(427)	n.d.	n.d.

n.d.= not determined

[a]E to MS fate transformation was determined by killing every blastomere but E with laser beam. The terminal fate of the partial embryo was then identified as described in the Material and Methods.

[b]C or Cp blastomere was isolated by laser beam.

[c]The multinucleated embryos from abnormal ovulation were excluded.

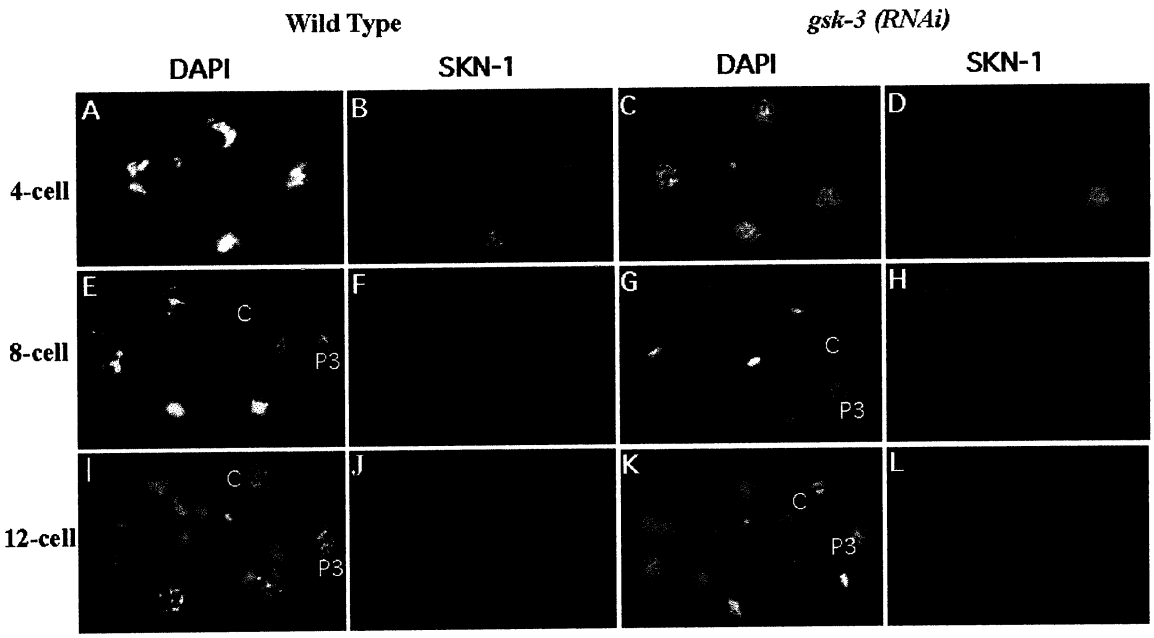

Figure III-8. Delayed SKN-1 protein degradation in *gsk-3* embryos. First two columns from the left show wild type embryos stained with DAPI (A, E and I) and SKN-1 antibody (B, F and J), the two columns on the right show *gsk-3* embryos stained with DAPI (C, G and K) and SKN-1 antibody (D, H and L). Embryonic stages are indicated on the left. Note that in a wild type 12-cell stage embryo SKN-1 protein is not detectable in any of the cells while in *gsk-3* embryos, SKN-1 protein is localized to C and P3 cells.

DISCUSSION

GSK-3 is a multifunctional kinase that is involved in a wide range of cellular functions. Here we describe the phenotypes of a probable null allele of gsk-3 during *C. elegans* development. During germ line development, GSK-3 is required for the differentiation of somatic gonadal cells and it functions to down-regulate MAPK activity during oocyte maturation in the germ cells. During embryogenesis, it is required for intestine cell fate specification. Yet, none of the defects caused by *gsk-3* mutation is fully penetrant. Our results suggest that GSK-3 modulates multiple signaling pathways during *C. elegans* development. We identified that duo-specificity phosphatase LIP-1 as a factor functions redundantly to down-regulate MAPK activity during oogenesis and Wnt signaling as a parallel pathway for intestine specification during embryogenesis.

Somatic function of GSK-3 in the gonad

One of the many functions of GSK-3 is to phosphorylate transcription factors such as c-Jun, NFAT and CREB et al, increasing or decreasing their transcription activity directly or indirectly (Doble and Woodgett, 2003; Frame and Cohen, 2001; Grimes and Jope, 2001). Here we show that GSK-3 is localized in the nuclei of DTC and sheath cells and is required for the differentiation of these cells. It is plausible that GSK-3 affects the differentiation of these cells through regulating the activity of some transcription factor(s). The POU-domain containing homeo box protein CEH-18 has been reported to be localized to the nuclei of DTC and the somatic sheath cells and is required for the differentiation of these cells (Greenstein et al., 1994). CEH-18 therefore makes a potential candidate for GSK-3 regulation. CEH-18 protein localization in gsk-3 (nr2047) mutants remains the same as the wild type worms. Moreover, we found that *gsk-3 (RNAi) carriers of* a

presumptive null allele of *ceh-18 (mg57)* are fully penetrant for the emo phenotype, compared to 27% and 11% for *ceh-18 (mg57)* and *gsk-3 (RNAi)* carriers respectively (Table 3& data not shown). This genetic synergy suggests that *gsk-3* and *ceh-18* function redundantly to control G2/M checkpoint during oogenesis. In addition, *gsk-3 (RNAi)* carriers of *ceh-18 (mg57); rrf-1 (pk1417)* fail to enhance the penetrance of emo phenotype in ceh-18 (mg57) suggesting that GSK-3 and CEH-18 function redundantly in the somatic gonad cells for their differentiation and therefore the cell cycle control of oocytes. The transcription factor (s) that is (are) regulated by GSK-3 and is (are) required redundantly with CEH-18 in the somatic sheath cells is (are) yet to be identified. It is also possible that GSK-3 modulates the activity of a few transcription factors including CEH-18 in the somatic sheath cells. In this case, removing the modulator, GSK-3, or one of the transcription factors, CEH-18, alone exhibits partial penetrant phenotypes, but removing both causes complete penetrant phenotypes due to the additive effects.

The somatic sheath cells in *C. elegans* are smooth muscle like cells (Strome, 1986). In mammalian system GSK-3 has also been reported to be involved in smooth muscle growth (Kuemmerle, 2004) and myogenesis in skeletal muscle (Rochat et al., 2004). In contract to the fact that GSK-3 activity is required for the sheath cell differentiation in *C. elegans*, growth factor stimulation mediated mammalian muscle cell growth or differentiation is achieved by inactivating GSK-3 activity via phosphorylation on the conserved ser-9 residue (Kuemmerle, 2004; Rochat et al., 2004). Interestingly, the conserved ser-9 residue does not exist in *C. elegans* GSK-3 protein (Fig. 1in supplementary material), which is also the case in *Dictyostelium*, in which GSK3 homolog, GSKA, has been shown to be required for cell fate specification and needs to be activated by a novel tyrosine kinase ZAK1 (Harwood et al., 1995; Kim et al., 1999a). It will be interesting to see if GSK-3 in *C. elegans* is regulated in a way similar to its *Dictyostelium*

counterpart instead of being a constitutively active kinase and needs to be down regulated during gonadal sheath cell differentiation.

Germline function of GSK-3 in the gonad

Here we show that GSK-3 functions in parallel with LIP-1in the germ cells to control G2/M transition during oocyte development by down-regulating MAPK activity. LIP-1 is a duo-specificity phosphatase that could inactivate MAPK by dephosphorylating it directly (Berset et al., 2001; Hajnal and Berset, 2002). How GSK-3 functions to down-regulate MAPK is not yet clear. In *Xenopus*, GSK-3 has also been shown to negatively regulate MAPK during oocyte development (Fisher et al., 1999) and does so by inhibiting CPEB mediated mRNA translation of C-mos, which encodes a MAP kinase kinase kinase and initiates MOS/MEK/MAPK activation cascade (Gebauer and Richter, 1996, 1997; Mendez et al., 2000; Sarkissian et al., 2004). It is possible that in *C. elegans*, GSK-3 down-regulates MAPK through a similar translational control mechanism. *C. elegans* CPEB homologs, however, have been shown to have key functions in spermatogenesis and are dispensable for oogenesis. One of them, *cpb-3* mRNA, is enriched in the oocytes although *cpb-3 (RNAi)* has no phenotype (Luitjens et al., 2000). Isolation of *cpb-3* genetic mutations would help address its function for oocyte maturation and a possible link between GSK-3 and CPEB during this process.

The role of MAPK activation during oocyte development has been well studied in *Xenopus* (Karaiskou et al., 2001). In *C. elegans* hermaphrodite germline, MAPK is activated in the pachytene germ cells as well as the maturing oocytes. The former is required for the germ cells to exit pachytene and enter diakinesis and the latter is a sperm dependant event correlated with oocyte maturation and ovulation but its function remains to be determined in *C. elegans* because null mutations in MAP kinase pathway cause germ cells to arrest in the pachytene stage of meiosis (Church et al., 1995; Miller et al., 2003; Page et

al., 2001). Here we show that the ectopic MAPK activity that persists in the diakinesis germ cells leads to accelerated cell cycle progression causing these cells fail to arrest at G2/M checkpoint resulting in endomitotic (emo) oocytes. In *C. elegans*, failed ovulation due to defective sheath and/or spermathecal function also leads to endomitotic oocytes (Aono et al., 2004; Bender et al., 2004; Clandinin et al., 1998; Iwasaki et al., 1996; McCarter et al., 1997; Myers et al., 1996; Rose et al., 1997). High levels of MAPK activation are not sufficient for oocyte ovulation rate (this paper & Hajnal and Berset, 2003). So the emo phenotype of *gsk-3; lip-1* double mutants could also be a combination of accelerated cell cycle progression in *gsk-3; lip-1* double mutants coupled with normal ovulation rate causing more matured oocytes trapped in proximal gonad arms and entering mitotic cell cycle without cytokinesis.

gsk-3 (nr2047) mutants produce less sperms in both hermaphrodites and males. This is more likely caused by disruption of somatic gonad cell differentiation, which affects sperm production (McCarter et al., 1997), instead of a direct effect on the germ cells as evidenced by *gsk-3 (RNAi)* carriers of *rrf-1 (pk1417)*. They still produce the same amount of embryos as *rrf-1 (pk1417)* and wild type worms do, suggesting that GSK-3 is not essential in the germ cells for sperm production.

GSK-3 and Wnt signaling pathway during *C. elegans* embryogenesis

GSK-3 is conventionally a negative regulator in the conserved Wnt/Wg signaling pathway. It forms a complex with scaffolding protein Axin and APC. In the absence of Wnt signaling, this complex facilitates GSK-3 to phosphorylate β-catenin, leading to β-catenin degradation (Jones and Bejsovec, 2003; Peifer and Polakis, 2000a). In *C. elegans*, similar complex consisting PRY-1, an Axin homolog, GSK-3 and APR-1, an APC homolog, regulates BAR-1/β-catenin stability during post-embryonic development (Gleason et al., 2002; Korswagen et al., 2002). During embryogenesis, however, our genetic data have shown that GSK-3 function positively and in parallel with the Wnt/Wg signaling for

intestine induction as APR-1 (APC) does (Rocheleau et al., 1997). Moreover, GSK function in parallel with APR-1 for endoderm specification. Live imaging of WRM-1::GFP signal during early embryogenesis shows that WRM-1(β-catenin) protein is regulated by nuclear accumulation instead of protein stability by the P2/EMS signaling components. Furthermore, GSK-3 appears to promote WRM-1nuclear accumulation (Nakamura et al., in preparation). Based on these observations, it is possible that GSK-3 phosphorylates WRM-1 and this phosphorylation when in combination with other modifications of WRM-1 by APR-1 or in response to MOM-2/Wnt signal promotes a net nuclear accumulation of WRM-1, which together with LIT-1 down-regulates POP-1 activity in the E cell.

GSK-3 and C cell fate specification

In *gsk-3* embryos, C blastomere adopts an EMS cell-like fate with Cp adopting the fate of E, posterior daughter of EMS. We show that GSK-3 is required for the timely removal of SKN-1 protein, a transcription factor required for the specification of EMS cell fate, from C blastomere after 8-cell stage. SKN-1 protein harbors several potential GSK-3 phosphorylation sites and it binds to GSK-3 protein *in vitro* (data not shown). It is possible that GSK-3 phosphorylates SKN-1 protein, targeting it for SCF mediated proteolysis in C blastomere. How *gsk-3* is spatially and temporally regulated in C blastomere for SKN-1 degradation is not clear since GSK-3 protein is uniformly localized to each blastomere during early embryogenesis. Hypomorphic alleles of *cdk-1* and *cks-1* embryos have similar phenotypes to *gsk* embryos with Cp blastomere producing intestine (Soto and Mello, unpublished results; Nakamura and Mello results). GSK-3 has a conserved CDK-1 phosphorylation site, GSK-3 activity therefore may be regulated by CDK-1, which in turn is regulated by different factors within each blastomere to ensure the invariantly ordered cell division of each cells during embryogenesis.

It has been proposed that in *gsk-3* embryos C blastomere makes intestine independent of signaling (Maduro et al., 2001; Schlesinger et al., 1999a). Our genetic data suggest that both C and EMS require the P2/EMS like signaling for intestine induction. Apparently, C and EMS responds to the signaling components a little differently. For example, in the absence of gsk-3, 56% of E fails to make gut while only ~10% of C fails to do so. The different requirement for signaling between C and EMS may be explained by the fact that C is smaller in cell volume than EMS so that it may be more responsive to the several parallel pathways upstream of WRM-1/LIT-1 and it does not require the presence of every single one of the parallel pathways for intestine induction.

MATARIAL AND METHODS

Strains and alleles

The Bristol strain N2 was used as the standard wild-type strain. Marker mutations, deficiencies and balancer chromosomes used are listed by chromosome as follows: *LGI: rrf-1(pk1417), unc-13(e51), mom-4(ne19), mom-5(zu193), unc-101(m1), gsk-3(nr2047), hIn1[unc-54(h1040)], hT2 [qIs48] (I;III); LGIV: lip-1(zh15), fem-1(hc17), let-60(n1046); LGV: neIs1 [Y18D10 (gsk-3::gfp); pRF4 (rol-6)], mom-2(ne141); X: ceh-18(mg57), ajm-1(ok160); jcEx44[pJS191(ajm::gfp); pRF4(rol-6)]. C. elegans* culture and genetics were done as described (Brenner, 1974).

RNAi and Molecular Biology

RNAi injection was performed as described (Fire et al., 1998; Rocheleau et al., 1997). *gsk-3* dsRNA was prepared from a full-length *gsk-3* cDNA (GenBank Accession Number NM_060842). RNAi feeding was done as described in Timmons et al., (2001). *gsk-3* full-length cDNA was cloned into vector L1440 for expression of *gsk-3* dsRNA in *E.*

coli strain HT115 (DE3). The *gsk-3 (nr2047)* deletion was identified by nested PCR. First round primers, forward: 5'-GCCTGCAAATTAAATTCAACGATG-3', reverse: 5'TCTAAGCGGTTTTGTGAG GGGG-3'; second round primers, forward: 5'-CGGCAGATATGGCGATTTTTGTC-3', reverse: 5'-TGGCTGCTTTTTTTTTGAGCGATC-3'. *gsk-3(nr2047)* deletion allele displays a 1.5kb product. The translational fusion *gsk-3::gfp* transgene was constructed in yeast artificial chromosome (YAC) as described in Rocheleau et al. (1999). The *gfp* gene with *sup4°* selection marker embedded within a synthetic *C. elegans* intron was inserted in frame in front of the stop codon of *gsk-3*. The recombinant YAC, Y18D10 (*gsk-3::gfp*) rescued *gsk-3 (nr2047)* fully. The GFP imaging was done from the rescued strain, its full genotype is *gsk-3 (nr2047) I, neIs1 [Y18D10 (gsk-3::gfp); pRF4 (rol-6)] V*.

Microscopy

Light and immunofluorescence microscopy and laser microsurgery were described previously (Rocheleau et al., 1997). DAPI staining of intact animals was done as described in Church et al. (1995). Endomitotic (Emo) phenotype was scored by intense DAPI staining of germ cell nuclei. Nematode gonads were dissected as described (Francis et al., 1995) with slight modifications. Briefly, young adult worms were placed in a drop of PBS containing 0.15mM of levamisole on a glass slide for gonad extrusion. The dissected gonads were then fixed and stained with the following antibodies/dyes as described, DAPI (Church et al., 1995), FITC-phalliodin (Molecular Probes) (McCarter et al., 1997), CEH-18 (Greenstein et al., 1994), K-76 (Strome and Wood, 1982a), diphosphorylated MAPK (Sigma, #M8159) (Page et al., 2001).

Tissue differentiation of *gsk-3* embryos was analyzed using morphological criteria at the light microscope and with tissue-specific antibodies. Antibodies used include: monoclonal antibodies 9.2.1 and J126 to visualize pharyngeal muscle and intestinal valve

cells respectively. The E, C or Cp cell fate was determined by isolating the respective blastomere using laser-microsurgery to kill other blastomeres. POP-1 and SKN-1 staining was done as described (Bowerman et al., 1993; Lin et al., 1995).

CHAPTER IV

GENERAL DISCUSSION

Collaboration between Wnt and Src signaling

The studies presented in this thesis have shown a genetic link between Wnt and Src pathways in a rather simple developmental interaction between two early embryonic cells, P2 and EMS, during early *C. elegans* embryogenesis. Wnt and Src pathways redundantly control both the mitotic spindle orientation of EMS cell as well as cell fate specification of its posterior daughter cell, E. The former event involves in cytoskeletal rearrangement independent of transcription while the latter requires gene transcription. It is more likely that Wnt and Src pathways converge on multiple factors to regulate these two events.

Both Wnt and Src signaling are very conserved and well studied among animal species. However, do they collaborate during other *C. elegans* developmental processes as well as development in other systems? Several recently published reports have shed some light on this question.

Recently members of Ryks (**r**elated to **t**yrosine **k**inases) (Hovens et al., 1992), a subfamily of atypical RTKs (**r**eceptor **t**yrosine **k**inases), have emerged as Wnt receptors or co-receptors (Lu et al., 2004, Inoue et al., 2004; Yoshikawa et al., 2003). The kinase domain of Ryks, however, is atypical because it contains mutations in the evolutionarily conserved tyrosine kinase residues and lacks protein tyrosine kinase activity (Hovens et al., 1992), which suggests that Ryks transduce the signal together with another catalytically active kinase (Yoshikawa et al., 2001). In *C. elegans*, LIN-18, the only member of Ryk, mediates Wnt signaling in parallel to LIN-17/Fz to establish P7p polarity during vulva development. Interestingly, the entire cytoplasmic domain of LIN-18 is completely dispensable for its activity (Inoue et al., 2004). How is the signal mediated within the cell then? It has been shown that ephrin-A5, which is attached to the outer leaflet of the plasma membrane by a

glycosyl-phosphatidylinositol (GTI)-anchor, when stimulated recruits Src family kinase, Fyn protein tyrosine kinase, to mediate cellular response (Davy et al., 1999). It will be interesting to examine if LIN-18, upon Wnt signal binding, activates Src family kinase mediated signaling, which functions in parallel with LIN-17/Fz to regulate P7p polarity.

In *Drosophila*, the sensory organ precursor (SOP) lineage cells undergo asymmetric cell division along the anterior-posterior axis (A/P). The GoLoco domain containing protein, Pins (**p**artner of **ins**cuteable) acts redundantly with Fz to orient the mitotic spindle to control the asymmetric cell divison in SOP cells (Bellaiche et al., 2001). In *C. elegans*, two closely related GoLoco domain containing proteins, GPR-1/GPR-2 (**G**-**p**rotein **r**egulator), are required for proper spindle positioning in the asymmetric cell division of the one-cell stage embryo which has the intrinsic polarity set up by the PAR proteins (Gonczy et al., 2000). In the 4-cell stage embryo, GPR-1 and GPR-2 proteins are asymmetrically localized at the P2/EMS boundary and this asymmetric localization is dependent on MES-1/SRC-1 (Polakis, 2000; Srinivasan et al., 2003; Tsou et al., 2003). So it is possible that in the *Drosophila* SOP cells, an Src like signaling regulates Pins asymmetric localization, which together with Fz establishes the cell polarity along the A/P axis.

In some cancers, both Wnt and Src pathways are deregulated. For example, in colon cancer, Wnt signaling is constitutively active due to inactivating mutations in APC or activation mutations in β-catenin (reviewed in Polakis, 2000). Aberrant activation of c-Src also occurs during colon tumor progression. It is conceivable that there is collaboration between Wnt and Src signaling pathways in these cancer cells. It has been shown that Src can activate β-catenin-TCF mediated transcription (Haraguchi et al., 2004). Wnt and Src pathways may converge on β–catenin directly to upregulate gene transcription in cancer cells (reviewed in Nelson and Nusse, 2004).

Identification of new components in either pathway or factor(s) on which Wnt and SRC pathways converge upon should help better understand the genetic link between these two pathways. Mutations of some of these factors in *C. elegans* might be zygotic lethal or sterile. The availability of RNAi feeding library together with isolation of temperature sensitive (*ts*) mutants would help identify these types of factors. In addition, some of the factors might also be required for the asymmetric cell divisions before the 4-cell stage, such as GRP-1/GRP-2. Isolation and reexamination of *ts* mutants with earlier asymmetry defects at restrictive temperature after the embryos reach the 4-cell stage might help identify some factors used in the EMS cell that are under the control of Wnt or (and) SRC-1 signaling in the 4-cell stage embryo during *C. elegans* embryogenesis. Identification of the common effectors for both Wnt and Src pathway may help develop some drugs against cancers in which both Wnt and Src pathways are deregulated.

Genetic interactions between the known components of Wnt pathway in P2/EMS signaling

Wnt signaling pathway plays an essential role in animal development and disease. It has been the subject of intense investigation due to its ubiquity and importance. A textbook model of linear canonical Wnt signaling has been proposed. In this model, Wnts induce the stabilization of cytosolic β-catenin, which subsequently translocates into the nucleus and associates with TCF/LEF family transcription factors to regulate expression of Wnt-target genes. In the absence of Wnt stimulation, β-catenin is phosphorylated by GSK-3, whose activity is facilitated by Axin and APC. The phosphorylated β-catenin is then targeted by protein degradation.

In *C. elegans*, Wnt signaling controls cell fate decisions, cell migrations and cell polarity. A canonical Wnt signaling pathway appears to regulate several post-embryonic events in *C. elegans* (Reviewed in Herman, 2002; Korswagen, 2002). However, during

P2/EMS signaling, although most of the components of the canonical Wnt signaling pathways have been identified, they exhibit rather different genetic interactions.

First of all, both APR-1 and GSK-3 are positive regulators. In the canonical Wnt signaling, APC/APR-1, GSK-3 together with Axin (PRY-1 in *C. elegans*) negatively regulate β-catenin stability, thus, the Wnt-responsive gene transcription. WRM-1, the β-catenin homolog of β-catenin responsive to P2/EMS polarity signaling has a low homology to β-catenin and lacks the conserved so called "destruction" box on the N-terminus of the protein. Furthermore, LIN-23, the *C. elegans* homolog of β–TrCP/Slimb, does not have an apparent function in WRM-1 function (This study, data not shown and Kipreos et al., 2000). These findings suggest that WRM-1 is unlikely predominantly regulated by protein stability. Indeed, through live imaging of WRM-1::GFP signal during early embryogenesis, Nakamura et al in the lab (in preparation) have found that WRM-1 is mainly regulated by nuclear localization rather than protein stability. P2/EMS signaling promotes a net nuclear accumulation of WRM-1 protein. GSK-3, APR-1 and MOM-2/Wnt pathways all positively promote the nuclear retention of WRM-1 proteins. How these inputs enables WRM-1 stay inside the nucleus is not known. For GSK-3, it is likely through phosphorylation. It has been reported that GSK-3 could regulate protein nuclear localization through phosphorylation (Beals et al., 1997; Diehl et al., 1998). Utilizing cell culture system may help address the molecular mechanism on WRM-1 regulation by different inputs in P2/EMS signaling (Rocheleau et al., 1999).

Another interesting difference is that PRY-1, the Axin homolog in *C. elegans*, is involved in a canonical Wnt signaling event during post-embryonic development but does not have an apparent function in P2/EMS signaling. As genetic data presented in Chapter II, although SRC-1 and MES-1 are required for E cell fate specification, their effects on this event could only be detected for phenotype enhancement in another partially penetrant mutant background while single mutation of *src-1* or *mes-1* does not exhibit any E cell fate

specification defect. It will be interesting to see if Axin has a similar genetic trait in P2/EMS signaling.

Where is LRP/Arrow in *C. elegans*? The role of LRP/Arrow as the co-receptor with Fz at the cell surface has been well established in both vertebrates and *Drosophila* (reviewed in He et al., 2004). In *C. elegans*, three LRP related genes, *lrp-1* (F29D11.1), *lrp-2* (T21E3.3) and *rme-2* (**r**eceptor-**m**ediated **e**ndocytosis) have been identified. But they do not encode the typical LRP5, 6/Arrow like proteins and do not appear to be involved in any Wnt signaling events during *C. elegans* developments (Kamikura and Cooper, 2003; Grant and Hirsh, 1999; Yochem et al., 1999). However, it has been shown that the extracellular domain of LRP/Arrow is not required for its activity. In fact, the membrane-tethered intracellular domain of LRP/Arrow is constitutively active (Mao et al., 2001). Furthermore, a PPPSP motif within its intracellular domain is the minimal module that is necessary and sufficient for LRP signaling function in mammalian cells and *Xenopus* embryos (Tamai et al., 2004). So an LRP/Arrow "like" receptor could exist in *C. elegans*, but it is so diverged from LRP/Arrow that conventional blast search would miss it out. Comprehensive searches through the essentially complete *C. elegans* genome for transmembrane proteins with (a) PPPSP motifs followed by RNAi analysis may help identify the LRP/Arrow "like" molecule in *C. elegans*. The important insight into the function of LRP/Arrow came from the finding that the LRP intracellular domain binds Axin (Mao et al., 2001, Tolwinski et al., 2003). PRY-1, the Axin like protein in *C. elegans*, is only remotely homologous to Axin molecules from other systems. It is possible that in *C. elegans* the LRP/Arrow molecule is not evolutionarily conserved and the worm utilizes a different mechanism from LRP/Arrow-Axin interaction.

Is the regulation of WRM-1/β-catenin by multiple parallel inputs so unique to P2/EMS signaling during *C. elegans* embryogenesis? In fact, certain well-characterized Wnt signaling pathways in both vertebrates and invertebrates show no obvious changes in

the level of β-catenin in response to Wnt ligand, and appear to operate instead by controlling β-catenin nuclear localization (Logan et al., 1999; Novak et al., 1998; Schneider et al., 1996). In addition, some components that govern β-catenin phosphorylation might also affect its subcellular localization. APC and Axin have been proposed to function in the nuclear export of β-catenin (Cong and Varmus, 2004; Henderson, 2000; Rosin-Arbesfeld et al., 2000), and Axin may also play a role as cytoplasmic anchors to retain β-catenin in the cytoplasm (Tolwinski and Wieschaus, 2001). So in systems other than *C. elegans*, β-catenin is also likely to be regulated by different inputs at many levels. In some sensitive background, these inputs might have some parallel outcome for a certain biological event, just like E cell fate specification during *C. elegans* embryogenesis.

GSK-3, a multi-tasking kinase

GSK-3 is a multifunctional serine/threonine kinase found in all eukaryotes. It is a key regulator of numerous sigaling pathways and is involved in a wide range of cellular processes. In Chapter III, we characterized GSK-3 function in E cell fate specification and C cell fate specification during embryogenesis, somatic gonadal cell differentiation and cell cycle control of the germ cells during germline development. Our genetic data suggest that GSK-3 functions in parallel with other pathways/factors to regulate each biological event.

GSK-3 is considered constitutively active in resting cells and needs to be inactivated upon signaling. One of the inactivating mechanisms is phosphorylation of GSK-3 on serine-9, which is conserved among most animal species; however, in *C. elegans*, serine-9 or a serine residue with a similar surrounding does not exit. So it is possible that GSK-3 is active and phosphorylates its available substrates wherever it is localized in *C. elegans*. This phosphorylation by GSK-3 renders its substrates susceptible to other signaling molecules present in different cells. So GSK-3 itself has no polarity ability, but could enhance the signaling. This could explain the genetic interactions between GSK-3 and other components

of the known Wnt pathway that put GSK-3 in parallel with every one of them for E cell fate specification. A similar mechanism also exists in Wnt signaling in *Drosophila*. Recent results in a weak (hypomorphic) allele of Arm/β-catenin and *zw3*/Gsk-3 null allele background have shown that cells still respond to Arm/β-catenin-responsive Wnt-responsive gene transcription (Tolwinski et al., 2003). These results indicate that in *Drosophila*, Wnt signal is too transmitted independent of Zw3/GSK-3 activity and the role of Zw3/GSK-3 in the Wnt pathway is to maintain Arm/β-catenin levels low and in a critical range amenable to rapid change in response to signaling.

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