THE STUDY OF WNT SIGNALING EFFECTOR POP-1/TCF

IN *C. ELEGANS* EARLY EMBRYOS

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To my parents,

who were always proud of me

THE STUDY OF WNT SIGNALING EFFECTOR POP-1/TCF IN *C. ELEGANS* EARLY EMBRYOS

by

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DISSERTATION

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Publication No. _____

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In *C. elegans* embryos, the combined Wnt/MAPK pathway polarizes the founder cell of mesendoderm, EMS blastomere, such that EMS produces two daughters with distinct developmental fates. The posterior daughter E, whose fate is specified by Wnt/MAPK, generates intestinal tissues (endoderm), whereas the anterior daughter MS generates pharynx and muscle cells (mesoderm). The downstream Wnt/MAPK effector POP-1 is asymmetrically localized in the nuclei of A-P sisters including the MS/E pair, with a higher level in the anterior cells. This phenomenon is called POP-1 nuclear asymmetry. The Wnt/MAPK signaling is required for POP-1 nuclear asymmetry. It is believed that POP-1

represses endoderm fate in MS and Wnt/MAPK allows endoderm fate in E by downregulating the nuclear level of POP-1.

In this study, the potential mechanisms for POP-1 nuclear asymmetry are presented. POP-1 nuclear asymmetry requires a 14-3-3 protein PAR-5 and at least three POP-1 potential phosphorylation sites for the MAPK LIT-1. LIT-1 activity is required for both POP-1/PAR-5 interaction and phosphorylation of at least two of the three potential LIT-1 sites *in vivo*. Nuclear export is also required for POP-1 nuclear asymmetry. The nuclear level of LIT-1 is higher in the E blastomere, which is regulated by the upstream kinase and Wnt signaling. All together, I propose that in the E blastomere, Wnt/MAPK signaling promotes PAR-5-mediated nuclear export of POP-1, thereby lowering its nuclear level. In addition to this differential nuclear export mechanism, POP-1 nuclear asymmetry may also be regulated by differential protein degradation.

This study also shows that POP-1 functions to activate a Wnt/MAPK-responsive gene, *sdz-23*, in the E blastomere. This challenged the commonly accepted model of Wnt/MAPK-induced gene expression in E, which is based upon the alleviation of the repressive activity of POP-1. The activation of *sdz-23* in E requires the β-catenin binding domain of POP-1 and a low nuclear level of POP-1. These results suggest that Wnt/MAPK converts the repressor POP-1 into a transcriptional activator and therefore, the non-canonical Wnt signaling in *C. elegans* early embryos is found to regulate its downstream effector POP-1 in a more canonical way than previously realized.

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PRIOR PUBLICATIONS

Shetty, P., **Lo, M.-C.**, Robertson, S.M., and Lin, R. Remodeling the Wnt signaling pathway for endoderm induction in *C. elegans*. *Submitted.*

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Gay, F., Calvo, D., **Lo, M.-C.**, Ceron, J., Maduro, M., Lin, R., and Shi, Y. (2003). Acetylation regulates subcellular localization of the Wnt signaling nuclear effector POP-1. *Genes Dev. 17*, 717-722.

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LIST OF ABBREVIATIONS

A-P – Anterior-Posterior

APC – Adenomatous Polyposis Coli

APR – APC-Related protein

bar or BAR – *b*eta-catenin/*a*rmadillo-*r*elated gene or protein

β-TrCP – β-Transducin repeat Containing Protein

Brg – protein of the *brahma/SWI2-related gene*

bZIP – basic leucine ZIPper

CBP – CREB-Binding Protein

CKII – Casein Kinase II

crm or CRM – *c*hromosome *r*egion *m*aintenance

CtBP – C-terminal Binding Protein

DIC – Differential Interference Contrast

dpr – *d*auer *p*heromone *r*esponsive

dpy – *d*um*py*

Dsh – Dishevelled

dTcf – *Drosophila* Tcf

EDR – Endoderm-Determining Region

EGF – Epidermal Growth Factor

egl or EGL – *eg*g-*l*aying defective

eh – En homology region

end or END – *end*oderm determining

ftt or FTT – *f*ourteen-*t*hree-*t*hree protein

Fz – Frizzled

gfp or GFP – *g*reen *f*luorescence *p*rotein

Grg – Groucho-related gene

GSK – Glycogen Synthase Kinase

GTPase – Guanosine TriPhosphate hydrolase

HDA – Histone DeAcetylase

HDAC – Histone DeACetylase

him – *h*igh *i*ncidence of *m*ales

hLEF1 – human *LEF1* gene

HMG – High Mobility Group

HMP – HuMPback

HMR – HaMmeRhead embryonic lethal

Hox – Homeobox genes

hTCF1E – human TCF1E isoform

hTCF4 – human TCF4

ICAT – Inhibitor of β-Catenin And TCF-4

IκBα – Inhibitor of NFκB α

JNK – c-Jun N-terminal Kinase

kin – protein *kin*ase

LEF – Lymphoid Enhancer-binding Factor

LG – Linkage Group

lin or LIN – *lin*eage defective

lit or LIT – *l*oss of *in*t*e*stine

LRP – Low density lipoprotein receptor-Related Protein

mab – *m*ale *ab*normal

MAPK – Mitogen-Activated Protein Kinase

MAPKKK – MAPK Kinase Kinase

med or MED – *m*esoderm and *e*ndoderm *d*etermination

mex – *m*uscle *ex*cess

mig – *mig*ration defective

mLef1 – mouse Lef1

mom or MOM – *mo*re *m*esoderm

NES – Nuclear Export Sequence

NLK – Nemo-Like Kinase

NLS – Nuclear Localization Sequence

par or PAR – *par*titioning defective

PCP – Planar Cell Polarity

pie or PIE – *p*haryngeal and *i*ntestinal *e*xcess

pop or POP – *po*sterior *p*harynx defective

por – *porcupine*

pry or PRY – *p*oly*r*a*y*

RNAi – RNA interference

sdz – *s*kn-1-*d*ependent *z*ygotic genes

SGG – ShaGgy-like Gene

Six – Sine oculis homeobox homolog

skn or SKN – excess *skin*

SNF – Sucrose Non-Fermenting

So – Sine oculis

SOX – Sry-related HMG-bOX protein

SWI – mating-type SWItching

TAK – Transforming growth factor (TGF)-β-Activated Kinase

TCF – T Cell-specific transcription Factor

UBF – Upstream Binding Factor

unc or UNC – *unc*oordinated

UTR – UnTranslated Region

wrm or WRM – *w*orm a*rm*adillo

XARP – *Xenopus* Axin-Related Protein

XLef-1 – *Xenopus* Lef-1

XTcf-3 – *Xenopus* Tcf-3

XWnt – *Xenopus* Wnt

CHAPTER ONE
Introduction and Literature Review

WNT SIGNALING

During the development of multicellular organisms, signaling between cells plays an essential role in the elaboration of cell types and tissues, triggering cell fate decisions and morphogenetic movements that are critical for the generation of a normally patterned embryo. A key player in the regulation of these important developmental processes is the Wnt family of signaling molecules. Wnt signaling is highly conserved from the simple metazoan *Hydra* to the most complex vertebrates (Figure 1.1). The developmental processes regulated by Wnt signaling include cell proliferation, cell polarity, cell migration, cell fate specification, and differentiation. In addition to playing an important role in development, Wnt signaling has also been implicated in adult tissue homeostasis. The importance of Wnt signaling is also signified by the fact that many key regulatory genes of Wnt signaling are mutated in human cancers (see reviews by Polakis, 2000; Moon *et al.,* 2002; Logan and Nusse, 2004).

Wnt signaling pathways have been intensively investigated for more than two decades since the initial observation that in mice the activation of Wnt-1 by viral insertion leads to the development of mammary tumors (Nusse and Varmus, 1982). It was quickly discovered that *Wnt-1* was in fact the vertebrate counterpart of the *Drosophila* segment polarity gene *wingless* (Sharma and Chopra, 1976; Cabrera *et al.,* 1987; Rijsewijk *et al.,*

1987). Wingless is involved in numerous developmental events including embryonic and larval patterning (see review by Cadigan and Nusse, 1997) and synaptic differentiation (Packard *et al.,* 2002). Ectopic expression of Wnt-1 in fertilized *Xenopus* eggs leads to duplication of the embryonic axis (McMahon and Moon, 1989). It was then shown that an endogenous Wnt signaling pathway did exist and played an important role in axis formation in *Xenopus* embryos (Heasman *et al.,* 1994; Molenaar *et al.,* 1996). During vertebrate development, perturbation in Wnt signaling can cause dramatic phenotypes such as embryonic lethality, CNS abnormalities and kidney and limb defects (see review by Logan and Nusse, 2004).

Wnt signaling regulates different developmental events by changing the transcription of specific target genes or affecting the cytoskeleton within responding cells. In *Drosophila* and vertebrates, these different outcomes are mediated by distinct signaling pathways: a canonical Wnt pathway that changes gene transcription via the T cell-specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF) family of transcription factors (see reviews by Polakis, 2000; Moon *et al.,* 2002; Logan and Nusse, 2004) and a planar cell polarity (PCP) pathway that activates the JNK cascades to modify cytoskeletal organization (see reviews by Kühl, 2002; Strutt, 2002; Tada *et al.,* 2002; Tree *et al.,* 2002; Veeman *et al.,* 2003).

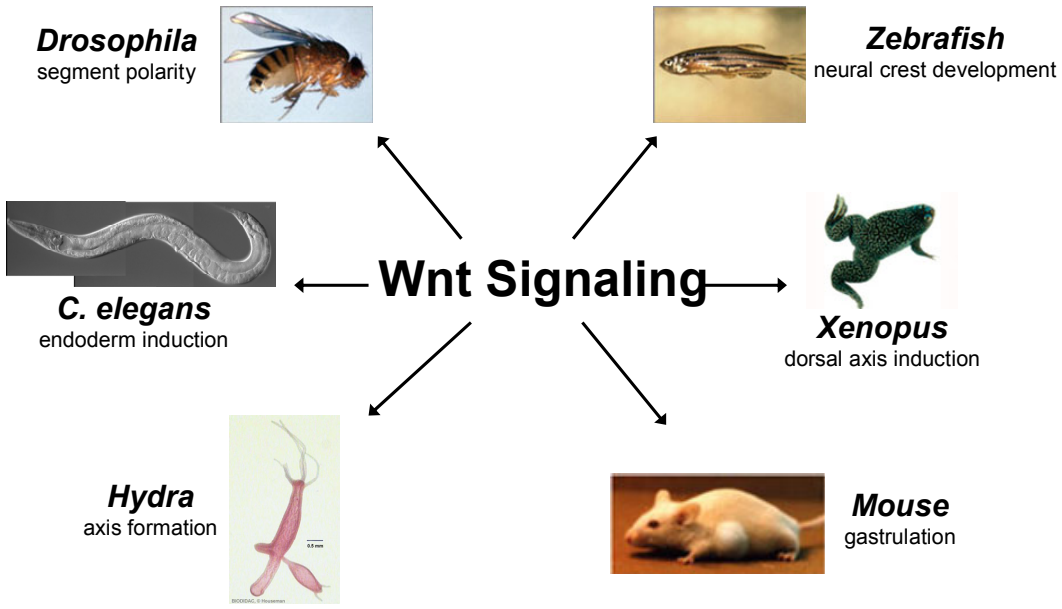


Figure 1.1 - Wnt signaling is evolutionarily conserved from *Hydra* to vertebrates. One example of the developmental processes regulated by Wnt signaling is listed for each organism.

Canonical Wnt Signaling Pathway

The first Wnt pathway to be discovered, and also the best characterized, is the *Drosophila* Wingless signaling pathway (Sharma and Chopra, 1976). Based on the *wingless* mutant phenotype, genetic screens in *Drosophila* have identified many components in this pathway and epitasis analyses have established a general pathway of Wingless signal transduction (see reviews by Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Seto and Bellen, 2004). Studies of Wnt signaling in other organisms such as *C. elegans*, *Xenopus* and mouse have generally confirmed the findings in *Drosophila*. This pathway is often referred to as the canonical Wnt signaling pathway (Figure 1.2) and involves the signaling through Frizzled (Wnt receptor) leading to the stabilization of a key downstream component β-catenin.

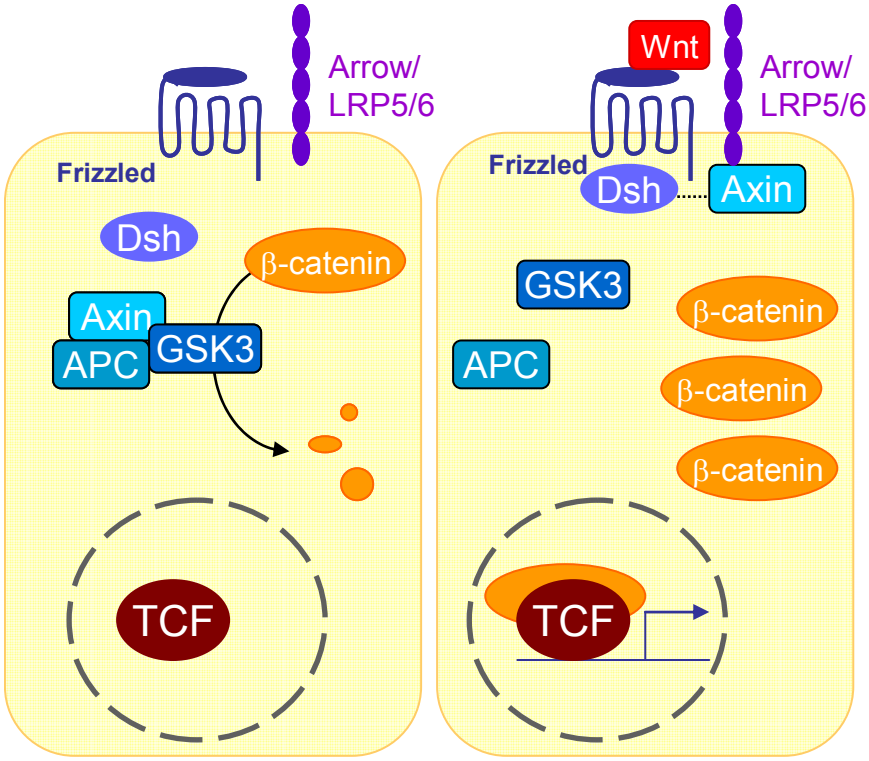


Figure 1.2 – (adapted from Logan and Nusse, 2004) The canonical Wnt signaling pathway. In the absence of Wnt signaling (left panel), β-catenin is degraded by the GSK3/APC/Axin destruction complex. When Wnt proteins bind to the Frizzled/LRP receptor complex (right panel), the activity of GSK3/APC/Axin is inhibited through the signaling protein Dishevelled (Dsh). Therefore, β-catenin is stabilized and accumulates in the cytoplasm and nucleus. β-catenin then interacts with the transcription factor TCF to activate gene expression.

Wnt Proteins Are Lipid Modified

Wnt genes are defined by their sequence homology to the first isolated members of this family, mouse *Wnt-1* and *Drosophila wingless* (Nusse and Varmus, 1982; Cabrera *et al.,* 1987; Rijsewijk *et al.,* 1987). They encode secreted glycoproteins that share a nearly invariant pattern of twenty-three cysteine residues (see review by Nusse and Varmus, 1992). Wnt proteins are lipid modified (palmitate) and therefore more hydrophobic than initially predicted from the primary amino acid sequence (Willert *et al.,* 2003). The palmitoylation is

found on a conserved cysteine residue and is critical for signaling (Willert *et al.,* 2003). The enzymes responsible for the palmitoylation of Wnts are likely to be Porcupine in *Drosophila* (Kadowaki *et al.,* 1996) and MOM-1 in *C. elegans* (Rocheleau *et al.,* 1997).

porcupine/mom-1 encode a protein with sequence similar to membrane-bound acyltransferases, endoplasmic reticulum resident enzymes that acylate a variety of substrates (Hofmann, 2000). *wnt* and *por/mom-1* mutants show similar phenotypes suggesting that Porcupine and MOM-1 function in Wnt signaling (Kadowaki *et al.,* 1996; Rocheleau *et al.,* 1997). All these genes are required in the Wnt-producing cells and not in the Wnt-receiving cells (Kadowaki *et al.,* 1996; Rocheleau *et al.,* 1997). The hydrophobicity of Wingless is lost when Porcupine is eliminated in *Drosophila* (Zhai *et al.,* 2004). Therefore, it is likely that Porcupine/MOM-1 catalyze the palmitoylation of Wnt proteins. Although the precise role of palmitoylation is not clear, the loss of membrane localization of Wingless when Porcupine is eliminated genetically or when acyltransferase activity is inhibited biochemically suggests that palmitate targets Wnt proteins to membranes (Zhai *et al.,* 2004).

Frizzled/LRP Complex: Wnt Receptors

The secreted or membrane-bound Wnt proteins bind to the Frizzled (Fz)/low density lipoprotein receptor-related protein (LRP) complex at the surface of Wnt-receiving cells. Fz proteins are the primary receptors for the Wnts (Bhanot *et al.,* 1996). They are seven-transmembrane receptors with a long N-terminal extension called cysteine-rich domain, which interacts directly with Wnt proteins (Bhanot *et al.,* 1996; Hsieh *et al.,* 1999; Dann *et al.,* 2001). LRPs are single-pass transmembrane proteins. They probably function as

coreceptors for Wnt ligands. In *Drosophila*, the LRP family member Arrow is required for Wingless signaling (Wehrli *et al.,* 2000). In *Xenopus*, LRP6 binds to Wnt-1 and associates with Fz in a ligand-dependent manner (Tamai *et al.,* 2000). Upon binding to Wnts, the receptor complex transduces a signal to several intracellular proteins, which leads to the stabilization and accumulation of an important downstream component β-catenin. This event is a hallmark of the canonical Wnt signaling (Figure 1.2).

Wnt Signaling Stabilizes β-Catenin

β-catenin, also known as Armadillo in *Drosophila*, was originally identified through its interaction with the adhesion molecule cadherin (McCrea *et al.,* 1991). In the absence of Wnt signaling, β-catenin associates primarily with the cell membrane, where it contributes to adherens junctions (see review by Bullions and Levine, 1998), and the cytoplasmic levels of β-catenin are kept low as a result of its targeted degradation mediated by the ubiquitin-proteosome pathway. The targeting involves a "destruction complex" containing glycogen synthase kinase 3 (GSK3), Adenomatous Polyposis Coli (APC) and Axin through a yet unknown mechanism (Aberle *et al.,* 1997; Ikeda *et al.,* 1998; Sakanaka *et al.,* 1998). Basically Axin is proposed to function as a scaffolding protein that brings GSK3 and β-catenin together, which promotes the phosphorylation of β-catenin. The phosphorylated β-catenin is recognized by the F-box protein β-TrCP, which subsequently targets it for ubiquitination and degradation by the proteosome.

Wnt signaling somehow antagonizes the activity of the destruction complex to stabilize β-catenin. This process requires a cytoplasmic protein, Dishevelled (Dsh). The

binding of Wnts to their receptors induces the phosphorylation of Dsh probably through a direct binding between Dsh and Fz (Yanagawa *et al.,* 1995; Chen *et al.,* 2003; Wong *et al.,* 2003). Dsh acts to inhibit the GSK3/APC/Axin complex, thereby allowing the stable accumulation of β-catenin in the cytoplasm and nucleus (Riggleman *et al.,* 1990; Yanagawa *et al.,* 1995). Dsh and a *Xenopus* Axin, XARP, have been shown to interact with each other and this interaction is required for Wnt signal transduction, suggesting that Wnt binding of Fz/LRP may promote direct interaction between Dsh and Axin, thereby disrupting the protein complex that downregulates β-catenin levels (Figure 1.2; Itoh *et al.,* 2000).

Once in the nucleus, β-catenin interacts with TCF/LEF transcription factors and activates the expression of Wnt target genes (Behrens *et al.,* 1996; Molenaar *et al.,* 1996; Brunner *et al.*, 1997; van de Wetering *et al.,* 1997).

TCF/LEF Proteins

The family of TCF/LEF proteins, which in human comprises TCF1, LEF1, TCF3 and TCF4, forms a subgroup of the high mobility group (HMG) box-containing superfamily of transcription factors. Multiple TCF/LEF isoforms with diverse functional domains are derived from alternative splicing and promoter usage (van de Wetering *et al.*, 1996; Korinek *et al.*, 1998; Duval *et al.*, 2000; Hovanes *et al.*, 2000, 2001). TCF/LEF proteins are highly conserved through evolution. The conservation of these factors allowed for the identification of TCF/LEF proteins in lower organisms. Two factors were identified in *Xenopus*, XTcf-3 and XLef-1 (Molenaar *et al.*, 1996, 1998). Only one factor appears to exist in *Drosophila*, dTcf or Pangolin (Brunner et al., 1997; van de Wetering *et al.*, 1997). Even in *C. elegans*, a

distant TCF/LEF family member POP-1 was identified (Lin *et al.*, 1995). For simplicity, the collective TCF/LEF family members will be refered to as TCF proteins hereafter.

HMG boxes are a novel type of DNA-binding domains found in a diverse group of proteins (Jantzen *et al.,* 1990; Laudet *et al.,* 1993). They can be divided into two groups. The HMG/UBF subfamily comprises of proteins with multiple HMG boxes, which bind DNA with relatively low specificity and the TCF/SOX subfamily consists of proteins with a single sequence-specific HMG box (Laudet *et al.,* 1993). TCF proteins bind DNA as monomers. The HMG boxes of TCF1 and LEF1 have been shown to mediate binding to a core consensus motif AGATCAAAGGG through contacts made predominantly within the minor groove of the DNA helix (Giese *et al.*, 1991; van de Wetering *et al.*, 1991; van Beest *et al.*, 2000; also see reviews by Clevers and van de Wetering, 1997; Roose and Clevers, 1999; Hurlstone and Clevers, 2002). Like other HMG box-containing transcription factors, TCF proteins have been implicated as architectural proteins due to their ability to induce substantial DNA bends, which may facilitate the assembly of functional nucleoprotein complexes and thereby activate transcription (Giese *et al.*, 1992, 1997). However, TCF proteins are not able to modulate transcription by themselves. Instead they bind specific binding partners and recruit essential functional domains to the regulatory regions of their target genes.

TCF proteins have been shown to regulate target gene expression in a bimodal fashion: activating the target genes in one subset of cells, while simultaneously repressing the same genes in a different subset (Brannon *et al.*, 1997; Cavallo *et al.*, 1998; Merrill *et al.*, 2001). Whether a gene is activated or repressed by TCF is determined by the cofactors that interact with TCF. Studies in LEF1 and TCF1 have identified β-catenin as a binding partner

of TCF proteins (Behrens *et al.*, 1996; Molenaar *et al.*, 1996). When bound to TCF, β-catenin could provide transcriptional activation domains, resulting in target gene expression. The binding occurs between the N-terminal region (the first 50 amino acids or so) of TCF and Armadillo repeats 3-10 of β-catenin. The β-catenin binding domain and the HMG box are the best-conserved regions among TCF proteins. These two domains are connected by a region of lower degree of homology, which mediates the interaction between TCF and another binding partner, Groucho (Roose *et al.*, 1998). Groucho is a transcriptional corepressor, and thus the TCF/Groucho complex represses transcription (Cavallo *et al.*, 1998). The most divergent region of the TCF family members is the C-terminus, which in certain longer isoforms contains two conserved motifs, KKCRARFG and WCXXCRRKKKC (Atcha *et al.*, 2003), and two CtBP binding sites (Brannon *et al.*, 1999).

Wnt Signaling Promotes the Formation of β-Catenin/TCF Activator Complex

In the absence of Wnt signaling, TCF functions as a transcriptional repressor by interacting with Groucho (Cavallo *et al.,* 1998). Groucho mediates the repressive effect by recruiting histone deacetylase (HDAC), which are thought to make DNA more compact and less accessible to the transcriptional machinery (Chen *et al.,* 1999).

Upon Wnt stimulation, the binding of β-catenin converts TCF repressor complex into a transcriptional activator complex. This may occur through displacement of Groucho from TCF and recruitment of the histone acetyltransferase CBP/p300 and the SWI/SNF chromatin remodeling complex. The SWI/SNF complex together with CBP may induce chromatin remodeling that favors gene transcription. CBP and Brg-1, a component of the SWI/SNF

complex, have been shown to interact with β-catenin to activate gene expression (Hecht *et al.,* 2000; Takemaru and Moon, 2000; Barker *et al.,* 2001).

Regulation of β-Catenin/TCF Complex

The nuclear β-catenin/TCF complex can be regulated by a number of proteins. For example, the nuclear protein Chibby binds to the C-terminus of β-catenin and inhibits the β-catenin-mediated transcriptional activation (Takemaru *et al.,* 2003). Another β-catenin-binding protein, ICAT, not only prevents β-catenin from binding to TCF (Tago *et al.,* 2000), but also dissociates complexes of β-catenin, TCF, and CBP/p300 (Daniels and Weis, 2002; Graham *et al.,* 2002). Regulation can also happen via post-translational modification of TCF proteins. TCF can be phosphorylated by the MAP kinase (MAPK)-related protein kinase NLK/Nemo, and it is believed that this phosphorylation reduces the DNA-binding affinity of the β-catenin/TCF complex, thereby affecting the Wnt target gene expression (Ishitani *et al.,* 1999, 2003).

Given the wide range of biological processes that Wnts control, the ability of TCF to interact with DNA and its binding partners must be highly regulated to ensure proper and distinct transcriptional outputs of Wnt signaling in different biological contexts.

Non-canonical Wnt Signaling Pathways

Recently, it has become evident that Wnt ligands can activate signaling pathways other than the canonical Wnt/β-catenin pathway. These pathways are called non-canonical Wnt

signaling pathways and have been observed in *C. elegans*, *Drosophila*, and vertebrates (see reviews by Herman, 2002; Kühl, 2002; Strutt, 2002; Tada *et al.,* 2002; Tree *et al.,* 2002; Veeman *et al.,* 2003; Wang and Malbon, 2004). The major difference between canonical and non-canonical Wnt pathways is that the activation of β-catenin or the formation of a β-catenin/TCF activator complex is absent in non-canonical Wnt pathways.

The existence of non-canonical Wnt pathways was proposed based on work with the *Drosophila* PCP pathway in the early 1990s (Wong and Adler, 1993; Theisen *et al.*, 1994; Krasnow *et al.*, 1995). PCP is a phenomenon where the epithelia in multicellular organisms are polarized in the plane orthogonal to their apical-basal axis. Like the canonical Wnt/β-catenin pathway, the PCP pathway requires Fz receptors and the cytoplasmic signal transduction molecule Dsh. However, it is not clear whether a Wnt is required to activate Fz in the PCP pathway. The PCP pathway diverges from the Wnt/β-catenin pathway downstream of Dsh in that it does not involve Axin, GSK3 or β-catenin. Instead, it activates JNK cascades via the small GTPases of the Rho family to modify cytoskeletal organization (see reviews by Strutt, 2002; Tree *et al.,* 2002; Veeman *et al.,* 2003). It has been shown that Dsh discriminates between β-catenin pathway in Wingless signaling and JNK pathway in PCP by distinct domain interactions (Boutros *et al.,* 1998).

Experiments in *Xenopus* fertilized eggs revealed that non-canonical Wnt signaling also existed in vertebrates to regulate cell movements during gastrulation. Overexpression of some Wnts, including XWnt1 and XWnt8, causes a duplication of the embryonic axis in *Xenopus* that has been shown to depend on the canonical Wnt signaling (Heasman *et al.,* 1994; Molenaar *et al.,* 1996). However, overexpression of other Wnts, such as XWnt4,

XWnt5a, and XWnt11, does not induce axis duplication but instead perturbs gastrulation movements (Moon *et al.*, 1993; Du *et al.*, 1995). This vertebrate non-canonical Wnt pathway also does not involve the activation of β-catenin. Recent studies have shown that this non-canonical Wnt signaling regulates vertebrate gastrulation movements at least in part through a pathway similar to the *Drosophila* PCP pathway (see reviews by Kühl, 2002; Tada *et al.,* 2002; Veeman *et al.,* 2003).

Non-canonical Wnt signaling is also involved in diverse cellular processes. In vertebrates, non-canonical Wnt pathways have been shown to play roles in cochlear hair polarity, tissue separation, cardiogenesis, myogenesis, neuronal migration, dorsoventral patterning, and cancer (Veeman *et al.,* 2003). In addition to the activation of JNK cascades, potential mechanisms of non-canonical Wnt pathways include signaling through calcium flux, small GTPases, and heterotrimeric G proteins.

Wnt Signaling Pathways in *C. Elegans*

As in *Drosophila* and vertebrates, *C. elegans* has both canonical and non-canonical Wnt signaling pathways (see reviews by Thorpe *et al.,* 2000; Herman, 2002; Korswagen, 2002). The best-characterized canonical Wnt pathway in *C. elegans* is the process that controls the migration of the descendants of the QL neuroblast (Harris *et al.,* 1996; Sawa *et al.,* 1996; Maloof *et al.,* 1999; Korswagen *et al.,* 2000; Herman, 2001; Korswagen *et al.,* 2002). Canonical Wnt signaling pathways are also involved in the cell fate decisions of the P12 neuroectoblasts (Jiang and Sternberg, 1998) and the vulval precursor cells (Eisenmann *et al.,* 1998). As in other organisms, these *C. elegans* canonical Wnt pathways regulate the

expression of homeobox-containing genes (Hox genes) through the *C. elegans* TCF homolog POP-1. In both of the two cell fate decisions, Wnt signaling synergizes with a Ras pathway to control Hox gene expression.

Canonical EGL-20/Wnt Pathway Controls QL Neuroblast Migration

The Q neuroblasts are migratory cells born at similar anteroposterior positions on the left (QL) and right (QR) side of the animal (Sulston and Horvitz, 1977). During the first larval stage, each Q neuroblast divides to generate a total of three neurons and two cells that undergo apoptosis. Despite the similarity in lineage, the Q neuroblasts and their descendants of the two sides migrate in opposite directions. The QL neuroblast and its descendants migrate in a posterior direction, whereas the QR neuroblast and its descendants migrate towards the anterior. The difference of Q daughter cell migration between the two sides is controlled by the asymmetric expression of the Hox gene *mab-5* (Chalfie and Sulston, 1981; Kenyon, 1986; Salser and Kenyon, 1992). Only the QL lineage expresses *mab-5*, which directs the migration towards the posterior. The QR lineage does not express *mab-5* and migrates in the default anterior direction (Salser and Kenyon, 1992).

The expression of *mab-5* in the QL lineage is in turn regulated by a Wnt signaling pathway. This Wnt pathway was characterized as a canonical Wnt pathway based on the following observations. First, mutations in *egl-20/Wnt*, *lin-17/Fz*, *mig-5/Dsh*, *bar-1/β-catenin* and *pop-1/TCF*, all positive regulators in the canonical Wnt signaling, disrupt *mab-5* expression in the QL lineage and induce anterior migration of QL daughters (Harris *et al.,* 1996; Maloof *et al.,* 1999; Guo, 1995; Antebi *et al.*, 1997; Korswagen *et al.,* 2000; Herman,

2001), whereas mutations in the negative regulator *pry-1/Axin* result in ectopic expression of *mab-5* in QR and posterior migration of QR daughters (Maloof *et al.,* 1999; Korswagen *et al.,* 2002). Second, the β-catenin BAR-1 physically interacts with POP-1 and this interaction is required for the activation of *mab-5* (Korswagen *et al.,* 2000; Herman, 2001; Natarajan *et al.*, 2001). These observations indicate that like the canonical Wnt signaling in other organisms, this *C. elegans* Wnt pathway promotes the formation of a β-catenin/TCF activator complex, thereby activating the expression of Wnt target genes.

Non-Canonical Wnt Pathways in C. Elegans

Non-canonical Wnt pathways primarily control the polarities of asymmetric cell divisions in *C. elegans*. These include the polarities of the EMS blasotmere in the early embryo, the T and B cells in the tail, and the Z1 and Z4 cells in the developing gonad. The first non-canonical Wnt pathway to be identified in *C. elegans* was the LIN-44/Wnt pathway, which controls the polarities of T cells (Sternberg and Horvitz, 1988; Herman and Horvitz, 1994; Herman *et al.,* 1995; Sawa *et al.,* 1996; Herman, 2001).

TL and TR, collectively known as the T cells, lie in the tail on each side of the animal. T cells divide asymmetrically. The anterior daughter, T.a, generates primarily epidermal cells and the posterior daughter, T.p, generates primarily neural cells. Certain divisions within the T cell lineage also generate daughters of different sizes. This T cell polarity is controlled by LIN-44/Wnt, LIN-17/Fz and POP-1/TCF (Sternberg and Horvitz, 1988; Herman and Horvitz, 1994; Herman *et al.,* 1995; Sawa *et al.,* 1996; Herman, 2001). However, a β-catenin homolog may not be involved (Herman, 2001). Mutations in *lin-44*

cause the polarities of the T cells to be reversed (Herman and Horvitz, 1994; Herman *et al.*, 1995), but mutations in *lin-17* and *pop-1* cause a loss of polarity in these cells such that both T daughters generate epidermal cells (Sternberg and Horvitz, 1988; Sawa *et al.,* 1996; Herman, 2001). This Wnt pathway is characterized as non-canonical because it differs from the canonical Wnt pathway in many respects. First, in the canonical Wnt pathway, mutations in TCF cause a similar phenotype as loss of Wnt signaling (Molenaar *et al.*, 1996; Brunner *et al.*, 1997; van de Wetering *et al.*, 1997). However, mutations in POP-1 cause a different phenotype than mutations in LIN-44. Second, none of the three *C. elegans* β-catenin homologs appeared to function with POP-1 to control T cell polarity (Herman, 2001). Third, a MAPK pathway is also involved in this LIN-44/Wnt signaling to control T cell polarity probably by regulating POP-1 (Rocheleau *et al.*, 1999).

The best-studied non-canonical Wnt pathway in *C. elegans* is the MOM-2/Wnt pathway, which polarizes the EMS blastomere and induces endoderm in the early embryo (see reviews by Thorpe *et al.,* 2000; Korswagen, 2002). This MOM-2/Wnt signaling pathway will be described in more detail later.

ENDODERM INDUCTION IN *CAENORHABDITIS ELEGANS*

In *C. elegans*, the endoderm arises from a single progenitor cell, the E blastomere, in the seven-cell embryo. Upon fertilization, the *C. elegans* zygote (P0) divides asymmetrically to produce two daughter cells called AB and P1. The P1 blastomere divides into the mesendodermal precursor EMS and the germline/mesectodermal precursor P2. The anterior

daughter of EMS, called MS, is the mesodermal precursor, which produces many mesodermal cell types, including body wall muscle and the posterior half of the feeding organ, pharynx. The posterior daughter of EMS is the endodermal precursor, E blastomere (Figure 1.3). The E blastomere undergoes no more than five rounds of division during embryogenesis, generating the twenty clonally derived cells of the juvenile intestine (Sulston *et al.,* 1983).

The cell identity of the E blastomere requires two maternal regulatory pathways (see reviews by Schnabel and Priess, 1997; Bowerman, 1998; Maduro and Rothman, 2002). Both pathways function at the four-cell stage, within the mother of E, the EMS blastomere. The first pathway specifies the identity of EMS through the action of cell-autonomous maternal factors, including the transcription factor SKN-1 (Bowerman *et al.,* 1992). The second pathway is an inductive interaction (P2 signaling) between EMS and P2, which is required to make the E cell different from MS (Schierenberg, 1987; Goldstein, 1992, 1993, 1995). When the maternal SKN-1 activity or the maternal P2 signaling pathway is inhibited, the E cell adopts the fate of another early blastomere and the subsequent endoderm tissues are not properly generated.

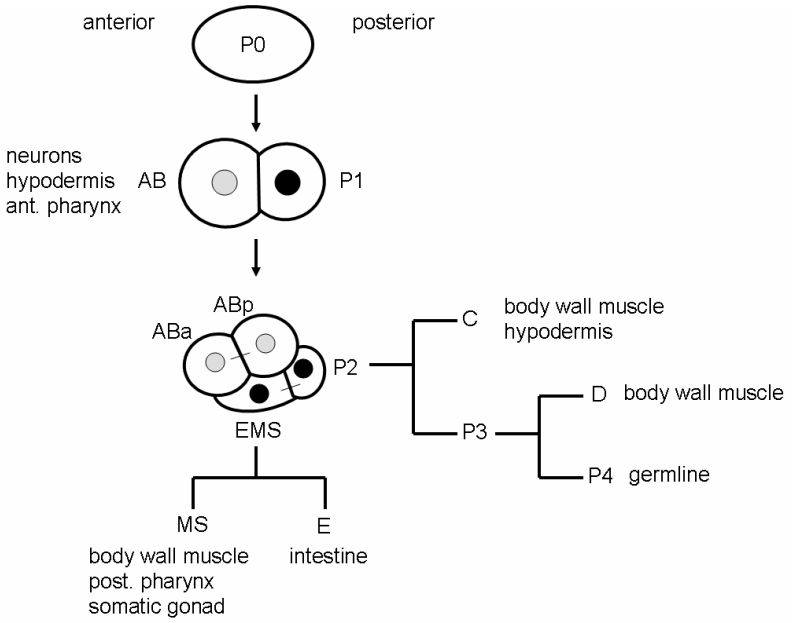


Figure 1.3 – Early cell lineages in *C. elegans*. Nuclei of the early blastomeres are shown as closed circles inside cells. Different shadings represent different levels of nuclear SKN-1 protein, with the darker being higher. P0 is the zygote (the first germline cell). Asymmetric divisions of the germline cells (P0-P3) lead to the formation of five somatic founder cells (AB, MS, E, C, D) and the germline progenitor cell P4. The fates of the founder cells are indicated.

Specification of the Mesendoderm Progenitor, EMS Blastomere

SKN-1, A Maternal Factor Important For EMS Fate

SKN-1 is a composite bZIP/homeodomain transcription factor that plays an important role in the EMS fate specification (Bowerman *et al.,* 1992; Blackwell *et al.,* 1994). In 60% of the *skn-1* mutant embryos, EMS adopts a C-like fate, which produces ectoderm and muscle (Bowerman *et al.,* 1992). Although the maternal *skn-1* mRNA is distributed equally throughout the early embryo, SKN-1 protein has an unequal distribution due to asymmetrical translation (Bowerman *et al.,* 1993; Seydoux and Fire, 1994). In a two-cell embryo, AB has a

low level of SKN-1, while P1, the EMS precursor, has a high level of SKN-1 (Figure 1.3). The maternal genes *par-1* and *mex-1* are required for this unequal distribution (Bowerman *et al.,* 1993).

When P1 divides, its daughters EMS and P2 both have equal levels of SKN-1 protein. However, SKN-1 is important for the identity of, and functions only in, the EMS blastomere. In P2 blastomere, SKN-1 activity is inhibited by a maternally provided transcription factor PIE-1, which acts as a global repressor of transcription throughout the germline (P) lineage (Mello *et al.,* 1996; Seydoux *et al.,* 1996; Batchelder *et al.,* 1999).

SKN-1 Activates the Zygotic Genes, med-1/2, to Promote EMS Fate

In EMS, SKN-1 activates its zygotic targets, the *med-1* and *med-2* genes, which was proposed to switch the control in mesendoderm specification from maternal to zygotic (Maduro *et al.,* 2001). *med-1/2* act redundantly to promote EMS fate. Inhibiting the function of these two genes simultaneously results in EMS cell fate transformation similar to that in the *skn-1* mutant. *med-1* and *med-2* encode two nearly identical GATA-type transcription factors (Maduro *et al.,* 2001). GATA factors are named for the degenerate consensus DNA binding site HGATAR to which they bind (Lowry and Atchley, 2000).

med-1 and *med-2* appear to be direct targets of SKN-1 as the *med* promoters contain clusters of SKN-1 binding sites (Blackwell *et al.,* 1994) that bind SKN-1 protein *in vitro* and are required for reporter expression (Maduro *et al.,* 2001). Inappropriate expression of SKN-1 in non-EMS lineages in certain maternal mutants results in ectopic expression of the *med* genes, which in turn are able to convert those non-EMS cells into mesendodermal

progenitors. Moreover, forced expression of the *med* genes can direct non-EMS cells into mesendodermal progenitors independent of SKN-1 activity (Maduro *et al.,* 2001). These observations suggest that a primary function of the maternal SKN-1 protein is to activate the zygotic expression of the *med* genes, which is sufficient to promote the EMS fate.

Specification of the Endoderm Progenitor, E Blastomere

MED-1 and MED-2 regulate two very different developmental fates, mesoderm (MS blastomere) and endoderm (E blastomere). The separation of these two fates is determined by the P2 signaling that polarizes the EMS blasotmere (Schierenberg, 1987; Goldstein, 1992, 1993, 1995).

P2 Signaling and Endoderm Specification

At the four-cell stage, P2 blastomere signals anteriorly to polarize the adjoining EMS blastomere such that EMS undergoes an asymmetric cell division to produce daughter cells with different developmental fates. The daughter derived from the part of EMS that contacted P2 (the posterior daughter E) will adopt the endoderm fate, whereas the unsignaled anterior daughter MS will adopt the mesoderm fate (Figure 1.4A; Goldstein, 1992).

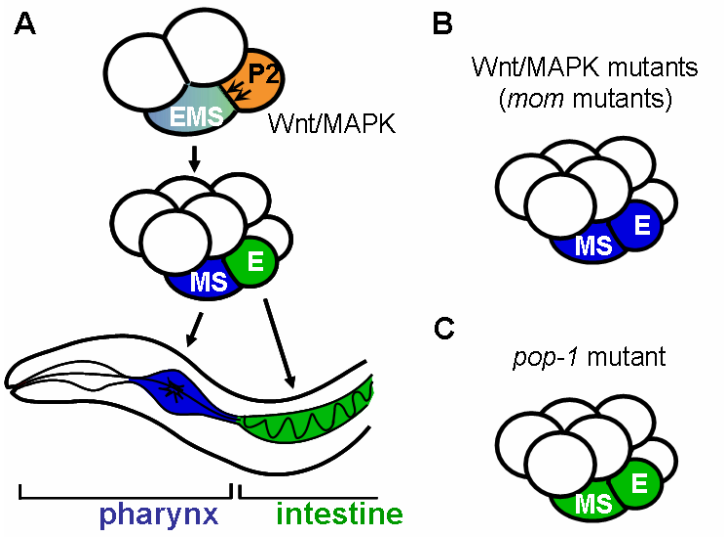


Figure 1.4 – Wnt/MAPK signaling is required for endoderm induction in *C. elegans*. A. P2 signaling (Wnt/MAPK) polarizes EMS blastomere to specify E fate. B. In Wnt/MAPK mutants (*mom* mutants), E adopts MS fate (blue). C. In *pop-1* mutant, MS adopts E fate (green).

The endoderm induction in *C. elegans* early embryos is now known to be mediated by two convergent signaling pathways (Figure 1.5). One is a non-canonical Wnt signaling pathway, and the other is a MAPK pathway. Genetic and molecular analyses have identified the components involved in this endoderm-inducing signaling. They are all maternally contributed and include MOM-1/Porcupine (Rocheleau *et al.,* 1997), MOM-2/Wnt (Rocheleau *et al.,* 1997; Thorpe *et al.,* 1997), MOM-5/Frizzled (Rocheleau *et al.,* 1997; Thorpe *et al.,* 1997), SGG-1/GSK3 (Schlesinger *et al.,* 1999), APR-1/APC (Rocheleau *et al.*, 1997), WRM-1/β-catenin (Rocheleau *et al.,* 1997), LIT-1/MAPK (Kaletta *et al.,* 1997; Ishitani *et al.,* 1999; Meneghini *et al.,* 1999; Rocheleau *et al.,* 1999), and MOM-4/MAPKKK (Ishitani *et al.,* 1999; Meneghini *et al.,* 1999; Shin *et al.,* 1999).

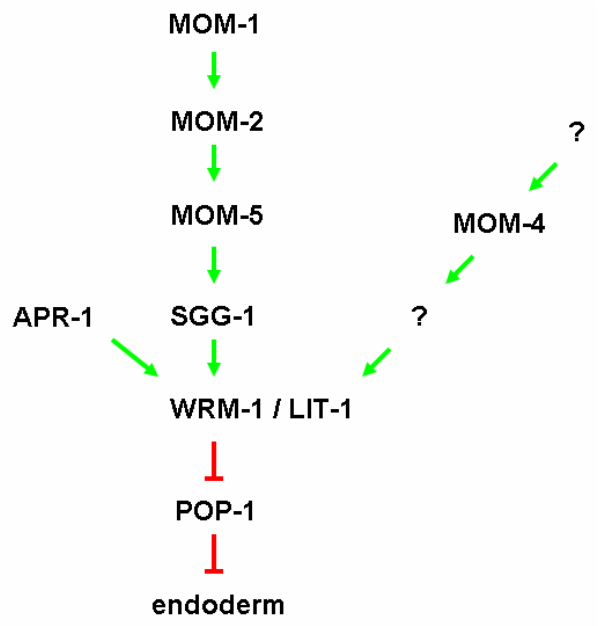


Figure 1.5 – Genetic model for the role of the Wnt/MAPK components in endoderm development. The Wnt/MAPK signaling acts to downregulate POP-1, which otherwise would repress the endoderm fate. Activation is shown in green, and inhibition is shown in red.

Both SGG-1 and APR-1 appear to be involved in this P2 to EMS signaling (Rocheleau *et al.,* 1997; Schlesinger *et al.,* 1999). However, unlike their mammalian counterparts GSK3 and APC, which act as negative regulators in the canonical Wnt pathway, both SGG-1 and APR-1 play a positive role in the P2 signaling during endoderm development in *C. elegans*.

lit-1 encodes a protein related to the *Drosophila* tissue polarity protein Nemo, which together with LIT-1 and NLK, form a small subfamily of serine/threonine protein kinases that are distinct from, but closely related to MAPKs (Choi and Benzer, 1994; Brott *et al.,* 1998; Rocheleau *et al.,* 1999). *mom-4* encodes a *C. elegans* homolog of mammalian TAK1,

which is thought to function as a MAPKKK (Ishitani *et al.,* 1999; Meneghini *et al.,* 1999; Shin *et al.,* 1999).

Mutations of any component in this Wnt/MAPK signaling result in MS and E both generating tissue types normally derived from the MS blastomere (Figure 1.4B; Kaletta *et al.,* 1997; Rocheleau *et al.,* 1997; Thorpe *et al.,* 1997; Meneghini *et al.,* 1999; Rocheleau *et al.,* 1999; Shin *et al.,* 1999).

P2 Signaling and POP-1 Asymmetry

The P2-to-EMS Wnt/MAPK signaling appears to result in a change in the nuclear level of the TCF homolog POP-1 in the E blastomere (Rocheleau *et al.,* 1997; Thorpe *et al.,* 1997; Lin *et al.*, 1998; Meneghini *et al.,* 1999; Rocheleau *et al.,* 1999; Shin *et al.*, 1999). In MS, which does not receive the endoderm-inducing Wnt/MAPK signal, the nuclear level of POP-1 is high, whereas in E, which receives the signal, the nuclear level of POP-1 is low (Lin *et al.,* 1995, Rocheleau *et al.,* 1997; Thorpe *et al.,* 1997; Lin *et al.,* 1998). Mutations in the Wnt/MAPK components result in a high level of POP-1 in the E nucleus that is similar to the level in the MS nucleus. This difference of nuclear POP-1 levels between MS and E is called POP-1 asymmetry and will be described in more detail later.

In embryos lacking the maternal *pop-1* expression, the MS blastomere adopts the fate of a wild-type E blastomere, producing excess intestinal tissues (Figure 1.4C; Lin *et al.,* 1995). All these observations suggest that POP-1 represses endoderm fate in MS and the Wnt/MAPK signaling downregulates the nuclear level of POP-1 in E, thereby allowing the endoderm fate.

Endoderm Formation Requires the Zygotic Expression of end Genes

The specification of endoderm fate in *C. elegans* also requires the zygotic expression of a genomic region (the endoderm-determining region or EDR; Zhu *et al.,* 1997). Deletions of the EDR completely block endoderm formation, causing the E cell to adopt the fate of its cousin, C blastomere (Zhu *et al.,* 1997). There appear to be multiple genes in the EDR [*end-1*, *dpr-1* (formerly *end-2*) and *end-3*] that regulate endoderm development. These genes might play redundant roles in endoderm development as expression of each of the three genes can rescue the endoderm differentiation defects in embryos homozygous for EDR deletions (Zhu *et al.,* 1997; Maduro and Rothman, 2002).

end-1/3 are the earliest expressed zygotic genes known in the endoderm lineage. Their transcripts are first detected specifically in the E cell and early E lineage, consistent with a direct role for *end-1/3* in endoderm development (Zhu *et al.,* 1997; Maduro and Rothman, 2002). Ectopic expression of *end-1* during a critical period in embryogenesis causes nonendodermal lineages to produce endoderm instead of ectoderm and/or mesoderm, and forced *end-1* expression bypasses the requirement for maternal SKN-1 and the maternal Wnt signaling pathway in endoderm formation (Zhu *et al.,* 1998). These results suggest that a major function of these maternal factors is to promote the zygotic expression of the *end* genes, which is sufficient to initiate endoderm differentiation.

end-1/3 appear to be direct targets of MED-1/2. There are multiple GATA binding sites in the promoter regions of both *end* genes (Zhu *et al.,* 1997; Maduro and Rothman, 2002; Broitman-Maduro *et al.*, 2005), and widespread expression of *med-1* in early embryos is sufficient to activate the expression of both (Maduro *et al.,* 2001). In fact, MED-1 interacts

directly with the *end-1* and *end-3* promoters in *C. elegans* early embryos (Maduro *et al.,* 2002).

However, the interaction between MED-1 and *end* promoters is found in both MS and E. It appears that POP-1 also binds to the *end* promoters in the MS blastomere, preventing their activation by MED-1, and binding of POP-1 to the *end* genes in the E blastomere is blocked by the Wnt/MAPK signaling (Figure 1.6; Maduro *et al.,* 2002). Therefore, the endoderm specification in *C. elegans* appears to be directed by the combined action of a positive regulator SKN-1 through its zygotic targets MED-1/2 and the Wnt/MAPK signaling which downregulates a negative regulator POP-1 (Figure 1.6). The final output is the expression of the *end* genes in the E lineage.

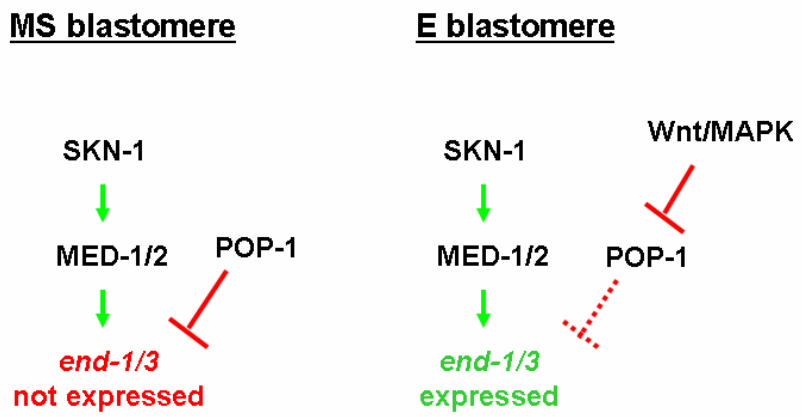


Figure 1.6 – Model for specification of E fate. POP-1 represses *end-1/3* activation by SKN-1 and MED-1/2 in the MS blastomere. In the E blastomere, the repressive effect of POP-1 is blocked by the Wnt/MAPK signaling. Activation is shown in green, and inhibition is shown in red.

Like *med-1* and *med-2*, both *end-1* and *end-3* encode GATA-type transcription factors, which contain a single zinc finger domain (Zhu *et al.,* 1997; Maduro and Rothman, 2002). Another gene in the EDR, *dpr-1*, encodes a nuclear receptor type transcription factor, and further studies show that *dpr-1* plays a later role in endoderm development (Maduro and Rotheman, 2002). GATA factors have also been implicated in endoderm development in *Drosophila* (Reuter, 1994; Rehorn *et al.,* 1996) and vertebrates (Laverriere *et al.,* 1994; Soudais *et al.,* 1995). These findings suggest that endoderm is specified by a conserved mechanism in all triploblastic animals (Shoichet *et al.*, 2000).

The MOM-2/Wnt Pathway

The endoderm-inducing MOM-2/Wnt pathway was characterized as a non-canonical pathway due to several significant differences from the canonical Wnt pathway described above. First, in the canonical Wnt pathway, the activity of TCF is required in the cells that receive the Wnt signal (Wnt-responsive cells), and mutations in TCF cause a similar phenotype as loss of Wnt signaling (Molenaar *et al.*, 1996; Brunner *et al.*, 1997; van de Wetering *et al.*, 1997). However, the *C. elegans* TCF homolog POP-1 is not required for the Wnt-responsive E cell fate, and mutations in POP-1 cause the opposite phenotype as loss of MOM-2 signaling (Lin *et al.*, 1995; Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). Second, the canonical Wnt pathway converts the transcriptional repressor TCF into a transcriptional activator and activates the expression of Wnt target genes in Wnt-responsive cells. However, it is believed that Wnt signaling lowers the nuclear level of POP-1, which alleviates the repressive activity of POP-1 and allows *end-1* expression and endoderm formation in the E

blastomere (Rocheleau *et al.,* 1997; Thorpe *et al.,* 1997; Calvo *et al.,* 2001). Third, the role of β-catenin in this Wnt pathway is not clear.

C. elegans has three β-catenin homologs, BAR-1, WRM-1, and HMP-2. It appears that the signaling and adhesion functions performed by a single β-catenin in *Drosophila* and two in vertebrates are distributed over the three β-catenins in *C. elegans* (Korswagen *et al.,* 2000; Natarajan *et al.,* 2001). BAR-1 interacts with POP-1 to function in the canonical Wnt signaling pathway. HMP-2 is the only β-catenin that interacts with HMR-1/cadherin and functions in cell adhesion. WRM-1 is the only β-catenin shown to be required for the specification of the endoderm fate (Rocheleau *et al.,* 1997). In the canonical Wnt pathway, Wnt signal leads to the accumulation of β-catenin in the nucleus where it complexes with TCF. However, the localization of WRM-1 has not been reported. Besides, no physical interaction between POP-1 and WRM-1 has been detected despite several attempts (Rocheleau *et al.,* 1999; Korswagen *et al.,* 2000; Natarajan *et al.,* 2001). These results suggest that WRM-1 may function in a non-canonical way in this MOM-2/Wnt signaling.

POP-1 ASYMMETRY

POP-1 Is Required for MS Fate Specification

pop-1 was first identified in a genetic screen for maternal effect lethal mutants with defects in pharyngeal development (Lin *et al.,* 1995). *pop-1* gene is expressed both maternally and zygotically. The *pop-1* mutation identified in this genetic screen *(zu189)* affects only the

maternal expression. The embryos produced from *pop-1(zu189)* homozygous mothers lack the posterior pharynx, which is normally generated by the MS blastomere. The partial pharynx (the anterior portion) in *pop-1(zu189)* mutant embryos is derived from the AB blastomere as in the wild-type. *pop-1(zu189)* mutant embryos produce about twice the size of a wild-type intestine, and it has been shown that the extra intestine is derived from MS. These results indicate that the MS blastomere adopts the fate of its sister E blastomere in *pop-1(zu189)* mutant embryos and POP-1 activity is required for the MS fate specification (Figure 1.4C; Lin *et al.,* 1995).

POP-1 Is an HMG Box Protein

pop-1 encodes a member of the HMG box superfamily of transcription factors. Based on sequence similarity, POP-1 belongs to the TCF/SOX subfamily of the HMG box proteins (Lin *et al.,* 1995). Within this subfamily, POP-1 is most closely related to TCF1 and LEF1 (Travis *et al.,* 1991; van de Wetering *et al.,* 1991; Waterman *et al.,* 1991; Lin *et al.,* 1995). POP-1 contains two features conserved with other TCF proteins, a centrally located DNA-binding HMG box and an N-terminal β-catenin binding domain (Figure 1.7; Lin *et al.*, 1995; Maduro *et al.*, 2002). The region between these two domains shares weak similarity to the Groucho-related gene (Grg) interaction domain of *Xenopus* Tcf-3 and has been proposed to be a putative Groucho binding domain (Maduro *et al.*, 2002). POP-1 shares about fifty percent sequence identity with TCF1 and LEF1 in the HMG box, and all these three proteins have a proline-rich region N-terminal to their HMG boxes (Lin *et al.,* 1995). The C-terminus of POP-1 has little sequence homology to any known protein.

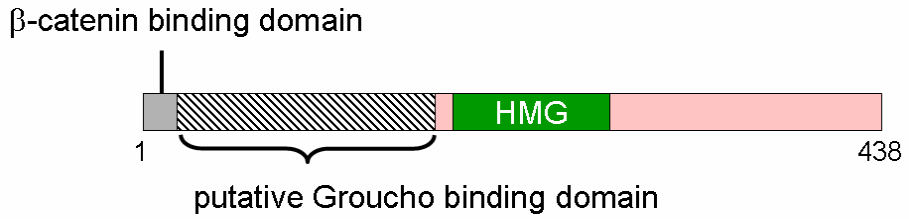


Figure 1.7 – POP-1 structure. POP-1 protein has 438 amino acids. The β-catenin binding domain is located at the N-terminus of POP-1. The single HMG DNA-binding domain is in the middle of the protein. There is a putative Groucho binding domain between these two domains.

POP-1 Asymmetry and A-P Fate Decisions

With antibody staining, POP-1 protein is first detected in the nuclei of maturing oocytes in the gonad. After fertilization, POP-1 is also detected in the nuclei of all early blastomeres (Lin *et al.,* 1995, 1998). The POP-1 staining detected before the 28-cell stage represents the maternal expression. After the 28-cell stage, the staining represents the zygotic expression of the *pop-1* gene (Lin *et al.,* 1995, 1998). In late-stage embryos, the nuclear POP-1 staining is prominent in the developing nervous system but absent from some other tissues such as the hypodermis (Lin *et al.,* 1998). During postembryonic development, the nuclear staining of POP-1 is detected in tissues such as seam cells (the hypodermal cells along the lateral surfaces of the body), the migratory Q neuroblasts and the developing gonad and vulva (Lin *et al.,* 1998).

Although POP-1 protein is present in all early blastomeres, a difference in staining intensities is seen in some pairs of sister blastomeres. The axes of cell divisions during *C. elegans* embryogenesis are either anterior-posterior (A-P) or transverse (dorsal-ventral or left-right). POP-1 nuclear staining is equal in sister cells born of transverse divisions.

However, a lower POP-1 staining is detected in the nuclei of posterior cells in almost all A-P divisions (Lin *et al.,* 1995, 1998). The asymmetric nuclear POP-1 pattern is also observed in postembryonic tissues (Lin *et al.,* 1998). This asymmetric nuclear POP-1 pattern in A-P sisters is called POP-1 asymmetry (Lin *et al.,* 1998).

In *C. elegans*, most cell divisions are oriented along the A-P axis, and most of the early A-P cell divisions result in sister cells with different developmental fates. Although different pathways determine cell fate difference in different sister pairs, several studies have suggested that cells throughout the embryo share a mechanism in recognizing a common A-P coordinate system to choose their fates (Mello *et al.,* 1992; Hutter and Schnabel, 1994; Mango *et al.,* 1994; Moskowitz *et al.,* 1994; Way *et al.,* 1994). POP-1 has been shown to be required for the MS/E cell fate difference (Lin *et al.,* 1995). Similarly, POP-1 is also responsible for the A-P fate difference in many pairs of sisters (Lin *et al.,* 1998; Schroeder and McGhee, 1998; Herman, 2001). Conditions that result in a high nuclear POP-1 level in the posterior sister cause a P to A cell fate transformation, whereas the absence of POP-1 in the anterior sister causes an A to P cell fate transformation (Lin *et al.,* 1995; Rocheleau *et al.,* 1997; Thorpe *et al.,* 1997; Lin *et al.,* 1998; Meneghini *et al.,* 1999; Rocheleau *et al.,* 1999; Shin *et al.,* 1999). Therefore, high nuclear POP-1 levels correlate with anterior cell fates, and low nuclear POP-1 levels correlate with posterior cell fates. These observations suggest that POP-1 asymmetry may provide a general A-P coordinate system that collaborates with other transcription factors to diversify sister cells.

Regulation of POP-1 Asymmetry

As described above, POP-1 asymmetry in the MS/E sister blastomeres requires the Wnt/MAPK signaling (Rocheleau *et al.,* 1997; Thorpe *et al.,* 1997; Meneghini *et al.,* 1999; Rocheleau *et al.,* 1999; Shin *et al.*, 1999). This Wnt signal appears to be provided by the P2 blastomere as revoming P2 at early four-cell stage leads to equivalent levels of POP-1 between MS and E (Lin *et al.*, 1998). The signaling source for POP-1 asymmetry outside of MS/E has not been identified. It appears that neither the P2 blastomere nor the Wnt molecule MOM-2 is required (Lin et al., 1998; Park and Priess, 2003; R.O. and R.L., personal communication). Within the AB lineage, POP-1 asymmetry is first detected after the third division of AB when there are four A-P pairs of AB descendants (the AB^8 cells) (Lin *et al.*, 1998). It has been shown that several descendants of the P1 blastomere, including E, C, and P3 and to a lesser extent MS, can induce POP-1 asymmetry in the AB^8 cells when grafted onto the isolated AB^8 precursors (Park and Priess, 2003). In these experiments, MOM-2 is essential for signaling from C, but not MS, to establish POP-1 asymmetry (Park and Priess, 2003). Since the AB^4 cells (the mother cells of the AB^8 cells) normally contact one or more of these P1 descendants in the intact embryo, it is likely that these P1 descendants function as signaling cells to induce POP-1 asymmetry observed in the AB^8 cells. However, the AB descendants after the AB^8 stage (AB^{16} and AB^{32} cells) as well as the A-P descendants of MS and E are able to generate POP-1 asymmetry without prior exposure to the signaling cells P2, MS, E, C, or P3 (Park and Priess, 2003). These results suggest that these older embryonic cells might be able to generate an intrinsic polarity along the A-P axis to establish POP-1 asymmetry independent of the MOM-2/Wnt signaling.

The intrinsic POP-1 asymmetry observed in the isolated AB descendants described above requires MOM-5/Fz (Park and Priess, 2003). This result suggests that MOM-5 may function to establish POP-1 asymmetry independent of the MOM-2 signaling. It is not clear whether a Wnt molecule is required to activate MOM-5 to generate POP-1 asymmetry in these cells. Consistent with a role for MOM-5 to lower the nuclear POP-1 level in posterior cells, an enrichment of a MOM-5::GFP fusion protein at the posterior pole of AB descendants prior to division has been observed (Park *et al.*, 2005). Similarly, this MOM-5::GFP asymmetry does not require MOM-2 activity, further supporting the hypothesis that MOM-5 can regulate POP-1 asymmetry without MOM-2 signal. One puzzle is that in intact embryos depleted of MOM-5 activity, the AB^{16} and AB^{32} cells usually show POP-1 asymmetry, whereas in embryos depleted of both MOM-2 and MOM-5, POP-1 asymmetry is lost (Park and Priess, 2003). These results suggest that in intact embryos MOM-2 and MOM-5 may function redundantly to generate POP-1 asymmetry in the AB^{16} and AB^{32} cells through independent pathways.

Unlike MOM-2 or MOM-5, WRM-1 and LIT-1 are required to establish POP-1 asymmetry throughout the early embryo (Lin *et al.*, 1998; Meneghini *et al.,* 1999; Rocheleau *et al.,* 1999; R.O. and R.L., personal communication). MOM-4 is also required for POP-1 asymmetry in AB descendants (Meneghini *et al.*, 1999; Shin *et al.*, 1999). However, MOM-4 seems to play less of an important role in POP-1 asymmetry in the MS/E pair (Meneghini *et al.*, 1999). Consistent with this, *mom-4* mutant embryos exhibit only partially penetrant losses of endoderm (Meneghini *et al.*, 1999).

The role of WRM-1 in the MOM-2/Wnt pathway is not clear. Althogh WRM-1 is required for POP-1 asymmetry, the mechanism by which WRM-1 leads to a lower nuclear POP-1 level in posterior cells is not known. As mentioned earlier, unlike the β-catenin in canonical Wnt pathways, WRM-1 does not seem to interact with POP-1 physically. Moreover, it has been shown that the β-catenin binding domain of POP-1 is dispensable for both its nuclear asymmetry and function in early embryos (Maduro *et al.*, 2002), making it unlikely that WRM-1 regulates the nuclear POP-1 level through direct binding to POP-1. It has been shown that WRM-1 and LIT-1 form a stable complex *in vivo* and that WRM-1 can activate the LIT-1 kinase activity when coexpressed in mammalian tissue culture cells (Rocheleau *et al.,* 1999). Therefore, it is likely that the only function of WRM-1 in the MOM-2 signaling is to activate the LIT-1 kinase activity.

The LIT-1 kinase activated by WRM-1 can phosphorylate POP-1 *in vitro*. WRM-1 and LIT-1 are also shown to promote POP-1 to redistribute from the nucleus to the cytoplasm in mammalian cells (Rocheleau *et al.,* 1999). These results suggest that phosphorylation of POP-1 by LIT-1 results in the nucleocytoplasmic redistribution of POP-1 in mammalian cells. A similar regulation may function in *C. elegans* embryos, as estimates of combined nuclear and cytoplasmic POP-1 in anterior and posterior cells were similar, suggesting nucleocytoplasmic redistribution as a mechanism for the observed POP-1 asymmetry (Maduro *et al.,* 2002). Consistent with this, a slightly higher cytoplasmic POP-1 level was seen in posterior cells than in their anterior sisters (Maduro *et al.*, 2002). Nevertheless, the mechanism changing the subcellular localization of POP-1 following the Wnt/MAPK stimulation remains to be determined.

THE AIM OF THIS STUDY

In addition to a difference in the nuclear POP-1 levels, A-P sisters also exhibit a difference in the cytoplasmic POP-1 levels. To be more specific, I will refer to the asymmetric nuclear POP-1 pattern as POP-1 nuclear asymmetry in this study.

The major aim of this study was to investigate the mechanism by which the Wnt/MAPK signaling downregulates POP-1 in posterior cells. In chapter two, I described the identification of the *cis*-elements important for POP-1 nuclear asymmetry and/or repressor activity. The proposed mechanisms for POP-1 nuclear asymmetry and data supporting them were presented in chapters three and four. In chapter five, the sufficiency for POP-1 domains in conferring A-P nuclear asymmetry in *C. elegans* embryos was tested in heterologous TCF proteins. Finally, the regulation of a Wnt-responsive gene by POP-1 and some components of the Wnt/MAPK signaling was described in chapter six.

Most experiments presented here were conducted in the Lin laboratory at University of Texas Southwestern Medical Center from 2001 to 2005. Some experiments were accomplished through collaboration with the Shi laboratory at Harvard Medical School.

CHAPTER TWO
Identifying *cis*-Elements Required for POP-1 Nuclear Asymmetry and/or Repressor Activity

INTRODUCTION

The Wnt/MAPK-responsive E blastomere has a low nuclear level of POP-1. This low nuclear POP-1 level seems to correlate with the specification of E fate because in many Wnt/MAPK mutants, the nuclear level of POP-1 is high in E and the subsequent endoderm tissues are not formed properly (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997; Lin *et al.*, 1998; Rocheleau *et al.*, 1999). This observation raises several questions. First, is a low nuclear level of POP-1 required for the E fate or is it just a consequence of the Wnt/MAPK signaling? Second, do different nuclear levels of POP-1 determine different fates? Third, how is the nuclear level of POP-1 regulated by the Wnt/MAPK signaling? Immunostaining of POP-1 in a *lit-1(t1534)* strain showed that the nuclear level of POP-1 was high in the E blastomere (Maduro et al., 2002). However, almost all the *lit-1(t1534)* mutant embryos produce endoderm (Rocheleau *et al.*, 1999). These results suggest that POP-1 nuclear asymmetry can be uncoupled from endoderm specification (Maduro *et al.*, 2002) and perhaps a low nuclear level of POP-1 is not absolutely required for the E fate as long as its endoderm repressive function is blocked in the E blastomere.

To study how the nuclear level of POP-1 is regulated by the Wnt/MAPK signaling, Maduro *et al.* have expressed GFP::POP-1 fusion constructs in *C. elegans* early embryos using the *med-1* promoter, which is proposed to be transcribed only in the EMS blastomere

($P_{med\text{-}1}$*gfp::pop-1*; Figure 2.1; Maduro *et al.,* 2001, 2002). The expression of the $P_{med\text{-}1}$ *gfp::pop-1* transgene is restricted to the EMS lineage and first detected in the MS and E blastomeres at the eight-cell stage. The GFP fluorescence is present at several subsequent stages even when the transgene mRNA is no longer detectable (Maduro *et al.*, 2002). This GFP::POP-1 fusion protein can respond to the Wnt/MAPK signaling to exhibit asymmetric nuclear levels between A-P sisters, and like the endogenous POP-1 protein, the nuclear asymmetry of GFP::POP-1 is lost in many Wnt/MAPK mutants such as *mom-2*, *mom-4* and *lit-1* as well as in *wrm-1(RNAi)* embryos (Maduro *et al.*, 2002; R.O. and R.L., personal communication). Expression of $P_{med\text{-}1}$*gfp::pop-1* in *pop-1(zu189)* mutant embryos can rescue the MS fate defect, suggesting that GFP::POP-1 is able to repress the endoderm fate in the MS blastomere. Furthermore, in the rescued embryos, the intestine appears normal, demonstrating that the Wnt/MAPK signaling is capable of blocking the endoderm repressive function of GFP::POP-1 in the E blasotmere (Maduro *et al.*, 2002).

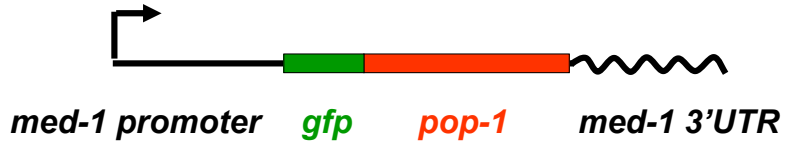


Figure 2.1 – Diagram of the transgene $P_{med\text{-}1}$*gfp::pop-1*.

The fact that the GFP::POP-1 recapitulates the asymmetric nuclear POP-1 pattern observed with antibody staining reflects that POP-1 nuclear asymmetry is, at least in part, resulted from genuine differences in nuclear protein levels. Quantification of the GFP::POP-1

intensities in both nuclear and cytoplasmic compartments of A-P sisters revealed that both anterior and posterior cells have a similar total amount of GFP::POP-1 (Maduro *et al.*, 2002). Therefore, the observed POP-1 nuclear asymmetry is likely a result of nucleo-cytoplasmic redistribution of POP-1 upon the Wnt/MAPK signaling, rather than differential stability, synthesis, or immunoreactivity. However, the mechanism that leads to this differential distribution of POP-1 between A-P sisters remains unclear.

In a deletion analysis, Maduro *et al*. showed that the β-catenin binding domain, the HMG box, and the region C-terminal to the HMG box are not required for the A-P nuclear asymmetry, whereas the region between the β-catenin binding domain and the HMG box (the putative Groucho binding domain) is critical for the A-P nuclear asymmetry (Maduro *et al.*, 2002).

Although the general structural requirements that allow POP-1 to respond to the Wnt/MAPK signaling and exhibit asymmetric nuclear levels were shown, a detailed dissection of the region critical for POP-1 nuclear asymmetry is still lacking. The amino acids responsive to the regulation by the signaling have also not been identified. Although it has been suggested that POP-1 nuclear asymmetry can be uncoupled from its function as a repressor (Maduro *et al.*, 2002), the regions and amino acids important for the endoderm repressive function of POP-1 have not been reported either. The region between the β-catenin binding domain and the HMG box serves as a good candidate, as this region is a potential interaction domain for the corepressor Groucho (Maduro *et al.*, 2002). The function of the C-terminus of POP-1 is also poorly understood, as it shows little sequence similarity to any known protein.

To further study the roles of different POP-1 domains in nuclear asymmetry and endoderm repression, I decided to map the *cis*-elements important for the regulation of POP-1 nuclear levels and/or repressor activity. Identifying these *cis*-elements will also help us understand the mechanism(s) by which the Wnt/MAPK signaling downregulates POP-1 in posterior cells. I expressed GFP::POP-1 fusions in which different domains of POP-1 were deleted or different amino acids of POP-1 were mutated in *C. elegans* early embryos using the *med-1* promoter. The cellular localization of these GFP::POP-1 fusions were examined and their endoderm repressive function was tested in the *pop-1(zu189)* mutant background.

RESULTS

DELETION ANALYSIS

Assay for POP-1 Nuclear Asymmetry

The First 100 Amino Acids of POP-1 Are Not Essential for POP-1 Nuclear Asymmetry

I and a lab member, Raanan Odom, generated two transgenic strains carrying the wild-type $P_{med\text{-}1}$*gfp::pop-1* transgene. The strain TX352 maintains the transgene as an extra-chromosomal array, and the strain TX300 contains the transgene integrated into chromosome V of the genome. Consistent with previous reports, the wild-type GFP::POP-1 in both strains showed asymmetric nuclear levels between A-P sisters, with a higher level in the anterior nuclei (Figure 2.2 A and B; Maduro *et al.*, 2002; Gay *et al.*, 2003). In addition to having different nuclear levels between A-P sisters, the wild-type GFP::POP-1 formed prominent

punta specifically in the nuclei of anterior but never in those of posterior sisters (Figure 2.2 A and B; Maduro *et al.*, 2002). Similar puncta have also been reported for endogenous POP-1 by immunostaining in wild-type embryos but not observed with an overexpressed GFP::MED-1 transgene, suggesting that these structures are likely a real property of POP-1 in unsignaled anterior nuclei (Maduro *et al.*, 2002).

Several deletions of *pop-1* coding sequences were introduced into the *med-1gfp* construct and these POP-1 deletions were assayed for localization in embryos. Among all the POP-1 deletions I tested, two still retained the A-P nuclear asymmetry (Figure 2.2 and Figure 2.6 A). POP-$1_{\Delta 1\text{-}47}$, where the β-catenin binding domain was deleted, showed normal nuclear asymmetry, and also formed puncta in the anterior nuclei (Figure 2.2 C and D). This result is consistent with the report by Maduro *et al.* that the N-terminal β-catenin binding domain is not required for POP-1 nuclear asymmetry (Maduro *et al.*, 2002). POP-$1_{\Delta 1\text{-}100}$ also showed asymmetric nuclear levels between A-P sisters, although the A-P difference was less obvious than that in the wild-type (Figure 2.2 E and E). The nuclear GFP signal for POP-$1_{\Delta 1\text{-}100}$ was more homogeneous as I did not observe nuclear punctate structures in the transgenic embryos expressing GFP::POP-$1_{\Delta 1\text{-}100}$.

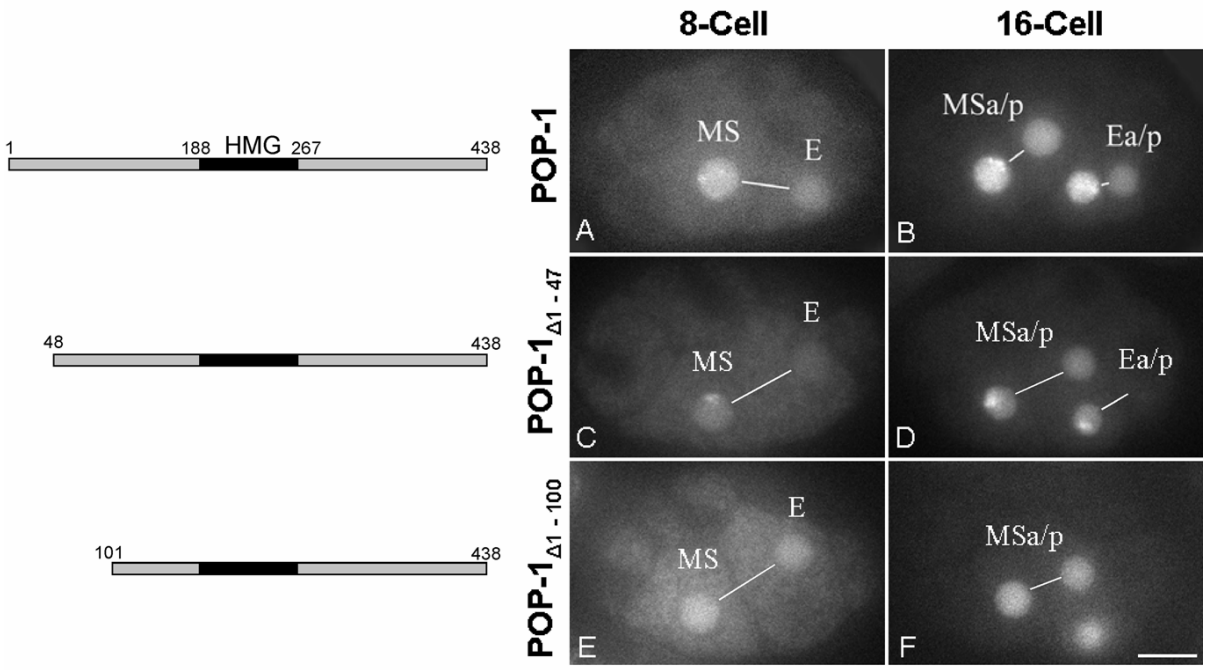


Figure 2.2 – Amino acids 1 through 100 of POP-1 are not essential for POP-1 nuclear asymmetry. GFP fluorescence micrographs of eight-cell (A, C, and E) or 16-cell (B, D, and F) transgenic embryos harboring $P_{med\text{-}1}$*gfp::pop-1* (TX352; A and B), $P_{med\text{-}1}$*gfp::pop-1*$_{\Delta 1\text{-}47}$ (TX540; C and D), or $P_{med\text{-}1}$*gfp::pop-1*$_{\Delta 1\text{-}100}$ (TX608; E and F). A-P sisters are connected by lines and their names are indicated. Except the Ep in D, the nuclei of each A-P sister blastomeres are equally focused at that particular focal plane. The high background in A, C, and E is due to the low GFP signal at the eight-cell stage of those embryos. Scale bar, 10 μm.

aa$_{101\text{-}130}$ *and aa*$_{400\text{-}438}$ *Regions of POP-1 Are Required for POP-1 Nuclear Asymmetry*

I found that the POP-1 protein construct with a 43-amino acid deletion within the putative Groucho interaction domain (POP-1$_{\Delta 88\text{-}130}$) lost its nuclear asymmetry. Two C-terminal deletions of POP-1, POP-1$_{\Delta 281\text{-}438}$ and POP-1$_{\Delta 400\text{-}438}$, also exhibited symmetric nuclear patterns (Figure 2.3 and Figure 2.6 A). Although all these three versions of POP-1 showed equal levels between A-P nuclei, I observed some minor differences in their nuclear GFP fluorescence. I found that both GFP::POP-1$_{\Delta 281\text{-}438}$ (Figure 2.3 E and F) and GFP::POP-1$_{\Delta 400\text{-}}$

$_{438}$ (Figure 2.3 G and H) were mostly nuclear, and both of them formed clear puncta in both anterior and posterior nuclei. However, GFP::POP-$1_{\Delta 88\text{-}130}$ showed a homogeneous nuclear pattern (Figure 2.3 C and D). In addition to the difference in nuclear GFP appearance, POP-$1_{\Delta 88\text{-}130}$ seemed to have a slightly elevated cytoplasmic level compared to either wild-type POP-1 or the two POP-1 C-terminal deletions. However, it is not clear why POP-$1_{\Delta 88\text{-}130}$ has a higher cytoplasmic level and whether its nuclear level is lower than that of wild-type POP-1 because different transgenic lines may have very different expression levels of the transgenes they carry.

Based on all the results described above, it is possible that the regions essential for POP-1 nuclear asymmetry are $aa_{101\text{-}130}$ and $aa_{400\text{-}438}$ (Figure 2.6 B). Without a more detailed deletion analysis, I do not know whether $aa_{281\text{-}399}$ is required for POP-1 nuclear asymmetry. In contrast to my results, however, Maduro *et al.* showed that a POP-1 deletion lacking the entire C-terminus (POP-$1_{\Delta 169\text{-}438}$), including the HMG box, still exhibited A-P nuclear asymmetry (Maduro *et al.*, 2002). However, POP-$1_{\Delta 169\text{-}438}$ was localized predominantly in the cytoplasm. It may be more difficult to unambiguously judge the nuclear asymmetry when the nuclear level is low.

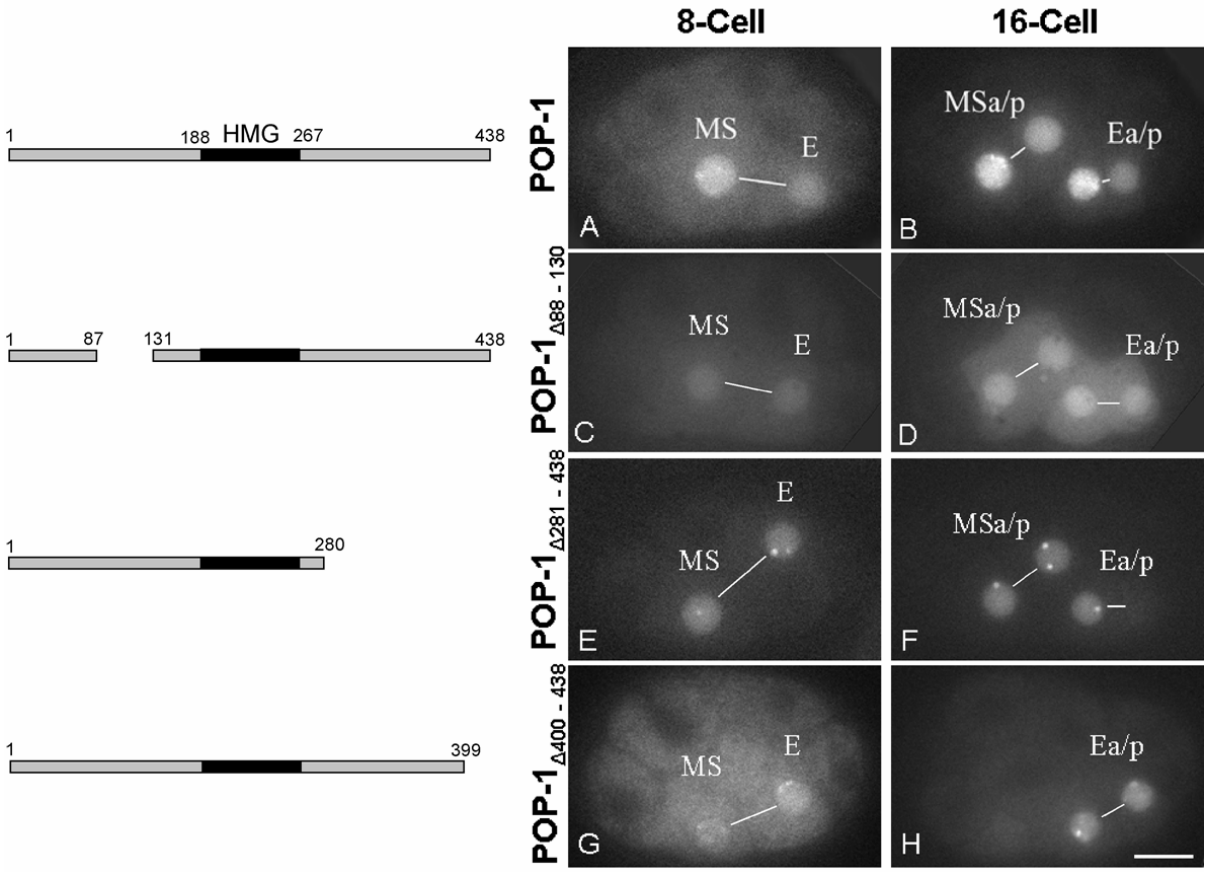


Figure 2.3 – POP-1 deletions that abolish A-P nuclear asymmetry. GFP fluorescence micrographs of eight-cell (A, C, E, and G) or 16-cell (B, D, F, and H) transgenic embryos harboring $P_{med\text{-}1}$ *gfp::pop-1* (TX352; A and B), $P_{med\text{-}1}$*gfp::pop-1*$_{\Delta 88\text{-}130}$ (TX348; C and D), $P_{med\text{-}1}$*gfp::pop-1* $\Delta_{281\text{-}438}$ (TX589; E and F), or $P_{med\text{-}1}$*gfp::pop-1*$\Delta_{400\text{-}438}$ (TX616; G and H). A-P sisters are connected by lines and their names are indicated. Except the Ep in F, the nuclei of each A-P sister blastomeres are equally focused at that particular focal plane. The high background in A and G is due to the low GFP signal at the eight-cell stage of those embryos. POP-$1_{\Delta 88\text{-}130}$ has a higher cytoplasmic level compared to the wild-type POP-1 (C and D). However, it is not clear whether the nuclear level of POP-$1_{\Delta 88\text{-}130}$ is lower than that of wild-type POP-1 because different transgenic lines may have very different transgene expression levels. Scale bar, 10 μm.

$aa_{101\text{-}130}$ of POP-1 Is Not Sufficient for POP-1 Nuclear Asymmetry

Because neither POP-$1_{\Delta 88\text{-}130}$ nor POP-$1_{\Delta 400\text{-}438}$ exhibit POP-1 nuclear asymmetry, it is likely that neither $aa_{101\text{-}130}$ nor $aa_{400\text{-}438}$ is sufficient for POP-1 nuclear asymmetry between A-P

sisters. However, to test their sufficiency in conferring A-P nuclear asymmetry, I also generated GFP fusion proteins containing different fragments of POP-1. I found that although required to respond to the Wnt/MAPK signaling for POP-1 to be lowered in the posterior nuclei, $aa_{101-130}$ region was not sufficient for POP-1 nuclear asymmetry. The three GFP::POP-1 fusion proteins containing $aa_{101-130}$, POP-1_{81-297}, POP-1_{61-180}, and POP-1_{81-200}, all showed equal nuclear levels between A-P sisters (Figure 2.4). POP-1_{81-297} was mostly nuclear and formed puncta in the nuclei of both anterior and posterior cells (Figure 2.4 C and D). Both POP-1_{61-180} and POP-1_{81-200} were localized predominantly in the cytoplasm, probably due to the lack of the DNA-binding HMG domain and the putative nuclear localization sequence (NLS, $aa_{272-280}$; Prieve *et al.*, 1996) located immediately C-terminal to the HMG domain (Figure 2.4 E-H). So far, no GFP::POP-1 fusion containing only $aa_{400-438}$ has been tested to conclude the sufficiency for $aa_{400-438}$ in conferring POP-1 nuclear asymmetry.

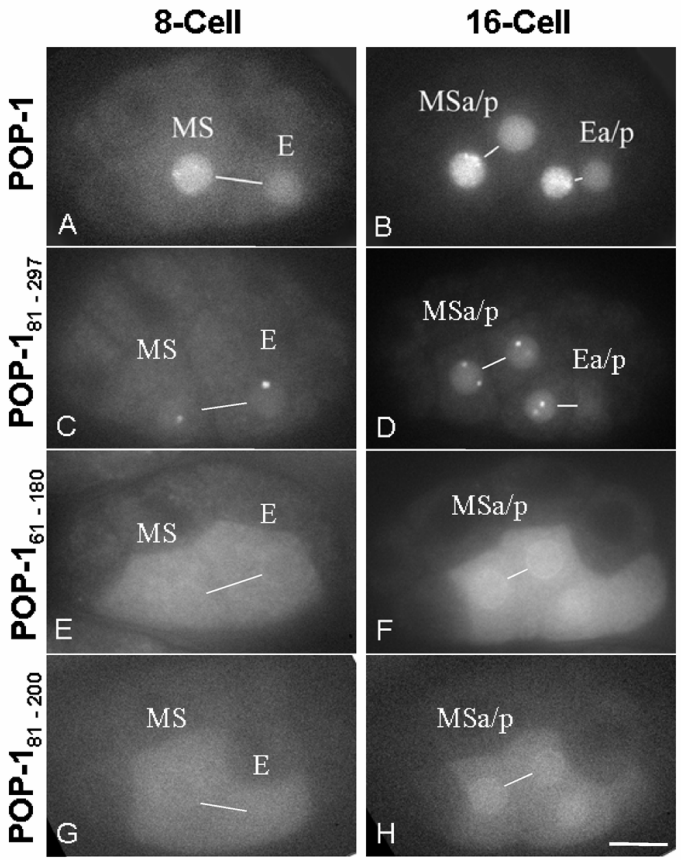


Figure 2.4 – The amino acid 101-130 region of POP-1 is not sufficient for POP-1 nuclear asymmetry. GFP fluorescence micrographs of eight-cell (A, C, E, and G) or 16-cell (B, D, F, and H) transgenic embryos harboring $P_{med\text{-}1}$*gfp::pop-1* (TX352; A and B), $P_{med\text{-}1}$*gfp::pop-1*$_{81\text{-}297}$ (TX618; C and D), $P_{med\text{-}1}$*gfp::pop-1*$_{61\text{-}180}$ (TX521; E and F), or $P_{med\text{-}1}$*gfp::pop-1*$_{81\text{-}200}$ (TX410; G and H). A-P sisters are connected by lines and their names are indicated. Except the Ep in D, the nuclei of each A-P sister blastomeres are equally focused at that particular focal plane. The high background in A, C, E and G is due to the low GFP signal at the eight-cell stage of those embryos. Scale bar, 10 μm.

HMG Box Alone Is Not Sufficient for POP-1 Nuclear Asymmetry

I also generated three GFP::POP-1 fusions containing the HMG box. Although these three fusion proteins all exhibited symmetric nuclear levels between A-P sisters, they had some differences in their subcellular localization (Figure 2.5 and Figure 2.6 A). POP-1$_{178\text{-}297}$, which

contains the entire HMG box and the putative NLS, was mostly nuclear with puncta in both anterior and posterior cells (Figure 2.5 B). Removing the putative NLS in this POP-1 fragment (POP-$1_{178\text{-}270}$) resulted in a dramatic increase in its cytoplasmic level (Figure 2.5 C), suggesting that $aa_{272\text{-}280}$ is likely to function as an NLS as predicted by analysis of the corresponding region of the human LEF1 (Prieve *et al.*, 1996). Another GFP fusion construct, which contains POP-$1_{189\text{-}308}$, also had an elevated cytoplasmic level (Figure 2.5 D), probably due to a defect in nuclear retention. POP-$1_{189\text{-}308}$ contains the putative NLS and most of the HMG box, except its first amino acid, lysine 188. Lysine 188 and two other lysines (185 and 187) of POP-1 have been shown to be required for POP-1 nuclear retention. Mutations of these three lysines to alanines dramatically increase the cytoplasmic level of POP-1 (Figure 3.3 E and G; Gay *et al.*, 2003), which can be rescued by blocking the nuclear export machinery (see chapter three; Lo *et al.*, 2004).

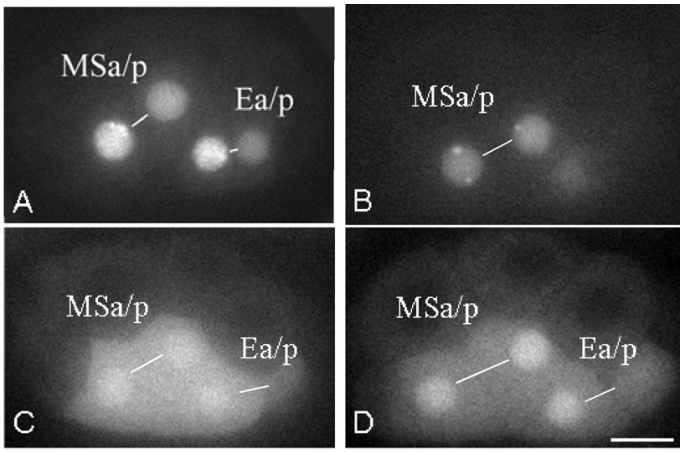


Figure 2.5 – Subcellular localization of POP-1 fragments containing the HMG box. GFP fluorescence micrographs of 16-cell transgenic embryos harboring (A) $P_{med\text{-}1}$*gfp::pop-1* (TX352), (B) $P_{med\text{-}1}$*gfp::pop-*$1_{178\text{-}297}$ (TX426), (C) $P_{med\text{-}1}$*gfp::pop-*$1_{178\text{-}270}$ (TX648), or (D) $P_{med\text{-}1}$*gfp::pop-*$1_{189\text{-}308}$ (TX522). A-P sisters are connected by lines and their names are indicated. Except the Ep in C and D, the nuclei of each A-P sister blastomeres are equally focused at that particular focal plane. Scale bar, 10 μm.

Assay for Endoderm Repressive Activity

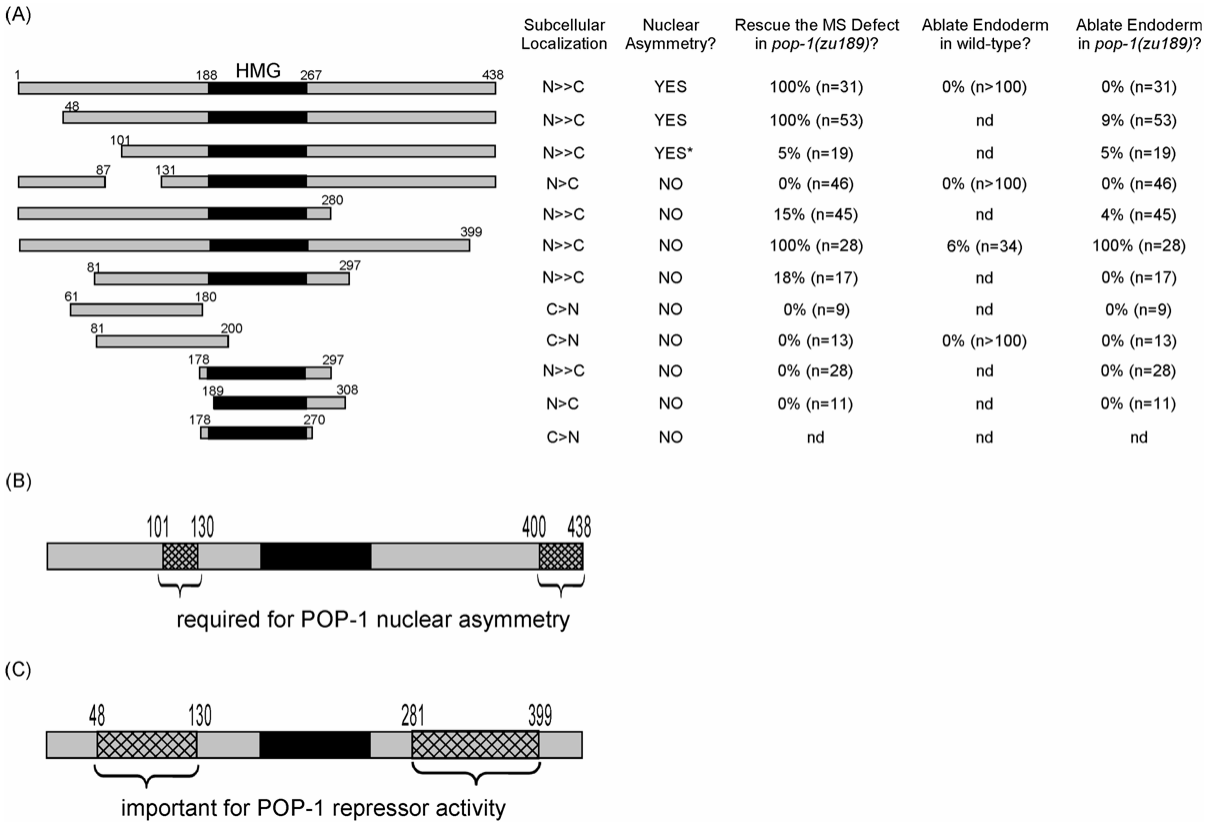
aa_{48-130} and $aa_{281-399}$ Regions of POP-1 Are Important for POP-1 Repressor Activity

I also assayed the endoderm repressive function of each GFP::POP-1 fusion protein in *pop-1 (zu189)* mutant embryos. The endoderm repressive function of POP-1 was determined by whether the MS fate defect in *pop-1(zu189)* embryos was rescued, that is, repression of MS-derived endoderm and formation of the posterior pharynx, when the various *gfp::pop-1* transgenes were introduced into *pop-1(zu189)* mutant background (Figure 2.6 A). The wild-type POP-1 was able to rescue the MS fate defect in all the *pop-1(zu189)* embryos carrying the transgene (100%, n = 31; data not shown). POP-$1_{\Delta 1-47}$ also rescued the MS fate defect comparable to the wild-type POP-1 construct (100%, n = 53; data not shown). However, the ability to repress the MS-derived endoderm was significantly affected when the first 100 amino acids of POP-1 were deleted (POP-$1_{\Delta 1-100}$, 5%, n = 19; data not shown). Deletion of aa_{88-130}, in addition to abolishing POP-1 nuclear asymmetry, totally blocked the endoderm repressive activity of POP-1 (0%, n = 46; data not shown). Without a more detailed deletion analysis, I can only conclude that the aa_{88-130} region of POP-1 is essential for, but aa_{48-87} may also play an important role in the endoderm repression (Figure 2.6 C). These results also suggest that the putative Groucho interaction domain contains elements critical for POP-1 repressor activity.

I found that aa_{48-130} is not the only region important for the endoderm repressive function of POP-1. When $aa_{281-438}$ of POP-1 was deleted, the endoderm repressive function of POP-1 also decreased dramatically (15%, n = 45; data not shown). However, although required for POP-1 nuclear asymmetry, $aa_{400-438}$ of POP-1 is not required for its endoderm

repressive activity, as all the *pop-1(zu189)* embryos carrying the *gfp::pop-1*$_{\Delta 400\text{-}438}$ transgene formed the posterior pharynx (100%, n = 28; data not shown). Therefore, POP-1 contains at least two regions functioning in the endoderm repression, aa$_{48\text{-}130}$ and aa$_{281\text{-}399}$ (Figure 2.6 C). aa$_{48\text{-}130}$ appears to play a more important role in the endoderm repression than aa$_{281\text{-}399}$, as the deletion of aa$_{88\text{-}130}$ abolishes the endoderm repressive activity of POP-1 completely, whereas POP-1$_{\Delta 281\text{-}438}$ still retains some repressor activity.

The ability of POP-1$_{81\text{-}297}$ to repress endoderm was similar to that of POP-1$_{\Delta 281\text{-}438}$ (18%, n = 17; data not shown). Both of the two POP-1 fragments that do not contain the HMG box but contain the region essential for endoderm repression, POP-1$_{61\text{-}180}$ and POP-1$_{81\text{-}200}$, failed to rescue the MS fate defect in *pop-1(zu189)* (0%, n = 9 and 13, respectively; data not shown). Neither did the two POP-1 fragments that contain mostly the HMG box, POP-1$_{178\text{-}297}$ and POP-1$_{189\text{-}308}$ (0%, n = 28 and 11, respectively; data not shown). These results suggest that both a DNA binding domain and a “repressor” domain(s) are required for POP-1 to repress endoderm fate.



Subcellular Localization	Nuclear Asymmetry?	Rescue the MS Defect in *pop-1(zu189)*?	Ablate Endoderm in wild-type?	Ablate Endoderm in *pop-1(zu189)*?
N>>C	YES	100% (n=31)	0% (n>100)	0% (n=31)
N>>C	YES	100% (n=53)	nd	9% (n=53)
N>>C	YES*	5% (n=19)	nd	5% (n=19)
N>C	NO	0% (n=46)	0% (n>100)	0% (n=46)
N>>C	NO	15% (n=45)	nd	4% (n=45)
N>>C	NO	100% (n=28)	6% (n=34)	100% (n=28)
N>>C	NO	18% (n=17)	nd	0% (n=17)
C>N	NO	0% (n=9)	nd	0% (n=9)
C>N	NO	0% (n=13)	0% (n>100)	0% (n=13)
N>>C	NO	0% (n=28)	nd	0% (n=28)
N>C	NO	0% (n=11)	nd	0% (n=11)
C>N	NO	nd	nd	nd

Figure 2.6 – Domains required or important for POP-1 nuclear asymmetry and/or repressor activity. (A) Schematic diagram of full-length POP-1 and various POP-1 deletions. The HMG domain (188-267) is shown as a black box. Numbers above the proteins indicate positions of amino acids. The subcellular localization and effect on POP-1 nuclear asymmetry as GFP fusions, the ability to rescue the MS defect in *pop-1(zu189)* embryos, and the effect on endoderm formation in wild-type and *pop-1(zu189)* backgrounds are indicated to the right. The asterisk indicates that POP-1 nuclear asymmetry is slightly affected. N, nucleus. C, cytoplasm. nd, not determined. (B and C) Schematic diagram of POP-1 protein. The regions required for POP-1 nuclear asymmetry (B) or important for repressor activity (C) are shown as hatched boxes. The numbers indicate the positions of amino acids.

POINT MUTATION ANALYSIS

The $aa_{101-130}$ and $aa_{400-438}$ Regions of POP-1

The deletion analysis showed that both the $aa_{101-130}$ and $aa_{400-438}$ regions of POP-1 are essential for the low nuclear level of POP-1 in posterior cells. To investigate the roles that these two regions might play in the regulation of nuclear POP-1 levels in posterior cells, I decided to analyze these two regions further. Mutational analyses of these two regions were performed. The results from these mutational analyses and other experiments suggest that the mechanism by which $aa_{101-130}$ mediates POP-1 nuclear asymmetry is differential nuclear export of POP-1 protein between A-P sisters and that $aa_{400-438}$ might confer POP-1 nuclear asymmetry through a differential protein degradation mechanism. To not disrupt the flow of this chapter, the data are presented in chapter three (for the $aa_{101-130}$ region) and chapter four (for the $aa_{400-438}$ region).

Miscellaneous Point Mutations

Serines 71 and 82 Are Not Important for POP-1 Nuclear Asymmetry or Repressor Activity

Serines 71 and 82 are the two amino acids corresponding to the NLK phosphorylation sites on mammalian TCF4, T_{178} and T_{189} (Ishitani *et al.*, 2003). It has been shown that the phosphorylation of TCF4 at T_{178} and T_{189} inhibits the DNA binding activity of the β-catenin/TCF complex *in vitro* (Ishitani *et al.*, 2003). Therefore, it is possible that POP-1 is also regulated by LIT-1 in a similar way. LIT-1 may phosphorylate POP-1 at S_{71} and S_{82} to affect its DNA binding activity. To test whether S_{71} and S_{82} play any roles in POP-1 nuclear

asymmetry and repressor activity, I mutated these two sites to either alanines (un-phosphorylated state) or aspartates (mimicking phosphorylated state). In either case, POP-1 nuclear asymmetry was not affected (data not shown). Besides, both POP-$1_{S71/82A}$ and POP-$1_{S71/82D}$ rescued the MS fate defect in *pop-1(zu189)* embryos comparable to wild-type POP-1 (Table 2.1; data not shown). Therefore, S_{71} and S_{82} do not appear essential for POP-1 nuclear asymmetry or repressor activity. Although aspartates may not exactly mimic phosphorylation in this case, these results suggest that LIT-1 may not regulate POP-1 on S_{71} and S_{82} in the same way that NLK regulates TCF4 on T_{178} and T_{189}.

Phenylalanines 93, 97, 112, 121, and 124 Are Important for Both POP-1 Nuclear Asymmetry and Repressor Activity

In the absence of Wnt signaling, TCF functions as a transcriptional repressor by interacting with members of the Groucho-related family of corepressors (Cavallo *et al.,* 1998). Although the interaction between POP-1 and a Groucho-like protein has not been reported in *C. elegans*, the region between the β-catenin-binding domain and the HMG domain of POP-1 has been proposed to be a putative Groucho binding domain due to its weak similarity to the Grg interaction domain of *Xenopus* Tcf-3 (Maduro *et al.*, 2002). Besides, a corepressor complex containing the histone deacetylase HDA-1 and the Groucho-like protein, UNC-37 has been shown to be required for the repression of *end-1* in the MS blastomere (Calvo *et al.*, 2001). Therefore, it is likely that POP-1 represses *end-1* expression by recruiting UNC-37 through the putative Groucho binding domain. I show earlier that aa_{88-130} is essential for the

endoderm repressive activity of POP-1, and thus this region is likely to mediate the binding between POP-1 and UNC-37.

Groucho-related proteins have been shown to interact with many different transcription factors (Paroush *et al.*, 1994; Jimenez *et al.*, 1997; Roose *et al.*, 1998; Zhu *et al.*, 2002). One such factor is Six3, a member of the evolutionarily conserved So/Six homeodomain family, which plays an important role in the development of the visual system in vertebrates (Oliver *et al.*, 1996; Loosli *et al.*, 1999; Lagutin *et al.*, 2001; Zhu *et al.*, 2002). Members of the So/Six gene family encode proteins that have a conserved Six domain and a homeodomain. Six3 interacts with mouse Grg5, and this interaction requires the Q domain of Grg5 and a conserved phenylalanine residue present in an eh1-like motif located in the Six domain of Six3 (Zhu *et al.*, 2002). Although POP-$1_{88\text{-}130}$ does not contain any eh1-like motif, there are five phenylalanine residues (F_{93}, F_{97}, F_{112}, F_{121} and F_{124}) in this region. To test whether these phenylalanine residues play any roles in POP-1 function, a POP-1 construct with all the five sites mutated to alanines (POP-$1_{5F\text{-}A}$) was examined.

I observed a slightly increased cytoplasmic GFP level in transgenic embryos expressing GFP::POP-$1_{5F\text{-}A}$ and a variable defect in the nuclear asymmetry of GFP::POP-$1_{5F\text{-}A}$ (Figure 2.7). The nuclear level of GFP::POP-$1_{5F\text{-}A}$ is symmetric between some A-P sisters. In addition to a defect in POP-1 nuclear asymmetry, GFP::POP-$1_{5F\text{-}A}$ did not form any nuclear puncta in most nuclei examined (Figure 2.7). The endoderm repressive activity was also affected in GFP::POP-$1_{5F\text{-}A}$. Only five percent of the *pop-1(zu189)* embryos expressing GFP::POP-$1_{5F\text{-}A}$ produced the posterior pharynx (n = 61, Table 2.1; data not shown). Although I can not rule out the possibility that the observed defects in POP-1 nuclear

asymmetry and repressor activity are caused by an incorrect folding of this mutated POP-1 protein, these results suggest that the phenylalanine residues within POP-$1_{88\text{-}130}$ are important for the *in vivo* function of POP-1. However, I do not know why POP-$1_{5F\text{-}A}$ has a higher cytoplasmic level.

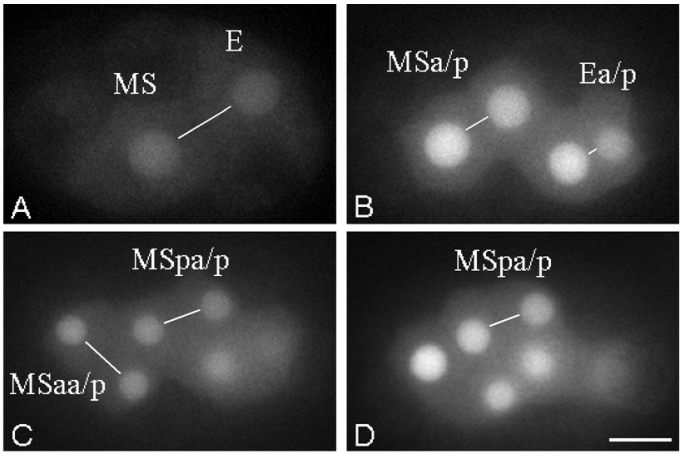


Figure 2.7 – POP-1 nuclear asymmetry is variably affected in POP-$1_{5F\text{-}A}$. GFP fluorescence micrographs of transgenic embryos harboring $P_{med\text{-}1}$*gfp::pop-*$1_{5F\text{-}A}$ (TX569). A-P sisters are connected by lines and their names are indicated. The A-P nuclear asymmetry is abolished in (A) and (C), but some asymmetry is still seen in (B) and (D). The nuclei of each A-P sister blastomeres are equally focused at that particular focal plane. Scale bar, 10 μm.

POP-1 Point Mutations	Subcellular Localization	Nuclear Asymmetry?	Rescue the MS Defect in *pop-1(zu189)*?	Ablate Endoderm in Wild-Type?	Ablate Endoderm in *pop-1(zu189)*?
within aa_{48-130}:					
Single amino acid mutations:					
S71A	N >> C	Yes	91% (n=23)	0% (n>100)	0% (n=23)
S107A	N >> C	Yes	87% (n=40)	0% (n>100)	0% (n=40)
S118A	N >> C	Yes	94% (n=33)	0% (n>100)	0% (n=33)
S127A	N >> C	Yes	78% (n=9)	0% (n>100)	0% (n=9)
Double amino acid mutations:					
S71/82A	N >> C	Yes	86% (n=21)	nd	5% (n=21)
S71/127A	N >> C	Yes	93% (n=14)	0% (n>100)	0% (n=14)
S107/109A	N >> C	Yes	nd	0% (n>100)	nd
S107/127A	N >> C	Yes	67% (n=6)	0% (n>100)	0% (n=6)
S118/T120A	N >> C	Yes	94% (n=32)	0% (n>100)	0% (n=32)
S71/82D	N >> C	Yes	100% (n=16)	nd	0% (n=16)
S107/109D	N >> C	Yes	88% (n=25)	nd	0% (n=25)
Triple amino acid mutations:					
S107/109/127A	N >> C	Variable*	nd	9% (n=23)	nd
S118/T120/S127A	N >> C	Variable*	93% (n=29)	nd	7% (n=29)
S107/118/127A	N >> C	No	nd	nd	nd
Quatruple amino acid mutations:					
S107/109/118/T120A	N >> C	Variable*	nd	3% (n=30)	nd
S107/118/T120/S127A	N >> C	No	nd	27% (n=11)	nd
S109/118/T120/S127A	N >> C	No	93% (n=14)	37% (n=8)	36% (n=14)
S107/109/T120/S127A	N >> C	Variable*	nd	4% (n=23)	nd
S107/109/118/127A	N >> C	No	nd	nd	nd
Penta amino acid mutations:					
S107/109/118/T120/S127A	N >> C	No	91% (n=32)	82% (n=22)	62% (n=32)
S107/109/118/T120/S127D	N >> C	No	41% (n=22)	nd	0% (n=22)
F93/97/112/121/124A	N > C	Variable*	5% (n=61)	nd	0% (n=61)
Other regions:					
D9E	N >> C	Yes	98% (n=119)	nd	8% (n=119)
S397A	N >> C	Yes	100% (n=4)	2% (n=43)	25% (n=4)
S404/406/408A	N >> C	Variable*	100% (n=13)	5% (n=22)	38% (n=13)

Table 2.1 – Effects of point mutations of POP-1 on its subcellular localization, nuclear asymmetry, ability to rescue the MS fate defect in *pop-1(zu189)* embryos, and endoderm formation in the wild-type or *pop-1(zu189)* background. N, nucleus. C, cytoplasm. Asterisks indicate a variable defect and that asymmetry is still detected in some A-P sisters. nd, not determined.

DISSUSSION

I show in this chapter that the $aa_{101-130}$ and $aa_{400-438}$ regions of POP-1 are essential for a low nuclear level of POP-1 in posterior cells, and the aa_{48-130} and $aa_{281-399}$ regions are important for the endoderm repressive activity of POP-1. Therefore, the two properties of POP-1, nuclear asymmetry and endoderm repression, appear to be mediated by both overlapping and distinct regions of POP-1. While $aa_{101-130}$ is required for both nuclear asymmetry and endoderm repression, $aa_{400-438}$ is only required for nuclear asymmetry. The aa_{48-100} and $aa_{281-399}$ regions are important for endoderm repression but not nuclear asymmetry. Since different *cis*-elements of POP-1 mediate its nuclear asymmetry and repressor function, it is possible that POP-1 nuclear levels and repressor activity can be regulated separately by either the same or distinct pathways. Both of the two *lit-1* alleles, *t1512* and *t1534*, have equal nuclear levels of POP-1 between MS and E (Rocheleau *et al.*, 1999; Maduro *et al.*, 2002; R.O. and R.L., personal communication). While *t1512* has a nearly fully penetrant loss of endoderm, almost all the *t1534* embryos produce gut (Rocheleau *et al.*, 1999). Therefore, it is likely that the Wnt/MAPK signaling downregulates POP-1 to permit endoderm fate by both lowering its nuclear level and inhibiting its repressor activity.

Is A Low Nuclear Level of POP-1 Absolutely Required for the E (Posterior) Fate?

The initial observation that the low nuclear level of POP-1 in E correlates with the specification of E fate led to the proposal that a low nuclear level of POP-1 is required for E fate (Lin *et al.*, 1995, 1998; Rocheleau *et al.*, 1997, 1999; Thorpe *et al.*, 1997). In A-P sister

cells other than MS/E, the nuclear level of POP-1 is also lower in posterior compared to anterior cells (Lin *et al.*, 1995, 1998), and in many of these cases, this low nuclear level of POP-1 also correlates with the posterior fates (Lin *et al.,* 1998; Schroeder and McGhee, 1998; Herman*,* 2001). These observations suggest that perhaps a high level of POP-1 determines the anterior fate, and a low level of POP-1 determines the posterior fate. However, I showed in this chapter that overexpression of GFP::POP-1 from the $P_{med\text{-}1}$ *gfp::pop-1* transgene in wild-type embryos does not disturb embryogenesis, a result consistent with a previous report by Maduro *et al.* (2002). The amount of GFP::POP-1 in the nucleus of E is estimated to be higher than the amount of the endogenous POP-1 in the nucleus of MS (Maduro *et al.*, 2002). Nevertheless, I never see any defect in gut formation in the wild-type embryos expressing GFP::POP-1. Therefore, perhaps the absolute abundance of POP-1 is not critical for its function in MS/E specification. The studies of POP-1 function in T cell polarity also indicate that the POP-1 levels *per se* are not necessarily responsible for the difference in T cell fates, as both high POP-1 levels and the absence of POP-1 are associated with the same anterior epidermal fate (Herman, 2001).

The above statement seems to argue that a low nuclear level of POP-1 is not absolutely required for the posterior cell fate. However, I found that some POP-1 deletions (Figure 2.6 A) and point mutations (Table 2.1; also see chapters three and four) that caused a high POP-1 level in the E nucleus repressed E-derived endoderm in either wild-type or *pop-1 (zu189)* mutant background, resulting in gutless embryos. Although further studies are needed to elucidate the functions of these POP-1 domains and amino acids, these results suggest that a low nuclear level of POP-1 is somewhat important for the E cell fate. Perhaps

the Wnt/MAPK signaling inhibits POP-1 repressor activity and at the same time lowers its nuclear level to ensure proper endoderm development.

POP-1 Is Qualitatively Different between A-P Nuclei

The fact that overexpression of POP-1 in the E nucleus does not disturb embryogenesis suggests that POP-1 is qualitatively different between MS and E and perhaps between all the A-P sisters. Observations of the dynamics of GFP::POP-1 provided additional evidence for the qualitative difference of POP-1 between A-P sisters. GFP::POP-1 forms prominent puncta in the nuclei of anterior but not in those of posterior cells. These structures are observed even at the E^8 stage, when the levels of GFP::POP-1 per nucleus are significantly diluted (Maduro *et al.*, 2002). Thus, the lack of these structures in the nuclei of posterior cells is probably not due to the lower nuclear level compared to that in their anterior sisters (Maduro *et al.*, 2002). These nuclear puncta may be a property of POP-1 in the unsignaled anterior cells (Maduro *et al.*, 2002), where POP-1 appears to function as a repressor for the posterior fate (Lin *et al.*, 1998). Consistent with this, I observed a close correlation between the presence of these nuclear puncta and the ability of POP-1 deletions or mutations to repress the endoderm fate. This qualitative difference is likely due to the differential presence of a POP-1-interacting protein, the differential modification of nuclear POP-1 between A-P sisters, or both.

A Positive Role for POP-1 in Endoderm Formation?

The endoderm ablation phenomenon associated with certain POP-1 deletions or mutations described above can be explained by the fact that the nuclear level of these POP-1 variants is elevated in the E blastomere. However, I also observed a gutless phenotype in *pop-1(zu189)* embryos expressing POP-$1_{\Delta 1\text{-}47}$ (Figure 2.6 A). Because POP-$1_{\Delta 1\text{-}47}$ exhibits normal POP-1 nuclear asymmetry, this endoderm ablation effect may be due to an increased endoderm repressive function or loss of a novel endoderm activating function in the E blastomere when $aa_{1\text{-}47}$ is deleted. Because the endoderm repressive activity of POP-$1_{\Delta 1\text{-}47}$ in the MS blastomere is comparable to that of wild-type POP-1, I favor the explanation that POP-1 has a positive role in endoderm formation in the E blastomere, which requires the $aa_{1\text{-}47}$ region. It is possible that the Wnt/MAPK signaling regulates this POP-1 function, in addition to lowering its nuclear level, to allow endoderm fate. Because $aa_{1\text{-}47}$ corresponds to the β-catenin binding domain, it is an intriguing question whether β-catenin or a β-catenin-like coactivator is involved in this hypothesized POP-1 function in endoderm formation (see chapter six).

It has been shown that the interaction between TCF and β-catenin requires a conserved aspartate residue in the β-catenin-binding domain of TCF (Hsu *et al.*, 1998; Graham *et al.*, 2000; von Kries *et al.*, 2000). A *pop-1* reduction-of-function mutation (*q645*) that changes the corresponding aspartate residue to glutamate (D_9E) has been isolated and shown to affect the POP-1 function in gonadogenesis (Siegfried and Kimble, 2002). Although it is not clear whether D_9E mutation affects the interaction between POP-1 and a β-

catenin protein, this result suggests that aspartate 9 is essential for POP-1 function, at least during postembryonic development. I showed that POP-1_{D9E} also caused a gutless phenotype in *pop-1(zu189)* embryos similar to POP-$1_{\Delta 1\text{-}47}$ (Table 2.1), suggesting that aspartate 9 may be important for the hypothesized endoderm activating function mediated by $aa_{1\text{-}47}$ (see chapter six).

The hypothesis that POP-1 acts positively in endoderm formation in E is supported by the observations with the POP-1 deletion POP-$1_{\Delta 400\text{-}438}$. Most of the POP-1 mutant constructs that showed a gut ablation activity repressed the E-derived endoderm in ≤ 62% of *pop-1 (zu189)* embryos carrying the transgenes (Figure 2.6 A and Table 2.1). These POP-1 constructs, when examined, had either similar or higher gut ablation activities in the wild-type background. Surprisingly, POP-$1_{\Delta 400\text{-}438}$ inhibited the E-derived endoderm in all the *pop-1(zu189)* embryos but in only 6% of the wild-type embryos expressing this protein. This is a very intriguing result because (1) removing $aa_{400\text{-}438}$ makes POP-1 a much better repressor for the endoderm fate than the other POP-1 constructs, (2) POP-$1_{\Delta 400\text{-}438}$ can only inhibit the E-derived endoderm efficiently when there is no endogenous POP-1. So far, I do not know whether $aa_{400\text{-}438}$ has other functions in addition to playing a role in POP-1 nuclear asymmetry. If deleting $aa_{400\text{-}438}$ increases the repressor activity of POP-1 (gain-of-function), a similar or more severe gut ablation effect should be observed in wild-type embryos than in *pop-1(zu189)* embryos. Therefore, deleting $aa_{400\text{-}438}$ may result in a loss of POP-1 function. It is possible that POP-1 normally functions as an activator of endoderm in the E blastomere, and $aa_{400\text{-}438}$ of POP-1 is required for this activator function (see chapter six). If this hypothesis is true, in *pop-1(zu189)* early embryos, where no endogenous POP-1 is present,

POP-$1_{\Delta 400\text{-}438}$, which lacks the activator function and has a high nuclear level in the E blastomere (elevated repressor activity), would repress the endoderm fate in E as in the MS blastomere. In wild-type embryos expressing POP-$1_{\Delta 400\text{-}438}$, the proposed activator function of the endogenous POP-1 may antagonize the repressor function of POP-$1_{\Delta 400\text{-}438}$ in the E blastomere. Therefore, the endoderm ablation effect of POP-$1_{\Delta 400\text{-}438}$ is not as severe in the wild-type embryos as in the *pop-1(zu189)* embryos.

MATERIALS AND METHODS

Strains

N2 was used as the wild-type strain. Genetic markers used in this chapter are: LGI, *pop-1 (zu189)*, *dpy-5(e61), hT1(I;V)*; LGIII, *unc-119(ed3)*; LGIV, *him-3(e1147)*. Most of the transgenic strains analyzed in this chapter were generated by injecting either *unc-119(ed3)III* or *unc-119(ed3)III;him-3(e1147)IV* (TX576) worms with *pop-1* transgenes together with *unc-119* rescuing plasmids (pDPmm016), at a concentration of 100 μg/ml for each. All the transgenes are maintained as extrachromosomal arrays. The following are the transgenes in their respective strains: $P_{med\text{-}1}$*gfp::pop-1* (pRL769) in TX352(*teEx72*); $P_{med\text{-}1}$*gfp::pop-*$1_{\Delta 1\text{-}47}$ (pRL1235) in TX540(*teEx187*); $P_{med\text{-}1}$*gfp::pop-*$1_{\Delta 1\text{-}100}$ (pRL1306) in TX608(*teEx235*); $P_{med\text{-}1}$ *gfp::pop-*$1_{\Delta 88\text{-}130}$ (pRL728) in TX348(*teEx68*); $P_{med\text{-}1}$*gfp::pop-*$1_{\Delta 281\text{-}438}$ (pRL1309) in TX589 (*teEx218*), $P_{med\text{-}1}$*gfp::pop-*$1_{\Delta 400\text{-}438}$ (pRL1298) in TX616(*teEx243*); $P_{med\text{-}1}$*gfp::pop-*$1_{81\text{-}297}$ (pRL1095) in TX618(*teEx245*); $P_{med\text{-}1}$*gfp::pop-*$1_{61\text{-}180}$ (pRL828) in TX521(*teEx177*); $P_{med\text{-}1}$

gfp::pop-1$_{81\text{-}200}$ (pRL826) in TX410(*teEx103*); $P_{med\text{-}1}$*gfp::pop-1*$_{178\text{-}297}$ (pRL825) in TX426 (*teEx110*); $P_{med\text{-}1}$*gfp::pop-1*$_{189\text{-}308}$ (pRL827) in TX522(*teEx178*); $P_{med\text{-}1}$*gfp::pop-1*$_{178\text{-}270}$ (pRL1096) in TX648(*teEx252*); $P_{med\text{-}1}$*gfp::pop-1*$_{S71A}$ (pRL654) in TX338(*teEx58*); $P_{med\text{-}1}$ *gfp::pop-1*$_{S107A}$ (pRL655) in TX344(*teEx64*); $P_{med\text{-}1}$*gfp::pop-1*$_{S118A}$ (pRL1019) in TX445 (*teEx122*); $P_{med\text{-}1}$*gfp::pop-1*$_{S127A}$ (pRL656) in TX341(*teEx61*); $P_{med\text{-}1}$*gfp::pop-1*$_{S71/82A}$ (pRL1050) in TX477(*teEx143*); $P_{med\text{-}1}$*gfp::pop-1*$_{S71/127A}$ (pRL698) in TX369(*teEx85*); $P_{med\text{-}1}$ *gfp::pop-1*$_{S107/109A}$ (pRL1015) in TX451(*teEx128*); $P_{med\text{-}1}$*gfp::pop-1*$_{S107/127A}$ (pRL699) in TX371(*teEx87*); $P_{med\text{-}1}$*gfp::pop-1*$_{S118/T120A}$ (pRL1056) in TX483(*teEx148*); $P_{med\text{-}1}$*gfp::pop-1* $_{S71/82D}$ (pRL1290) in TX574(*teEx210*); $P_{med\text{-}1}$*gfp::pop-1*$_{S107/109D}$ (pRL1198) in TX525 (*teEx180*); $P_{med\text{-}1}$*gfp::pop-1*$_{S107/109/127A}$ (pRL1023) in TX464(*teEx134*); $P_{med\text{-}1}$*gfp::pop-1* $_{S118/T120/S127A}$ (pRL1055) in TX497(*teEx159*); $P_{med\text{-}1}$*gfp::pop-1*$_{S107/118/127A}$ (pRL1197) in TX559(*teEx198*); $P_{med\text{-}1}$*gfp::pop-1*$_{S107/109/118/T120A}$ (pRL1053) in TX479(*teEx145*); $P_{med\text{-}1}$ *gfp::pop-1*$_{S107/118/T120/S127A}$ (pRL1054) in TX489(*teEx154*); $P_{med\text{-}1}$*gfp::pop-1*$_{S109/118/T120/S127A}$ (pRL1063) in TX498(*teEx160*); $P_{med\text{-}1}$*gfp::pop-1*$_{S107/109/T120/S127A}$ (pRL1064) in TX551 (*teEx193*); $P_{med\text{-}1}$*gfp::pop-1*$_{S107/109/118/127A}$ (pRL1065) in TX554(*teEx196*); $P_{med\text{-}1}$*gfp::pop-1* $_{AAAAA}$ (pRL1024) in TX466(*teEx136*); $P_{med\text{-}1}$*gfp::pop-1*$_{DDDDD}$ (pRL1237) in TX629(*teEx248*); $P_{med\text{-}1}$*gfp::pop-1*$_{5F\text{-}A}$ (pRL1245) in TX569(*teEx206*); $P_{med\text{-}1}$*gfp::pop-1*$_{D9E}$ (pRL1317) in TX658(*teEx259*). TX300 (*teIs3*$P_{med\text{-}1}$*gfp::pop-1*) was derived from integrating the transgene in JR2308 (a gift from Joel Rothman and Morris Maduro) after EMS mutagenesis, and screening for F2 segregating 100% non-Unc progeny. TX576 [*unc-119(ed3)III;him-3(e1147) IV*] was generated by crossing CB1256 [*him-3(e1147)IV*] males with *unc-119(ed3)III* and screening for Unc Him F2.

Plasmid Construction

Most GFP fusions were constructed using the Gateway cloning technology (Invitrogen) (Lo *et al.*, 2004; Robertson *et al.*, 2004). All the GFP fusions containing POP-1 fragments were generated by introducing the corresponding *pop-1* coding sequences into the $P_{med\text{-}1}$*gfp* destination vector pRL707. pRL707 was generated by replacing the BamHI-NcoI *gfp::pop-1* fragment of pMM414 (Maduro *et al.*, 2002) with *gfp* and then blunt-end ligating the Gateway cassette (reading frame A) via an introduced EcoRV site downstream of the *gfp* sequence. The *pop-1*$_{\Delta 88\text{-}130}$ coding sequence is a gift from Yang Shi. The GFP fusions containing POP-1 point mutation(s) were generated by site-directed mutagenesis on pMM414, pRL769 or pRL683 (*pop-1* entry clone) and then subcloning the SacI-BclI fragment back into the parental pMM414, pRL769 or pRL683 after mutagenesis. All site-directed mutageneses were performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene).

Assay for POP-1 Repressor Activity

Various *gfp*::*pop-1* transgenes were introduced into *pop-1(zu189)* mutant embryos by mating the males carrying the transgenes with hermaphrodites homozygous for the *pop-1(zu189)* mutation at 20ºC for 24 hours. The embryos were collected onto 2% agar pads, checked for the presence of *gfp* transgenes using fluorescence microscopy, and allowed to develop at 15ºC for 16 hours. Then the embryos were checked with differential interference contrast (DIC) optics for the production of posterior pharynx and scored as rescued if they produced wild-type amount of posterior pharyngeal tissues. The formation of intestine was viewed with both DIC and polarizing optics.

Analysis of Embryos and Imaging

Imaging of live embryos was performed using an Axioplan microscope equipped with epifluorescence, polarizing, and DIC optics, and a MicroMax-512EBFT CCD camera as described previously (Rogers *et al.*, 2002). Intestinal cells were identified by their birefringent gut-specific granules under polarizing optics. At each time point of live imaging, GFP fluorescence and DIC images were collected from a selected focal plane to follow particular anterior-posterior sister pairs. All live images were collected as 16-bit with the raw pixel values within the linear range (0-4095) of the CCD camera and scaled to 8-bit using the custom program EditView4D (Rogers *et al.*, 2002).

CHAPTER THREE
POP-1 Nuclear Asymmetry Is Regulated by Differential Nuclear Export Mediated by 14-3-3 Protein

INTRODUCTION

In order to identify additional proteins that may function in the regulation of POP-1 nuclear asymmetry, the lab members Raanan Odom and Rueyling Lin introduced the transgene $P_{med\text{-}1}$ *gfp::pop-1* into various mutant backgrounds shown to be defective in embryonic polarity. Such mutants include various *par* mutants. They found that one of the mutants that showed the abolishment of POP-1 nuclear asymmetry in early embryos was *par-5(it55)* mutant (Lo *et al.*, 2004).

PAR-5 is required for the normal partition of cytoplasmic contents and timing of divisions in early blastomeres (Morton *et al.*, 2002). In the *par-5(it55)* mutant embryos, the *med-1* promoter is transcribed ubiquitously, resulting in the expression of GFP::POP-1 in all blastomeres starting at the eight-cell stage. In all the eight-cell *par-5(it55)* mutant embryos examined, equal levels of nuclear GFP::POP-1 between sisters were observed, regardless of their division axes (Lo *et al.*, 2004). When these eight cells divide, each produces two daughters with equal levels of nuclear GFP::POP-1 (Lo *et al.*, 2004). These results suggest that PAR-5 is required for GFP::POP-1 nuclear asymmetry in early embryos. The asymmetric nuclear GFP::POP-1 pattern is gradually re-established in *par-5(it55)* embryos after the 16-cell stage. GFP::POP-1 nuclear asymmetry is observed in almost all A-P sisters after the 64-cell stage (Lo *et al.*, 2004).

The *par-5* gene, also known as *ftt-1*, encodes a 14-3-3 protein (Wang and Shakes, 1997; Morton *et al.*, 2002). 14-3-3 proteins are evolutionarily conserved proteins that have been shown to regulate diverse cellular processes via interaction with different binding partners (see review by Fu *et al.*, 2000). *C. elegans* has two closely related 14-3-3 proteins. PAR-5 (FTT-1) is expressed maternally, and the second *C. elegans* 14-3-3 protein, FTT-2, is expressed zygotically after the eight-cell stage (Wang and Shakes, 1997). Gradual restoration of POP-1 nuclear asymmetry in *par-5(it55)* mutant embryos is likely due to the expression of FTT-2 (Lo *et al.*, 2004).

One of the cellular processes that 14-3-3 proteins have been shown to regulate is nuclear export of their binding partners (Lopez-Girona *et al.*, 1999). Therefore, it is an intriguing possibility that POP-1 nuclear asymmetry is regulated by differential nuclear export mediated by PAR-5 between A-P sisters. To further test this hypothesis, the following questions are asked. First, does PAR-5 play a direct role in POP-1 nuclear asymmetry? Second, is POP-1 nuclear asymmetry regulated by nuclear export? Finally, if nuclear export regulates POP-1 nuclear asymmetry, what is the mechanism leading to the differential nuclear export of POP-1 between A-P sisters?

RESULTS

Expression of PAR-5 in the Four-Cell Stage *par-5* Mutant Embryos Restores POP-1 Nuclear Asymmetry

To eliminate the possibility that the defect in POP-1 nuclear asymmetry in *par-5(it55)* mutant embryos is a nonspecific consequence of the abnormal early cleavages in those mutant embryos, I asked whether POP-1 nuclear asymmetry could be restored without the early cleavage defect being rescued. *par-5* mutant embryos exhibit observable defects as early as the one-cell stage. I generated a transgenic strain homozygous for the *par-5(it55)* mutation but carrying a transgene expressing wild-type GFP::PAR-5 under the control of the *med-1* promoter. The *med-1* promoter is not expressed until the four-cell stage (Maduro *et al.*, 2001), by which stage the cleavage defect in *par-5(it55)* embryos is already apparent.

The nuclear asymmetry for the endogenous POP-1 was then assayed in these transgenic embryos by immunostaining using mabRL2 (Lin *et al.*, 1998). The immuno-staining experiments were performed by Rueyling Lin. As I mentioned earlier, the GFP signal will not be seen until eight-cell stage and POP-1 nuclear asymmetry would be re-established in some A-P sisters after 16-cell stage in *par-5(it55)* (Lo *et al.*, 2004). Therefore, the restoration of POP-1 nuclear asymmetry was scored in 16-cell stage embryos. Approximately 69% of the *par-5(it55)* 16-cell stage embryos examined had an equal level of nuclear POP-1 throughout the embryo (n = 32; Figure 3.1 B). The remaining 31% of the embryos showed asymmetric nuclear POP-1 staining between adjacent nuclei. Because *par-5* mutant embryos have abnormal cell divisions, it is difficult to unambiguously identify sister

cells in fixed embryos. Therefore, an embryo was scored as having asymmetric levels of nuclear POP-1 if adjacent nuclei had different levels of POP-1. When GFP::PAR-5 was expressed in the *par-5(it55)* mutant embryos, the percentage of embryos with symmetric nuclear POP-1 staining was decreased (8%, n = 11). The other 92% showed a clear difference in nuclear POP-1 staining between adjacent nuclei (Figure 3.1 D-F). The conclusion from these results is that PAR-5 is likely to regulate POP-1 nuclear asymmetry independent of its function in the one-cell stage embryo.

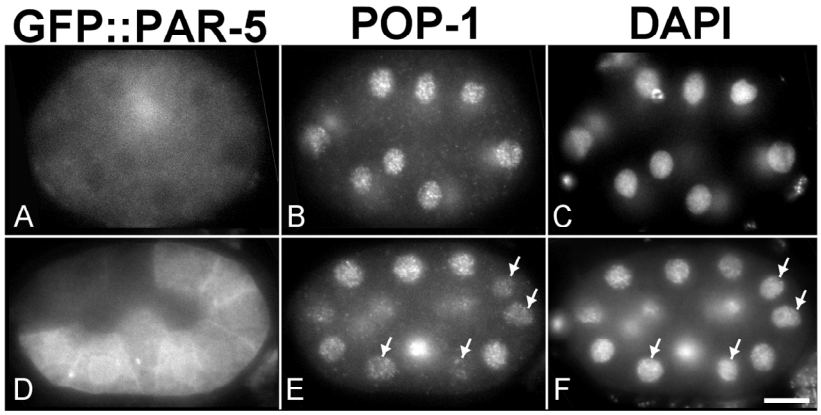


Figure 3.1 – Expression of PAR-5 in the four-cell stage *par-5(it55)* mutant embryos restores POP-1 nuclear asymmetry. *par-5(it55)* mutant embryos with (D, E, and F) or without (A, B, and C) the $P_{med\text{-}1}$*gfp::par-5* transgene were stained with mabRL2 (B and E) and DAPI (C and F). Arrows in (E) and (F) point to nuclei with low levels of nuclear POP-1. The immuno-staining experiments were performed by Rueyling Lin. Scale bar, 10 μm.

POP-1 and PAR-5 Interact in Wild-Type *C. elegans* Embryos and This Interaction Depends on the MAP Kinase LIT-1

POP-1 and PAR-5 Interact in Wild-Type C. elegans Embryos

To test whether PAR-5 regulates POP-1 nuclear asymmetry directly, I examined their physical interaction in *C. elegans* embryo extracts by coimmunoprecipitation (co-IP). Co-IP was performed using a polyclonal antibody against POP-1 protein, 94I (Lin *et al.*, 1995). Small but consistent amount of PAR-5 was detected in the immunoprecipitates (Figure 3.2 B). Very little or no PAR-5 was detected when co-IP was performed using the control, preimmune antibody (Figure 3.2 B).

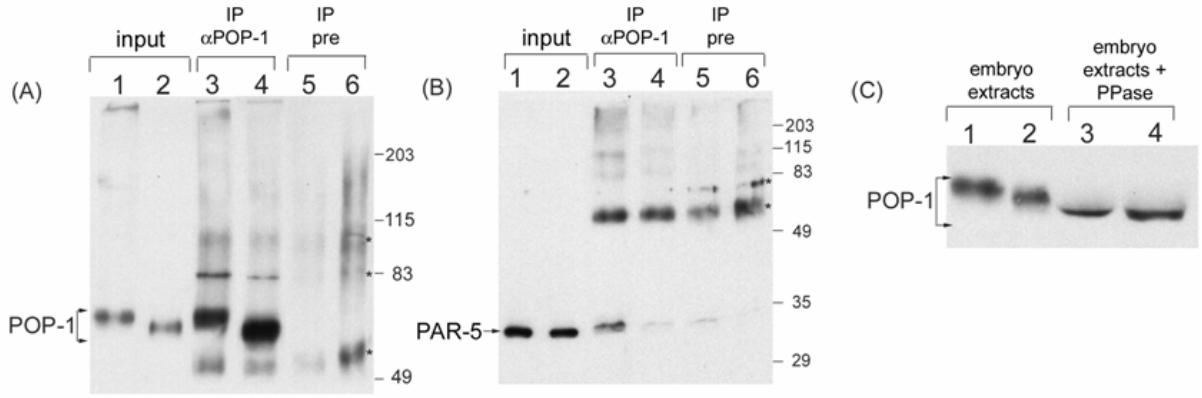


Figure 3.2 – POP-1 and PAR-5 interact in a LIT-1-dependent manner in *C. elegans* embryos. (A and B) Embryo extracts derived from either wild-type (lanes 1, 3 and 5) or *lit-1(t1512)* mutants (lanes 2, 4 and 6) were immunoprecipitated with either 94I (lanes 3 and 4) or preimmune antibody (lanes 5 and 6), resolved on either a 10% (A) or 15% (B) PAGE, and probed with either 94I (A) or anti-PAR-5 antibody (B), respectively. Lanes 1 and 2 correspond to 5% (A) or 0.06% (B) of input extracts. Asterisks, nonspecific bands detected by the secondary antibody. (C) A higher-resolution gel demonstrating that POP-1 is hyperphosphorylated in wild-type (lanes 1 and 3) but hypophosphorylated in *lit-1(t1512)* mutant extracts (lanes 2 and 4). Extracts in lanes 3 and 4 were treated with phosphatase before loading.

Hyperphosphorylation of POP-1 and POP-1/PAR-5 Interaction in vivo Requires LIT-1 Kinase Activity

Many 14-3-3 binding partners are phosphoproteins, and the phosphorylation regulates the interaction between these partners and 14-3-3 proteins. Therefore, I asked whether the interaction between POP-1 and PAR-5 is affected by the phosphorylation state of POP-1. In wild-type embryo extracts, POP-1 is highly phosphorylated, as indicated by a reduction of its electrophoretic mobility and a disappearance of these slow-migrating bands upon phosphatase treatment (Figure 3.2 C). This phosphorylation is greatly reduced in extracts derived from *lit-1(t1512)* mutant or *wrm-1(RNAi)* embryos (Figure 3.2 A and C and Figure 3.6). These results suggest that LIT-1 kinase activity and WRM-1 function are at least partially responsible for POP-1 hyperphosphorylation *in vivo*.

Although POP-1 is hypophosphorylated in *lit-1(t1512)* embryo extracts, it can still be efficiently immunoprecipitated with 94I (Figure 3.2 A). However, very little or no PAR-5 was detected in the immunoprecipitates (Figure 3.2 B). Because both POP-1 hyper-phosphorylation and its interaction with PAR-5 are dependent on LIT-1 kinase activity, it is likely that LIT-1 phosphorylates POP-1, thereby promoting its interaction with PAR-5 in *C. elegans* embryos.

GFP::POP-1 Nuclear Asymmetry Requires a CRM-1-Dependent Nuclear Export in *C. elegans* Embryos

The above results suggest that LIT-1-dependent phosphorylation of POP-1 promotes the interaction between POP-1 and PAR-5, which may directly regulate POP-1 nuclear asymmetry. The next question was how PAR-5 regulated POP-1 nuclear asymmetry. Because differential nucleocytoplasmic distribution of POP-1 protein in anterior versus posterior cells has been proposed to be a mechanism of POP-1 nuclear asymmetry, the role of nuclear export in regulating POP-1 nuclear asymmetry was examined. Nuclear export in the embryo was perturbed by targeting the *C. elegans* homolog (named CRM-1) of the vertebrate exportin CRM1, via RNAi. CRM1 is a highly conserved protein of the karyopherin β superfamily and has been shown to mediate nuclear export of leucine-rich nuclear export sequence (NES)-containing proteins in a variety of organisms (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997). The following *crm-1* RNAi experiments were performed by Rueyling Lin.

CRM-1 Regulates POP-1 Nuclear Export in C. elegans Embryos

Rueyling first examined whether *crm-1(RNAi)* inhibits nuclear export of POP-1 in *C. elegans* embryos by taking advantage of a transgenic strain expressing predominantly cytoplasmic GFP::POP-1 (TX283; Gay *et al.*, 2003). TX283 expresses mutant POP-1 in which lysines 185, 187, and 188 were mutated to alanine (GFP::POP-1_{MutAAA}). Mutation of these three lysines dramatically increases the cytoplasmic level of GFP::POP-1 in all blastomeres without affecting GFP::POP-1 nuclear asymmetry between A-P sister cells (Figure 3.3 E and

G; Gay *et al.*, 2003; Lo *et al.*, 2004). The cytoplasmic accumulation of GFP::POP-1_{MutAAA} was due to a defect in nuclear retention and can not be rescued by addition of an SV40 NLS (Figure 3.3 F and H). When CRM-1 was depleted by RNAi in TX283, a significant increase in the level of nuclear GFP::POP-1_{MutAAA} was observed (Lo *et al.*, 2004), suggesting that CRM-1 regulates POP-1 nuclear export in *C. elegans* embryos.

POP-1 Nuclear Asymmetry Is Regulated by CRM-1

Rueyling next depleted CRM-1 in the strain TX300, which carries integrated wild-type P_{med-1} *gfp::pop-1* transgenes [will be referred to as *crm-1(RNAi)*;TX300 hereafter]. In *crm-1(RNAi);* TX300 embryos, approximately 40% of the A-P sisters in the EMS lineage had equal and high levels of nuclear GFP-fluorescence (Lo *et al.*, 2004). Although the effect of *crm-1* RNAi on POP-1 nuclear asymmetry was variable, this result suggested that the low nuclear level of POP-1 in Wnt/MAPK-responsive cells was resulted from a CRM-1-dependent nuclear export. Consistent with this, addition of an SV40 NLS to GFP::POP-1 did not significantly alter GFP nuclear asymmetry (Figure 3.3 B and D), suggesting that POP-1 nuclear asymmetry is not due to a decreased nuclear import in the posterior cells. The incomplete penetrance of the defect in POP-1 nuclear asymmetry could be due to the incomplete depletion of CRM-1 by RNAi. Alternatively, it could mean that POP-1 nuclear asymmetry is only partially regulated by CRM-1.

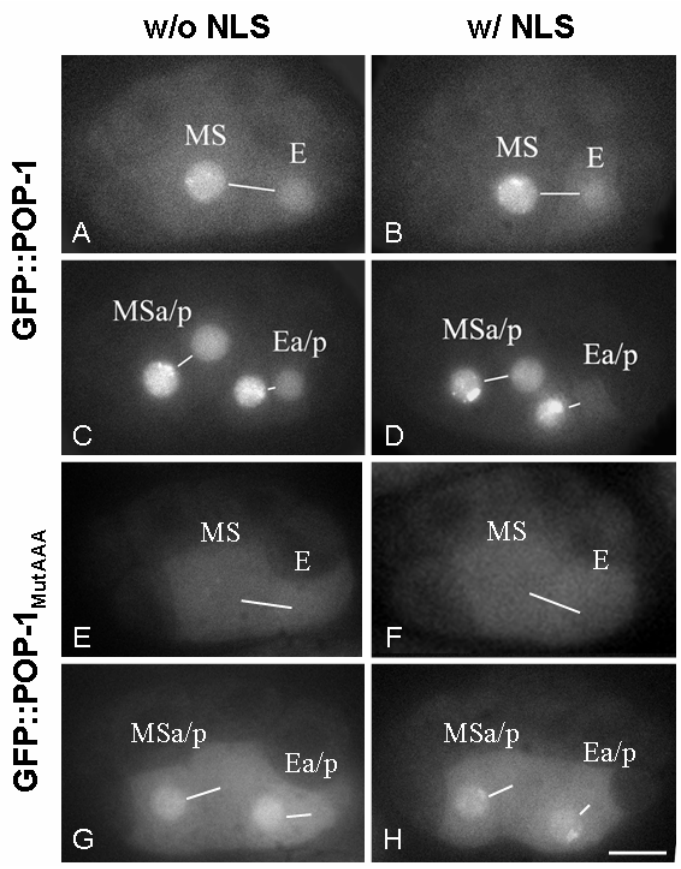


Figure 3.3 – Addition of an SV40 NLS to GFP::POP-1 does not affect its nuclear asymmetry. GFP fluorescence micrographs of eight-cell (A, B, E, and F) or 16-cell (C, D, G, and H) transgenic embryos harboring $P_{med\text{-}1}$*gfp::pop-1* (TX352; A and C), $P_{med\text{-}1}$*NLSgfp::pop-1* (TX350; B and D), $P_{med\text{-}1}$*gfp::pop-1*$_{MutAAA}$ (TX283; E and G), or $P_{med\text{-}1}$ *NLSgfp::pop-1*$_{MutAAA}$ (TX438; F and H). A-P sisters are connected by lines and their names are indicated. Except the Ep in (D) and (G), the nuclei of each A-P sister blastomeres are equally focused at that particular focal plane. Scale bar, 10 μm.

Potential Phosphorylation Sites within the $aa_{101-130}$ Region of POP-1 Are Important for POP-1 Nuclear Asymmetry

It has been suggested that phosphorylation of POP-1 by LIT-1 regulates POP-1 nucleo-cytoplasmic redistribution, resulting in the observed A-P nuclear asymmetry (Rocheleau *et al.,* 1999; Maduro *et al.*, 2002). Because LIT-1 is closely related to MAP kinases (Rocheleau *et al.*, 1999), I therefore performed a motif search for potential MAPK phosphorylation sites in the region essential for POP-1 nuclear asymmetry, $aa_{101-130}$ (see chapter two), using the Scansite available at http://scansite.mit.edu/.

Serines 107/109, 118, and 127 Are Redundantly Required for POP-1 Nuclear Asymmetry

There are only five serine(S)/threonine(T) residues within $aa_{101-130}$, S_{107}, S_{109}, S_{118}, T_{120}, and S_{127}. I found that S_{107}, S_{118}, and S_{127} are all potential MAPK sites, and interestingly S_{109} is a potential binding site for 14-3-3 proteins. To test the importance of these potential phosphorylation sites in POP-1 nuclear asymmetry, I mutated them to alanine (A), which can not be phosphorylated, in different combinations and examined the effect of these mutations on GFP::POP-1 nuclear asymmetry. A summarized table for the results is shown in Table 2.1. I found that the A-P nuclear asymmetry was abolished when all the five amino acids were mutated ($POP\text{-}1_{AAAAA}$, Figure 3.4 B). A similar abolishment of nuclear asymmetry was observed when three combinations of four sites ($S_{107,\ 118,\ 127}\ T_{120}$, $S_{109,\ 118,\ 127}\ T_{120}$, and $S_{107,\ 109,\ 118,\ 127}$) or one combination of three sites ($S_{107,\ 118,\ 127}$) were mutated (Figure 3.4 C-F). However, the other two quadruple mutations ($S_{107,109,118}T_{120}A$ and $S_{107,109,127}T_{120}A$) or two triple mutations ($S_{107,109,127}A$ and $S_{118,127}T_{120}A$) did not abolish the A-P nuclear asymmetry

(data not shown). This result demonstrates that serines 107/109, 118, and 127 are redundantly required for POP-1 nuclear asymmetry. For all the point mutation constructs that abolished POP-1 nuclear asymmetry, the GFP fluorescence was high in both anterior and posterior nuclei with clear punctate structures. These results suggest that the nuclear export of POP-1 in posterior cells is blocked when these potential MAPK phosphorylation sites are mutated to alanine.

If phosphorylation of these serine/threonine residues promotes POP-1 to undergo nuclear export, a lower nuclear GFP::POP-1 level should be observed in both anterior and posterior cells when these residues are phosphorylated. I tried to mimic the phosphorylation state by changing those serine/threonine residues to aspartate (D). When S_{107} and S_{109} were changed to aspartates, I still detected A-P nuclear asymmetry (Figure 3.4 G). However, when all the five sites were mutated to aspartate (POP-1_{DDDDD}), I detected equal nuclear levels between A-P sisters (Figure 3.4 H), further demonstrating the redundant roles of these amino acids in POP-1 nuclear asymmetry. In some transgenic embryos expressing GFP::POP-1_{DDDDD}, I detected a slightly increased GFP level in the cytoplasm, suggesting an increased nuclear export for POP-1_{DDDDD}. However, I could not tell whether the nuclear level of GFP::POP-1_{DDDDD} is lower than that of GFP::POP-1 because the transgenes in different transgenic lines may have very different expression levels. Nevertheless, the nuclear GFP::POP-1_{DDDDD} appeared homogeneous in both anterior and posterior cells and did not form any punctate structures (Figure 3.4 H). This result strongly suggests that the property of POP-1_{DDDDD} is more similar to that of the wild-type POP-1 in posterior cells.

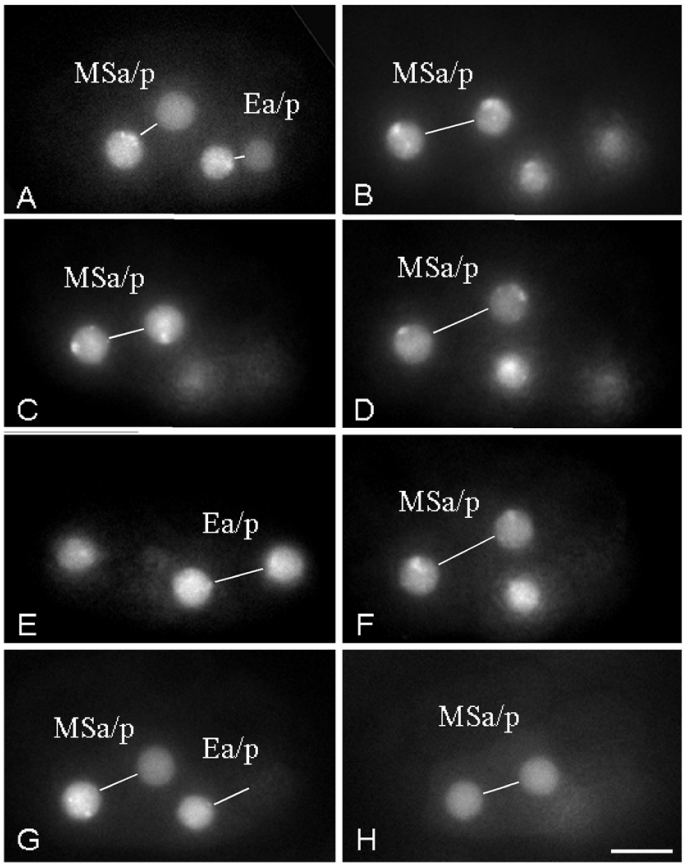


Figure 3.4 – Serines 107/109, 118, and 127 are redundantly required for POP-1 nuclear asymmetry. GFP fluorescence micrographs of 16-cell transgenic embryos harboring (A) $P_{med\text{-}1}$*gfp::pop-1* (TX352), (B) $P_{med\text{-}1}$*gfp::pop-1*$_{AAAAA}$ (TX465), (C) $P_{med\text{-}1}$*gfp::pop-1*$_{S107/S118/T120/S127A}$ (TX489), (D) $P_{med\text{-}1}$*gfp::pop-1*$_{S109/S118/T120/S127A}$ (TX498), (E) $P_{med\text{-}1}$*gfp::pop-1*$_{S107/S109/S118/S127A}$ (TX554), (F) $P_{med\text{-}1}$*gfp::pop-1*$_{S107/S118/S127A}$ (TX559), (G) $P_{med\text{-}1}$*gfp::pop-1*$_{S107/S109D}$ (TX525), or (H) $P_{med\text{-}1}$*gfp::pop-1*$_{DDDDD}$ (TX629). A-P sisters are connected by lines and their names are indicated. Except the Ep in (G), the nuclei of each A-P sister blastomeres are equally focused at that particular focal plane. Scale bar, 10 μm.

GFP::POP-1$_{AAAAA}$ *Is Defective in Nuclear Export but Not Repressor Activity*

I also assayed the effect of these S/T to A or S/T to D mutations on the endoderm repressive function of POP-1. All the transgenes containing S/T to A mutation(s) examined, including POP-1$_{AAAAA}$, rescued the MS fate defect in *pop-1(zu189)* embryos comparable to a wild-type

gfp::pop-1 transgene (Table 2.1; Figure 3.5 and data not shown). In rescued embryos, the MS-derived posterior pharynx, characterized by a structure called the grinder, was clearly produced (Figure 3.5). This result suggests that phosphorylation at any of these five sites is not needed for POP-1 to function as a transcriptional repressor in the MS blastomere. All the S/T to A mutations that affected POP-1 nuclear asymmetry examined also ablated E-derived endoderm in either wild-type or *pop-1(zu189)* embryos (Table 2.1; Figure 3.5 and data not shown). In 62% of *pop-1(zu189)* (n = 32; data not shown) and 91% of wild-type (n = 22; Figure 3.5, C-F) embryos expressing GFP::POP-1_{AAAAA}, endoderm fate is repressed in the E blastomere as well. The repression of endoderm fate in embryos expressing GFP::POP-1_{AAAAA}, together with a high level of nuclear POP-1 in the E blastomere, demonstrates that mutations of these potential phosphorylation sites affect specifically POP-1 nuclear export but not its repressor function.

Some wild-type embryos expressing GFP::POP-1_{AAAAA} (23%, n = 22) produced an excess of pharyngeal tissue (data not shown), suggesting an E to MS fate transformation. In wild-type embryos, two of the earliest observable differences between the MS and E blastomeres, in addition to different levels of nuclear POP-1, are the division rates and cleavage axes of their daughter cells. The MS daughters divide along the A-P axis 15 minutes after they are born, whereas the E daughters divide left-right 20-25 minutes later. In mutants defective in the specification of the E blastomere, including mutants in the Wnt/MAPK signaling pathway, E daughters divide along the A-P axis within 5 minutes of the MS daughter divisions (Goldstein, 1992; Rocheleau *et al.*, 1997). Similarly, in all embryos expressing GFP::POP-1_{AAAAA} analyzed (n = 7; data not shown), the E daughters divided

along the A-P axis within 5 minutes of the MS daughter divisions. These results together demonstrate that expressing GFP::POP-1_{AAAAA} can cause the E blastomere to adopt the fate of its sister blastomere MS.

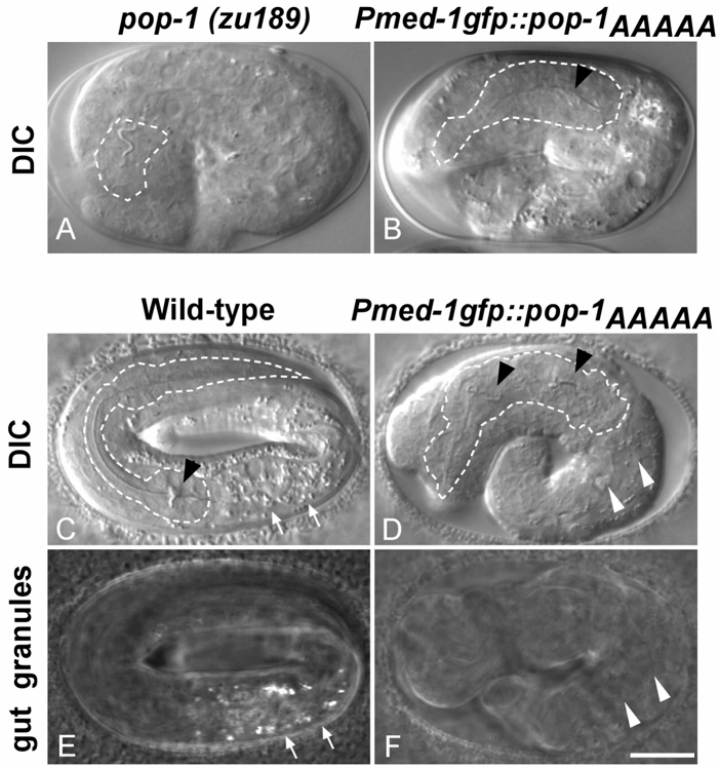


Figure 3.5 – Five potential phosphorylation sites within the $aa_{101-130}$ region of POP-1 are not required for POP-1 repressor activity. Micrographs of non-transgenic (left-hand column) and transgenic (right-hand column) embryos carrying P_{med-1}*gfp::pop-*1_{AAAAA}. (A and B) *pop-1 (zu189)* mutant embryos. (C-F) Wild-type embryos. (C-F) Micrographs of terminally differentiated embryos viewed with DIC (C and D) or polarized light (E and F). The wild-type embryo has a well-differentiated intestine (white arrows), whereas >90% of transgenic embryos have no apparent intestinal cells. White arrowheads in (D) and (F) point to the position where intestine would have been formed in a wild-type embryo. Pharyngeal tissues are outlined in (A)-(D). Black arrowheads point to grinder-like structures characteristic of the posterior pharynx. Scale bar, 10 μm.

POP-1$_{DDDDD}$ Has A Lower Endoderm Repressive Activity

In contrast to S/T to A mutations, the endoderm repressive activity of POP-1 is significantly affected when these five serine/threonine residues were changed to aspartate (Table 2.1). Only 41% of the *pop-1(zu189)* embryos expressing GFP::POP-1$_{DDDDD}$ produced the posterior pharynx (n = 22, data not shown). This result suggests that changing these five amino acids to aspartate lowers the endoderm repressive activity of POP-1. Therefore, mutations of these five sites to aspartate not only abolish POP-1 nuclear asymmetry but also lower its repressor activity.

Despite the A-P nuclear asymmetry was abolished for POP-1 $_{DDDDD}$, I did not observe any gut ablation effect associated with this mutated POP-1. Although it is possible that changing these five sites to aspartate causes an incorrect folding of POP-1 or aspartates do not exactly mimic phosphorylated serines/threonines in this case, these results suggest that phosphorylation of these amino acids, in addition to promoting POP-1 nuclear export, may inhibit the repressor activity of POP-1.

crm-1(RNAi);TX300 Embryos Fail to Produce Endoderm

A defect in endoderm formation was also observed in *crm-1(RNAi)*;TX300 embryos (Lo *et al.*, 2004). Every RNAi embryo examined produced no intestinal granules and had an accelerated division rate of E daughters (Lo *et al.*, 2004). This endoderm repression is dependent on the *P$_{med-1}$gfp::pop-1* transgene, because 100% of *crm-1(RNAi)* embryos produced gut granules (Lo *et al.*, 2004). Immunofluorescence using the POP-1 antibody confirmed that the nuclear asymmetry of endogenous POP-1 protein is variably affected in

crm-1(RNAi) embryos similar to GFP::POP-1 in *crm-1(RNAi)*;TX300 embryos (Lo *et al.*, 2004). This result strongly suggests that a high level of POP-1 in the E nucleus (rather than an equal level of POP-1 in the nuclei of MS and E) is important for endoderm repression. Consistent with this, I observed a more severe endoderm repression effect in transgenic animals expressing higher levels of GFP::POP-1_{AAAAA} (data not shown).

LIT-1/WRM-1 Promotes POP-1/PAR-5 Interaction and POP-1 Nuclear Export via Phosphorylation at S_{118} and S_{127} of POP-1

The results so far suggest that LIT-1-dependent phosphorylation of POP-1 mediates the interaction between POP-1 and PAR-5, and this interaction may facilitate export of POP-1 from the posterior nucleus. To further test this model, antibodies that recognize phosphorylated POP-1 specifically at either S_{118} (anti-S_{118}-P) or S_{127} (anti-S_{127}-P) were raised. The specificity of each antibody to its respective phosphoepitope was confirmed using GFP::POP-1 with either S_{118}A or S_{127}A mutation (data not shown).

S_{118} and S_{127} of POP-1 Are Phosphorylated in vivo, which Requires LIT-1 and WRM-1

Both anti-S_{118}-P and anti-S_{127}-P detect POP-1 protein in wild-type but not in *lit-1(t1512)* mutant or *wrm-1(RNAi)* embryo extracts (Figure 3.6). This result demonstrates that POP-1 is phosphorylated at S_{118} and S_{127} in *C. elegans* embryos, and phosphorylation of these two sites requires the LIT-1 kinase activity and the β-catenin WRM-1.

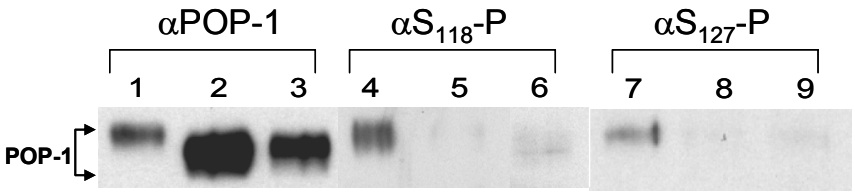


Figure 3.6 – S_{118} and S_{127} of POP-1 are phosphorylated *in vivo* in a LIT-1/WRM-1-dependent manner. Extracts derived from wild-type (lanes 1, 4, and 7), *lit-1(t1512)* mutant (lanes 2, 5, and 8), or *wrm-1(RNAi)* (lanes 3, 6, and 9) embryos were probed with 94I (αPOP-1), anti-S_{118}-P (αS_{118}-P), or anti-S_{127}-P (αS_{118}-P).

The following experiments in mammalian tissue culture cells were performed by Frédérique Gay in the laboratory of Yang Shi at Harvard Medical School.

LIT-1/WRM-1 Promotes POP-1/PAR-5 Interaction via Phosphorylation at S_{118} and S_{127} of POP-1

All of the S/T to A mutations in the $aa_{101-130}$ region of POP-1 that abolished nuclear asymmetry also ablated endoderm, resulting in dead embryos. I had difficulty obtaining sufficient transgenic embryos to perform biochemical analyses. Therefore, the importance of S_{118} and S_{127} and their phosphorylation in POP-1/PAR-5 interaction was tested using transfected COS-7 mammalian tissue culture cells. Our collaborators Frédérique Gay and Yang Shi at Harvard Medical School showed that S_{118} and S_{127} were phosphorylated only when POP-1 was cotransfected into COS-7 cells with LIT-1 and WRM-1 and not when POP-1 was cotransfected with LIT-1 alone (Lo *et al.*, 2004). This result is consistent with a previous report that WRM-1 is required for LIT-1 kinase activity (Rocheleau *et al.*, 1999).

Although I can not rule out an indirect effect, this result strongly suggests that the LIT-1/WRM-1 complex phosphorylates POP-1 at S_{118} and S_{127} in COS-7 cells.

Frédérique then tested whether phosphorylation of POP-1 at S_{118} and S_{127} was required for POP-1/PAR-5 interaction. She detected a strong interaction between POP-1 and PAR-5 in a pull-down experiment only when POP-1 was isolated from cells cotransfected with LIT-1 and WRM-1 and was phosphorylated at S_{118} and S_{127} (Lo *et al.*, 2004). No PAR-5 was pulled down with POP-1_{AAAAA} (Lo *et al.*, 2004). These results demonstrate that the LIT-1/WRM-1 complex stimulates POP-1/PAR-5 interaction *in vitro* via phosphorylation of at least two of these five sites.

LIT-1/WRM-1 Stimulates POP-1 Nuclear Export via Phosphorylation at S_{118} and S_{127} of POP-1

Finally, Frédérique tested whether LIT-1 and WRM-1 stimulate POP-1 nucleocytoplasmic redistribution in transfected mammalian tissue culture cells via the potential phosphorylation sites within the $aa_{101-130}$ region of POP-1. When *pop-1* is transfected into mammalian tissue culture cells, POP-1 protein localizes predominantly in the nucleus in approximately 50% of the transfected cells (N > C). In the other 50% of the transfected cells, POP-1 distributes either predominantly in the cytoplasm or equally between the cytoplasmic and nuclear compartments (N ≤ C). Upon cotransfection with LIT-1 and WRM-1, POP-1 distribution is changed from N > C to N ≤ C in 10% of the transfected cells (Lo *et al.*, 2004), similar to what was reported previously (Rocheleau *et al.*, 1999). On the contrary, the distribution of POP-1_{AAAAA} is not significantly changed in cells cotransfected with LIT-1 and WRM-1 (Lo

et al., 2004). All together, the results presented here demonstrate that LIT-1 and WRM-1 stimulate POP-1 nucleocytoplasmic redistribution primarily via phosphorylation of at least two of these five sites.

Nuclear GFP::LIT-1 Exhibits an Asymmetric Pattern between A-P Sisters Reciprocal to that of Nuclear GFP::POP-1

GFP::LIT-1 Is Enriched in the Posterior Nuclei in C. Elegans Early Embryos

If phosphorylation of POP-1 by LIT-1 promotes POP-1/PAR-5 interaction and the subsequent nuclear export in the posterior Wnt/MAPK-responsive cells, POP-1 must be differentially phosphorylated by LIT-1 between the nuclei of A-P sisters. I could not prove this hypothesis directly, because anti-S_{118}-P and anti-S_{127}-P detected primarily a non-POP-1 nuclear protein in the immunofluorescence assay (data not shown). However, an elevated level of nuclear GFP::LIT-1 was detected in the posterior cells compared to their anterior sisters (Figure 3.7, also see next paragraph), a pattern reciprocal to that of nuclear POP-1. This result supports the hypothesis that POP-1 is differentially phosphorylated by LIT-1 between the nuclei of A-P sisters.

The GFP::LIT-1 was detected in every blastomere, both cytoplasmic and nuclear. The nuclear GFP::LIT-1, despite a low level, was detected in a dynamic pattern both temporally and spatially. GFP::LIT-1 was consistently enriched in the interphase nuclei of only a subset of early blastomeres. Comparison with the corresponding DIC images revealed that at the eight-cell stage, nuclear GFP::LIT-1 was enriched in the interphase E blastomere but not in

the MS blastomere (Figure 3.7 A, B, and I). At the 12-cell stage, interphase nuclear GFP::LIT-1 was enriched in four of the great granddaughters of the AB blastomere (ABarp, ABalp, ABprp, and ABplp, all posterior daughters of A-P divisions) but not their anterior sister cells (Figure 3.7 C, D, and I and data not shown). Nuclear enrichment was also observed at the 14-cell stage in the posterior daughters of MS and E, MSp and Ep, respectively, but not their anterior daughters (Figure 3.7 E and F and data not shown). After the 14-cell stage, the GFP signal was too low for us to continue evaluating. This result strongly supports the model that the elevated POP-1 nuclear export in the posterior daughter cells of A-P divisions is a result of a higher level of LIT-1 kinase in the posterior nuclei.

GFP::LIT-1 Nuclear Asymmetry Is Regulated by the Wnt/MAPK Pathway

If POP-1 nuclear asymmetry is regulated through phosphorylation by LIT-1, whose own nuclear level is reciprocally asymmetric between A-P sisters, the question then becomes what regulates the asymmetry of LIT-1. I have examined the effect on GFP::LIT-1 in embryos depleted of some components of the Wnt/MAPK signaling pathway, *mom-2*, *mom-5*, *wrm-1*, or *mom-4*. I observed that depletion of any of these four genes resulted in a loss of the enrichment for interphase nuclear GFP::LIT-1 in E (88%, n = 16; 93%, n = 15; 100%, n = 19; 100%, n = 28, respectively) (Figure 3.7 G, H, and I and data not shown). These results suggest that the level of nuclear LIT-1 in E is regulated by both Wnt and MAPK signaling pathways. The enrichment for interphase nuclear GFP::LIT-1 in ABxxp was also abolished when *mom-5* (93%, n = 15), *wrm-1* (100%, n = 19), or *mom-4* (100%, n = 28) was depleted (Figure 3.7 I and data not shown). However, the enrichment in ABxxp was still observed in

mom-2(RNAi) embryos (100%, n = 16; Figure 3.7 I and data not shown), suggesting that while some components in the Wnt/MAPK signaling pathway are involved in regulating the level of nuclear LIT-1 in ABxxp, the MOM-2 Wnt signaling molecule is not required. Taken together, these results demonstrate that the upstream components of the Wnt/MAPK pathway regulate POP-1 nuclear asymmetry in part by regulating the level of nuclear LIT-1 in posterior cells.

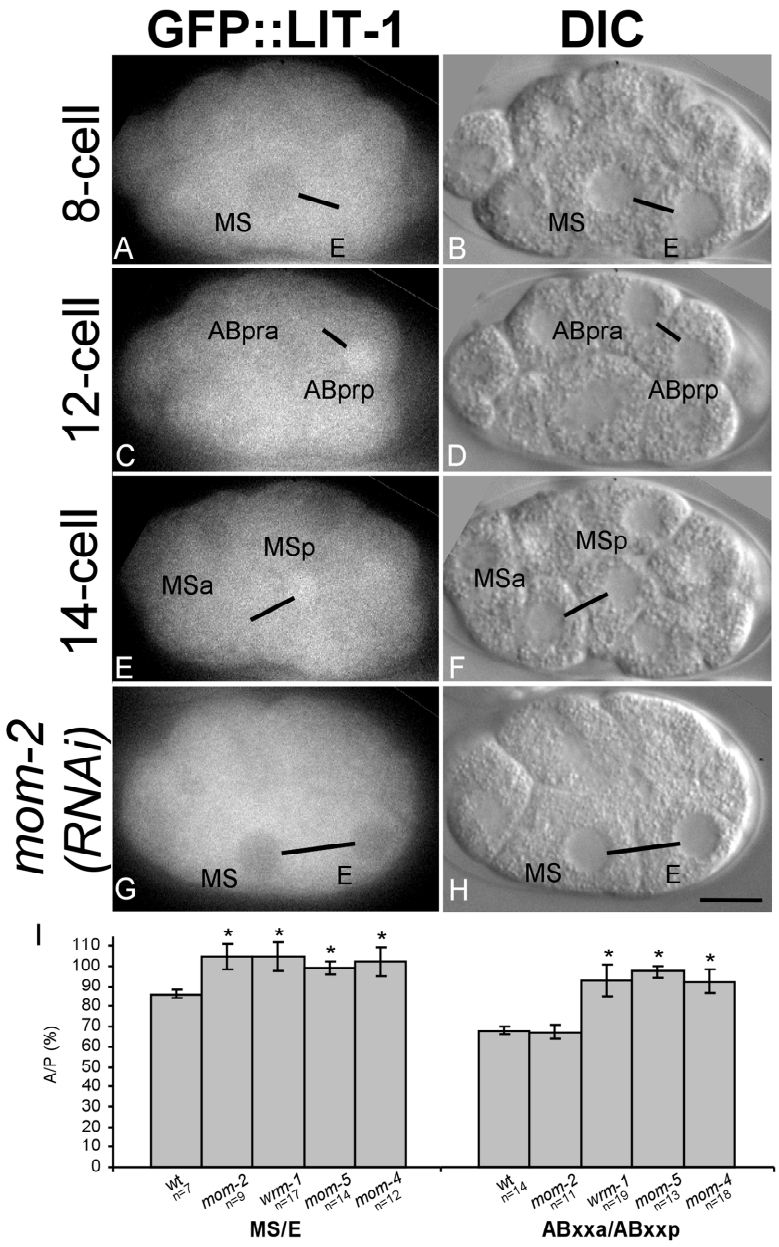


Figure 3.7 – GFP::LIT-1 nuclear asymmetry between A-P sisters is reciprocal to GFP::POP-1 nuclear asymmetry. Micrographs showing GFP::LIT-1 fluorescence (A, C, E, and G) and DIC images (B, D, F, and H) in wild-type or *mom-2(RNAi)* backgrounds. (A, B, G, and H) eight-cell, (C and D) 12-cell, (E and F) 14-cell. Images are selected from movie recordings. The black line in each panel connects a pair of A-P sister nuclei whose names are indicated and which are equally focused at that particular focal plane. (I) Relative levels (A/P%) of nuclear GFP::LIT-1 between MS and E (left-hand graphs) and ABxxa and ABxxp (right-hand graphs) in wild-type, *mom-2(RNAi)*, *wrm-1(RNAi)*, *mom-5(RNAi)*, and *mom-4(RNAi)* embryos. *$p < 0.001$. Scale bar, 10 μm.

DISCUSSION

I show here that the Wnt/MAPK signaling regulates POP-1 nuclear asymmetry in early embryos by regulating its interaction with PAR-5. First, POP-1 nuclear asymmetry is disrupted in *par-5* mutant embryos. Reintroduction of PAR-5 into four-cell staged *par-5* mutant embryos restores POP-1 nuclear asymmetry. Second, POP-1 is hyperphosphorylated and interacts with PAR-5 in embryo extracts. Both POP-1 hyperphosphorylation and its interaction with PAR-5 depend on the LIT-1 kinase. Third, the potential POP-1 phosphorylation sites essential for POP-1 nuclear asymmetry are also required for PAR-5 interaction and POP-1 nuclear export following stimulation by the LIT-1/WRM-1 complex *in vitro*. At least two of these potential phosphorylation sites are phosphorylated in a LIT-1/WRM-1-dependent manner *in vivo*. Fourth, inhibition of nuclear export also leads to a loss of POP-1 nuclear asymmetry. These data support the model that PAR-5 regulates POP-1 nuclear asymmetry by facilitating its nuclear export. In addition, I show that a GFP::LIT-1 reporter also exhibits nuclear asymmetry between A-P sisters, but in a reciprocal pattern to that of POP-1, and that the GFP::LIT-1 nuclear asymmetry requires regulation by the upstream kinase as well as Wnt signaling.

It is clear that a Wnt-independent mechanism regulates the steady-state level of nuclear POP-1 in every embryonic cell. This is supported by two observations. (1) In the absence of input from Wnt/MAPK signaling (either in signaling mutants or in transgenic animals expressing GFP::POP-1_{AAAAA}), POP-1 remains nuclear in every cell. (2) Mutations of lysines 185, 187, and 188 to alanines result in a defect in POP-1 nuclear retention in every cell but do not affect A-P asymmetry (Gay *et al.*, 2003). What our results present here is a

Wnt/MAPK-dependent mechanism that facilitates nuclear export of POP-1 preferentially in the signal-responsive cell, resulting in the observed A-P nuclear asymmetry (Figure 3.8). Our data demonstrate that the Wnt/MAPK signaling results in a higher level of nuclear LIT-1 kinase in the posterior cell. The LIT-1/WRM-1 complex promotes POP-1/PAR-5 interaction via phosphorylation of POP-1 at two and perhaps more of the five identified potential phosphorylation sites.

The mechanism by which PAR-5 results in a low level of nuclear POP-1 remains unclear, although a role for the exportin CRM-1 is suggested. It has been shown previously that acetylation of POP-1 by CBP-1 at K_{185} (and likely K_{187} and K_{188}) facilitates POP-1 nuclear retention (Gay *et al.*, 2003). Therefore, PAR-5 could result in a low level of nuclear POP-1 by antagonizing the effect of acetylation at these three lysines, by sequestering POP-1 in the cytoplasm, or by promoting POP-1 nuclear export. 14-3-3 proteins have been shown to promote nuclear export through different mechanisms. These include interfering with the NLS of the target protein, allowing recognition or binding by the CRM-1-dependent nuclear export machinery, and providing an NES located at their own C-termini (Lopez-Girona *et al.*, 1999). Despite our efforts, we have not yet identified an NES of POP-1 that functions in its export in *C. elegans* embryos (M-C.L., F.G., Y.S. and R.L., unpublished data). Because of their roles in POP-1 nuclear retention, acetylated lysines 185, 187, and 188 could interact with nuclear export machinery and complicate the identification of an NES *in vivo*. Further analyses are underway to elucidate the mechanism by which PAR-5 binding facilitates POP-1 nuclear export.

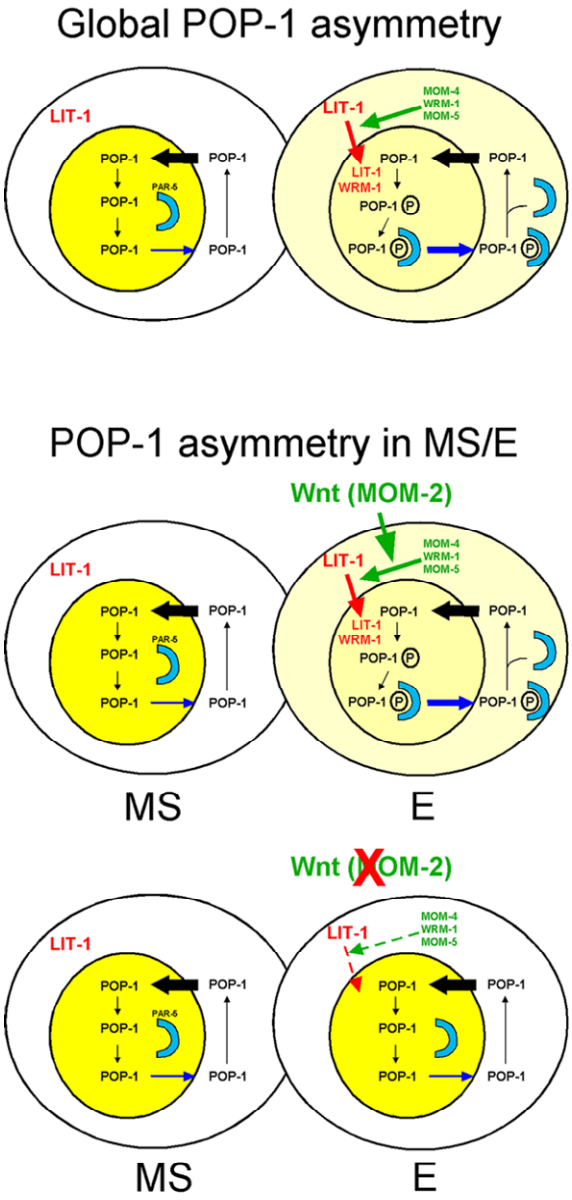


Figure 3.8 – Model
We propose that POP-1 nuclear asymmetry throughout the early *C. elegans* embryo is a result of a PAR-5-mediated nuclear export that occurs predominantly in the posterior cells. In this model, the upstream Wnt/MAP kinase components result in a high nuclear LIT-1 level in posterior cells. A higher level of nuclear LIT-1, in combination with the presence of the WRM-1 protein, results in hyperphosphorylation of POP-1 in the posterior nuclei. Phosphorylation of POP-1 by LIT-1 creates a binding site(s) for PAR-5 (blue crescent). Binding to PAR-5 facilitates the nuclear export of POP-1, resulting in the observed lower level of nuclear POP-1 in posterior cells. In the E blastomere, input from the Wnt signaling (MOM-2) is also required for a high level of nuclear LIT-1. The level of POP-1 is indicated by different shades of yellow, with the brightest yellow representing the highest level.

What Is the First Asymmetry?

POP-1 nuclear asymmetry is observed between all sisters derived from A-P divisions throughout early embryogenesis (Lin *et al.*, 1998). LIT-1 appears to function in regulating

POP-1 nuclear asymmetry throughout all lineages examined (Meneghini *et al.,* 1999; Rocheleau *et al.*, 1999; R.O. and R.L., personal communication). I now show that WRM-1 and MOM-4 regulate nuclear LIT-1 levels in the posterior cells, which alone could account for how these genes regulate POP-1 nuclear asymmetry. However, it is unlikely that WRM-1 and MOM-4 regulate POP-1 nuclear asymmetry solely by regulating the level of LIT-1 in the posterior nuclei, because WRM-1 is essential for and MOM-4 enhances LIT-1 kinase activity *in vitro* (Rocheleau *et al.*, 1999; Shin *et al.*, 1999), suggesting multiple levels of regulation. How do WRM-1 and MOM-4 lead to a high nuclear LIT-1 level in posterior cells is not clear. The localization of WRM-1 and MOM-4 has not been determined. It will be interesting to know whether these two proteins also exhibit A-P asymmetry.

Anterior-posterior polarity in *C. elegans* embryos is established upon fertilization and is propagated through the subsequent divisions. A key challenge is to identify the first cue that establishes A-P differences recognized by the Wnt/MAPK signaling pathway and to understand how this cue is propagated through subsequent divisions. In most eukaryotes, cells in a tissue exhibit a common polarity within the plane of the tissue, a phenomenon known as planar cell polarity (see review by Adler and Lee, 2001). Many key members functioning in regulating the planar cell polarity have been identified in other organisms, including Frizzled and Nemo, which are homologs of MOM-5 and LIT-1, respectively (Choi and Benzer, 1994; Adler and Lee, 2001). This suggests the intriguing possibility that the global POP-1 A-P polarity in *C. elegans* embryos may be regulated by a mechanism similar to that regulating the planar cell polarity.

The level of nuclear LIT-1 (as well as nuclear POP-1) in E requires additional input from P2 via Wnt signaling (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997; Lin *et al.*, 1998; and this work). It is not clear why Wnt signaling is needed for the E blastomere or how it achieves this regulation. One possible convergent point for Wnt and MAPK pathways is the WRM-1 protein, which (1) is an integral part of LIT-1 kinase activity, (2) is required for the enrichment of nuclear LIT-1 in the posterior cells, and (3) shares similarity to the known Wnt component, β-catenin. Perhaps Wnt signaling activates WRM-1, which otherwise is inactive in E, promoting a high level of nuclear LIT-1. In the canonical Wnt pathway, β-catenin translocates into the nucleus upon Wnt signal (Riggleman *et al.,* 1990; Yanagawa *et al.,* 1995). Since WRM-1 physically interacts with LIT-1 in *vivo* (Rocheleau *et al.*, 1999), it is an intriguing possibility that WRM-1 may import LIT-1 into the nuclei of posterior cells following Wnt stimulation.

Phosphorylation of POP-1 by LIT-1 May Cause A Qualitative Change of POP-1

It was mentioned in chapter two that POP-1 is qualitatively different between MS and E. In the *crm-1* RNAi experiments, a GFP::POP-1-dependent endoderm repression was observed in *crm-1(RNAi)* embryos. Although the levels of the endogenous POP-1 are equal between the nuclei of MS and E in some of the RNAi-treated embryos, all the embryos produce intestine. However, no *crm-1(RNAi)* embryos that express GFP::POP-1 produce intestine (Lo *et al.*, 2004). Furthermore, although GFP::POP-1_{AAAAA} is detected equally between the nuclei of MS and E, it represses MS-derived endoderm in 91% of *pop-1 (zu189)* embryos and E-derived endoderm in only 62% of *pop-1(zu189)* embryos. These results further support that

POP-1 is qualitatively different between MS and E (and perhaps all the A-P sisters) and that a higher level of POP-1 is needed to repress endoderm in the E blastomere than in the MS blastomere.

This qualitative difference is likely due to the differential modification of POP-1 between MS and E. The MAPK LIT-1 is differentially localized between the nuclei of MS and E, with a higher level in the E nucleus, which may lead to POP-1 phosphorylation preferentially in the E nucleus. When the potential LIT-1 phosphorylation sites within POP-$1_{101\text{-}130}$ were changed to aspartates (POP-1_{DDDDD}), I observed a decrease in the ability of this mutated POP-1 to rescue the MS fate defect in *pop-1(zu189)* embryos. This result suggests that the phosphorylation of POP-1 at these amino acids by LIT-1, in addition to promoting its nuclear export, may inhibit the endoderm repressive activity of POP-1. This may explain why a higher nuclear level of POP-1 is needed to repress endoderm in the E blastomere than in the MS blastomere. The reasons that the endoderm repressive activity of POP-1_{DDDDD} in the MS blastomere is not completely blocked could be: (1) aspartates may not exactly mimic the phosphorylation state, (2) phosphorylation at these amino acids may not be the only qualitative difference of POP-1 between MS and E, and other modifications of POP-1 may occur in the E blastomere to inhibit POP-1 repressor activity, (3) there are other differences between MS and E in addition to the quality of nuclear POP-1, such as the differential presence of a POP-1 interacting protein.

Nuclear Export of TCF Transcription Factors: A Conserved Mechanism?

Is the Wnt/MAPK induced nuclear export of a TCF protein described here a *C. elegans* specific phenomenon? *C. elegans* POP-1 is the only TCF protein known to undergo nucleo-cytoplasmic redistribution upon Wnt signaling. TCF proteins appear to be constitutive nuclear proteins in all other organisms examined so far. In addition, the canonical Wnt signaling pathway results in the activation of Wnt-responsive genes via a TCF/β-catenin complex. It would seem counterintuitive to lower the level of nuclear TCF proteins in order to activate transcription in this model. Therefore, it is possible that the Wnt-induced nuclear export of TCF proteins only occurs in *C. elegans* embryos where POP-1 functions mainly as a repressor. However, the following observations suggest that the Wnt signaling-induced nuclear export of TCF proteins may not be limited to *C. elegans* embryos. First, the *Drosophila* TCF homolog dTcf has been observed to undergo nuclear export in the wing imaginal disc in response to Wingless stimulation (Chan and Struhl, 2002). Although this paper was retracted (Struhl, 2004), personal communication indicated that this result was still valid. Second, it has been shown that reduction of dTcf partially suppresses whereas its overexpression enhances the *wingless* mutant phenotype (Cavallo *et al.*, 1998). This is consistent with a model where Wnt signaling lowers the level of TCF proteins. Third, in the development of *C. elegans* male tail, Wnt signaling lowers the nuclear level of POP-1 in the cell T.p, whose fate is specified by POP-1 (Herman, 2001). LIT-1 homologs have been shown to regulate the activity of TCF proteins in a variety of organisms (Choi and Benzer, 1994; Hyodo-Miura et al., 2002; Ishitani et al., 2003), and 14-3-3 proteins are highly

conserved among eukaryotes. Therefore, it is an intriguing possibility that LIT-1 homologs and 14-3-3 proteins may also regulate nuclear export of TCF proteins in other organisms.

MATERIALS AND METHODS

Strains

N2 was used as the wild-type strain. Genetic markers used in this chapter are: LGI, *pop-1 (zu189)*, *dpy-5(e61), hT1(I;V)*; LGIII, *unc-119(ed3)*, *unc32(e189)*, *lit-1(t1512)*; LGIV, *par-5 (it55)*, *unc-22(e66), dnT1(IV;V)*. Most transgenic strains analyzed in this chapter were generated by injection [the *unc-119(ed3)III* strain] and maintained the transgenes as extrachromosomal arrays. The following are the transgenes and strains that were not described in chapter two: $P_{med\text{-}1}$*gfp::par-5* (pRL779) in TX385(*teEx95*); $P_{med\text{-}1}$*SV40NLS::gfp::pop-1* (pRL768) in TX350(*teEx70*); $P_{med\text{-}1}$*gfp::pop-1*$_{mutAAA}$ (pRL626) in TX283(*teEx26*); $P_{med\text{-}1}$*SV40NLS::gfp::pop-1*$_{mutAAA}$ (pRL777) in TX438(*teEx115*); TX324(*teEx48*) and TX326 (*teEx50*) were generated by microparticle bombardment (Praitis *et al.*, 2001) of the plasmid $P_{pie\text{-}1}$*gfp::lit-1* (pRL710) and show identical GFP::LIT-1 patterns. The *par-5(it55)unc-22 (e66)(IV)/dnT1(IV;V);teEx95* strain (TX374) was generated by crossing TX385 males with KK299 hermaphrodites [*par-5(it55)unc22(e66)(IV)/dnT1(IV;V)*, a gift from Ken Kemphues], backcrossing the F1 non-Unc males to KK299, and picking F2 Unc hermaphrodites segregating *unc-22*, *dnT1* and GFP.

Plasmid Construction

Most GFP fusions were constructed using the Gateway cloning technology (Invitrogen) as described in chapter two (Lo *et al.*, 2004; Robertson *et al.*, 2004). The destination vector containing $P_{med\text{-}1}$*SV40NLS::gfp* (pRL706) sequence was generated by replacing the BamHI-NcoI *gfp::pop-1* fragment of pMM414 (Maduro *et al.*, 2002) with *NLS::gfp* (from the Fire vector kit pPD104.53) and then blunt-end ligating the Gateway cassette (reading frame A) via an introduced EcoRV site downstream of the *gfp* sequence. The *par-5* coding sequence is a gift from Ken Kemphues. The *pop-1*$_{mutAAA}$ coding sequence is a gift from Yang Shi. The GFP::LIT-1 expressing plasmid was generated by introducing the *lit-1* coding sequence into the $P_{pie\text{-}1}$*gfp* destination vector pID3.01B (a gift from Geraldine Seydoux). All site-directed mutageneses were performed using the QuikChange Site-Directed Mutagenesis kit as described in chapter two (Stratagene).

Analysis of Embryos and Imaging

Embryos of the *unc-22* animals segregated from KK299 and TX374 were processed for POP-1 immunostaining as described previously (Lin *et al.*, 1998). Imaging of live embryos and immunofluorescence and data analyses were performed as described previously (Rogers *et al.*, 2002; chapter two). Quantification of GFP::LIT-1 was performed using the ImageJ program and analyzed using the Student's t-test for the statistical significance.

RNA Interference

The dsRNA for *mom-2, mom-4*, and *mom-5* were synthesized from corresponding cDNA or genomic DNA using the *In Vitro* Transcription Kit from Amershan, and RNAi was performed by injection (Rogers *et al.*, 2002). *wrm-1(RNAi)* was performed by feeding L3 or L4 larvae to adulthood at 25ºC (Timmons and Fire, 1998). Embryos laid from 16 to 30 hours after injection or 24 to 30 hours after feeding were collected and either allowed to differentiate for 8 more hours, a period long enough for wild-type control embryos to hatch, or processed for imaging. RNAi with all the four genes resulted in 100% dead embryos. A decrease in the overall level of GFP::LIT-1 in the *mom-4(RNAi)* and *wrm-1(RNAi)* embryos was observed (n>100), suggesting that MOM-4 and WRM-1 might also be required for LIT-1 stability.

Antibody Production and Biochemical Analyses

Anti-S_{118}-P and anti-S_{127}-P antibodies were generated by immunizing rabbits with peptides phosphorylated at respective residues. The antibodies were precleared with non-phosphorylated peptides then affinity-purified using phosphorylated peptides (Bethyl Laboratory, Inc.). The affinity-purified antibodies react with corresponding phosphorylated peptides with a >1000-fold higher affinity compared to the unphosphorylated peptides by ELISA assays.

Embryos were collected from gravid adults and sonicated in the co-IP buffer to yield extracts (Calvo *et al.*, 2001). *lit-1(t1512)* mutant animals were shifted to 25°C at L3 prior to

the embryo collection. For each co-IP reaction, 400 μg of extracts were incubated overnight at 4°C with protein A beads coated with either affinity-purified 94I (Lin *et al.*, 1995) or preimmune IgG. The beads were washed three times in the co-IP buffer with 500 mM KCl and twice in the co-IP buffer. The immunoprecipitates were subjected to western blotting analyses using either anti-PAR-5 antibody (a gift from Andy Golden and Diane Shakes) or 94I and visualized using an ECL system (Amersham). For the gel shown in Figure 2C, 20 μg of embryo extracts were incubated with 20 U of calf intestinal alkaline phosphatase (Roche) at 37°C for 1.5 hours prior to SDS-PAGE.

CHAPTER FOUR
POP-1 Nuclear Asymmetry May Also Be Regulated by Differential Protein Degradation

INTRODUCTION

In chapter two, I showed that the $aa_{400-438}$ region of POP-1 is essential for the low POP-1 nuclear level in posterior cells. The $aa_{400-438}$ region of POP-1 is poorly understood due to its poor sequence similarity to any known protein. I was surprised to find out that deleting this region caused a complete loss of POP-1 nuclear asymmetry. Therefore, I took a closer look at the amino acid sequence of this region. Using a computer program called PEST-FIND available at http://www.biu.icnet.uk/projects/pest/, I found that a potential PEST region, which extends from arginine 396 to histidine 427, overlapped with $aa_{400-438}$ (Figure 4.1).

PEST regions were first described when Rogers and Rechsteiner attempted to find common sequences of a small group of rapidly degraded proteins in 1986 (Rogers and Rechsteiner, 1986). They found that these proteins contained regions enriched in proline (P), glutamate (E), serine (S) and threonine (T), which were uninterrupted by positively charged residues. PEST-FIND defines PEST regions as hydrophilic stretches of amino acids greater than or equal to twelve residues in length, which contain at least one P, one E or D and one S or T. These regions are flanked by lysine, arginine or histidine residues, but positively charged residues are not allowed within the PEST sequence (see review by Rechsteiner and Rogers, 1996).

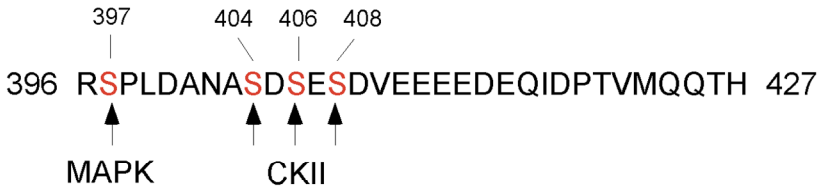


Figure 4.1 – The amino acid sequence of the potential PEST region located at the C-terminus of POP-1. The amino acid sequence of the $aa_{396-427}$ region is shown as single-letter code, and the potential MAPK and CKII phosphorylation sites are in red. Numbers above the sequence indicate positions of amino acids.

PEST regions have been shown to serve as proteolytic signals for the PEST-containing proteins (see review by Rechsteiner and Rogers, 1996), and most studies support that PEST sequences target proteins for degradation through the ubiquitin-proteosome pathway (see reviews by Rechsteiner, 1991; Rechsteiner and Rogers, 1996). Therefore, it is an intriguing possibility that the low POP-1 nuclear level in posterior cells is resulted from increased POP-1 degradation specifically in posterior cells. Some PEST sequences are constitutive proteolytic signals, but some appear to be inducible. There are many ways to activate inducible PEST sequences (see review by Rechsteiner and Rogers, 1996). For example, binding of phorbol ester to protein kinase C leads to a conformational change of the protein and exposure of a hidden PEST motif. There are many examples where phosphorylation controls the stability of a protein. So it is likely that addition of phosphates to serine/threonine residues serves as a mechanism for activating a latent PEST signal. Phosphorylation could target proteins for ubiquitination and subsequent binding of the ubiquitinated proteins to the proteosome complex.

To further study the importance of the PEST sequences located at the C-terminus of POP-1, I searched for possible phosphorylation sites in this region using the Scansite (see

chapter three). Several potential phosphorylation sites were identified. First, three potential casein kinase II (CKII) sites are located at serines 404, 406 and 408. Second, one potential MAPK site is located at serine 397 (Figure 4.1). The importance of these amino acids in POP-1 nuclear asymmetry and repressor function was examined.

RESULTS

The Potential CKII Sites, S_{404}, S_{406} and S_{408}, Are Important for POP-1 Nuclear Asymmetry

To test the importance of these potential CKII sites in POP-1 nuclear asymmetry and repressor function, I mutated them to alanines (POP-$1_{S404/406/408A}$). I observed a variable defect in POP-1 nuclear asymmetry when these three serines were changed to alanines. The nuclear asymmetry was abolished or less obvious in some A-P sisters (Figure 4.2). However, the endoderm repressive activity of POP-$1_{S404/406/408A}$ was not affected. The MS fate defect was rescued in all the *pop-1(zu189)* embryos expressing POP-$1_{S404/406/408A}$ (100%, n = 13; Table 2.1; data not shown). These results suggest that S_{404}, S_{406} and S_{408} are important for POP-1 nuclear asymmetry but not its repressor activity. I also observed an endoderm ablation effect associated with POP-$1_{S404/406/408A}$. In five percent of the wild-type (n = 22) and 38% of the *pop-1(zu189)* (n = 13) embryos expressing POP-$1_{S404/406/408A}$, the endoderm fate was repressed in both MS and E (Table 2.1; data not shown).

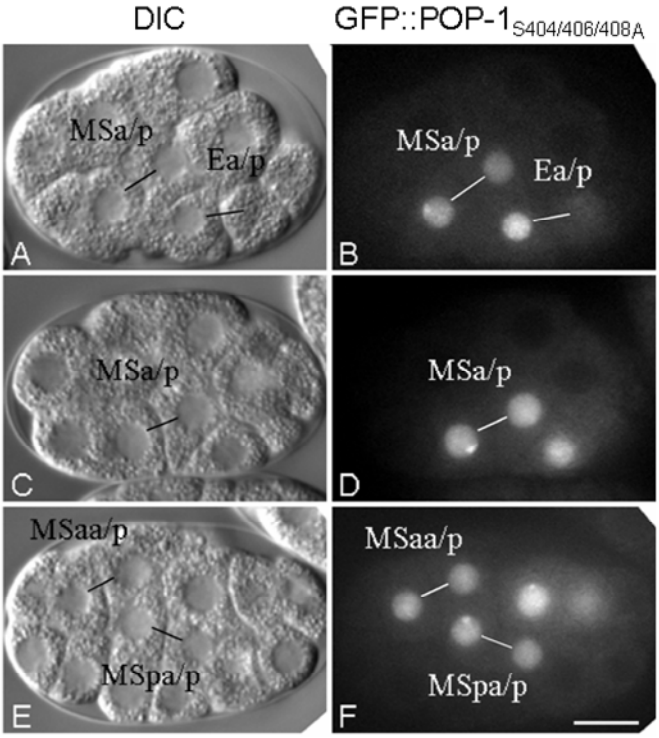


Figure 4.2 – POP-1 nuclear asymmetry is variably affected in POP-1$_{S404/406/408A}$. Micrographs of transgenic embryos harboring $P_{med\text{-}1}$*gfp::pop-1*$_{S404/406/408A}$ (TX722). (A, C, and E) DIC images. (B, D, and F) GFP fluorescence images. A-P sisters are connected by lines and their names are indicated. The A-P nuclear asymmetry is abolished in the MSa/p sisters in (D) and in the MSaa/p sisters in (F), but some asymmetry is still seen in the MSa/p sisters in (B) and in the MSpa/p sisters in (F). Except the Ep in (A) and (B), the nuclei of each A-P sister blastomeres are equally focused at that particular focal plane. Scale bar, 10 μm.

CKII RNAi Causes A Gutless Phenotype in Embryos Expressing GFP::POP-1

To know whether CKII plays a role in POP-1 nuclear asymmetry, I performed RNAi against the regulatory subunit of CKII (*kin-10*) in the strain TX300, which carries integrated $P_{med\text{-}1}$ *gfp::pop-1* transgenes. *kin-10* RNAi in TX300 resulted in dead embryos (22%, n = 143; data not shown), but I did not observe any obvious defect in POP-1 nuclear asymmetry (data not shown). Although POP-1 nuclear asymmetry was largely normal, I observed a gutless

phenotype in these RNAi-treated embryos. About 10% of the TX300 embryos treated with *kin-10* RNAi failed to produce intestine (n = 143, Figure 4.3), suggesting a role for CKII in the endoderm formation in *C. elegans*.

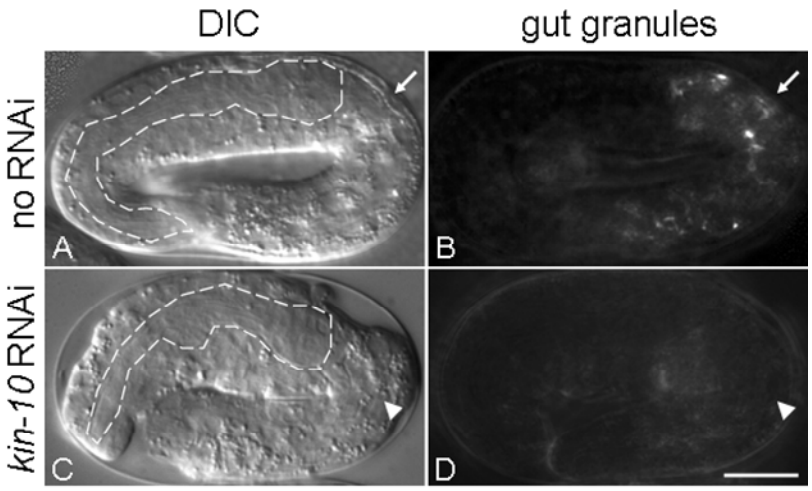


Figure 4.3 – *kin-10* RNAi causes a gutless phenotype in the embryos expressing $P_{med\text{-}1}$ *gfp::pop-1*. Transgenic embryos harboring $P_{med\text{-}1}$*gfp::pop-1* (TX300) with (C and D) or without (A and B) *kin-10* RNAi treatment. Terminally differentiated embryos are viewed with DIC (A and C) or polarized light (B and D). Ten percent of the TX300 embryos treated with *kin-10* RNAi do not produce intestine. Pharyngeal tissues are outlined in (A) and (C). White arrows in (A) and (B) point to the well-differentiated intestine in the TX300 embryo, and white arrowheads in (C) and (D) point to the position where intestine would have been formed in a wild-type embryo. Scale bar, 10 μm.

S_{397} Is Not Important for POP-1 Nuclear Asymmetry or Repressor Activity

Changing S_{397} to alanine did not affect POP-1 nuclear asymmetry or endoderm repressive function (Table 2.1; data not shown). However, an endoderm ablation effect was also observed with POP-1_{S397A}. In two percent of the wild-type (n = 43) and 25% of the *pop-1*

(zu189) (n = 4) embryos expressing POP-1_{S397A}, the endoderm fate was repressed in both MS and E (Table 2.1; data not shown).

DISCUSSION

The mechanism by which $aa_{400-438}$ mediates POP-1 nuclear asymmetry may be different from that by $aa_{101-130}$. I showed in chapter three that $aa_{101-130}$ may mediate POP-1 nuclear asymmetry via differential nuclear export of POP-1 protein between A-P sisters. The $aa_{400-438}$ region overlaps with a potential PEST region, suggesting that POP-1 nuclear asymmetry may also be regulated by differential protein degradation between A-P sisters. Consistent with this hypothesis, depleting a component of the proteosome complex by RNAi resulted in a defect in POP-1 nuclear asymmetry (R.O. and R.L., personal communication).

There are three potential CKII phosphorylation sites within this potential PEST region. In *Drosophila*, CKII has been shown to associate with and phosphorylate Dishevelled in response to Wingless stimulation (Willert *et al.*, 1997), suggesting a role for CKII in Wg/Wnt signal transduction. CKII has also been shown to phosphorylate a constitutive phosphorylation site of IκBα, which resides in the C-terminal PEST region of IκBα (Schwarz *et al.*, 1996). This constitutive phosphorylation is required for rapid degradation of free IκBα (Schwarz *et al.*, 1996). Therefore, it is likely that phosphorylation of the potential CKII sites at the C-terminus of POP-1 by CKII targets POP-1 for degradation in the Wnt/MAPK-responsive posterior cells, resulting in POP-1 nuclear asymmetry. Consistent

with this hypothesis, I show that the potential CKII sites, S_{404}, S_{406} and S_{408}, are important for POP-1 nuclear asymmetry. However, changing these three serines to alanines does not abolish POP-1 nuclear asymmetry completely, whereas the nuclear asymmetry is completely abolished in POP-$1_{\Delta 400\text{-}438}$. These results suggest that other amino acids within $aa_{400\text{-}438}$ may also be important for the differential POP-1 degradation required for POP-1 nuclear asymmetry.

It is not clear whether S_{404}, S_{406} and S_{408} are indeed phosphorylated by CKII. I did not observe any obvious defect in POP-1 nuclear asymmetry when I blocked CKII activity by *kin-10* RNAi in TX300 strain. Although *kin-10* RNAi may only cause a minor defect in POP-1 nuclear asymmetry, this result suggests that S_{404}, S_{406} and S_{408} may not be the real phosphorylation sites for CKII. Other explanations for this result could be: (1) *kin-10* RNAi did not completely block CKII activity, and the residual kinase activity is enough to phosphorylate those potential CKII sites. (2) CKII may not be the only kinase that can phosphorylate S_{404}, S_{406} and S_{408}.

Although *kin-10* RNAi did not cause a detectable defect in POP-1 nuclear asymmetry, it resulted in a gutless phenotype, suggesting that CKII plays a role in the endoderm formation in *C. elegans*. Because CKII has been implicated in transducing the Wg signal in *Drosophila* (Willert *et al.*, 1997), it is possible that CKII functions in the Wnt/MAPK signaling to induce the endoderm fate in *C. elegans*. However, it is not clear whether CKII acts on POP-1 or other Wnt/MAPK components to achieve this function. It is possible that CKII functions to downregulate the POP-1 nuclear level or POP-1 repressor activity in the E blastomere. It is also possible that CKII is required for the proposed

endoderm-activating function of POP-1 (see chapters two and six). Alternatively, CKII may act on other factors in the Wnt/MAPK signaling. It is not clear whether the endoderm ablation effect of *kin-10* RNAi is dependent on the $P_{med\text{-}1}$*gfp::pop-1* transgene in the TX300 strain because a similar RNAi experiment was not performed in the wild-type N2 strain. As in the *crm-1* RNAi experiment (see chapter three; Lo *et al.*, 2004), the overexpressed GFP::POP-1 from the $P_{med\text{-}1}$*gfp::pop-1* transgene may account for the endoderm ablation phenotype caused by *kin-10* RNAi. Further experiments are required to elucidate the role of CKII in *C. elegans* endoderm development.

MATERIALS AND METHODS

Strains

Genetic markers used in this chapter are: LGI, *pop-1(zu189)*, *dpy-5(e61), hT1(I;V)*; LGIII, *unc-119(ed3)*; LGIV, *him-3(e1147)*. Most transgenic strains analyzed in this chapter were generated by injection [the *unc-119(ed3)III; him-3(e1147)IV* (TX576) strain] and maintained the transgenes as extrachromosomal arrays. The following are the transgenes and strains that were not described in previous chapters: $P_{med\text{-}1}$*gfp::pop-1*$_{S404/406/408A}$ (pRL1490) in TX722 (*teEx283*); $P_{med\text{-}1}$*gfp::pop-1*$_{S397A}$ (pRL1491) in TX721(*teEx282*).

RNA Interference

kin-10 RNAi was performed by feeding L1 or L2 larvae of TX300 strain to adulthood at 20ºC (Timmons and Fire, 1998). The feeding bacteria strain for *kin-10* RNAi was from Julie Ahringer's feeding RNAi library. About 48 hours after feeding, embryos were collected and either allowed to differentiate for 16 more hours at 15ºC, or processed for imaging.

CHAPTER FIVE
The Study of Sufficiency for POP-1 Domains in Conferring A-P Nuclear Asymmetry Using Heterologous TCF Proteins

INTRODUCTION

In chapter three, I showed that the 14-3-3-mediated nuclear export of POP-1 induced by the Wnt/MAPK signaling in posterior cells resulted in the observed POP-1 nuclear asymmetry in *C. elegans* early embryos (Lo *et al.*, 2004). I then asked whether a heterologous TCF protein can be regulated by the Wnt/MAPK signaling in *C. elegans* embryos to undergo nuclear export in posterior cells. If it can not be regulated in *C. elegans* embryos, then it will be a great tool to assay the sufficiency for POP-1 domains in conferring A-P nuclear asymmetry. To test whether other TCF proteins exhibit A-P nuclear asymmetry in *C. elegans* embryos, various GFP::TCF fusions were expressed using the *med-1* promoter.

POP-1 and other TCF proteins share a very high sequence homology in their DNA-binding HMG domains and β-catenin binding domains, and a truncated POP-1 containing the HMG domain has been shown to bind a TCF target sequence (Korswagen *et al.*, 2000). Therefore, it is very likely that other TCF proteins may be able to bind POP-1 target sequences and regulate POP-1 target genes in *C. elegans* embryos. To test this possibility, the ability of some TCF proteins to rescue the MS fate defect was also assayed in the *pop-1 (zu189)* mutant background.

RESULTS

hTCF4, mLef1 and XTcf-3 Do Not Exhibit A-P Nuclear Asymmetry in *C. Elegans* Embryos

Three vertebrate TCF proteins were randomly selected, human TCF4 (hTCF4), mouse Lef1 (mLef1) and *Xenopus* Tcf-3 (XTcf-3). To examine their localization in *C. elegans* embryos, the gfp fusion constructs of each under the control of *med-1* promoter were generated ($P_{med\text{-}1}$*gfp::hTCF4*, $P_{med\text{-}1}$*gfp::mLef1*, and $P_{med\text{-}1}$*gfp::XTcf-3*) and expressed in *C. elegans* embryos. None of these three TCF proteins exhibited asymmetric nuclear levels between A-P sisters (Figure 5.1 and data not shown; M.-C.L., R.O., and R.L., unpublished data). These results suggest that hTCF4, mLef1 and XTcf-3 are not able to respond to the Wnt/MAPK signaling in *C. elegans* embryos. They also suggest that POP-1 contains unique *cis*-elements that allow POP-1 to be downregulated by the Wnt/MAPK signaling in *C. elegans* early embryos.

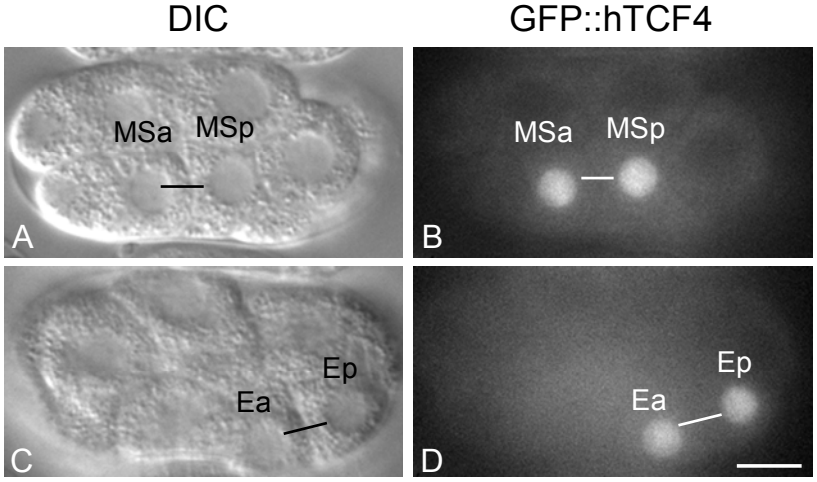


Figure 5.1 – hTCF4 does not exhibit A-P nuclear asymmetry in *C. elegans* embryos. Micrographs showing 16-cell transgenic embryos harboring $P_{med\text{-}1}$*gfp::hTCF4* (TX469). (A and C) DIC images. (B and D) GFP fluorescence images. A-P sisters are connected by lines and their names are indicated. The nuclei of each A-P sister blastomeres are equally focused at that particular focal plane. Scale bar, 10 μm.

hTCF4 Is Able to Rescue the MS Fate Defect in *pop-1(zu189)* Mutant Embryos

To test whether these TCF proteins can functionally substitute POP-1, the transgenes were introduced into *pop-1(zu189)* embryos and their ability to rescue the MS fate defect was assayed. Three transgenic lines expressing $P_{med\text{-}1}$*gfp::mLef1* were tested, including one with a low expression level (TX423) and two with a high expression level (TX424 and TX425). None of these three lines was able to rescue the MS fate defect in *pop-1(zu189)* embryos [0%, n = 27 (TX423), 16 (TX424), or 28 (TX425); data not shown; M.-C.L., R.O., and R.L., unpublished data]. Three lines expressing $P_{med\text{-}1}$*gfp::hTCF4* were also tested. TX468 was a low expression line, and both TX469 and TX470 expressed the transgene at a high level. While TX468 only rescued the MS fate partially in a small percentage of the *pop-1(zu189)* embryos examined (9%, n = 22; data not shown; M.-C.L., R.O., and R.L., unpublished data), both TX469 and TX470 showed a strong MS-fate rescuing activity [75% (n = 32) and 68% (n = 19), respectively; Figure 5.2 and data not shown]. These results suggest that when expressed at a high level, hTCF4 can repress endoderm fate in the MS blastomere in *pop-1 (zu189)* embryos. Moreover, hTCF4 also repressed the E-derived endoderm in *pop-1(zu189)*, resulting in gut ablation in 19% (TX469, n = 32) and 47% (TX470, n = 19) of the *pop-1 (zu189)* embryos expressing GFP::hTCF4 (Figure 5.2 and data not shown). This endoderm ablation effect was probably due to the high nuclear level of hTCF4 in the E blastomere. Only one transgenic line expressing $P_{med\text{-}1}$*gfp::XTcf-3* was tested, which expressed the transgene at a low level. No *pop-1(zu189)* embryos examined were rescued with this line (0%, n = 11; data not shown; M.-C.L., R.O., and R.L., unpublished data). Because none of

the high expression lines for XTcf-3 has been tested, it is not clear whether XTcf-3 can rescue the MS fate when expressed at a high level.

These results demonstrate that a human homolog of POP-1 can functionally substitute POP-1 to repress endoderm fate in the MS blastomere, probably through binding to POP-1 target sequences and regulating the expression of its target genes. I currently do not have data supporting this hypothesis. The ability of hTCF4 to rescue the derepression of E-specific genes such as *end-1*, *end-3*, *sdz-23*, and *sdz-26* (Robertson *et al.*, 2004; also see chapter six) in the MS blastomere in *pop-1(zu189)* mutant embryos is under investigation.

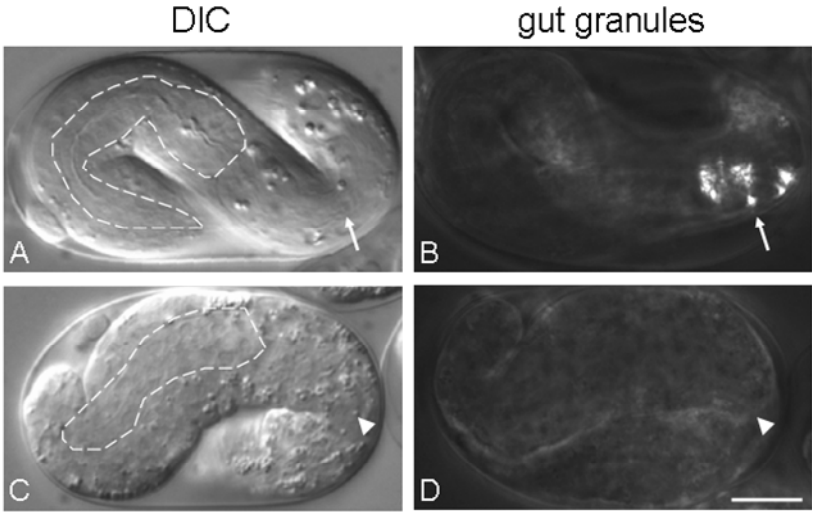


Figure 5.2 – hTCF4 is able to rescue the MS fate defect in *pop-1(zu189)* mutant embryos. Micrographs showing *pop-1(zu189)* embryos harboring $P_{med\text{-}1}$*gfp::hTCF4* (from TX469). Terminally differentiated embryos are viewed with DIC (A and C) or polarized light (B and D). MS fate is rescued in 75% of these embryos (dashed outline in A and C). (C and D) 19% of the embryos harboring the transgene do not produce intestine. White arrows in (A) and (B) point to the well-differentiated intestine in this particular embryo, and white arrowheads in (C) and (D) point to the position where intestine would have been formed in a wild-type embryo. Scale bar, 10 μm.

Swapping POP-1 Domains Required for A-P Nuclear Asymmetry into hTCF4

I have previously identified POP-1 domains required for the A-P nuclear asymmetry (see chapter two). To test the sufficiency for these POP-1 domains in conferring A-P nuclear asymmetry, I asked whether I can swap these domains into the corresponding regions of hTCF4 and generate a hTCF4/POP-1 chimera that is able to respond to the Wnt/MAPK signaling to exhibit A-P nuclear asymmetry in *C. elegans* embryos.

POP-1$_{48-192}$ Is Not Sufficient to Make hTCF4 Asymmetric

I showed in chapter two that aa$_{101-130}$ of POP-1 is required for POP-1 nuclear asymmetry. However, POP-1 shares very little homology with hTCF4 in this region as well as in the entire putative Groucho binding domain (see Figure 1.7). I could not determine the corresponding region for POP-1$_{101-130}$ in hTCF4. Therefore, I decided to swap the entire region between the β-catenin binding domain and the HMG domain. To do this, I first generated an EcoRI site at POP-1$_{47-48}$, which changed leucine 48 to phenylalanine, and a PmlI site at POP-1$_{191-192}$ without changing any amino acid. The same restriction enzyme sites were generated at aa$_{53-54}$ and aa$_{326-327}$ of hTCF4, respectively. The generation of restriction enzyme sites resulted in a point mutation at leucine 48 of POP-1, which would also be introduced into hTCF4 after the swap. To test whether this mutation would affect POP-1's function, I first examined the effect of this L$_{48}$F change on POP-1 nuclear asymmetry and repressor activity. I found that a GFP::POP-1$_{L48F}$ fusion protein expressed from the control of the *med-1* promoter still exhibited normal A-P nuclear asymmetry in *C. elegans* early embryos (data not shown). The ability of GFP::POP-1$_{L48F}$ to rescue the MS fate defect in

pop-1 (zu189) embryos was also comparable to a wild-type GFP::POP-1 (91%, n = 11; data not shown). These results indicate that the $L_{48}F$ mutation does not affect POP-1 nuclear asymmetry or repressor activity.

I then replaced the EcoRI-PmlI region of hTCF4 with the EcoRI-PmlI fragment of POP-1. The resultant chimera was named hTCF4/POP-$1_{48\text{-}192}$, in which $aa_{54\text{-}327}$ of hTCF4 was replaced with $aa_{48\text{-}192}$ of POP-1. This replacement also shortened hTCF4 protein from 596 amino acids to 467 amino acids. When GFP::hTCF4/POP-$1_{48\text{-}192}$ was expressed using *med-1* promoter in *C. elegans* embryos, I observed equal nuclear GFP levels between A-P sisters, similar to that of GFP::hTCF4 (Figure 5.3). This result suggests that although required for POP-1 nuclear asymmetry, $aa_{101\text{-}130}$ of POP-1 is not sufficient to confer the nuclear asymmetry property to hTCF4. Consistent with this, I have shown in chapter two that the region between the β-catenin binding domain and the HMG domain and its sub-regions are not sufficient for the A-P nuclear asymmetry in the context of POP-1 protein sequences.

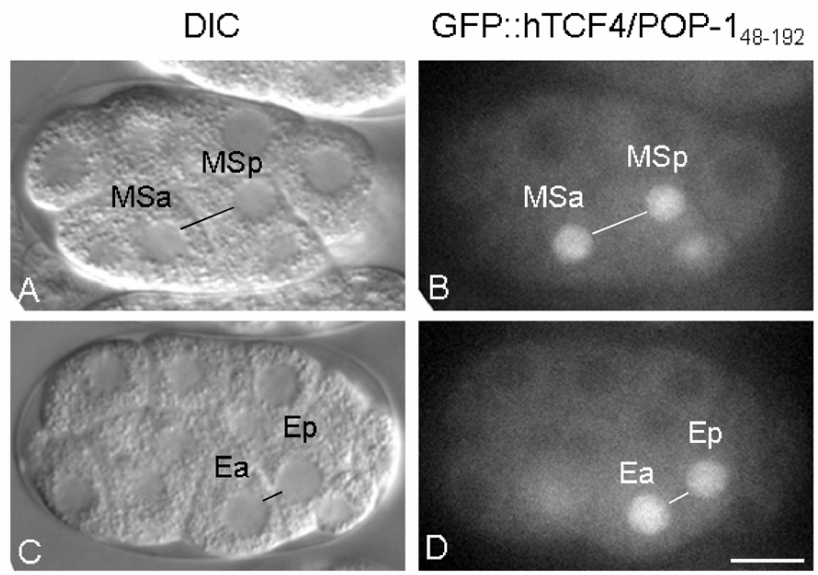


Figure 5.3 – POP-$1_{48\text{-}192}$ is not sufficient to confer A-P nuclear asymmetry to hTCF in *C. elegans* embryos. Micrographs showing transgenic embryos harboring $P_{med\text{-}1}$*gfp::hTCF4 /POP-*$1_{48\text{-}192}$ (TX614). (A and C) DIC images. (B and D) GFP fluorescence images. A-P sisters are connected by lines and their names are indicated. The nuclei of each A-P sister blastomeres are equally focused at that particular focal plane. Scale bar, 10 μm.

hTCF4/POP-$1_{48\text{-}192}$ had a better MS-fate rescuing activity than hTCF4 even though it was expressed at a lower level than hTCF4. In all of the *pop-1(zu189)* embryos expressing hTCF4/POP-$1_{48\text{-}192}$ examined, the MS-derived endoderm was repressed and the posterior pharynx was produced (100%, n = 53; data not shown). hTCF4/POP-$1_{48\text{-}192}$ also repressed the E-derived endoderm in 57% of the *pop-1(zu189)* embryos expressing this protein (n = 53; data not shown). These results demonstrate that the putative Groucho binding domain of POP-1 has a better capacity to repress endoderm fate than the corresponding region of hTCF4. Perhaps it has a stronger affinity for the corepressors such as UNC-37 (Groucho homolog) or other factors required for the endoderm repression in the MS blastomere. This

may also explain why a higher level of wild-type hTCF4 is needed to rescue the MS fate defect in *pop-1(zu189)* mutant embryos.

hTCF4/POP-1$_{48\text{-}192+315\text{-}438}$ Exhibits A-P Nuclear Asymmetry

I showed in chapter two that aa$_{400\text{-}438}$ of POP-1 is also required for POP-1 nuclear asymmetry. Therefore, hTCF4/POP-1$_{48\text{-}192}$ is likely to exhibit nuclear asymmetry if POP-1$_{400\text{-}438}$ is introduced into this protein. The C-terminus of TCF proteins is the most divergent region among the TCF family members. The sequence conservation between POP-1 and hTCF4 in the region distal to the HMG domain is restricted to the NLS located immediately C-terminal to the HMG domain (Prieve *et al.*, 1996; Hurlstone and Clevers, 2002; Hecht and Stemmler, 2003) and two motifs (KKCRARFG and WCXXCRRKKKC) located C-terminal to the NLS (Hurlstone and Clevers, 2002; Atcha *et al.*, 2003). I could not determine the corresponding region for POP-1$_{400\text{-}438}$ in hTCF4. Therefore, I decided to first swap the entire region distal to the two conserved motifs in the C-terminus. To do this, a truncated version of hTCF4/POP-1 $_{48\text{-}192}$ was first generated [hTCF4/POP-1$_{48\text{-}192}$(aa$_{1\text{-}334}$)], which contains the first 334 amino acids of hTCF4/POP-1$_{48\text{-}192}$. hTCF4/POP-1$_{48\text{-}192}$(aa$_{1\text{-}334}$) lacks the region C-terminal to the KKCRARFG and WCXXCRRKKKC motifs of hTCF4 and does not show A-P nuclear asymmetry (data not shown). Then a POP-1 fragment containing aa$_{315\text{-}438}$ was fused to the C-terminus of hTCF4/POP-1$_{48\text{-}192}$(aa$_{1\text{-}334}$), resulting in hTCF4/POP-1$_{48\text{-}192+315\text{-}438}$.

The GFP::hTCF4/POP-1$_{48\text{-}192+315\text{-}438}$ fusion protein was expressed in *C. elegans* early embryos using *med-1* promoter. Most embryos expressing GFP::hTCF4/POP-1$_{48\text{-}192+315\text{-}438}$ examined showed asymmetric nuclear GFP levels between A-P sisters with higher levels in

the anterior cells (Figure 5.4). Like hTCF4/POP-$1_{48\text{-}192}$, hTCF4/POP-$1_{48\text{-}192+315\text{-}438}$ was able to rescue the MS fate defect in *pop-1(zu189)* mutant embryos (100%, n = 6; Figure 5.5). Surprisingly, this hTCF4/POP-1 chimera showed a strong endoderm repressive activity in the E blastomere as well. 83% of the *pop-1(zu189)* embryos expressing hTCF4/POP-$1_{48\text{-}192+315\text{-}438}$ did not produce any intestinal tissue (n = 6, Figure 5.5). I currently do not know the reason for this strong endoderm ablation effect caused by hTCF4/POP-$1_{48\text{-}192+315\text{-}438}$.

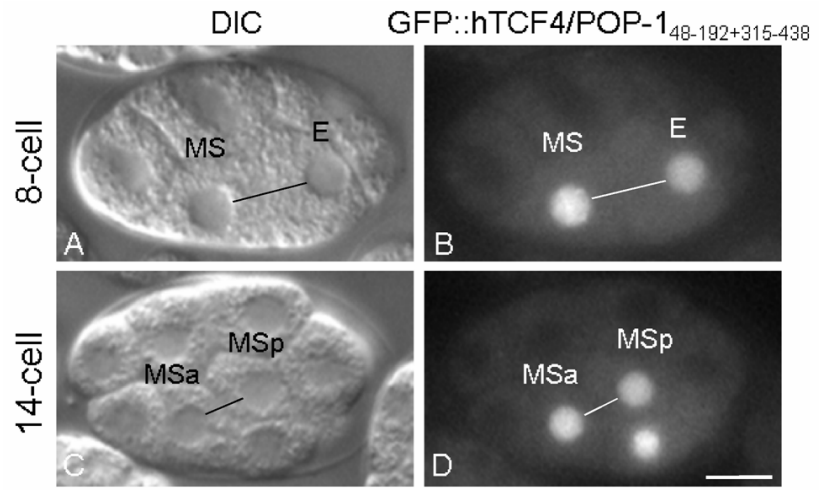


Figure 5.4 – hTCF4/POP-$1_{48\text{-}192+315\text{-}438}$ exhibits A-P nuclear asymmetry in *C. elegans* embryos. Micrographs showing 8-cell (A and B) and 14-cell (C and D) transgenic embryos harboring $P_{med\text{-}1}$*gfp::hTCF4/POP-*$1_{48\text{-}192+315\text{-}438}$ (TX712). (A and C) DIC images. (B and D) GFP fluorescence images. A-P sisters are connected by lines and their names are indicated. The nuclei of each A-P sister blastomeres are equally focused at that particular focal plane. Scale bar, 10 μm.

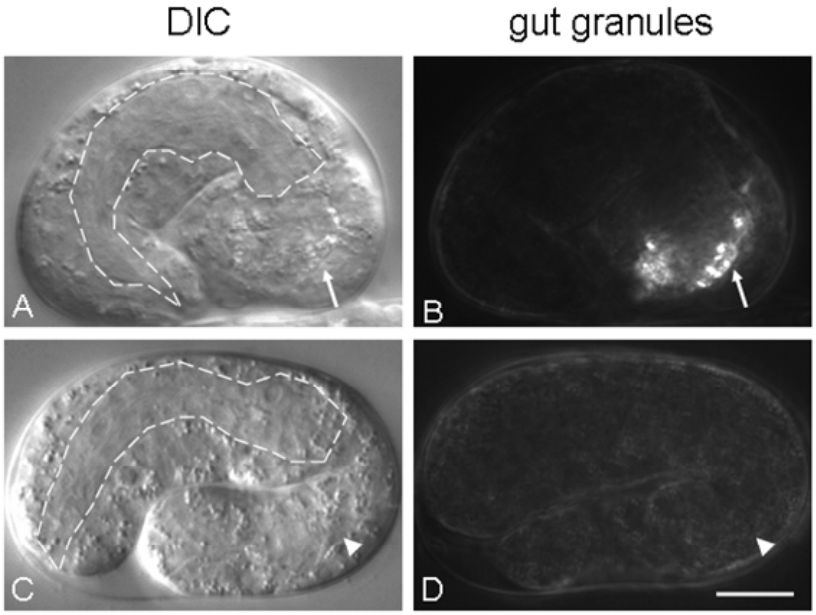


Figure 5.5 – hTCF4/POP-$1_{48\text{-}192+315\text{-}438}$ rescues the MS fate defect in *pop-1(zu189)* mutant embryos. Micrographs showing *pop-1(zu189)* embryos harboring $P_{med\text{-}1}$*gfp::hTCF4/POP-*$1_{48\text{-}192+315\text{-}438}$ (from TX712). Terminally differentiated embryos are viewed with DIC (A and C) or polarized light (B and D). MS fate is rescued in all of these embryos (dashed outline in A and C). (C and D) 83% of the embryos harboring the transgene do not produce intestine. White arrows in (A) and (B) point to the intestinal tissues, and white arrowheads in (C) and (D) point to the position where intestine would have been formed in a wild-type embryo. Scale bar, 10 μm.

DISCUSSION

I show in this study that hTCF4, mLef1 and XTcf-3 expressed in *C. elegans* embryos do not exhibit nuclear asymmetric patterns between A-P sisters, suggesting that these POP-1 homologs do not respond to the Wnt/MAPK signaling in *C. elegans* embryos to undergo nuclear export in Wnt-responsive cells. However, hTCF4 is able to substitute POP-1's function in repressing endoderm fate in the MS blastomere. This result suggests that hTCF4

is able to perform POP-1's function in the Wnt-nonresponsive MS blastomere, that is, binding to POP-1 target sequences and regulating their expression. While hTCF4 rescues the MS fate defect in only ≤75% of *pop-1(zu189)* embryos, a hTCF4/POP-1 chimera containing the putative Groucho binding domain of POP-1 has an MS-fate rescuing activity comparable to a wild-type POP-1 protein, suggesting that: (1) the DNA-binding HMG domains of POP-1 and hTCF4 are interchangeable, (2) the putative Groucho binding domain of POP-1 harbors important elements for endoderm repression. Evidence supporting the second point was shown in chapter two.

By domain swapping, I also showed that a hTCF4/POP-1 chimera containing the region between the β-catenin binding domain and the HMG domain of POP-1 did not exhibit A-P nuclear asymmetry in *C. elegans* embryos. However, this region together with the C-terminal region of POP-1 makes hTCF4 asymmetric between A-P nuclei. These results confirm the previous finding that one of the regions required for POP-1 nuclear asymmetry, $aa_{101\text{-}130}$, is not sufficient for conferring A-P nuclear asymmetry. However, there is no direct evidence showing whether $aa_{400\text{-}438}$ is sufficient for POP-1 nuclear asymmetry.

hTCF4 Contains Elements Important for Endoderm Repression that mLef1 lacks

While hTCF4 has a strong MS-fate rescuing activity in *pop-1(zu189)* embryos, neither mLef1 nor XTcf-3 seems to be able to rescue the MS fate defect in *pop-1(zu189)*. Since hTCF4, mLef1 and XTcf-3 have nearly identical DNA-binding HMG domains and bind the same DNA sequences, it is likely that the endoderm repressive activity of hTCF4 is located outside this region. I have shown previously that at least two regions of POP-1 are important

for its endoderm repressive activity. One is $aa_{48\text{-}130}$, which is located within the putative Groucho binding domain, and the other is $aa_{281\text{-}399}$, which is located C-terminal to the HMG domain (see chapter two). None of these three TCF proteins share significant sequence homology with POP-1 in their Groucho binding domains. However, the Groucho binding domains of these three TCF proteins are quite conserved with one another. Therefore, the region that makes hTCF4 a better repressor than mLef1 or XTcf-3 in the MS blastomere probably does not reside in its Groucho binding domain.

The above statement argues that a "repressor" domain other than the Groucho binding domain may exist in hTCF4, and this domain is likely to reside in the C-terminus of hTCF4. As I mentioned earlier, the C-terminus is the most divergent region among the TCF family members (see review by Hurlstone and Clevers, 2002). When I compared the C-terminal regions of POP-1, hTCF4, mLef1 and XTcf-3, I found that the conserved KKCRARFG and WCXXCRRKKKC motifs are only present in POP-1 and hTCF4 (Figure 4.6). These two motifs are only found in certain longer TCF isoforms (see review by Hurlstone and Clevers, 2002) and have been shown to be required for the activation of *hLEF1* promoter by hTCF1E isoform (Atcha *et al.*, 2003). The involvement of these two motifs in repressor function of TCF proteins has not been reported. I do not know whether they are required for the endoderm repressive activity of hTCF4 in *C. elegans* embryos. However, the endoderm repressive activity of POP-$1_{\Delta 281\text{-}438}$, which lacks these two motifs was greatly compromised, suggesting that they may contribute to the repressor function of POP-1. The C-terminus of hTCF4 also contains two binding sites for the transcriptional corepressor CtBP, which are located downstream of the two conserved motifs (Brannon *et al.*, 1999). These two CtBP

binding sites may not play a role in the endoderm repressive activity of hTCF4 because they are also found in XTcf-3 but not in POP-1 (Brannon *et al.*, 1999). However, although XTcf-3 failed to repress the MS-derived endoderm at a low level, I do not know whether it has the endoderm repressive activity when expressed at a high level as in the case of hTCF4. Therefore, it is still possible that the CtBP binding sites in hTCF4 provide a function similar to the C-terminus of POP-1 and contribute to the endoderm repressive activity of hTCF4 in *C. elegans* embryos. The C-terminal truncated hTCF4/POP-1 chimera [hTCF4/POP-$1_{48\text{-}192}$($aa_{1\text{-}334}$)], which lacks these two CtBP binding sites may provide an answer to this question. Further investigations are required to elucidate the roles of these motifs in the endoderm repressive function of hTCF4.

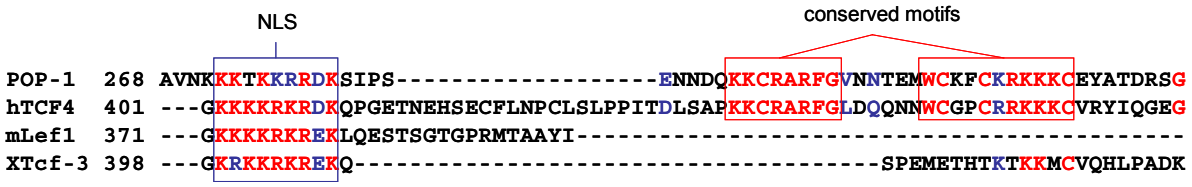


Figure 5.6 – Amino acid sequence comparison of the C-terminal region of POP-1, hTCF4, mLef1 and XTcf-3. Red letters indicate identity, blue letters conserved residues. Note that the NLS (blue box) is highly conserved among these four TCF members. The KKCRARFG and WCXXCRRKKKC motifs (red boxes) are only conserved between POP-1 and hTCF4.

MATERIALS AND METHODS

Strains

N2 was used as the wild-type strain. Genetic markers used in this chapter are: LGI, *pop-1(zu189)*, *dpy-5(e61)*, *hT1(I;V)*; LGIII, *unc-119(ed3)*; LGIV, *him-3(e1147)*. The transgenic strains studied in this chapter were generated by injection [either the *unc-119(ed3)III* or *unc-119(ed3)III;him-3(e1147)IV* strain]. All the transgenes were maintained as extra-chromosomal arrays in the following strains: $P_{med\text{-}1}$*gfp::mLef1* (pRL1002) in TX423 (*teEx107*), TX424(*teEx108*), and TX425(*teEx109*); $P_{med\text{-}1}$*gfp::XTcf-3* (pRL1001) in TX429 (*teEx113*); $P_{med\text{-}1}$*gfp::hTCF4* (pRL1034) in TX468(*teEx138*), TX469(*teEx139*), and TX470 (*teEx141*); $P_{med\text{-}1}$*gfp::pop-1*$_{L48F}$ (pRL1291) in TX575(*teEx211*); $P_{med\text{-}1}$*gfp::hTCF4/POP-1*$_{48\text{-}192}$ (pRL1334) in TX614(*teEx241*); $P_{med\text{-}1}$*gfp::hTCF4/POP-1*$_{48\text{-}192}$*(aa*$_{1\text{-}334}$*)* (pRL1408) in TX688(*teEx271*); $P_{med\text{-}1}$*gfp::hTCF4/POP-1*$_{48\text{-}192+315\text{-}438}$ (pRL1409) in TX712(*teEx279*).

Plasmid Construction

All the expression clones used in this chapter were generated using the Gateway cloning technology (Invitrogen) (Lo *et al.*, 2004; Robertson *et al.*, 2004; chapter two). The coding sequences of TCF variants and chimeras were introduced into the $P_{med\text{-}1}$*gfp* destination vector pRL707. EcoRI and PmlI sites were generated using Site-Directed Mutagenesis (Stratagene) in pRL769 ($P_{med\text{-}1}$*gfp::pop-1*) and pRL1034. After mutagenesis, an EcoRV-EcoRV fragment containing the *pop-1* or *hTCF4* coding sequence with the introduced restriction enzyme sites was subcloned back into non-mutagenized pRL769 resulting in pRL1291 or pRL1292 ($P_{med\text{-}1}$

gfp::hTCF4$_{T54F/I327V}$). pRL1334 was generated by replacing the EcoRI-PmlI region of pRL1292 with the EcoRI-PmlI fragment of pRL1291. To make pRL1408, an entry clone for *hTCF4/POP-1*$_{48\text{-}192(aa_{1\text{-}334})}$ (pRL1405) was first generated using the Gateway cloning technology. The *hTCF4/POP-1*$_{48\text{-}192(aa_{1\text{-}334})}$ coding sequence was derived from pRL1334 by PCR, where an FspI site was introduced right after the codon for aa_{334}. To generate pRL1409, a *pop-1* PCR fragment containing $aa_{315\text{-}438}$ was then cloned into this FspI site, generating pRL1407 (the entry clone for *hTCF4/POP-1*$_{48\text{-}192+315\text{-}438}$).

CHAPTER SIX
Regulation of *sdz-23* by POP-1 and Wnt/MAPK signaling

INTRODUCTION

The specification of MS and E cell fates requires both positive and negative regulation. The positive factors involved in E fate determination include the maternal components of the Wnt/MAPK signaling pathway (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997; Meneghini *et al.*, 1999; Rocheleau *et al.*, 1997; Shin *et al.*, 1999) as well as transcriptional activators such as SKN-1 (Bowerman *et al.*, 1992), MED-1/2 (Maduro *et al.*, 2001) and CBP-1, a histone acetyltransferase (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996; Shi and Mello, 1998). An important function of these positive regulators is the activation of an endoderm-determining gene, *end-1* (Zhu *et al.*, 1998; Calvo *et al.*, 2001; Maduro *et al.*, 2001, 2002). The MS and E fate specification process also requires mechanisms that suppress *end-1* expression and hence E fate in the MS blastomere. A mutation in the *pop-1* gene (*zu189*) results in the transformation of an MS fate to an E fate, indicating that POP-1 functions as a repressor of E fate in the MS blastomere (Lin *et al.*, 1995).

POP-1 appears to antagonize the activity of CBP-1 and MED-1/2, resulting in the repression of *end-1* in the MS blastomere (Calvo *et al.*, 2001; Maduro *et al.*, 2002). Repression by POP-1 involves a complex containing the Groucho-like corepressor UNC-37 and a histone deacetylase HDA-1 (Calvo *et al.*, 2001). It is believed that the Wnt/MAPK signaling blocks the endoderm repressive activity of POP-1 by lowering its nuclear level in

the E blastomere, thereby allowing *end-1* expression and endoderm formation (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997; Calvo *et al.*, 2001; Lo *et al.*, 2004; chapter three; also see Figure 1.6).

Recent studies indicate that POP-1 may not act exclusively as a repressor in endoderm formation. SKN-1 and MED-1/2 are important for both MS and E fates. However, while all *skn-1* and *med-1/2* mutant embryos lack MS-derived tissues, only 80% of *skn-1(-)* and 50% of *med-1/2(RNAi)* embryos do not make intestine (Bowerman *et al.*, 1992; Maduro *et al.*, 2001). About 90% (or less) of embryos lacking the maternal Wnt ligand, MOM-2, fail to make gut (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). However, all the embryos of either *skn-1; mom-2* or *med-1/2; mom-2* mutant are gutless (Thorpe *et al.*, 1997; Maduro *et al.*, 2001). These results suggest that a Wnt-dependent and SKN-1/MED-1/2-independent input exists to activate endoderm development (Maduro and Rothman, 2002). Because TCF proteins function as downstream activators of Wnt signaling in other systems (see reviews by Polakis, 2000; Moon *et al.,* 2002; Logan and Nusse, 2004), it is possible that POP-1 functions as an activator of E fate upon Wnt/MAPK signaling. Consistent with this hypothesis, embryos lacking both SKN-1 and POP-1 have a more penetrant gutless phenotype compared to *skn-1* mutants alone (Maduro and Rothman, 2002).

Recent studies in our lab also suggest a positive role for POP-1 in endoderm development. By microarray analyses conducted with cDNA prepared from individual four-cell or 12-cell wild-type embryos, 275 newly transcribed genes were identified during this approximately 30 minute window, during which the MS and E cell fates are specified (Robertson *et al.*, 2004). By comparing individual 12-cell wild-type embryos with 12-cell

skn-1 mutant embryos, fifty of these 275 genes were found to be downregulated in *skn-1* mutant and designated as *skn-1*-dependent zygotic (*sdz*) genes (Robertson *et al.*, 2004). Since SKN-1 is required for MS and E cell fates, these *sdz* genes may function as key regulators of MS and/or E-derived tissues. Indeed, promoter fusion to a GFP::histone H2B reporter gene indicated that several of these *sdz* genes are expressed specifically in the MS, E, or EMS lineage (Robertson *et al.*, 2004).

One of the E-specific genes identified in the microarray analyses is *sdz-23*. *sdz-23* is predicted to encode a novel protein containing an EGF-like domain. The GFP::histone H2B reporter construct driven by the 5' regulatory sequence of *sdz-23* ($P_{sdz\text{-}23}$*gfp::H2B*) showed that *sdz-23* was expressed specifically in the E lineage starting at the 12-cell stage (Robertson *et al.*, 2004). Depletion of POP-1 results in the derepression of *sdz-23* in the MS blastomere (P.S., M-C.L., S.R. and R.L. unpublished data), an effect similar to that seen with two previously identified E-specific genes, *end-1* and *end-3* (Zhu *et al.*, 1997; Calvo *et al.*, 2001; Maduro and Rothman, 2002). These results confirm the previous observation that POP-1 represses E-specific genes, directly or indirectly, in MS.

Although *sdz-23* is derepressed in the MS blastomere in *pop-1* mutant embryos, the microarray results surprisingly indicated that *sdz-23* was downregulated in 12-cell *pop-1* mutant embryos compared to 12-cell wild-type embryos (Robertson *et al.*, 2004). This inconsistency was resolved when carefully-staged wild-type and *pop-1* mutant embryos, each carrying the GFP reporter transgene, were examined side-by-side under the fluorescence microscope. Although similar GFP intensities from the reporter transgene were observed in both MS and E lineages in the *pop-1* mutant embryos, these GFP intensities were much

weaker than the E-restricted expression in the wild-type embryos (P.S., M-C.L., S.R. and R.L. unpublished data). Similar results were seen with *end-1* and *end-3* GFP reporters (P.S., M-C.L., S.R. and R.L. unpublished data). Two main conclusions can be drawn from these results: first, POP-1 not only represses the expression of *sdz-23* (as well as *end-1* and *end-3*) in MS, but also has a positive role in its expression in the E blastomere; and second, in the absence of POP-1, this normally E-specific gene exhibits low level basal expression in both MS and E.

Our results challenge the traditional view of POP-1's role in endoderm development, which is a repressor of E fate and E-specific genes. Our new model is that in the Wnt-nonresponsive MS blastomere, POP-1 functions as a repressor of E-specific genes, but in the Wnt-responsive E blastomere, the Wnt/MAPK signaling converts POP-1 to an activator of E-specific genes. To test this model, I asked whether *sdz-23* expression is regulated by the Wnt/MAPK signaling. To further understand the mechanism by which POP-1 activates the expression of *sdz-23* in the E blastomere, I also examined the requirement for some POP-1 domains in the high expression level of *sdz-23* in the E blastomere.

RESULTS

sdz-23 Is A Wnt-Responsive Gene

The expression of *sdz-23* is not only restricted to the E blastomere and its descendants, but also dependent on the Wnt signaling. WRM-1, the β-catenin functioning in the MOM-2/Wnt

signaling of *C. elegans* early embryos, is required to downregulate the POP-1 nuclear level and perhaps the repressor activity of POP-1 in the E blastomere to specify E fate, and the depletion of WRM-1 results in a fully penetrant gutless phenotype (Rocheleau *et al.*, 1997). Therefore, WRM-1 is likely to be involved in the POP-1-mediated activation of the E-specific gene, *sdz-23*, in the E lineage. When WRM-1 was depleted by RNAi, the expression of the $P_{sdz\text{-}23}$*gfp::H2B* transgene was abolished (P.S., M-C.L., S.R. and R.L. unpublished data). The repression of *sdz-23* by *wrm-1* RNAi is dependent upon POP-1 protein. Removal of POP-1 in *wrm-1(RNAi)* embryos restored the expression of *sdz-23* to the low basal level observed in *pop-1(zu189)* or *pop-1(RNAi)* embryos (P.S., M-C.L., S.R. and R.L. unpublished data). These results suggest that although the basal level expression of the Wnt-responsive gene, *sdz-23*, does not require POP-1 protein, its expression can be either repressed or activated by POP-1, and the Wnt signaling switches POP-1, in a β-catenin-dependent manner, from being a negative regulator to being a positive regulator of the expression of *sdz-23*.

The Expression Level of *sdz-23* Is Reduced in *lit-1* and *mom-4* Mutants

The MAPK LIT-1 and MAPKKK MOM-4 converge with the Wnt signaling to downregulate POP-1 in the E blastomere to allow endoderm fate (Meneghini *et al.,* 1999; Rocheleau *et al.,* 1999; Shin *et al.*, 1999). Embryos lacking the maternal LIT-1 or MOM-4 exhibit equal and high POP-1 nuclear levels between MS and E and have variable defects in endoderm formation (Thorpe *et al.*, 1997; Meneghini *et al.*, 1999; Rocheleau *et al.*, 1999; Shin *et al.*, 1999). To test whether these two kinases are also involved in the regulation of *sdz-23*

expression, I examined the expression pattern of *sdz-23* in various *lit-1* or *mom-4* mutants. These mutants include *lit-1(t1512)*, *lit-1(t1534)*, *mom-4(or11)*, *mom-4(or39)*, and *mom-4 (ne19)*. *lit-1(t1512)* is a temperature-sensitive allele with an almost fully penetrant gutless phenotype at the restrictive temperature (25ºC) (Rocheleau *et al.*, 1999). The percentages of embryos lacking gut in other mutants are variable ranging from 1% to 43% (Thorpe *et al.*, 1997; Rocheleau *et al.*, 1999; Shin *et al.*, 1999; also see Figure 5.1).

The integrated $P_{sdz\text{-}23}$*gfp::H2B* transgene was introduced into these *lit-1* and *mom-4* mutants. The expression of *sdz-23* in these mutants is restricted to the E lineage. All of these mutants except the *lit-1(t1512)* mutant express *sdz-23* in every embryo examined, including those embryos which failed to make gut (n > 150, Figure 5.1). I observed a significant reduction of *sdz-23* reporter gene expression in the *lit-1(t1512)* mutant embryos at 25ºC (Figure 5.1 A). In some of the *lit-1(t1512)* embryos, the GFP signal was almost undetectable. The *sdz-23* expression was also reduced but to a lesser extent in the weaker *lit-1* allele *(t1534)*, *mom-4(or39)* and *mom-4(ne19)* mutants (Figure 5.1 B, D, and E). However, the expression level of *sdz-23* was only slightly reduced in the *mom-4(or11)* mutant embryos (Figure 5.1 C). These results suggest that both LIT-1 and MOM-4 are required for the high expression level of *sdz-23* in the E lineage, and the mutant alleles with a more severe gutless phenotype have a stronger defect in *sdz-23* expression. However, the lack of gut formation in these mutants does not seem to correlate with the lack of *sdz-23* expression.

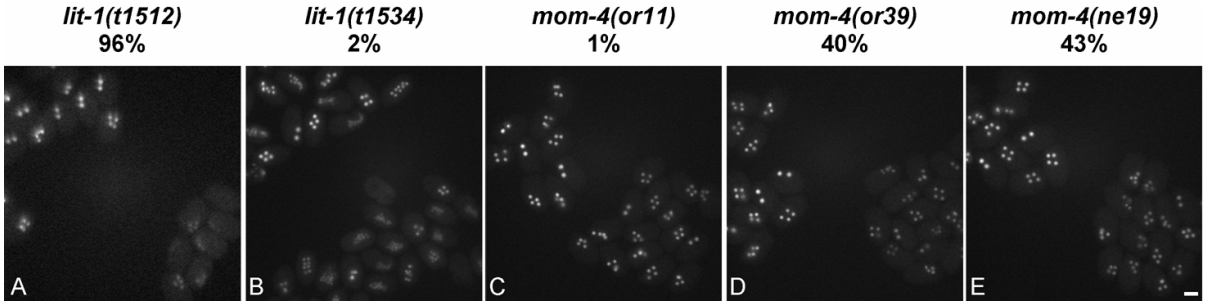


Figure 6.1 – The expression level of *sdz-23* in the E lineage is reduced in *lit-1* and *mom-4* mutants. GFP fluorescence images of wild-type (the cluster of embryos located at the upper left corner in each panel) and *lit-1* or *mom-4* mutant (the cluster of embryos located at the lower right corner in each panel) embryos expressing the $P_{sdz\text{-}23}$*gfp::H2B* transgene photographed together. The mutant alleles are noted above each panel, and the numbers indicate the percentages of embryos lacking gut in the respective mutant strains. Most embryos shown here are at the stage when two E daughters or four E granddaughters are present. The GFP is only seen in the E lineage. The wild-type embryos in (B), (C), (D), and (E) are heterozygous for the mutant alleles indicated above each panel. Scale bar, 20 μm.

The Activation of *sdz-23* by POP-1 Requires the β-Catenin Binding Domain of POP-1

TCF proteins in *Drosophila* and vertebrates activate Wnt-responsive genes upon binding of β-catenin to their N-terminal domains (Behrens *et al.*, 1996; Molenaar *et al.*, 1996; Brunner *et al.*, 1997; van de Wetering *et al.*, 1997). Deletion of this N-terminal domain results in a dominant negative effect, as the TCF protein remains bound by corepressors (Cavallo *et al.*, 1998; Roose *et al.*, 1998). A similar regulation functions for POP-1 in a canonical Wnt pathway regulating cell migration in postembryonic tissues, where POP-1 activates the expression of a homeobox gene, *mab-5*, upon Wnt stimulation (Korswagen *et al.*, 2000). To study the mechanism of POP-1-mediated activation of *sdz-23* in the E blastomere, I asked whether the high level expression of *sdz-23* in the E blastomere requires the N-terminal domain of POP-1. This domain (amino acids 1 to 47) was previously shown to be

dispensable for both POP-1 nuclear asymmetry and POP-1 activity in rescuing the MS fate defect in *pop-1(zu189)* mutant embryos (Maduro *et al.*, 2002; Lo *et al.*, 2004; chapter two). The rescue of the MS fate defect, however, would address only the ability of POP-1 to repress the endoderm fate in MS (i.e. the repression of E-specific genes in MS), but not the potential activation of E-specific genes by POP-1 in E.

Using a *pop-1(zu189)* strain carrying integrated $P_{sdz\text{-}23}$*gfp::H2B* transgenes, I assayed the effect upon reporter gene expression following the introduction of either full-length POP-1 or ΔN POP-1 (POP-$1_{\Delta 1\text{-}47}$) (Figure 5.2). The expression of the GFP reporter in MS was repressed in all the *pop-1(zu189)* embryos containing the full-length POP-1 (100%, n = 32; Figure 5.2 A and F). Consistent with previous findings that the N-terminal domain is dispensable for POP-1 rescuing activity, POP-$1_{\Delta 1\text{-}47}$ was also able to repress the reporter gene expression in MS (100%, n = 33; Figure 5.2 B and G). On the contrary, while full-length POP-1 restored a high level expression of the GFP reporter in E (44%, n = 32; Figure 5.2 A and F), POP-$1_{\Delta 1\text{-}47}$ did not (0%, n = 33; Figure 5.2 B and G). A similar result was observed with POP-1_{D9E} in which an N-terminal invariant aspartate was mutated to glutamate (0%, n = 24; Figure 5.2 C and H). This D_9E change corresponds to the *pop-1(q645)* mutation, which was isolated as a reduction-of-function mutation affecting somatic gonad morphogenesis, a larval Wnt-dependent process that requires WRM-1 (Siegfried and Kimble, 2002). It has been shown that the interaction between TCF and β-catenin requires the corresponding aspartate residue in the β-catenin-binding domain of TCF (Hsu *et al.*, 1998; Graham *et al.*, 2000; von Kries *et al.*, 2000). Seventy and fifty percent of embryos expressing POP-$1_{\Delta 1\text{-}47}$ (n = 33) and POP-1_{D9E} (n = 24), respectively, have GFP expression levels repressed to below

the basal level observed in the *pop-1(zu189)* mutant (Figure 5.2 G and H). A dominant negative effect in endoderm formation was also observed in those embryos with reduced GFP expression levels, with some embryos exhibiting complete endoderm ablation (9% and 12%, respectively; Figure 5.2 L, M, Q, and R). These results suggest that while the N-terminal β-catenin binding domain of POP-1 is not required for the repression of *sdz-23* in MS, it is required for the Wnt-dependent activation of *sdz-23* in E. With this domain deleted or mutated, POP-1 can further repress *sdz-23* to below the basal level in the E blastomere.

The Activation of *sdz-23* Requires A Low Nuclear POP-1 Level in the E Blastomere

The results so far suggest that Wnt signal converts the transcriptional repressor POP-1 into a transcriptional activator for Wnt-responsive genes. However, this hypothesis raises a new question. Why downregulate the POP-1 level in the E nucleus in response to Wnt signal, when POP-1 might be required in the E nucleus as a transcriptional activator of E-specific genes? Therefore, I asked whether POP-1 nuclear levels have to be low in order to activate Wnt-responsive genes. To answer this question, I examined the effect upon *sdz-23* expression of a POP-1 mutant (POP-$1_{S107/118/127A}$) that exhibits high nuclear levels in Wnt-responsive cells. I have shown previously that POP-$1_{S107/118/127A}$ is present at a high level in the E nucleus due to a defect in Wnt-induced nuclear export (Lo *et al.*, 2004; chapters two and three). Nonetheless, this export-defective POP-1 protein was able to rescue the MS fate defect in *pop-1(zu189)* embryos. I found that in embryos expressing POP-$1_{S107/118/127A}$, *sdz-23* was repressed properly in the MS blastomere (100%, n = 14; Figure 5.2 D and I), but activation of *sdz-23* in E did not occur (0%, n =14; Figure 5.2 D and I). In fact, *sdz-23* in E

was often repressed to below the basal level observed in *pop-1(zu189)* mutant embryos (71%, n = 14; Figure 5.2 I). Consistent with a high level of nuclear POP-1 functioning as a repressor in E, about 29% of *pop-1(zu189)* embryos expressing POP-$1_{S107/118/127A}$ failed to produce endoderm from the E blastomere (Figure 5.2 N and S; Lo *et al.*, 2004; chapter two).

I also examined the effect of another POP-1 mutant on *sdz-23* expression. This POP-1 mutant (POP-$1_{\Delta 400-438}$) is also defective in A-P nuclear asymmetry with equal and high levels in both MS and E nuclei (see chapter two). The mechanism by which $aa_{400-438}$ regulates POP-1 nuclear asymmetry is not clear although differential protein degradation has been suggested. Deleting this region does not affect the repressor activity of POP-1, as the MS-derived endoderm is repressed in all the *pop-1(zu189)* embryos expressing POP-$1_{\Delta 400-438}$. Interestingly, POP-$1_{\Delta 400-438}$ represses the E-derived endoderm in all the *pop-1(zu189)* embryos expressing this protein (see chapter two). Consistent with the E-fate repressive activity of POP-$1_{\Delta 400-438}$ present in both MS and E, the expression of the E-specific gene, *sdz-23*, was abolished in both MS and E of all the *pop-1(zu189)* embryos expressing POP-$1_{\Delta 400-438}$ (100%, n = 9; Figure 5.2 E and J). An endoderm ablation effect was also observed in the embryos expressing POP-$1_{\Delta 400-438}$ (67%, n = 9; Figure 5.2 O and T).

The above results indicate that a low nuclear POP-1 level in the Wnt-responsive E blastomere is needed to activate the Wnt-responsive gene *sdz-23*.

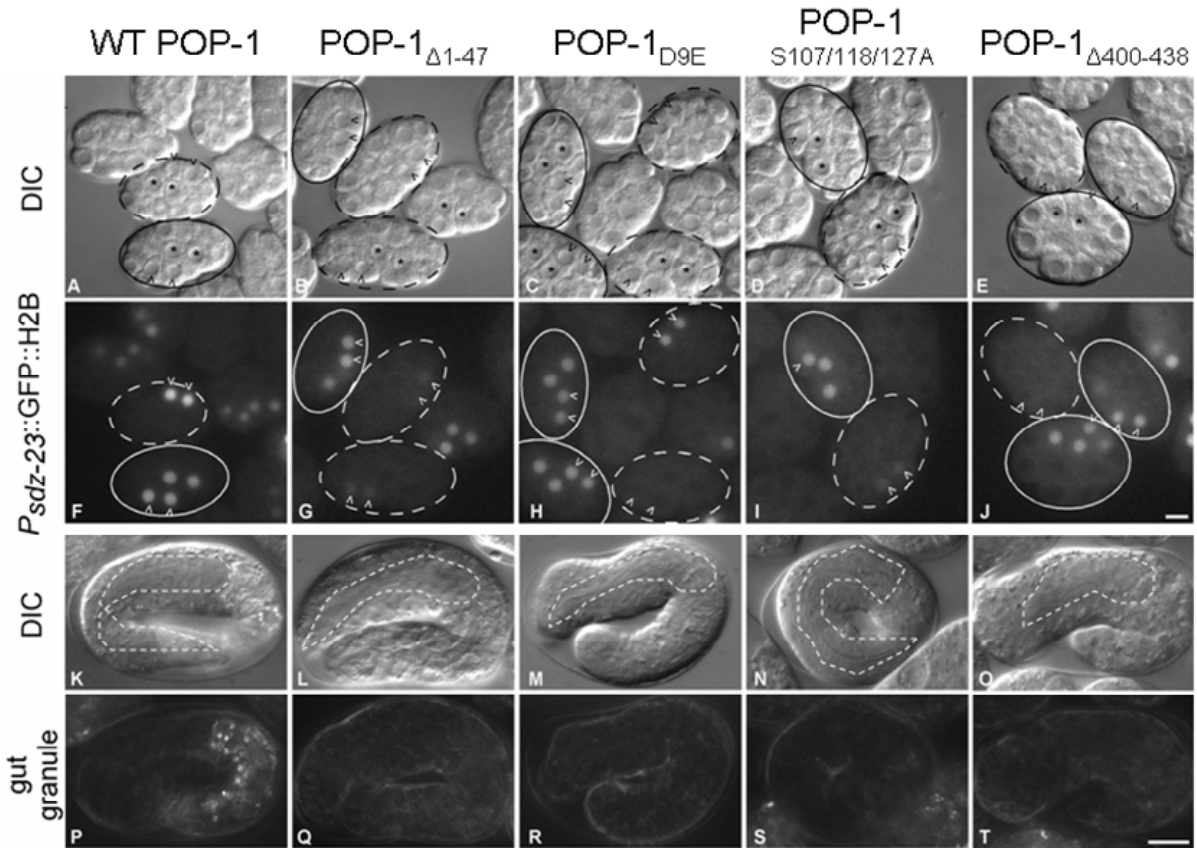


Figure 6.2 – The activation of *sdz-23* in E requires POP-1 N-terminal domain and a low nuclear POP-1 level in E. *pop-1(zu189)* with integrated $P_{sdz\text{-}23}$*gfp::H2B* expressing CFP-fused wild-type POP-1 (A, F, K, and P), POP-$1_{\Delta 1\text{-}47}$ (B, G, L, and Q), POP-1_{D9E} (C, H, M, and R), POP-$1_{S107/118/127A}$ (D, I, N, and S), or POP-$1_{\Delta 400\text{-}438}$ (E, J, O, and T) (see Materials and Methods). The transgenes of POP-1 variants are not integrated. (A-J) DIC and corresponding GFP fluorescence images of the above embryos. Embryos expressing *pop-1* transgenes are in dashed outline, and embryos without *pop-1* transgenes are in solid outline. Open arrowheads, Ea and Ep. Asterisks, MSa and MSp. While all the POP-1 variants are capable of repressing GFP expression in the MS lineage, only wild-type POP-1 is capable of restoring high level GFP expression in the E lineage (F). (K-T) Micrographs of terminally differentiated embryos viewed with DIC (K-O) or polarized light (P-T). All the POP-1 variants restore the generation of posterior pharynx (dashed outline). All embryos expressing wild-type POP-1 form intestine, viewed as gut-specific birefringent granules (P), whereas a proportion of embryos expressing the other POP-1 variants are gutless (Q-T). Scale bar, 10 μm.

DISCUSSION

Our results present the first demonstration that POP-1 functions to activate Wnt-responsive target genes in the Wnt-responsive cell in the early *C. elegans* embryo, which challenges the commonly accepted non-canonical Wnt signaling model, based upon alleviation in E of the demonstrated capacity of POP-1 to repress Wnt-responsive genes in MS. We propose that, in conjunction with corepressors (Calvo *et al.*, 2001) POP-1 normally functions to repress Wnt-responsive genes to well below the basal levels observed in the absence of POP-1. In the Wnt-responsive cell, POP-1 instead selectively interacts with coactivators, thereby activating transcription of target genes to well above the basal levels (P.S., M-C.L., S.R. and R.L. unpublished data). Therefore, Wnt signaling in the early *C. elegans* embryo is found to share much more in common with canonical Wnt pathways in *C. elegans* postembryonic tissues and other organisms than previously realized (Bienz, 1998; Brantjes *et al.*, 2002; Herman and Wu, 2004).

POP-1 and other TCF proteins display a very high amino acid homology in their DNA-binding HMG domains. Therefore, POP-1 may bind similar sequence motifs to regulate gene transcription. Indeed, a truncated POP-1 protein containing the HMG domain has been shown to bind a TCF target sequence (Korswagen *et al.*, 2000). Two optimal TCF binding sites were identified in the promoter region of *end-1* -- GTTCAAAG beginning at -164 relative to the AUG translation initiation, and GAACAAAG at -953. When the -164 TCF binding site was mutated to GTTCGGGG, a change shown to abolish binding by the HMG domain (Korswagen *et al.*, 2000), the *end-1* GFP reporter exhibited low level basal expression in both the MS and E lineages, similar to the expression observed for the wild-

type *end-1* reporter in the *pop-1* mutant (P.S., M-C.L., S.R. and R.L. unpublished data). This low level expression is still detected in *wrm-1(RNAi)* embryos (P.S., M-C.L., S.R. and R.L. unpublished data), consistent with our conclusion above that both activation and repression of a Wnt-responsive gene are POP-1 dependent. These results suggest that regulation of *end-1* expression requires direct binding by POP-1. The genomic promoter region of *sdz-23* used in the reporter construct does not contain the consensus TCF binding sites, suggesting that *sdz-23* may be an indirect target of POP-1.

Wnt and MAPK Pathways Converge to Activate E-Specific Genes

I show here that the high level expression of the Wnt-responsive gene, *sdz-23*, in the E lineage requires the Wnt signaling as well as the MAP kinases LIT-1 and MOM-4. Consistent with the convergent function of Wnt and MAPK signaling pathways in down-regulating POP-1 repressor activity and specifying E fate (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997; Meneghini *et al.,* 1999; Rocheleau *et al.,* 1999; Shin *et al.*, 1999), these results suggest that Wnt and MAPK pathways also work together to activate E-specific genes. It has been shown that forced *end-1* expression can bypass the requirement for the maternal SKN-1 and Wnt signaling pathway in endoderm formation (Zhu *et al.,* 1998), suggesting that a major function of these maternal factors is to promote the zygotic expression of *end* genes, which is sufficient to initiate endoderm development.

A complete abolishment of *sdz-23* expression was observed upon *wrm-1* RNAi, which had a 100% gutless phenotype. The expression of *sdz-23* in a severe *lit-1* mutant strain *(t1512)*, which has a nearly 100% gutless phenotype, was also repressed to an almost

undetectable level. In fact, a strong *lit-1* RNAi resulted in a complete loss of *sdz-23* expression (P.S., personal communication). However, there was only a moderate reduction of *sdz-23* expression in *mom-4* mutants including a possibly null allele (*ne19*, Shin *et al.*, 1999). These results suggest that while WRM-1 and LIT-1 are essential for *sdz-23* expression, MOM-4 may play less of an important role. This is consistent with the observation that MOM-4 is in one of the multiple parallel pathways for activating the WRM-1/LIT-1 complex to downregulate POP-1 and specify E fate (Rocheleau *et al.*, 1997; Shin *et al.*, 1999). The function of *sdz-23* is not known yet. Depletion of *sdz-23* by RNAi did not cause any abnormal phenotype probably due to functional redundancy with other early zygotic genes (Robertson *et al.*, 2004). Although some of the *mom-4* mutant embryos fail to produce intestine, all the *mom-4* embryos examined still express *sdz-23*, suggesting that *sdz-23* may not function in endoderm formation. It will be interesting to know how mutations in *mom-4* affect the expression of *end-1*, a known endoderm-determining gene.

The Relative Amount of TCF Protein to Its Coactivator May Determine Transcriptional Repression or Activation

I show here that the activation of *sdz-23* requires a low nuclear POP-1 level in the E blastomere. A lowering of nuclear POP-1 levels has also been observed in several post-embryonic cells whose fates require activation by POP-1 in response to Wnt signaling (Herman, 2001; Siegfried *et al.*, 2004). It was suggested that if a coactivator for POP-1 was limiting, the majority of POP-1 would be bound to the coactivator in cells with low POP-1 levels, but in cells with high POP-1 levels, the excess unbound POP-1 could function as a

repressor (Siegfried *et al.*, 2004). It is interesting to note that in *Drosophila*, *wingless* and *armadillo* mutant phenotypes can be partially suppressed by a reduction of dTcf activity, whereas the phenotype of a weak *wingless* allele is enhanced by overexpression of wild-type dTcf (Cavallo *et al.*, 1998). These results suggest that Wnt signal strength is determined, at least in part, by relative levels of TCF to β-catenin. We propose that Wnt signaling induces the transcription of target genes by decreasing the relative amount of TCF protein to its coactivator, thereby allowing most or all of the TCF protein to be complexed with coactivator (Figure 5.3; P.S., M-C.L., S.R. and R.L. unpublished data). This can be achieved by either increasing the level of nuclear coactivator (as in the canonical Wnt model, Figure 5.3 B), decreasing the amount of nuclear TCF protein (as seen in *C. elegans* E blastomere, Figure 5.3 C), or both.

The β-catenin binding domain of POP-1 is required for the activation of *sdz-23* in the E lineage. A POP-1 version with a point-mutation in this domain, which was shown to disrupt binding between TCF and β-catenin (Hsu *et al.*, 1998; Graham *et al.*, 2000; von Kries *et al.*, 2000) also failed to activate *sdz-23* expression in the E lineage. These results suggest that a β-catenin or a β-catenin-like protein may interact with POP-1 to activate Wnt target gene expression upon Wnt signaling. However, this proposed coactivator for POP-1 remains to be identified because no physical interaction has yet been demonstrated between POP-1 and WRM-1, the only *C. elegans* β-catenin shown to function in endoderm induction.

Why convert POP-1 into an activator of endoderm-specific genes in the E nucleus if, in the absence of POP-1, simple derepression and basal level expression is sufficient to induce endoderm formation? Indeed, the fact that basal level expression of Wnt target genes

in *pop-1* mutant embryos was sufficient to induce endoderm is the likely reason why the role of POP-1 in the activation of Wnt-responsive genes went undetected until now. We suggest that endoderm formation is only a partial readout of the effect of Wnt signaling on the E blastomere. Wnt signaling might, for example, also modify how E and/or E descendants interact with neighboring blastomeres, independent of E-derived endoderm formation. In fact, *sdz-23* is predicted to encode a variant Notch ligand (Robertson *et al.*, 2004), and a possible function in cell-cell interactions is currently under investigation.

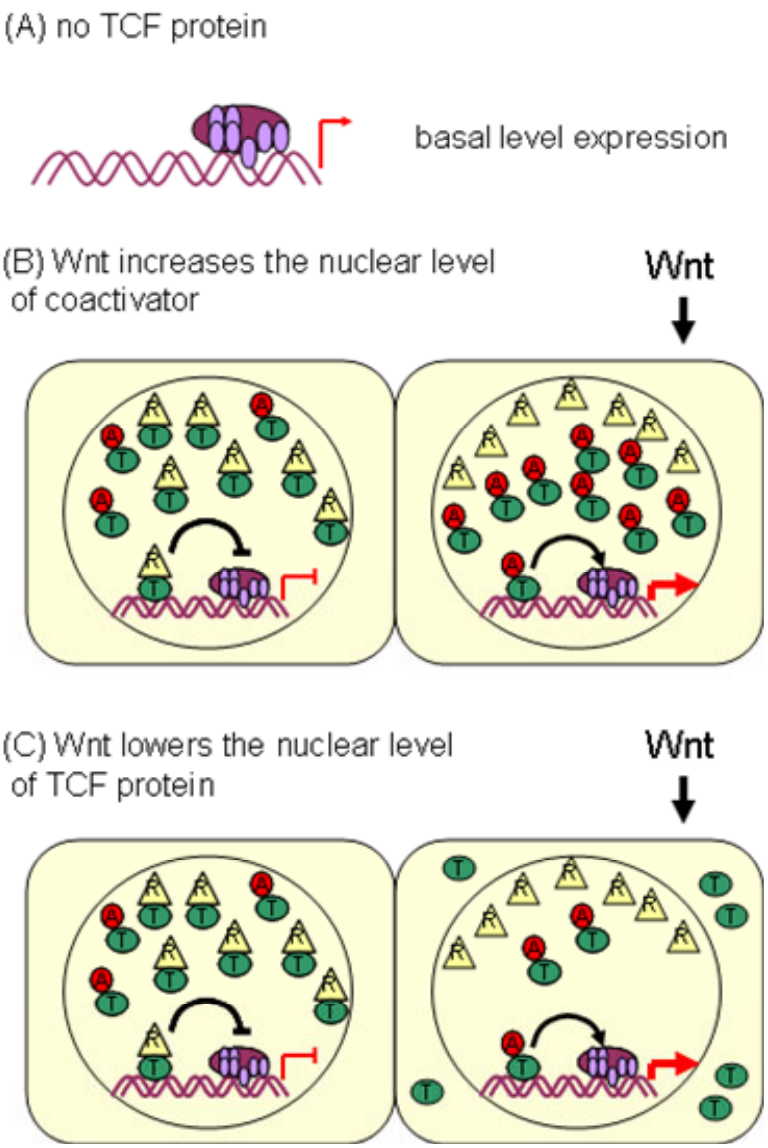


Figure 6.3 – Model for how Wnt signaling activates target gene expression. (A) In the absence of TCF protein, basal transcription machinery (purple ovals) and other ubiquitous transcription factors engage the promoter of Wnt-responsive genes, resulting in low-level basal expression. (B and C) In the absence of Wnt signaling (cells on the left), TCF protein (green oval) complexes with corepressor(s) (yellow triangle) to repress the expression of Wnt target genes to below the basal level. Wnt signaling (cells on the right) activates the expression of target genes by increasing the amount of TCF/coactivator (red circle) complexes. This can be achieved by increasing the nuclear level of coactivator (B), decreasing the nuclear level of TCF protein (C), or both.

The C-terminus of POP-1: A Potential Transactivation Domain?

The requirement for a low nuclear POP-1 level in the activation of *sdz-23* expression in the E blastomere was demonstrated with two POP-1 mutants, POP-$1_{S107/118/127A}$ and POP-$1_{\Delta 400-438}$. Both of these two POP-1 variants have a similar ability to repress *sdz-23* in MS. However, POP-$1_{\Delta 400-438}$ results in a stronger repression of *sdz-23* in E compared to POP-$1_{S107/118/127A}$. Similarly, while the ability of these two proteins to repress endoderm fate in MS appears to be equal, POP-$1_{\Delta 400-438}$ has a stronger E-fate repressive activity in the E blastomere than POP-$1_{S107/118/127A}$ (see Table 2.1). Since both of these two POP-1 proteins exhibit a high nuclear level in E, the better ability of POP-$1_{\Delta 400-438}$ to repress *sdz-23* expression and E fate is likely not simply due to the elevated nuclear level. I propose that $aa_{400-438}$ of POP-1 is required for POP-1 to activate Wnt target genes and induce endoderm formation upon Wnt signaling. I do not know how POP-$1_{400-438}$ might perform this function. Because POP-$1_{400-438}$ is highly enriched in acidic residues aspartate (D) and glutamate (E), it is likely that this region functions as a transcriptional activation domain similar to the acidic transactivation domains found in many transcription factors (Hope and Struhl, 1986; Struhl, 1987; Ma and Ptashne, 1987). Acidic transactivation domains have not been described in the C-terminus of TCF proteins. However, the C-terminus is the most divergent region of the TCF family members (see review by Hurlstone and Clevers, 2002). It is possible that POP-1 harbors an acidic transactivation domain in its C-terminus that is not conserved among TCF proteins. Further investigations are needed to prove this hypothesis.

MATERIALS AND METHODS

Strains

N2 was used as the wild-type strain. Genetic markers used in this chapter are: LGI, *pop-1 (zu189)*, *dpy-5(e61)*, *hT1(I;V)*, *unc-13(e1091)*, *mom-4(or11)*, *mom-4(or39)*, *mom-4(ne19)*, *hT2(I;III)*; LGIII, *unc-119(ed3)*, *unc-32(e189)*, *lit-1(t1512)*, *lit-1(t1534)*, *qC1(III)*; LGIV, *him-3(e1147)*. TX585(*teIs18*) was generated by microparticle bombardment (Praitis *et al.*, 2001), in which the $P_{sdz\text{-}23}$*gfp::H2B* reporter was stably integrated into chromosome V of the genome. All the other strains were generated by injection and maintained the transgenes as extrachromosomal arrays. These strains and transgenes include: $P_{med\text{-}1}$*cfp::pop-1* (pRL1011) in TX652(*teEx256*); $P_{med\text{-}1}$*cfp::pop-1*$_{\Delta 1\text{-}47}$ (pRL1386) in TX654(*teEx258*); $P_{med\text{-}1}$*cfp::pop-1*$_{D9E}$ (pRL1387) in TX683(*teEx266*); $P_{med\text{-}1}$*cfp::pop-1*$_{S107/118/127A}$ (pRL1412) in TX690(*teEx273*), $P_{med\text{-}1}$*cfp::pop-1*$_{\Delta 400\text{-}438}$ (pRL1410) in TX696(*teEx276*); $P_{med\text{-}1}$*yfp::pop-1* (pRL1012) in TX583(*teEx215*); $P_{med\text{-}1}$*yfp::pop-1*$_{\Delta 1\text{-}47}$ (pRL1325) in TX604(*teEx231*); $P_{med\text{-}1}$*yfp::pop-1*$_{D9E}$ (pRL1322) in TX606(*teEx233*). TX622 [*unc-13(e1091)mom-4(or11)(I)/hT2(I;III);teIs18*], TX623 [*unc-13(e1091)mom-4(or39)(I)/hT2(I;III);teIs18*], and TX716 [*unc-13(e1091)mom-4 (ne19)(I)/hT2(I;III);teIs18*] were generated by crossing TX585 males with EU443, EU446 (both are gifts from Bruce Bowerman), and the *unc-13(e1091)mom-4(ne19)(I)/hT2(I;III)* strain, respectively, backcrossing the F1 males to EU443, EU446, and *unc-13(e1091)mom-4 (ne19)(I)/hT2(I;III)*, respectively, and picking F2 non-Unc segregating wild-type, *unc-13*, *hT2* dead embryos and GFP. TX621 [*unc-32(e189)lit-1(t1534)(III)/qC1(III);teIs18*] was generated in a similar way. The *unc-32(e189)lit-1(t1534)(III)/qC1(III)* strain is a gift from

Ralph Schnabel. *qC1* homozygotes are Dpy Ste. TX713 [*unc-32(e189)lit-1(t1512)III;teIs18*] was generated by crossing TX585 males with the *unc-32(e189)lit-1(t1512)III* strain (generated by Rueyling Lin) and screening for F2 Unc segregating 100% GFP positive embryos at 15ºC and 100% dead embryos at 25ºC. *wrm-1* RNAi was performed by feeding as described (Lo *et al.*, 2004; chapter three).

Plasmid Construction

All the expression clones used in this chapter were generated using the Gateway cloning technology (Invitrogen) (Lo *et al.*, 2004; Robertson *et al.*, 2004; chapter two). Destination vectors containing $P_{med\text{-}1}$*cfp* (pRL1006) and $P_{med\text{-}1}$*yfp* (pRL1008) sequences were generated by replacing the BamHI-NcoI *gfp::pop-1* fragment of pMM414 (Maduro *et al.*, 2002) with *cfp* (from the Fire vector kit pPD134.96) and *yfp* (from the Fire vector kit pPD134.99), respectively, and then blunt-end ligating the Gateway cassette (reading frame A) via an EcoRV site downstream of the *cfp* or *yfp* sequence. The coding sequences of *pop-1* variants were then introduced into pRL1006 or pRL1008. The reporter construct for *sdz-23* was a transcriptional fusion containing a 1.2 kb presumptive *sdz-23* promoter and GFP::H2B (Robertson *et al.*, 2004), whereas the *end-1* reporter was a translational fusion containing a 2.2 kb *end-1* promoter, a 1.7 kb genomic sequence downstream of the AUG, and GFP::H2B.

Analysis of Embryos and Imaging

Imaging of live embryos was performed as described in chapter two (Rogers *et al.*, 2002). The filter wheels (Ludl Electronic Product) and shutter controller were driven by a custom software package (*os4d 1.0*, freely available on request to jwaddle@mail.smu.edu). The experiments shown in Figure 5.2 were performed with either CFP::POP-1 or YFP::POP-1. The folding of CFP is too slow for the rapid division cycles observed during early *C. elegans* embryogenesis and therefore only very low CFP signals were seen with all CFP fusions constructed. The separation of YFP::POP-1 and GFP::H2B was performed using an LSM 510 Meta confocal microscope (Zeiss). Similar results were obtained with either CFP::POP-1 or YFP::POP-1.

CHAPTER SEVEN
Conclusion

In summary, this study shows that POP-1 nuclear asymmetry requires two POP-1 regions, $aa_{101-130}$ and $aa_{400-438}$. The $aa_{101-130}$ region appears to mediate a low nuclear level of POP-1 in Wnt/MAPK-responsive cells via increased nuclear export of POP-1, whereas the $aa_{400-438}$ region is likely to mediate a low nuclear level of POP-1 through increased POP-1 degradation. Neither region by itself is sufficient to confer POP-1 nuclear asymmetry because deletion of either region resulted in equal POP-1 levels between A-P nuclei. Also, by domain swapping the $aa_{101-130}$ region of POP-1 was not sufficient to confer A-P nuclear asymmetry to a heterologous TCF protein (hTCF4) in *C. elegans* embryos. However, a hTCF4 chimera containing both $aa_{101-130}$ and $aa_{400-438}$ of POP-1 exhibits A-P nuclear asymmetry. These results further confirm the requirement for both domains in POP-1 nuclear asymmetry. This study also shows that POP-1 acts as a transcriptional activator for Wnt/MAPK-responsive genes in the E blastomere. The activation of Wnt/MAPK-responsive genes by POP-1 requires the β-catenin binding domain of POP-1 and a low nuclear level of POP-1 in the E blastomere.

It is known that Wnt/MAPK signaling downregulates POP-1 in the E blastomere, thereby allowing endoderm fate (Rocheleau *et al.,* 1997; Thorpe *et al.,* 1997; Lin *et al.*, 1998; Meneghini *et al.,* 1999; Rocheleau *et al.,* 1999; Shin *et al.*, 1999). Because Wnt/MAPK signaling is required for a low nuclear level of POP-1 in E, it is believed that Wnt/MAPK signaling induces endoderm by lowering the nuclear level of POP-1. The data presented in

this study suggest that Wnt/MAPK signaling promotes a 14-3-3-mediated nuclear export of POP-1 in the E blastomere, thereby lowering its nuclear level. However, 14-3-3-mediated nuclear export is probably not the only mechanism that lowers the level of POP-1 in the E nucleus. The $aa_{400-438}$ region of POP-1 contains putative PEST sequences, suggesting that protein degradation may play a role in regulating the nuclear level of POP-1. This putative PEST region contains potential casein kinase II phosphorylation sites, which are important for a low nuclear level of POP-1 in posterior cells. Because casein kinase II has been implicated in Wingless signal transduction in *Drosophila* (Willert *et al.*, 1997), it is likely that casein kinase II functions in the Wnt/MAPK signaling in *C. elegans* to regulate the nuclear level of POP-1 and endoderm development. A role for casein kinase II in endoderm formation was also shown in this study. All together, these results suggest that POP-1 nuclear asymmetry is regulated by at least two mechanisms and the Wnt/MAPK signaling may be directing both mechanisms to initiate the lowering of nuclear POP-1 levels in Wnt/MAPK-responsive cells.

This study and a previous report by Maduro *et al.* (2002) showed that POP-1 is qualitatively different between MS and E. The data presented here suggest that the Wnt/MAPK signaling may cause this qualitative difference by phosphorylating POP-1 preferentially in the E nucleus. This phosphorylation may lower the endoderm repressive activity of POP-1. Therefore, the Wnt/MAPK signaling may lower the nuclear level of POP-1 and at the same time inhibit its repressor activity to ensure proper endoderm development.

The activation of Wnt/MAPK-responsive genes by POP-1 in the E blastomere requires the β-catenin binding domain of POP-1 and a low nuclear level of POP-1. Why is a

low level of POP-1 associated with its function as a transcriptional activator? This is in agreement with previous reports which showed that the nuclear level of POP-1 is lowered in postembryonic cells whose fates require POP-1 upon Wnt signaling (Herman, 2001; Siegfried *et al.*, 2004). All of these results are consistent with a model where a low nuclear level is required for POP-1 to function as a transcriptional activator. We propose that the expression of Wnt-responsive genes is determined, at least in part, by the ratio of TCF to its coactivator and perhaps Wnt signaling activates gene expression by decreasing this ratio, thereby allowing most or all of the TCF protein to be complexed with coactivator. Wnt signaling could decrease the TCF to coactivator ratio by either increasing the level of nuclear coactivator (as in the canonical Wnt model), decreasing the amount of nuclear TCF protein (as seen in *C. elegans* E blastomere), or both.

The results presented here force us to reinterpret the non-canonical Wnt signaling that induces endoderm in *C. elegans* early embryos. As in the canonical Wnt model, the Wnt/MAPK signaling appears to convert the repressor POP-1 into an activator in the Wnt/MAPK-responsive E blastomere. However, this study raised two interesting questions. First, is the Wnt/MAPK-induced nuclear export of a TCF protein shown here a *C. elegans*-specific phenomenon? Second, is the nuclear level of TCF proteins also lowered in order to activate Wnt target gene expression in other organisms? The *Drosophila* TCF homolog dTcf has been observed to undergo nuclear export in the wing imaginal disc in response to Wingless stimulation (Chan and Struhl, 2002). Although this paper was retracted (Struhl, 2004), personal communication indicated that this result was still valid, suggesting that perhaps a similar Wnt/MAPK-induced nuclear export mechanism regulates TCF proteins in

organisms other than *C. elegans*. It has also been shown that in *Drosophila*, *wingless* and *armadillo* mutant phenotypes can be partially suppressed by a reduction of dTcf activity, whereas the phenotype of a weak *wingless* allele is enhanced by overexpression of wild-type dTcf (Cavallo *et al.*, 1998). This is consistent with a model where the level of TCF is lowered in order to transduce Wnt signaling. Therefore, it seems that the Wnt signaling in the early *C. elegans* embryo has more in common with the canonical Wnt signaling pathways in other systems than previously realized.

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VITAE

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