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The Wnt effector POP-1 and the PAL-1/Caudal homeoprotein collaborate with SKN-1 to activate *C. elegans* endoderm development

Morris F. Maduro[1,2], Jodie J. Kasmir[2], Jiangwen Zhu, Joel H. Rothman*

Department of MCD Biology and Neuroscience Research Institute, University of California at Santa Barbara, Santa Barbara, CA 93106, USA

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Abstract

POP-1, a Tcf/Lef-1-like target of the convergent Wnt and MAP kinase (MAPK) signaling pathways, functions throughout *Caenorhabditis elegans* development to generate unequal daughters during asymmetric cell divisions. A particularly prominent such asymmetric division occurs when the EMS blastomere divides to produce MS, a mesoderm precursor, and E, the sole endoderm progenitor. POP-1 allows mesoderm development in the MS lineage by repressing the endoderm-promoting *end-1* and *end-3* genes. This repression is relieved in the E lineage by Wnt/MAPK signaling, which results in phosphorylation and export of POP-1 from the E nucleus. Here, we report that, in addition to repressing E development in MS, POP-1 also functions positively in endoderm development, in conjunction with the well-characterized endoderm-promoting SKN-1→MED regulatory cascade. While removal of POP-1 alone results in derepression of endoderm development in the MS lineage, mutations in several genes that result in impenetrant loss of endoderm are strongly enhanced by loss of *pop-1* function. A Lef-1-like binding site is essential for activation of an *end-1* promoter fusion, suggesting that POP-1 may act directly on *end-1*. Thus, POP-1 may generate developmental asymmetry during many cell divisions in *C. elegans* by reiteratively switching from repressive and activating states. Furthermore, we report that the Caudal-like homeodomain protein PAL-1, whose role in early embryogenesis was thought to be exclusive specification of mesectodermal development in the lineage of the C blastomere, can act with POP-1 to activate endoderm specification in the absence of the SKN-1→MED transcriptional input, accounting for the impenetrance of mutants lacking SKN-1 or MED-1,2 activity. We conclude that the combined action of several separate transcriptional regulatory inputs, including SKN-1, the MEDs, PAL-1, and the Wnt/MAPK-activated form of POP-1, are responsible for activating *end* gene transcription and endoderm development.

Keywords: *Caenorhabditis elegans*; Wnt pathway; Endoderm; TCF/LEF factors; POP-1; PAL-1; Asymmetric cell division; Germ layers

Introduction

The Wnt signaling pathway functions in a large number of cellular activities in metazoans, including cell type specification, cell polarization, spindle orientation, control of cell division, and morphogenesis (Cadigan and Nusse, 1997; Miller and Moon, 1996; Moon et al., 2002; Roose and Clevers, 1999). In the nematode *Caenorhabditis elegans*, the Wnt pathway acts reiteratively throughout development to direct the developmentally asymmetric division of cells resulting in dissimilar daughters (Eisenmann et al., 1998; Herman, 2001; Herman and Wu, 2004; Lin et al., 1998; Maloof et al., 1999). The generation of unequal daughter cells by Wnt signaling during *C. elegans* development is first seen in the four-cell embryo (Fig. 1) (Rocheleau et al., 1997; Thorpe et al., 1997). At this stage, the posterior-most blastomere, P_2, sends a polarizing inductive signal to its neighbor, the EMS mesendoderm progenitor. Upon division of EMS, the side that had received the P_2 signal gives rise to the endoderm progenitor, or E cell; the opposite side, which received no signal, generates the mesoderm-generating MS cell (Goldstein, 1992, 1993, 1995; Schierenberg, 1987). The inductive signals emanating from P_2 activate a Wnt pathway, a MAP kinase (MAPK) signaling cascade, and an Src-

* Corresponding author. Fax: +1 805 893 2005.
E-mail address: rothman@lifesci.ucsb.edu (J.H. Rothman).
[1] Present address: Department of Biology, University of California, Riverside, Riverside, CA 92521, USA.
[2] These authors were equal contributors.

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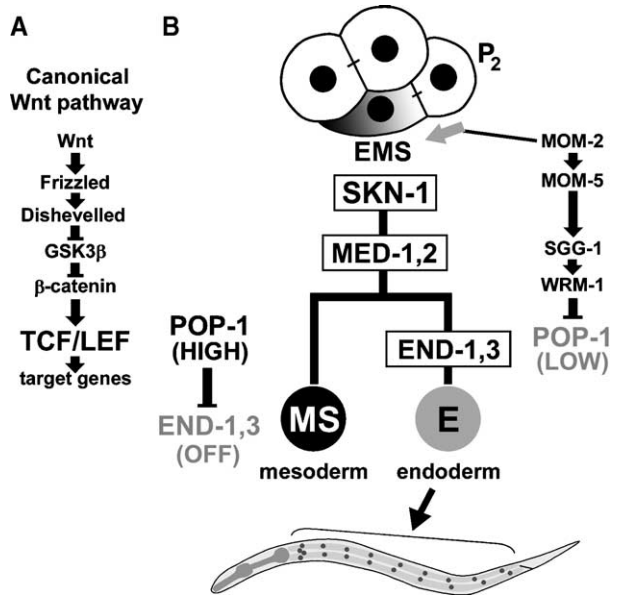


Fig. 1. Canonical Wnt pathway and *C. elegans* embryonic Wnt pathway. (A) In the canonical Wnt pathway, Wnt signaling results in activation of a bipartite transcription factor consisting of β-catenin and a TCF/Lef factor, which activates target genes (reviewed in Cadigan and Nusse, 1997). In the absence of the Wnt signal, TCF/Lefs can function as repressors (not shown on the figure) (Kim et al., 2000; Merrill et al., 2001). A similar pathway is proposed to exist for postembryonic *C. elegans* signaling events (Herman, 2001; Herman and Wu, 2004; Korswagen et al., 2000). (B) The Wnt pathway as previously described for *C. elegans* E specification. At the 4-cell stage, P_2 polarizes the ventral blastomere EMS (depicted by shading of the EMS cytoplasm) such that its posterior daughter becomes E. The terminal TCF/Lef-1-like regulator, POP-1, represses E specification in MS by preventing activation of *end-1,3* by the SKN-1/MED-1,2 pathway (Calvo et al., 2001; Maduro et al., 2002; Maduro et al., 2001). A newly hatched L1 animal is diagrammed showing the 20 E nuclei (dots) that constitute the intestine.

type tyrosine kinase signal, which collaborate to polarize EMS (Bei et al., 2002; Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1997; Thorpe et al., 1997).

Wnt/MAPK/Src signaling apparently functions in endoderm induction by inactivating POP-1, a TCF/LEF-related repressor of E-specific gene activity. In the MS cell, unsignaled POP-1 represses endoderm-specific gene activity, allowing mesoderm development to occur (Lin et al., 1995; Rocheleau et al., 1997; Thorpe et al., 1997). Several lines of evidence led to the view that the Wnt/MAPK/Src signaling functions to abrogate the endoderm-repressing activity of POP-1 rather than acting positively to direct endoderm development. First, in the absence of POP-1 activity, both E and MS produce endoderm independent of Wnt/MAPK/Src signaling (Lin et al., 1995, 1998). Second, a complex of the β-catenin-like molecule, WRM-1, and an MAPK component, LIT-1, can phosphorylate POP-1 in vitro (Rocheleau et al., 1999), and phosphorylation has been shown to disrupt the DNA-binding properties of other TCF/Lef factors (Ishitani et al., 2003). Third, nuclear POP-1 levels are reduced in the E cell in response to Wnt/MAPK signaling as a result of redistribution of the protein to the cytoplasm mediated by the 14-3-3 protein PAR-5 (Ishitani et al., 1999; Lin et al., 1998; Lo et al., 2004; Maduro et al., 2002; Meneghini et al., 1999). Similarly, expression of WRM-1 and LIT-1 results in relocalization of POP-1 from the nucleus to the cytoplasm in cultured mammalian cells (Rocheleau et al., 1997).

Induction of endoderm by a double negative pathway originally suggested that the Wnt pathway functions differently in *C. elegans* than in other organisms (Lin et al., 1995, 1998; Rocheleau et al., 1997; Thorpe et al., 1997). More recently, it has been shown that, in the absence of signaling, TCF/LEF proteins can function as repressors through their association with Groucho-like co-repressors (Brantjes et al., 2001; Cavallo et al., 1998). Indeed, the repression of endoderm fate in the MS cell appears to involve a similar complex formed by POP-1, the Groucho-like protein UNC-37, and the histone deacetylase HDA-1 (Calvo et al., 2001), showing that the repressive role of non-Wnt signaled POP-1 has been evolutionarily conserved. However, while nuclear POP-1 levels decrease in response to Wnt/MAPK signaling, significant levels of POP-1 are nonetheless detected in the E nucleus (Lin et al., 1995, 1998; Maduro et al., 2002), suggesting that it may also function in signaled cells. Indeed, postembryonically, POP-1 associates with the β-catenin BAR-1 to activate the Wnt target gene *mab-5* (Korswagen et al., 2000), and in gonadogenesis, WRM-1 and LIT-1 are required to activate POP-1 function rather than to abrogate its function (Siegfried and Kimble, 2002). It therefore remains a possibility that Wnt-signaled POP-1 may also perform an as yet undetected function in endoderm development.

Endoderm is specified by two redundant, zygotically expressed GATA-type transcription factors that are sufficient and together essential for endoderm development (Maduro and Rothman, 2002; Zhu et al., 1997). The *end* genes are expressed exclusively in the early E lineage in response to activation by the zygotically expressed MED-1,2 proteins, redundant, non-canonical GATA transcription factors. Expression of the *med* genes in the E and MS lineage is initiated by the maternally provided SKN-1 transcription factor, and the SKN-1→MED pathway specifies development of both E and MS (Bowerman et al., 1992; Maduro et al., 2001; Maduro and Rothman, 2002). While the SKN-1→MED pathway is absolutely essential for MS development, ~30% of embryos lacking SKN-1 and ~50% of embryos lacking MED-1,2 generate gut, indicating that an SKN-1, MED-independent mechanism also specifies endoderm.

In embryos that do not make endoderm as a result of defects in the SKN-1→MED→END pathway, the E cell adopts the fate of its cousin, the C blastomere, which gives rise to mesectoderm (muscle, epidermis, and neurons). Conversion to a C-like blastomere, as well as the normal C fate, requires maternal contribution of the Caudal-like homeodomain protein PAL-1 (Hunter and Kenyon, 1996). While maternal PAL-1, like SKN-1, is expressed in both the

C and E lineages, no function has been ascribed to endoderm-expressed PAL-1.

We report here that, although the SKN-1→MED cascade is required for high levels of *end-1* expression, *end-1* is expressed at low levels in the absence of SKN-1 or MED-1,2, consistent with the impenetrance of the endoderm specification defect in *skn-1* and *med-1,2* mutants. We found that Wnt-modified POP-1 is responsible in part for this SKN-1, MED-independent activation of *end-1* and endoderm development and that a Lef-1-like site is essential for activation of *end-1*, consistent with a positive role for POP-1 in endoderm development. These findings suggest that POP-1 is converted from a repressor to an activator of endoderm gene expression. We also made the unexpected finding that PAL-1 activates endoderm in the E lineage when the SKN-1→MED pathway is inactive. Thus, the positive action of POP-1 and PAL-1 on *end* gene expression accounts for the impenetrance of mutants lacking SKN-1, and the SKN-1→MED, Wnt-activated POP-1, and PAL-1 pathways can function independently to activate endoderm development in *C. elegans*.

Materials and methods

C. elegans strains and genetics

The following strains were used: N2 [wild type], EU1 [*skn-1(zu67) IV/nT1[unc-?(n754) let-?(m435)] (IV;V)*], JJ1057 [*pop-1(zu189) dpy-5(e61) I/hT1 (I;V); him-5(e1490) V/hT1 him-5(e1490) V*], EU384 [*dpy-11(e1180) mom-2(or42) V/nT1 [let-?(m435)] (IV;V)*], EU353 [*skn-1(zu67) IV/nT1[unc-?(n754) let-?(m435)] (IV;V); mom-2(or42) unc-42(e70) V/nT1*], JJ762 [*end-3(zu247) V*], JR1798 [*pop-1(zu189) dpy-5(e61)/hT1 (I;V); end-3(zu247) V/hT1 him-5(e1490) V*], and MS162 [*med-1(ok804) X; dpy-17(e164) sDf127 unc-32(e189) III; irDp1 (III;f)*]. The *ok804* mutation deletes the entire *med-1* locus, and the deficiency *sDf127* deletes *med-2* (data not shown). The free duplication *irDp1* balances *dpy-17* and *sDf127* and includes an integrated array containing *unc-32(+)*, an *unc-119*::YFP reporter used to identify animals carrying *irDp1*, and additional copies of *med-1(+)*. The deletion *ok1448* deletes the DNA-binding domain of END-3 (data not shown). A detailed description of MS162 and RB1331 will be presented elsewhere.

Constructs and transgenic animals

The 5′-deleted promoter constructs were amplified by PCR from pJZ21, an *end-1* minigene reporter containing 1.7 kbp of noncoding 5′ DNA fused to an *end-1* cDNA fragment encoding amino acids 1–191 of the 221-aa END-1 coding region inserted into plasmid pPD96.04 (NLS::GFP::*lacZ*::*unc-54*_3′UTR; a gift from A. Fire). Animals containing 5′-deleted promoter::reporter fusions were obtained by injecting gel-purified PCR products derived from pJZ21 as template. The SKN-1 and GATA sites in construct E265 were substituted by use of an imperfect 3′ oligonucleotide, and deletion of the −165 Lef site was performed using a PCR-based cloning strategy with the enzyme *Ear*I; both constructs were cloned into pPD96.04, the critical regions were sequenced, and then the promoter::reporter segment was amplified by PCR for injection. Oligonucleotide sequences and cloning details are available on request. For injections, the marker pRF4 (*rol-6D*) was used, and DNA mixtures were injected at a concentration of 10–100 ng/μl. Chromosomal integrants for the constructs E1720, Er880, and Er880ΔLef were obtained from an extrachromosomal line following irradiation at 400 J/m^2 using a UV crosslinker (Stratagene) and identification of integrants in the F_2 generation.

RNA interference (RNAi)

dsRNA was synthesized using the MEGAscript kit (Ambion) from T7 promoter-tagged fragments of cDNA clones as described (Maduro et al., 2001). For experiments involving *skn-1(RNAi)* and *pop-1(RNAi)*, soaking of parent animals in dsRNA (Timmons and Fire, 1998) was found to be as effective as direct gonadal injection (Fire et al., 1998). For soaking experiments, 10 to 50 gravid adult hermaphrodites were immersed in 4 μl dsRNA (~4 μg/μl) in RNase-free water for 8–12 h then recovered on seeded plates for 12–15 h prior to embryo collection for phenotype analysis or X-gal staining. For injection, dsRNA at a concentration of ~4 μg/μl was injected into both gonad arms of 10–20 hermaphrodites, and animals were allowed to recover for 12–15 h. For RNAi soaking or injection experiments targeted to multiple genes, equimolar mixtures of the dsRNAs were used. For dsRNA delivery into strain MS162, animals were fed *E. coli* HT115 expressing dsRNA corresponding to a fragment of the *pop-1* or *pal-1* cDNAs (Timmons et al., 2001). All experiments were performed at 20°C.

X-gal staining

Ten to 100 adult gravid worms were placed in the lid of an microfuge tube, and all liquid was removed before placing on dry ice for a minimum of 10 min. Worms were then lyophilized for 1 h, then cold ethanol was added, followed by the slow addition of PBST. Worms were washed 2×, and then 100 μl of X-gal staining solution was added (Fire, 1992). Stain was developed at 37°C until visible. Strength of reporter expression was judged based on both intensity of staining as well as time taken to see signal.

Antibody staining

Staining was performed on methanol–acetone fixed embryos as described (Zhu et al., 1997). β-galactosidase

(*lacZ*) was detected using a monoclonal β-gal Ab (Promega #Z3781), and intestine was detected with the monoclonal Ab MH33 (a gift from R. Waterston). Secondary antibodies were obtained from Sigma.

Results

Endoderm-specific expression of end-1 is regulated by SKN-1, the MEDs, POP-1, and the Wnt pathway

We sought to characterize the regulatory inputs that direct the expression of *end-1* to the E lineage by examining expression of an *end-1* gene reporter in embryos defective for genes required for normal endoderm development. A 1.7 kb *end-1* sequence upstream of the apparent transcriptional start site is sufficient to direct robust expression of a *lacZ* reporter in the early E lineage, similar to that of the endogenous RNA revealed by in situ hybridization (Zhu et al., 1997); however, while transcripts are first detectable in the E cell, reporter expression is not apparent until after E has divided, likely owing to the short length of the E cell cycle and a temporal requirement for transgene product accumulation. This sequence, coupled to the *end-1* coding region and 3′UTR, is sufficient to restore endoderm development in transgenic embryos homozygous for a deficiency that eliminates *end-1* and its redundant partner *end-3* and which otherwise never produces endoderm (Maduro and Rothman, 2002; Zhu et al., 1997).

We first assessed the requirement for the SKN-1→MED pathway on *end-1* expression. We previously found that depletion of *med-1,2* function by RNAi results in reduced expression of an *end-1* reporter (Maduro et al., 2001). To test the effect of removing the contribution of *med-1*, *med-2*, and *skn-1* together, we examined expression of an *end-1* reporter in embryos depleted for *skn-1*; such embryos show no detectable expression of *med-1,2*, which are apparent direct targets of SKN-1 action (Maduro et al., 2001). 49% (n = 350) of *skn-1(RNAi)* embryos fail to express the *end-1* reporter entirely, while the remainder express it at substantially reduced levels, consistent with the impenetrant lack of gut in *skn-1* mutants (see below). These observations also suggest that *end-1* can activate endoderm development even when expressed at diminished levels.

A comparison of the requirement for SKN-1 and the MEDs suggests that SKN-1 provides both MED-dependent and -independent inputs into *end* gene transcription. While a large fraction of both *skn-1* and *med-1,2* mutant embryos lack endoderm, ~30% of *skn-1(zu67)* mutants and ~50% of *med-1,2(RNAi)* embryos contain a differentiated gut (Bowerman et al., 1992; Maduro et al., 2001). This difference in penetrance might be attributable to the lower efficacy of RNAi compared to a chromosomal mutation or may reflect a greater requirement for SKN-1 than the MEDs for endoderm specification. To address these alternatives, we examined embryos lacking the chromosomal copies of both *med-1* and *-2* by constructing a strain carrying the *med-1(ok804) X* deletion identified by the *C. elegans* Knockout Consortium and the deficiency *sDf127 III*, which removes many genes including *med-2* (data not shown). Consistent with our observations of *med-1,2(RNAi)* embryos, we found that 45% (n = 260) of *med-1(ok804); sDf127* embryos, which appear to lack all MED activity, make endoderm (Table 1). These findings suggest that some SKN-1 may be independent of the MEDs; for example, it may act directly on the *end* genes, consistent with the presence of SKN-1 sites in these genes (see below; Zhu et al., 1997).

We next examined the effect of eliminating the endoderm-inducing Wnt pathway on *end-1* expression. We found that the *end-1* reporter is often expressed in embryos lacking maternal MOM-2, the Wnt-like molecule apparently produced by P_2 that induces endoderm in EMS (Rocheleau et al., 1997; Thorpe et al., 1997). As with the *skn-1* mutants, the fraction of embryos expressing *end-1* is greater than the fraction that produces endoderm: in the *mom-2(or42)* mutant, ~28% of the embryos contain a gut (Thorpe et al., 1997), yet ~50% express the *end-1* reporter, albeit at a reduced level (Fig. 2E). Embryos lacking both MOM-2 and SKN-1 virtually never contain a gut (Thorpe et al., 1997),

Table 1
Intestinal differentiation in mutant embryos

Genotype	% Intestine[a] (n)
Wild type	100% (n > 500)
skn-1(zu67)	36% (164)
skn-1(RNAi)[b,c]	28% (731)
skn-1(RNAi); unc-22(RNAi)[b]	30% (60)
pop-1(zu189)	96% (45)
pop-1(RNAi)[b,c]	96% (437)
pop-1(RNAi); unc-22(RNAi)[b]	95% (100)
pal-1(RNAi)[c]	100% (235)
pal-1(RNAi); pop-1(RNAi)[c]	100% (437)
skn-1(zu67); pop-1(zu189)	30% (118)
skn-1(zu67); pop-1(RNAi)[b]	5% (323)
skn-1(RNAi); pop-1(zu189)[b]	32% (394)
skn-1(RNAi); pop-1(RNAi)[b,c]	11% (1245)
skn-1(RNAi); pal-1(RNAi)[c]	9% (323)
skn-1(RNAi); pop-1(RNAi); pal-1(RNAi)[c]	0% (519)
med-1(ok804); sDf127[d]	45% (260)
med-1(ok804); sDf127; pop-1(RNAi)[e]	6% (95)
med-1(ok804); sDf127; pal-1(RNAi)[e]	7% (190)
end-3(zu247)	91% (247)
end-3(ok1448)	95% (155)
end-3(zu247); pal-1(RNAi)[c]	68% (327)
end-3(zu247); pop-1(zu189)	14% (78)
end-3(zu247); pop-1(RNAi)[c]	3% (175)
end-3(ok1448); pop-1(RNAi)[c]	1% (262)
end-3(zu247); pop-1(RNAi); Ex[end-3(+)][c]	99% (79)

[a] Scored by presence of birefringent gut granules in terminal embryos.
[b] Soaking of parent hermaphrodites in dsRNA was used. The proportion of gutless embryos was found to be comparable to results obtained by direct injection.
[c] Direct injection of dsRNA into parent hermaphrodites was used.
[d] *sDf127* deletes *med-2* and is linked to recessive mutations in *dpy-17* and *unc-32*.
[e] Growth of hermaphrodite parents on *E. coli* HT115 bacteria expressing dsRNA was used.

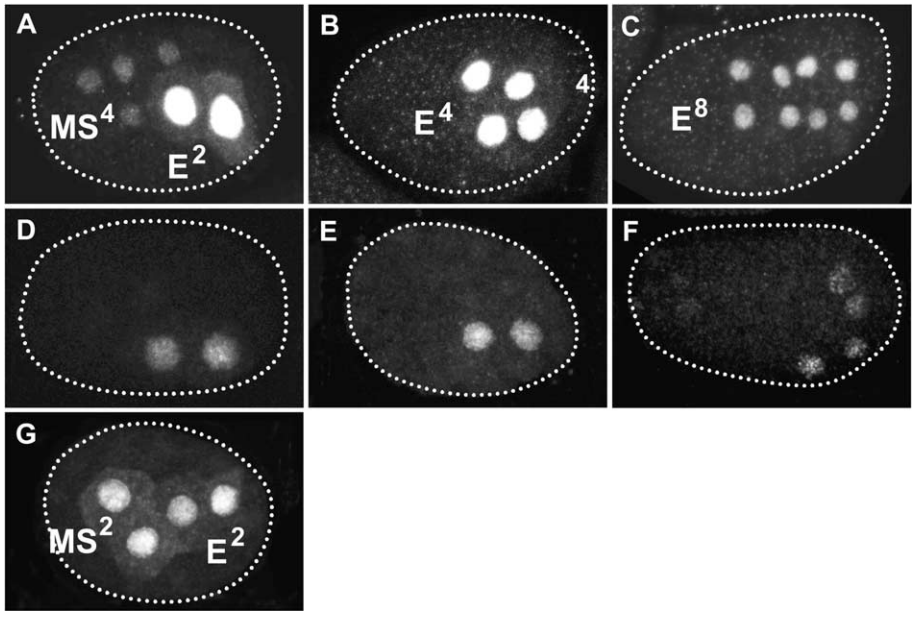


Fig. 2. Expression of *end-1* in maternal mutants. Confocal micrographs of fixed embryos show expression of a full-length promoter *end-1*::NLS::GFP::*lacZ* transgene in wild-type (A–C) and mutant (D–G) backgrounds, as detected by staining for immunoreactive β-galactosidase. (A) Strong expression in the two E daughters at the 28-cell stage. Weak expression of the reporter is detectable in the four MS descendants. (B) 4E (~64-cell) stage. (C) 8E (~128-cell) stage. (D) Reduced *end-1* expression was observed in 51% (n = 350) of *skn-1(RNAi)* embryos as shown here at the 2E stage. (E) 50% (n = 86) of *mom-2(or42)* mutant embryos express *end-1* as shown for the 2E stage. Because gastrulation fails in these mutants, the endoderm cells remain at the ventral–posterior surface of the embryo. (F) 19% (n = 27) of *skn-1(zu67); mom-2(or42)* embryos display some *end-1* expression (shown at the 4E stage). (G) Derepression of *end-1* in the MS lineage is observed in 100% (n = 138) of *pop-1(RNAi)* embryos. The embryos shown in panels (F) and (G) carry an extrachromosomal *end-1*::NLS::GFP::*lacZ* array, while the remaining embryos carry the chromosomal *end-1*::NLS::GFP::*lacZ* insertion *wIs28*. The percentage of expressing embryos given above has been adjusted for transmission frequency of the array in panels (F) and (G).

suggesting that the Wnt pathway and SKN-1 function in parallel to activate endoderm development. We found that ~19% of *skn-1(zu67); mom-2 (or42)* embryos express the *end-1* reporter (Fig. 2F). Thus, the Wnt pathway and SKN-1 collaborate to activate *end-1* expression; however, the residual expression in the double mutant suggests that there is yet another factor that activates *end-1* (see below).

Maternal POP-1, which represses endoderm differentiation in the MS lineage, is the target of the endoderm-inducing Wnt signal. We found that elimination of maternal POP-1 by either a chromosomal *pop-1* mutation or by RNAi results in expression of the *end-1* reporter in descendants of both the E and MS blastomeres (Fig. 2G), supporting the view that the major role of POP-1 in endoderm specification is as a repressor of *end-1* (and *end-3*) in MS (Calvo et al., 2001; Maduro et al., 2002).

Potential conserved regulatory elements in end-1 include consensus binding sites for SKN-1, POP-1, and GATA factors

The foregoing studies establish that *end-1* transcription is regulated by the SKN-1→MED pathway and POP-1. Immunoreactive SKN-1 and POP-1 are both found in the nucleus of the E blastomere (Bowerman et al., 1993; Lin et al., 1995), as are GFP- or myc-tagged versions of MED-1 (Maduro et al., 2001). MED-1 binds to two sites in the *end-1* promoter both in vitro and in vivo (Broitman-Maduro et al., 2005; Maduro et al., 2002). To assess the potential of POP-1, SKN-1, and END-1/3 to interact directly with the *end-1* promoter, we examined the regulatory regions of *end-1*, *end-3*, and homologs of *end-1* and *end-3* in the related species *C. briggsae* (Fig. 3) (Maduro et al., in press; Zhu et al., 1997). *C. briggsae* is estimated to have diverged from *C. elegans* ~50–120 Myr ago, and comparisons of noncoding regions between these species can be used to establish sites important for regulation (Coghlan and Wolfe, 2002; Kennedy et al., 1993). A number of sequences corresponding to consensus recognition sites for known transcription factors are apparent in the regulatory regions of all four *end* genes. Consensus binding sites for SKN-1 (RTCAT) (Blackwell et al., 1994), GATA factors (HGATAR) (Lowry and Atchley, 2000), and the newly identified MED-1 binding site (RAGTATAC) (Broitman-Maduro et al., 2005) are present at several positions, often in close proximity (Fig. 3). Within *C. elegans end-1*, for example, a cluster of five SKN-1 sites extends from −570 to −1000 bp, suggesting that this region might act as a SKN-1-responsive module. In addition, two sequences that match the consensus binding site for the mammalian homolog of POP-1, Tcf/Lef-1 (CTTTGWW) (Lin et al., 1995; Travis et al., 1991) are present in *end-1*; we will refer to these as Lef-1 sites. *Ce-end-1*, *Ce-end-3*, and *Cb-end-1* all contain at least one Lef-1 site within the proximal-most 200 bp. As POP-1 can bind to Lef-1 sites (Korswagen et al., 2000), these are excellent candidates for sites of direct POP-1

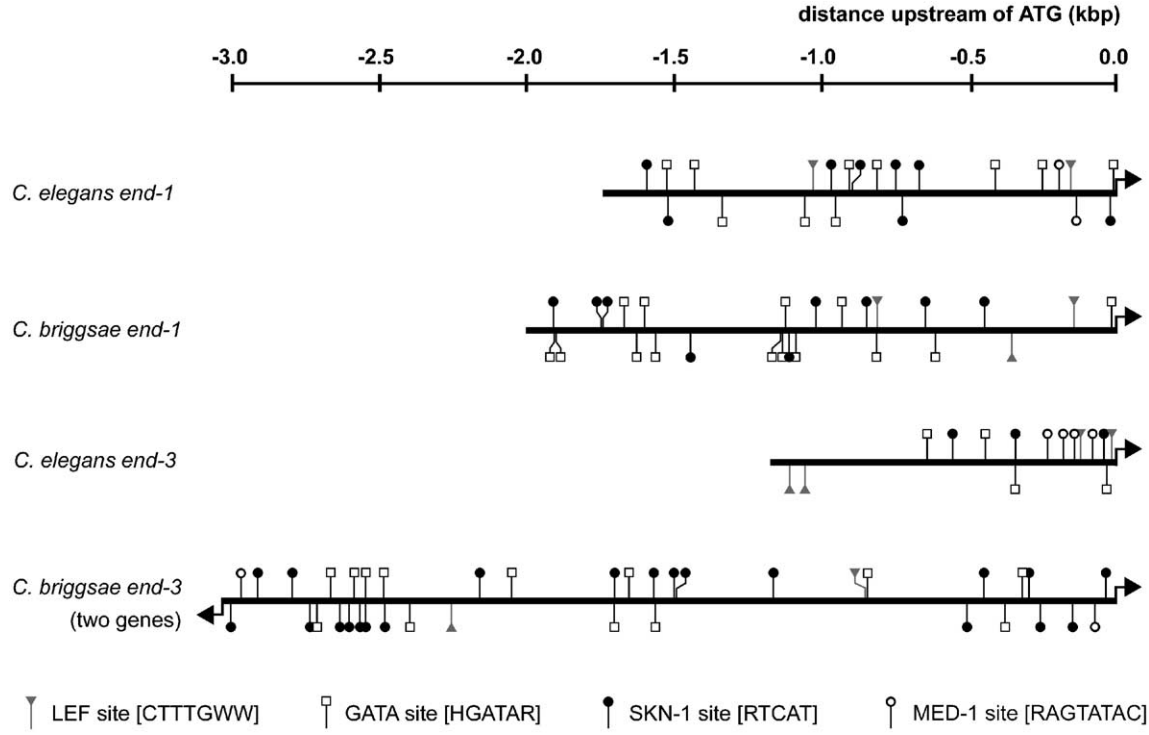


Fig. 3. Putative regulatory sites in the upstream sequences of the *end* genes of *C. elegans* and *C. briggsae.* Sites are denoted by symbols above each promoter (thick line) if they reside on the top strand and below if they reside on the bottom strand. A legend for the symbols used, and the corresponding consensus sequence, is shown at the bottom of the figure. Based on 5′RACE analysis, the 5′ ends of the *end-1* and *end-3* transcripts are within <5 bp of their respective translation start sites (Maduro et al., in press; Zhu et al., 1997). For consistency, coordinates are given relative to translation start sites (arrows). In *C. briggsae*, there are two *end-3* homologs expressed as divergent transcripts from an intervening 3024 bp promoter. All five *end* genes show E lineage-specific expression in *C. elegans* (Maduro et al., in press; Zhu et al., 1997).

regulation. Based on the sequences of their upstream regulatory regions, we conclude that the *end* genes in *C. elegans* and *C. briggsae* may be directly controlled by the regulatory inputs described above, including POP-1.

Requirement for a Lef-1 site in end-1 activation

As there are numerous putative transcription factor binding sites in *end-1*, we sought to simplify the analysis of the promoter by identifying a minimal region capable of directing E lineage-specific expression of *end-1*. A series of 5′ deletions was created in the *end-1*::*lacZ* reporter and transgenic embryos assayed for β-galactosidase expression. Sequential removal of distal promoter segments revealed that each contributes incremental activating functions; however, none of these is essential for proper spatial and temporal regulation of *end-1* in the E lineage (Fig. 4 and data not shown). Removal of a cluster of SKN-1 sites between −570 and −1000 bp results in greatly reduced reporter expression levels, consistent with the aforementioned genetic data suggesting that SKN-1 provides MED-independent endoderm specifying activity. Furthermore, comparison of deletion constructs differing in retention of a single GATA site at −414 bp (E1000 vs. Er880 and E487 vs. E402) reveals that this site contributes positively to *end-1* activation. While MED-1 does not bind to a canonical GATA site (Broitman-Maduro et al., 2005), the contribution of this site to *end-1* activation implicates autoregulation by END-1 or activation by END-3. Recombinant END-1 protein can bind a canonical GATA site, suggesting that END-1 recognizes a GATA site in *C. elegans* (Shoichet et al., 2000).

These analyses demonstrated that the proximal-most 310 bp of the *end-1* promoter (construct E310) is sufficient for expression of the reporter specifically in the E lineage. Further removal of an additional 95 bp results in sporadic ectopic expression outside of the E and MS lineages later in embryogenesis. However, this minimal 215 bp construct contains all elements sufficient for activation of some *end-1* expression in the E lineage, as well as repression in the MS lineage, as it does not show increased expression in MS above the trace amount detected with the full-length promoter (not shown).

The consensus binding sites in the minimal 215 bp segment include Lef-1, SKN-1, MED-1, and GATA sites (Fig. 4). We predicted that POP-1 might repress *end-1* by acting through this Lef-1 site; in such an event, removal of the site would be expected to cause a *pop-1(−)*-like phenotype, resulting in expression in both the E and MS lineages. To our surprise, either upstream deletion (E158) or precise removal (E310ΔLef) of the Lef-1 site abolished expression of the *end-1* reporter. This result indicates that the Lef-1 site may also be required for activation, in addition

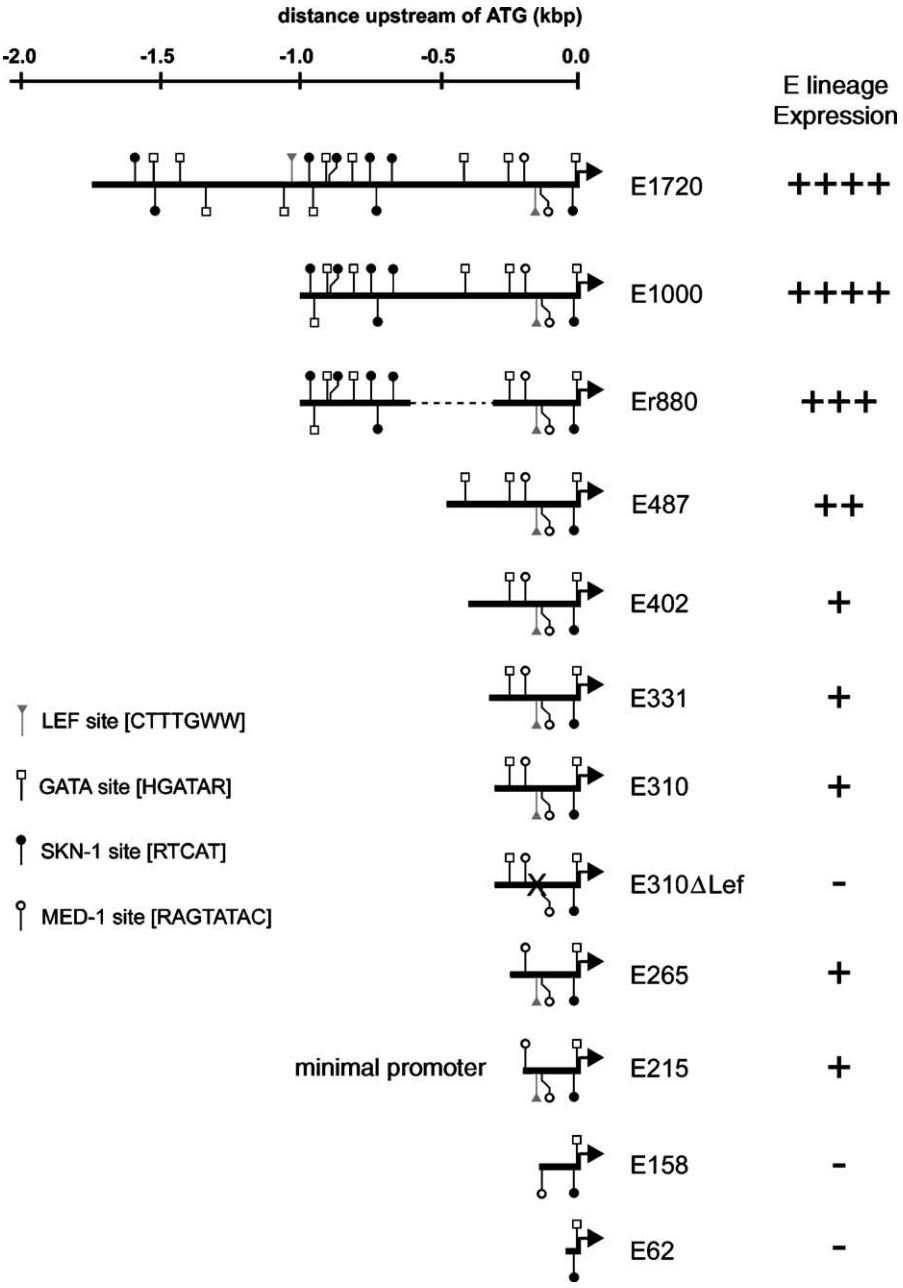


Fig. 4. Identification of a minimal *end-1* promoter and requirement for candidate *cis*-acting sites. Fragments of the 1.7-kbp *end-1* promoter that were cloned into the vector pPD96.04 are shown alongside the construct name (i.e. 'E1720' for the full-length promoter) and a representation of *end-1* reporter levels, based on the X-gal staining of fixed embryos. –, +, ++, +++, and ++++ denote successive levels of signal (++++, strongest signal; +, weak signal; –, no signal detectable). Putative transcription factor binding sites are shown as in Fig. 3.

to repression, of *end-1*. If indeed this site is recognized by POP-1, it would contrast with the prevailing view that POP-1 function as a repressor of endoderm is merely down-regulated in E and hence lacks a function when modified by Wnt/MAPK signaling.

POP-1 acts synergistically with SKN-1 to activate end-1 expression and endoderm development

The finding that an Lef-1 site is essential for *end-1* expression led us to consider the possibility that POP-1, which binds to Lef-1 target sequences (Korswagen et al., 2000), may function as an activator of *end-1* in the E cell. This activating function could account for the ability of embryos to make endoderm in the absence of SKN-1 or the MEDs (Bowerman et al., 1992; Maduro et al., 2001). Such an activating role for POP-1 would likely have eluded detection in *pop-1* mutants owing to robust activation of *end-1* by SKN-1 and MED-1,2. In a mutant lacking maternal *pop-1* function, MS-specific repression is eliminated and SKN-1 and MED-1,2 can activate *end-1* in both cells (Fig. 2G), thereby masking the requirement for a positive role for

POP-1. An activating role for POP-1 in the E lineage would therefore be evident only when SKN-1 is absent.

We found that simultaneous depletion of SKN-1 and POP-1 by RNAi reveals a requirement for POP-1 in activation of *end-1*. While 88% of POP-1-depleted and 51% of SKN-1-depleted embryos express the *end-1* reporter, only 21% of embryos depleted for both SKN-1 and POP-1 expressed the reporter (Fig. 5). The decrease in the fraction of embryos expressing the reporter was highly significant ($P < 0.00001$ for *skn-1* vs. *skn-1; pop-1* mutants). This synergistic effect of removing both POP-1 and SKN-1 was reflected not only in the fraction of embryos expressing the reporter, but also in the expression levels observed: *skn-1(RNAi); pop-1(RNAi)* embryos express the reporter at substantially lower levels than embryos depleted for the function of either gene alone (Fig. 6). Together, these findings demonstrate that POP-1 contributes positively to *end-1* expression in the E lineage.

We next determined whether the decrease in *end-1* reporter expression in *pop-1(−); skn-1(−)* double mutants compared to *skn-1(−)* mutants alone is reflected as a decrease in the proportion of embryos producing endoderm. We scored for production of differentiated gut by gut granule birefringence, expression of an intestine-specific marker (*elt-2*::GFP) (Fukushige et al., 1998), and presence of an intestine-specific antigen detected with the antibody (Bossinger et al., 2004) in terminally differentiated embryos. In all three cases, *pop-1(RNAi); skn-1(RNAi)* mutants showed a highly significant ($P < 0.0003$), 2- to 3-fold decrease in the number of embryos producing gut compared to *skn-1(RNAi)* single mutants, while controls with *unc-22(RNAi); skn-1(RNAi)* did not result in such a decrease. The proportion of *skn-1(RNAi); pop-1(RNAi)* embryos that produce differentiated gut was less than the fraction expressing the *end-1* reporter, similar to the results obtained with *skn-1* and *mom-2* mutant embryos.

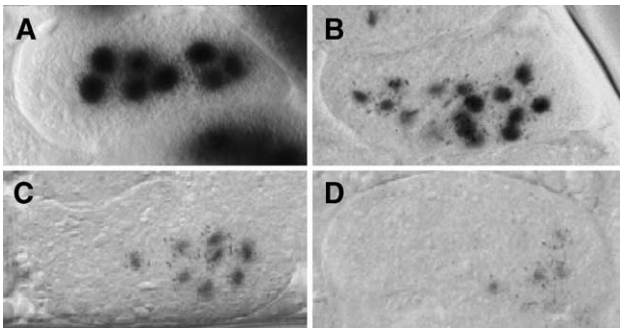


Fig. 6. Synergistic effect of SKN-1 and POP-1 on *end-1* expression levels. *end-1*::NLS::GFP::*lacZ* expression was revealed by X-gal staining of 64-cell stage embryos in utero. (A) Wild-type. (B) *pop-1(RNAi)* showing ectopic expression in both MS (smaller nuclei) and E descendants (larger nuclei). (C) *skn-1(RNAi)*. (D) *pop-1(RNAi); skn-1(RNAi)*. The signal in the double mutant is much weaker than in *skn-1(RNAi)* alone. The wild-type embryo in panel (A) has been stained for a longer period of time than in panels (B–D).

It was previously reported that a *skn-1(zu67); pop-1(zu189)* double chromosomal mutant showed a phenotype that was indistinguishable from that of the single *skn-1(zu67)* mutant alone (Lin et al., 1995), revealing no interaction between the two mutations. This conflicting result might indicate that the double RNAi phenotype does not accurately reflect the simple loss of both gene functions or alternatively that one of the chromosomal mutations insufficiently debilitates the corresponding gene to reveal the synergistic requirement for both SKN-1 and POP-1. To resolve these conflicting observations, we compared chromosomal double mutant embryos with embryos mutant for one of the chromosomal mutations and depleted for the other gene function by RNAi (Table 1). Consistent with the findings of Lin et al. (1995), the fraction of embryos lacking gut, ~30%, was similar in *skn-1(zu67)* and *skn-1(zu67); pop-1(zu189)* double mutants and was also similar to that in *skn-1(RNAi); pop-1(zu189)* double mutants.

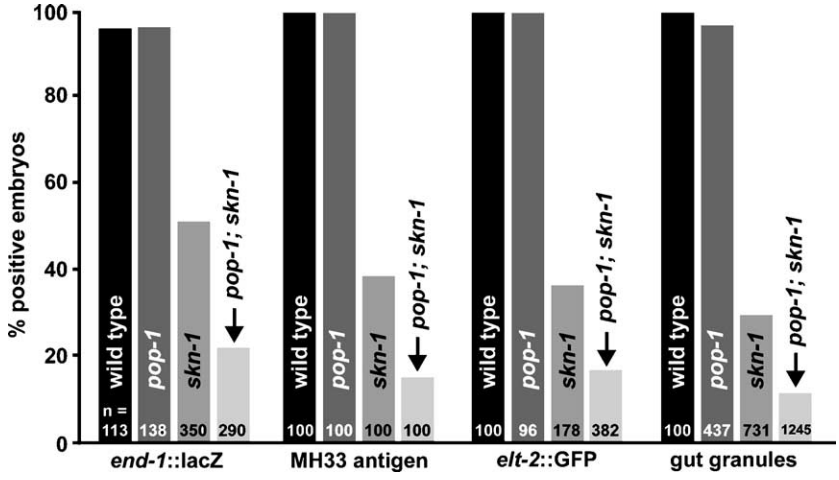


Fig. 5. Loss of *skn-1* and *pop-1* function leads to a synergistic defect in endoderm development. Expression of *end-1* and intestine production in *pop-1(−)* and *skn-1(−)* are shown for single and double mutant embryos generated by RNAi. Bars show percentage of 28- to 128-cell stage embryos (*end-1*::*lacZ*) or terminally arrested embryos (MH33, *elt-2*::GFP, gut granules) positive for each respective marker. The RNAi phenotypes are shown on or above the bars, and the number of embryos scored is indicated at the base of each bar.

However, only 5% (P = 0.001) of *skn-1(zu67); pop-1(RNAi)* embryos produced gut, implying that RNAi targeted to *pop-1* is more effective at eliminating *pop-1* function than is the *zu189* mutation. The *zu189* lesion is a transposon insertion in the 3′UTR of the gene, specifically affecting maternal expression of POP-1 (Lin et al., 1995, 1998), suggesting that there may be residual POP-1 activity in *zu189* embryos. The difference we observed accounts for why the synergistic requirement for POP-1 and SKN-1 in specification of the endoderm is apparent with *pop-1(RNAi)*, but not *pop-1(zu189)*. We also found that, when *skn-1* and *pop-1* are inactivated by RNAi simultaneously, 11% (n = 1245) of embryos make endoderm. As the *skn-1(zu67)* lesion is a nonsense mutation (Bowerman et al., 1993), the reduced penetrance of *skn-1(RNAi); pop-1(RNAi)* (11%) over *skn-1(zu67); pop-1(RNAi)* (5%) may reflect the limited efficacy of targeting two genes by RNAi simultaneously.

Synergy of the endoderm-promoting function of POP-1 with other genes that act in endoderm development

To examine whether the positive requirement for POP-1 in endoderm development is specifically seen only when SKN-1 function is reduced, we tested the ability of *pop-1* mutants to synergize with other mutants in which *end* activity is reduced. The *zu247* mutation alters a residue in the zinc finger of END-3, the redundant partner of END-1 (Maduro and Rothman, 2002), and appears to be hypomorphic (Maduro et al., in press), while the *ok1448* lesion deletes the END-3 DNA-binding domain and is predicted to be a molecular null (our unpublished observations). We found that, while 91% (n = 247) of *end-3(zu247)* mutants made intestine, only 14% (n = 78) of *end-3(zu247); pop-1(zu189)* animals and 3% (n = 175) of *pop-1(RNAi)*; *end-3(zu247)* animals did (Table 1). Similar results were obtained with the *end-3* molecular null (Table 1): only 1% of *pop-1(RNAi)*; *end-3(ok1448)* double mutants made endoderm. Thus, elimination of *pop-1* function is highly synergistic with reduced *end-3* function, which otherwise results in a very mild phenotype.

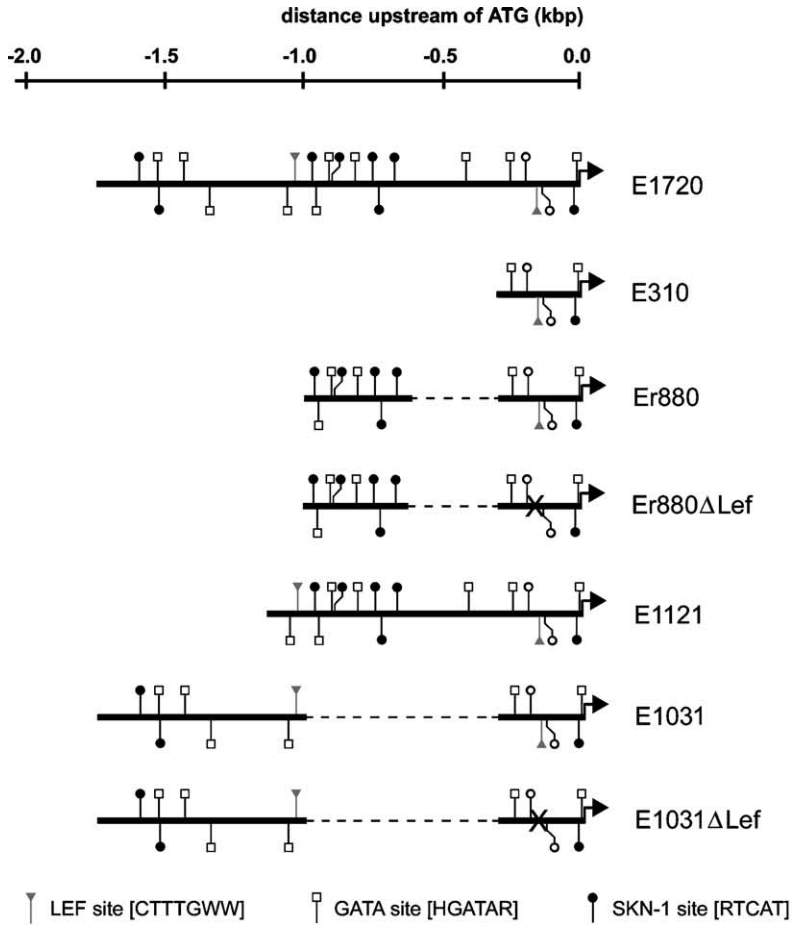
We also found that loss of *pop-1* function strongly synergizes with absence of zygotic MED activity: while 45% (n = 260) of *med-1(ok804); sDf127* embryos make endoderm, only 6% (n = 95) of *med-1(ok804); sDf127; pop-1(RNAi)* embryos do. Together, these results confirm the positive contribution of POP-1 in endoderm specification and show that the activating function of POP-1 is apparent even in the presence of functional SKN-1 (see Discussion).

A Lef-1 site is required for POP-1-dependent end-1 reporter expression

Given the requirement for both an Lef-1-like site and an Lef-1-like protein, POP-1, in *end-1* activation, it seems likely that Wnt-activated POP-1 may promote *end-1* expression by directly interacting with the *end-1* promoter. While we have previously shown that GFP-tagged POP-1 can form subnuclear ‘spots’ in vivo by binding to extrachromosomal arrays containing the *end-1* or *end-3* promoters in the MS lineage (Maduro et al., 2002), we do not know if POP-1 can also act directly on *end-1,3* promoters in the E lineage, where it is present at a lower concentration. To assess the requirement for POP-1 and the Lef-1 site in *end-1* reporter expression, we attempted to identify the smallest segment of the *end-1* promoter that might reveal a requirement for both POP-1 function (in the absence of SKN-1) and the −165 Lef-1 site. A small promoter segment consisting of 310 bp of upstream DNA, either in isolation (E310) or combined with additional upstream sequences (Er880), drives E lineage expression dependent on the SKN-1→MED pathway and the −165 Lef-1 site (Fig. 7). The most informative construct, E1031, retains sufficient residual expression in *skn-1(RNAi)* mutants that the positive contribution of POP-1 can be measured: while 18% (n = 201) of *skn-1(RNAi)* embryos express a reporter derived from E1031, 0% (n = 114) of *skn-1(RNAi); pop-1(RNAi)* embryos express this construct, demonstrating the POP-1 dependence of transcription from this construct. In contrast, E lineage-specific expression of a reporter carrying a deletion of the −165 Lef-1 site (E1031ΔLef) depends almost exclusively on SKN-1: only 4% (n = 120; $P < 0.0002$) of *skn-1(RNAi)* embryos express this reporter. Hence, the −165 Lef-1 site is important for the positive contribution of POP-1, supporting a direct interaction of Wnt-signaled POP-1 with this site.

Activation of endoderm by the mesectodermal-specifying PAL-1 homeodomain protein

Simultaneous removal of SKN-1 and POP-1 function greatly attenuates but does not abolish, endoderm development, suggesting that at least one additional factor can contribute to endoderm specification. One candidate for such a factor is Caudal/PAL-1, which is present in all descendants of P_1, including the E cell (Hunter and Kenyon, 1996). However, PAL-1 is required to specify the fates of C and D, somatic founder cell descendants of P_2, and *pal-1* mutants show no conspicuous defect in endoderm specification (Hunter and Kenyon, 1996). In embryos that fail to make endoderm as a result of a defect in the SKN-1→MED→END pathway, the E cell adopts a C-like fate, producing body wall muscle and hypodermis (Bowerman et al., 1992; Maduro et al., 2001); this mesectodermal C-like fate, as with that of the normal C cell, requires PAL-1, demonstrating that PAL-1 is functional in the E cell (Hunter and Kenyon, 1996; Maduro et al., 2001). To test whether PAL-1 contributes to endoderm specification in the absence of SKN-1 and POP-1, we depleted all three activities by RNAi.



E lineage expression			
	untreated	*skn-1(RNAi)*	*skn-1(RNAi); pop-1(RNAi)*
E1720	88% (n=26)	51% (n=350)	21% (n=290)
E310	100%* (n=157)	0%* (n=85)	0%* (n=109)
Er880	96% (n=47)	0% (n=78)	0% (n=31)
Er880ΔLef	11% (n=40)	0% (n=60)	4% (n=75)
E1121	100%* (n=123)	11%* (n=164)	3%* (n=62)
E1031	100%* (n=102)	18%* (n=201)	0%* (n=114)
E1031ΔLef	100%* (n=128)	4%* (n=120)	0%* (n=101)

Fig. 7. Expression of *end-1* promoter variants in *skn-1* and *pop-1* mutants. The −165 Lef-1 site accounts for most of the positive POP-1-dependent input into *end-1* expression. Strains bearing different *end-1* reporters were tested for E lineage expression dependence on SKN-1 and/or POP-1 activity. Constructs E1720, E1121, and E1031 show a reduction in expression in *pop-1(RNAi); skn-1(RNAi)* compared to *skn-1(RNAi)* alone. In construct E1031ΔLef, in which the −165 Lef-1 site has been deleted, expression is almost completely dependent upon SKN-1 alone, implicating a requirement for the Lef-1 site in activation by POP-1. An asterisk (*) indicates data for an extrachromosomal reporter transgene in which the proportion of untreated embryos that express the reporter was normalized to 100%. The remaining reporters (E1720, Er880, and Er880ΔLef) were analyzed as integrated arrays.

Unexpectedly, we found that endoderm development was completely abrogated in this triple mutant (n = 519; Table 1), revealing a positive role for PAL-1 in E specification.

We further tested the effect of depleting *pal-1* function in conjunction with *skn-1(RNAi)* alone, the double mutant strain lacking zygotic *med-1* and *-2*, and the *end-3(zu247)* mutant. These experiments revealed that PAL-1 function is required for much of the residual endoderm made in each of these mutants (Table 1). As with POP-1, this requirement for PAL-1 in endoderm specification is normally masked by the SKN-1→MED pathway, and depletion of either *pal-1* and *pop-1* function alone, or simultaneous removal of both, fails to result in an endoderm specification defect. These results lead to the surprising conclusion that, while PAL-1 is normally required for the development of the mesectodermal progenitor, C, it can also activate endoderm development in the E lineage and that SKN-1, MED-1,2, POP-1, and PAL-1 can all contribute to endoderm specification in *C. elegans*.

Discussion

We have shown here that the *end-1* gene and endoderm specification are regulated by several independent transcriptional inputs, including SKN-1→MED, POP-1, acting both positively (in E) and as a repressor (in MS), and PAL-1, which normally functions to regulate mesectodermal development in the P_2 lineage. Collectively, these findings reveal that a complex transcriptional network is required to establish a relatively simple pattern of expression: activation of *end-1* (and, presumably *end-3*) in a simple clonal lineage descending from the E cell.

Dual action of POP-1 in both negative and positive regulation of endoderm

The terminal Wnt pathway regulator POP-1 establishes transcriptional differences between sister cells throughout *C. elegans* development (Herman, 2001; Jiang and Sternberg, 1999; Korswagen et al., 2000; Lin et al., 1995, 1998).

At the 8-cell embryonic stage, POP-1 is required to make the MS cell different from its sister, E, the endoderm progenitor (Lin et al., 1995). The role of POP-1 in this asymmetry was thought to be exclusively as a repressor in MS, where non-Wnt-signaled POP-1 blocks activation of the endoderm specification genes *end-1* and *end-3* by the SKN-1→MED pathway (Calvo et al., 2001; Maduro et al., 2002). Although POP-1 is detectable in both the MS and E cells, it does not appear to be required for endoderm specification per se (Calvo et al., 2001; Lin et al., 1995; Maduro et al., 2002). However, we now report several lines of evidence for an activating role of POP-1 in endoderm development. First, expression of a minimal *end-1* reporter is dependent on POP-1 and a presumptive POP-1 binding site for expression. Second, in mutant backgrounds that reduce expression or activity of the *end* genes, POP-1 is required for endoderm specification: in *skn-1(zu67)*, *skn-1(RNAi)*, *med-1,2(−)*, *end-3(zu247)*, and *end-3(ok1448)* mutant backgrounds, depletion of POP-1 activity results in a profound decrease in the levels of *end-1* expression and in the proportion of embryos producing endoderm. In the most extreme case, loss of POP-1 leads to the nearly complete (1%) elimination of endoderm in a mutant (*end-3(ok1448)*) that otherwise makes endoderm in >90% of embryos. This latter finding shows that the positive action of POP-1 in endoderm development is not only required when the SKN-1→MED pathway is inactivated, but also likely functions to elevate *end* gene expression in the E lineage of normal embryos. We conclude that POP-1 acts dually as a repressor and an activator of *end* gene expression, likely by directly interacting with *end* regulatory sequences.

The original analysis of *pop-1* (Lin et al., 1998) did not reveal its positive contribution to *end* gene expression and endoderm development for two reasons. First, the parallel robust contribution of the SKN-1→MED pathway to *end-1,3* activation masks the positive input of POP-1 in *pop-1* mutants. Second, depletion of *pop-1* function by RNAi is more effective at eliminating POP-1 function than is the maternal-specific mutant *zu189*; hence, the first experiments investigating the phenotype of the *pop-1; skn-1* double mutants did not reveal the synergy of the two genes.

Our findings explain the observation that, although *skn-1* and *med-1,2* mutant embryos invariably fail to produce MS-derived tissues, a substantial fraction still produce endoderm (Bowerman et al., 1992; Maduro et al., 2001): Wnt-activated POP-1 stimulates *end-1* expression even in the absence of the SKN-1→MED pathway. By genetic criteria, the parallel regulatory inputs of these two pathways are redundant; however, they act by quite distinct mechanisms. Moreover, we have shown that the homeodomain protein PAL-1 can promote endoderm development in parallel with SKN-1→MED and POP-1, accounting for the small fraction of *skn-1; pop-1* mutant embryos that still produce endoderm.

Wnt-signaled and Wnt-unsignaled POP-1 may function at distinct regulatory sites

While we were able to identify a putative POP-1 site that is required for positive regulation of an *end-1* reporter, we have been unable to identify a regulatory element that is required for repression in the MS lineage. Of particular significance, the −165 Lef-1 site is essential for E-specific activation of *end-1* but is not required for repression in the MS lineage: construct Er880ΔLef, in which this site is deleted, gives E lineage-specific expression, albeit at a low frequency. If POP-1 represses *end-1* by acting exclusively through this Lef-1 site, then we would expect to observe expression in both the E and MS lineages. Evidence has been obtained for a POP-1 repressive complex that includes the histone deacetylase HDA-1 and the Groucho-like co-repressor UNC-37 (Calvo et al., 2001). Derepression of an *end-1* reporter is observed in the MS lineage in embryos depleted for HDA-1; this effect is enhanced when UNC-37 is simultaneously depleted. One possibility, therefore, is that the SKN-1→MED pathway cannot activate *end-1* when POP-1 is present in this repressive complex. We have previously shown that GFP-tagged forms of MED-1 and POP-1 localize to the *end-1* promoter in the MS cell, suggesting that the repressive function of POP-1 does not preclude simultaneous binding by MED-1 (Maduro et al., 2002). We postulate that low affinity sites distributed throughout the *end-1* promoter might account for localization of the repressive POP-1 complex; such a possibility would explain why *end-1* promoter sub-fragments do not show significant derepression.

How does POP-1 function as an activator in the E cell?

TCF/LEF transcription factors are known to activate transcription only when bound to a β-catenin-like protein (Cadigan and Nusse, 1997). The *C. elegans* genome encodes three recognizable β-catenins, WRM-1, HMP-2, and BAR-1 (Korswagen et al., 2000; Natarajan et al., 2001), and a fourth protein, SYS-1, that appears to carry out a similar function (Kidd et al., 2005). HMP-2 appears to function in cell adhesion as it is the only *C. elegans* β-catenin that interacts with the cadherin HMR-1 (Costa et al., 1998; Korswagen et al., 2000; Natarajan et al., 2001). While POP-1 does not activate transcription of a Tcf target reporter gene in tissue culture, co-expression of POP-1 with SYS-1, BAR-1, or *Drosophila* Armadillo, but not WRM-1 or HMP-2, does result in activation (Kidd et al., 2005; Korswagen et al., 2000). Of the four β-catenins, BAR-1 and SYS-1 have been shown to interact directly with POP-1 (Kidd et al., 2005; Korswagen et al., 2000; Natarajan et al., 2001); indeed, a BAR-1/POP-1 complex activates postembryonic expression of the Wnt target gene *mab-5*, and a SYS-1/POP-1 complex is proposed to activate Wnt-dependent genes required for asymmetrical cell divisions in the somatic gonad (Kidd et al., 2005). Loss of *bar-1* function does not

cause an endoderm defect, and *bar-1(RNAi)* does not enhance *skn-1(−)* gutlessness (data not shown), suggesting that POP-1 associates with another β-catenin or that BAR-1 and another β-catenin function redundantly. One possibility is that WRM-1 is the β-catenin that functions with POP-1. WRM-1 possesses an activation domain and demonstrates a weak but detectable interaction with POP-1 in a yeast two-hybrid assay (Natarajan et al., 2001); moreover, when expressed under control of the *bar-1* promoter, WRM can substitute for BAR-1 in *C. elegans* (Natarajan et al., 2001). In gonadogenesis, depletion of WRM-1 and the Nemo-like kinase LIT-1 results in the same lineage defects as zygotic *pop-1* mutants, suggesting that WRM-1 and LIT-1 can contribute to a positive function for POP-1 (Siegfried and Kimble, 2002). In the early embryo, however, WRM-1/LIT-1 activity is required to block the repressive function of POP-1 (Lo et al., 2004; Rocheleau et al., 1999). It is therefore not possible to detect the positive contribution of WRM-1 to *end-1* activation, as depletion of *wrm-1* or *lit-1* activity results in the complete absence of endoderm (Rocheleau et al., 1997; Thorpe et al., 1997). It may be possible, therefore, that WRM-1 fulfils the requirement of the β-catenin–TCF interaction involved in transcriptional activation, as seen in other systems.

A better candidate for the POP-1 coactivator in endoderm specification is the novel Wnt coactivator SYS-1 (Kidd et al., 2005). A predicted null mutant of *sys-1*, *q736*, shows an embryonic lethal phenotype, suggesting that SYS-1 does function in the embryo (Kidd et al., 2005). However, it is not known when putative zygotic *sys-1* activity begins nor is it known how *sys-1* may function maternally as weaker *sys-1* mutants are sterile (Miskowski et al., 2001). As we have shown that positive POP-1 activity is detectable only when endoderm specification has been partially compromised, a role for SYS-1 in endoderm specification may yet be revealed. Therefore, while the mechanism remains to be elucidated, our findings nonetheless implicate a previously unrecognized mechanism for embryonic POP-1 activation in the *C. elegans* embryo.

A mesectodermal regulatory factor can promote endoderm development

Our experiments also establish an unexpected role for the Caudal-like transcription factor PAL-1 in endoderm development. PAL-1 is normally required for specification of mesectoderm in the P_2 lineage, and *pal-1* mutants show no discernible endoderm phenotype (Hunter and Kenyon, 1996). Why, then, is PAL-1 required for the residual endoderm made in *skn-1*; *pop-1* mutants (see Table 1)? One possible explanation for this finding is that PAL-1 may bind to the *end* genes as a means of keeping them repressed in the C lineage; in so doing, it would ensure that SKN-1, which is also present in the C lineage, does not activate the *end* genes inappropriately. Such a mechanism would be part of the system used to ensure exclusivity of cell fate assignments to early blastomeres (e.g., Cowan and McIntosh, 1985): a cell in which PAL-1 is active and promotes mesectoderm development is strongly repressed for other pathways of differentiation, including endoderm, thereby ensuring adoption of an exclusive cell fate. GSK-3β, which prevents SKN-1 from activating the *med*s in the C lineage (Maduro et al., 2001), thus allowing PAL-1-dependent mesectoderm development, may poise this PAL-1/*end* interaction toward repression. In the E cell, in which GSK-3β is apparently not active in blocking the SKN-1→MED pathway, PAL-1 may lose its *end*-repressive activity, allowing PAL-1 bound to the *end* promoters to *activate* rather than repress endoderm development.

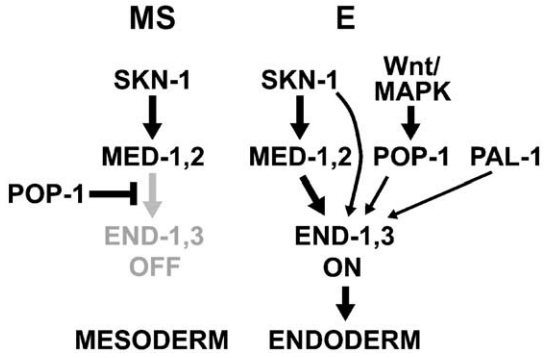


Fig. 8. Revised model for *C. elegans* endoderm specification. In the absence of a Wnt signal, an EMS daughter will produce an MS like fate since unsignaled POP-1 blocks activation of the endoderm-specifying genes *end-1,3* by the SKN-1→MED cascade. The E fate is specified when an overlapping Wnt/MAPK signal modifies POP-1, resulting in its conversion into an activator of endoderm. The combination of SKN-1→MED, PAL-1, and Wnt/MAPK-modified POP-1 results in activation of *end-1,3* and specification of an endodermal fate.

A revised model for C. elegans endoderm specification

Our results suggest a revised view of endoderm specification (Fig. 8), in which POP-1, in response to Wnt signaling, is converted from a repressor to an activator of the endoderm-promoting *end* genes. This POP-1-dependent activating function is not essential for endoderm formation since the comparatively stronger contribution by the SKN-1→MED cascade is unaltered by Wnt signaling in the E cell. In parallel with these factors, Caudal/PAL-1 also contributes to endoderm specification. We propose that the use of POP-1 as both an activator and repressor may occur recursively throughout embryonic development to establish differences between daughter cells arising from anterior–posterior asymmetric cell division.

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