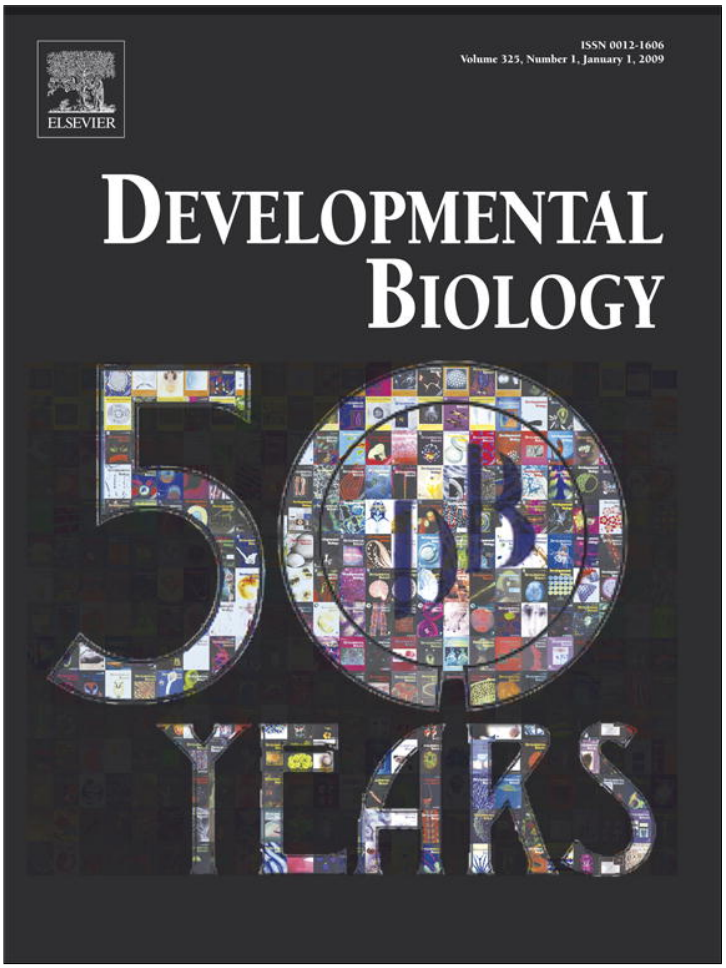

Developmental Biology 325 (2009) 296–306

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Evolution of Developmental Control Mechanisms

Knockdown of SKN-1 and the Wnt effector TCF/POP-1 reveals differences in endomesoderm specification in *C. briggsae* as compared with *C. elegans*

Katy Tan-Hui Lin [a,b], Gina Broitman-Maduro [a], Wendy W.K. Hung [b], Serena Cervantes [b], Morris F. Maduro [a,*]

[a] Department of Biology, University of California, 3380 Spieth Hall, Riverside, Riverside, CA 92521, USA
[b] Graduate Program in Cell, Molecular and Developmental Biology, UC Riverside, USA

ARTICLE INFO

Article history:
Received for publication 15 July 2008
Revised 29 September 2008
Accepted 1 October 2008
Available online 19 October 2008

Keywords:
Endomesoderm
C. elegans
C. briggsae
POP-1
SKN-1
Evolution
Gene networks

ABSTRACT

In the nematode, *C. elegans*, the bZIP/homeodomain transcription factor SKN-1 and the Wnt effector TCF/POP-1 are central to the maternal specification of the endomesoderm prior to gastrulation. The 8-cell stage blastomere MS is primarily a mesodermal precursor, giving rise to cells of the pharynx and body muscle among others, while its sister E clonally generates the entire endoderm (gut). In *C. elegans*, loss of SKN-1 results in the absence of MS-derived tissues all of the time, and loss of gut most of the time, while loss of POP-1 results in a mis-specification of MS as an E-like cell, resulting in ectopic gut. We show that in *C. briggsae*, RNAi of *skn-1* results in a stronger E defect but no apparent MS defect, while RNAi of *pop-1* results in loss of gut and an apparent E to MS transformation, the opposite of the *pop-1* knockdown phenotype seen in *C. elegans*. The difference in *pop-1(−)* phenotypes correlates with changes in how the endogenous endoderm-specifying *end* genes are regulated by POP-1 in the two species. Our results suggest that integration of Wnt-dependent and Wnt-independent cell fate specification pathways within the *Caenorhabditis* genus can occur in different ways.

Introduction

Appropriate spatiotemporal activation of cell type-specific gene networks in metazoans must be robust and yet flexible over evolutionary time (Davidson and Erwin, 2006). During speciation, genetic drift and natural selection may lead to changes in cell fate specification mechanisms, even if the phenotype does not change overall (Felix and Barriere, 2005). The related nematodes *C. elegans* and *C. briggsae* are emerging as good comparative systems for probing molecular differences in similar developmental pathways (Haag and Pilgrim, 2005). Recent estimates for the divergence time of the two species range from as low as 4–30 MYA to as high as 80–110 MYA (Cutter, 2008; Stein et al., 2003). Similar experimental tools, including a sequenced genome and ability to perform RNAi, are available in both species (Baird and Chamberlin, 2006). In this study, we examine the maternal specification of two embryonic precursors, MS and E.

The embryonic development of *C. elegans* and *C. briggsae* is remarkably similar (Zhao et al., 2008). In both, the zygote undergoes a series of asymmetric cell divisions that establish the so-called 'founder cells'. The MS and E blastomeres are the two daughters of EMS, the ventralmost cell at the 4-cell stage (Fig. 1A). While descendants of E will generate the 20 cells of the larval endoderm (gut), MS gives rise to four times as many cells that are largely mesodermal, including body muscle cells and pharynx cells found primarily in the posterior half (Priess et al., 1987; Sulston et al., 1983). The remaining cells in the pharynx are descended from ABa; specification of these precursors requires a GLP-1/Notch-mediated cell induction from MS to descendants of ABa (Priess et al., 1987). In both *C. elegans* and *C. briggsae*, loss of *glp-1* results in absence of anterior pharynx, suggesting that the molecular basis for this induction event is conserved (Rudel and Kimble, 2001).

Correct specification of MS and E in *C. elegans* requires the combinatorial activity of two maternal pathways (Fig. 1B): The SKN-1 pathway assigns the fate of EMS as endomesodermal, while the Wnt/MAPK pathway, through the TCF-like regulator POP-1, functions primarily to make E and MS different (Maduro and Rothman, 2002). SKN-1 is a bZIP/homeodomain transcription factor that specifies EMS fate (Bowerman et al., 1992). In the absence of *skn-1*, MS-derived tissues are absent all the time, and gut is absent in ~70% of embryos (Bowerman et al., 1992). In *skn-1* mutants, MS and E adopt the fate of their lineal cousin C, which makes body muscle and hypodermis as a result of cryptic activity of the Caudal-like homeoprotein PAL-1 (Bowerman et al., 1992; Hunter and Kenyon, 1996). Loss of *skn-1* also results in the absence of ABa-derived pharynx, so *skn-1* mutants lack pharynx entirely (Bowerman et al., 1992).

The second maternal pathway in endomesoderm acts to make MS and E different. EMS becomes polarized through a cell–cell interaction with its sister cell, P_2 (Goldstein, 1992). In the absence of this interaction, EMS divides symmetrically to produce two MS-like cells, a phenotype which can also be obtained by mutations in the Wnt/MAPK

* Corresponding author. Fax: +1 951 827 4286.
E-mail address: mmaduro@citrus.ucr.edu (M.F. Maduro).

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doi:10.1016/j.ydbio.2008.10.001

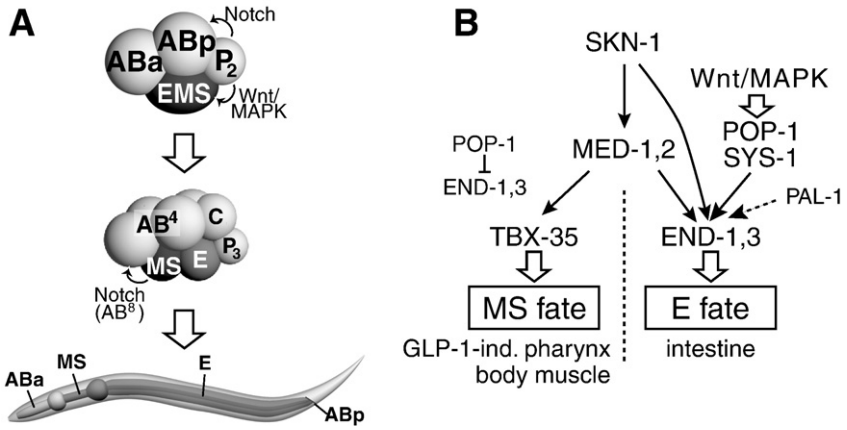

Fig. 1. The early *C. elegans* embryo, L1 larva and simplified endomesoderm specification pathway. (A) Diagrams of 4-cell and 8-cell embryos and L1 larva, showing blastomere names, Notch and Wnt/MAPK pathway cell–cell interactions, and lineal origin of portions of the digestive tract. Here and in other single embryo images, anterior is shown to the left and dorsal is up. A *C. elegans* embryo and larva are approximately 50 μm and 150 μm long, respectively. (B) Abbreviated *C. elegans* endomesoderm network (Huang et al., 2007; Maduro, 2006; Maduro and Rothman, 2002; Phillips et al., 2007). Solid, lined arrows, and the repression of *end-1,3* by POP-1, denote direct regulatory interactions. GLP-1-ind., GLP-1 independent.

pathway (Goldstein, 1992; Rocheleau et al., 1997; Thorpe et al., 1997). The nuclear effector of Wnt/MAPK signaling is the TCF homolog POP-1, which functions as a repressor in the absence of Wnt signaling, and as an activator following Wnt-dependent association with the divergent β-catenin SYS-1 (Calvo et al., 2001; Kidd et al., 2005; Lin et al., 1998; Phillips et al., 2007; Shetty et al., 2005). POP-1 forms part of a binary switching mechanism that is used multiple times throughout development to generate asymmetric fates from sister cells (Kaletta et al., 1997; Lin et al., 1998; Mizumoto and Sawa, 2007). The primary role of POP-1 in MS/E specification is to repress endoderm fate in MS, revealed by the phenotype of loss of maternal *pop-1* function as a transformation of MS to an E-like cell (Lin et al., 1995). More recently, however, it has become clear that Wnt/MAPK-signaled POP-1 makes a weak, but significant contribution to endoderm specification in E itself, as *pop-1(RNAi)* is able to significantly enhance the endoderm phenotype of *skn-1* mutant embryos (Maduro et al., 2005b; Phillips et al., 2007; Shetty et al., 2005). In the current model, Wnt/MAPK signaling lowers the nuclear concentrations of POP-1 and raises the nuclear concentrations of SYS-1, allowing the bipartite POP-1/SYS-1 factor to activate, rather than repress, endoderm specification (Huang et al., 2007; Phillips et al., 2007).

Both the SKN-1 pathway and the Wnt/MAPK pathway (via POP-1) converge on the lineage-specific activation of zygotic regulators. Within EMS, the *med-1,2* divergent GATA factor genes are directly activated by SKN-1 (Maduro et al., 2001). Loss of *med-1,2* together results in a penetrant loss of MS-derived tissues and a partial loss of endoderm (Goszczynski and McGhee, 2005; Maduro et al., 2007, 2001). In E, SKN-1, MED-1,2 and POP-1 activate expression of the GATA factor genes *end-1* and *end-3* (Maduro et al., 2007, 2005b, 2001; Shetty et al., 2005). In MS, POP-1 directly represses *end-1,3*, while MED-1,2 directly activate *tbx-35*, contributing to MS specification (Broitman-Maduro et al., 2006; Maduro et al., 2007, 2002). There is additional evidence that *med-1,2* are activated maternally, that *end-3* contributes to *end-1* activation, and that PAL-1 also contributes to endoderm, further showing that the endoderm specification pathway is not strictly linear, but contains multiple, parallel inputs (Maduro et al., 2007, 2005b; Shetty et al., 2005).

Candidate orthologs of all of the known *C. elegans* (*Ce*) endomesoderm regulators exist in the *C. briggsae* (*Cb*) genome (WormBase, release WS187). At least two *Cb-med* orthologs can complement loss of *Ce-med-1,2* when expressed as transgenes, and these also demonstrate endogenous activation in the early EMS lineage (Maduro et al., 2007). Similarly, the function of the *Cb-end* orthologs appears to be conserved in *C. briggsae* (Maduro et al., 2005a). In this study we examine the contribution of SKN-1 and Wnt/POP-1 to endoderm specification in *C. briggsae*, and find unexpected differences in phenotype as compared with *C. elegans*. First, RNAi of *Cb-skn-1* results in embryos that have a stronger endoderm phenotype, and these embryos continue to make MS-derived pharynx. Second, RNAi of *Cb-pop-1* results in an E to MS transformation in most embryos, and persistence of MS-derived pharynx, contrary to *Ce-pop-1* depletion. Knockdown of *Cb-pop-1* and *Cb-skn-1* simultaneously results in a synergistic absence of MS-derived pharynx. While activation of the *C. elegans end* genes occurs in both the MS and E lineages in *Ce-pop-1(RNAi)*, *Cb-end* expression is abolished in *Cb-pop-1(RNAi)*, showing that the different phenotypes can be attributed to differences in POP-1-dependent regulation of the *end* genes. Knockdown of *C. remanei pop-1* results in an ectopic gut phenotype similar to *C. elegans*, suggesting that the differences with *C. briggsae* have occurred on a short timescale. Our results show that molecular events in maternal blastomere specification pathways in *Caenorhabditis* can be different despite a similar developmental output.

Materials and methods

Strains and genetics

Wild-type strains were as follows: *C. elegans*, N2; *C. briggsae*, AF16; *C. remanei*, PB4641; *C. sp. 9*, JU1325; *C. brenneri*, CB5161 and PB2801; *C. japonica*, DF5081. The following mutations were used: *C. elegans*: *med-1(ok804) X, pop-1(zu189) I, dpy-5(e61) I, hT1(I;V), med-2 (cx9744) III, unc-119(ed4) III, him-8(e1489) IV, him-5(e1490) V, end-1 (ok558) V, end-3(ok1448) V*. *C. briggsae*: *Cb-unc-4(sy5341) II, Cb-dpy (ir12)*. The following transgene strains were used: *C. elegans*: *ruIs37 III* [*Ce-myo-2*::GFP], *cuIs1 V* [*Ce-ceh-22*::GFP], *irIs81* [*Cb-end-3.2*::GFP]; *C. briggsae*: *qtIs21* [*Ce*-SID-2::GFP], *irIs7* [*Ce-ceh-22*::YFP], *irIs44* [*Ce-elt-2*::GFP].

C. briggsae transgenics were identified using rescue of *Cb-unc-4 (sy5341)* with plasmid pNC4-21 (gifts from Takao Inoue and Paul Sternberg). Integrants were made from stable lines following 3500 rad of gamma irradiation from a ^{137}Cs source and screening of F_3 animals for 100% transmission. Injections for DNA and dsRNA delivery were performed according to standard protocols (Mello et al., 1991). Experimental manipulations were performed at 23 °C with incubation of strains at 20 °C.

POP-1 and SKN-1 orthologs

C. briggsae orthologs of *skn-1* (CBG19887), *pop-1* (CBG04236) and *unc-22* (CBG06205) were identified by WormBase (release

WS187) and confirmed by independent TBLASTN searches of the *C. briggsae* genome. Sequencing of cloned RT-PCR products revealed minor splicing differences with the original gene models for CBG19887 and CBG04236 and the corrections have been reported to WormBase. In *C. elegans*, *skn-1* encodes multiple isoforms, of which we have confirmed the structure of the *C. briggsae* ortholog of only the longest of these (T19E7.2a). Orthologs for other genes were identified by BLAST searches or through WormBase; further information on these is available on request. A partial *C. sp. 9 pop-1* cDNA was amplified by RT-PCR using primers that work in *C. briggsae*. The sequence of this fragment was found to be nearly identical to the corresponding portions of the *Cb-pop-1* cDNA (data not shown).

Plasmids and cloning

A *ceh-22*::YFP reporter (pMM526) was made by inserting an NcoI–ApaI fragment of pPD132.112 (*myo-2*::YFP, a gift from David Miller) into the larger portion of pCW2.1 (*ceh-22*::GFP, a gift from Peter Okkema) digested with the same enzymes. pMM526 and pCW2.1 thus carry the same *Ce-ceh-22* promoter and *Ce-unc-54* 3′ UTR regions. The *med-1*::GFP::*Cb*-POP-1::*med-1*_3′UTR fusion pGB291 was made from a *Cb-pop-1* cDNA using a strategy similar to the construction of the corresponding *Ce*-POP-1 fusion (Maduro et al., 2002). Further cloning details and oligonucleotide sequences are available on request.

Rescue of pop-1(zu189) mutants

To assess ability of *med-1*::GFP::CbPOP-1 (mgCbPOP-1) to rescue maternal *pop-1* mutants, *unc-119(ed4)* males rescued with an array carrying an *unc-119(+)* plasmid (pDP#MM016B) and pGB291, or wild-type N2 males as controls, were mated with *pop-1(zu189) dpy-5(e61); him-5(e1490); ruIs37* progeny of *pop-1 dpy-5/hT1 I*; *him-5/him-5 hT1 V; ruIs37* mothers, similar to a previously described assay (Maduro et al., 2002). Ratios of GFP::CbPOP-1 in nuclei were calculated from average pixel intensities of regions of interest (Adobe Photoshop 7.0).

RNA interference

Unless otherwise indicated, RNAi by dsRNA injection was used for experiments on *C. remanei, C. brenneri, C. sp. 9* and *C. japonica*, and for experiments on *C. briggsae* and *C. elegans* in which multiple genes were targeted. dsRNA was synthesized and injected as described (Maduro et al., 2001). For feeding-based RNAi experiments, hermaphrodites were grown for at least 36 h on *E. coli* strain HT115 engineered to express specific dsRNAs using plasmid pPD129.36 (Timmons and Fire, 1998). The *C. briggsae* HC189 strain carries an integrated *C. elegans* SID-2::GFP transgene (*qtIs21*) that enables RNAi to be achieved by ingestion (Winston et al., 2007). To control for RNAi specificity, non-overlapping fragments of *Cb-pop-1* and *Cb-skn-1* were tested and found to produce similar results.

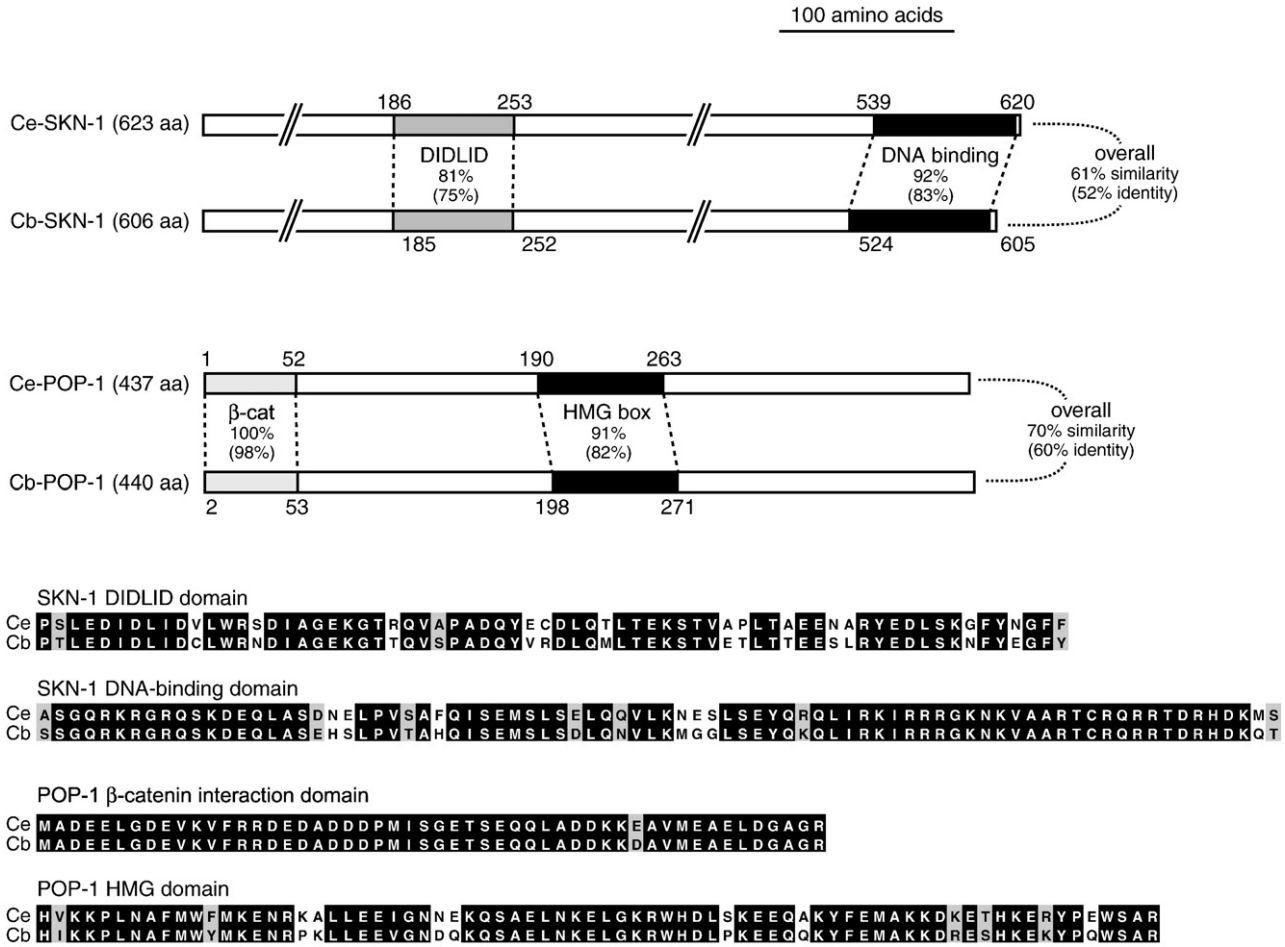

Fig. 2. Orthologs of SKN-1 and POP-1 in *C. briggsae*. Shown are schematic alignments of the predicted coding regions, indicating conservation among regions important for binding to DNA (POP-1 HMG box and SKN-1 DNA binding domain) and for transcriptional activation (POP-1 β-catenin interaction domain and SKN-1 DIDLID region) (Blackwell et al., 1994; Grosschedl et al., 1994; Kidd et al., 2005; Walker et al., 2000). Below are amino acid alignments of the indicated regions showing identities (white text on black background) and similarities (black text on gray background) identified by a positive score in the BLOSUM62 substitution matrix (Henikoff and Henikoff, 1992).

In addition to RT-PCR (Fig. 3I), we confirmed depletion of *Cb-skn-1* transcripts following dsRNA injection of ~100 AF16 animals with *Cb-skn-1* dsRNA. One day after injection, injected animals and untreated *Cb-dpy(ir12)* adults were stained on the same slide by in situ hybridization. Consistent with specific depletion of endogenous *Cb-skn-1* mRNA, germline signal with a *Cb-skn-1* antisense probe was seen in only 1/19 animals injected *vs.* 17/19 uninjected animals, but comparable for a *Cb-pop-1* probe between *skn-1*-injected (12/14) and uninjected (16/16).

We attempted species-specific *pop-1(RNAi)* in *C. brenneri* and *C. japonica* but could not make any reliable conclusions due to a high level (35–50%) of embryonic lethality. Nonetheless, *Cj-pop-1(RNAi)* appeared to cause a reduction in the amount of embryos producing endoderm, from 68% (n=60, uninjected) to 32% (n=166, *Cj-pop-1* dsRNA-injected).

In situ hybridization

Whole-mount in situ hybridization was performed using species-specific probes as described (Broitman-Maduro et al., 2006). To facilitate isolation of early embryos from gravid *C. briggsae* hermaphrodites, we used a weak egg-laying defective mutant, *Cb-dpy(ir12)*. Strain MS1042 [*dpy(ir12); qtIs21*] was used for feeding-based RNAi prior to in situ hybridization. For in situ hybridization on *Cb-skn-1 (RNAi); pop-1(RNAi)* embryos, we grew MS1042 animals on *E. coli* expressing a dsRNA fusion of portions of the *Cb-skn-1* and *Cb-pop-1* cDNAs.

Microscopy, laser ablations and imaging

Embryos were mounted on agar pads or directly on slides and imaged on an Olympus BX-51 Epifluorescence Microscope equipped with a Canon 350D camera. For scoring of *ceh-22*::YFP in embryos that also expressed intestinal SID-2::GFP, signal was confirmed to be *ceh-22*::YFP by looking for lack of signal in a CFP filter set (Miller et al., 1999). A lack of strict additivity in numbers of *ceh-22*-expressing cells counted, both within and between species, may result from a combination of mosaicism of the *ceh-22* reporter plus the difficulty of resolving individual cells that are very close together; alternatively, there may be subtle differences in the expression of the *ceh-22* reporter in *C. briggsae* or variability in the stage of embryonic arrest in RNAi embryos. Images were processed in Adobe Photoshop. For fluorescence images, images of multiple focal planes were merged. Cell ablations were performed using a Photonic Microsystems Pulsed Laser on a Zeiss Axioskop2 at the Core Instrument Facility at UC Riverside.

Results

Identification of C. briggsae orthologs of skn-1 and pop-1

Orthologs of *skn-1* and *pop-1* can be identified in the *C. briggsae* genome as CBG19887 and CBG04236, respectively (release WS188). The WormBase synteny browser shows that the genes B0547.1(*Ce*)/CBG19889(*Cb*) and T19E7.3(*Ce*)/CBG19886(*Cb*) flank *skn-1* in both

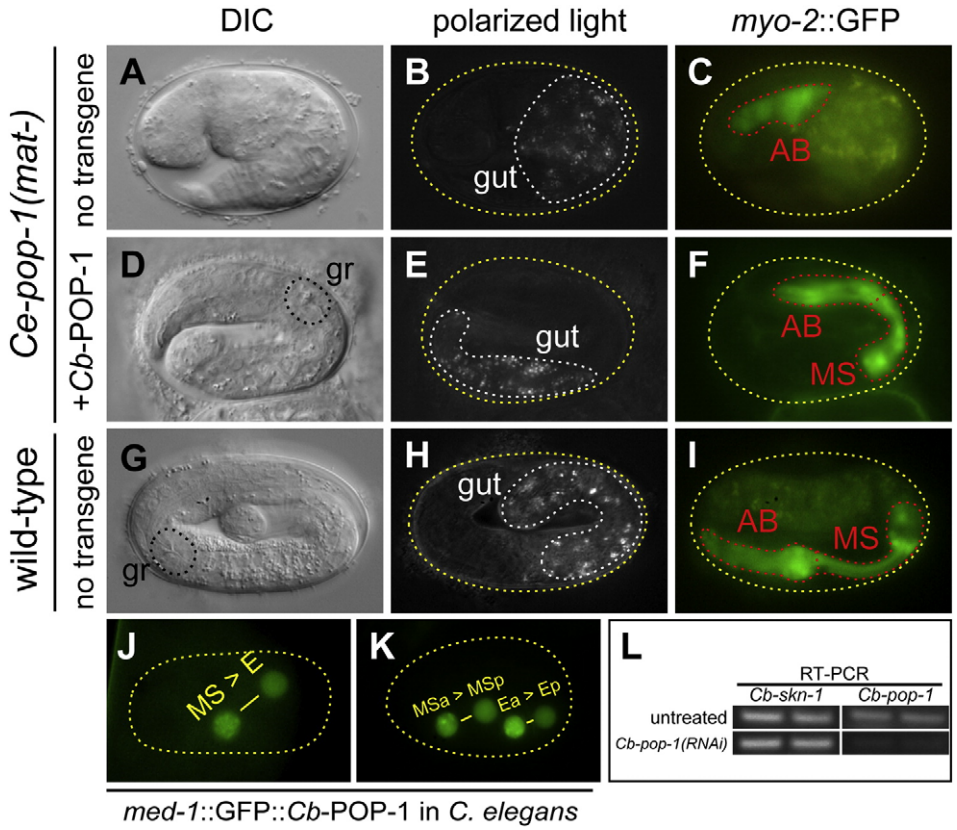

Fig. 3. Similarity of transgenic *Cb*-POP-1 to *Ce*-POP-1 when expressed in *C. elegans* and evidence for specificity of RNAi in *C. briggsae*. (A–C), arrested *Ce-pop-1(−)* embryo showing 2-fold elongation, ectopic gut and small AB-derived pharynx. (D–F), embryo lacking maternal *pop-1* (*mat-*) and carrying transgenic *Ce-med-1*-driven *Cb*-POP-1 fused to GFP (mgCbPOP-1). The embryo has elongated to ~2.5-fold, contains a normal amount of gut and exhibits restored MS-derived pharynx as evidenced by the grinder (gr) and increased domain of *myo-2*::GFP expression. (G–I), wild-type *C. elegans* embryo elongated to ~3.5-fold, exhibiting normal gut and pharynx. (J–K) Expression of mgCbPOP-1 in *C. elegans* in the early MS and E lineages shows puncta and asymmetric signal (anterior nuclei>posterior nuclei) similar to mgCePOP-1 (Maduro et al., 2002). Measurement of pixel intensities for images of 10 A–P sister nuclei in the early EMS lineage, from multiple embryos, produced an average ratio of 1.5±0.1 (anterior:posterior), slightly lower than the ratio of ~1.8 previously reported for GFP::*Ce*-POP-1 (Maduro et al., 2002). (L) RT-PCR of *Cb-pop-1* and *Cb-skn-1* in *C. briggsae* HC189 grown on *E. coli* OP50 (untreated) or *E. coli* HT115 expressing *Cb-pop-1* dsRNA. The regions of the *Cb-pop-1* and *Cb-skn-1* cDNAs that were amplified do not overlap with the fragments that were used for RNA interference.

species, although conservation of synteny was not apparent with *pop-1*. We validated the gene models for *Cb-pop-1* and *Cb-skn-1* by sequencing of RT-PCR products. Between species, the orthologs show 75%–98% identity across functional domains associated with protein–DNA interaction and transcriptional activation function, with overall sequence identities of 52% (SKN-1) and 60% (POP-1) (Fig. 2). Comparable degrees of protein sequence similarity are seen between the functionally-conserved *C. elegans* and *C. briggsae* END-1 parologs, which exhibit 79% identity within the DNA-binding domain and 51% identity overall (Maduro et al., 2005a). Consistent with maternal function, *Cb-skn-1* and *Cb-pop-1* are expressed in the germline as assessed by in situ hybridization (data not shown).

As conservation of the *pop-1* chromosomal context was not apparent between the two species, we tested for functional conservation of POP-1 in two ways. First, we expressed a GFP::*Cb*-POP-1 fusion in *C. elegans* under the control of the *Ce-med-1* promoter (a construct abbreviated mgCbPOP-1), which drives expression in the early EMS lineage (Maduro et al., 2002). As with endogenous POP-1 (Lin et al., 1998), anterior cells in the E and MS lineages showed higher nuclear levels than their posterior sisters (Figs. 3J, K). Furthermore, puncta were visible in anterior nuclei, a property of GFP::*Ce*-POP-1 fusions (Maduro et al., 2002; Siegfried et al., 2004). Hence, when expressed as GFP fusions, *C. elegans* and *C. briggsae* POP-1 appear to undergo similar post-translational processing in *C. elegans*.

We next tested the ability of the *C. briggsae pop-1* transgene to complement maternal loss of *C. elegans pop-1* function. The maternal-effect *zu189* allele of *pop-1* specifically compromises maternal *pop-1* activity, resulting in a transformation of MS to an E-like cell (Lin et al., 1995). Homozygous *pop-1(zu189)* progeny of heterozygous mothers were mated with males carrying the mgCbPOP-1 fusion, an assay previously used to test rescue with a similar *Ce*-POP-1 transgene (Maduro et al., 2002). Of 28 embryos scored, 12 (~40%) showed the presence of normal gut and pharynx as scored by Nomarski optics and expression of a pharyngeal myosin reporter (*myo-2*::GFP) (Figs. 3D–F). In contrast, control matings with wild-type males failed to result in rescue of the posterior pharynx or ectopic endoderm defects in the progeny (n=40). The proportion of rescued animals is similar to results obtained with a *C. elegans* POP-1 transgene (Maduro et al., 2002), and is likely to be an underestimate given that the mgCbPOP-1 transgene is transmitted as an extrachromosomal array. We conclude that zygotic expression of *Cb-pop-1* in *C. elegans* can functionally complement maternal loss of *Ce-pop-1* function.

Cb-skn-1(RNAi) results in a loss of endoderm and partial loss of pharynx

To assess the contributions of *Cb*-SKN-1 and *Cb*-POP-1 to MS and E specification in *C. briggsae*, we used RNA interference (RNAi) (Fire et al., 1998). RNAi of *Cb-glp-1*, *Cb-pal-1* or the *C. briggsae* orthologs of the

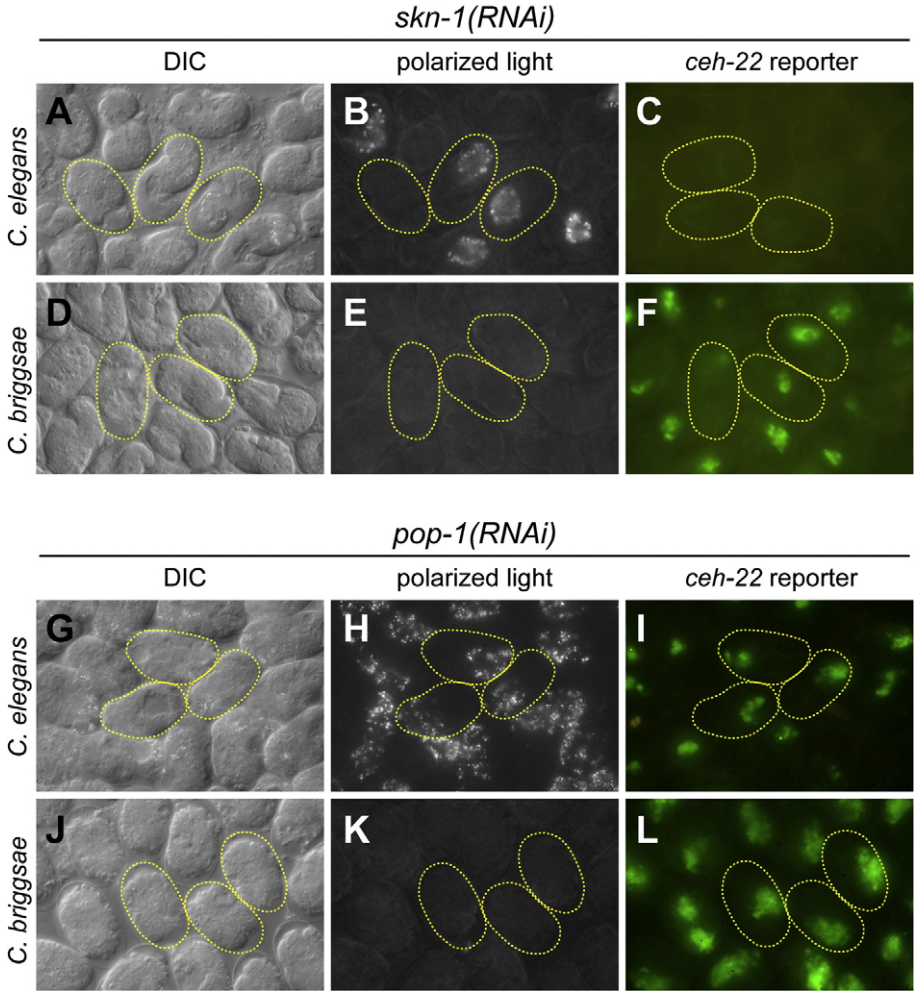

Fig. 4. Phenotypes of *Cb-skn-1(RNAi)* and *Cb-pop-1(RNAi)*. In all rows except for panel (C), the same three embryos were outlined to facilitate a comparative assessment of the phenotype. In panels A, B, the DIC and endoderm analysis was done with N2, as the *ceh-22*::GFP transgene *culs1* causes a gut defect enhancement with *skn-1(RNAi)*. This results from an event associated with integration of the *ceh-22* reporter, as differences in endoderm defects were not observed in *C. briggsae* RNAi experiments with or without *ceh-22*::YFP. Absence of pharynx in *Ce-skn-1* mutant embryos (C) has been independently confirmed by antibody staining of pharynx muscle (Bowerman et al., 1992). The reporter in *C. elegans* is an integrated *ceh-22*::GFP reporter, and in *C. briggsae* an integrated *ceh-22*::YFP reporter. In this figure and Fig. 6, a YFP filter set was used to detect both reporters (Miller et al., 1999).

Table 1
Production of gut and pharynx in *Caenorhabditis* embryos

Genotype[a]	% (total) of embryos with:	
	Gut[b]	Pharynx[c]
C. elegans (wild-type)	100% (>200)	100% (>200)
C. briggsae (wild-type)	100% (>200)	100% (>200)
Cb-pal-1(RNAi) inj	100% (84)	100% (85)
Cb-lit-1(RNAi) inj	3% (188)	nd[d]
Cb-lit-1(RNAi) feeding	3% (249)	100% (249)
Cb-wrm-1(RNAi) inj	2% (178)	nd
Cb-wrm-1(RNAi) feeding	0% (105)	100% (105)
Cb-sys-1(RNAi) inj	50% (124)	nd
Cb-hmp-2(RNAi) inj	100% (235)	nd
Cb-bar-1(RNAi) inj	93% (141)	nd
Cb-mom-2(RNAi) inj	18% (228)	nd
Cb-cwn-1(RNAi) inj	94% (133)	nd
Cb-cwn-2(RNAi) inj	94% (145)	nd
Cb-lin-44(RNAi) inj	98% (138)	nd
Cb-egl-20(RNAi) inj	99% (158)	nd
Cb-cfz-2(RNAi) inj	86% (149)	nd
Cb-mig-1(RNAi) inj	94% (256)	nd
Cb-mom-5(RNAi) inj	90% (225)	nd
Cb-mom-2(RNAi); mom-5(RNAi) inj	18% (229)	nd
Cb-apr-1(RNAi) inj	91% (242)	nd
Cb-mom-2(RNAi); apr-1(RNAi) inj	9% (194)	nd
Ce-skn-1(RNAi) feeding	8% (731)[e]	0% (165)
Cb-skn-1(RNAi) inj	9% (227)	100% (105)
Cb-skn-1(RNAi) feeding	4% (213)	97% (166)
Ce-pop-1(RNAi) feeding	96% (437)[e]	100% (>100)
Cb-pop-1(RNAi) inj	17% (159)[f]	100% (199)
Cb-pop-1(RNAi) feeding	4% (127)	100% (127)
Ce-glp-1(RNAi) inj	100% (197)	100% (197)
Cb-glp-1(RNAi) inj	100% (79)	100% (33)
Ce-glp-1(RNAi); pop-1(RNAi)	100% (20)	7% (43)
Cb-glp-1(RNAi); pop-1(RNAi)	9% (67)	100% (67)
Ce-skn-1(RNAi); glp-1(RNAi)	nd	0% (134)
Cb-skn-1(RNAi); glp-1(RNAi)	nd	100% (36)
Ce-skn-1(RNAi); pop-1(RNAi)	11% (1245)[e]	0% (126)
Cb-skn-1(RNAi); pop-1(RNAi)	0% (187)	15% (97)[g]
C. remanei (wild-type)	97% (195)	nd
C. remanei E ablated	0% (19)	nd
Cr-pop-1(RNAi)	93% (180)	nd
Cr-pop-1(RNAi) E ablated	88% (17)	nd

[a] RNAi experiments in *C. remanei*, or those in *C. elegans* or *C. briggsae* with multiple RNAi targets, were performed by injection.
[b] Gut was scored by presence of birefringent gut granules. An *elt-2*::GFP reporter or the intestine-specific *Ce*-SID-2::GFP transgene present in the strains corroborated the identification of gut(+) embryos by granules.
[c] Pharynx was scored by expression of an integrated *ceh-22* reporter.
[d] nd, not determined.
[e] From Maduro et al., 2005b. For *Ce-skn-1; pop-1*, another group reported higher degrees of synergy, approaching 0% of embryos making gut (Phillips et al., 2007).
[f] Among embryos with endoderm, none had more than 20 *elt-2*::GFP-expressing cells.
[g] Among embryos with pharynx, the average number of cells was 3.1±0.6. inj, injection of dsRNA; feeding, growth on *E. coli* HT115 expressing dsRNA.

C. elegans end genes results in phenotypes similar to the corresponding *C. elegans* mutants (Maduro et al., 2005a; Rudel and Kimble, 2001; Winston et al., 2007), suggesting that RNAi of *Cb-skn-1* and *Cb-pop-1* should work similarly in *C. briggsae*. We used both injection of dsRNA and feeding of *E. coli* expressing dsRNA into *Ce*-SID-2::GFP transgenic *C. briggsae* animals, and performed controls to validate specific and robust knockdown of the endogenous transcripts (Fig. 3L and Materials and methods).

Cb-skn-1(RNAi) produced developmentally arrested embryos with a similar overall appearance as in *C. elegans skn-1* mutant and *Ce-skn-1(RNAi)* embryos (Figs. 4A and D) (Bowerman et al., 1992). Approximately 70% of embryos (n=43) contained internal cavities that appeared to be lined with hypodermal cells, similar to those found in *Ce-skn-1* mutants, and which are attributed in *C. elegans* to ectopic hypodermal tissue (Bowerman et al., 1992). We found, however, that production of pharynx and endoderm was different in two ways. First, whereas *Ce-skn-1(RNAi)* embryos failed to make endoderm ~73% of the time, *Cb-skn-1(RNAi)* embryos lacked gut 91–96% of the time, as scored by accumulation of birefringent gut granules (Table 1). The lack of endoderm and appearance of internal cavities together suggest that E adopts a C-like fate in the *Cb-skn-1(RNAi)* embryos (Bowerman et al., 1992).

Second, we observed the continued presence of pharynx in all *Cb-skn-1(RNAi)* embryos as evidenced by appearance of a small clump of pharyngeal cells surrounded by a basement membrane (not shown), and which was confirmed by expression of a *Ce-ceh-22*::YFP reporter and accumulation of endogenous pharyngeal myosin *Cb-myo-2* mRNA (Table 1; Figs. 4F, 5B). To determine whether this pharynx tissue is derived from ABa or MS, we first examined the requirement for GLP-1 function. RNAi of *glp-1* in *C. briggsae* produces a similar loss of anterior pharynx as seen in *C. elegans glp-1* mutants (Figs. 6B, G) (Rudel and Kimble, 2001). We found that *Cb-skn-1(RNAi); glp-1(RNAi)* embryos contained similar amounts of pharynx as *Cb-glp-1(RNAi)* embryos (Figs. 6G, H), suggesting that the pharynx present in *Cb-skn-1(RNAi)* embryos is MS-derived. We next used laser ablation to confirm the origin of pharynx tissue in these embryos (Table 2). While ABa-ablated or MS-ablated *Cb-unc-22(RNAi)* embryos and ABa-ablated *Cb-skn-1(RNAi)* embryos continued to make pharynx muscle, ablation of MS in *Cb-skn-1(RNAi)* resulted in almost no pharynx (n=10; one embryo made a single *ceh-22*::YFP-expressing cell). Hence, in addition to a slightly stronger endoderm defect, *Cb-skn-1(RNAi)* embryos specifically lack ABa-derived pharynx cells but continue to make pharynx from MS. We note that this does not mean that MS specification occurs without input by *Cb*-SKN-1; rather, there appears to be at least one parallel input by *Cb*-POP-1, as discussed below.

Cb-pop-1(RNAi) results in a loss of endoderm and excess pharynx

In *C. elegans* MS and E specification, the predominant role of POP-1 is the repression of endoderm fate in MS, as loss of maternal *pop-1* function results in a highly penetrant transformation of MS to an E-like precursor, resulting in absence of MS-derived pharynx and production of excess gut (Lin et al., 1995). As in *C. elegans*, *Cb-pop-1 (RNAi)* resulted in arrested one-fold embryos (Figs. 4G, J). However,

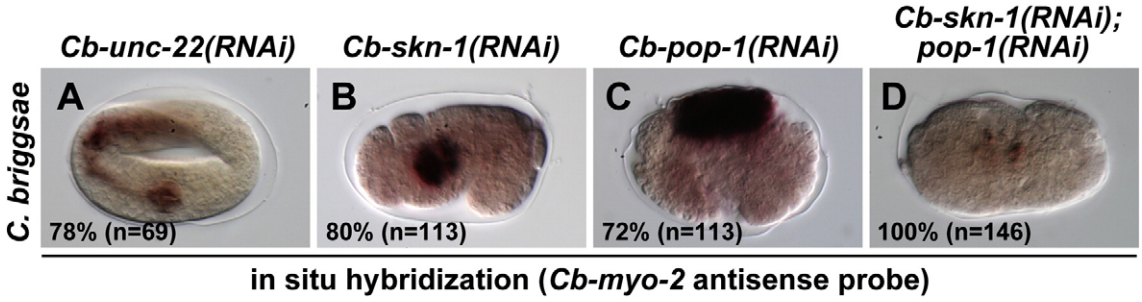

Fig. 5. Endogenous *Cb-myo-2* expression in wild-type and RNAi-knockdown *C. briggsae* embryos. In panels A–C, the percentages (out of total) represent the proportion of embryos demonstrating the staining shown, with the remaining embryos showing very little or no signal. In panel D, all embryos demonstrated either very little signal as shown here, or no staining.

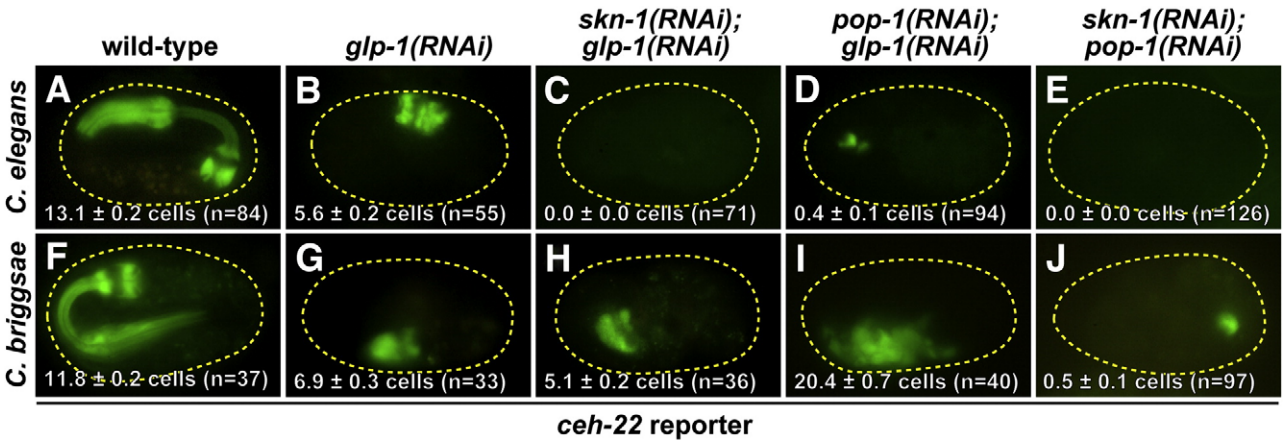

Fig. 6. Appearance of pharynx muscle (*ceh-22* reporter) in wild-type and RNAi-knockdown *C. elegans* and *C. briggsae* embryos. Mean numbers of cells counted (±SEM) are shown on the figures. In the top row (*C. elegans*), an integrated *ceh-22*::GFP reporter is used, and in the bottom row (*C. briggsae*), it is *ceh-22*::YFP.

the vast majority of these (83–96%) lacked endoderm, and those that contained endoderm did not have an apparent excess (Table 1, Fig. 4K and data not shown). In greater than 80% of *Cb-pop-1(RNAi)* embryos, we observed an abnormally large pharynx-like organ that expressed the *ceh-22*::YFP marker (Fig. 4L), and which corresponded in size and shape to accumulation of endogenous *Cb-myo-2* mRNA (Fig. 5C). The majority of this pharynx tissue appears to be GLP-1-independent, as *Cb-pop-1(RNAi); glp-1(RNAi)* embryos continued to make excess pharynx (Table 1 and Fig. 6I). One possibility is that only MS continues to make pharynx in *Cb-pop-1(RNAi)* embryos, but a requirement for POP-1 in the later MS lineage causes non-pharynx precursors to adopt a pharynx-like fate. Using laser ablation analysis, however, we found that both MS and E make pharynx tissue in *Cb-pop-1(RNAi)* embryos, with MS making pharynx all of the time (17/17 embryos) and E making pharynx most of the time (21/24 embryos; Table 2). Hence, the *Cb-pop-1(RNAi)* phenotype is similar to the Mom phenotype, an E to MS transformation characteristic of *C. elegans* embryos depleted for activity in upstream components of the Wnt/MAPK pathway (Rocheleau et al., 1997; Thorpe et al., 1997). We note that as we have not checked for expression of additional markers, the transformation of E to an MS-like precursor in *Cb-pop-1(RNAi)* embryos may not be complete.

In contrast to both *Cb-skn-1(RNAi)* and *Cb-pop-1(RNAi)* single mutants, which always produced some pharynx, production of pharynx was blocked in the majority of *Cb-pop-1(RNAi); skn-1(RNAi)* double mutants (Table 1 and Figs. 5D, 6J), and reduced to an average of 3.1 ± 0.6 *ceh-22*::YFP-expressing cells among those that made pharynx. The persistence of a small amount of pharynx in some embryos suggests that there may be an additional input into pharynx in addition to *Cb*-SKN-1 and *Cb*-POP-1, or that RNAi targeted to both genes was not completely effective. These results nonetheless confirm that the MS- and E-derived pharynx in *Cb-pop-1(RNAi)* embryos is largely SKN-1-dependent, and conversely, that the majority of the pharynx produced by MS in *Cb-skn-1(RNAi)* embryos is POP-1-dependent. Hence, compared with *C. elegans*, *C. briggsae* exhibits differences in the way in which regulatory input from *Cb*-SKN-1 and *Cb*-POP-1 is integrated to produce specification of MS and E (see Discussion).

Table 2
Production of tissues from partial *C. briggsae* embryos

Blastomeres ablated or isolated[a]	# of partial embryos with pharynx muscle[b] (/total)	Mean number of pharynx muscle cells (±SEM)[b]
Cb-unc-22(RNAi)[c]		
MS ablation	21/21	5.8±0.4
ABa ablation	19/19	5.8±0.4
ABa+MS ablation	2/19	0.1±0.1
EMS isolation	3/3	7.3±1.5
MS isolation	6/6	6.7±0.7
E isolation	1/5	0.4±0.4
Cb-skn-1(RNAi)		
No ablation	17/17	5.1±0.4
ABa ablation	12/12	4.6±0.4
MS ablation	1/10	0.1±0.1
Cb-pop-1(RNAi)		
No ablation	54/54	19.3±0.5
EMS ablation	3/14	0.6±0.3
MS ablation	5/5	11.6±2.6
ABa ablation	13/13	10.5±0.5
ABa+MS ablation	15/23	2.6±0.5
EMS isolation	13/13	12.1±1.0
MS isolation	17/17	6.1±0.4
E isolation	21/24	5.4±0.7

[a] To isolate a blastomere, all other blastomeres or their precursors were ablated. Embryos were allowed to develop for a further 8–10 h prior to scoring.
[b] Pharynx muscle was scored with an integrated *ceh-22*::YFP reporter.
[c] RNAi for all cases was performed by feeding on dsRNA-expressing bacteria.

The positive contribution of Cb-POP-1 to endoderm is Wnt-dependent

Although *Ce*-POP-1 is almost completely dispensable for endoderm in *C. elegans*, the positive contribution made to E specification is dependent on the Wnt/MAPK pathway (Huang et al., 2007; Phillips et al., 2007; Thorpe et al., 1997). *C. elegans* encodes five Wnt ligands (*lin-44*, *egl-20*, *cwn-2*, *mom-2* and *cwn-1*), three Wnt receptors (*mig-1*, *mom-5* and *cfz-2*), and four known β-catenins (*hmp-2*, *bar-1*, *wrm-1* and *sys-1*), and a single, unambiguous ortholog exists for each gene in *C. briggsae* (Zhao et al., 2008). RNAi of the divergent β-catenin gene, *Cb-wrm-1*, and the Nemo-like kinase gene *Cb-lit-1*, resulted in >95% loss of endoderm (Table 1), similar to loss of function of the orthologous *C. elegans* genes (Goldstein, 1992; Kaletta et al., 1997; Rocheleau et al., 1999). Among the remaining Wnt component genes, RNAi of only *Cb-mom-2* gave a strong defect of 82% gutless (n=228) (Table 1). RNAi of the other components gave milder effects ranging from 0% (*Cb-hmp-1*) to 14% (*Cb-cfz-2*). We and others have obtained only weak effects of *Ce-mom-2(RNAi)* of 11%–14% gutless (Maduro et al., 2005a; Rocheleau et al., 1997). Previous studies have revealed synergistic interactions among these Wnt components when targeted simultaneously by RNAi (Rocheleau et al., 1997). We found that *Cb-mom-2(RNAi); apr-1(RNAi)* demonstrated synergy (91% gutless, n=194, p<0.01) similar to results reported for *C. elegans*, but we detected no synergy with *Cb-mom-2(RNAi); mom-5(RNAi)* (82%

gutless, $n=229$, $p>0.5$). Taken together, these results suggest that the main Wnt factors that participate in endoderm in *C. elegans* (WRM-1, LIT-1 and MOM-2) also do so in *C. briggsae*.

Positive function of *Ce*-POP-1 has been shown to require the divergent β-catenin, *Ce*-SYS-1, which is proposed to form a bipartite activator with *Ce*-POP-1 in Wnt-signaled cells (Huang et al., 2007; Kidd et al., 2005; Phillips et al., 2007). Consistent with dispensability of *Ce*-POP-1/SYS-1 in activation of endoderm specification in E, RNAi of *Ce-sys-1* results in only a 1–4% endoderm defect (Huang et al., 2007; Phillips et al., 2007). *Ce*-SYS-1 interacts with the POP-1 amino-terminal β-catenin interaction domain (Kidd et al., 2005), and this region is 98% identical between *Ce*-POP-1 and *Cb*-POP-1 (Fig. 2), suggesting that the *Cb*-POP-1-*Cb*-SYS-1 interaction might be conserved. If the endoderm-promoting contribution of *Cb*-POP-1 functions similarly to *C. elegans*, depletion of *Cb-sys-1* should have a much stronger phenotype. Indeed, 50% ($n=124$) of *Cb-sys-1(RNAi)* embryos were found to lack endoderm, suggesting that *Cb*-POP-1 and *Cb*-SYS-1 work together at least part of the time to promote endoderm, just as in *C. elegans*, but in *C. briggsae*, this contribution is much more critically required.

Changes in pop-1 phenotype correlate with differences in end regulation

In *C. elegans*, endoderm specification requires *end-1* and *end-3*, which encode GATA-type transcription factors with overlapping function (Maduro et al., 2005a). Loss of *end-1,3* together in *C. elegans* results in a complete loss of endoderm (Zhu et al., 1997). In *C. briggsae*, the *Cb-end-1* ortholog and the two *Cb-end-3* orthologs, *Cb-end-3.1* and *Cb-end-3.2*, have similar, redundant roles in E specification (Maduro et al., 2005a). RNAi of the *Cb-end* genes results in elimination of endoderm from >95% of embryos. Conversely, overexpression of *Cb-end-3.2* in *C. elegans* is sufficient to convert many blastomeres into endoderm precursors, similar to overexpression of *Ce-end-1* and *Ce-end-3* (Maduro et al., 2005a; Zhu et al., 1998). By in situ hybridization, the *C. briggsae* and *C. elegans end* genes demonstrate similar activation in the early E lineage (Figs. 7A, C, E, G).

In *C. elegans*, the MS-to-E transformation that is seen in *pop-1(−)* embryos correlates with de-repression of the *end* genes in MS (Maduro et al., 2005b; Shetty et al., 2005). We examined *Cb-end-1* and *Cb-end-3.1/3.2* expression in wild-type and *Cb-pop-1(RNAi)* embryos using in situ hybridization. As anticipated by the absence-of-gut phenotype, *Cb-pop-1(RNAi)* embryos showed no detectable *Cb-end-1* or *Cb-end-3.1/3.2* expression (Figs. 7F, H), contrary to the MS+E expression obtained in *C. elegans* (Figs. 7B, D). Hence, the endoderm defect of *Cb-pop-1(RNAi)* embryos can be attributed to a failure to activate *Cb-end-1* and *Cb-end-3.1/3.2*.

The difference in *end* regulation of the *Cb-end* genes could result solely from changes in *cis*-regulatory sites within the *end* promoters, or from other differences. We have previously reported that a *Cb-end-3.2*::GFP reporter carrying 1.7 kbp of promoter sequence is expressed in *C. elegans* in the early E lineage (Fig. 7I) (Maduro et al., 2005a). We found that this reporter showed a decrease in expression in *Ce-skn-1(RNAi)* and *Ce-med-1,2(−)* double mutants, but was unaffected in *Ce-end-1,3(−)* double mutants (data not shown), similar to the behavior of *end-1* or *end-3* transgene reporters (Maduro et al., 2007, 2005b). If the differences in POP-1-dependent regulation of the *end* genes in the two species results solely from changes in *cis*-regulation, then depletion of *Ce-pop-1* in *C. elegans* harboring *Cb-end-3.2*::GFP should show a loss of expression. We found, however, that *Ce-pop-1(RNAi)* of *C. elegans* carrying *Cb-end-3.2*::GFP resulted in ectopic activation of the reporter in the early MS lineage (Fig. 7J) (Maduro et al., 2007). We were unable to make conclusions based on *end* reporters in *C. briggsae*, as even following transgene integration, only a small percentage of embryos (<5%) showed any expression, although this was found to be in the expected cells (i.e., the early E lineage for *Ce-end-3*::GFP and *Cb-end-3.2*::GFP). These results suggest that changes in *cis*-regulatory sites alone may not account for the differences in *pop-1* phenotype between the two species. However, our *Cb-end-3.2* reporter may not carry all of the regulatory sites of importance, or as a transgene array (Mello et al., 1991) the reporter may behave differently than it would in a single-copy chromosomal context. Nonetheless, the cryptic ability of *Cb-end-3.2*::GFP to be activated in MS in *C. elegans* suggests that the POP-1-independent activators of endoderm specification in both species act through qualitatively similar *cis*-regulatory sites.

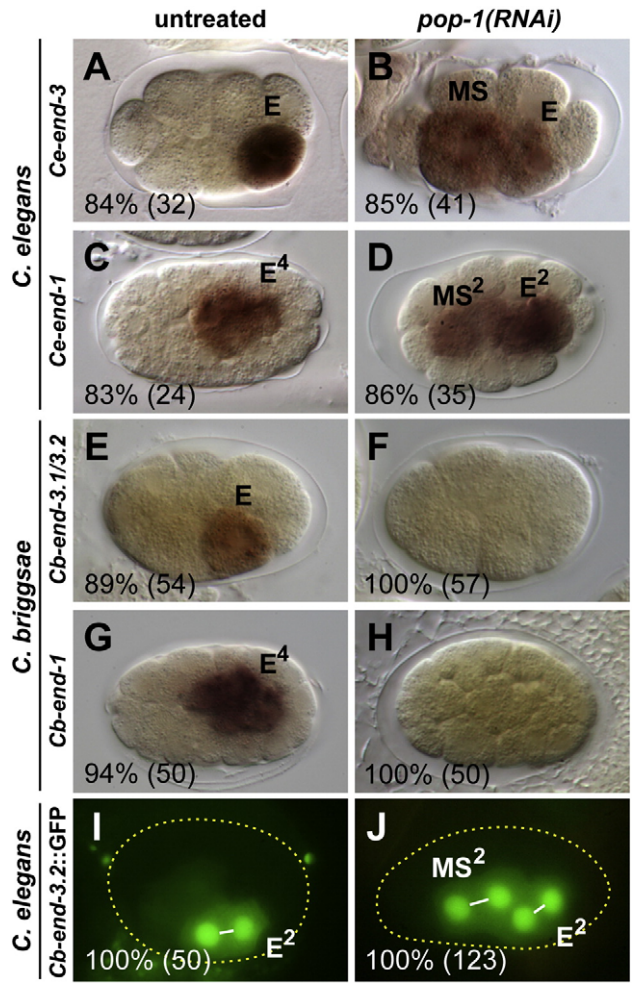

Fig. 7. *end* gene expression in wild-type and *pop-1(RNAi)* embryos. (A–H) Detection of endogenous *end* gene transcripts in wild-type and *pop-1(RNAi)* embryos of *C. elegans* and *C. briggsae*. Numbers indicate proportion of embryos showing staining similar to that shown in the image; remaining embryos showed either no signal or nonspecific staining, except in panel B, where 3/41 (7%) showed apparent E lineage-specific staining. (I–J) Expression of an integrated *Cb-end-3.2*::GFP reporter in *C. elegans*. The reporter carries 1.7 kbp of sequence 5′ of the *Cb-end-3.2* ATG and is fused to an NLS::GFP coding region in the middle of the second *Cb-end-3.2* exon (Maduro et al., 2005a). WormBase predicts a 1.4 kbp intergenic region between *Cb-end-3.2* and the nearest upstream gene, CBG11405, suggesting that this reporter captures all upstream regulatory elements. Cytoplasmic expression in panels I and J results from incomplete nuclear localization of NLS::GFP. Weak MS lineage expression as seen in panel I is also frequently observed with reporters for *Ce-end-1* or *end-3* (unpublished observations) although in situ hybridizations fail to detect endogenous *end* transcripts in the MS lineage (Maduro et al., 2007; Zhu et al., 1997). Data in panels A and C were previously published (Maduro et al., 2007).

The pop-1(RNAi) phenotype in C. remanei is similar to C. elegans

A molecular phylogeny of several species within the *Caenorhabditis* genus has been established (Kiontke and Fitch, 2005). To try to

determine which of the *pop-1(RNAi)* phenotypes might be an ancestral condition, we examined other *Caenorhabditis* species, of which *C. remanei* gave the most decisive result (see Materials and methods). This species is more closely related to *C. briggsae*, while *C. elegans* is similarly diverged from, and an outgroup to, both *C. briggsae* and *C. remanei* (Cutter, 2008; Kiontke and Fitch, 2005). Untreated wild-type *C. remanei*, or animals injected with *Cb-unc-22* dsRNA as a control, gave a similar number of animals producing gut [97% (n=195) for uninjected, 94% (n=109) for *Cb-unc-22* dsRNA-injected]. In contrast, *Cr-pop-1(RNAi)* resulted in 100% embryonic lethality (n=180), with 93% of embryos containing endoderm occupying an apparently larger volume than in wild-types. Using laser ablation, we found that wild-type *C. remanei* embryos with E ablated did not make gut (n=11 embryos), while 15 of 17 *Cr-pop-1(RNAi)* embryos with E ablated continued to make endoderm. We have also performed RNAi by injection targeted to the *pop-1* ortholog of *Caenorhabditis sp. 9*, a very close relative of *C. briggsae* (Marie-Anne Félix, personal communication). *C. sp.9 pop-1(RNAi)* by injection resulted in 79% of embryos failing to make endoderm (n=170), comparable to results obtained in *C. briggsae* with *Cb-pop-1* dsRNA injection (83% gutless, n=159; p=0.40). Taken together, these results suggest that with respect to POP-1 function in endoderm specification, *C. remanei* is more like *C. elegans* than *C. briggsae*, and that the molecular changes associated with a difference in *pop-1* function were derived following the divergence of *C. remanei* and *C. briggsae* from their last common ancestor.

Discussion

Differences in regulatory logic of endomesoderm specification in C. elegans and C. briggsae

Changes in gene regulatory networks drive evolutionary change (Davidson and Erwin, 2006). Here we have shown that knockdown of the maternal SKN-1 and POP-1 pathways results in different phenotypes in *C. elegans* and *C. briggsae*. The most conspicuous aspect is an apparent E to MS transformation in *Cb-pop-1(RNAi)*, rather than the MS to E transformation seen in *C. elegans*. From these differences we can extrapolate the manner in which SKN-1 and POP-1 contribute to correct spatial activation of lineage specification genes in MS and E in the two species (Figs. 8A, C). In the *C. elegans* MS cell, POP-1 represses the E genes *Ce-end-1* and *Ce-end-3* (Huang et al., 2007; Maduro et al., 2007), while SKN-1 activates the MS specification gene *Ce-tbx-35* through the intermediate regulators *Ce*-MED-1,2 (Broitman-Maduro et al., 2006; Maduro et al., 2001) (Fig. 8C). Loss of function of *Ce-skn-1* or *Ce-med-1,2* results in the penetrant loss of MS-derived tissues, suggesting these are the sole (or primary) input into MS specification. In the E cell, *Ce*-POP-1 and *Ce*-SKN-1 contribute to activation of *Ce-end-1* and *Ce-end-3* (Maduro et al., 2005b; Phillips et al., 2007; Shetty et al., 2005). These contributions appear to occur with primarily an 'OR' type of regulatory logic as revealed by loss-of-function analysis: Loss of either *Ce-skn-1* or *Ce-pop-1* individually results in continued specification of endoderm, while loss of both synergistically results in an increase in absence of endoderm (Bowerman et al., 1992; Huang et al., 2007; Lin et al., 1995; Maduro et al., 2005b; Phillips et al., 2007).

In *C. briggsae*, the combinatorial logic of SKN-1 and POP-1 regulatory input exhibits fundamental differences (Fig. 8C). First, we found no evidence that *Cb-pop-1* acts in MS to repress specification of endoderm. *Cb-pop-1(RNAi)* did not result in ectopic gut, and we could not detect ectopic expression of *Cb-end-1* and *Cb-end-3.1/3.2* in MS in such embryos. Rather, there is evidence that *Cb*-SKN-1 and *Cb*-POP-1 work through 'OR' logic to specify MS: Depletion of either factor alone resulted in a persistence of GLP-1-independent pharynx muscle from MS, while depletion of both together resulted in a synergistic absence of pharynx. Hence, unlike its *C. elegans* counterpart, *Cb*-POP-1 makes

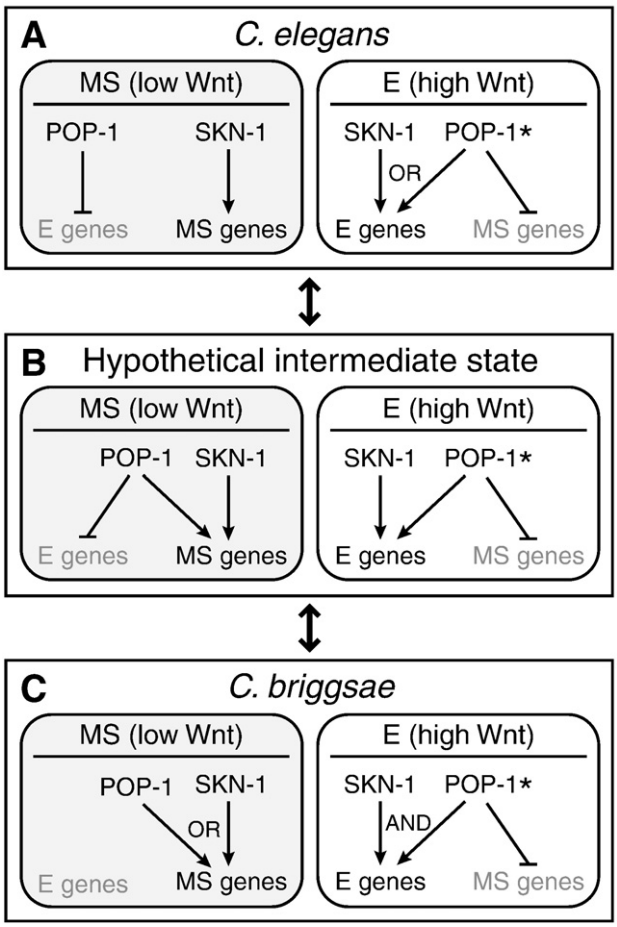

Fig. 8. Regulatory logic of SKN-1 and POP-1 in MS and E specification in *C. elegans*, *C. briggsae* and a hypothetical intermediate state. (A–C) Simplified models to indicate changes in combinatorial input of SKN-1 and POP-1. Arrows indicate the 'activating' or 'repressing' nature of a contribution to regulation of MS or E specification genes and do not necessarily indicate a direct *cis*-regulatory interaction. The MS cells are shaded to indicate a Wnt-unsignaled state (low Wnt). In the E cells, POP-1 is shown with an asterisk (*) to indicate a Wnt-signaled state (high Wnt).

an apparent positive contribution to MS specification rather than a repressive one. Second, in E, *Cb*-SKN-1 and *Cb*-POP-1 both demonstrate 'AND' logic in endoderm specification: Depletion of either regulator alone resulted in the near absence of endoderm, suggesting that neither input in isolation is sufficient to specify E. An inability of *Cb*-SKN-1 to activate endoderm specification in the absence of Wnt-signaled *Cb*-POP-1 also accounts for why the E genes are not activated in MS.

Within E, does *Cb*-POP-1 function as an endoderm activator or MS repressor? Our experiments cannot rule out both, and evidence from *C. elegans* and *Drosophila* suggests both are possible. A positive (though weaker) role for *Ce*-POP-1 in E specification has already been demonstrated (Huang et al., 2007; Maduro et al., 2005b; Phillips et al., 2007), making it likely that this same positive contribution occurs in *C. briggsae*. We have also found that although *Ce-pop-1(RNAi)* embryos express *Ce-end-1,3* in both the MS and E cells, such embryos also misexpress the MS-specific gene Y80D3A.3 in both MS and E (G.B.-M. and M.M., unpublished). Hence, the models in Fig. 8 include repression of MS genes as a POP-1 regulatory input in E. Evidence from *Drosophila* indicates that Wnt-signaled TCF, through interaction with β-catenin/Armadillo, can repress target genes via novel TCF-binding sites (Blauwkamp et al., 2008), suggesting that such a mechanism is at least possible with Wnt-signaled POP-1.

Origin of regulatory differences: transition through an intermediate state?

The common features of the SKN-1 and POP-1 regulatory contributions suggest a hypothetical intermediate configuration that is the sum of the regulatory inputs found in the two species (Fig. 8B). In this network, POP-1 has clear dual roles in both MS and E, contributing to both activation of the correct fate and repression of the alternate fate. We propose that the network configurations in the two species might be able to evolve into one another through this intermediate state, driven primarily by changes at the level of *cis*-regulatory sites in the promoters of MS- and E-specifying genes. Changes in *cis*-regulation underlie phenotype changes in other systems (Wray, 2007), and it is reasonable to speculate that in the endomesoderm network, gain and loss of *cis*-regulatory sites would be tolerated over time as long as the final output – lineage-specific activation of MS- and E-specifying genes – is robustly maintained.

In many contexts, alternate pathways of specification of similar structures have been discovered in nematodes (Felix and Barriere, 2005). For example, in studies of specification of the vulval lineages within *Caenorhabditis*, a full spectrum of quantitative variation was observed in the relative contributions of different signaling pathways (Felix, 2007). For *Caenorhabditis* endomesoderm specification, alternate network configurations might exist that generate novel phenotypes that differ from the two *pop-1* phenotypes seen in *C. elegans* and *C. briggsae*. For example, if Wnt-unsignaled POP-1 played a more critical role in activating MS-specific genes, loss of *pop-1* might result in absence of MS tissues, but no endoderm phenotype. In *C. remanei* and *C. sp. 9* we observed that the *pop-1* knockdown phenotype was either *C. elegans*-like (ectopic gut) or *C. briggsae*-like (loss of gut). Although a wider sample of species is clearly needed, the existence of these two network configurations among four species suggests that not all possible networks may be as stable as the extant *C. briggsae* or *C. elegans* states, or that some constraints prevent all possible network configurations from evolving. Hybridization of the zygotic genomes of *C. briggsae* and *C. remanei*, two species that show opposite *pop-1* knockdown phenotypes, is apparently compatible with normal E specification: Hybrid embryos resulting from crosses between *C. briggsae* males and *C. remanei* females appear to specify gut normally, although developmental defects appear later (Baird and Yen, 2000). Hence, it is plausible that at least some intermediate states could arise and become stably maintained in one of the two forms (i.e. *C. elegans*-like or *C. briggsae*-like).

Changes in SKN-1 and POP-1 are not as likely as cis-regulatory changes

In our model in which the endomesoderm network undergoes changes in architecture, it is assumed that the functional properties of the SKN-1 and POP-1 orthologs remain largely unchanged. Here we have obtained only indirect experimental evidence for conservation of *Cb*-POP-1: A GFP-tagged version can complement the MS specification defect of *C. elegans pop-1* mutants, and the fusion protein demonstrates asymmetric nuclear localization in vivo (POP-1 asymmetry; Fig. 3). Additional constraints may prevent major changes in SKN-1 or POP-1 function, as both proteins have additional roles in other contexts. *Ce*-SKN-1 has an ancestral role in stress response in the intestine, while *Ce*-POP-1 functions in other anterior-posterior cell divisions to produce transcriptional regulatory differences (An and Blackwell, 2003; Lin et al., 1998). Hence, functional differences in SKN-1 and POP-1 between the two species might be less likely to evolve because of the large number of target genes that would be affected. The most likely explanation of the *skn-1* and *pop-1* phenotype differences, therefore, rests on changes in how combinatorial input of SKN-1 and POP-1 is integrated at the level of promoters in target genes. It should ultimately be possible to identify a *cis*-regulatory basis for the unexpected phenotype differences observed between *C. briggsae* and *C. elegans*, accounting for how substantive changes in the endomesoderm gene network can arise in the absence of a change in developmental output.

Acknowledgments

We thank Marie-Anne Félix, Karin Kiontke, David Fitch, Jessica Smith, Craig Hunter, Peter Okkema, David Miller, Jim McGhee, Takao Inoue and Paul Sternberg for sending us nematode strains and plasmids, James Gosses for amplification of a *C. sp. 9 pop-1* cDNA fragment, David Carter for help with laser ablations, and two anonymous reviewers for helpful comments. Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). This work was funded by grants from the NSF (IBN#0416922 and IOS#0643325) and NIH (1R03HD054589-01) to M.M.

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