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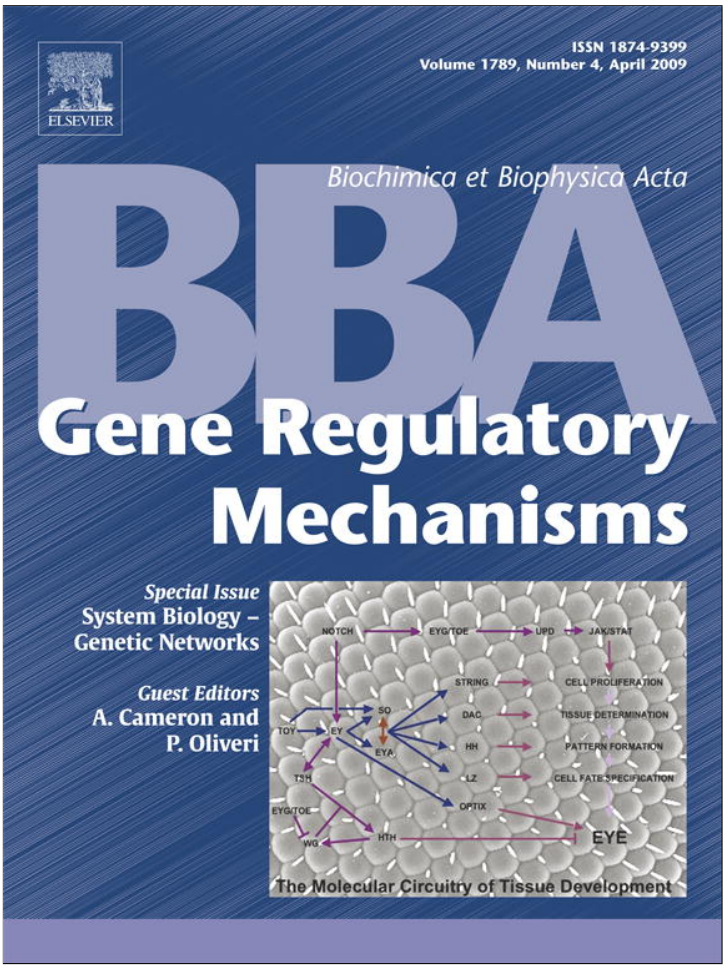

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Review

Structure and evolution of the *C. elegans* embryonic endomesoderm network

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ABSTRACT

The specification of the *Caenorhabditis elegans* endomesoderm has been the subject of study for more than 15 years. Specification of the 4-cell stage endomesoderm precursor, EMS, occurs as a result of the activation of a transcription factor cascade that starts with SKN-1, coupled with input from the Wnt/β-catenin asymmetry pathway through the nuclear effector POP-1. As development proceeds, transiently-expressed cell fate factors are succeeded by stable, tissue/organ-specific regulators. The pathway is complex and uses motifs found in all transcriptional networks. Here, the regulators that function in the *C. elegans* endomesoderm network are described. An examination of the motifs in the network suggests how they may have evolved from simpler gene interactions. Flexibility in the network is evident from the multitude of parallel functions that have been identified and from apparent changes in parts of the corresponding network in *Caenorhabditis briggsae*. Overall, the complexities of *C. elegans* endomesoderm specification build a picture of a network that is robust, complex, and still evolving.

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1. Introduction

Triploblastic animals begin life as a single cell, which after many rounds of mitosis will ultimately consist of a multitude of genetically equivalent cells. By adulthood, the majority of these will have selected a particular pathway of differentiation, each expressing a subset of the genes in the organism's genetic complement that uniquely defines its type. At some point in embryogenesis, precursor cells become specified, and acquire transcriptional differences that set them apart from their neighbors. These differences will instruct their descendants as to their ultimate cell type, or at least restrict their choices until a later decision is made.

In the nematode, *Caenorhabditis elegans*, cells acquire these differences very early, as seen in the stereotyped cleavage patterns that are the hallmark of its nearly-invariant cell lineage [1]. The point of sperm entry sets the posterior of the embryo, defining one of the three embryonic axes (reviewed in [2]). The first division produces a larger cell, AB, and a smaller posterior cell, P_1. Following division of AB and P_1, the embryo consists of the anterior and posterior daughters of AB (ABa and ABp, respectively), and the two daughters of P_1, called EMS and P_2 (Fig. 1). EMS, situated ventrally, is an endomesoderm precursor: it will divide to produce a posterior daughter, called E, and an anterior daughter, MS. The E cell will clonally generate the 20 larval cells of the midgut (endoderm), while MS generates many cells that are primarily mesodermal, which includes most cells in the posterior half of the pharynx, and many of the animal's body muscles. The remaining portion of the pharynx is made by the anterior daughter of AB (ABa). Because many cells in the *C. elegans* lineage undergo anterior–posterior divisions to produce daughters that will acquire different fates [1], specification of MS and E makes a good platform for examining mechanisms that may operate throughout much of the animal's development.

Work over the past 15+ years has identified multiple factors that specify the *C. elegans* endomesoderm. Essentially, there are two pathways that converge on EMS specification: the SKN-1/MED-1,2 pathway assigns an endomesodermal fate to EMS, while the Wnt/β-catenin asymmetry pathway makes E different from MS [3,4]. Although the pathways that lead to MS and E specification look superficially like a simple cascade, the network contains much subtlety, crosstalk, redundancy, and flexibility. This review will examine the genes that specify MS and E, how deployment of their developmental programs is restricted to the appropriate lineages, and how the overall network may be evolving. A diagrammatic summary of the information flow in the network is presented in Fig. 1, and a summary of the relevant genes is given in Table 1.

2. The endomesoderm network

The rapid development of *C. elegans* is considered derived within the phylum, and the rapid divisions in the early embryo are proposed to be correlated with the use of maternal factors to drive much of the early cell specification events [5–7]. Screens for maternal embryonic lethals, in which arrested embryos lack one or more major tissue types but still contain many differentiated nuclei, led to the identification of multiple factors, including the gene *skn-1* [8].

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2.1. Getting it all started: Maternal SKN-1 specifies EMS

Embryos from *skn-1(−)* mothers undergo a developmental arrest and lack pharynx 100% of the time, while endoderm is absent in approximately 70% of embryos [8]. Pharynx originates from descendants of both MS and ABa [1]; the absence of AB- derived pharynx in *skn-1* mutant embryos is attributed to the failure of a GLP-1/Notch-mediated cell induction that normally occurs between the MS cell and descendants of ABa [8,9].

Antibody staining shows that SKN-1 protein is present in the EMS and P_2 nuclei at the 4-cell stage, placing it in the correct time and place to directly act in EMS specification [10]. As discussed below, SKN-1 is blocked in P_2 due to the function of another maternal gene, *pie-1* [11–13]. The *skn-1* locus encodes a transcription factor that has domains similar to those found in bZIP and homeodomain proteins [14]. As its expression normally disappears during the MS and E cell cycles [10], SKN-1 is likely to be a factor that initiates a zygotic gene cascade that will specify MS and E. The observation that many *skn-1(−)* embryos still make endoderm points to the existence of parallel pathways that are capable of contributing to gut specification in the absence of SKN-1; these pathways will be discussed later.

2.2. Zygotic specification of endoderm by END-1 and END-3

Mutagenic screens for penetrant zygotic mutations that resulted in the absence of endoderm identified only a large genomic region on chromosome V, named the 'Endoderm Determining Region' or EDR [15]. EDR-deficient [EDR(Df)] embryos lack endoderm and show a transformation of E to a C-like cell [15]. Within a 30-kbp region located within the EDR, two GATA factor genes, *end-1* and *end-3*, were identified that could individually restore endoderm development to EDR(Df) embryos, suggesting that they share overlapping function [15,16]. Consistent with this, overexpression of either *end-1* or *end-3* can reprogram non-endodermal cells into gut precursors [16,17]. Transgene fusion reporters for both genes are also expressed in the early E lineage, though expression goes away after several cell divisions [16,18]. Hence, *end-1* and *end-3* are clearly paralogous, likely having arisen from an ancient gene duplication [16].

Two observations suggest that *end-1* and *end-3* have diverged somewhat, which might account for their maintenance [19]. First, while a null mutation of *end-1* has no discernible phenotype, mutation of *end-3* results in a weak endoderm specification defect, and in those embryos making endoderm, the number of gut cells frequently

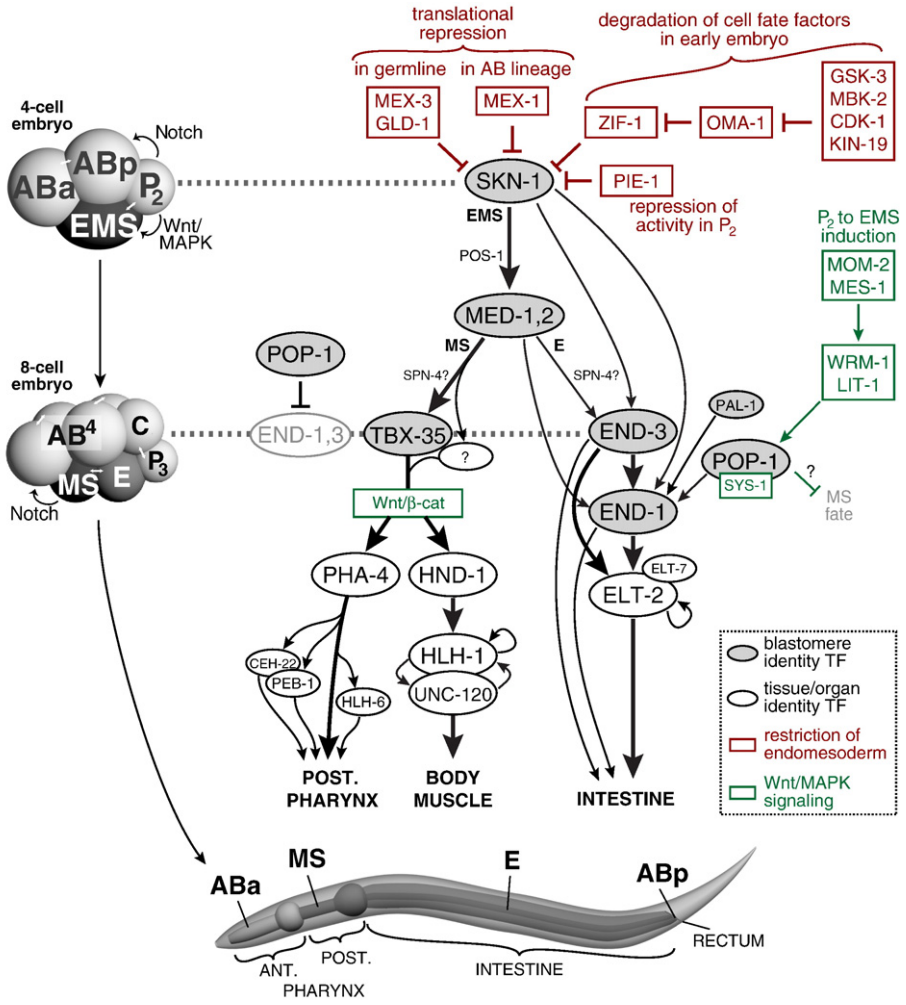

Fig. 1. The *C. elegans* endomesoderm gene regulatory network. Ovals represent transcription factors, while rectangles indicate other types of proteins. Question marks denote hypothesized co-regulators or functions. Arrows denote direct regulatory interactions. Thicker arrows denote stronger inputs as defined by the phenotype of loss of the input, while thinner arrows denote weaker parallel, autoregulatory or feed-forward inputs. Overlapping transcription factor symbols denote common function and are not meant to imply physical interaction. Diagrams of the *C. elegans* embryo (4-cell and 8-cell stages), and anatomy of the digestive tract in a larva are shown, after Ref. [3], with anterior to the left, and dorsal upwards. Sections of the digestive tract are labeled with the name of the blastomere whose descendants contribute to that region. The association of 'anterior pharynx' with ABa, and 'posterior pharynx' with MS is in reality not a precise distinction [9] and is shown here as such for simplicity. GLP-1/Notch-dependent cell–cell interactions are shown as 'Notch' with an arrow; the MS-to-AB induction actually occurs later than shown [9]. Only some of the pharynx and intestinal regulators are shown as examples; for more comprehensive descriptions see references [77,75].

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deviates from the 20 normally seen in wild-type [16,20]. Second, both *in situ* hybridization studies and whole-embryo transcriptome experiments show that *end-3* is activated slightly earlier than *end-1* within the E cell cycle [20,21]. There is further genetic evidence, discussed below, that END-3 also contributes to activation of *end-1* [20].

Are there any other zygotic genes that specifically contribute to E specification? RNAi targeted to both *end-1* and *end-3* resulted in only approximately 43% of embryos that lacked gut, although this appears to be the result of a weak RNAi effect on *end-1* [16]. To resolve the question of whether or not other genes can specify endoderm in addition to *end-1* and *end-3*, we generated an *end-1(−) end-3(−)* double mutant using putative null mutants for both loci. All *end-1,3 (−)* embryos lack differentiated endoderm (Fig. 2E), although the overall phenotype is surprisingly mild: most embryos become properly enclosed in epidermis and undergo elongation, and some can hatch into arrested larvae as shown in Fig. 2B (M.M., unpublished results). This phenotype is very different from EDR(Df) embryos, which arrest well before elongation due to the simultaneous loss of many genes in addition to *end-1* and *end-3* [15,22].

Table 1
Gene products that act in *C. elegans* endomesoderm specification

Gene (product)/ortholog[a]	Role[b]	Reference(s)
Maternal gene products — blastomere identity		
SKN-1 (bZIP/homeodomain TF)/NRF1	EMS specification	[8]
PAL-1 (homeodomain TF)/caudal	E specification	[69,102]
POP-1 (TCF TF)/pangolin	MS, E specification	[59,67,69]
MOM-2 (Wnt ligand)/Wnt	E specification	[53,54]
MES-1 (receptor tyrosine kinase)	E specification	[44]
SRC-1 (Src tyrosine kinase)	E specification	[44]
SYS-1 (β-catenin)/armadillo	E specification	[65,66]
WRM-1 (β-catenin)/armadillo	E specification	[53,54]
LIT-1 (Nemo-like kinase)	E specification	[101]
POS-1 (CCCH zinc finger)	E, MS specification	[48]
SPN-4 (RNP-type RNA-binding domain)	E, MS specification	[47]
Maternal gene products — restriction of endomesoderm specification		
MEX-3 (KH-domain RNA-binding)	Germline repression of specification	[46]
GLD-1 (K homology RNA-binding)	Germline repression of specification	[46]
GSK-3 (glycogen synthase kinase)	Degradation of maternal factors	[42]
MBK-2 (Yak1-related kinase)	Degradation of maternal factors	[42]
CDK-1 (cyclin-dependent kinase)	Degradation of maternal factors	[42]
KIN-19 (serine/threonine kinase)	Degradation of maternal factors	[42]
OMA-1 (CCCH zinc finger)	Timely activation of specification factors	[41,42]
ZIF-1 (SOCS-box protein)	Degradation of maternal factors	[42]
MEX-1 (CCCH zinc finger)	Blocks SKN-1 expression in AB lineage	[12]
PIE-1 (CCCH zinc finger)	Blocks SKN-1 activity in P_2	[12]
Zygotic gene products — blastomere identity		
MED-1,2[c] (divergent GATA TF)	MS, E specification	[25]
TBX-35 (T-box TF)/brachyury	MS specification	[33]
END-1,3 (GATA TF)/GATA4,5,6	E specification	[15,16]
Zygotic gene products — tissue identity		
ELT-2,4,7 (GATA TFs)/GATA4,5,6	Intestine	[73,78,87]
PHA-4 (FoxA TF)/HNF-3	Pharynx	[90,91]
CEH-22 (homeodomain TF)	Pharyngeal muscle	[92]
PEB-1 (novel DNA-binding TF)	Pharyngeal muscle	[94]
HLH-6 (bHLH TF)	Pharynx gland	[95]
HND-1 (bHLH TF)/HAND	Body muscle	[97,119]
HLH-1 (bHLH TF)/MyoD	Body muscle	[97,120]
UNC-120 (MADS-box TF)/SRF	Body muscle	[97]

[a] For some gene products a likely vertebrate or *Drosophila* ortholog is not immediately apparent. Some ortholog names were obtained from Wormbase (http://www.wormbase.org, release WS192).
[b] Only the contribution to MS and E specification is shown; many of these gene products have additional roles in other lineages.
[c] There is also evidence of a maternal contribution of MED-1,2 [20]. Abbreviation: TF, transcription factor.

The behavior of early E-lineage cells may explain the mild *end-1,3 (−)* phenotype. During normal gastrulation, the two E daughter cells (Ea and Ep) move into the interior of the embryo [23]. Examination of the early E descendants in *end-3(−)*, *end-1,3(−)* and EDR(Df) embryos, alone and in combination with Wnt pathway mutants, shows that Wnt components and the ENDs share overlapping function in gastrulation [15,16,23] [Jacob Sawyer and Bob Goldstein, personal communication]. Hence, loss of *end-1,3(−)* may still permit normal morphogenesis. This is consistent with the notion that in general, pathways that control morphogenesis appear to exhibit greater redundancy than those that specify cell fate [24].

2.3. The MED-1 and MED-2 factors

The involvement of the GATA factors *end-1* and *end-3* in E specification led to the hypothesis that a similar regulator might be responsible for specification of MS [25]. Searches of the partially-assembled *C. elegans* genome sequence led to the identification of *med-1* and *med-2*, two unlinked but nearly-identical putative GATA factors [25]. Rather than function in MS specification analogous to the *end* genes, however, the *med-1,2* genes appear to function between SKN-1 and *end-1,3*. RNA interference and double mutant studies with null alleles of both genes have shown that *med-1,2(−)* embryos lack MS-derived tissues all of the time, but also lack endoderm some of the time (15–50%) [3,25–27]. Unlike loss of *end-1,3*, *med-1,2(−)* mutant embryos do not complete elongation, and arrest before hatching. Reporter gene, gel shift and *in situ* hybridization studies showed that the *med* genes are activated in the EMS cell, and that their expression is transient [20,25]. Expression is dependent on the presence of SKN-1 sites in the *med* promoters, implying that SKN-1 directly activates *med-1,2* in EMS [20,25]. An additional *med-1,2* expression component occurs in the maternal germ line that also is dependent on SKN-1 [20]. The germline expression appears to account for the observation that embryos only zygotically lacking in *med-1,2* are more likely to specify endoderm, though these findings have been disputed by others based on indirect evidence [20,26].

Sufficiency experiments show that overexpression of MED-1 throughout the embryo results in widespread expression of *end-1*::GFP and *end-3*::GFP reporters, and embryonic lethality with excess pharynx muscle or endoderm [18,25]. These results suggest that *med-1* is sufficient to initiate a program of MS or E specification, perhaps in combination with other factors, and that with respect to endoderm, the MEDs function upstream of the *end* genes [25]. Terminal *med-1,2; end-1,3* quadruple mutant embryos (Fig. 2C) lack gut and arrest with an appearance similar to the most profoundly affected *med-1,2(−)* embryos, consistent with placement of *end-1,3* downstream of *med-1,2* [25]. The weak endoderm phenotype of *med-1,2(−)* embryos is consistent with involvement of parallel inputs into *end-1,3* activation, discussed later.

Additional evidence supports a role for MED-1,2 in activation of *end-1,3*. Both in vitro studies (DNaseI footprinting and gel shift assays) and in vivo experiments (visualization of subnuclear spots representing interaction of GFP-tagged MED-1 with *end* target arrays) confirm a direct interaction of MED-1 with the promoters of *end-1* and *end-3* [28,29]. Two sites in *end-1*, and four in *end-3*, were identified as regions of MED-1 interaction [28]. Unexpectedly, MED-1 appears to not recognize a canonical GATA binding site (HGATAR), but rather a related sequence (RAGTATAC) [28,30].

A recent study of GATA factors has shown that those involved in *C. elegans* endomesoderm have arisen recently, though the precise relationship with vertebrate and arthropod GATA factors, and the reason for the expansion, remain unresolved [31]. Molecular and genetic evidence suggests that *med-1* and *med-2*, like the *end-1,3* pair, are not completely redundant despite their near-identity. First, germline *med-2* mRNAs accumulate to apparently higher levels than those of *med-1*, although both show similar expression in EMS [20].

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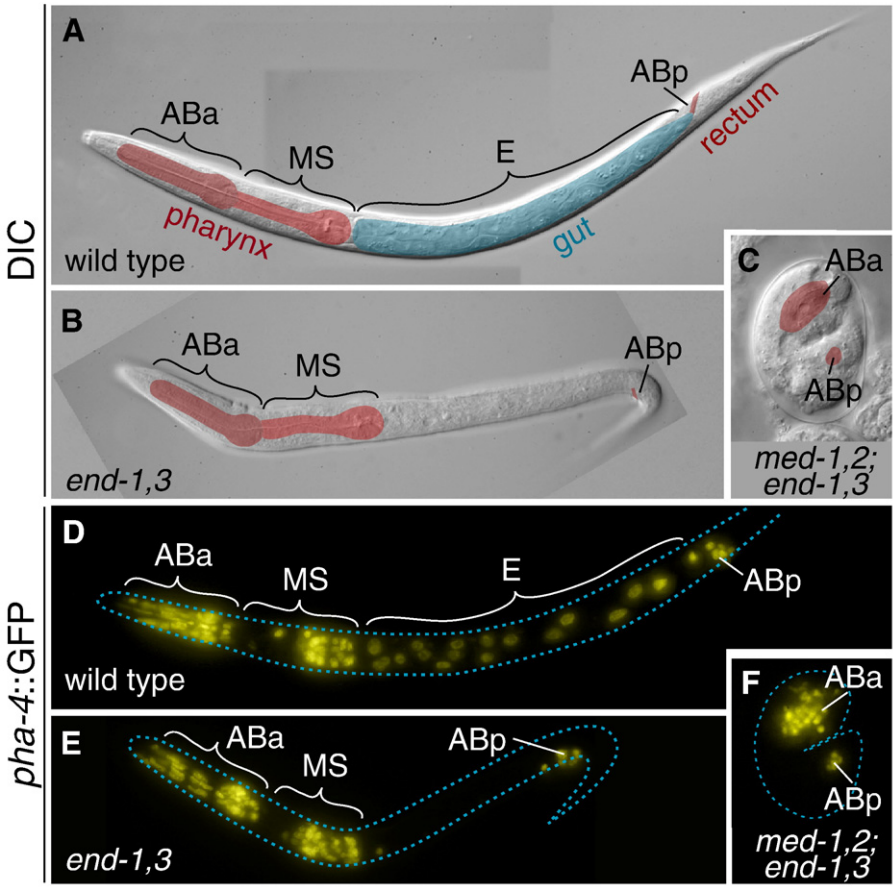

Fig. 2. Appearance of wild-type and zygotic cell fate specification mutants and associated expression of a *pha-4*::GFP reporter (pseudocolored yellow; a gift from Jeb Gaudet, University of Calgary). (A) Wild-type L1, showing pharynx and rectum (red) and intestine (blue) and associated blastomeres as in Fig. 1. (B) Arrested *end-1(ok558) end-3(ok1448)* double mutant embryo, showing absence of gut and slightly reduced body length as compared with wild-type larvae. The majority of *end-1,3(−)* embryos elongate within the eggshell, but only some of these hatch. A more detailed characterization of these mutants will be presented elsewhere. (C) Arrested *med-1(ok804); med-2(cx9744); end-1(ok558) end-3(ok1448)* quadruple-null mutant. The most severely affected *med-1,2(−)* embryos arrest lacking gut and at a similar stage as the embryo shown here [25]. (D–F) Expression of *pha-4*::GFP in pharynx, rectum and intestine cells, corresponding to the DIC images in A–C. The hypodermis is indicated with a dotted blue line. Anterior is to the left in panels A, B, E and F, and up in panels C and F. All micrographs are shown at the same scale. In A–C, Adobe Photoshop was used to colorize regions. A *C. elegans* embryo is approximately 50 μm along its long axis.

Second, of the four possible *med*-x; *end*-y double mutant combinations, only *med-1; end-3* demonstrates a synergistic effect, resulting in a ~58% gutless phenotype, and a high proportion of embryos containing less than the wild-type number of 20 gut cells [20]. In contrast, a *med-2; end-3* double mutant strain makes endoderm >95% of the time, similar to loss of *end-3* alone. Therefore, even though the *med* genes are undoubtedly the result of a recent duplication, they have nonetheless diverged [20,32]. Hence, subfunctionalization of duplicate GATA genes may play a role in their retention.

2.4. MS specification and TBX-35

Under the hypothesis that MED-1,2 would likely bind putative MS regulators in the same manner as with the E targets *end-1,3*, the *C. elegans* genome sequence was searched for MED-1 binding site clusters [28]. This identified (among other genes) *tbx-35*, a gene that contains seven putative MED-1 sites and which encodes a putative T-box transcription factor [28,33]. Consistent with *med-1,2*-dependent activation, *tbx-35* transcripts are found in the MS cell, and a *tbx-35*::GFP reporter is expressed in the early MS lineage [33]. *tbx-35* was also identified as an early MS-specific gene in a study that identified downstream embryonic targets of SKN-1 [34]. Recombinant MED-1 protein is able to shift fragments of the *tbx-35* promoter, confirming a direct interaction [33]. Mutant *tbx-35(−)* animals undergo developmental arrest as embryos or larvae, with the most affected animals demonstrating a lack of MS-derived pharynx and body wall muscle [33]. The variable phenotype contrasts with the more stereotyped embryonic arrest of *med-1,2(−)* embryos, suggesting that many *tbx-35(−)* embryos can still make MS-derived tissues. Consistent with the existence of factors that work in parallel with TBX-35, we have found that the four embryonically-derived coelomocytes, which normally arise from MS descendants [1], are still present in *tbx-35(−)* embryos [Melissa Owraghi and M.M., unpublished observations]. Consistent with an ability to promote MS specification, overexpression of TBX-35 leads to the appearance of ectopic pharynx and body muscle [33] as well as coelomocytes [M.O. and M.M, unpublished]. Therefore, while TBX-35 is clearly an important regulator in MS specification, there are additional factors that work in parallel.

3. Restriction of SKN-1 activity to EMS

Just as there are factors that assure timely activation of lineage-specific specification genes, other gene products act to prevent inappropriate activity of such factors in other cells. In the endomesoderm, these factors all act, collectively, in three functions: to restrict SKN-1 activity to the EMS nucleus, to prevent its translation in inappropriate parts of the embryo (or in the germline), and to promote its timely degradation. Hence, these components do not act directly in the endomesoderm specification network but are nonetheless vital for assuring its deployment in the EMS lineage alone.

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3.1. PIE-1 and MEX-1

One of the factors that restricts SKN-1 function is the maternal factor PIE-1 [12]. In embryos lacking *pie-1* function, P_2 develops like an EMS cell, generating SKN-1-dependent MS and E fates in its descendants [12]. Consistent with ectopic activation of the EMS specification pathway by the SKN-1 protein normally found in P_2, *pie-1* mutant embryos display ectopic activation of *med-1* and *end-3* reporters in P_2 descendants [16,25]. Whole-genome transcriptome analysis also detected elevated *med-1,2* and *end-1* transcripts in *pie-1* mutant embryos, consistent with ectopic activation of the pathway downstream of SKN-1 [35]. PIE-1 is a CCCH zinc finger protein that is found in the P lineage, and functions by inhibiting transcription, explaining how the SKN-1 in P_2 normally does not activate EMS development [13,36,37].

At the other end of the embryo, the maternal factor MEX-1 restricts appearance of ectopic MS-like fates by preventing appearance of high levels of SKN-1 protein in the early AB lineage [10,12,38]. In embryos lacking *mex-1* function, the AB granddaughters adopt MS-like fates, concomitant with ectopic expression of *med-1,2* and *tbx-35* [12,25,33,39]. As with *pie-1*, SKN-1 is required for the appearance of ectopic MS-derived tissues in *mex-1* mutant embryos, consistent with the ectopic activation of the normal MS specification pathway in the AB lineage [12]. MEX-1 is a CCCH-type zinc finger protein similar to PIE-1, and like PIE-1, is found in the P lineage, where it functions in PIE-1 localization [40]. Hence, the role of MEX-1 in preventing in AB-specific accumulation of SKN-1 is apparently indirect.

3.2. Regulation of OMA-1 degradation

A gain-of-function (gf) mutation in *oma-1*, a gene encoding another CCCH-type zinc finger protein, results in ectopic mis-specification of C, a somatic daughter of P_2 (Fig. 1), as an EMS-like cell [41]. In *oma-1(gf)* embryos, SKN-1 protein degradation is delayed compared with the wild-type, and ectopic expression of a *med-1*::GFP reporter is observed in the early C lineage [41]. Other maternal proteins (e.g. PIE-1 and MEX-1) are found to perdure in *oma-1(gf)* embryos, consistent with a role for OMA-1 in negative regulation of timely degradation of cell fate specification factors in general [41]. A group of kinases (CDK-1, GSK-3, KIN-19 and MBK-2) has been identified that act upstream of OMA-1 [42,43]. Loss of function of any of these components results in stabilization of OMA-1 and an ectopic SKN-1-dependent, ectopic endoderm phenotype similar to *oma-1(gf)* [42,43]. Timely degradation of OMA-1 permits the proteolysis of cell fate determinants, dependent upon the SOCS-box protein ZIF-1 [42]. These results provide an explanation for how loss of GSK-3 function was previously found to result in ectopic expression of a *med-1* reporter in the C lineage, and specification of Cp as an E-like cell [25,44]. As GSK-3 functions in post-embryonic regulation of SKN-1 nuclear localization, there may also be a more direct role for GSK-3 in regulation of SKN-1 activity in C, rather than only through regulation of OMA-1 stability [45].

3.3. Maintenance of germline totipotency

The germline must assure that cell fate pathways are not activated. Intestine-like cells have been observed within the germline in approximately 25% of animals lacking function of both *mex-3* and *gld-1*, genes that encode RNA-binding proteins that function as translational repressors [46]. Although the appearance of intestine-like cells was not specifically associated with ectopic activity of SKN-1, transdifferentiation of other somatic cell types in this background was correlated with ectopic activation of other known tissue regulators (e.g., *hlh-1* for body muscle, as discussed below), suggesting that this is likely to be the case [46].

4. Permissive functions for SKN-1 activity

Loss of function of either of two genes, *pos-1* and *spn-4*, results in defects in EMS specification [47,48]. In *pos-1* mutant embryos, SKN-1 localization appears normal, but *med-1*::GFP is not expressed [25,48]. *spn-4* mutants also show normal SKN-1 localization, but apparently normal activation of a *med-1* reporter, suggesting that *spn-4* plays a different role in EMS development [47]. As *pos-1* and *spn-4* mutants have a number of other developmental defects outside of the EMS lineage, their roles would appear to be more permissive for EMS specification [47,48]. Indeed, POS-1 and SPN-4 have been shown to physically interact, and have a role in regulating translation of maternal *glp-1* mRNA [49].

5. Making MS and E different

The genes described above participate in specification of EMS as an endomesodermal precursor, but not in choosing between the alternate fates of mesoderm and endoderm. To make an MS or E cell from EMS, the SKN-1 pathway works with an evolutionarily conserved switching system that acts on sister cells to specify their fates as different, through a signaling cascade that is now called the Wnt/β-catenin asymmetry pathway [4,50].

5.1. The Wnt/β-catenin asymmetry pathway and POP-1

When cultured in isolation, EMS divides asymmetrically to produce two MS-like cells, giving rise to excess pharynx muscle and no endoderm [51,52]. When EMS is allowed to contact P_2 before it divides, it becomes polarized, such that the side of EMS that was in contact with P_2 will become the E cell, while its sister becomes MS [52]. Screens for mutations that could produce the same phenotype in intact embryos identified components of overlapping Wnt, MAPK and Src pathways [44,53,54]. The Wnt ligand MOM-2 and the receptor tyrosine kinase MES-1 function in parallel in the interaction between P_2 and EMS, though in slightly different ways: while MOM-2 is required only in P_2, MES-1 functions in both P_2 and EMS in an apparent dynamic interaction between the two cells [44,54]. Through a network of downstream signal transduction, which also participates in the reorientation of the EMS spindle [55], the Wnt/MAPK and Src pathways ultimately converge on the differential localization and activity of the nuclear Wnt effector TCF/POP-1 [44,56–58]. *pop-1* was first identified by a maternal-effect mutation that results in the MS cell adopting the fate of E [59]. Antibody staining of POP-1 showed that it is widely expressed, and in sister cells that are born along an anterior–posterior cleavage axis, there is a higher level of POP-1 in the anterior daughter nucleus than the posterior one [60]. This 'POP-1 asymmetry' is the result of the nuclear export of POP-1 in Wnt-responsive cells [29,61]. In addition to the MS/E decision, POP-1 has been found to be involved in a number of asymmetric cell divisions, including those of the post-embryonic T hypodermal blast cell [62], and the Z1 and Z4 somatic gonad precursor cells [63]. Recently, it has been shown that the reduced levels of POP-1 in Wnt-signaled cells permits POP-1 to form a bipartite activator with limiting concentrations of the divergent β-catenin SYS-1, which shows a reciprocal posterior–anterior asymmetry compared with POP-1 [64–66]. POP-1 thus forms part of a binary switching system that can establish transcriptional differences of lineage-specific factors.

5.2. Multiple functions for POP-1

How does POP-1 participate in the MS/E decision? The phenotype of *pop-1* loss is a transformation of MS to E, and the ectopically-specified MS blastomeres in *mex-1* and *pie-1* mutant embryos adopt an E-like fate when *pop-1* function is simultaneously lost [59]. These results suggest that the main function of POP-1 is to repress endoderm

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specification in cells specified as MS. Indeed, *in situ* and transgene reporter assays show that *end-1* and *end-3* are activated in the MS and E lineages in *pop-1* mutant embryos [16,20,67]. A GFP-tagged form of POP-1 can interact in vivo with extrachromosomal arrays carrying the *end-1* or *end-3* promoters, suggesting that this repression is direct [29], and a repressor complex that includes the Groucho homolog UNC-37 mediates repression of *end-1* in MS [68].

Multiple studies have shown that POP-1 also promotes endoderm fate in the E cell in parallel with SKN-1 and MED-1,2. Genetically, depletion of *pop-1* greatly enhances the endoderm phenotype of *skn-1* and *med-1,2* mutants [16,66,69]. Consistent with a role in activation of endoderm when these genes are not mutated, expression of multiple E-specific reporter transgenes, including *end-1*, occurs in both the MS and E lineages at reduced levels in *pop-1* mutants as compared with expression in wild types [67]. An *end-1*::GFP reporter requires optimal TCF/POP-1 binding sites to exhibit POP-1-dependent repression in MS and activation in E, confirming that POP-1 likely exerts both effects through direct interaction with *end-1* [67]. As predicted by association of SYS-1 with POP-1 to form a bipartite activator in E, depletion of *sys-1* significantly enhances the endoderm defect of *skn-1* mutant embryos [65,66]. Accumulation of endogenous *end-1* transcripts is slightly reduced in *pop-1* mutant embryos, and almost eliminated in *end-3; pop-1* mutants, confirming overlapping roles of both POP-1 and END-3 in *end-1* activation in E [20]; as an aside, this synergistic effect on *end-1* activation is consistent with greatly enhanced gutlessness in *end-3; pop-1* double mutants [16].

There is further evidence that POP-1 is capable of additional functions besides repression of endoderm in MS and contribution to activation of endoderm fate in E. Expression of a *tbx-35*::GFP reporter [34] is diminished in MS, and activated in E, in *pop-1* mutant embryos, suggesting that some gene(s) might be able to respond to POP-1 in a reciprocal manner from *end-1,3* [Premnath Shetty and Rueyling Lin, personal communication]. However, expression of endogenous *tbx-35* transcripts, and a slightly different *tbx-35*::GFP reporter, are apparently unaffected by loss of *pop-1*, suggesting that there may be additional nuclear factors that respond to Wnt signaling [33].

To test for a possible requirement for *pop-1* in MS specification beyond repression of *end-1,3*, we examined the phenotype of *end-1,3 (−)* embryos in which *pop-1* function was eliminated by RNAi. Preliminary results suggest that both MS and E adopt some properties of MS in *pop-1(RNAi); end-1,3(−)* embryos, suggesting that POP-1 is not strictly required to initiate a program of MS development [Melissa Owraghi and M.M., unpublished observations]. The apparent adoption of MS-like properties by the E cell in such embryos contrasts with the penetrant transformation of E to C in *end-1,3(−)* embryos [15,16], and suggests a cryptic MS repression role for POP-1 in E. Future work will no doubt shed light on these additional roles.

6. Moving from lineage-to tissue-based gene networks

As development proceeds, two types of specification mechanisms are thought to drive the process forward: those that are lineage-based, in which mutation affects all descendants of a cell irrespective of the tissue types it produces, and those that are organ/tissue-based, in which mutation results in a defect in an entire organ/tissue irrespective of its lineal origin [70]. Global expression studies have identified several hundred genes activated in the pharynx [71], several thousand in the intestine [72,73], and more than a thousand in muscle [74]. Activation of these tissue-specific networks results from the activation, in multiple lineages, of a small number of organ/tissue identity factors. In contrast with early cell fate specification genes, most of these factors remain active through the lifespan of animal. These will be described only briefly below, as there are recent reviews that cover these networks [75–77].

6.1. Intestine fate: ELT-2 and gut identity

The GATA factor ELT-2 appears to be the intestine identity factor. *elt-2* expression begins in the E daughter cells, downstream of *end-1* and *end-3*, and continues through adulthood in all intestinal cells [16,17,78]. Consistent with the placement of ELT-2 at the top of an intestine differentiation network, overexpression of *elt-2* can drive specification of gut throughout the embryo, similar to ectopically-expressed *end-1* or *end-3* [16,17,78], and it maintains its own expression through positive autoregulation [79]. Independent studies found that the vast majority of genes that are activated in the intestine have a recognizable GATA binding site, with a core sequence of TGATAA [72,73]. Recombinant ELT-2 has been repeatedly shown to be able to interact with GATA sites required for ELT-2-dependent expression of numerous intestinal genes, for example *ges-1*, *ftn-1*, *pho-1* and the intestine-specific, Notch-dependent component of *ref-1* [80–83]. *elt-2* has also been shown to be required for an innate immune response to microbial pathogens such as *Pseudomonas* [84,85].

Within the intestine, ELT-2 works in combination with other genes to generate more restricted patterns of gene expression, either autonomously within the gut, or as a result of external inductions. For example, ELT-2 and POP-1 appear to collaborate to restrict expression of *pho-1* to the posterior gut, or for anterior-specific expression of a deleted *ges-1* promoter::reporter construct [82,86]. An increase in the SYS-1::POP-1 ratio by an early E-lineage transgene can result in changes in anterior *pho-1* expression, consistent with a role of the Wnt/β-catenin asymmetry pathway in patterning expression within the gut [65]. Extrinsic cell signaling also interacts with gut-intrinsic factors, as Notch signaling and ELT-2 collaborate to activate expression of *ref-1* in the left side of the primordial gut at the 4E and 8E stages, and the right side at the 16E stage [80].

There is evidence that in the embryo, ELT-2 activates intestinal development with at least one other gene. Loss of *elt-2* does not prevent gut formation in the embryo, though it does result in larval lethality from a failure to maintain gut integrity [78]. A tandem partial duplication of *elt-2*, called *elt-4*, exhibits some later intestinal expression but does not appear to contribute to endoderm [87]. In earlier models of the endoderm specification pathway, it was hypothesized that another intestinal GATA factor, ELT-7, might work in parallel with ELT-2 in the early embryo [18]. This hypothesis made sense in light of the fact that *elt-7* is apparently coexpressed with *elt-2* through adulthood [18]. However, loss of *elt-7* does not detectably affect intestine development, and only a slight enhancement of the *elt-2* phenotype is reported to be seen in an *elt-2; elt-7* double mutant, not a complete loss of gut [73]. Instead, perdurance of END-1 or END-3, or both, might be responsible for activating early expression of downstream intestine-specific factors [75]. This is plausible, as *glo-1* and *pgp-2*, both of which function in gut granule biogenesis, commence transgene expression in the two E daughter cells prior to activation of an *elt-2* reporter, suggesting that at least some gut-specific 'differentiation' factors are targets of END-1,3 [78,88,89]. As these regulators are all GATA factors, they would be expected to be able to bind to the same target sites. Hence, early intestine gene expression could be initiated by END-1/3 and ELT-2, and maintained by ELT-2 later, with some function contributed by ELT-7.

6.2. MS and pharynx, body muscle fates

Unlike E, the MS blastomere generates descendants of very different types, including pharynx cells, body muscle cells, the four embryonically-derived coelomocytes, the somatic gonad precursors Z1 and Z4, and even some neurons [1]. The gene networks that drive specification of these various cell types are undoubtedly complex, though there are two major differentiation pathways that can be considered: of the 80 cells made by MS in the embryo, 28 are body

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muscle cells and 31 are pharynx cells, which together comprise the majority of the embryonic MS descendants [1].

The regulator FoxA/PHA-4 was identified by mutations that resulted in the absence of pharynx [90,91]. Consistent with the placement of PHA-4 at the top of a network that specifies pharynx fate, ectopic PHA-4 is sufficient to induce formation of ectopic pharynx tissue [90]. *pha-4* is also expressed throughout the intestine and rectum (Fig. 2), and is required for rectum development, though *pha-4* loss does not profoundly affect gut development [91]. The pharynx itself contains numerous cell types, which includes muscles, neurons and epithelia [77], implying that there are additional factors that work with PHA-4 to generate the pharynx. Within pharynx muscle, the gene *myo-2*, which encodes a pharynx-specific myosin, is regulated by distinct *cis*-regulatory modules that function within distinct pharynx muscle groups [92]. Both PHA-4 and the homeodomain transcription factor CEH-22 recognize *cis*-regulatory sites in *myo-2*, but whereas *pha-4* is expressed throughout the pharynx, *ceh-22* is activated only in pharynx muscle [91,93]. Another factor, PEB-1, is found both within and outside the pharynx, but also contributes directly to *myo-2* regulation [94]. The pharynx gland-specific gene *hlh-6* contains three regulatory elements, one of which binds PHA-4, and all three *cis*-regulatory modules work in concert to produce cell type-specific activation [95]. Multiple other pharynx regulators have been identified, suggesting that pharynx organogenesis in general involves the activation of many complex sub-networks [71,77,96].

Development of body muscles has been shown to be the result of three-way redundancy among the factors HLH-1, HND-1 and UNC-120 [35,97,98]. Consistent with overlapping function, loss of any one of these factors produces only mild muscle phenotypes, but loss of all three together results in a profound failure of muscle specification [97]. Similarly, overexpression of *hlh-1*, *hnd-1* or *unc-120* can specify cells as muscle precursors [97,99]. Within the C lineage, the factor Caudal/PAL-1 is proposed to activate zygotic *pal-1*, which activates a 'muscle module' consisting of *hnd-1*, *unc-120* and *hlh-1*, specifying muscle progenitors [35,76,97]. HND-1 is proposed to act in early embryogenesis, participating in activation of *hlh-1*, while *hlh-1* and *unc-120* act later, mutually enforcing their expression [76]. Enrichment of embryonic muscle transcripts has identified more than 1300 muscle-enriched genes, suggesting that just as with pharynx and intestine, there are complex sub-networks that remain to be identified [100].

The mechanisms by which the muscle and pharynx tissue networks are activated in appropriate MS-derived precursors, downstream of MED-1/2 and TBX-35, are not yet fully understood. It is likely that the Wnt/β-catenin asymmetry pathway plays a role based on multiple observations. First, POP-1 asymmetry is found in the MS daughters and grand-daughters [29,60], and lower levels of POP-1 are permissive for specification of muscle fates by ectopic HLH-1 [99]. Second, transgenic SYS-1 in the early MS lineage can produce apparent MSa to MSp fate transformations that are manifested as changes in muscle fates within the pharynx [65]. Third, loss of the divergent β-catenin WRM-1, required for Wnt-dependent modification of POP-1, causes defects within the MS lineage [53]: mutants in *lit-1*, a gene whose product works with WRM-1 to modify POP-1, show posterior-to-anterior transformations in multiple lineages, including MS [101]. These transformations are consistent with a fourfold, rather than twofold, increase in the number of *ceh-22*::GFP-expressing cells produced from E + MS in *wrm-1(RNAi)* embryos [33].

Analogous to the MS/E decision, there must be factors that work in combination with the Wnt/β-catenin asymmetry pathway to segregate fate potential within the early MS lineage. TBX-35 is a candidate for such a factor, as overexpression of TBX-35 promotes widespread pharynx or muscle development [33]. Our laboratory has found that a homeodomain protein, CEH-51, acts downstream of TBX-35 to promote muscle fate (Wendy Hung, Gina Broitman-Maduro and M. M., unpublished). The C cell, cousin to MS and E, generates primarily muscle fates among its posterior grand-daughters (Cxp), similar to the muscle fates made by the MS posterior grand-daughters (MSxp) [1]. PAL-1, which normally promotes C fate, is found in the early MS and E lineages as well [69,102]. An intriguing possibility, therefore, is that PAL-1 contributes to muscle specification in the MS lineage. Other candidates for such factors might also be found among the genes identified by their expression in the early EMS lineage [21,34].

7. Conservation of endomesoderm genes among nematodes

In perhaps the most well-characterized gene regulatory network, that of the sea urchin endomesoderm [103], evolutionary changes are driven by mutations in *cis*-regulatory sites [104]. This phenomenon seems to be generalizable as in multiple taxa, there are many examples of evolution being driven by changes in *cis*-regulatory sites [105]. Based on the extant *C. elegans* endomesoderm network, is it possible to make any conclusions about how it may have evolved? In a recent review, homologs of genes in the network were sought using genome sequence information from related species [3]. In the nematodes *Haemonchus contortus* and *Brugia malayi*, maternal factors and tissue/organ identity factors were found to be the most conserved [3]. This might be expected as SKN-1 and POP-1 have additional roles in the animal [50,60,106], and the organ identity factors (e.g. PHA-4, ELT-2) have so many targets that it would seem unlikely that their functions could be transferred to another regulator. Among nematodes as a group, there are significant differences in the way fates are assigned to early blastomeres, although later embryogenesis tends to be more similar [7]. For example, in *Romanomermis*, it is the AB blastomere that produces gut [107], while in *Acrobeloides*, endoderm fate, normally derived from E, is reassigned to another blastomere if the AB cell is ablated [108]. These differences mean that gene networks upstream of tissue/organ identity factors would be expected to be the highly divergent among nematode species.

Within the 'Elegans group' in the *Caenorhabditis* genus (*elegans*, *briggsae*, *sp. 5*, *remanei*, and *brenneri*), there are in most cases one-to-one homologs for all the known genes in the endomesoderm network, although duplications appear to be occurring more frequently among the early zygotic regulators [3,109]. For example, *elt-4* is a partial duplication of *elt-2* that is found only within *C. elegans*, and *C. briggsae* carries a very recent, inverted duplication of *end-3* [16,31,87]. In the nematode *Pristionchus pacificus* there appears to be a pair of linked *end*-like genes whose transcripts accumulate in the early E lineage, suggesting that the *end-1,3* pair is at least as old as the time to the common ancestor of *Caenorhabditis* and *Pristionchus* (George Hsu and M.M., unpublished).

The *med* genes show a unique pattern of evolution among nematode GATA factors. First, outside of *Caenorhabditis*, there are no known *med*-like GATA factor genes at all, suggesting that they evolved very recently [32]. Second, all known *med* genes are intronless [32]. Third, in contrast to the other nematode GATA factors, the *med*s are undergoing rapid duplications: while *C. elegans* has only two *med* paralogs, the *med*s in *C. remanei* number at least seven genes, and in *C. briggsae*, at least four [31,32]. There is no reason to think that the frequency of duplications of *med* genes should be any different than other genes in the network, suggesting that some other selective pressure, as yet unknown, is maintaining the duplicates.

8. Evolution of the network

With the limited studies done in other nematodes, it is premature to make any conclusions about how connectivity, gene hierarchies and robustness arose in the *C. elegans* endomesoderm network. Here, speculation is made as to how parts of the network might have evolved by gene duplication and loss/gain of transcription factor binding sites, while brief mention is made of evidence for flexibility in how the Wnt/β-catenin asymmetry pathway functions in the MS/E decision.

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8.1. Intercalation of genes into a pathway

Studies of gene networks in other systems find that particular types of local connections, or motifs, occur at a high frequency [110]. Within the *C. elegans* endomesoderm network, many of these types of motifs, such as the regulatory chain, can be found [3]. Based on recent studies of motif evolution in the yeast transcriptional network [111], two speculative models are presented below to account for the formation of two motifs within the *C. elegans* endomesoderm network (Fig. 3). The evolutionary changes proposed are plausible based on findings in other systems [104,105,111].

Consider first the regulatory chain of SKN-1 activating *med-1,2*, which then leads to activation of *tbx-35* (Fig. 3A) [33]. Given that the *med*s are not found outside of *Caenorhabditis*, the ancestral pathway might very well have been direct activation of *tbx-35* by SKN-1. If a *med*-like gene arose from duplication of a GATA factor, this gene could have acquired sites for SKN-1, forming a single-input motif. Next, *tbx-35* could have acquired sites that allowed it to be recognized by the MED product, forming a feed-forward motif. If *tbx-35* then lost the original sites for SKN-1, while the number of sites for MED-1 was increased, the result would be the regulatory chain SKN-1 → MED-1 → TBX-35. Duplication of *med-1* would result in the extant pathway in *C. elegans*.

Next, consider the feed-forward loop whereby SKN-1 activity leads to activation of *end-3*, and SKN-1 and END-3 together activate *end-1* (Fig. 3B) [20]. By analogy to the first example, the ancestral pathway could be taken to be SKN-1 activating a single ancestral, autoregulatory *end* gene. A duplication of this ancestral *end* gene occurs, and following subfunctionalization, a feed-forward loop is established. The intercalation of MED-1,2 could have occurred prior to *end* duplication (similar to the example above), or afterwards, to produce the additional relationships in the extant network.

8.2. Evidence for flexibility in how POP-1 influences the MS/E decision

In the above examples, the initial input (SKN-1) and final output (MS or E specification) remain the same after the pathways have undergone changes, and the evolutionary steps required to change motif types are simple enough that they might occur over short time periods. Post-embryonically, changes in relative importance of overlapping signal transduction pathways has been demonstrated in the vulval lineages, despite no apparent differences in developmental output, suggesting that such informational connectivity changes can and do occur [112].

We have begun to look for cryptic differences in the endomesoderm network in *C. briggsae*. *C. elegans* and *C. briggsae* diverged approximately 100 million years ago, but their embryonic cell lineages have remained highly similar [113,114]. Both genomes encode a single *pop-1* ortholog. While loss of *pop-1* in *C. elegans* leads to a mis-specification of MS as an E-like cell [59], RNA interference of *C. briggsae pop-1* leads to a loss of endoderm, and an apparent transformation of E to an MS-like cell [Katy Lin, Gina Broitman-Maduro, Wendy Hung, Serena Cervantes and M.M., under revision]. Surprisingly, the two *end-3* paralogs in *C. briggsae* are not expressed ectopically in the MS lineage, and are also undetectable in the E lineage, in *Cb-pop-1(RNAi)* embryos. One explanation is that the positive input by POP-1 into endoderm specification, apparent in *C. elegans* only under certain conditions, is now a primary input in *C. briggsae*. Similarly, the role for POP-1 in repression of the *end* genes in MS might simply have been lost, if the *Cb-end* genes were no longer as responsive to the SKN-1 pathway due to changes in *cis*-regulatory sites. Regardless of the molecular details, it is clear that there is flexibility in how factors such as POP-1 participate in the same embryonic cell fate decision in related species. In other developmental events in nematodes, such differences are thought to underlie the basis for robustness [115]. The prediction is that other such cryptic differences in phenotypes will be discovered as more comparative studies are performed.

9. Conclusion

When it comes to gene regulatory networks, the *C. elegans* embryonic endomesoderm network seems to have it all. Gene interactions have been characterized by forward genetics, reverse genetics, whole-genome transcriptional profiling, bioinformatics, transgene analysis and biochemistry. The network is replete with

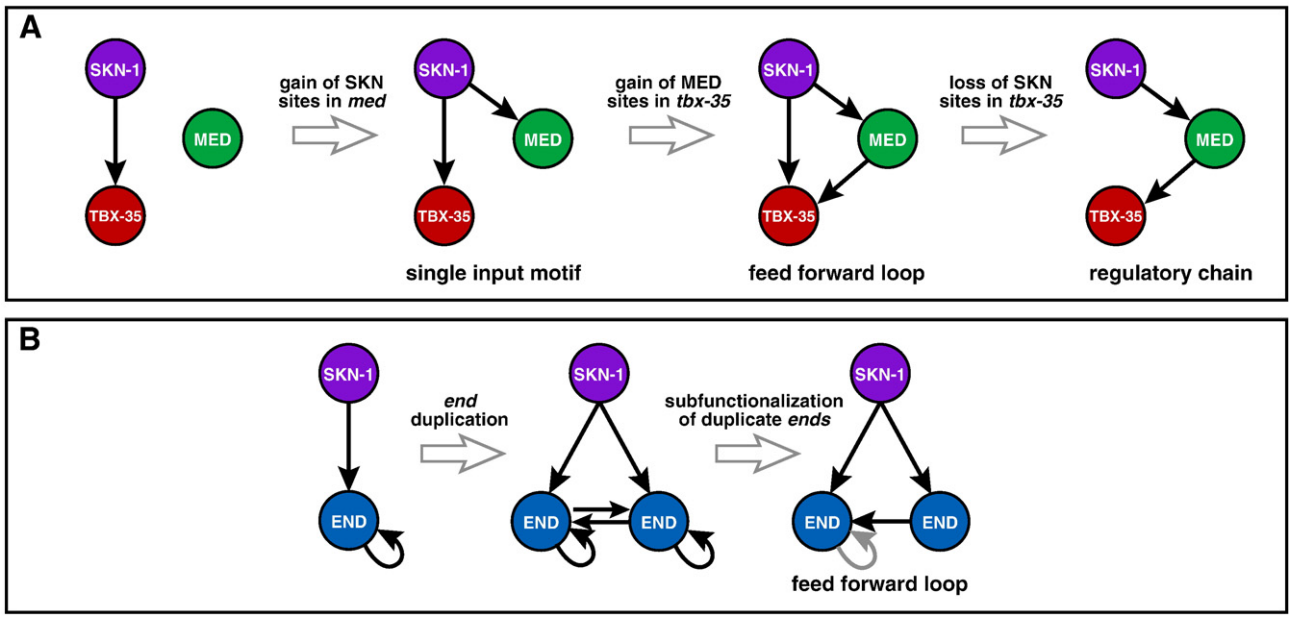

Fig. 3. Speculative models for stepwise evolution of two extant motifs in the *C. elegans* endomesoderm network, starting with single activator, single target interactions. (A) Stepwise formation of the SKN-1 → MED-1,2 → TBX-35 regulatory chain. (B) Formation of a feed-forward loop involving SKN-1 and two ENDs, through subfunctionalization of duplicate, autoregulatory *end* paralogs. Note that extant autoregulation of the first *end* gene is inferred but has not been shown for *end-1*. Intercalation of the MEDs could occur at an early or later step by acquisition of MED sites in the *end* genes.

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parallel pathways, redundant genes, combinatorial specification, feed-forward loops, regulatory chains, maternal genes, zygotic genes, repression, activation, cell-autonomous mechanisms and cell–cell interactions. Parallel pathways and redundancies almost certainly contribute to the robustness of the network, and they also enable alternate gene interactions to arise over time, even in the absence of any obvious outward phenotypic change. Other gene networks tend to use similar motifs and gene interactions, e.g. [110,116,117], suggesting that comparative studies with *C. elegans* and other related animals will yield new insights into their evolution. More specifically, it will be interesting to compare embryonic gene regulatory networks from related animals outside the nematode phylum, to test if hypotheses of the mechanisms seem to be the main driving forces within the protostomes. To this end, studies in other ecdysozoa that partition fates early, such as the tardigrade *Hypsibius dujardini*, may prove fruitful [118].

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