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Developmental Biology 284 (2005) 509 – 522

DEVELOPMENTAL BIOLOGY

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Genomes & Developmental Control

Genetic redundancy in endoderm specification within the genus *Caenorhabditis*

Morris F. Maduro[a,1], Russell J. Hill[b,2], Paul J. Heid[c,3], Erin D. Newman-Smith[a], Jiangwen Zhu[a,c], James R. Priess[b,d], Joel H. Rothman[a,c,*]

[a]*Department of Molecular, Cellular and Developmental Biology and Neuroscience Research Institute, University of California, Santa Barbara, CA 93106, USA*

[b]*Fred Hutchinson Cancer Research Center, Howard Hughes Medical Institute, Seattle, WA 98109, USA*

[c]*Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706, USA*

[d]*Zoology Department, University of Washington, Seattle, WA 98195, USA*

Received for publication 11 April 2005, revised 14 May 2005, accepted 16 May 2005
Available online 24 June 2005

Abstract

Specification of the endoderm precursor, the E cell, in *Caenorhabditis elegans* requires a genomic region called the Endoderm Determining Region (EDR). We showed previously that *end-1*, a gene within the EDR encoding a GATA-type transcription factor, restores endoderm specification to embryos deleted for the EDR and obtained evidence for genetic redundancy in this process. Here, we report molecular identification of *end-3*, a nearby paralog of *end-1* in the EDR, and show that *end-1* and *end-3* together define the endoderm-specifying properties of the EDR. Both genes are expressed in the early E lineage and each is individually sufficient to specify endodermal fate in the E cell and in non-endodermal precursors when ectopically expressed. The loss of function of both *end* genes, but not either one alone, eliminates endoderm in nearly all embryos and results in conversion of E into a C-like mesectodermal precursor, similar to deletions of the EDR. While two putative *end-1* null mutants display no overt phenotype, a missense mutation that alters a residue in the zinc finger domain of END-3 results in misspecification of E in approximately 9% of mutant embryos. We report that the EDR in *C. briggsae*, which is estimated to have diverged from *C. elegans* ~50–120 myr ago, contains three *end*-like genes, resulting from both the ancient duplication that produced *end-1* and *end-3* in *C. elegans*, and a more recent duplication of *end-3* in the lineage specific to *C. briggsae*. Transgenes containing the *C. briggsae end* homologs show E lineage-specific expression and function in *C. elegans*, demonstrating their functional conservation. Moreover, RNAi experiments indicate that the *C. briggsae end* genes also function redundantly to specify endoderm. We propose that duplicated *end* genes have been maintained over long periods of evolution, owing in part to their synergistic function.

Keywords: *C. elegans*; Endoderm; GATA factor; Genetic redundancy; Germ layers; Transcription

Introduction

During the early development of triploblastic metazoans, embryonic cells undergo a dramatic rearrangement to generate the three germ layers, ectoderm, mesoderm, and endoderm. Nuclear differences must emerge during this process, resulting in the activation of appropriate regulatory gene networks that then direct patterns of cell division and morphogenesis specific to each germ layer type. *Caenorhabditis elegans*, typical of nematodes in general, has solved this problem in part by assigning the generation of

* Corresponding author. Department of Molecular, Cellular, and Developmental Biology and Neuroscience Research Institute, University of California, Santa Barbara, Santa Barbara, CA 93106, USA. Fax: +1 805 893 2005.

E-mail address: rothman@lifesci.ucsb.edu (J.H. Rothman).

[1] Present address: Department of Biology, UC Riverside, Riverside, CA 92521, USA.

[2] Present address: Department of Molecular Genetics, Ohio State University, Columbus, OH 43210, USA.

[3] Present address: W.M. Keck Dynamic Image Analysis Facility, Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, USA.

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.05.016

the entire endodermal germ layer to a single blastomere at the 7-cell stage, the E cell (Fig. 1) (Sulston et al., 1983).

Studies from many laboratories over the last decade have revealed that many of the salient molecular events of E specification occur in the mother cell of E, called EMS, and involve the convergence of cell-intrinsic and -extrinsic regulatory pathways, the SKN-1/MED-1,2 transcriptional pathway and convergent Wnt, MAP kinase, and Src/tyrosine kinase signaling pathways, respectively (reviewed in Maduro et al., 2002). In the 4-cell embryo, the maternal bZIP/homeodomain factor SKN-1 activates zygotic transcription of the atypical, redundant GATA factors MED-1 and -2 in the EMS cell (Bowerman et al., 1992, 1993; Broitman-Maduro et al., 2005; Maduro et al., 2001). Simultaneously, EMS becomes polarized through a cell–cell interaction with its neighbor, P_2 (Goldstein, 1992). This polarizing event causes the daughter arising from the side of EMS that was in contact with P_2 to adopt an E cell (endoderm) fate (Goldstein, 1992), while the more distal sister adopts the unsignaled fate, that of the mesodermal progenitor MS. The components of the P_2 signal include Wnt, MAPK, and Src signaling systems, which regulate the state of a Tcf-like transcription factor, POP-1, in the daughters of EMS (Bei et al., 2002; Lin et al., 1995; Maduro et al., 2002; Meneghini et al., 1999; Rocheleau et al., 1997, 1999; Thorpe et al., 1997, 2000). After EMS divides into the mesoderm precursor MS and the endoderm precursor E, the combined action of MED-1,2 and POP-1 dictates their fate: in the unsignaled cell, MS, POP-1 represses endoderm specification, allowing MED-1,2 to activate MS-specific gene activity and MS fate, while in E, the repressive action of POP-1 is blocked by the P_2 inducing signal, and MED-1,2 activate a cascade of endoderm-specific genes and fate (Broitman-Maduro et al., 2005; Maduro and Rothman, 2002). This difference in POP-1 involves a change in its nuclear localization and its ability to bind DNA (Lo et al., 2004; Maduro et al., 2002).

We have previously shown that endoderm specification downstream of the SKN-1/MED-1,2 and Wnt/MAPK/Src pathways requires a genomic region called the endoderm-determining region (EDR). Moreover, we found that the *end-1* gene, located in the EDR, can restore endoderm to embryos deleted for the EDR (Zhu et al., 1997). Consistent with an instructive role for *end-1* in directing endoderm specification, *end-1* transcripts are detected in the E cell, and ectopic expression of *end-1* can reprogram normally non-endodermal cells into endoderm (Zhu et al., 1997, 1998). *end-1* encodes a GATA type transcription factor, named for the consensus HGATAR sequence found in canonical GATA factor binding sites (Lowry and Atchley, 2000). The involvement of GATA factors in endoderm specification is conserved throughout the metazoans (Patient and McGhee, 2002; Shivdasani, 2002; Stainier, 2002). In *Drosophila*, the GATA factor SERPENT is required for specification of the midgut endoderm (Rehorn et al., 1996). In *Xenopus*, GATA5 has been shown to have a role in endoderm development, and can reprogram ectodermal and mesodermal cells towards endoderm in animal caps (Weber et al., 2000). Expression of *C. elegans end-1* in *Xenopus* ectoderm can activate endoderm development, while expression of a form of END-1 containing a repressor domain can block endoderm formation, demonstrating apparent conservation of END-1 function in vertebrates (Shoichet et al., 2000).

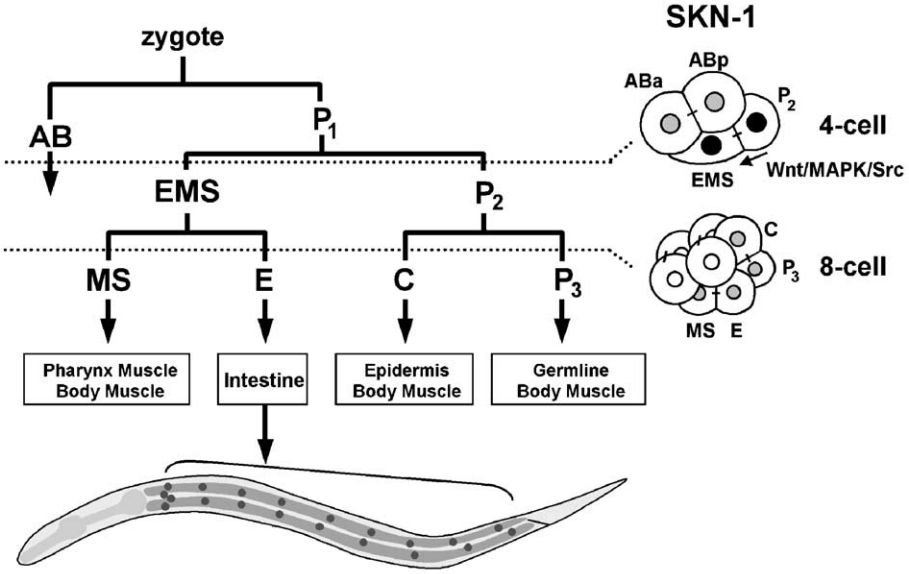

Fig. 1. Lineal origin of the MS, E, and C blastomeres. The zygote (P_0) undergoes a series of stereotyped cleavages (horizontal lines). The arrangement of cells at the 4- and 8-cell stages is schematized with anterior to the left and dorsal up. Short lines indicate sister cells, and an arrow indicates the endoderm-inducing $P_2 \rightarrow$ EMS signal. The antibody expression pattern of nuclear SKN-1 is represented as black (strong expression) or gray (weak) circles within cells (based on Bowerman et al., 1992). The differentiated cell types produced by the P_1-derived founder cells are indicated. BWM, body wall muscle. The E cell gives rise to the 20 cells of the differentiated intestine in L1 stage larvae.

Genetic evidence suggested that *end-1* in *C. elegans* shares endoderm-promoting function with at least one other gene (Zhu et al., 1997). Here, we report that an *end-1* paralog located in the EDR, *end-3*, functions redundantly with *end-1*, and show that END-1 and END-3 together define the endoderm-specifying properties of the EDR. Like *end-1*, *end-3* rescues endoderm formation in embryos lacking the EDR, is expressed in the E cell, and when ectopically expressed can specify endoderm fate ectopically. However, we find that while mutation of *end-1* results in no detectable phenotype, a point mutation in *end-3* results in misspecification of endoderm in a small fraction of embryos. Mutation of either *end-1* or *end-3* enhances the incompletely penetrant endoderm defects of *skn-1(RNAi)* and *mom-2(RNAi)*, suggesting that the activities of *end-1* and *end-3* are additive. Finally, we show that the related nematode, *C. briggsae*, contains three *end*-like genes, which also function redundantly to specify endoderm. Our results reveal that genetic redundancy is an ancient feature of *Caenorhabditis* endoderm specification.

Materials and methods

C. elegans strains and genetics

The following *C. elegans* strains were used: N2 [wild type]; JJ762 [*end-3(zu247) V*]; JR1798 [*pop-1(zu189) dpy-5(e61)/hT1 (I;V); end-3(zu247) V / hT1 him-5(e1490) V*]; JR2417 [*unc-119(ed4) III; ced-1(e1735) I; him-8(e1489) IV*]; JR2276 [*wIs139* (*end-3*::END-3[P202L]::GFP) *?*]; JR2554 [*wIs152* (*end-3*::END-3::GFP) *?*]; JR70 [*ced-1(e1735) I; itDf2 / unc-42(e270) dpy-21(e428) V*]; EG2894 [*end-1 (ox134) ric-7(ox134) V; lin-15(n765ts) X; oxEx396[lin-15 (+), ric-7(+)]*]; JR1130 [*wIs84* (*elt-2*::GFP) *X*]; VC271 [*end-1(ok558)*]; NP97 [*cat-4(e1141) V; otIs77 II* (*unc-122*::GFP, *ttx-3*::*kal-1*)]. The *C. briggsae* strain used was AF16.

Integrated GFP reporters for *ceh-22*, *hlh-1*, and *lin-26* were obtained from P. Okkema, A. Fire and M. Labouesse, respectively. Rescue of *itDf2* was assessed by obtaining JR2417 animals transgenic for an *end-1* or *-3* transgene, the *unc-119(+)* clone pDP#MM016B (Maduro and Pilgrim, 1995), and an *unc-119*::YFP fusion (pMM531). Non-Unc males were mated to JR70 hermaphrodites, and YFP-expressing F_1s that segregated dead eggs were obtained. Homozygous *itDf2* embryos were identified as arrested embryos that lacked cell corpses (Zhu et al., 1997). Expression of the transgene in strains carrying *end-3(zu247)* or hs-*end-3(zu247)* was confirmed by sequencing RT-PCR products obtained from early embryos (data not shown). For analysis of hs-*end-3(+)*, hs-*end-3(zu247)* and hs-*Cb-end-3*, gravid hermaphrodites were incubated at 33°C for 30 min and allowed to lay eggs for 3 h at 20°C. Eggs were counted and then analyzed for phenotype after a further 12 h.

Isolation of mutant alleles

The *ox134* mutation was isolated in an ethylnitrosourea (ENU) screen by K. Schuske and E. Jorgensen (University of Utah) and deletes bases corresponding to nucleotides 679-15502 on the cosmid F58E10. This deletion removes part or all of F58E10.1/*ric-7*, F58E10.7, and F58E10.2/*end-1* (Nick Andersen and E. Jorgensen, personal communication). The *ok558* mutation was isolated by the *C. elegans* Gene Knockout Consortium. For both *ok558* and *ox134*, *end-1*-specific primers within the deletions failed to amplify products from homozygous strains. The *zu247* mutation was isolated in a *lin-2(e1309)* background in an EMS screen for mutations that resulted in one-quarter dead embryos (Page et al., 1997). Upon backcrossing, the strain demonstrated reduced penetrance of the End phenotype and was found to be viable as a homozygote. All *zu247* strains, even after extensive backcrossing and recombination with nearby markers *rol-4(sc8)* and *unc-61(e228)*, exhibit an occasional transient increase in the severity of embryonic lethality and the endoderm phenotype. We have not found conditions that reliably reproduce this state. Therefore, for the genetic experiments reported here, *zu247* strains were first verified as being in the more stable "reduced penetrance" state. The *zu247* lineages were obtained before the existence of these two states was noted.

Plasmids and cloning

PCR and cloning were performed according to standard protocols. Oligonucleotide sequences and cloning details are available upon request. The *zu247* lesion was identified by sequencing independent genomic and cDNA clones of *end-3* amplified from JJ762. A PCR-RFLP strategy was used to confirm the presence of the same lesion in multiple strains derived from JJ762. At the time that we identified *end-3* from the preliminary sequence of cosmid F58E10, we were not aware of the locus *aip-1*, whose 3′ end is 1.2 kbp upstream of the *end-3* start codon (Sok et al., 2001). Consequently, reporter fusions and transgenes contain *aip-1* as well as *end-3*. We have since found that *end-3* genomic fragments lacking *aip-1* drive reporter expression in the early E lineage (not shown).

Identification of C. briggsae end genes

The *end* sequences were identified from the *C. briggsae* genome sequence using the TBLASTN search algorithm (Altschul et al., 1990) using the amino acid sequences of *C. elegans* END-1 and END-3. This approach identified only the highly conserved DNA binding domains; the complete coding regions were predicted by manually examining the sequence for intron donor and acceptor sites. Embryonic expression and the predicted coding region of all three genes were confirmed by sequencing of RT-PCR products.

RNA interference

dsRNA for RNAi was synthesized from genomic subclones or cDNA fragments as described (Maduro et al., 2001). RNAi of *Cb-end-3* is expected to target both *Cb-end-3.1* and *Cb-end-3.2* since both genes share substantial nucleotide identity. For most experiments, dsRNA was injected directly into the gonad (Mello et al., 1991). For *C. elegans end-1/-3(RNAi)* embryos used in laser ablations and 4-D time lapse analysis, interference was obtained by coexpressing sense and antisense transcripts from an extrachromosomal array (Maduro et al., 2001). For *end-3*::GFP; *pop-1(RNAi)*, the *end-3*::GFP strain was grown on *E. coli* strain HT115 expressing *pop-1* dsRNA (Timmons and Fire, 1998).

Laser ablation and cell lineage analysis

Embryonic blastomeres were isolated using a VSL-337 Nitrogen Laser (Laser Science, Inc.) as described (Maduro et al., 2001). Cell lineage analysis was performed using 4D time lapse video microscopy (Thomas et al., 1996) as described elsewhere (Zhu et al., 1997).

In situ hybridization

In situ hybridization was performed according to published protocols (Seydoux and Fire, 1995). For detection of endogenous *end-3* mRNA, a tyramide signal amplification kit (Molecular Probes, Eugene, OR) was used according to the manufacturer's instructions.

Results

end-1 alone is not essential for endoderm specification in C. elegans

A ~250 kbp region on LG *V* defined by several overlapping chromosomal deficiencies, the "endoderm determining region" or EDR, is essential to specify E cell identity and endoderm development in *C. elegans* (Zhu et al., 1997). The *end-1* gene was identified on the basis of its ability to rescue endoderm specification in *Df(EDR)* embryos (Zhu et al., 1997). *end-1* is expressed in the early E lineage, starting in E itself, and is capable of re-specifying cells outside the E lineage into endoderm precursors when ectopically expressed (Zhu et al., 1997, 1998). To analyze the requirement for *end-1* in endoderm development, we performed a screen for lethal mutations targeted to the EDR, but were unable to identify any point mutations that cause a defect in endoderm formation. In a separate genome-wide screen for zygotic embryonic lethal mutations, we recovered an apparent point mutant mapping to the EDR, *zu247*, in which a small percentage (<10%) of embryos fail to make intestine (see below). We found that the *zu247* phenotype is efficiently rescued by an *end-1* transgene (Table 1); however, analysis of DNA from the *zu247* homozygous strain failed to identify any sequence alterations in the entire *end-1* gene and regions flanking it. A direct assessment of the requirement for *end-1* was made possible when two apparent null *end-1* mutations, *ok558* (identified by the *C. elegans* Gene Knockout Consortium) and *ox134* (a gift from N. Andersen and E. Jorgensen) were subsequently identified (Fig. 2). We analyzed both alleles and found that neither leads to any conspicuous defect in endoderm formation or to any other phenotype (Fig. 2B and Table 1). These findings demonstrate that, although it is sufficient to do so, *end-1* is not

Table 1
Intestinal differentiation in *end* and *Df*(EDR) mutant embryos

Genotype	% embryos with intestine[a]
C. elegans	
wild type	100
wild type; *Ex[end-3(zu247)]*	100 (>200)
end-1(ox134)	100 (364)
end-1(ok558)	100 (322)
end-3(RNAi)	95 (236)
end-3(zu247)	91 (247)
end-3(zu247); Ex[end-3(zu247)][b]	93 (267)
end-3(zu247); Ex[end-1(+)][b]	100 (136)
end-3(zu247); Ex[end-3(+)][b]	100 (384)
end-1(RNAi); end-3(RNAi)	43 (67)
end-1(RNAi); end-3(zu247)	25 (65)
end-1(ok558); end-3(RNAi)	11 (471)
end-1(ox134); end-3(RNAi)[c]	7 (191)
itDf2; ced-1(e1735)[d]	0 (>200)
itDf2; ced-1(e1735); Ex[end-1(+)][b,d]	100 (22)
itDf2; ced-1(e1735); Ex[end-3(+)][b,d]	95 (20)
itDf2; ced-1(e1735); Ex[end-3(zu247)][b,d]	0 (45)
pop-1(zu189)	100 (235)
pop-1(zu189); end-3(zu247)	14 (78)
pie-1(RNAi)	100 (143)
pie-1(RNAi); end-3(zu247)	88 (344)
lit-1(RNAi)	2 (312)
lit-1(RNAi); hs-end-3	37 (234)
C. briggsae	
wild type	100 (>200)
Cb-end-1(RNAi)	100 (381)
Cb-end-3.1,3.2(RNAi)	100 (455)
Cb-end-1(RNAi); Cb-end-3.1,3.2(RNAi)	4 (341)

[a] Total number of embryos is shown in brackets. Intestine was scored by gut granule birefringence under polarized light or expression of an integrated *elt-2*::GFP transgene (Fukushige et al., 1998). All animals were grown at 20°C.

[b] Embryos carrying *end* transgenes were identified by expression of an *unc-119*::YFP reporter present on the same array.

[c] The *ox134* lesion also deletes part of the adjacent gene F58E10.1/*ric-7*, which results in an uncoordinated defect. For RNAi experiments with this allele, an *ox134*; *lin-15(−)* strain carrying *ric-7*(+) and *lin-15(+)* on an extrachromosomal array (EG2894) was used (N. Andersen and E. Jorgensen, personal communication).

[d] The *ced-1* mutation allows scoring of *itDf2* homozygotes, which lack *egl-1* and hence do not accumulate cell corpses (Conradt and Horvitz, 1998; Ellis et al., 1991; Zhu et al., 1997).

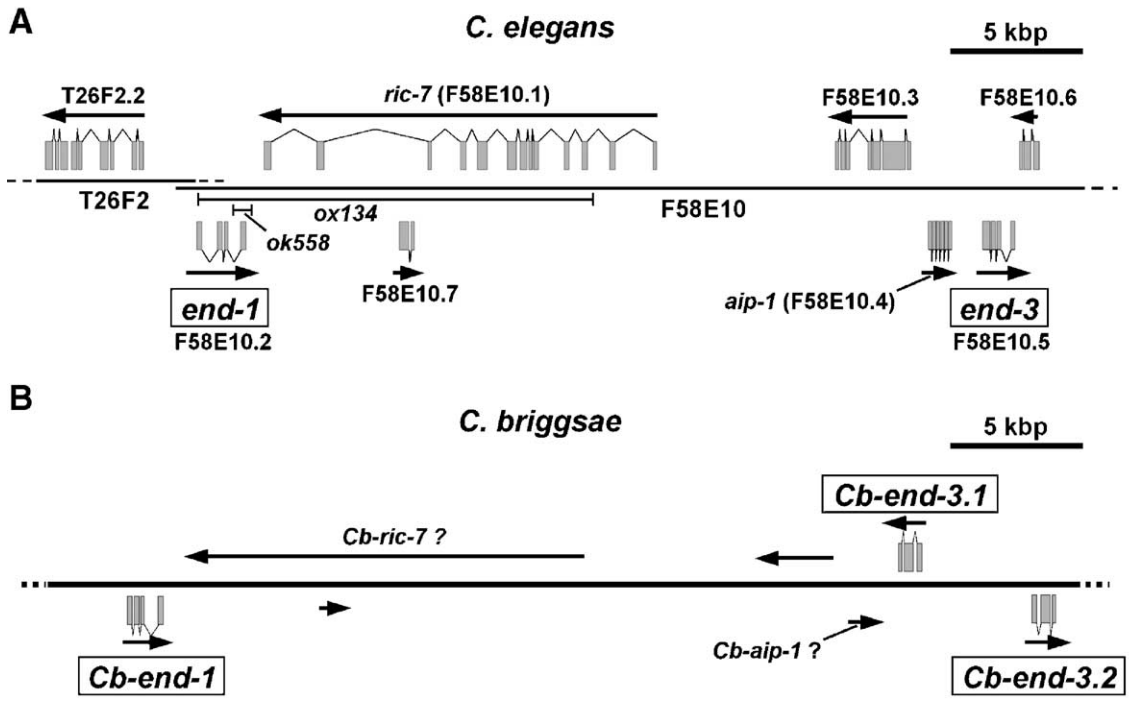

Fig. 2. The *end* gene regions of *C. elegans* and *C. briggsae*. (A) The *end* genes are located on the right arm of LG *V* near the overlap between cosmids T26F2 and F58E10. Gene models are shown with spliced exons, and an arrow indicates the direction of transcription. Exons for *end-3* (F58E10.5) were confirmed by sequencing of RT-PCR products (not shown). The regions deleted by *ox134* and *ok558* are indicated. *end-1* and *end-3* are separated by ∽28 kbp. (B) The corresponding region in *C. briggsae*, showing the relative arrangement of the three *end* genes. Genome alignments displayed on Wormbase (www.wormbase.org) indicate that the genes between *C. elegans end-1* and *-3* show a similar structure in *C. briggsae* (represented as arrows only).

required for endoderm development, and that at least one other gene in the EDR must therefore contribute to this process.

The end-3 gene functions redundantly with end-1

Given the compelling evidence for genetic redundancy in the EDR, we sought to identify the postulated redundant partner of END-1, reasoning that it may be another GATA factor. Indeed, we were able to identify a second EDR gene, located ∽28 kbp to the right of *end-1* that, like *end-1*, encodes a single-finger GATA factor (Fig. 2A). We named this gene *end-3*. (Another EDR gene, originally named *end-2*, was subsequently found not to be required for endoderm development and has been renamed *dpr-1*, as will be reported elsewhere; E. Newman-Smith, T. Suzuki, G. Broitman-Maduro, M. Len, and J. Rothman, unpublished). END-1 and END-3 share ∽40% identity (48% similarity) along their lengths, and ∽50% identity (∽53% similarity) within their DNA binding domains. All other GATA factors in *C. elegans* are more divergent from either END-1 or -3, with virtually no sequence relatedness outside the DNA binding domains. Although other apparent transcription factors are encoded in the EDR, none is a putative GATA factor. The proximity of *end-1* to *end-3* and their sequence similarity suggests that they are paralogs, raising the possibility that they may share endoderm specifying activity. Indeed, we found that a 3.7-kbp genomic segment including *end-3* rescues the endoderm defect of homozygous *itDf2* embryos (Table 1 and Figs. 6A–D).

Both in situ hybridization and reporter transgene fusions revealed that *end-3* is expressed in the early E lineage (Figs. 5A–C). The *end-3* reporter is not expressed in maternal mutants in which E fate is not specified (Fig. 5F) and is ectopically expressed in the predicted cells in mutants in which endoderm is made ectopically (Figs. 5D and E); in all cases, *end-3* expression marks cells that are specified to produce endoderm. Moreover, in embryos in which the mesendoderm-specifying *med-1* gene is expressed ubiquitously, *end-3* expression is seen throughout the embryo (Fig. 5G), confirming that *end-3* is downstream of *med-1* (Maduro and Rothman, 2002). Subsequent to these studies, we showed that GFP-tagged MED-1 can bind the *end-1,3* promoters in vivo and that these promoters contain binding sites recognized by recombinant MED-1 (Broitman-Maduro et al., 2005; Maduro et al., 2002). The congruent expression patterns and activity of *end-3* and *end-1* indicate that they are functional paralogs.

We next asked whether *end-3* is sufficient to specify endoderm when expressed outside of its normal context, by driving its expression under control of heat-shock (hs)-activated promoters. We found that heat shock-induced ubiquitous expression of *end-3* results in extra endoderm, with many embryos producing virtually exclusively gut, as assayed by expression of the gut marker *elt-2*::GFP (Fig. 6H). This widespread activation of endoderm development

was also evident in heat-shocked *hs-end-3* embryos depleted for the Nemo-like kinase LIT-1, which is required to transduce the $P_2 \rightarrow$ EMS signal (Meneghini et al., 1999; Rocheleau et al., 1999) (Table 1 and data not shown). Thus, like *end-1* (Zhu et al., 1998), *end-3* is apparently sufficient to activate endoderm development in any somatic cell precursor, independent of its lineal origin, and in the absence of the Wnt/MAPK/Src pathway component LIT-1.

To test the requirement for *end-3* in endoderm specification, we inhibited its function by RNAi (Fire et al., 1998). While the apparent null phenotype of *end-1* indicates that it is not essential for endoderm development, we found that a small fraction (5%; $n = 236$) of *end-3(RNAi)* embryos reproducibly lack differentiated gut (Table 1). The sequences of *end-1* and *end-3* are sufficiently divergent that this effect is not likely to be the result of cross-reactivity of the *end-3* dsRNA with *end-1*. Thus, by itself, *end-3* performs an essential, albeit incremental role in endoderm formation.

Their similar pattern of expression, rescuing activity, and endoderm-promoting activity in non-endodermal cells suggested that the functional requirements for *end-1* and *end-3* might overlap. To address this possibility, we examined whether the impenetrant phenotype of *end-3(RNAi)* is enhanced by the *end-1(ox134)* deletion mutant. Indeed, while 100% of *end-1(ox134)* embryos make intestine, only 7% ($n = 191$) of *end-1(ox134); end-3(RNAi)* do so (Table 1). Some double mutant animals elongate and hatch into slightly misshapen, but elongated larvae completing lacking an intestine. Many of the gutless larvae contain structures that resemble the cuticle-lined cavities found in *skn-1* mutants (Bowerman et al., 1992), presumably as a result of inappropriate specification of epidermal cells within the gut region (Fig. 6F; see below). The possibility that the residual gut made in some of these double mutant embryos is attributable to a failure of RNAi to completely abolish *end-3* activity is supported by our finding that 43% ($n = 67$) of embryos make gut when the function of both *end-1* and *end-3* is reduced by RNAi. We conclude that depletion of *end-1* and *-3* together strongly synergizes to block endoderm development.

To further assess the role of *end-1* and *-3* in endoderm development, we compared the fate of E descendants in *end-1,3(RNAi)* and *Df(EDR)* embryos. We analyzed the differentiated fate of the E cell by ablating all other blastomeres in mutant embryos with a laser microbeam. In the wild type, partial embryos that develop from an isolated E cell always produce intestinal cells (Table 2). In contrast, in embryos homozygous for the EDR deficiency *zuDf2*, descendants of an isolated E cell always produce body wall muscle and epidermis, characteristic of the C cell, a cousin of E (Fig. 1 and Table 2). Similarly, we found that in *end-1,3(RNAi)* embryos, isolated E blastomeres that do not produce endoderm instead produce body wall muscle and epidermis (Table 2). This putative E → C transformation was confirmed by analyzing the cell lineage of Ea (the anterior daughter of E) in an *end-1,3(RNAi)* embryo from 4-D time-lapse recordings (Thomas et al., 1996). This cell followed a cell division pattern that strongly resembles that of Cp (the posterior daughter of C) (Fig. 4A), similar to what we found for homozygous *itDf2* embryos (Fig. 4A) (Zhu et al., 1997). While we did not assess whether the E cell gave rise to pharynx tissue in such embryos, the foregoing data are most consistent with an E → C transformation in *end-1,3(RNAi)*. We conclude that the E → C transformation observed when the entire EDR is deleted is attributable to simultaneous removal of *end-1* and *-3*. Taken together, these data strongly suggest that *end-1* and *end-3* account for most or all of the endoderm-specifying properties of the EDR.

Table 2
Fates of isolated blastomeres in wild-type and mutant embryos

Genotype	Blastomere	Marker		
	Isolated[a]	Gut[b]	Epidermis[c]	Muscle[d]
wild type	E	9/9	0/4	0/5
	C	0/17	9/9	8/8
zuDf2[e]	E	0/6	5/6	6/6
end-1,3(RNAi)[f]	E	4/16	7/9	5/7

[a] E was isolated by ablating ABa, ABp and P_2 in a 4-cell embryo, then ablating MS after EMS divided. C was isolated by ablating ABa, Abp, and EMS in a 4-cell embryo, then ablating P_3 after P_2 divided.
[b] Gut was scored by gut granule birefringence.
[c] Epidermis was scored by expression of a *lin-26*::GFP reporter (Labouesse et al., 1996).
[d] Muscle was scored by *hlh-1*::GFP expression (Krause et al., 1990).
[e] Data from Zhu et al. (1997). The single embryo that did not stain for epidermis may have been damaged by the laser-ablation procedure.
[f] These embryos were obtained in an interval of 11–13 h after coexpression of sense/antisense *end-1* and *end-3* transcripts from a transgene array in the mother, which results in maximal recovery of mutant embryos.

A missense mutation in end-3 leads to an impenetrant loss of endoderm

While genome-wide and targeted screens for penetrant zygotic mutations that prevent endoderm development recovered only deletions of the EDR, the *zu247* mutation eliminates gut in a small percentage (<10%) of animals (Table 1). We found that the *zu247* is a transition mutation that causes a proline to leucine (C*C*G → C*T*G) substitution at position 202 in the predicted END-3 protein (Fig. 3A). This amino acid immediately precedes the fourth zinc-coordinating cysteine in the C4 zinc finger of END-3. As the corresponding positions in the vertebrate GATA factor cGATA1 and the fungal GATA AreA are known to be important for DNA binding (Omichinski et al., 1993; Starich et al., 1998a), this lesion is consistent with a reduction-of-function mutation of END-3.

A number of genetic observations confirm that *zu247* is a strong hypomorphic or amorphic allele of *end-3*. The mutation is fully recessive and, in contrast to intact *end-1* or *end-3*, either of which can rescue the endoderm defect of the mutant, a transgene expressing *end-3(zu247)* is not

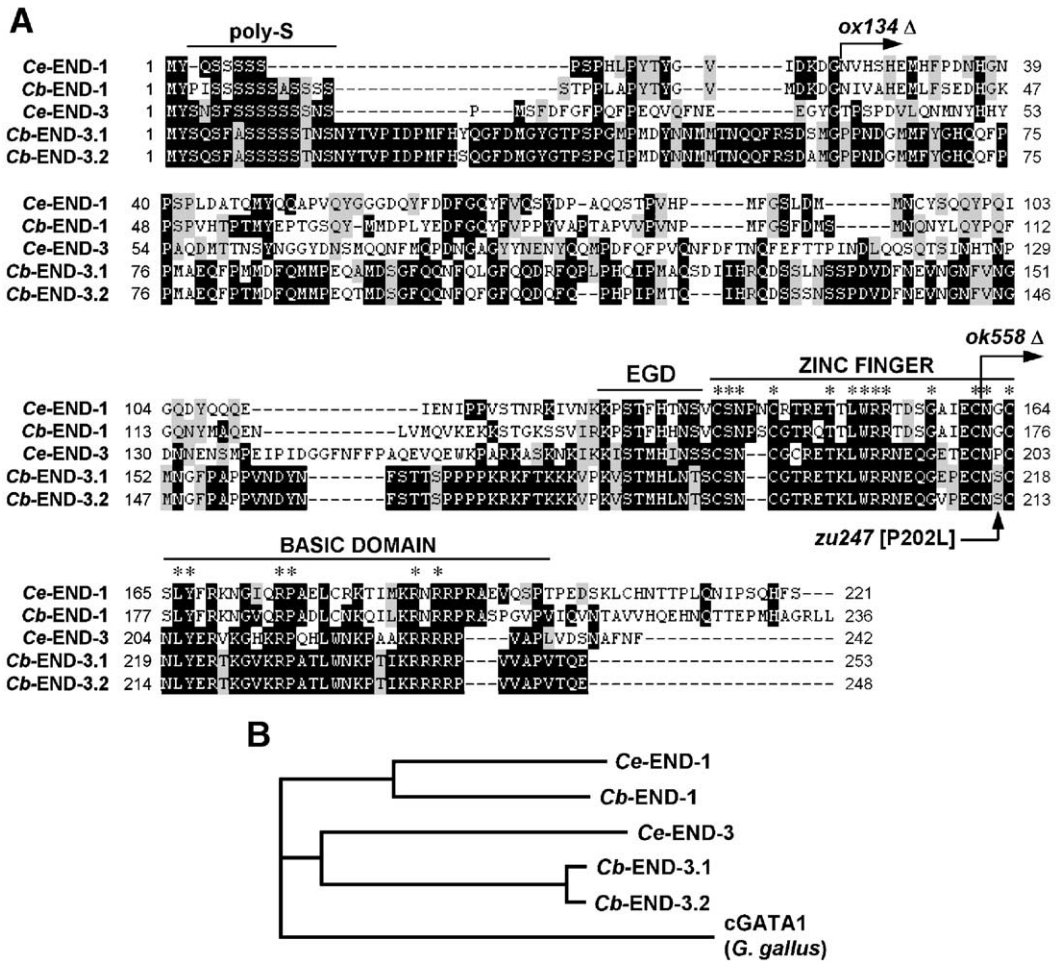

Fig. 3. Similarity of *C. elegans* and *C. briggsae* END proteins. (A) Alignments of the two *C. elegans* and three *C. briggsae* END amino acid sequences. All coding regions were confirmed by RT-PCR. The left breakpoints of the *end-1* mutations *ox134* and *ok558* (both of which extend beyond the last coding exon), as well as the lesion in *end-3(zu247)*, are indicated. The conserved zinc fingers, basic regions, serine-rich regions (poly-S), and END family GATA domain (EGD) are indicated. Asterisks (*) denote amino acids conserved among all END proteins and the vertebrate GATA factor cGATA1 (*G. gallus*; Accession number A32993). White text on a black background indicates identities among three or more proteins, while gray background denotes either conservative substitutions or positions conserved between two proteins using the AlignX blosum62mt2 scoring matrix (Vector NTI Suite, InforMax, North Bethesda, MD). (Note that with this scheme, some paired conservations are not indicated.) (B) AlignX tree showing evolutionary relationship of the *C. briggsae*/*C. elegans* END proteins and cGATA1.

capable of rescuing either *itDf2* or the chromosomal *end-3 (zu247)* mutation (Table 1). Moreover, ectopic expression of the END-3[P202L] mutant protein under heat-shock control does not result in ectopic expression of *elt-2*::GFP or endoderm (not shown); it also does not block normal endoderm development, showing that it does not act as a dominant negative. RNAi of *end-1* strongly enhances the phenotype of the *end-3(zu247)* mutation, consistent with an overlapping function for *end-1* and *end-3*, and further evidence that *zu247* is a strong reduction-of-function *end-3* mutation (Table 1). We found that a transgenically-expressed END-3[P202L]::GFP fusion protein is present in the early E lineage and exhibits strong nuclear localization similar to the wild-type fusion protein, indicating that the mutation does not alter the localization of END-3. Thus, although it is apparently present in the nucleus, the mutant END-3[P202L] protein must possess very little, if any, residual endoderm-promoting activity.

end-3(zu247) is homozygous viable, although a fraction of the animals die as embryos or larvae. Of the embryos that make endoderm, many are defective in ingression of Ea and Ep (i.e., the onset of gastrulation) and show severe morphogenesis defects, perhaps as a secondary consequence (not shown); some of these abnormally formed embryos arrest with a differentiated gut, suggesting that the requirements for *end* function in gastrulation and in specification of the endoderm may be separable. In contrast, some *end-3(zu247)* embryos that completely lack a gut hatch and arrest as relatively well-formed L1 larva, and other arrested larvae contain a partial gut appearing in the anterior or posterior region normally occupied by the gut (not shown). Many of the animals that escape lethality often appear

unhealthy, possibly suggesting impaired gut function, though defects in the morphology of the gut were not conspicuous by Nomarski microscopy.

We performed cell lineage analysis on several *end-3 (zu247)* embryos to assess the fate of the E cell in animals lacking endoderm. As seen in mutants lacking the function of both *end-1* and *end-3*, two gutless embryos analyzed showed an apparent E → C transformation. We were surprised, however, to find in a third such embryo evidence for an apparent E → MS transformation (as was most striking in the lineage of an E granddaughter, shown in Fig. 4B). This observation may reflect a rare event or incomplete transformation in cell fates, as we were unable to detect the presence of extra cells normally made by the MS lineage (pharynx cells or coelomocytes) by analyzing differentiation markers in *end-3(zu247)* embryos (not shown).

end-1 and end-3 single mutants enhance mutations in maternal endoderm specification genes

While elimination of both *end* genes appears to abolish endoderm formation, apparent null mutations in *end-1* show no discernible effect on endoderm formation and a strong loss-of-function mutation of *end-3* results in a very impenetrant endoderm phenotype. Thus, each *end* gene expressed at its normal level may be sufficient to reliably activate endoderm formation. Alternatively, *end-1* and *-3* may mutually influence each other's expression, such that elimination of one results in compensatory increases in the expression level of the other. To assess whether either gene contributes to endoderm formation when both are expressed, we asked whether a mutation in either enhances the impenetrant phenotypes of mutations in the SKN-1 and Wnt/MAPK/Src pathways. Depletion of maternal SKN-1 or MOM-2/Wnt results in impenetrant loss of endoderm (Table 3; Bowerman et al., 1992; Rocheleau et al., 1997). We found that both *end-1* mutations strongly synergize with *skn-1(RNAi)*, reducing the proportion of embryos that make endoderm from 27% to 2% (Table 3). A somewhat reduced synergy was also observed with *mom-2(RNAi)*: the 89% of embryos making endoderm in *mom-2(RNAi)* was reduced to 53% (for *ox134*) and 62% (for *ok558*). This enhancement by the *end-1* null mutations indicates that *end-3* cannot completely substitute for *end-1* in the absence of these maternal functions.

The *end-3(zu247)* mutation generally shows even greater synergy with the maternal mutants. While *zu247* reduces the proportion of *skn-1(RNAi)* embryos that produce endoderm to 4%, similar to the *end-1* mutations (P = 0.39), this mutation completely eliminates endoderm in the otherwise weakly penetrant *mom-2(RNAi)* mutant: 0% of *mom-2(RNAi); end-3(zu247)* embryos make endoderm (Table 3). Moreover, although *src-1(RNAi)* embryos show almost no defect in production of endoderm, except when combined with mutations in other components of the Wnt/MAPK signal (Bei et al., 2002), we found that the *end-3(zu247)* mutation shows significant synergy with *src-1(RNAi)* (Table 3). In contrast, *end-1(ox134)* has no effect on *src-1(RNAi)*. The disparity in the degree of synergy between the *end-3* and *end-1* mutations is consistent with the observation that only *zu247* shows a significant endoderm defect in isolation.

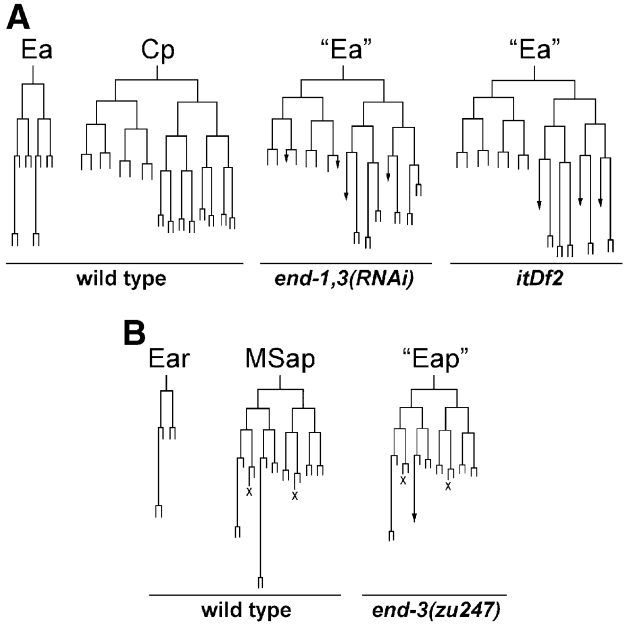

Fig. 4. Partial cell lineages from 4-D time-lapse micrographs of wild-type and mutant embryos. (A) Lineage of wild-type E and C daughters compared to "E" daughters in *end-1,3(RNAi)* and *itDf2*(EDR deficiency) embryos. The transformed Ea lineages are strikingly similar to those of the wild-type Cp, consistent with the apparent E → C transformation. (B) Lineage of wild-type E and MS granddaughters compared to "E" granddaughter in an *end-3(zu247)* embryo. The occurrence of two cell deaths and the pattern of cell divisions arising from this cell clearly resemble the corresponding sublineage of MS. In lineage diagrams, a horizontal line indicates a cell division, a vertical line indicates a cell, and the vertical axis is time. An arrow indicates a cell whose position became ambiguous during the recording, 'X' indicates a cell death, and the end of a line indicates a cell that has differentiated.

Collectively, these data suggest that E specification is partially compromised in the absence of either *end-1* or *end-3* alone.

Endoderm specification in C. briggsae: conservation of redundancy

While *end-1* and *-3* are largely functionally redundant under normal growth conditions, the foregoing findings suggest that each provides a significant input into endoderm specification, perhaps accounting for maintenance of both genes over long evolutionary time spans. We sought to determine whether the shared action of these genes is conserved by examining the degree of conservation of the *end* genes in *C. briggsae*, estimated to have diverged from *C. elegans* approximately 50–120 myr ago (Coghlan and Wolfe, 2002). From the nearly complete *C. briggsae* genome sequence, we identified three apparent *end* homo-

Table 3
Synergy between *end* mutants and maternal genes

Genotype	% embryos with intestine[a]
wild type	100
end-1(ox134)	100 (364)
end-1(ok558)	100 (322)
end-3(zu247)	91 (247)
skn-1(RNAi)	27 (876)
skn-1(RNAi); end-1(ox134)[b]	2 (215)
skn-1(RNAi); end-1(ok558)	2 (235)
skn-1(RNAi); end-3(zu247)	4 (207)
mom-2(RNAi)	89 (78)
mom-2(RNAi); end-1(ox134)[b]	53 (110)
mom-2(RNAi); end-1(ok558)	62 (429)
mom-2(RNAi); end-3(zu247)	0 (137)
src-1(RNAi)	99 (312)
src-1(RNAi); end-1(ox134)[b]	100 (181)
src-1(RNAi); end-3(zu247)	78 (289)

[a] Intestinal cells were scored by gut granule birefringence or *elt-2*::GFP expression (Fukushige et al., 1998).

[b] These strains were also homozygous for *lin-15(−)* and carried *lin-15(+)* and *ric-7(+)* on an extrachromosomal array (N. Andersen and E. Jorgensen, personal communication).

logs and confirmed the predicted coding regions by RT-PCR (Fig. 3A and data not shown). Two of the genes, which we have named *Cb-end-3.1* and *Cb-end-3.2* appear to be relatively recent duplications of an ancestral *end-3* locus, based on their adjacent inverted orientation and nearly identical sequences (Figs. 2 and 3). The *C. briggsae end-1* homolog, *Cb-end-1*, is located ~27 kbp away from the two *end-3* homologs, similar to the ~28 kbp distance between *end-1* and *end-3* in *C. elegans* (Fig. 2). An alignment of the predicted proteins implies that the duplication of *end-1* and *end-3* predates the *elegans-briggsae* evolutionary split, while *end-3* apparently underwent a further duplication in the *C. briggsae* lineage (Fig. 3B). Alignment of all five END proteins reveals two additional regions of conservation in addition to the C_4 zinc finger and basic domain common to all GATA factors (Lowry and Atchley, 2000): the first is a serine-rich region at the amino terminus, and the second is a 10-aa domain found immediately upstream of the zinc finger, which we have called the EGD (END family GATA domain; Fig. 3A). The significance of these conserved domains is not known, but their absence in other GATA factors suggests an involvement in functions unique to the ENDs.

Several experiments indicate that the *C. briggsae end* genes are functionally conserved. A *Cb-end-3.2*::GFP transgene introduced into *C. elegans* is expressed in the early E lineage, similar to *C. elegans end-3* (Fig. 5H) and heat-shock-mediated overexpression of *Cb-end-3.2* in *C. elegans* is sufficient to promote ectopic endoderm and activation of *elt-2*::GFP (Fig. 6J). Further, a genomic fragment containing the *Cb-end-1* homolog can restore endoderm to EDR deficiency embryos (R. Hozak, J. Zhu, and J. H. R., unpublished data). Finally, RNAi experiments indicate that these three genes are apparently functionally

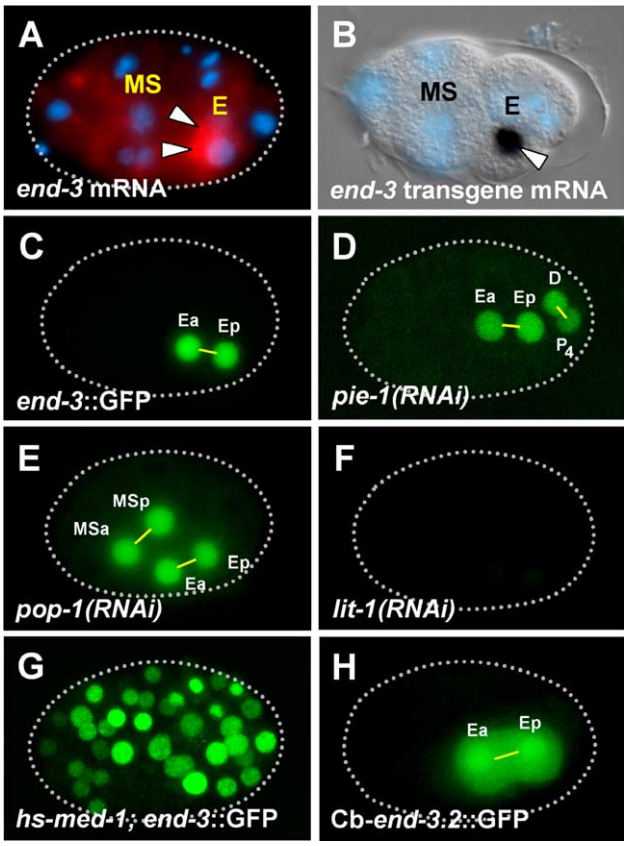

Fig. 5. Expression of *end-3*. (A) Whole-mount in situ (WMISH) hybridization using an anti-sense *end-3* probe shows signal (red) in the E cell (arrowheads). Nuclei, detected by DAPI staining of DNA, are blue. (B) WMISH of an *end-3*::GFP transgene strain with an antisense GFP probe shows nuclear accumulation of transcripts in the E cell, an indication of active transcription (Seydoux and Fire, 1995). An image of DAPI-stained nuclei (in blue) has been merged with the DIC image. (C) Expression of an *end-3*::END-3::GFP reporter in the daughters of E (Ea and Ep). (D) Confocal micrograph of *end-3*::END-3::GFP in a *pie-1(RNAi)* genetic background shows appearance of additional *end-3*-expressing cells correlated with the generation of an ectopic E cell from P_3 (the parent of D and P_4) (Mello et al., 1992). We also observed additional *end-3* expression from the C descendants in some *pie-1* mutant embryos (not shown), consistent with the fraction of *pie-1* mutants in which both C and P_3 adopt E-like fates (Mello et al., 1992). Supporting a requirement for *end* function in the ectopic E-like blastomeres made in *pie-1(RNAi)* embryos, we observed many embryos that failed to make endoderm in *end-3(zu247); pie-1(RNAi)* double mutants (Table 1). (E) Fluorescence micrograph showing *end-3*::END-3::GFP in both the MS and E lineages in a *pop-1 (RNAi)* background, in which MS adopts the fate of the E cell (Lin et al., 1995). As with *pie-1(RNAi)*, many *end-3(zu247); pop-1(zu189)* embryos lacked intestine (Table 1). We note that depletion of *pop-1* actually synergizes with *end-3(zu247)* as a result of the positive activating function of POP-1 in endoderm specification (M.M. et al., manuscript in review). (F) Expression of *end-3*::END-3::GFP is greatly reduced or eliminated in a *lit-1(RNAi)* background, in which E adopts an MS-like fate (Meneghini et al., 1999; Rocheleau et al., 1999). (G) Ectopic expression of *med-1* under heat-shock control is sufficient to drive ectopic expression of *end-3*::END-3::GFP, demonstrating that *med-1* is upstream of *end-3*. (H) Expression of *C. briggsae end-3.2*::NLS::GFP in *C. elegans*. The cytoplasmic signal results from incomplete nuclear localization of GFP from the reporter. In some panels, sister cells are indicated with a line.

redundant for endoderm specification in *C. briggsae*. As *Cb-end-3.1* and *Cb-end-3.2* are nearly identical, dsRNA targeted to one gene is expected to interfere with both (Fire et al., 1998). We found that while RNAi of *Cb-end-1* or *Cb-end-3.1,3.2* failed to show a detectable endoderm defect, the triple *Cb-end-1,3.1,3.2(RNAi)* showed a nearly fully penetrant absence of endoderm in which only 4% of embryos made gut (Table 1 and Fig. 6I). Taken together, these data reveal that the structure, expression, and most notably, the redundant function of the *end* genes have been conserved over at least 50–100 million years of evolution.

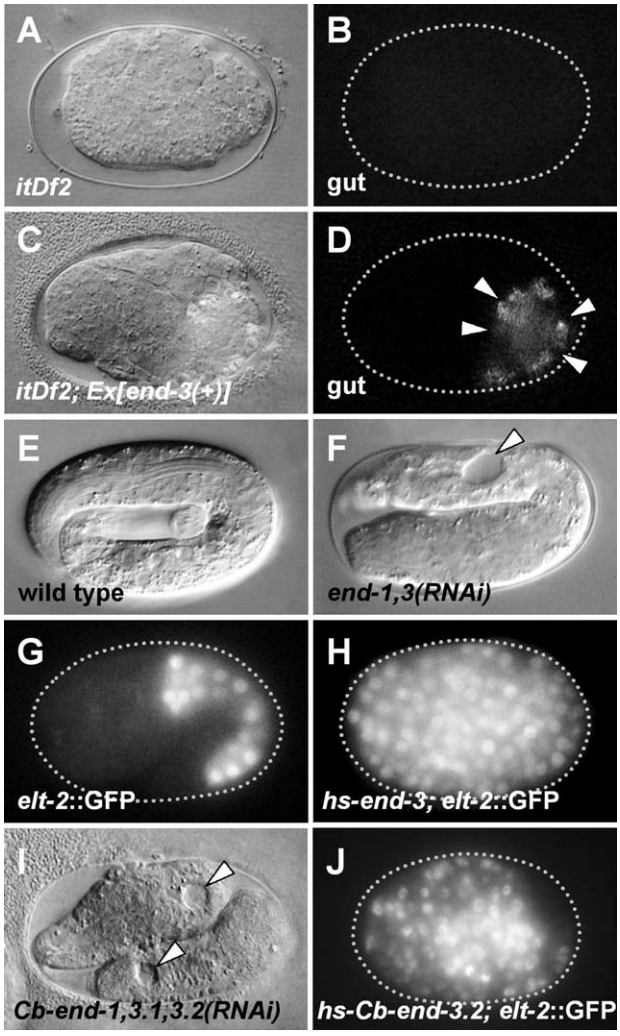

Fig. 6. END-3 promotes endoderm development and functions redundantly with *end-1*. (A–B) An arrested *itDf2* embryo is defective in elongation (A, differential interference contrast (DIC) image) and does not exhibit birefringent gut granules (B, dark field polarized light image). (C–D) An extrachromosomal array containing *end-3* restores endoderm to an *itDf2* homozygous embryo. Although rescued embryos still fail to elongate (C), owing to the absence of other genes deleted by *itDf2*, they display gut granules typical of differentiated endoderm (D). (E) Wild-type three-fold embryo. (F) *end-1,3(RNAi)* embryo arrested at the two-fold stage showing an internal cuticle-lined cavity, resulting from the E → C transformation. A partial lineage of this embryo is shown in Fig. 4A. (G) Fluorescence micrograph showing expression of the intestine marker *elt-2*::GFP (Fukushige et al., 1998) in the developing embryonic gut of a wild-type embryo. (H) Widespread, ectopic expression of *elt-2* occurs throughout the embryo following forced ubiquitous expression of *end-3* from a heat shock (hs) construct. Ectopic ELT-2 expression was also observed in *hs-end-3* embryos lacking an *elt-2* transgene, as detected using an anti-ELT-2 antibody (not shown). (I) Arrested *C. briggsae end-1,3.1,3.2(RNAi)* embryo. Internal cavities similar to those seen for *C. elegans end-1,3(RNAi)* embryos (panel F) are present (arrowheads). (J) Ectopic expression of *Cb-end-3* in *C. elegans* causes generation of ectopic intestine and widespread expression of *elt-2*::GFP. The eggshell is indicated with a dotted line in some panels. *C. elegans* embryos are approximately 50 μm long.

Discussion

Our previous studies provided evidence that the endoderm in *C. elegans* is specified by genetically redundant factors. Here, we present multiple lines of evidence establishing that *end-1* and *end-3* are paralogs that together function to specify the E cell. First, *end-1* and *end-3* are nearby loci separated by ∽28 kbp, consistent with the possibility that they arose from a duplication event. Second, their encoded protein sequences share substantial homology both within the conserved DNA binding domain and in two additional regions (Fig. 3A). Third, deletions that remove a segment of LG *V* (the EDR) containing both genes result in the penetrant absence of endoderm and the conversion of E into a C-like cell (Zhu et al., 1997). Either gene alone is capable of restoring endoderm specification in these deficiency embryos (Table 1 and Figs. 6A–D). Fourth, both *end-1* and *end-3* are expressed in the E cell at the time of its specification (Zhu et al., 1997; Fig. 5). Ectopic expression of either gene is sufficient to initiate a program of endoderm development in non-endodermal cells (Zhu et al., 1998). Finally, we report that E specification in the related nematode *C. briggsae* involves one *end-1*-like gene and two *end-3*-like genes, consistent with a duplication event that preceded the *elegans–briggsae* divergence, estimated to have occurred over 50 myr ago (Coghlan and Wolfe, 2002).

Duplicate genes and genetic redundancy appear at several stages in endoderm development

The action of apparently duplicated, redundant genes during *C. elegans* endoderm development is not restricted to *end-1,3*. The *med-1* and *med-2* genes, whose products directly activate *end-1,3*, are 98% identical but are located on different chromosomes (Maduro et al., 2002, 2001). The ENDs are substantially more divergent than the nearly identical MEDs. In fact, the DNA binding domain of END-1 is less similar to that of END-3 (43% identical) than it is to the ectodermal GATA factor ELT-3 (55% identical) (Gilleard et al., 1999 and data not shown). Apparent targets of the ENDs include another pair of partially redundant GATA factor-encoding genes, *elt-2* and *elt-7* (Fukushige et al., 1998; Maduro and Rothman, 2002). ELT-2 and ELT-7 are more functionally and structurally divergent than are END-1 and -3: mutation of *elt-2* results in fully penetrant larval

lethality (Fukushige et al., 1998) and defective gut differentiation. This phenotype is enhanced when *elt-7* function is depleted in an *elt-2* mutant (K. Strohmaier and J. H. R., unpublished observations). Hence, the endoderm gene regulatory cascade progresses through sequentially acting pairs of GATA factors that appear to share successively less function (Maduro and Rothman, 2002).

In addition to these six GATA factors that act in mesendoderm development, some of the remaining five GATA factors encoded in the *C. elegans* genome appear to function redundantly. Two adjacent genes (*egl-18* and *elt-6*) have overlapping function in the ectoderm (Koh and Rothman, 2001; Koh et al., 2002). The ELT-3 GATA factor, which can specify epidermal fates when ectopically expressed, shows no phenotype when deleted, suggesting that it may function redundantly with another factor (Gilleard et al., 1999; Gilleard and McGhee, 2001). The tiny GATA factor-encoding gene *elt-4*, an apparent duplication of part of *elt-2*, appears to lack function (Fukushige et al., 2003). The remaining GATA factor, ELT-1, is essential for ectodermal fate specification (Page et al., 1997). Curiously, of the GATA factors known to be required for some aspect of *C. elegans* development, only ELT-1 contains two zinc fingers, and only ELT-1 is known to act non-redundantly. In *Drosophila*, the *serpent* (*srp*) gene, which appears to perform the same function in endoderm development as the two *end* genes in *C. elegans*, encodes two GATA factors, one with a single zinc finger (SrpC), and one with two zinc fingers (SrpNC) (Waltzer et al., 2002). The amino-terminal finger of SrpNC isoform allows interaction with the cofactor U-shaped and stabilizes the interaction of SrpNC with palindromic GATA sites (Waltzer et al., 2002). An intriguing possibility, therefore, is that GATA factor pairs in *C. elegans* generate functional versatility through homotypic and heterotypic interactions or differential association with cofactors.

Structural clues to END GATA factor function

The END proteins appear to be highly specific for their endoderm-promoting activity; for example, ectopic expression of these proteins at high levels is able to activate the network of gene activity appropriate for endoderm development in non-endodermal progenitors. In similar experiments, another GATA factor, ELT-3, shows a distinct activity, the ectopic activation of epidermal development (Gilleard and McGhee, 2001). While it seems likely that the DNA binding domains of these transcription factors account for their differences in specificity, we have been unable to identify a signature sequence that is suggestive of endoderm-specific action of the END proteins. Indeed, the DNA binding domains of END-1 and ELT-3 are more similar than are these domains in the two END proteins (data not shown). However, comparisons of the two *elegans* and three *briggsae* proteins reveal two elements outside the DNA binding domain that are likely to be critical for their activity. One might speculate that the serine-rich sequence at the extreme amino termini of all the proteins might be a site for phosphorylation, for example, while the EGD immediately upstream of the DNA binding domain might be a site for interactions with other proteins that collaborate with the ENDs to direct its endoderm-specific activation function.

The END-3[P202L] mutation may provide some clues as to the important structural elements in the protein. This lesion would be expected to abrogate wild-type END-3 function, since it occurs in the DNA binding domain (Fig. 3), consistent with our genetic experiments showing that it acts as a strong loss-of-function mutation. However, it is somewhat surprising that this particular position (immediately upstream of the fourth cysteine in the C4 zinc finger) is not conserved. Within *C. elegans*, only ELT-5 and END-3 contain a proline at this position, while END-1 contains glycine, and the remaining eight GATA factors alanine, the residue that is typical for the vertebrate GATA factors (Lowry and Atchley, 2000). There is a serine in this position in *Cb*-END-3.1/3.2, while the single *Cb*-END-1 homolog retains glycine. The occurrence of proline in the wild-type ELT-5 and END-3 zinc fingers is somewhat paradoxical, as this residue would be expected to disrupt the α-helical structure formed by the corresponding regions in chicken cGATA1 and *Aspergillus* AreA, which contain alanine at this position (Omichinski et al., 1993; Starich et al., 1998a,b). Indeed, an alanine to proline mutation at this site causes loss of AreA function (Kudla et al., 1990; Platt et al., 1996). The context of this amino acid is evidently important, and the relevance of this residue within the DNA binding domain of END-3 should become clear once the three-dimensional structure of the protein has been determined.

Unequal but synergistic contributions of END-1 and END-3

Our data suggest that although *end-1* and *end-3* share overlapping functions, they make unequal contributions to endoderm specification. Two mutations of *end-1* that remove part (*ok558*) or all (*ox134*) of the DNA binding domain show no discernible phenotype (Table 1). In contrast, *end-3(RNAi)* and the *end-3(zu247)* point mutation result in an impenetrant defect in endoderm specification (Table 1). Moreover, stronger synergy is observed with *zu247* in combination with *mom-2(RNAi)* or *src-1(RNAi)*, than with either *end-1* mutant.

A similar unequal requirement is seen with other examples of genetically redundant gene pairs that have arisen by duplication. For example, the *C. elegans* Notch proteins LIN-12 and GLP-1 perform an essential, but genetically redundant zygotic function in the embryo: a double mutation in both *lin-12* and *glp-1*, but not either mutation alone, gives a penetrant lethal phenotype (the "Lag" phenotype) owing to misspecification of particular epidermal and rectal cells (Lambie and Kimble, 1991; Moskowitz and Rothman, 1996). However, while zygotic loss-of-function mutations in *glp-1* do not lead to an

obvious embryonic phenotype, *lin-12* null mutations give rise to Lag animals at a low frequency, in analogy to what we have observed with *end-3* mutants. Similarly, the duplicated *Drosophila* genes *engrailed* and *invected*, which share common transcriptional regulatory domains, are genetically redundant for regulation of segmentation (Gustavson et al., 1996). Mutants lacking *engrailed* alone show a moderate segmentation phenotype, whereas *invected* single mutants appear wild-type.

Does the difference in requirement for *end-1* and *-3* reflect simply differences in their levels of expression or fundamentally distinct activities in their encoded proteins? Although we have not rigorously quantified expression levels, *end-3* reporter fusions appear to be expressed at somewhat higher levels than *end-1* reporters in several lines examined. Indeed, there are four sites for MED-1 in *end-3*, and only two in *end-1*, suggesting that *end-3* may be more efficiently targeted for activation than *end-1* (Broitman-Maduro et al., 2005). Regardless of the mechanism responsible, it will be of interest to learn whether the unequal requirement for the *end* genes, with a somewhat more critical role for *end-3*, is a conserved feature of their action. Our initial results with RNAi experiments in *C. briggsae* do not support a differential requirement for the *end* genes; however, it is curious to note that *end-3*, which appears to be somewhat more critical in *C. elegans*, is the gene that is duplicated in *C. briggsae*.

Evolutionary conservation of genetic redundancy

The mild (or non-existent) phenotypes of the individual *end* mutations, and the ability of either *end-1* or *-3* to rescue the endoderm specification defects in *Df(EDR)* and *end-3(zu247)* embryos, prompt the question as to why both genes have been maintained through evolution. One hypothesis is that each gene may have accumulated degenerative mutations that are mutually complementary (Force et al., 1999); however, this does not seem to account for the complete absence of phenotype of apparent null mutations in *end-1*. Alternatively, there may be conditions encountered by embryos in their natural environment, but not in the laboratory, in which each gene provides a crucial input. Such a possibility would argue that the redundancy engenders robustness in the system, ensuring fidelity under widely varying conditions. Finally, it is conceivable that the two genes truly are redundant for endoderm specification even under extreme growth conditions and the duplication of the genes reflects their co-option into other processes distinct from specification of the E lineage. Arguing for such a possibility is the finding that, based on many microarray experiments performed under very different conditions, the patterns of expression of the two genes are not correlated: i.e., the genes reside in different “mountains” of the *C. elegans* expression topomap (Kim et al., 2001). An example of such deployment of a transcription factor in both early specification and in an environmental response system is provided by SKN-1. The protein, which initiates the mesendoderm gene regulatory network, acts in a completely different guise to regulate oxidative stress response in the fully differentiated intestine and chemosensory neurons (An and Blackwell, 2003). Analysis of *end* gene expression under varying conditions might identify a role for either *end-1* or *end-3* that is distinct from its action in endoderm specification.

Acknowledgments

We thank A. Fire for reporter plasmids, Y. Bei for the *src-1* cDNA clone, J. McGhee and T. Fukushige for the anti-ELT-2 antibody, K. Schuske and E. Jorgensen for the *end-1 (ox134)* mutation, and R. Weimer and E. Jorgensen for the *ric-7*-rescued strain EG2894. We thank Rebecca Hozak for identifying the first clone of *C. briggsae end-1* and providing evidence that it rescues *itDf2*. We are grateful to many members of the Rothman lab over the many years that this project developed for useful discussions. 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). M.F.M. was supported in part by a fellowship from the Natural Sciences and Engineering Research Council of Canada (NSERC), E.D.N.-S. by an NIH NRSA fellowship, R.J.H. by a Jane Coffin Childs Memorial Fund fellowship and the Howard Hughes Medical Institute, and P.J.H. by an NIH Cell and Molecular Biology Training Grant. This work was supported by the Howard Hughes Medical Institute and a grant from the NIH to J.P. and from the NIH (HD37487) and March of Dimes Birth Defects Foundation (FY96-1081 and FY98-0391) to J.H.R. Some of the *end-3* expression analysis was supported by University of California, Riverside, startup funds to M.F.M.

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