Developmental Biology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Endoderm development in *Caenorhabditis elegans*: The synergistic action of ELT-2 and -7 mediates the specification→differentiation transition

Erica M. Sommermann [1], Keith R. Strohmaier [1], Morris F. Maduro [2], Joel H. Rothman *

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

ARTICLE INFO

Article history:
Received for publication 4 June 2010
Revised 24 July 2010
Accepted 18 August 2010
Available online xxxx

Keywords:
Caenorhabditis elegans
Endoderm
Differentiation
GATA transcription factor
Autoregulation

ABSTRACT

The transition from specification of cell identity to the differentiation of cells into an appropriate and enduring state is critical to the development of embryos. Transcriptional profiling in *Caenorhabditis elegans* has revealed a large number of genes that are expressed in the fully differentiated intestine; however, no regulatory factor has been found to be essential to initiate their expression once the endoderm has been specified. These gut-expressed genes possess a preponderance of GATA factor binding sites and one GATA factor, ELT-2, fulfills the expected characteristics of a key regulator of these genes based on its persistent expression exclusively in the developing and differentiated intestine and its ability to bind these regulatory sites. However, a striking characteristic of *elt-2(0)* knockout mutants is that while they die shortly after hatching owing to an obstructed gut passage, they nevertheless contain a gut that has undergone complete morphological differentiation. We have discovered a second gut-specific GATA factor, ELT-7, that profoundly synergizes with ELT-2 to create a transcriptional switch essential for gut cell differentiation. ELT-7 is first expressed in the early endoderm lineage and, when expressed ectopically, is sufficient to activate gut differentiation in nonendodermal progenitors. *elt-7* is transcriptionally activated by the redundant endoderm-specifying factors END-1 and -3, and its product in turn activates both its own expression and that of *elt-2*, constituting an apparent positive feedback system. While *elt-7* loss-of-function mutants lack a discernible phenotype, simultaneous loss of both *elt-7* and *elt-2* results in a striking all-or-none block to morphological differentiation of groups of gut cells with a region-specific bias, as well as reduced or abolished gut-specific expression of a number of terminal differentiation genes. ELT-2 and -7 synergize not only in activation of gene expression but also in repression of a gene that is normally expressed in the valve cells, which immediately flank the termini of the gut tube. Our results point to a developmental strategy whereby positive feedback and cross-regulatory interactions between two synergistically acting regulatory factors promote a decisive and persistent transition of specified endoderm progenitors into the program of intestinal differentiation.

Introduction

Two central challenges facing metazoan embryos are imbuing progenitor cells that arise from a single fertilized egg with distinct properties and activating their ensuing differentiation into tissues with unique functions. For development to succeed, the differentiation program must ensure a rapid and robust transition from specification, coordinate the proper patterning of cells in organ systems, and lock down the terminally differentiated state of all cells. Understanding how these biological switches are controlled is pivotal to our understanding of animal development.

An effective model system for illuminating the mechanisms at the interface between the programs of specification and differentiation is provided by the *Caenorhabditis elegans* endoderm. As revealed over a century ago, the endoderm in nematodes arises exclusively from one blastomere, the E cell, in the early embryo (Boveri, 1893, 1899). Through a determinate pattern of 4–5 rounds of cell division, E gives rise to the 20 cells of the intestine, the sole endoderm-derived organ (Sulston et al., 1983). These 20 cells are organized into an epithelial tube consisting of 9 intestinal rings, or "ints," with four cells forming int1 and two in each of the remaining rings (Leung et al., 1999). The intestine comprises the midgut of the *C. elegans* alimentary tract, connecting to the pharynx (foregut) and rectum (hindgut) by interfacing with sets of valve cells on either termini of the gut tube. The differentiating intestine arising from the E lineage must coordinate with its neighbors to engender a functional digestive organ system.

The well-described regulatory pathway for endoderm links early maternal genes through a series of intermediary regulators to

* Corresponding author. Fax: +1 805 893 2005.
E-mail address: rothman@lifesci.ucsb.edu (J.H. Rothman).
[1] These authors contributed equally to this paper.
[2] Present address: Department of Biology, University of California, 2121A Genomics Building, Riverside, Riverside, CA 92521, USA.

0012-1606/$ – see front matter © 2010 Published by Elsevier Inc.
doi:10.1016/j.ydbio.2010.08.020

terminal structural proteins and enzymes comprising the intestine. A maternal transcription factor, SKN-1 (Bowerman et al., 1993, 1992), initiates the transcriptional cascade for endoderm development. Its immediate zygotic targets are two redundant genes encoding the atypical GATA transcription factors MED-1 and -2 that specify both E and its sister, the mesoderm-producing MS cell (Broitman-Maduro et al., 2005; Maduro et al., 2007, 2001). In the E lineage, the MEDs directly activate expression of two GATA factor-encoding genes, *end-1* and *end-3*, which are redundantly required to specify the entire endoderm (Maduro et al., 2005; Zhu et al., 1997). Removal of both genes causes E to adopt the fate of its cousin, C, a progenitor of mesectoderm (Bowerman et al., 1993; Maduro et al., 2005; Zhu et al., 1997). While *end-1* and *-3* are expressed only until the E4 and E8 cell stages, respectively (Baugh et al., 2003; Maduro et al., 2007), they activate expression of another GATA factor, *elt-2*, which maintains its own expression throughout development (Fukushige et al., 1998, 1999; Hawkins and McGhee, 1995; Maduro and Rothman, 2002). ELT-2 also binds directly to transcriptional regulatory elements of genes encoding structural components and enzymes of the differentiated gut (Fukushige et al., 1998, 1999; Hawkins and McGhee, 1995). This transcriptional cascade appears to be a conserved mechanism for endoderm specification and differentiation across metazoa. In *Drosophila*, for example, the SERPENT GATA factor, which specifies endoderm in the embryo, activates dGATAe, whose expression persists through adulthood and which initiates gene expression for terminal gut differentiation (Murakami et al., 2005; Okumura et al., 2005). GATA factors have also been found to specify endoderm throughout the vertebrates, implying a pan-triploblastic mechanism for endoderm formation (Murakami et al., 2005; Okumura et al., 2005; Shivdasani, 2002).

The first known terminal marker of intestinal differentiation in *C. elegans*, the GES-1 gut esterase, was identified nearly 25 years ago (Edgar and McGhee, 1986). Subsequent studies revealed that GATA-type regulatory sequences are required for endoderm-specific expression of the *ges-1* gene, leading to identification of the endoderm-specific ELT-2 GATA factor based on its ability to bind these sequences (Fukushige et al., 1998, 1999; Hawkins and McGhee, 1995). Comprehensive transcriptional profiling of isolated embryonic and adult intestines revealed that a common element linking all gut-expressed genes is an extended TGATAA-like consensus binding site sequence, which, in some cases, has been shown to be essential for gut-specific expression, suggesting that gut-specific differentiation is broadly controlled by GATA factors (McGhee et al., 2009; McGhee et al., 2007; Pauli et al., 2006). Confirming that it acts in gut formation or function, deletion of *elt-2* results in an obstructed gut at the anteriormost intestinal rings, resulting in L1 larval lethality. While two other GATA factors, ELT-7 and ELT-4, are also expressed in the developing endoderm (Baugh et al., 2003; Fukushige et al., 2003); this study), ELT-4 shows no discernible function *in vivo* or *in vitro* (Fukushige et al., 2003) and no phenotype is apparent in *elt-7(0); elt-4(0)* double mutants, leading to the suggestion that ELT-2 is the dominant, and perhaps sole required regulator of intestinal differentiation (McGhee et al., 2009, 2007). However, such a conclusion poses the challenge that the gut in *elt-2(0)* mutants appears morphologically as fully differentiated as that of wild-type worms, with a complete lumen, well-developed brush border, and characteristic rhabditin granules throughout all gut cells (Fukushige et al., 1998) (this work). Moreover, transcription of *ges-1* and other genes is robustly activated in *elt-2* mutant embryos (McGhee et al., 2009). These observations make it clear that other factor(s) likely function to mediate the critical specification-to-differentiation transition during endoderm development.

Here we report that ELT-7, acting with ELT-2, is a key component of the intestinal developmental program, explaining how gut differentiation is initiated. *elt-7* is activated by the END-1/3 GATA factors, is first expressed before *elt-2*, and is sufficient to activate gut differentiation in ectopic lineages. We find that *elt-7(0);elt-2(0)* double-knockout mutants fail to express a number of markers of gut differentiation, including GES-1, and are profoundly defective in gut differentiation in a regionalized manner, revealing an apparent underlying all-or-none differentiation switch. Finally, we find that ELT-7 and ELT-2 also synergize to repress transcription of a gene whose expression is normally limited to the valve cells flanking the gut tube, suggesting that activation of gut differentiation acts to exclude differentiation of non-gut cell types of the digestive tract. Our findings suggest a model in which the auto- and cross-regulatory action of ELT-2 and -7 initiates and locks down gut differentiation, thereby directing the transition from specification of endoderm fate to the persistent differentiated state of the intestine.

Materials and methods

elt-7 reporter constructs

Several different *elt-7*::GFP reporter constructs containing 1 kb or more of upstream sequence between the predicted translation start sites of *elt-7* and neighboring predicted protein-coding region C18G1.9 were created. (Oligonucleotide sequences are available on request.) The largest construct contained 2647-bp upstream of the *elt-7* ATG, which includes almost 90% of C18G1.9. Another construct was made by fusing GFP to the amino terminus of the entire *elt-7* protein coding region with 1 kb of upstream and 660 bp of downstream sequence, which includes the entire 3′-UTR found in a NEXTDB cDNA clone (Kohara, http://nematode.lab.nig.ac.jp/) plus an additional 500 bp. The transcriptional fusion reporters shown in this paper, containing 1 kb of upstream sequence, produced expression patterns identical to all other reporter constructs tested.

Ectopic expression of GATA factors

Gravid adult animals (for embryonic heat shock) or larvae growing on agar plates were incubated at 34 °C for 30 minutes. After incubation, adults were allowed to lay embryos for 2 hours before being removed from plates. Heat-shocked embryos or larvae were then placed at 20 °C overnight and were observed the following day.

Genetics

All genetic manipulations were performed according to standard techniques (Ahringer, 2006). Two methods were used to generate *elt-7(−);elt-2(−)* double mutants and the phenotypes seen with both approaches were indistinguishable. (1) RNAi knockdown of *elt-7* transcripts was performed on strain JR2531 *elt-2(ca15); wEx1527 [sur-5*::*GFP, elt-2(+)]* mothers using standard injection or feeding procedures (Fire et al., 1998; Timmons and Fire, 1998) and *elt-2(ca15)* homozygotes were identified as non-GFP-expressing embryos or larvae. (2) Strain MS851 *elt-2(ca15);irEx404 [unc-119*::*CFP, elt-2(+)]* was crossed with strain FX840 *elt-7(tm840)* to generate the double-mutant strain JR3295 *elt-7(tm840);elt-2(ca15);irEx404 [unc-119*::*CFP, elt-2(+)]* and homozygous double mutants were identified as those not expressing the CFP marker. The *ca15* deletion removes the entire *elt-2* coding sequence (Fukushige et al., 1998). The *tm840* deletion removes exons two and three, including the first 22 amino acids of the DNA-binding domain of *elt-7* (Supp. Fig. 1) (WormBase Web site, http://www.wormbase.org, release WS213, 31 May 2010). The *elt-2(+)* rescuing array is transmitted to ~75% of MS851 offspring and to ~90% of JR3295 offspring.

Immunofluorescence analysis

Embryos and L1 larvae were fixed and stained for immunofluorescence by methanol/acetone fixation on slides. Embryos were

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harvested from plates and washed with M9 buffer. L1 larvae were obtained by bleaching gravid adults and allowing embryos to hatch overnight and synchronize as L1s in the absence of food. Freeze-crack permeabilized embryos and larvae on poly-L-lysine slides were fixed in methanol and acetone at −20 °C in succession for 2 minutes each, air-dried, then rehydrated in a series of ethanol/PBST in 1-minute washes. Samples were blocked with 1% BSA in PBST (PBS + 0.1% Tween-20) for 30 minutes before incubation with primary antibodies. All primary antibodies were incubated overnight at 4 °C, rinsed three times in PBST, and incubated for 4 hours at room temperature in a 1:100 dilution of either TRITC-, Cy3-, or FITC-conjugated secondary antibodies. Slides were rinsed three times for 5 minutes in PBST and then mounted for microscopy with Vectashield with DAPI (Vector Labs, Burlingame, CA). Slides were viewed on a Zeiss Axioskop 2 microscope and pictures were taken with a DVC digital camera using DVC acquisition software or with MicroSuite Images software. Confocal images were taken with an Olympus Fluoview 500 confocal microscope (Center Valley, PA, USA). Some images were processed using Adobe Photoshop.

Detection of cdf-1 message by in situ hybridization

A probe template of approximately 600 bp was amplified from *cdf-1* cDNA and tagged at one end with a T7 RNA polymerase recognition site by PCR (Forward primer 5′-ggtcacagtcatgcaaatgg-3′ and reverse primer 5′-taatacgactcactatagggactccacacagacagcttttcca-3′). RNA probe was then generated using the Roche DIG RNA labeling mix (cat. no. 11277073910). Synchronized L1s were obtained by allowing bleached embryos to develop overnight in M9 in the absence of food. mRNA detection in L1 larvae was performed as follows: freeze-crack-permeabilized L1's were fixed in ice-cold methanol and then rehydrated in an ethanol/DEPC H_2O series; L1 larvae were treated with Streck Tissue Fixative (Streck, Inc.) for 1 hour at 37 °C and then washed once with DEPC H_2O and twice with 2× SSC (from Fisher 20× SSC #BP1325-1) before hybridization. Worms were prehybridized for 1 hour at 42 °C in humid chambers with prehybridization buffer (4× SSC, 10% dextran sulfate, 1× Denhardt's [0.02% Ficoll, 0.02% polyvinyl pyrolidone, 0.02% BSA], 2 mM EDTA, 50% deionized formamide (from Ultra Pure Grade, AMRESCO 0606-500 ml), 500 μg/ml herring sperm DNA, brought to volume with DEPC H_2O) and then hybridized with probe at 42 °C overnight. Posthybridization washes were performed at 42 °C twice with 2× SSC, twice with 60% formamide, and once with 0.2× SSC, then at room temperature twice with 2× SSC and once with TN (100 mM Tris pH 7.5 (Fisher BP-1757-500), 150 mM NaCl). Samples were blocked with <5% dried milk in TN for 30 minutes at room temperature and then incubated with 1:2000 dilution of anti-DIG-alkaline phosphatase antibody (Roche: anti DIG-AP # 1.093.274) in milk blocker for 2 hours at 37 °C. Samples were washed twice in TN and once in TNM (100 mM Tris, pH 9.5, 150 mM NaCl, 50 mM $MgCl_2$) and then developed overnight, protected from light, in freshly prepared 1× TNM, 5% PVA, 0.24 mg/ml levamisole with 200 μl each BCIP and NBT.

Results

elt-7 expression suggests a function in gut differentiation

Possessing a single C_4-GATA-type zinc finger and a C-terminal region enriched in basic residues, ELT-7 is one of eleven GATA transcription factors encoded in the *C. elegans* genome (Lowry and Atchley, 2000; Maduro and Rothman, 2002; Patient and McGhee, 2002). We analyzed *elt-7* expression by constructing GFP reporters and found that, as with *elt-2*::*gfp* (Fukushige et al., 1998), it is continuously expressed at high levels exclusively in all cells of the gut lineage, starting at the 2E cell stage and progressing through adulthood (Fig. 1 and data not shown). The expression appears strongest during embryogenesis (Figs. 1C and D) and diminishes somewhat after hatching, consistent with endogenous expression measured in genome-wide microarray expression studies of embryonic and adult intestines (Hill et al., 2000; McGhee et al., 2009, 2007). Further, genome-wide transcriptional profiling performed on staged embryos (Baugh et al., 2003) indicated that endogenous *elt-7* expression is first detectable approximately 1 hour before the stage at which *elt-2* transcripts are first detectable and presages activation of the gut differentiation program. These observations raise the possibility that ELT-7 may play a key role in initiating gut differentiation.

ELT-7 is sufficient to promote gut differentiation in normally non-endodermal lineages

If ELT-7 regulates intestinal differentiation, we reasoned that its ectopic expression might cause non-gut progenitor cells to differentiate into gut-like cells, as has been seen with END-1, END-3, and ELT-2 (Bossinger et al., 2004; Fukushige et al., 1998; Maduro et al., 2005; Zhu et al., 1998). Indeed, we found that ubiquitous *elt-7* expression driven by heat-shock-dependent transcriptional control of the ELT-7 coding sequence (*hs-elt-7*) not only results in arrested embryonic development but also causes most nuclei of the arrested embryos to adopt the characteristic morphology of differentiated intestinal cells (Fig. 2B). However, in marked contrast to what has been observed with heat shock-induced expression of *end-1* and *end-3*, where arrest, lethality, and ectopic expression of gut markers occurs only during a restricted window of time during early development when cells are still undifferentiated (Zhu et al., 1998) (and our unpublished results), we found that high ubiquitous levels of ELT-7 generated by heat shock later in development are sufficient to cause developmental arrest and lethality in both late-stage embryos and early to midstage larva, suggesting that ELT-7 is capable of affecting differentiated tissues late in development (not shown).

The gut-like morphology of nuclei present throughout arrested *hs-elt-7* embryos suggested that gut differentiation might be activated broadly in response to ubiquitous ELT-7 expression. Indeed, we found that heat-shocked *hs-elt-7* embryos show widespread expression of the gut differentiation markers IFB-2, an intermediate filament specific for the fully developed intestinal brush border (Bossinger et al., 2004), and the gut-expressed antigen recognized by antibody 1CB4 (Bossinger et al., 2004; Okamoto and Thomson, 1985). While in wild-type animals, IFB-2 is continuously expressed along length of the intestine flanking the lumen (Fig. 2B), it appears as a scattered group of rings throughout the entire embryo in terminal *hs-elt-7* embryos (Fig. 2D). This pattern of large rings may result from polarization of clusters of cells in the same orientation. In wild-type animals, 1CB4 staining is strongest along the lumen of the intestine, but less intense staining also occurs around the remaining periphery of the gut cells, as well as in the rhabditin granules (Fig. 2E). In *hs-elt-7* arrested embryos, most 1CB4 staining is reminiscent of the less intense staining seen at the basolateral periphery of wild-type intestinal cells, although occasionally some spots are observed with more prominent staining, as seen with the lumen of wild-type animals (Fig. 2F).

Further validating the capacity of ELT-7 to function as an activator of the broad program for gut differentiation, we found that GFP reporters for the later terminal gut markers PEP-2 and PHO-1 were also expressed throughout heat-shocked *hs-elt-7* embryos (Beh et al., 1991; Meissner et al., 2004). *pep-2*::GFP shows extremely high gut-specific expression in wild-type animals (Fig. 2G) and is strongly expressed throughout the entire embryo in *hs-elt-7* arrested embryos (Fig. 2H). *pho-1*::LacZ::GFP is highly expressed in the nuclei of all intestinal cells, with the exception of the six most anterior cells (Fig. 2I). In arrested *hs-elt-7* embryos, virtually all nuclei express this reporter (Fig. 2J). Taken together, these results indicate that ELT-7 is sufficient to drive a comprehensive program for intestinal differentiation.

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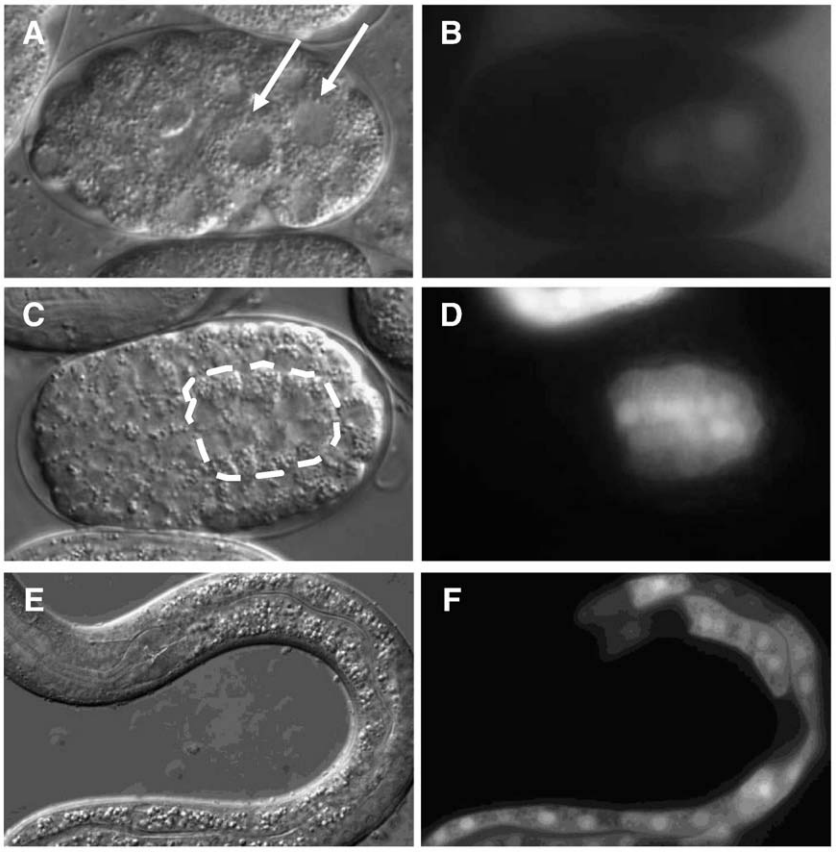

Fig. 1. *elt-7::GFP* is expressed strongly and exclusively in the endoderm. (A) Arrows point to the two gut progenitor cells that have just entered the interior of the embryo during the onset of gastrulation in this DIC image of a wild-type embryo. (B) Fluorescent image of the embryo in A shows expression of an *elt-7::GFP* transcriptional fusion reporter that is first detectable at the late 2E cell stage. (C) DIC image of a wild-type embryo at the 16E cell stage, just prior to elongation. The gut cells are outlined. (D) Fluorescence image of the embryo in C shows that expression of the reporter has greatly increased by this stage. (E and F) Reporter expression persists throughout the organ as seen in this L2 stage larva.

Cross-regulatory interactions between endodermal GATA factors

The presence of 11 GATA consensus binding sites around *elt-7*, including four within the 1 kb region upstream of the coding region and three within the first intron (Supp. Fig. 1), and the timing of its expression pattern, suggest that *elt-7* expression might be regulated by the endoderm-specifying END-1 or END-3 GATA factors. Indeed, we found that widespread expression of either END-1 or END-3 driven under heat-shock control results in activation of an *elt-7::lacZ::GFP* reporter throughout early embryos (Figs. 3A and B). This effect does not reflect a general ability of GATA factors to activate *elt-7* transcription, as we found that widespread expression of the ectoderm-specific GATA factor, ELT-1, under the same conditions, does not affect the normal expression of the *elt-7* reporter (data not shown).

As ELT-7 is first expressed before ELT-2 during endoderm development, it might be capable of activating *elt-2* transcription. We found that high levels of ELT-7 can indeed drive expression of *elt-2::LacZ::GFP* in both embryos and larvae (Figs. 3E and F), consistent with the placement of ELT-7 upstream of *elt-2* in a GATA factor transcriptional cascade. However, we also found that ELT-2 can activate an *elt-7::lacZ::GFP* reporter (Figs. 3C and D), demonstrating cross-regulatory interactions between these genes. We observed one unexpected and significant difference between these interactions: while heat shock-induced END-1, END-3, and ELT-2 can cause ectopic gut marker expression in only a small window of time during early development (Zhu et al., 1998) (our unpublished results), we found that late expression of *hs-elt-7*, even in larvae, could activate the *elt-2::lacZ::GFP* reporter (Fig. 3F). Thus, based on these experiments, there appears to be a special relationship between ELT-7 and its target, *elt-2*, in that the latter does not become refractory to activation by the former at any time during embryogenesis but remains susceptible to activation continuously throughout development.

elt-7 is expressed throughout development and in adults, raising the possibility that it might maintain its own expression through an autoregulatory activity. Indeed, based on heat-shock-induced expression experiments, we found that ELT-7 is capable of activating an *elt-7* transcriptional reporter (Fig. 3D). Thus, as has been shown for *elt-2* (Fukushige et al., 1999; Zhu et al., 1998), *elt-7* appears to be both a target of the gut specification factors and is capable of activating its own expression through a presumed autoregulatory loop. Further, the cross-regulatory activity of ELT-7 implicates it as a mediator of sustained *elt-2* activation via a feedback loop that is more complex in architecture than previously suggested.

ELT-2 and ELT-7 function synergistically to promote morphological differentiation of the intestine

In agreement with other reports (McGhee et al., 2007), we were unable to detect any effect on larval and embryonic viability, brood size, growth, movement, or morphology in either *elt-7(tm840)* knockout mutants or in RNAi-sensitized *rrf-3(pk1426)* worms in which *elt-7* is knocked down by RNAi. Of particular relevance, *elt-7 (tm840)* homozygotes contain a morphologically normal gut with a continuous intestinal lumen and gut granules of wild-type intensity and distribution (Figs. 4A and B). Similarly, while worms carrying the *elt-2(ca15)* knockout mutation die shortly after hatching, apparently as the result of an obstructed gut that cannot pass macerated bacteria arriving from the pharynx (Fukushige et al., 1998), morphological differentiation of the gut appears to proceed essentially normally: the

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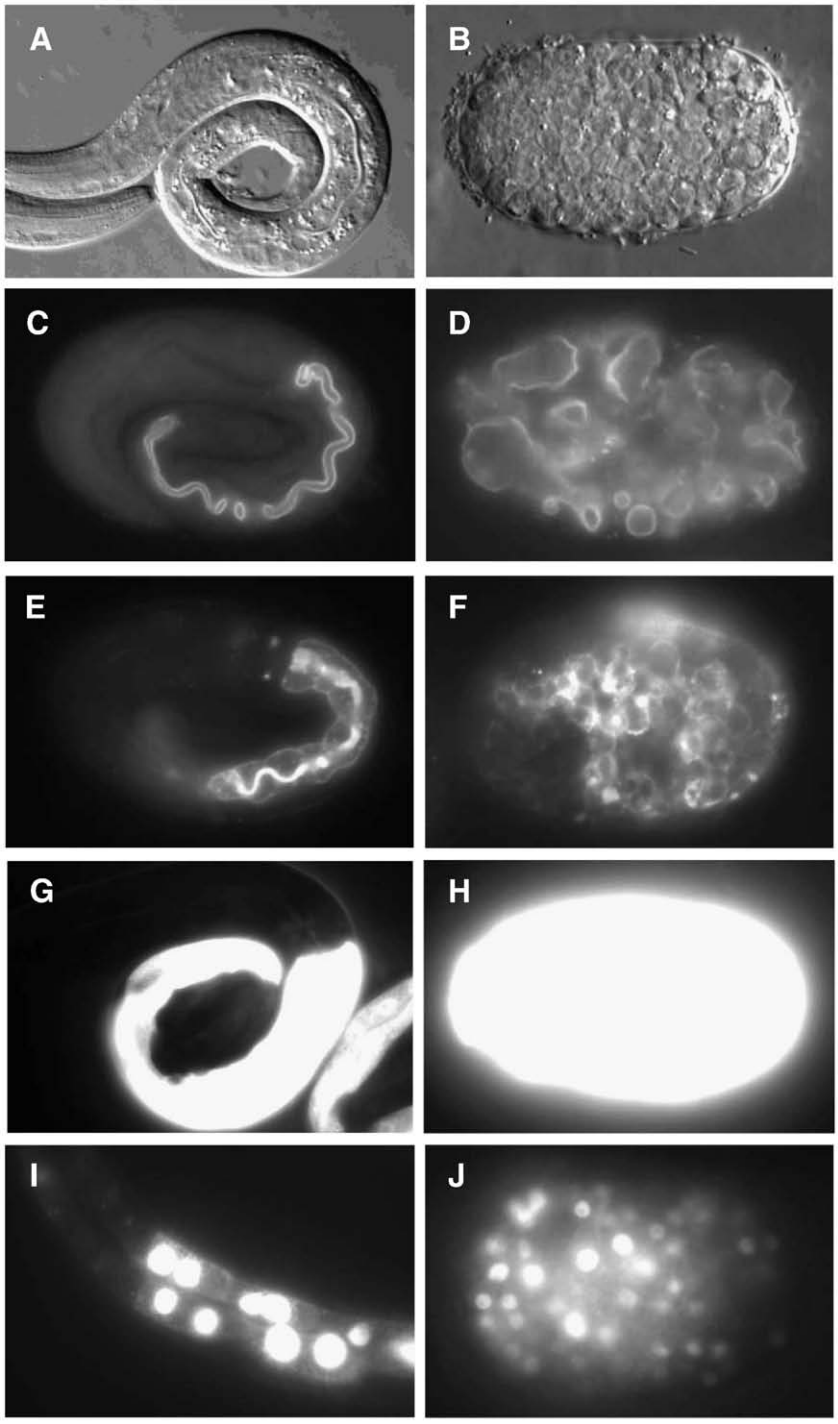

Fig. 2. ELT-7 is sufficient to activate ectopic gut differentiation. Early embryos carrying *hs-elt-7* were heat-shocked and allowed to develop overnight. (A) Wild-type embryos that are not heat-shocked continue development and hatch. (B) Virtually all cells in arrested transgenic embryos following a heat-shock contain nuclei with the "fried egg" morphology characteristic of differentiated intestinal cells. (C) MH33 staining of wild-type IFB-2 expression is gut-specific in an elongated embryo. (D) MH33 staining is seen throughout an arrested *hs-elt-7* embryo as a series of rings of varying size. (E) 1CB4 stains the intestine of wild-type embryos beginning at the onset of elongation, similar to MH33; pharyngeal gland cells also stain with this antibody, seen just anterior to the gut. (F) 1CB4 staining in an arrested *hs-elt-7* embryo extends throughout the embryo. Strong staining, similar in intensity to that seen in the gut lumen, is observed around individual cells. (G) *pep-2*::GFP in a wild-type L1 larva. (H) *pep-2*::GFP fills an arrested *hsp*::ELT-7 embryo. (I) *pho-1*::*lacZ*::*GFP* expression begins shortly before hatching and remains high throughout the rest of development, as shown in this L3 larva. (J) Virtually all nuclei of an arrested *hs-elt-7* embryo express *pho-1*::*lacZ*::*GFP*.

gut contains a typical continuous lumen throughout the remainder of the intestine, a well-developed brush border, and abundant rhabditin granules (Figs. 4C, D, and H).

Given the high degree of genetic redundancy observed in the endoderm specification pathway (Maduro et al., 2005, 2001), it seemed possible that the pattern of redundancy might extend to ELT-2 and ELT-7, whose expression overlaps throughout virtually all stages of endoderm development. We constructed double mutants carrying deletions of *elt-2* and *elt-7* (see Materials and methods) and found that, in stark contrast to either single mutant, morphological gut differentiation is dramatically disrupted in both *elt-2(ca15);elt-7 (tm840)* and *elt-2(ca15);elt-7(RNAi)* animals, which invariably arrest

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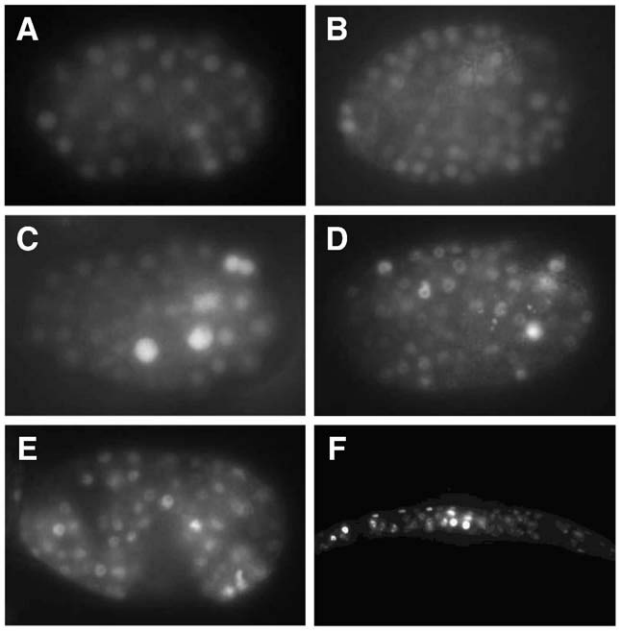

Fig. 3. Activation of *elt-7*::*GFP* and *elt-2*::*GFP* by gut-specific GATA factors. (A–D) Expression of an *elt-7*::*lacZ*::*GFP* transcriptional fusion reporter is driven by integrated *hs-end-1* (A), *hs-end-3* (B), *hs-elt-2* (C), and *hs-elt-7* (D) constructs following heat shock of early embryos and overnight development. The reporter is expressed throughout the embryo in each case. (E) Expression of an *elt-2*::*lacZ*::*GFP* reporter (as described in Fukushige et al., 1999) after being treated similarly in an embryo carrying *hs-elt-7* on a separate extrachromosomal element. (F) Expression of *elt-2*::*lacZ*::*GFP* throughout the entire body of an L1 larva observed several hours after it was subjected to heat shock.

as L1 larvae. As the chromosomal double mutants and those obtained by RNAi of *elt-7* in the *elt-2(ca15)* mutant are indistinguishable in appearance, we used both types of double mutants to analyze the synergistic effects of *elt-2* and *elt-7*. The double-mutant larvae show what appears to be an extensive, albeit sporadic, block to gut differentiation, with patches of apparently well-differentiated gut cells interspersed with extended regions of cells that exhibit no overt signs of differentiation (Figs. 4E–H and not shown). Moreover, the birefringent and autofluorescent rhabditin granules characteristic of differentiated gut cells are absent in clusters of cells in both *elt-2(ca15);elt-7(RNAi)* and *elt-2(ca15);elt-7(tm840)* L1 larvae. The most frequent patches lacking gut granules appear to be in the region of int5 (perhaps as a result of its interaction with the primordial germ cells (Sulston et al., 1983)) as well as at the anteriormost and posteriormost ints, indicating that the regions of interface with the pharyngeal and rectal valves are the most severely compromised. The arrested larvae largely lack gut lumen, with only isolated patches of lumen and brush border appearing infrequently with approximately uniform probability along the length of the worm (Figs. 4E and G). In contrast, the mutant worms appear entirely normal outside the gut region, with no apparent defects in other major organs, including the epidermis, which is generally disrupted in mutants lacking gut (e.g., Maduro et al., 2005). Thus, ELT-2 and ELT-7 show a highly synergistic requirement in morphological differentiation of the gut and there appears to be largely an all-or-none effect, in which clusters of cells in the gut show no hint of differentiation, while others activate a robust morphologically evident differentiation program.

The morphology of the undifferentiated gut regions in the double-mutant larvae is strikingly different from the gut region in mutants lacking *end-1* and *-3* function, in which the E cell undergoes a transformation into a C-like mesectodermal progenitor. In the latter case, the production of epidermal cells from the transformed E lineage (a differentiated cell type produced by the normal C cell) leads to cuticle-lined cavities as a result of epidermal differentiation within the interior of the animal (Bowerman et al., 1992; Maduro et al., 2005). Such cavities are never seen in the *elt-7(0);elt-2(0)* double mutants, consistent with a postspecification block in gut differentiation.

ELT-2 and ELT-7 collaborate to promote gut epithelialization

Given the profound disruption of gut differentiation in *elt-2(ca15);elt-7(tm840)* worms, particularly the widespread absence of a brush border and lumen, we examined whether the epithelial character of gut cells is established in the mutants by analyzing expression of the epithelial markers ERM-1B (an ezrin/radixin/moesin protein connecting membrane proteins with the actin cytoskeleton present in most epithelial cells (Gobel et al., 2004; Van Furden et al., 2004)), ITX-1 (a member of the neurexin superfamily mediating cell–cell interactions (L. Haklai-Topper, and E. Peles, personal communication), and AJM-1 (a component of adherens junctions in all epithelial cells (Koppen et al., 2001)). We found that the levels of *erm-1B*::*gfp* expression are unaffected by *elt-7* RNAi but are diminished to comparable subnormal levels in both *elt-2(ca15)* and *elt-2(ca15);elt-7(tm840)* larvae (Table 1). Expression of the *itx-1*::*gfp* reporter, which is strongly expressed in the gut continuously from elongation through adulthood interactions (L. Haklai-Topper, and E. Peles, personal communication), is indistinguishable in both *elt-7(RNAi)* and *elt-2(ca15)* single-mutant larvae (Figs. 5A and B and Table 1; not shown) but is reduced and sporadically expressed in the *elt-2(ca15);elt-7(RNAi)* double-mutant animals (Fig. 5C and Table 1). Finally, a more severe effect was seen with AJM-1, as detected both with the MH27 antibody (Priess and Hirsh, 1986) and an *ajm-1*::*gfp* reporter (Koppen et al., 2001). Although *elt-7(tm840)* and *elt-2(ca15)* single mutants show wild-type AJM-1 expression, we observed a dramatic reduction in expression in *elt-7(tm840);elt-2(ca15)* larvae: immunoreactive AJM-1 is largely undetectable throughout much of the intestine in these double mutants (Fig. 6 and Table 1). Interestingly, normal immunoreactive AJM-1 was nearly always observed in the anterior ints of the double mutants, suggesting a regional bias in this aspect of differentiation. It may be that the anterior cells of the E lineage experience additional cues that provide a more robust developmental environment for epithelialization, consistent with other observations suggesting that the gut termini are differentially affected (see below).

ELT-2 and ELT-7 synergistically activate gut-specific gene expression

The foregoing results establish a strong synergistic role for ELT-2 and -7 in morphological gut differentiation and in activating at least some elements of epithelial formation. Given the prevalence of consensus GATA factor binding sites in the large number of intestine-expressed genes (McGhee et al., 2007), we asked whether the ELT-2 and -7 GATA factors might be essential for expression of a spectrum of genes expressed in the differentiated gut by analyzing expression of a representative sampling of gut-expressed genes (Table 1). Consistent with the absence of any detectable phenotype associated with loss of ELT-7 function, we found that all of the genes analyzed were expressed normally in *elt-7(−)* single mutants (Table 1). However, we found that expression of all genes is diminished or abolished in the *elt-7(−);elt-2(−)* double mutants. In all but two cases, expression is dramatically altered in the double mutants compared to either single mutant (Table 1), demonstrating that ELT-2 and ELT-7 strongly synergize to activate gut-specific gene expression.

The discovery of the major gut esterase, GES-1, over two decades ago (Edgar and McGhee, 1986), launched extensive biochemical and molecular analysis of gut development in *C. elegans*. ELT-2 was identified as a factor that binds to regulatory sequences essential for gut-specific transcription of *ges-1*, which provided a key link between

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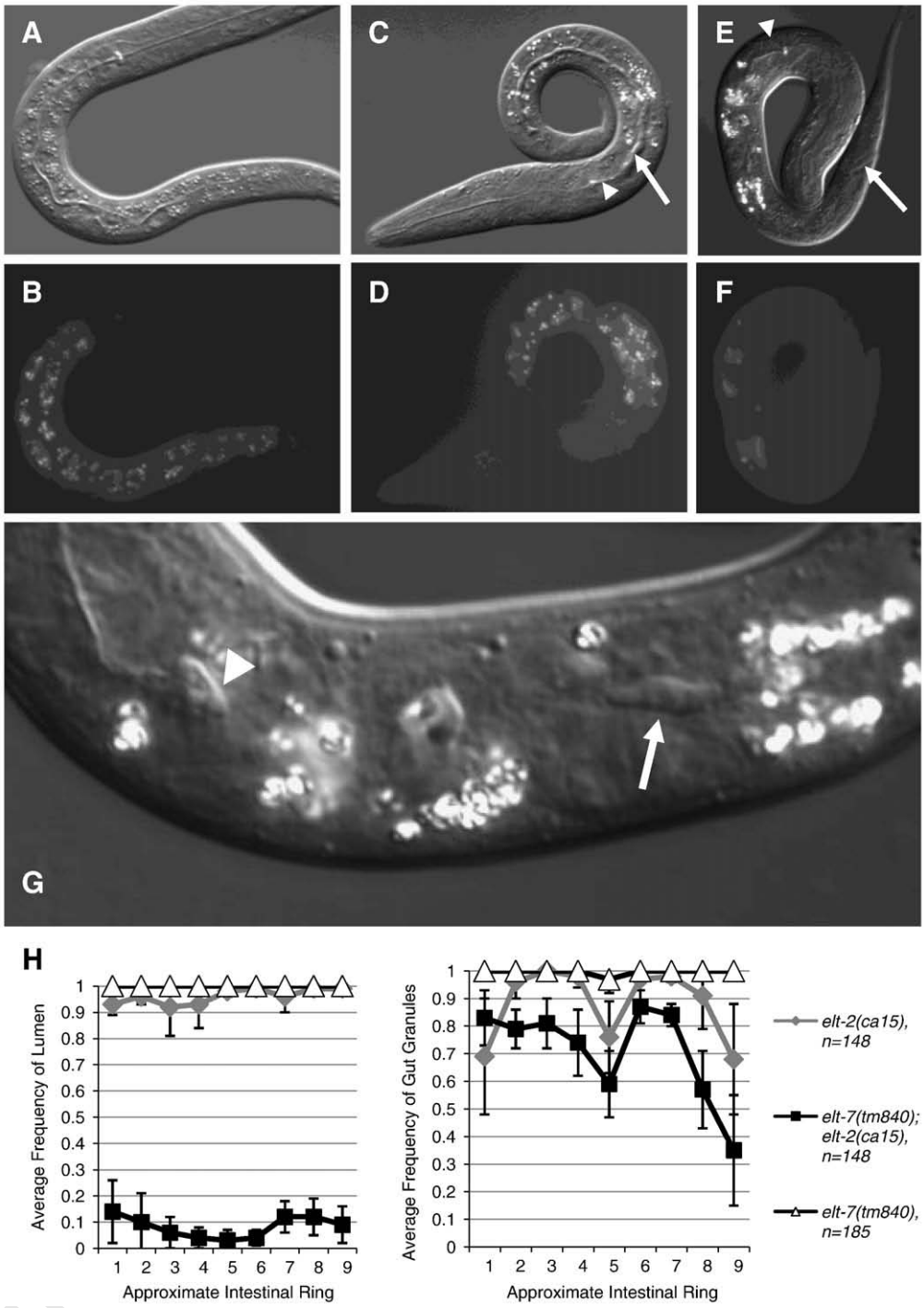


Fig. 4. Synergistic requirement of ELT-7 and ELT-2 in morphological gut differentiation. (A, B) A typical *elt-7(tm840)* L1 larva has a smooth, well-defined gut lumen and brush border, as observed by DIC microscopy (A) and autofluorescent gut granules evident throughout the intestine (B), appearing essentially wild-type. (C) The blockage and swelling of the brush border surrounding the gut lumen (arrow) is apparent in an *elt-2(ca15)* L1 larva, but morphological differentiation of the entire gut occurs normally. Note the lumen progressing continuously from the pharynx (arrowhead) throughout the length of the gut. (D) Autofluorescent gut granules are present throughout the intestine of the same *elt-2 (ca15)* larva. (E–G) A representative *elt-7(tm840); elt-2(ca15)* L1 larva lacks an evident brush border, lumen, and rhabditin granules in sporadic patches in the region between the pharynx (arrowhead in E) and rectum (arrow in E). These patches show no apparent signs of differentiation. (G) Magnified view of a portion of the larva in E shows that the pharynx lumen is continuous with a small anterior portion of the gut lumen and brush border, which end abruptly (arrowhead). Only a single small patch of brush border is present more posteriorly in this animal (arrow). Birefringent (E, G) and autofluorescent (F) gut granules are also observed only sporadically. (H) The average frequencies of visible lumen (left panel) and gut granules (right panel) are dramatically reduced in *elt-7(tm840); elt-2(ca15)* L1 larvae compared to those in *elt-2(ca15)* or *elt-7(tm840)* single mutant larvae.

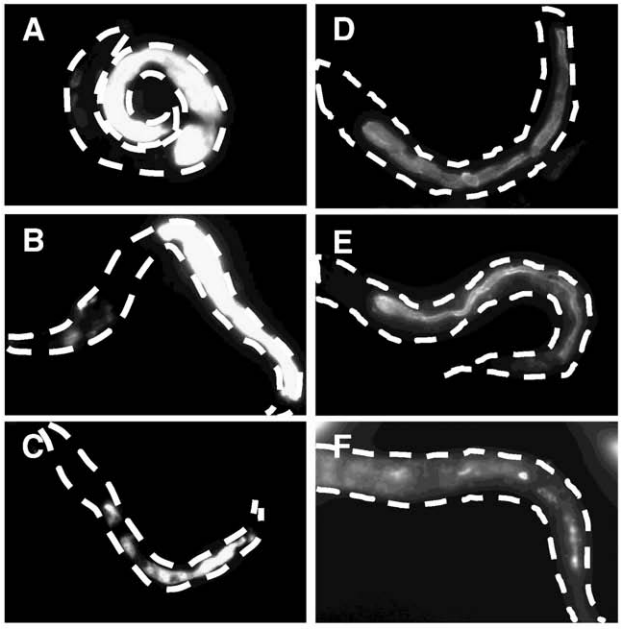
the mechanisms of endoderm specification and intestinal differentiation (Fukushige et al., 1998, 1999). However, although ELT-2 binds directly to the *ges-1* gene both *in vitro* and *in vivo* (Fukushige et al., 1999), GES-1 is expressed at high levels in animals deleted for *elt-2* (Fukushige et al., 1998). Given that the *ges-1* gene led to identification of ELT-2, it was of particular interest, therefore, to examine whether synergy of ELT-2 and -7 extends to expression of this gene. Confirming earlier reports that ELT-2 is not essential for *ges-1* expression, we observed that a *ges-1::lacZ::GFP* reporter is expressed at normal or slightly reduced levels in approximately 25% of embryos derived from

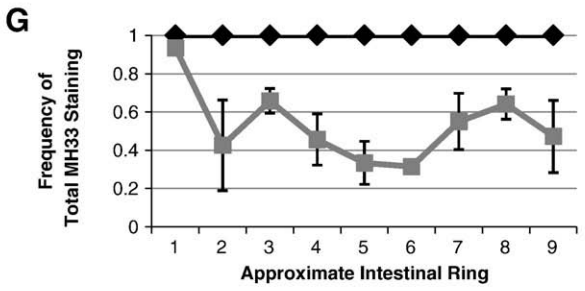
Table 1
Summary of expression of gut and valve markers in the gut region.

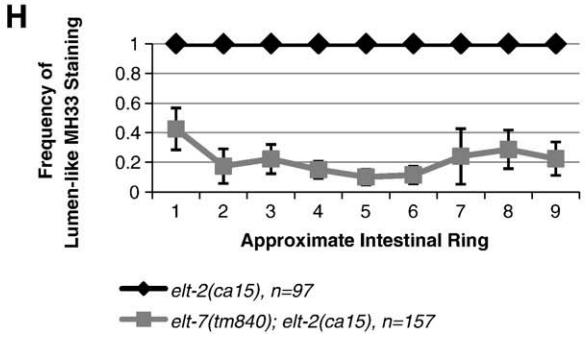
Marker	Stage scored	*elt-7(RNAi)*	*elt-2(ca15)*	*elt-7(RNAi);elt-2(ca15)*	*elt-7(tm840);elt-2(ca15)*
erm-1B::GFP	L1 larva	WT (103)	− − (19)	− − (11)	
itx-1::GFP	L1 larva	WT (89)	WT (25)	− (39)	
AJM-1[a]	Mid–late embryo	WT (82)	WT (35)	− (35)	
AJM-1[a]	L1 larva	WT[b] (60)	WT (101)		− − (150)
ges-1::lacZ::GFP	Early–mid embryo	WT (62)	− (28)	0 (29)	
itr-1C::GFP	L1 larva	WT (91)	− − (27)	0 (37)	
IFB-2[c]	Mid–late embryo	WT (87)	− − (27)	0 (11)	
IFB-2[c]	L1 larva		WT (97)		− −(157)
let-767::GFP	L1 larva	WT (61)	− − (29)	− − (33)	
cdf-1::GFP	L1 larva	WT (75)	+ (45)	+ + (42)	
cdf-1 mRNA	L1 larva		WT (58)		+ + (50)

+ + = strongly increased compared to wt; + = slightly increased compared to wt; wt = wild-type; − = slightly reduced or sporadic compared to wt; − − = reduced or sporadic compared to wt; 0 = absent/barely detectable; total number scored for each group is listed in parentheses; all animals showed the phenotype indicated for each genotype scored.

[a] AJM-1 detected by MH27.
[b] *elt-7(tm840)*.
[c] IFB-2 detected by MH33.







heterozygous *elt-2(ca15)* mothers (Table 1). In sharp contrast, we found that ~25% of the embryos from *elt-2(ca15)* heterozygotes show no detectable expression of this marker when *elt-7* function is eliminated by RNAi. Thus, although neither is required alone, ELT-2 and ELT-7 are together essential to initiate *ges-1* expression (Table 1).

A defect in expression of two other genes in *elt-2(0)* mutants is substantially enhanced in the *elt-7(−);elt-2(−)* double mutants (Table 1). Gut expression of a reporter for the mesendodermally expressed IP_3 receptor ITR-1C, which is normally specific to both the gut and the isthmus of the pharynx (Gower et al., 2001; Jee et al., 2004), is reduced in *elt-2(ca15)* larvae. *elt-7(RNAi)* of *elt-2(ca15)* animals virtually abolishes expression of this reporter in the gut but not in the pharynx (Table 1). Expression of the gut-specific terminal web protein IFB-2, which appears wild-type in *elt-2(ca15)* animals (Figs. 5D and E), is largely abolished in *elt-7(tm840);elt-2(ca15)* double mutants (Fig. 5F). The terminal web is clearly defined throughout the entire length of wild-type and *elt-2(ca15)* intestines, but double mutants show sporadic patches of the protein, with a slight anterior bias (Figs. 5G and H and Table 1), consistent with the patches of brush border and lumen seen by DIC microscopy in the double mutants (Figs. 4E and G).

These results, suggesting that ELT-2 and ELT-7 collaborate to activate a comprehensive set of gut-expressed genes, are consistent with the notion that general GATA factor input is required for proper gut-specific gene expression and provide no clear evidence of specificity differences between these two GATA factors. However, analysis of two other reporters suggests that ELT-2 and -7 may indeed carry out distinct functions. Expression of two genes, *erm-1* (described above) and *let-767*, which encodes a steroid modifying enzyme expressed specifically throughout the cytoplasm of gut cells (Kuervers et al., 2003), is reduced in the *elt-2(0)* mutant, but this defect is not exacerbated by elimination of ELT-7 function (Table 1). This finding reveals that while ELT-7 collaborates with ELT-2 to regulate gut differentiation and activation of *ges-1* and probably many other

Fig. 5. ELT-2 and ELT-7 function synergistically to activate markers of intestinal fate. (A–C) Expression of *itx-1*::GFP. (A) Wild-type worms and (B) *elt-2(ca15)* L1 larvae show similar levels of *itx-1*::*GFP* expression. (C) *itx-1*::GFP is expressed sporadically and at reduced levels in *elt-2(ca15);elt-7(RNAi)* L1 larvae. (D–H) MH33 staining of IFB-2. (D) A wild-type L1 shows uniform staining with antibody MH33. (E) MH33 staining appears wild-type in *elt-2(ca15)* L1 larvae. (F) Staining with MH33 reveals only sporadic patches of IFB-2 in *elt-7(tm840); elt-2(ca15)* double mutants. Images D and E were taken with a 100-ms exposure time; image F was taken with a 300-ms exposure. (G) *elt-2(ca15)* worms stain for IFB-2 along the entire length of the lumen, while *elt-7(tm840); elt-2(ca15)* worms show significant reduction in frequency of staining across the gut. (H) An even greater difference between single and double mutants is observed when comparing the frequency of MH33 staining that shows typical lumen-like morphology. Anterior is to the left in all images.

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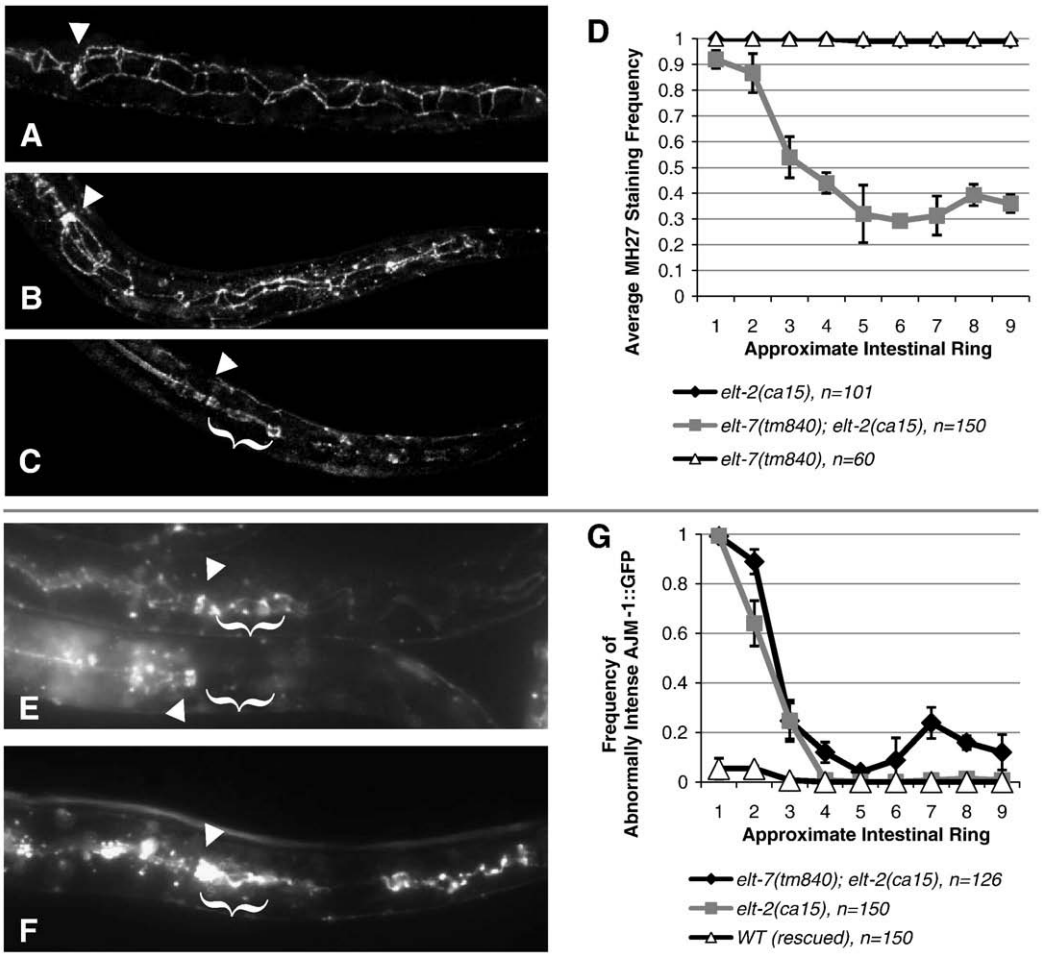

Fig. 6. ELT-2 and ELT-7 are required to form normal apical junctions within intestinal cells. MH27 staining of intestinal AJM-1 is not significantly different in wild-type (A) and *elt-2 (ca15)* L1 larvae (B). (C) MH27 reveals that only sporadic patches of AJM-1 are present in *elt-7(tm840); elt-2(ca15)* larvae and shows an intense valve-cell-like staining pattern at the anterior (bracket). (D) Quantification of MH27 staining frequency across the length of the intestine reveals and anterior bias in the double mutants. (E) AJM-1::GFP expression is observed in *elt-2(ca15)* mutants (top worm) and L1 worms rescued for the *elt-2* mutation (bottom worm). Rescued worms show wild-type expression, while *elt-2(ca15)* mutants show strong reporter expression extending caudally into the region of the anterior intestine (bracket). (F) Caudal extension of intense reporter expression is also virtually always seen in *elt-7(tm840); elt-2(ca15)* L1s (bracket), although the reporter expression is sporadic along the length of the gut. (G) Analysis of reporter signal reveals a strong anterior bias along the length of the intestine. Arrowheads mark the position of the pharyngeal–intestinal valves. A–C are confocal images.

genes, it does not synergize with ELT-2 in the activation of all gut genes, suggesting that these two GATA factors possess distinct transcription-activating properties.

ELT-2 and ELT-7 repress characteristics of valve cell fate at the gut termini

The finding that AJM-1 and IFB-2 are dramatically less affected in the anterior gut region of *elt-7(0);elt-2(0)* animals suggests that loss of ELT-2 and ELT-7 may uncover special characteristics of the gut cells at the pharyngeal–intestinal interface. Closer examination of the adherens junctions in *elt-2(0)* single and *elt-7(0);elt-2(0)* double mutants revealed that the anteriormost gut cells may adopt at least one characteristic of their anterior neighbors in the digestive tract. In wild-type worms, AJM-1::GFP signal is intense through the adherens junctions surrounding the lumen of the pharynx and pharyngeal–intestinal valve cells and drops off sharply to low levels starting at the anterior terminus, and continuing throughout the entire length of the intestine (Fig. 6E, bottom larva). We found that essentially all worms lacking *elt-2* function, however, generally show greatly elevated AJM-1 expression that continues from the valve cells through to the anterior portion of the intestine to approximately the level of int 3 (Figs. 6E, top larva; F and G). This caudal extension of intense, pharynx- and valve-like expression is also observed in *elt-7(tm840); elt-2(ca15)* double mutants, although expression along the remainder of intestine is sporadic. We note that, although AJM-1 and IFB-2 (Fig. 5G) are nearly always present in the anterior gut region in the double mutant, gut lumen is generally not visible (Fig. 4H), suggesting that despite the presence of these markers, the anterior gut cells are abnormal.

These observations raise the possibility that the anterior gut might adopt some characteristics of its anterior valve or pharynx cell neighbors in the absence of ELT function. Further analysis revealed that, in fact, both termini of the intestine may be affected in this way. In wild-type worms, a reporter for *cdf-1*, which encodes a cation diffusion facilitator protein (Bruinsma et al., 2002), is first expressed at high levels during embryonic elongation in the pharyngeal–intestinal and rectal–intestinal valves (Fig. 7A) and thus serves as a marker of valve cell identity. Expression of the reporter appears unchanged in *elt-7(RNAi)* (Figs. 7A and B and Table 1) animals and shows a very slight increase in the anterior gut in *elt-2(ca15)* L1 animals (Fig. 7C and Table 1). However, we observed a dramatic expansion of *cdf-1*::GFP expression into the anterior and posterior termini of the gut in *elt-2(ca15);elt-7(RNAi)* L1 larvae (Fig. 7D),

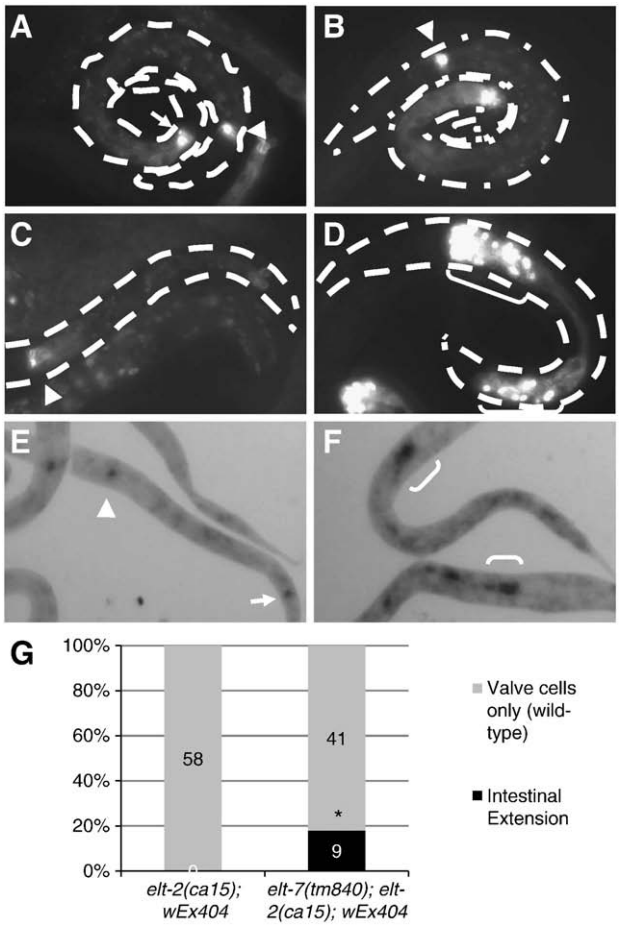

Fig. 7. ELT-7 synergizes with ELT-2 to repress markers of valve cell fate in the terminal gut regions. (A–D) Expression of the *cdf-1*::*GFP* reporter. (E–F) *In situ* hybridization of *cdf-1* transcripts. (A) Wild-type animals show strong *cdf-1*::*GFP* expression in the pharyngeal–intestinal (arrowhead) and rectal–intestinal (arrow) valve cells. The expression pattern is not substantially altered in *elt-7(RNAi)* (B) or *elt-2(ca15)* (C) animals. (D) Expression is greatly enhanced (brackets) at both termini of the intestine in *elt-2(ca15);elt-7(RNAi)* animals, while it remains very low in the middle of the organ. (E) *cdf-1* transcripts detected by *in situ* hybridization are restricted to the pharyngeal and rectal valve cells in all *elt-2(ca15)* worms (both with and without the *elt-2(+)* rescuing array, see Materials and methods). (F) Typical expansion of *cdf-1* mRNA hybridization seen in *elt-7(tm840); elt-2(ca15)* animals. (G) A fraction of *elt-7 (tm840); elt-2(ca15)* worms, corresponding to the percentage of non-rescued worms in this strain, showed strong expansion of *cdf-1* mRNA (brackets) into the terminal gut regions. $^{*}\chi^2$ test *P* value = 0.0007.

suggesting that ELT-7 and ELT-2 repress transcription of this normally valve-specific gene in the gut. Analysis of endogenous *cdf-1* expression in *elt-2(ca15)* and *elt-7(tm840);elt2(ca15)* L1 larvae by RNA *in situ* hybridization yielded similar results. While 100% of *elt-2(ca15)* larvae ($n = 58$, approximately 30% of which lack the *elt-2(+)* rescuing array) displayed wild-type *cdf-1* transcript staining restricted to the pharyngeal and rectal valve regions (Figs. 7E and G), expression of *cdf-1* transcripts expands into both anterior and posterior gut termini in 18% ($n = 50$) of *elt-7(tm840);elt2(ca15)* L1 larvae. This rate of ectopic expression is roughly comparable to that of the frequency of loss (~10%) of the *elt-2(+)*-containing extrachromosomal array (Figs. 7F and G), implying that most or all *elt-7(tm840);elt2(ca15)* arrested larvae show the effect. These observations reveal that in addition to functioning synergistically in activation of gene expression in the gut, ELT-7 and ELT-2 repress at least some characteristics of pharyngeal–intestinal and intestinal–rectal valve cell fate in the gut termini.

Discussion

We present several lines of evidence establishing ELT-7 and ELT-2 as redundant partners that function synergistically to activate expression of gut genes, promote morphological gut differentiation, and repress nongut gene expression. First, ELT-7 is expressed specifically in the endoderm during the period in which intestine differentiation is initiated. Second, ELT-7 is sufficient to activate expression of both early and late markers of gut differentiation when ectopically expressed in normally non-gut progenitors. Third, *elt-7*, like *elt-2*, can be activated by the endoderm-specifying factors END-1 and -3 and shows auto- and cross-regulatory activation with *elt-2*. Fourth, simultaneous loss of *elt-7* and *elt-2* function but not of either alone, eliminates all morphological features of differentiation in patches of the gut, as well as several markers of epithelial and gut fate. Finally, removal of both factors results in derepression of valve-specific gene expression in the intestinal cells that are adjacent to the valve cells, demonstrating a role in repression as well as activation. While our results show that ELT-2 and -7 act together, they also suggest that at least one additional factor is likely to function with ELT-2 and -7 to activate differentiation of the intestine.

The goal of identifying transcriptional regulators of *C. elegans* gut differentiation began approximately 25 years ago with the discovery of GES-1, the first protein known to be specifically expressed in the gut (Edgar and McGhee, 1986). While ELT-2 directly binds to and activates *ges-1*, the observation that *elt-2(0)* mutants retain both GES-1 activity and a well-differentiated intestine implied the existence of another factor that regulates terminal gut differentiation. The strong synergy observed between ELT-7 and ELT-2 explains the lack of differentiation defect in the *elt-2(0)* mutant and identifies ELT-7 as a key regulator of gut differentiation. Based on the lower level of expression of *elt-7* compared to *elt-2* in dissected intestines of starved adult worms and the lack of a phenotype of *elt-7(0)* mutants, it has been argued that ELT-2 is the dominant "organ selector" or "organ identity factor" for intestinal differentiation and that ELT-7 must play at most a subsidiary role in gut gene expression (McGhee et al., 2009, 2007). In fact, *elt-7* transcripts are first detectable approximately an hour before *elt-2* transcripts during embryogenesis (Baugh et al., 2003), raising the possibility that ELT-7 may function to initiate differentiation during normal development. In any event, the finding that ELT-7 and ELT-2 together are essential to activate gut differentiation has made it possible to link specification of the E cell to the subsequent events of activation of the terminal differentiation program, thereby helping to close a longstanding gap in the regulatory cascade for intestinal differentiation.

ELT-7 and ELT-2 as redundant partners in intestinal organogenesis

The strong synergy of *elt-2(−)* and *elt-7(−)* mutations points to their redundant action, a prominent theme throughout the entire endoderm regulatory pathway in *C. elegans* (Maduro et al., 2005, 2001; Maduro and Rothman, 2002). This widespread redundancy presumably serves to ensure robustness of developmental decisions to environmental variation. The triply redundant endoderm-inducing Wnt, MAPK, and Src-type signals, together with an intrinsic transcriptional program involving the SKN-1 maternal transcription factor acting through the redundant MED transcription factors, promotes expression of the redundant END-1 and -3 transcription factors (Bei et al., 2002; Maduro et al., 2001; Meneghini et al., 1999; Rocheleau et al., 1997, 1999; Shin et al., 1999; Thorpe et al., 1997). In the E lineage, the END-1 and -3 GATA factors, which specify endoderm, are apparently immediately upstream of the redundant ELT-2 and -7 GATA factors. No single factor at any stage is known to be completely essential for the endoderm developmental program. Rather, elimination of any one component alone results in either a weak or impenetrant phenotype, as is observed in mutants defective

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for SKN-1, any one component in the Wnt/MAPK/Src pathways, or END-3 (Bowerman et al., 1992; Maduro et al., 2005; Rocheleau et al., 1997; Thorpe et al., 1997), or in no phenotype at all, as in the case of mutations in either of the *med* genes, *end-3*, or *elt-7* (Maduro et al., 2005, 2001; McGhee et al., 2007).

In many cases of genetically redundant gene pairs, one partner shows a more substantial requirement than the other, in that its elimination leads to a partially penetrant phenotype that is not seen when its partner alone is removed. For example, the two Notch-type receptors, LIN-12 and GLP-1, perform a genetically redundant function in certain inductions in the late *C. elegans* embryo (Henderson et al., 1994; Lambie and Kimble, 1991), in which simultaneous loss of both genes results in a fully penetrant "Lag" phenotype. While *glp-1(−)* mutants alone do not result in a Lag phenotype, a small fraction of *lin-12(−)* mutants do show a phenotype that is indistinguishable from that of the fully penetrant double mutants. Similarly, loss of END-1 shows no phenotype, while that of its partner, END-3, results in an impenetrant (~5%) phenotype that is indistinguishable from that of the double mutant (abolishment of endoderm) (Maduro et al., 2005). However, the redundancy seen between ELT-2 and -7 presents a striking difference from these examples. While the mutant phenotypes observed at low frequency in *lin-12(−)* and *end-3(−)* single mutants are identical to the fully penetrant phenotype seen in their respective double mutants, the *elt-2(0)* mutation causes a fully penetrant obstructed gut phenotype that is entirely distinct from that seen in the *elt-7(−);elt-2(−)* double mutant (sporadic block to differentiation). Thus, while ELT-2 is a dominant factor required for gut function, it is not by itself essential for any morphological differentiation, owing to the redundancy with ELT-7 function. Of particular note, orthologs of both *elt-7* and *elt-2* are present in *Caenorhabditis briggsae*, *Caenorhabditis remanei*, and *Caenorhabditis japonica* (WormBase web site, http://www.wormbase.org, release WS213; February 16, 2010), suggesting that the synergistic relationship of the two ELT genes persists in these other species.

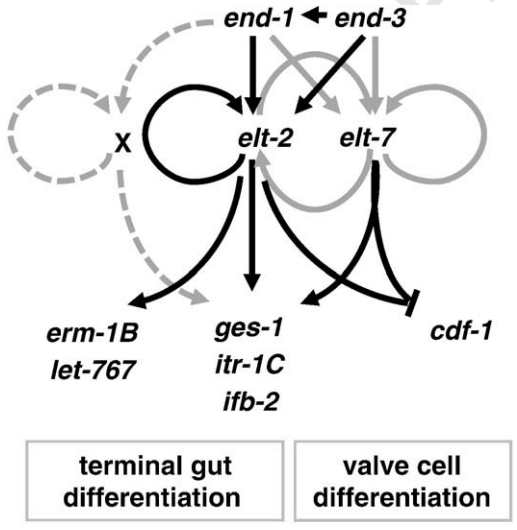

Fig. 8. Proposed regulatory network of endoderm-specific GATA factors. Solid grey arrows indicate a relationship implied by overexpression data. Solid black arrows denote a relationship demonstrated through overexpression data and either gel-shift analysis (END-1 and *elt-2*; ELT-2 and *ges-1*), nuclear-spot assay (ELT-2 and *elt-2*), or loss-of-function (END-1 and END-3 *elt-2*). All other black arrows are based on loss-of-function studies. Other factor(s) proposed to be involved in intestinal differentiation (see Discussion) are denoted X, and proposed relationships indicated with dashed grey lines.

Apparent auto- and cross-regulatory feedback system creates a dynamic specification-to-differentiation switch

Our data support a more complex circuitry for the genetic regulation of gut development (Fig. 8) involving a network of auto- and cross-regulatory interactions among the endoderm-specific GATA factors rather than a linear cascade of transcriptional activation. We suggest that END-1 and END-3 directly activate *elt-2* and *elt-7* through GATA-type binding sites (Supp. Fig. 1). Given their very transient expression (Baugh et al., 2003; Maduro et al., 2007; Zhu et al., 1997), END-1 and END-3 presumably function only to initiate expression of these *elt* genes and fade off rapidly thereafter. Once activated, we propose that ELT-2 and ELT-7 subsequently sustain their own expression by autoregulation and also help to maintain each other's expression by cross-regulatory interactions (Fig. 8).

This genetic circuitry bears remarkable similarity to other canonical dynamic feedback lockdown circuits underlying major developmental switches. In skeletogenic development of *Strongylocentrotus purpuratus*, a transient input signal is translated into an irreversible and sustained decision to proceed with differentiation (Davidson, 2009), similar to the case of *C. elegans* endoderm development. In *Xenopus*, the all-or-none, irreversible nature of cell fate switching during oocyte maturation similarly depends on positive feedback (Ferrell et al., 2009). Thus, it appears that ELT-7, together with ELT-2, provides a rich network of auto- and cross-regulatory positive feedback within the differentiation program to provide a rapid and robust lockdown of intestinal cell fate.

Sporadic differentiation in the absence of ELT-2/7 function implicates additional inputs required for differentiation

While morphological differentiation of gut cells is blocked in *elt-7(0); elt-2(0)* double mutants, differentiation of some gut cells does occur in a sporadic fashion, suggesting that yet other factors activate the program for gut differentiation. Obvious candidates for such other activators include END-1 and END-3. The simplest model, in which the END factors directly participate in intestinal differentiation, posits that all four factors work together to initiate expression of the next tier of genes at the 4E cell stage. However, the expression of both *end* genes never reaches the high levels seen for the *elt*s and rapidly wanes during the time that ELT-7 expression first ramps up (Baugh et al., 2003; Maduro et al., 2007; Zhu et al., 1997); thus, END-1 and -3 could act only briefly in this process and would be expected to give at best only a low input to the system. Given that the patches of gut cells that are observed in the *elt-7(0);elt-2(0)* double mutants appear fully differentiated (i.e., an "all-or-none" effect), one might expect that the levels of END factors would be insufficient to activate the full differentiation program so robustly. This raises the possibility that another factor that is a target of the ENDs ("X," Fig. 8) might operate to promote differentiation of gut cells. Such a factor, like the ELTs, would also be likely to show autoregulatory positive feedback which propels gut differentiation forward well after expression of the ENDs has subsided.

In our model of the gene regulatory network governing intestinal differentiation (Fig. 8), redundant inputs provide robust activation of downstream factors; these factors, being expressed at sufficient levels to initiate a positive feedback loop, ramp up to higher levels, and trigger the "on" state for differentiation. However, loss of redundancy would debilitate the positive feedback system, leading to stochastic switching into the differentiation program. The variation in transcription that results from noise in the system when components of the endoderm regulatory pathway are missing (Raj et al., 2010) could on occasion bring a single input (the hypothesized "X") up to the minimum threshold required to trigger the positive feedback loop and subsequent differentiation. In *elt-7(0);elt-2(0)* mutants, following a

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period of stochastic variation, transcripts for factor X (Fig. 8) might demonstrate "on" and "off" states on a cell-by-cell basis that presage the appearance of differentiated and undifferentiated cells.

Regionalized cell fate potential in the gut

Although all gut cells arise from a single progenitor and assemble into a tube of fundamentally one cell type, our findings show that cell fate potential varies along the length of the intestine in two respects, consistent with previous studies of gut-specific gene expression (Schroeder and McGhee, 1998). First, the frequency of gut cells undergoing morphological differentiation, and the incidence of expression of AJM-1 and IFB-2, is higher in the anterior intestinal rings (ints) than in the posterior ints in the *elt-7(0);elt-2(0)* double mutants. It is possible that the anterior cells experience cell-extrinsic cues that support differentiation. Further evidence for region-specific cell fate differences between gut cells is provided by our finding that expression of the valve-specific *cdf-1* gene expands into the termini of the gut region in *elt-7(−);elt-2(−)* animals. The anteriormost int1 and posteriormost int9 rings must locate and maintain tight physical connections with the pharyngeal and rectal valve cells while retaining their specific intestinal cell identity. When these connections are disrupted in *nDf25* and *nDf24* deletion mutants, the posterior end of an initially linear intestine migrates anteriorly to attach at the pharyngeal valve (Terns et al., 1997), suggesting that the terminal cells are specialized to seek connections with surrounding cells. It is possible that the valve cells might signal to the adjacent cells of either gut terminus, explaining the latent ability of these cells to express genes characteristic of valve cells in the absence of the ELTs. ELT-mediated repression of such genes might help to modulate the establishment of organ junctions and maintain boundaries of cell identity during development. The action of ELT-2 in repression of some genes was not evident from *elt-2(ca15)* single mutants (McGhee et al., 2009). Mutants lacking both ELT-7 and -2 may provide a system with which to examine how the two GATA factors function to repress gene expression.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.08.020.

Acknowledgments

We thank Kerry Kornfeld for the *cdf-1*::CDF-1::GFP reporter, Yasuji Sakube for the *erm-1B*::GFP reporter, Liat Haklai-Topper and Oliver Hobert for the *itx-1*::GFP reporter, Howard Baylis for the *itr-1C*::GFP reporter, Lynnette Kuervers and Dave Baillie for the BC6157 strain (containing *let-767*::GFP), and Shohei Mitani for the FX840 strain. Other nematode strains were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). The Olympus Fluoview 500 confocal microscope was provided by NCRR Shared Instrumentation grant no. 1S10RR017753-01. This work was supported by grants from the NIH and March of Dimes to J.H.R.

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