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Maternal deployment of the embryonic SKN-1 → MED-1,2 cell specification pathway in *C. elegans*

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Abstract

We have previously shown that the MED-1,2 divergent GATA factors act apparently zygotically to specify the fates of the MS (mesoderm) and E (endoderm) sister cells, born at the 7-cell stage of *C. elegans* embryogenesis. In the E cell, MED-1,2 activate transcription of the endoderm-promoting *end-1* and *end-3* genes. We demonstrate by *in situ* hybridization that *med* transcripts accumulate both in the EMS cell and in the maternal germline in a SKN-1-dependent manner. Removal of zygotic *med* function alone results in a weakly impenetrant loss of endoderm. However, *med-1,2(−)* embryos made by mothers in which germline *med* transcripts have been abrogated by transgene cosuppression fail to make endoderm 50% of the time, similar to the phenotype seen by RNAi. We also find that reduction of Med or End activity results in aberrant numbers of intestinal cells in embryos that make endoderm. We further show that regulation of the paralogous *end-1* and *end-3* genes consists of both shared and distinct inputs, and that END-3 activates *end-1* expression. Our data thus reveal three new properties of *C. elegans* endoderm specification: both maternal and zygotic activities of the *med* genes act to specify endoderm, defects in endoderm specification also result in defects in gut cell number, and activation of the paralogous *end-1* and *end-3* genes differs qualitatively in the relative contributions of their upstream regulators.
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Introduction

Embryos have evolved strategies to convert the information encoded in their genomes into a functional organism that resembles its parents. Fundamental among these is the correct spatiotemporal deployment of embryonic genes. Their differential activation is an otherwise unseen indication that qualitative differences exist among cells that display a relatively homogeneous appearance. In the nematode, *C. elegans*, a network of maternal and zygotic genes acts to assign identities to cells very early in development.

One such gene regulatory network is that which specifies the descendants of the 4-cell-stage blastomere EMS, which divides to produce the founder cells, MS and E, at the 7-cell stage. EMS can be considered to be an endomesoderm precursor, because E will clonally generate the entire endoderm (intestine or gut), while MS makes primarily mesodermal cell types, including body muscle and the posterior half of the pharynx (Sulston et al., 1983). Specification of MS and E is thought to occur through the sequential activation of a cascade of genes that starts with the maternal factor SKN-1, and proceeds through the activation of the nearly identical paralogous genes *med-1* and *med-2* in the EMS cell (Maduro et al., 2002). In turn, MED-1,2 activate the endoderm-specifying genes *end-1,3* in E, and are prevented from doing so in MS by the repressive activity of the TCF/LEF homolog POP-1 (Broitman-Maduro et al., 2005; Lin et al., 1995; Maduro et al., 2002). In the E cell, POP-1 is converted into an endoderm activator through the action of the Wnt/MAPK/Src pathway, transduced from a cell–cell interaction that occurs when EMS contacts its neighbor, P_2 (Goldstein, 1992; Lo et al., 2004; Maduro et al., 2005b; Maduro and Rothman, 2002; Rocheleau et al., 1997, 1999; Shetty et al., 2005;

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Thorpe et al., 1997). In the MS cell, MED-1,2 specify the MS fate primarily by activating the T-box gene *tbx-35* (Broitman-Maduro et al., 2006).

Specification of the E fate results from a surprising number of parallel activities that converge on the activation of *end-1* and *end-3*: SKN-1, MED-1,2, Wnt-modified POP-1, and the Caudal homolog PAL-1 all contribute to E specification (Maduro et al., 2005b; Shetty et al., 2005). Therefore, while descendants of MS are absent in all embryos lacking *skn-1* or *med-1,2* function, a significant fraction of these (~30% of *skn-1(−)* embryos, and ~50% of *med-1,2(−)* embryos) still make endoderm (Bowerman et al., 1992; Coroian et al., 2005; Maduro et al., 2001).

A recent study has challenged the notion that the MEDs are substantially required for endoderm specification (Goszczynski and McGhee, 2005). In that study, chromosomal *med-1,2(−)* embryos segregated from a mother carrying *med-2(+)* were found to specify endoderm between 80% and 97% of the time, contrasting with the 50% we have observed (Coroian et al., 2005; Maduro et al., 2001). While the most obvious explanation would be that a maternal contribution of the *med*s accounts for the difference, Goszczynski and McGhee reported that they could find no evidence for maternal *med* activity. Here we use *in situ* hybridization to show that the *med* genes are indeed expressed both maternally and zygotically in *C. elegans* and in the closely related species *C. briggsae*. Furthermore, *med-1,2(−)* embryos lacking both the maternal and zygotic components of *med* activity display a strong endoderm phenotype. We also find that *med-1,2(−)* embryos that make endoderm contain an abnormal number of gut cells, and that this phenotype is also a characteristic of *skn-1* and *end-3* mutants. We extend earlier observations suggesting that *end-3* is expressed earlier than *end-1* in the E lineage (Baugh et al., 2003) and show that END-3 activates *end-1* expression.

Our results validate our original model for *C. elegans* endoderm specification through *med-1,2* and add additional levels of complexity to our understanding of this unexpectedly complex gene regulatory network. These results also suggest that at least some embryonic specification genes are expressed in the *C. elegans* germ line, working against mechanisms that maintain germ cell totipotency.

Materials and methods

C. elegans strains and genetics

Genetic manipulation and crosses were performed as described (Brenner, 1974). The following mutations, rearrangements or transgenes were used: LGX: *lon-2(e678)*, and *med-1(ok804)*. LGIII: *dpy-17(e499)*, *sDf127*, *med-2(cx9744)*, *unc-36(e251)*, and *unc-32(e189)*. LGIV: *skn-1(zu67)*. LGV: *dpy-11(e224)*, *end-1(ok558)*, *end-1(ox134)*, and *end-3(ok1448)*. Free duplications: *sDp3(III;f)* [complements *dpy-17*, *sDf127*, and *unc-36*] and *irDp1(III;f)* [complements *dpy-17*, *sDf127*, *unc-36*, *unc-32*, and *med-1*]. Translocation: *nT1[unc-?(n754) let-?](IV;V)*. Integrated transgenes: *wIs84 X* [*elt-2*::GFP]; *irIs25 V* [*elt-2*::GFP]; *cuIs2 V* [*ceh-22*::GFP]. Unmapped integrants: *irIs21, irIs22, irIs23* [*elt-2*::YFP]; *qtIs12* [*pal-1*::YFP]. The *ok558*, *ok1448*, *ok804*, and *cx9744* lesions are described in Wormbase (http://www.wormbase.org, release WS160; *cx9744* is also known as *cxTi9744*).

The free duplication *irDp1* was constructed as follows. BC4638 [*dpy-17 sDf127 unc-32; sDp3*] hermaphrodites were made transgenic for an array (*irEx14*) carrying an *unc-119*::NLS::YFP::lacZ fusion (pMM531), the *unc-32*-rescuing plasmid pAIE5 (Pujol et al., 2001), and the *med-1(+)* plasmid pMM277. Expression of the *unc-119* reporter can be followed at all stages beginning in mid-embryogenesis (Maduro and Pilgrim, 1995). The balancer *irDp1* was recovered as spontaneous integrant of *irEx14* that had become linked to *sDp3*, allowing *irDp1*(+) animals to be identified by *unc-119*::YFP expression. PCR analysis showed that the *med-2* gene remained intact on *irDp1*. In addition to *unc-32* and *med-1*, *irDp1* complements *dpy-17*, *sDf127*, and *unc-36*, suggesting that it contains most of *sDp3*. To make MS162 [*med-1; dpy-17 sDf127 unc-32; irDp1*], *med-1* males were crossed with *lon-2; dpy-17 sDf127 unc-32; irDp1* hermaphrodites, and YFP(+) F_2 descendants were identified that segregated only dead eggs and viable non-Lon YFP(+) animals. Consistent with their being of similar size, *irDp1* and *sDp3* are inherited by approximately 60% of progeny (data not shown; Hedgecock and Herman, 1995).

To replace the *sDf127* chromosome with *med-2(cx9744)*, *med-2* males were crossed to *med-1; dpy-17 ncl-1 unc-36* hermaphrodites, and progeny males were then crossed to *med-1; dpy-17 ncl-1 unc-36; irDp1*. Wild-type F_2 animals from this cross were singled out and the line MS247 [*med-1; med-2; irDp1*] was one of those that segregated only dead eggs and viable non-DpyUnc YFP(+) animals. PCR analysis showed that MS247 is homozygous for *med-1(ok804)* and *med-2(cx9744)*. Construction of MS290, a strain derived from MS247 in which *irDp1* has been replaced by an array containing the *med-1(+)* plasmid pMM277 and the *unc-119*::CFP reporter pMM809, is described in Coroian et al. (2005). MS290 transmits the *med-1(+)* array to approximately 45% of progeny. Genotypes were confirmed by PCR where appropriate. Additional strain construction details are available on request.

Microscopy and imaging

An Olympus BX-71 microscope was used for fluorescence and Nomarski microscopy. Images were captured using either a monochrome Microfire CCD or a Canon EOS-350D digital SLR coupled to an LMscope DSLRC adapter (Micro Tech Lab, Austria). GFP, YFP, and CFP were detected using appropriate filter sets (Miller et al., 1999) obtained from Chroma Technology Corp. Images were processed with Adobe Photoshop 7, and figures compiled with Adobe Illustrator CS2.

In situ hybridization

Detection of mRNA *in situ* was performed as described, using antisense RNA probes of size 0.5 kb–0.8 kb (Coroian et al., 2005). Freeze-crack permeabilized embryos and hermaphrodites were treated with Streck Tissue Fixative (Streck, Inc.) for 1–2 h at 37°C prior to hybridization. RNA probes were prepared using the Roche DIG RNA labeling kit (#1 175 025) with PCR products tagged on one side with the T7 RNA polymerase recognition sequence. All experiments were performed a minimum of two times with three microscope slides in each experiment. In any given experiment, probe quality and concentration were assayed by staining wild-type animals fixed in parallel, and including only those experiments in which strong staining, with minimal background, was observed in these controls. Both antisense and sense *med-1* probes frequently detected weak signal in the anus and pharyngo-intestinal valve, but only antisense *med* probes detect signal in the gonad, anterior intestine, and early embryo.

RNA interference

RNA interference for *skn-1*, *pal-1*, and *pop-1* was performed by growing animals on *E. coli* HT115 bacteria expressing dsRNA (Timmons et al., 2001).

Results

SKN-1-dependent med expression occurs maternally and zygotically

med-1 and *med-2* translational reporter fusions display expression that can be detected in the nuclei of EMS, its

daughters MS and E, and their descendants for several divisions (Maduro et al., 2001). We performed RNA *in situ* hybridization using an antisense *med-1* probe, which detects both *med-1* and *med-2* transcripts owing to their 98% sequence identity. We confirmed that embryonic *med* transcripts do indeed accumulate in the EMS nucleus at the 4-cell stage, become cytoplasmic in MS and E, and are undetectable thereafter (Fig. 1A) (Coroian et al., 2005). Signal was also observed in the gonads of all adults, with 68% (174/257) showing very strong staining (Fig. 1A), while very little or no gonadal signal was observed using sense probes (not shown). In the related nematode, *C. briggsae*, we also found that *in situ* hybridization with a *Cb-med-1* probe reveals gonadal as well as embryonic signal in EMS (Fig. 1H) (Coroian et al., 2005). We did not detect significant levels of *med* transcripts in very early embryos or late oocytes (data not shown). We conclude that *med* expression has both a maternal and zygotic component, and that both components are evolutionarily conserved.

We compared maternal *med* expression with that of *pal-1*, a gene that is also expressed both maternally and zygotically, and which acts to specify the C and D lineages (Baugh et al., 2003; Hunter and Kenyon, 1996). Gonadal signal detected under similar conditions with a *pal-1* probe suggests that maternal *med* transcripts are significantly less abundant than those of *pal-1* (Fig. 1I). This observation is consistent with results from global microarray experiments, which detected significant germline signal for *pal-1* mRNA but not for *med-1* (Reinke et al., 2004). Hence, maternal *med* transcripts are of relatively low abundance.

The bZIP/homeodomain factor SKN-1 is required for embryonic expression of *med* transgenes (Bowerman et al., 1993; Maduro et al., 2001). Recombinant SKN-1 protein can

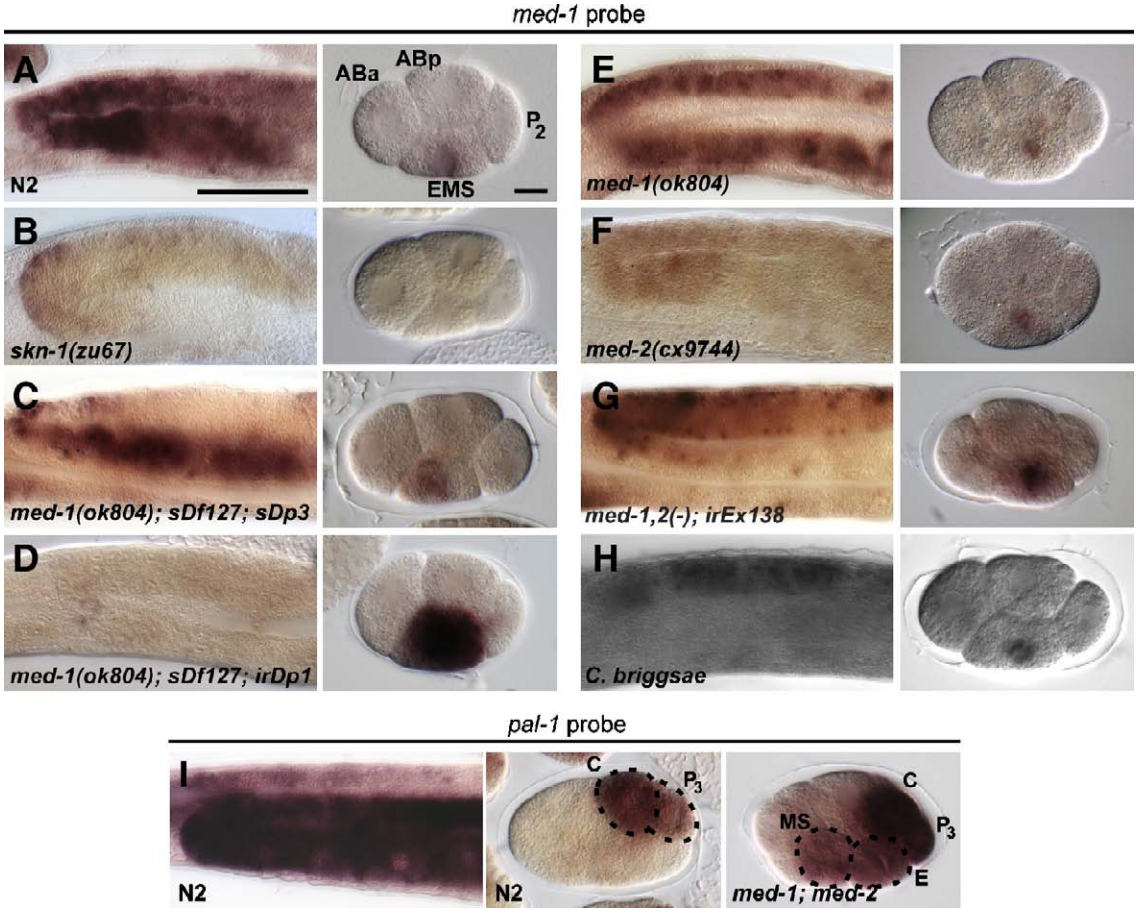

Fig. 1. Detection of *med* and *pal* mRNAs by *in situ* hybridization. (A) Wild-type (N2) animals show gonadal signal and activation in EMS at the 4-cell stage. (B) In parallel experiments, expression is strongly abrogated in the gonads of *skn-1(zu67)* hermaphrodites and in their progeny. *skn-1(RNAi)* embryos were similarly absent of zygotic *med* transcripts. (C) JM134 shows high levels of gonadal *med* signal similar to wild-type. Embryos carrying *sDp3* also express *med-2* zygotically. (D) MS162 demonstrates strongly abrogated maternal expression, but zygotic overexpression, due to an integrated *med-1(+)* transgene on *irDp1*. (E) Detection of *med-2* (in *med-1(ok804)* animals) shows both maternal and zygotic expression. (F) Detection of *med-1* (in *med-2(cx9744)* animals) shows decreased maternal expression, but normal zygotic expression. (G) An extrachromosomal array providing rescue to a *med-1(ok804); med-2(cx9744)* strain demonstrates maternal expression and zygotic overexpression. (H) Maternal and zygotic activation of the *med*s is conserved in the related nematode *C. briggsae*. (I) Detection of *pal-1* mRNA in the gonad and embryos at the 12-cell stage. In N2, *pal-1* accumulates stronger signal than *med-1* when detected with a similar-sized probe (left panel). At the 12-cell stage, zygotic *pal-1* activation is seen in the sister cells C and P_3. In the absence of *med-1,2*, an additional expression component appears in MS and E, consistent with the MS, E→C transformation seen in *med-1,2(RNAi)* (Maduro et al., 2001). Embryos are shown with the anterior to the left, and dorsal to the top in this and other figures. Scale bars in panel A are 100 μm (adult image) and 10 μm (embryo image).

directly bind SKN-1 sites in the *med-1* promoter, and these sites are required for *med-1* reporter expression (Blackwell et al., 1994; Maduro et al., 2001). To assess the requirement of SKN-1 for endogenous *med* activation, we performed *in situ* hybridization to detect *med* transcripts in embryos and adults depleted for *skn-1* activity. In the progeny of *skn-1(zu67)* mothers, or of animals fed *skn-1* dsRNA-expressing bacteria, we failed to detect embryonic *med* transcripts (Fig. 1B and data not shown). Furthermore, while gonadal expression was detectable in *skn-1 (zu67)/nT1* animals, the signal was almost undetectable in *skn-1 (zu67)* homozygotes (Fig. 1B). We conclude that SKN-1 is required for both maternal and zygotic activation of the *med* genes.

To determine whether *med-1* and *med-2* might be differentially regulated, we performed *in situ* hybridization on strains harboring a mutation in one gene but not the other. The mutation *ok804* deletes the entire *med-1* locus (Coroian et al., 2005). Staining of *med-1(ok804)*, which should detect only *med-2* transcripts, shows signal in the gonad and embryo that is comparable to that seen in wild-type, with 67% (108/161) of adults showing strong signal (Fig. 1E). The *cx9744* mutation is a Mos1 transposon insertion in the *med-2* open reading frame that is predicted to result in production of an aberrant mRNA containing an early nonsense mutation (Coroian et al., 2005). These aberrant transcripts would be expected to be rapidly degraded by the Smg mRNA surveillance system (Pulak and Anderson, 1993). Staining of the *med-2(cx9744)* strain, which should therefore specifically detect only *med-1* mRNA, shows a dramatic reduction in the gonad signal: only 15% (25/166) of adults showed staining, and this staining was significantly weaker than in wild-type or *med-2(−)* (Fig. 1F). We conclude that while both *med-1* and *med-2* are activated to roughly similar levels in the 4-cell embryo, *med-2* produces more germ line transcripts than *med-1*. Despite these differences, however, homozygosity for either *med* allele does not result in an endoderm defect (Table 1).

Table 1
Intestinal differentiation in mutant embryos

Genotype	% Intestine[a] (*n*)
Wild type	100 (*n*>500)
med-1(ok804)	100 (240)
med-2(cx9744)	100 (774)
dpy-17(e164) sDf127 unc-32(e189)[b]	100 (196)
dpy-17(e164) sDf127 unc-32(e189)[c]	100 (127)
unc-32(e189)	100 (249)
med-1(ok804); dpy-17(e164) sDf127 unc-32(e189)[b] [JM134]	77 (121)
med-1(ok804); dpy-17(e164) sDf127 unc-32(e189)[c] [MS162]	49 (525)
med-1(ok804); med-2(cx9744)[d] [MS290]	83 (165)
med-1(ok804); med-2(cx9744)[c] [MS247]	53 (263)
skn-1(RNAi)	28 (731)
med-1(ok804); skn-1(RNAi)	33 (220)
med-2(cx9744); skn-1(RNAi)	21 (394)
med-1(ok804); med-2(cx9744); skn-1(RNAi)	11 (102)
end-1(ok558)	100 (322)
end-1(ox134)	100 (364)
end-3(ok1448)	95 (155)
med-1(ok804); dpy-11(e224) end-1(ok558)	100 (181)
med-1(ok804); end-3(ok1448)	42 (251)
med-2(cx9744); dpy-11(e224) end-1(ok558)	98 (344)
med-2(cx9744); end-3(ok1448)	97 (150)
med-1(ok804); dpy-17(e164) sDf127 unc-32(e189); skn-1(RNAi)[c]	19 (68)
med-1(ok804); dpy-17(e164) sDf127 unc-32(e189); dpy-11(e224) end-1(ok558)[b]	32 (207)
med-1(ok804); dpy-17(e164) sDf127 unc-32(e189); dpy-11(e224) end-1(ok558)[c]	3 (123)
med-1(ok804); med-2(cx9744); dpy-11(e224) end-1(ok558)[c]	3 (121)
med-1(ok804); med-2(cx9744); end-3(ok1448)[c]	37 (258)

[a] Scored by presence of birefringent gut granules in terminal embryos.
[b] Segregated from mothers carrying *sDp3*, which provides maternal *med(+)* activity.
[c] Segregated from mothers carrying *irDp1*, which causes knockdown of maternal *med* transcripts.
[d] Segregated from mothers carrying *irEx138*, which provides maternal *med(+)* activity.

Maternal med activity rescues endoderm (E) but not mesoderm (MS) specification

The original assessment of *med* loss-of-function was based on knockdown of *med* expression by RNAi. Direct gonadal injection of *med-1* dsRNA (which targets both *med-1* and *med-2*) produces arrested progeny embryos, all of which lack posterior pharynx and nearly all PAL-1-independent body wall muscle, tissues normally made by descendants of MS (Maduro et al., 2001; Sulston et al., 1983). Approximately 50% of these embryos also lack differentiated endoderm. In contrast to these results, Goszczynski and McGhee (2005) reported that 80–97% of embryos chromosomally lacking *med-1* and *med-2* make endoderm. One strain the authors used to produce *med-1,2(−)* embryos, JM134, is homozygous for *med-1(ok804)* and *sDf127*, a large deletion that removes many genes including *med-2* (Figs. 2A, B). The lethality of *sDf127* in this strain is complemented by the free duplication *sDp3*, which carries *med-2(+)*. We confirmed that while *sDf127* embryos (derived from an *sDf127; sDp3* mother) made endoderm 100% of the time (*n*=196), 77% of *med-1; sDf127* embryos (*n*=121) segregated by *med-1; sDf127; sDp3* mothers (strain JM134) made endoderm (Figs. 3A, B and Table 1).

As RNAi can efficiently target maternal as well as zygotic transcripts (Fire et al., 1998), the simplest explanation for the lower percentage of gutless embryos from the JM134 strain compared to that obtained by *med-1,2(RNAi)* experiments is that maternal *med* expression can rescue endoderm. We performed *in situ* hybridization on JM134 and found that this strain accumulates *med* transcripts in the gonad similar to wild-type animals, with 75% (57/76) of adults showing strong gonadal staining (Fig. 1C). Hence, the JM134 strain demonstrates maternal *med-2* expression from *sDp3*.

The presence of gonadal *med* transcripts in the JM134 strain, whose *med-1,2(−)* progeny demonstrate a weaker endoderm phenotype than *med-1,2(RNAi)*, suggests that endoderm specification in *med-1,2(−)* embryos can be partially rescued by maternal *med* transcripts. We tested this hypothesis by assessing endoderm specification in *med-1,2(−)* embryos produced by mothers that lack gonadal *med* transcripts. To deplete maternal *med* expression reliably, we modified *sDp3* to contain an integrated *med-1(+)* array, generating the free

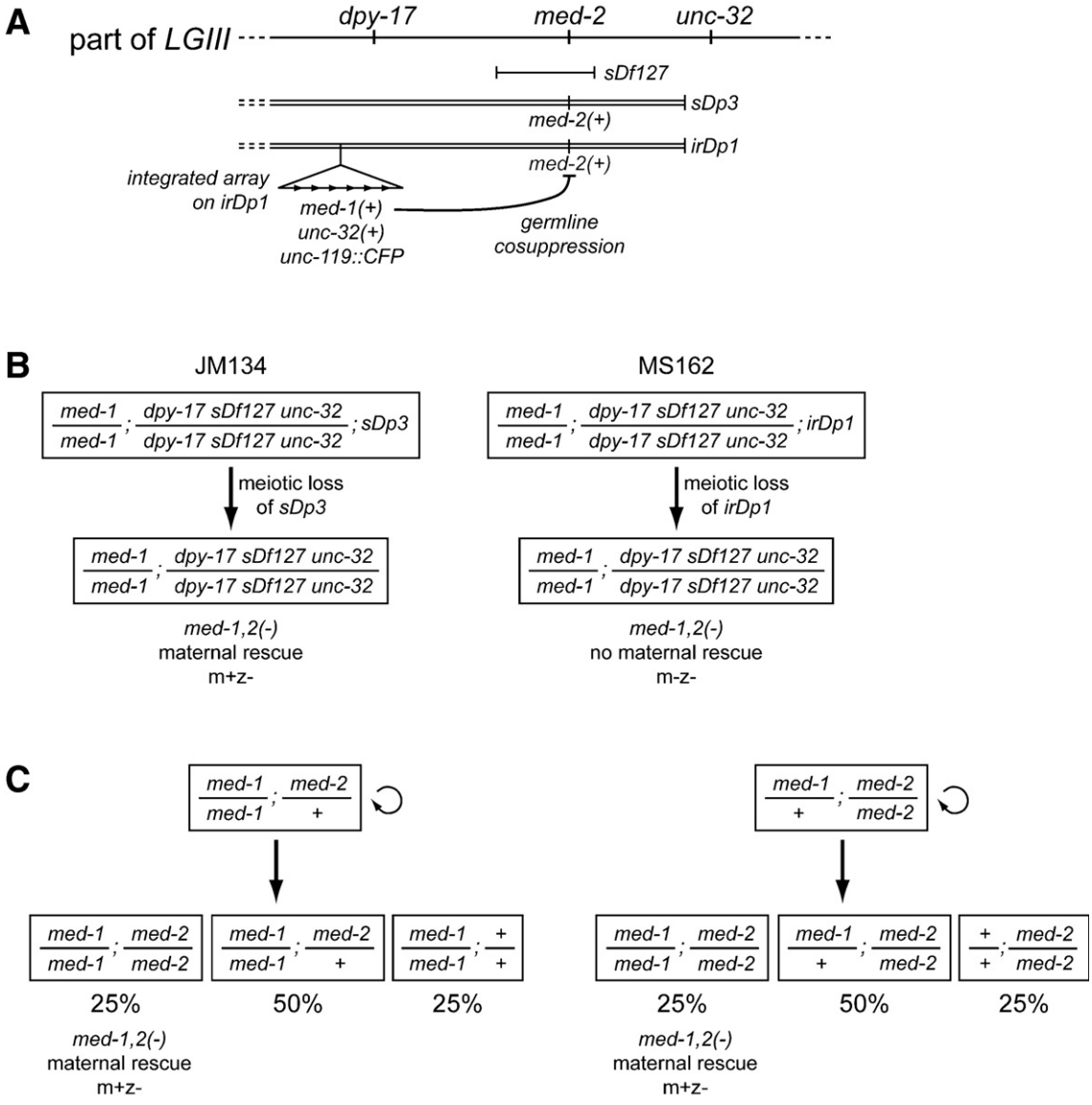

Fig. 2. Genotypes of *med* strains used to test maternal and zygotic *med* function. (A) Gene arrangement of part of *LGIII* containing *med-2*. *sDf127* is a ~1 MB deletion that removes ~240 genes including *med-2* (http://www.wormbase.org, release WS160). *sDp3* is a free duplication that complements *sDf127*, *dpy-17*, and a large portion of *LGIII* to the left (not shown). *irDp1* is *sDp3* carrying an unmapped integrated array containing *med-1(+)*, *unc-32(+)* and *unc-119*::YFP (see Materials and methods). The *med-1(+)* array causes germline cosuppression of *med-2* on *irDp1*. (B) Production of chromosomally *med-1,2(−)* embryos in JM134 (Goszczynski and McGhee, 2005) and in MS162, in which *sDp3* has been replaced by *irDp1*. While the JM134 strain has the Unc32 phenotype, MS162 animals are essentially wild-type. (C) Production of *med-1,2(−)* embryos to test maternal contribution of either *med* gene. Expected genotype ratios are shown. Abbreviations: *m+z−*, maternal product supplied, zygotic product absent; *m−z−*, maternal and zygotic products absent.

duplication *irDp1* (Figs. 2A, B). The presence of this array is predicted to cause depletion of maternal *med* transcripts by transgene-mediated cosuppression (Dernburg et al., 2000; Robert et al., 2005). The resultant strain, MS162, has the same genotype as JM134 (i.e. *med-1; sDf127*) but is balanced by *irDp1* instead of *sDp3*. We performed *in situ* hybridization on MS162 and found that gonadal *med* transcripts were undetectable in 99% (69/70) of adults, while embryonic levels were greatly enhanced in EMS (Fig. 1D). This enhanced expression is consistent with zygotic expression of the repetitive *med-1(+)* transgene, as a chromosomally integrated *med-1*::GFP::MED-1 strain produced a similarly strong EMS signal when stained with an antisense GFP probe (data not shown). We conclude that transgene cosuppression can be used to abrogate endogenous maternal *med-2* transcript accumulation, and that this cosuppression does not prevent zygotic activation.

If maternal *med* expression can rescue endoderm specification in some *med-1,2(−)* embryos derived from JM134 mothers, then *med-1,2(−)* embryos produced by MS162, which lacks germline *med* transcripts, would be predicted to make endoderm less frequently. Indeed, 51% of the *med-1,2(−)* embryos produced by MS162 lack endoderm (Fig. 3C, Table 1), similar to the results we obtained using RNAi (Maduro et al., 2001). To rule out any chromosomal differences between JM134 and

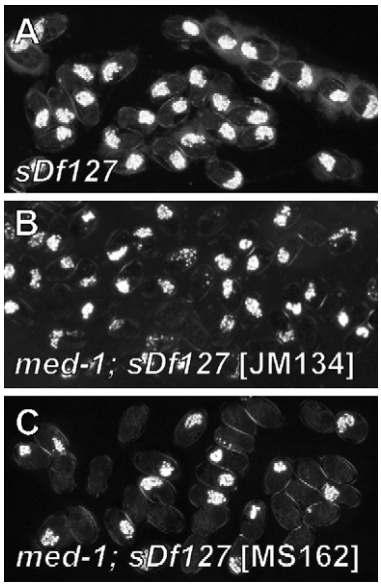

Fig. 3. Gut granules in *med* mutant embryos. (A) All embryos homozygous for a deletion that removes *med-2* make gut as seen by the similar-sized regions of gut granule birefringence. (B) JM134 generates *med-1; sDf127* embryos that contain varying degrees of gut. (C) MS162 generates genetically identical *med-1; sDf127* embryos, but fewer of these make endoderm. Fields are approximately 0.5 mm across.

MS162, we backcrossed MS162 males to JM134, and mated progeny males back to JM134 repeatedly. We obtained similar results (~50% endoderm defect among *med-1,2(−)* embryos) after 3× and 10× backcrosses, confirming that the increased severity of the endoderm defect is linked to *irDp1*, and is not due to a fortuitous background mutation. Replacement of the *sDf127* chromosome in MS162 with *med-2(cx9744)*, to create strain MS247, also resulted in a similarly strong endoderm defect (Table 1), showing that the increased endoderm defect with *irDp1* does not require the loss of other genes deleted by *sDf127*. We conclude that maternal *med-2* expression on *sDp3* can rescue endoderm specification in a significant fraction of *med-1,2(−)* embryos.

To rule out any contribution of the additional genes on the *sDp3* or *irDp1* balancers used to propagate the *med-1; med-2* strains, we replaced *irDp1* with an extrachromosomal array containing only *med-1(+)* and *unc-119*::CFP, to create MS290 (*med-1(ok804); med-2(cx9744); Ex[med-1(+)]*) (Coroian et al., 2005). As the vast majority of arrays in *C. elegans* do not show germline expression (Kelly et al., 1997), *med-1,2(−)* embryos produced by MS290 would not be expected to be maternally rescued for endoderm specification. Unexpectedly, *med-1,2(−)* embryos segregated from this strain made endoderm 83% (137/165) of the time, suggesting that they were maternally rescued. When we performed *med in situ* hybridization on MS290, we detected very high levels of gonadal *med* transcripts (Fig. 1G). This suggests that intrinsic differences between the *med-1(+)* array on *irDp1* and the extrachromosomal *med-1(+)* array in MS290 preclude germline transcript accumulation in one case but not the other. Nonetheless, these results provide a further correlation between the presence of germline *med* transcripts in a mother animal and an increased likelihood that its *med-1,2(−)* progeny will make endoderm.

As the MS blastomere is also specified by the activity of the *med*s, we assayed for maternal rescue of the MS defect in *med-1 (ok804); med-2(cx9744)* embryos. As anticipated by our prior RNAi results, all *med-1,2(−)* embryos from a mother carrying *irDp1* arrest without MS-derived posterior pharynx, as scored both by Nomarski optics and expression of the pharynx muscle marker *ceh-22*::GFP (Coroian et al., 2005; Okkema and Fire, 1994; data not shown). We then examined *med-1,2(−)* embryos made by MS290 mothers, which provide maternal *med(+)* activity, and found that 100% of the *med-1,2(−)* embryos lacked posterior pharynx. Hence, maternal *med* transcripts produced by a *med-1* transgene array cannot rescue MS fate in *med-1,2(−)* embryos.

To provide a maternal source of *med* expression from a normal chromosomal context, we constructed animals homozygous for a mutation in one *med* gene and heterozygous for a mutation in the other, such that 1/4 of the self-progeny are expected to be *med-1,2(−)* (Fig. 2C). We found that 26% (190/528) of the progeny of *med-1/+; med-2* mothers arrested, consistent with expected Mendelian segregation ($p=0.4$) and lethality of the *med-1; med-2* genotype. However, 42% (332/783; $p<10^{-6}$ compared with expected 1:3) of the progeny of *med-1; med-2/+* mothers arrested as embryos. As *med-1; +/+* embryos are almost always viable, we compute that approximately 35% of *med-1; med-2/+* embryos undergo embryonic arrest due to zygotic haploinsufficiency of *med-2* in the absence of *med-1*. All arrested embryos made from either *med-1/+; med-2* mothers or *med-1; med-2/+* mothers lacked posterior pharynx as scored by Nomarski optics, confirming that MS fate cannot be rescued by a maternal *med*(+) gene in its normal chromosomal context. The arrest of 35% of *med-1; med-2/+* embryos further suggests that a single zygotic copy of *med-2* cannot always rescue MS fate.

We also evaluated rescue of E specification by a single maternal dose of *med-1* or *med-2*. 31% (59/190) of the arrested progeny from *med-1/+; med-2* mothers lacked endoderm, while 13% (42/332) of the arrested progeny from *med-1; med-2/+* mothers lacked endoderm. Given that 1/4 of the progeny in this latter case are expected to be of the *med-1; med-2* genotype, we calculate that a maximum of 21% (42/196) of these lacked endoderm. The 31% [maternal *med-1(+)*] and 21% [maternal *med-2(+)*] gutless phenotypes contrast sharply with the ~50% of *med-1,2(−)* embryos that lack endoderm when germline *med* transcripts are reduced by transgene cosuppression or RNAi. We conclude that a single chromosomal copy of either *med-1* or *med-2* can maternally rescue specification of E, but not MS, in *med-1,2(−)* embryos. That more *med-1,2(−)* embryos are rescued when the mother carries *med-2(+)* is consistent with the observation that *med-2* accumulates more gonadal transcripts than *med-1*. The differences in gonadal expression and maternal rescue of endoderm suggest that despite 98% sequence identity between the *med* genes, there are qualitative differences in their contributions to embryonic development.

Evidence for transport of somatic med transcripts into the gonad

As an alternate approach to preventing *med* transcripts from being transmitted via the maternal gonad, we obtained *med-1,2(−)* embryos from mosaic animals that carry *med-1(+)* gene in the soma, but have a *med-1(−)* germline. For the strain MS290 (*med-1(ok804); med-2(cx9744); Ex[med-1(+)]*), such mothers can be recognized because they give rise to a brood consisting entirely of arrested embryos instead of an approximate 1:1 distribution of viable:arrested embryos. From 280 singled MS290 mothers, we obtained 8 (3%) germline mosaics and found, somewhat unexpectedly, that 73% of their progeny ($n=195$) contained endoderm. A similar result (73% endoderm, $n=125$) was also seen with the progeny from a single germline mosaic *med-1(ok804); med-2(cx9744); sDp3* mother. Hence, there is at most only a slight reduction in the degree of maternal *med* rescue of endoderm when the maternal germline lacks a *med(+)* gene, rather than a reduction to ~50% as seen with RNAi or when transgene cosuppression is used to abrogate gonadal *med* transcripts.

One possible explanation for this discrepancy is that somatic *med* transcripts are imported into the gonad from another tissue. Indeed, we observed that 80% of wild-type adults ($n=45$) stained with *med-1* antisense probe demonstrated strong expression in the anterior intestine (data not shown). In contrast, only 29% of *skn-1(RNAi)* animals ($n=42$) demonstrated qualitatively similar staining, though at a lower level. In controls using antisense probes for other genes (*myo-3*, *myo-2*, and *hlh-1*), we observed only weak anterior intestine expression in less than 10% of animals ($n>70$ for each case). These results suggest that the SKN-1-dependent transcripts found in the adult gonad may result from somatic transcription. This result is further supported by the finding that SKN-1 protein, which activates *med* expression in the EMS cell, is also present in the intestine (Bowerman et al., 1993).

med-1 and med-2 are essential for normal E development

The foregoing experiments and our previously published studies have established that while all *med-1,2(−)* embryos lack cell types made by the MS blastomere, a significantly large proportion of such embryos still make endoderm. As an epitope-tagged *med-1*::c-*myc*::MED-1 transgene reveals transgenic MED-1 protein in only the EMS, MS and E nuclei (Maduro et al., 2001), we wished to investigate whether there might be a role for the *med*s beyond specification of an endodermal fate.

There is evidence that some *med-1,2(RNAi)* embryos that make endoderm show defects in early E development. In wild-type embryos, the E daughter cells Ea and Ep migrate into the interior of the embryo at the onset of gastrulation (Sulston et al., 1983). In some *med-1,2(−)* embryos, Ea and Ep divide precociously on the ventral surface (Maduro et al., 2001). Similar defects are also seen in *skn-1(zu67)* mutant embryos, consistent with the participation of *skn-1* and the *med*s in the same genetic pathway (Bowerman et al., 1992; Maduro et al., 2001). When endoderm is not made in *med-1,2(−)* embryos, the E cell (as with MS in all *med-1,2(−)* embryos) adopts the fate of its lineal cousin, the mesectodermal precursor C (Maduro et al., 2001). Specification of C and the ectopic C-like cells made in *med-1,2(−)* embryos requires maternal activity of the Caudal-like homeodomain protein PAL-1 (Hunter and Kenyon, 1996). In the normal C cell, maternal PAL-1 activates zygotic expression of *pal-1*, providing an early marker for specification of this lineage (Baugh et al., 2005).

To test for activation of zygotic *pal-1* in *med-1,2(−)* embryos, we performed *pal-1 in situ* hybridization on wild-type embryos and those from the MS247 [*med-1; med-2; irDp1*] strain. *pal-1* mRNA was detected in C and P_3 in 87% (26/30) of wild-type embryos showing staining (Fig. 1I), and at low levels in C, P_3, MS, and E in the remaining 13%. This suggests that PAL-1 can activate a low level of zygotic *pal-1* in a small proportion of MS and E cells. In contrast, 47% (14/30) of stained MS247 progeny showed expression in C and P_3, and the remaining 53% showed staining in C, P_3, MS, and E (Fig. 1I). As the frequency of meiotic loss of *irDp1* is approximately 40%, this suggests that the majority of, if not all, *med-1,2(−)* embryos activate *pal-1* in MS and E. We also observed similar ectopic expression of a zygotic *pal-1*::YFP reporter in *med-1,2(−)* embryos, suggesting that these *pal-1* transcripts do not represent perdurance of maternal message (data not shown). Therefore, many (if not all) of the E cells that ultimately produce gut in *med-1,2(−)* embryos contain aberrant *pal-1* transcripts.

We hypothesized that division and expression defects in the early E lineage may manifest themselves in later embryonic development by altering the pattern of cell divisions in the E lineage. The E cell normally divides 4–5 times to produce 20 cells by the end of embryogenesis (Sulston et al., 1983). Terminal *sDf127* embryos contain a patch of gut granules that comprises ~25% of the cross-sectional area of the embryo, and which can be seen to be fairly consistent from embryo to embryo (Fig. 3A). In contrast, many *med-1; sDf127* embryos contain gut granule patches that occupy as little as 10% of the area, or consist of only a small number of puncta (Fig. 3B). We asked whether this reflects a change in the number of gut cells by examining the endoderm in various *med-1,2(−)* strains using a chromosomally integrated *elt-2* reporter transgene to mark intestinal nuclei, as has been used in similar studies of gut cell number (Fukushige et al., 1998; Kostic et al., 2003). A summary of our results is shown in Fig. 4. As expected, wild-type and single *med-1* or *med-2* embryos made an average of 20 *elt-2*-expressing cells with little variation. We found that *sDf127* embryos make endoderm with a similar mean number of cells as wild-type, although with a slightly higher variance. The variation was similar in embryos segregated from mothers carrying *sDp3* or *irDp1*, suggesting that the increased variance compared to that in *med-2(−)* embryos results from loss of other gene(s) in *sDf127*, rather than from differences in the two free duplications.

Among *med-1,2(−)* embryos that still made endoderm, the average number of gut cells was reduced, and the variance increased significantly. *med-1; sDf127* embryos segregated from mothers carrying either *sDp3* or *irDp1* showed a combined

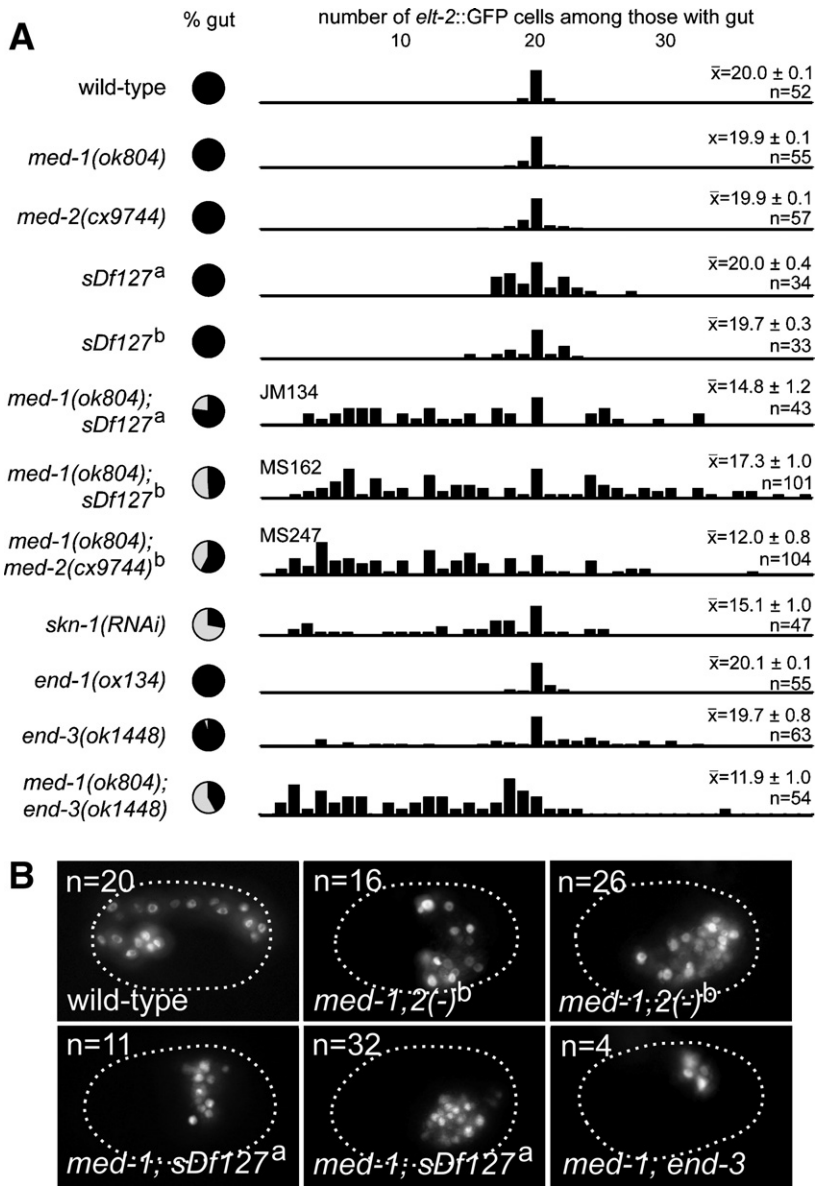

Fig. 4. Number of endoderm cells in late-stage embryos. (A) Distribution of gut cell numbers among embryos making endoderm. A small pie chart indicates the proportion of embryos of the given genotype that make endoderm (darker portion) as shown in Table 1. The histograms represent the numbers of embryos containing the number of gut cells indicated along the *X* axis. The height of the bars has been normalized to that of the modal class within each genotype. Mean±SEM and total embryos scored (*n*) are shown towards the right. (B) Examples of appearance of *elt-2*::GFP-expressing cells among 3× or terminal embryos. Multiple epifluorescence images at different focal planes were digitally stacked.

range of 3–40 gut cells with averages of 14.8±1.2 and 17.3± 1.0 cells, respectively. The majority of embryos contained less than 20 cells (67% in the case of *sDp3(+)* mothers, and 55% for *irDp1*). *med-1,2(−)* embryos segregated from *irDp1(+)* mothers showed a lower mean number (12.0±0.8) and an even higher proportion (81%) of embryos with subnormal numbers of gut cells. We found, overall, that only 10% of *med-1,2(−)* embryos with any endoderm contain a normal number of 20 gut cells. Thus, 93–95% of *med-1,2(−)* embryos either make no gut or make a gut that contains abnormal numbers of cells. We conclude, therefore, that *med-1* and *med-2* are important for normal development of the endoderm in the vast majority of *C. elegans* embryos.

Distinguishable contributions of end-1 vs. end-3

The paralogous *end-1* and *end-3* genes appear to function redundantly (Maduro et al., 2005a). Loss of *med-1*, *med-2*, or *end-1* individually has no detectable endoderm phenotype, while loss of *end-3* results in a weak endoderm specification defect, with approximately 5% of *end-3(−)* embryos failing to make gut (Table 1). We tested the ability of mutations in *end-1* or *end-3* to enhance mutations in the *med*s (Table 1). *med-1,2(−)* embryos lacking the maternal contribution were greatly enhanced for gutlessness by *end-1* (only 3% made gut) but not *end-3* (37% made gut). In combinations of *med* and *end* single mutants, three of the four possible *med; end* double mutants

showed very weak endoderm defects (>97% made endoderm). The *med-1; end-3* double mutant, however, showed strong synergy, as only 42% of embryos made gut. Among those that made endoderm, the number of gut cells made was nearly always abnormal (Fig. 4). These results show that *end-1* and *end-3* make different contributions to endoderm specification.

Global transcriptome analysis has suggested that *end-3* is activated slightly earlier than *end-1* (Baugh et al., 2003). We performed *in situ* hybridization to detect *end* transcripts in wild-type embryos. *end-3* mRNA was detected in 84% of embryos at the 1E stage, 63% of 2E stage embryos, and 0% of 4E stage embryos (Figs. 5A, C, E). *end-1* transcripts, by contrast, were detected in 19% of 1E, 90% of 2E, and 83% of 4E stage embryos (Figs. 5B, D, F). These differences confirm an earlier onset of *end-3* activation as compared with *end-1*. We note that, for both *end-1* and *end-3 in situ* hybridization at the 2E stage, approximately half of the stained embryos showed stronger signal in Ep than in Ea (Figs. 5C, D; discussed below).

We next tested for *end* activation differences in the absence of the SKN-1→MED-1,2 pathway by depleting *skn-1*, a treatment that results in abrogation of both *skn-1* and the *med*s (this work and Maduro et al., 2001). In *skn-1(RNAi)* embryos *end-3* mRNA is undetectable, while *end-1* is still expressed at high levels (Figs. 6A, C). We conclude that the early activation of *end-3* is accomplished primarily by the activity of SKN-1→MED-1,2, while *end-1* is still activated in their absence.

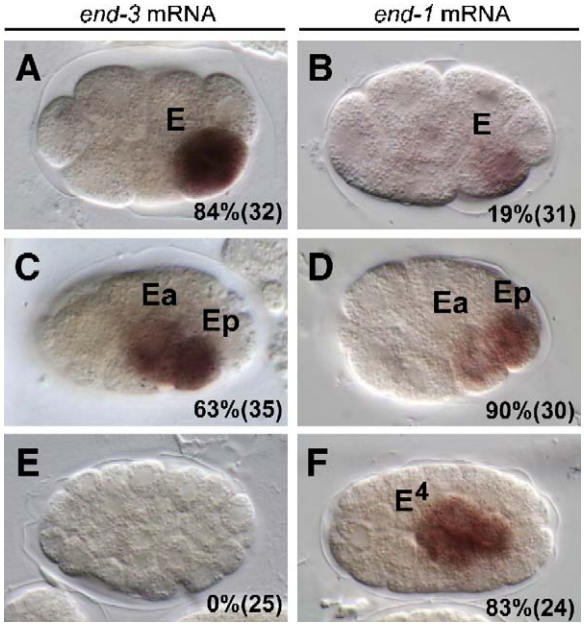

Fig. 5. *end-3* is activated earlier than *end-1*. Detection of transcripts by *in situ* hybridization is shown for *end-3* at the 1E (A), 2E (C), and 4E (E) stages, and *end-1* at similar stages in panels B, D, and F respectively. Percentages shown indicate the proportion of embryos of a given stage that showed expression, with total number scored in brackets. Note that in panels C and D, signal is stronger in Ep than in Ea. This was observed in approximately 50% of embryos; symmetrical signal was observed in the remainder. Slides for each probe were prepared from the same batch of embryos.

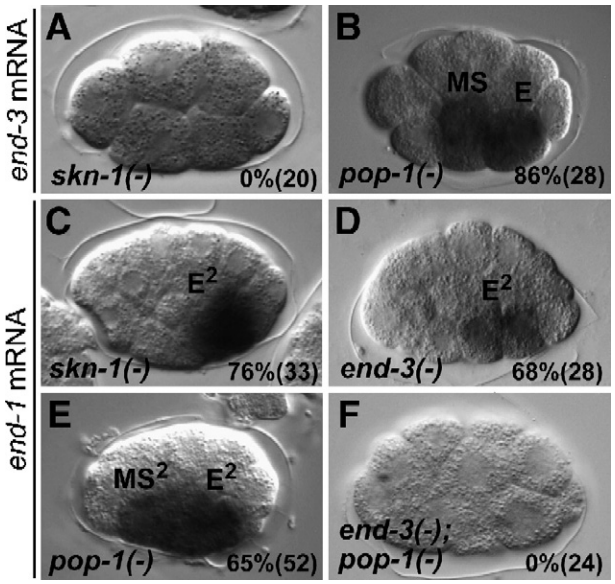

Fig. 6. Expression of the *end*s in mutant backgrounds. (A) In *skn-1(RNAi)* embryos, *end-3* mRNA is not detected. (B). *end-3* is derepressed in the MS cell in *pop-1(RNAi)*. A single additional embryo (not shown) had expression in only the E cell. (C) *end-1* mRNA is still detected in *skn-1(RNAi)*. (D) In *end-3 (ok1448)* mutant embryos, *end-1* mRNA levels are reduced. (E) Depletion of *pop-1* by RNAi results in derepression of endogenous *end-1* mRNA in the MS lineage. An additional seven embryos showed signal in Ea/Ep alone. (F) *end-3(ok1448); pop-1(RNAi)* embryos show no detectable *end-1* mRNA above background levels. Percentages and numbers shown are as in Fig. 5.

END-3 and POP-1 activate end-1

Previous experiments established that while the primary role of POP-1 in endoderm specification is the repression of endoderm fate in the MS cell, it also contributes to Wnt-dependent activation of the endoderm specification pathway in the E cell (Lin et al., 1995; Maduro et al., 2002, 2005b; Shetty et al., 2005). Consistent with this observation, in the absence of *end-3*, specification of the endoderm becomes largely dependent on POP-1 (Maduro et al., 2005b). We examined expression of the *end*s in a *pop-1(RNAi)* background, and found that transcripts for both genes become detectable to equal levels in the MS and E lineages in a manner that recapitulates their temporal differences in wild-type (Figs. 6B, E). A repressive role for POP-1 in the Ea cell also became apparent, as >90% of 2E stage *pop-1(RNAi)* embryos showed equal levels of *end-1* expression in Ea and Ep (visible in Fig. 6E). We have previously shown that transgenic GFP::POP-1 can interact directly with the *end-1* and *end-3* promoters *in vivo* at the 2E stage (Maduro et al., 2002). We conclude that *end-1,3* are still responsive to POP-1 in the E daughter cells.

Given the observation that *end-3* mRNA accumulates in E earlier than *end-1* mRNA, and that an *end-3; pop-1* double mutant shows a synergistic defect (only 3% make gut), we hypothesized that END-3 might activate *end-1* in parallel with POP-1. There is likely sufficient time for active END-3 to be synthesized from the *end-3* mRNA before the E cell divides, as the product of an *end-3*::c-*myc*::END-3 fusion transgene can be detected near the end of the E cell cycle (MM and JR,

unpublished). In *end-3(−)* embryos, we found that *end-1* mRNA is detectable, though at lower levels than in the wild type (Fig. 6D). In *end-3(ok1448); pop-1(RNAi)* embryos, however, expression of *end-1* becomes essentially undetectable (Fig. 5F). We conclude that both POP-1 and END-3 together are essential activators of *end-1*.

Discussion

A complex network specifies C. elegans endoderm

The advances we have made in this study are as follows: (1) The *med* genes are expressed maternally in both *C. elegans* and *C. briggsae*; (2) Like the zygotic activation of *med-1,2*, maternal expression requires SKN-1; (3) Endoderm (E) but not mesoderm (MS) fate is partially rescued by this maternal activity; (4) When specification of endoderm is partially compromised, abnormal numbers of intestinal cells are made; (5) *med-1* and *med-2* make different genetic contributions to E specification, as do *end-1* and *end-3*; (6) *end-3* is activated earlier in the E cell cycle than *end-1*; (7) In addition to acting in parallel with *end-1* in promoting endoderm fate, END-3 also activates *end-1*. Our data reveal new and unexpected complexities in the *C. elegans* endoderm gene cascade (Fig. 7).

Maternal deployment of the embryonic SKN-1→MED-1,2 pathway

We have previously shown that maternally supplied SKN-1 directly activates *med-1,2* transgenes in the early EMS lineage, placing *med-1,2* downstream of maternal SKN-1. We have shown here that a significant amount of *med* mRNA accumulates in the hermaphrodite germline, and that this component

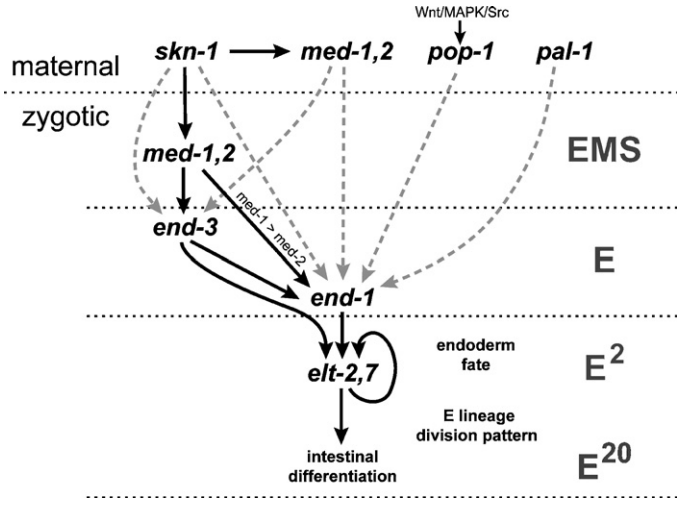

Fig. 7. A comprehensive model for *C. elegans* endoderm specification. Arrows indicate direction of positive regulation as follows: solid arrows indicate our subjective assessment of the strongest regulatory interactions in wild-type embryos, while dashed arrows indicate weaker contributions. All arrows shown have been substantiated by genetic interactions presented in this and other work (Bei et al., 2002; Maduro et al., 2001, 2005b; Rocheleau et al., 1997, 1999; Thorpe et al., 1997; Zhu et al., 1998).

of *med* expression also requires SKN-1. Although the *med* genes are 98% identical within their intronless coding regions and in several hundred base pairs of flanking genomic DNA (Maduro et al., 2001), suggesting they are regulated by similar mechanisms, higher levels of maternal *med-2* mRNA were detected than for *med-1*. As *med-1* is X-linked, and the X chromosome is specifically underrepresented for genes expressed in the maternal germline (Reinke et al., 2000), a global X chromosome repression mechanism may act to down-regulate maternal *med-1* transcription.

We were unable to detect *med* transcripts above background levels in the very early embryo, even though transcripts for other maternal genes such as *skn-1* are detectable this early (Seydoux and Fire, 1994). One possibility is that a low level of *med* transcripts in early embryos is simply beyond our ability to detect beyond background signal. Alternatively, some post-transcriptional contribution, such as MED protein, or even *end-1,3* mRNA, might be deposited into oocytes. We have seen very weak evidence for a maternal contribution of *end-3*: 98% of *end-3(zu247)* embryos ($n=689$) derived from an *end-3 (zu247)/+* mother made endoderm, while *end-3(zu247)* embryos from an *end-3(zu247)* mother made endoderm approximately 94% of the time ($n=518$, $p<0.001$; MM and JR, unpublished). Regardless of the mechanism, we have shown genetically that the maternal *med* component is capable of rescuing E specification, but not MS specification, in a fraction of *med-1,2(-)* progeny embryos.

We have further shown that accumulation of germline transcripts requires SKN-1 function in the mother. As regulation of the *med*s by SKN-1 is direct in the embryo, maternal activation of the *med*s is also likely to be direct. While SKN-1 protein has been detected in early embryos, it was not detected in the adult gonad (Bowerman et al., 1993). This may be because it is below threshold levels for detection. However, our germline mosaic and *in situ* hybridization data suggest that maternal transcription of the *med*s may take place outside the germline, perhaps in the intestine, where SKN-1 is also found (Bowerman et al., 1993). An intriguing model, then, is that SKN-1 in the soma activates *med* expression, and these transcripts are somehow transported into the maternal gonad.

What is the purpose of deploying the SKN-1→MED-1,2 pathway maternally, before it is used embryonically? The activity is clearly weak, as maternal *med* expression cannot rescue mesoderm specification in the absence of embryonic expression. The pathway that specifies the E fate must be deployed on a relatively short time scale, approximately 20 min (Baugh et al., 2003). It is possible, therefore, that maternal *med* activation primes the endoderm specification pathway. Our genetic data show that *med* activity is particularly critical for activation of *end-3*, which precedes that of *end-1*. As *end-3* contains four MED binding sites, compared to only two in *end-1*, *end-3* might be more responsive to MED-1,2. Hence, low levels of MED-1,2 in the very early embryo could bind the *end-3* promoter and set the stage for activation after E is born.

Accumulation of *med* transcripts in the germline requires that mechanisms exist to prevent specification of endoderm fate

and maintain germ cell totipotency. Translational regulation by the KH-domain proteins MEX-3 and GLD-1 plays a role in blocking somatic differentiation in the germline (Ciosk et al., 2006). In that study, a small number of germ cells acquired an endoderm fate in *mex-1; gld-1* mutants. During the oocyte to embryo transition, the CCCH-type zinc finger OMA-1 is required for oocyte maturation and the blockage of inappropriate activation of cell specification pathways (Shirayama et al., 2006). It is likely therefore, that multiple mechanisms act to prevent inappropriate specification of endoderm in the germline.

Splitting a redundant gene pair: a new function for END-3

Previous studies indicated that *end-3* acts in parallel with *end-1* to promote endoderm specification (Maduro et al., 2005a; Zhu et al., 1997). Null mutations of *end-1* showed no endoderm phenotype, while those for *end-3* showed a weak defect, suggesting that END-3 makes a stronger contribution to endoderm specification (Maduro et al., 2005a). Here, we have shown that activation of *end-3* is more dependent on the SKN-1→MED-1,2 pathway than is *end-1*. Furthermore, we have confirmed earlier findings by others suggesting that *end-3* is activated earlier in the E cell cycle than *end-1* (Baugh et al., 2003), and found that *end-1* expression is diminished in *end-3 (−)* embryos and essentially eliminated in *end-3(−); pop-1(−)* embryos, demonstrating a requirement for both POP-1 and END-3 in *end-1* activation. Hence, *end-3* mutants show a weak endoderm defect, and *end-1* mutants none, because loss of *end-3* also affects *end-1*. It will be of interest to see whether any of these aspects of endoderm specification have been conserved in *C. briggsae*, which also expresses maternal *med*s (this work), and which encodes three *end*-like genes (Maduro et al., 2005a).

E specification is coupled to the endoderm lineage

We have found that the genes that act to specify the E fate are also important for production of the correct number of gut cells. Mutations in the cell cycle regulators *cki-1* and *cdc-25.1* result in supernumerary intestinal cells despite correct E specification (Fukuyama et al., 2003; Kostic and Roy, 2002), suggesting that improper activation of cell cycle regulators accounts for the abnormal gut cell numbers we have observed in embryos that make gut in endoderm-specification mutant backgrounds. It has been proposed that specification of endoderm results from activation of the genes *elt-2* and *elt-7*, which then maintain their expression by positive autoregulation (Fukushige et al., 1998; Maduro and Rothman, 2002). The wee1 kinase homolog *wee-1.1*, activated in the E cell, is an apparent target of MED-1,2 (Broitman-Maduro et al., 2005; Wilson et al., 1999), and depletion of the *end*s by RNAi abrogates the expression of intestinal genes (Pauli et al., 2006). There must therefore be additional roles for the *med*s and *end*s in endoderm development in addition to the activation of *elt-2,7*, or else subtle defects in activation of *elt-2,7* may persist into later stages. Whatever the mechanism, correct deployment of the endoderm gene cascade is required for both E specification and normal development of the intestine, suggesting that E specification is not an all-or-none event.

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