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Note

Neither Maternal nor Zygotic *med-1/med-2* Genes Play a Major Role in Specifying the *Caenorhabditis elegans* Endoderm

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ABSTRACT

The *med-1* and *med-2* genes encode small, highly similar proteins related to GATA-type transcription factors and have been proposed as necessary for specification of both the mesoderm and the endoderm of *Caenorhabditis elegans*. However, we have previously presented evidence that neither maternal nor zygotic expression of the *med-1/2* genes is necessary to specify the *C. elegans* endoderm. Contradicting our conclusions, a recent report presented evidence, based on presumed transgene-induced cosuppression, that the *med-1/2* genes do indeed show an endoderm-specifying maternal effect. In this article, we reinvestigate *med-2(−); med-1(−)* embryos using a *med-2*- specific null allele instead of the chromosomal deficiences used previously and confirm our previous results: the large majority (~84%) of *med-2(−); med-1(−)* embryos express gut granules. We also reinvestigate the possibility of a maternal *med-1/2* effect by direct injection of *med* dsRNA into sensitized (*med*-deficient) hermaphrodites using the standard protocol known to be effective in ablating maternal transcripts, but again find no evidence for any significant maternal *med-1/2* effect. We do, however, show that expression of gut granules in *med-1/2*-deficient embryos is exquisitely sensitive to RNAi against the vacuolar ATPase-encoding *unc-32* gene [present on the same multicopy *med-1(+)*-containing transgenic balancer used in support of the maternal *med-1/2* effect]. We thus suggest that the experimental evidence for a maternal *med-1/2* effect should be reexamined and may instead reflect cosuppression caused by multiple transgenic *unc-32* sequences, not *med* sequences.

THE *med-1* and *med-2* genes encode a redundant pair of highly similar GATA-factor-related zinc-finger proteins that together are crucial for the early development of the *Caenorhabditis elegans* embryo (Maduro *et al.* 2001). The original model for *med-1/2* action (Maduro *et al.* 2001) was that both genes were strictly zygotic, acting downstream of the maternally transcribed but zygotically translated transcription factor SKN-1 and essential for specifying E and MS fate (where the E and MS blastomeres give rise to all of the worm endoderm and much of the worm mesoderm, respectively). In this article, we are concerned only with the question of whether *med-1/2* are indeed necessary to specify the *C. elegans* endoderm.

The evidence that *med-1/2* are essential for endoderm specification was based on two different protocols for administering *med-1/2* RNA interference (RNAi) (Maduro *et al.* 2001). In the first protocol, embryos were collected 7–9 hr following injection of concentrated double-stranded RNA (dsRNA) into the maternal gonad; the rationale for such an early and limited observation period was to more effectively target the transient expression of the *med-1/2* genes in the early embryo (Maduro *et al.* 2001; Coroian *et al.* 2006). In the more standard protocol, the RNAi effect is found to be maximally effective from 1 to 3 days following injection (Fire *et al.* 1998; Zipperlen *et al.* 2001; Goszczynski and McGhee 2005; Ahringer 2006); however, these more usual methods of administering RNAi show no effect with the *med-1/2* genes (Kamath *et al.* 2003; Goszczynski and McGhee 2005; Sonnichsen *et al.* 2005). A second method of administering *med-1/2* RNAi (Maduro *et al.* 2001) was by means of a transgenic heat-inducible promoter driving expression of double-stranded *med* RNA (Tavernarakis *et al.* 2000). Together, these two protocols were reported to produce a small number of embryos that arrested with a distinctive phenotype (approximately twofold elongation with absence of posterior pharynx); ~50% of these arrested embryos failed to show the standard marker for endoderm specification, the presence of birefringent gut granules (Maduro *et al.* 2001; Coroian *et al.* 2006).

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As reported in detail elsewhere (Goszczynski and McGhee 2005), we had only limited success producing *med*-type-arrested embryos by either of these two protocols. With the first protocol, we were able to produce fewer than one gut-granule-negative embryo for every two injected hermaphrodites, which is only marginally higher than that produced by injection of nonspecific control RNA (see also Coroian *et al.* 2006). In our hands, the second protocol produces either sterile mothers or embryos that arrest prior to the stage where they would ordinarily express gut granules. We thus proceeded with a more definitive test of the requirements of the *med-1/2* genes for *C. elegans* endoderm specification, constructing worm strains segregating embryos that genetically lacked both *med-1* and *med-2* zygotic function. In these strains, the *med-1* gene was removed by the gene-specific deletion *ok804*; the *med-2* gene was removed by either of two chromosomal deficiencies, *sDf127* or *nDf16*, both of which remove several hundred genes, including *med-2*. Our results were unexpected but clear: we found that only 16–17% or 0–3% of the doubly homozygous *med-2(null); med-1(null)* embryos did *not* express gut granules (depending on whether *sDf127* or *nDf16* was used to remove *med-2*). Both estimates were far lower than the >70% gut-granule-negative embryos predicted by the original model of Maduro *et al.* (2001). We thus concluded that the zygotic expression of the *med-1* and *med-2* genes could not play a major *necessary* role in specifying endoderm. Even though *med* transcripts cannot be detected in the maternal germline (Reinke *et al.* 2004), we nonetheless looked for (but did not find) any evidence for a possible maternal effect of the *med-1/2* genes, using the standard protocol of dsRNA injection into sensitized *med-1/2*-deficient hermaphrodites. Using the same protocol even with nonsensitized strains, we routinely produce 100% arrested embryos when targeting the maternal *skn-1* gene (Goszczynski and McGhee 2005) and 100% arrested larvae when targeting the relatively early zygotic gene *elt-2* (Fukushige *et al.* 2005).

Two unresolved issues remained. First, what is the correct estimate for the percentage of gut-granule-negative *med-2(−); med-1(−)* embryos? If the 16–17% estimated with the s*Df127*-containing strain is correct (and hence the 0–3% estimated with the *nDf16*-containing strain is incorrect), can an "endoderm suppressor" be identified among the genes removed by *nDf16* but not by *sDf127*? The second issue is that, in spite of our defense of chromosomal deficiencies (Goszczynski and McGhee 2005), use of gene-specific knockouts for both *med-1* and *med-2* would strengthen our conclusion that loss of the *med-1/2* genes causes only a weakly impenetrant loss of endoderm.

The recent availability of a Mos transposon insertion (Granger *et al.* 2004; Williams *et al.* 2005) into the *med-2* gene (allele *cxTi9744*) provides a means to reassess the importance of the *med-1/2* genes for specifying endoderm without relying on chromosomal deficiencies. Thus, we constructed the two balanced strains described in Table 1:JM142 [*med-2(cxTi9744); + med-1(ok804)/lin-2 +*)] lacks both copies of *med-2(+)* but has one copy of *med-1(+)*, and JM143 [*+ med-2(cxTi9744)/sma-3 +; med-1(ok804)*] lacks both copies of *med-1(+)* but has one copy of *med-2(+)*. Both *med* alleles are likely to be null (see footnote *a* to Table 1) and are hereafter referred to simply as *med-1(−)* or *med-2(−)*. One-quarter of the embryos produced by either strain should be *med-2(−); med-1(−)* and should arrest. This expectation is accurately met with strain JM142 but 42.2% of the embryos produced by JM143 arrest prior to hatching, suggesting a degree of *med-2* haplo-insufficiency (verified semiquantitatively by PCR on arrested embryos). In other words, an embryo with only a single copy of any *med* gene has a higher probability of surviving if that single copy is *med-1* (~100%) rather than *med-2* (~66%). The morphology of the arrested embryos is similar for both strains (Figure 1, A and C): many of the embryos are arrested at the twofold stage with a morphology like that originally reported by Maduro *et al.* (2001); others have arrested earlier and can be grossly vacuolated.

Figure 1, B and D, show that only a minor fraction of the arrested embryos produced by either strain do not express gut granules, the standard assay for specified endoderm. After correcting for haplo-insufficiency (Table 1), the proportion of gut-granule-negative homozygous *med-2(−); med-1(−)* embryos is estimated at 14.2 and 17.3% for the two different strains, in excellent agreement with the 16–17.3% gut-granule-negative *med-1/2*-deficient embryos previously measured segregating from the *sDf127*-containing strain JM134 (Goszczynski and McGhee 2005). Thus, our combined results remain incompatible with the original model of Maduro *et al.* (2001, p. 481), which proposed that the *med-1/2* genes "function downstream of SKN-1 in the EMS lineage and are essential to specify E (and MS) fates in any context."

Our final estimate of ~16% gut-granule-negative *med-2(−); med-1(−)* embryos differs significantly from the 0–3% gut-granule-negative *med-2(−); med-1(−)* embryos segregating from the *nDf16*-containing strain JM136 (Goszczynski and McGhee 2005). As noted above, this discrepancy raises the possibility that a potential "endoderm suppressor" resides among the ~413 genes (excluding microRNAs) deleted by *nDf16* but not by *sDf127*. We thus fed JM134 [*dpy-17 sDf127 unc-32 III; sDp3 (III,f); med-1(−)*] animals on individual *Escherichia coli* strains containing the 290 of these 413 genes that are present in the Ahringer RNAi library (Kamath *et al.* 2003). However, we were not able to identify any clone that could lower the proportion of gut-granule-negative embryos from the ~16% seen with *sDf127* to the <3% seen with *nDf16*.

As noted above, we had previously performed *med* RNAi in the sensitized strain JM134 [one copy of *med-2(+)* and no copies of *med-1(+)*] using the standard protocol that efficiently ablates maternal transcripts

TABLE 1

The majority (>80%) of *med-2(−); med-1(−)* embryos express the endoderm marker gut granules

Strain	Genotype[a]	% arrested embryos[b]	Observed % gut-granule-negative arrested embryos[c]	% gut-granule-negative *med-2(−); med-1(−)* embryos[d]
JM142	*med-2(−); + med-1(−)/lin-2 +*	25.2 (111)	14.1 (893)	14.2
JM143	*+ med-2(−)/sma-3 +; med-1(−)*	42.2 (2338)	10.2 (1652)	17.3
Average				15.8

[a] *med-2(−)* refers to Mos insertion allele *med-2(cxTi9744)*, previously designated *cxP9744*, which was outcrossed five times; we confirmed the site and sequence of the insertion event. *med-2(cxTi9744)* is predicted to introduce a stop codon upstream of the MED-2 DNA-binding domain and is likely to be a null. The *med-1(−)* allele refers to deletion allele *ok804*, which removes the *med-1*-coding sequence and has now been outcrossed a total of five times. The markers used for balancing are *lin-2(e1309)* and *sma-3(e491)*, located on cosmids adjacent to *med-1* and *med-2*, respectively.

[b] The Lin-2 phenotype can be slightly impenetrant so all the counts on percentage of arrest were performed on embryos produced by mothers whose genotype had been confirmed by PCR. The two strains *med-2(−); lin-2* and *sma-3; med-1(−)* produce 1.3 and 3.4% arrested embryos, respectively (data not shown). Total number of scored embryos is shown in parentheses.

[c] Birefringent gut granules were assayed as previously described (Goszczynski and McGhee 2005) by allowing balanced heterozygous mothers to lay eggs for several hours on a thin layer of seeded NGM agar poured on a microscope slide. After removal of mothers, embryos were incubated at 20° overnight; gut granules were scored by inspecting unhatched embryos with polarized light. In this manner, embryos are minimally manipulated and the possibility of selection of particular classes of embryos is avoided. The total number of arrested embryos scored is shown in parentheses.

[d] Corrected by assuming that arrested embryos in excess of the expected 25% are all gut granule positive.

(Goszczynski and McGhee 2005). We found no significant increase in the proportion of gut-granule-negative embryos following injection of dsRNA targeting both *med-1* and *med-2*, compared to injection of GFP dsRNA, and concluded that the *med-1/2* genes do *not* show a maternal effect. However, Maduro *et al.* (2007) have recently made the opposite claim, namely that the *med-1/2* genes show a previously unreported maternal rescue of endoderm specification (although apparently not maternal rescue of mesoderm specification). Maduro *et al.* (2007) did not address our RNAi results (Goszczynski and McGhee 2005) that contradict their proposal. However, because our previous results could possibly have been influenced by the use of chromosomal deficiencies and could always be criticized as being "negative" (*i.e.*, no effect was observed, even though both positive and negative controls behaved as expected), we repeated our experiments using a strain carrying the two gene-specific *med-1/2* nulls. *med-1* dsRNA (which should target both *med-1* and *med-2* transcripts) was injected into JM143 [*med-1(−); + med-2(−)/sma-3 +*] mothers, GFP dsRNA was injected as a control, and arrested embryos were scored (blind) for expression of gut granules. The results are collected in Table 2 and our conclusions remain unchanged: we could find no significant evidence that the *med-1/2* genes show a maternal effect.

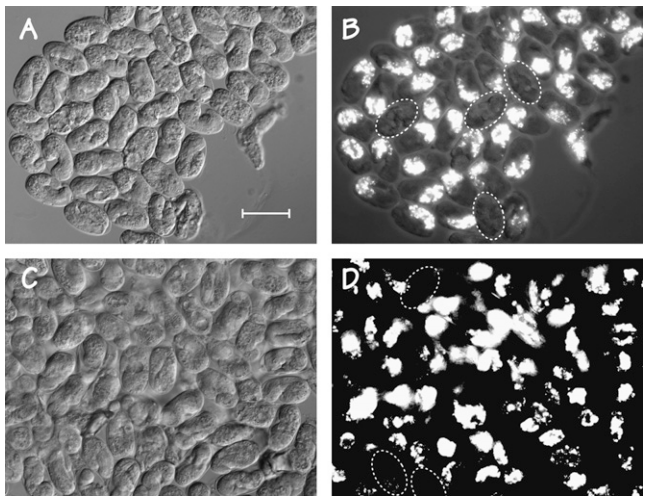

Figure 1.—Images of arrested embryos produced by (A and B) strain JM142 [*med-2(−); + med-1(−)/lin-2 +*] and (C and D) strain JM143 [*+ med-2(−)/sma-3 +; med-1(−)*]. In A and C, differential interference contrast optics were used. In B and D, polarization optics reveal birefringent gut granules (Z-projection of four to five different focal planes). Bar, 50 μm. Dotted ellipses indicate arrested embryos that are gut granule negative. Embryos produced by JM143 appear more heterogenous (more than might be expected from the observed *med-2* haplo-insufficiency) and more fragile than those produced by JM142.

We thus want to consider possible explanations for the differences between our results and those of Maduro *et al.* (2007). In spite of the large difference in interpretations and implications, the numerical differences between our observations are rather modest. We routinely observe that <20% of *med-2(−); med-1(−)* embryos do *not* express gut granules (Goszczynski and McGhee 2005; Table 1) and for all but two of their strains,

TABLE 2

RNAi against *med-1* and *med-2* has no significant effect but RNAi against *unc-32* greatly increases the percentage of gut-granule-negative *med-2(−); med-1(−)* embryos

Recipient strain	dsRNA injected[a]	n^b	% gut granule negative (±SD)[c]
JM143 [+ *med-2(−)/sma-3 +; med-1(−)*]	*med-1*	620	21.9 ± 5.8
JM143 [+ *med-2(−)/sma-3 +; med-1(−)*]	GFP	450	18.6 ± 6.6
JM143 [+ *med-2(−)/sma-3 +; med-1(−)*]	*unc-32*	1144	83.6 ± 7.1
N2 wild-type control	*unc-32*	1008	26.0 ± 3.4

[a] *med-1* dsRNA was injected at a concentration of ~1 mg/ml by the protocol described previously (Goszczynski and McGhee 2005), which is highly effective against either maternal or zygotic transcripts; *i.e.*, it produces 100% arrested embryos/larvae when *skn-1/elt-2* dsRNA is injected (Fukushige *et al.* 2005; Goszczynski and McGhee 2005). Because of their high sequence similarity (Maduro *et al.* 2001), injection of *med-1* dsRNA should also target *med-2* transcripts. Integrity and concentration of dsRNA was verified by gel electrophoresis following the injection.

[b] Total number of embryos scored.

[c] Presence or absence of birefringent gut granules was scored blind in unhatched embryos. Results are presented as the average (± standard deviation) of the percentage of gut-granule-negative embryos scored in two independent experiments; a total of 19–20 injected hermaphrodites and five to six separate broods were collected between 1 and 3 days following injection. The average values have not been corrected for possible haploinsufficiency of *med-2*. The 83.6 ± 7.1% gut-granule-negative embryos produced by injection of *unc-32* dsRNA into strain JM143 is close to the 81.5% expected if *unc-32* RNAi completely inhibits gut granule formation in *med-2(−); med-1(−)* homozygous embryos and in *med-2(−)/med-2(+); med-1(−)* heterozygous embryos and in only 26% of *med-2(+); med-1(−)* embryos (as in the wild-type controls).

Maduro *et al.* (2007) record a similar number. (Their numbers are usually slightly higher than ours, in the range of 20–25% gut granule negative; we ascribe no significance to this slight difference and suggest that it may be due to our gentler observation technique; see footnote *c* to Table 1.) We also note that when Maduro *et al.* (2007) assay embryos segregated from our strain JM134, they reproduce our results. Only two strains of Maduro *et al.* (2007), MS162 and MS247, produce *med-2(−); med-1(−)* embryos that are in the range of 40–50% gut granule negative. This ~20–30% difference in the proportion of gut-granule-negative embryos is the basis for their conclusion that the *med-1/2* genes show a maternal effect. We will propose an alternative explanation that is both simpler and experimentally supported.

The two strains in this study and Maduro *et al.* (2007) that can be most closely compared are our strain JM134 [*dpy-17(e164) sDf127(s2428) unc-32(e189) III; sDp3(III,f); med-1(ok804) X*], producing <20% gut-granule-negative embryos, and their strain MS162 [*dpy-17(e164) sDf127 (s2428) unc-32(e189) III; irDp1(III,f); med-1(ok804) X*], producing 40–50% gut-granule-negative embryos. Doubly homozygous arrested embryos produced by either strain should have exactly the same genotype: *dpy-17(e164) sDf127(s2428) unc-32(e189) III; med-1(ok804) X*. The difference lies in the manner in which the two strains are balanced. Our strain JM134 is balanced by the well-characterized free duplication *sDp3 (III,f)* (Rosenbluth *et al.* 1985; Hedgecock and Herman 1995), which contains a single copy of the wild-type *med-2* gene in its normal chromosomal context. Strain MS162 used by Maduro *et al.* (2007) is balanced by *irDp1*, a derivative of *sDp3* containing a spontaneously integrated transgenic array composed of multiple copies of *unc-119*::YFP, *med-1(+)* and a plasmid containing the wild-type *unc-32* gene (Pujol *et al.* 2001). Thus, the key observation is that *med-2(−); med-1(−)* embryos segregating from *irDp1*-containing MS162 mothers are 40–50% gut granule negative whereas genotypically identical arrested embryos segregating from *sDp3*-containing JM134 mothers are <20% gut granule negative. Maduro *et al.* (2007) interpret this difference to mean that putative maternal *med* transcripts are being removed by the RNAi-related phenomenon called cosuppression (Dernburg *et al.* 2000; Robert *et al.* 2005) caused by the multiple transgenic copies of *med-1(+)* integrated into *irDp1*. However, Maduro *et al.* (2007) did not test their model by standard methods of *med* RNAi. In contrast, as we have noted above, we performed *med* RNAi in sensitized strains carrying only a single copy of a *med* gene under conditions that should ablate maternal transcripts, but the proportion of gut-granule-negative embryos is not significantly increased (Goszczynski and McGhee 2005; Table 2). Thus, the interpretation of Maduro *et al.* (2007) requires that the putative maternal *med* transcripts are somehow susceptible to cosuppression and susceptible to RNAi for only a narrow window of several hours following injection (Maduro *et al.* 2001), but are not susceptible to conventional (and usually much more powerful) RNAi effects that routinely persist for days.

We will now propose a simple alternative explanation for the difference between our results and those of Maduro *et al.* (2007). We suggest that cosuppression caused by the multiple transgenic copies of the *unc*-32 gene (not the *med-1* gene) present on the *irDp1* balancer is the reason that strain MS162 produces the higher levels (40–50%) of gut-granule-negative *med-2(−); med-1(−)* embryos. *unc-32* is a complex locus with multiple transcripts expressed widely in the worm, including in the maternal germline and throughout the early embryo (Pujol *et al.* 2001); the null allele of *unc-32* is associated with a strict maternal-effect lethality and the arrested embryos often show vacuolated intestines (Pujol *et al.* 2001) [*unc-32(e189)*, present in both JM134 and MS162, is a weak allele primarily affecting the nervous system]. *unc-32* encodes a subunit of a vacuolar ATPase involved in acidifying intracellular organelles (Pujol *et al.* 2001), and since gut granules are lysosome derivatives (Clokey and Jacobson 1986; Hermann *et al.* 2005), it seemed possible that *unc-32*-mediated cosuppression in the maternal germline could weaken the subsequent formation of embryonic gut granules. To test this possibility, we synthesized dsRNA corresponding to a portion of the *unc-32* gene (completely included in the transgenic *unc-32* sequences present on *irDp1*), injected it into strain JM143 [+ *med-2(−)/sma-3 +; med-1(−)*] hermaphrodites (with wild-type hermaphrodites as controls), and then assayed gut granule formation in the subsequently produced embryos. The results are collected in Table 2 and strongly support our hypothesis. The three key observations are: (i) *unc-32* RNAi is effective and causes essentially complete embryonic arrest in both strains, as expected from the genetic experiments of Pujol *et al.* (2001); (ii) gut granule birefringence in arrested wild-type control embryos is markedly weakened such that ~25% of the embryos are scored as gut granule negative; and (iii) gut granules in embryos produced by the *med*-deficient strain JM143 [+ *med-2(−)/sma-3 +; med-1(−)*] are far more sensitive to *unc-32* RNAi than in control embryos. Over 80% of arrested JM143 *unc-32* RNAi embryos were scored as gut granule negative. We do not know whether the *unc-32* RNAi effect is directly on the formation of the gut granule marker (*e.g.*, a block in lysosome maturation because of aberrant acidification) or is indirect because it causes early embryonic arrest; in either case, the end result would be the same, namely that the affected embryos would be scored as gut granule negative. Thus, because of the strong effect caused by *unc-32* RNAi and because of the absence of any effect caused by *med* RNAi (Goszczynski and McGhee 2005; Table 2), we suggest that even mild cosuppression caused by the multiple transgenic *unc-32* sequences present on *irDp1* is a more likely explanation for the results of Maduro *et al.* (2007) than is cosuppression caused by *med* sequences.

One obvious experiment to distinguish between the two cosuppression-based explanations is to balance *med-2(−); med-1(−)* with a multicopy transgenic array containing *med-1(+)* sequences but not containing *unc-32* sequences. Maduro *et al.* (2007) have already performed this experiment. Their strain MS290 (*med-2(cxTi9744); med-1(ok804); Ex[med-1(+); unc-119::CFP]*) contains transgenic *med-1(+)* sequences but does not contain transgenic *unc-32* sequences. Strain MS290 clearly segregates low levels (~17%) of gut-granule-negative arrested embryos, agreeing precisely with our prediction. Maduro *et al.* (2007) find this result "unexpected" and suggest that there must be intrinsic differences in the cosuppression abilities of individual transgenic arrays. However, their explanation contradicts the findings of Dernburg *et al.* (2000), who showed that cosuppression by individual transgenic arrays, if it does occur, is highly reliable and reproducible.

The results of a second experiment performed by Maduro *et al.* (2007) also agree with our hypothesis but not with theirs. Maduro *et al.* (2007) identified eight MS290 hermaphrodites that had lost the *Ex[med-1(+); unc-119::CFP]* balancing array from the maternal germline, as well as a single hermaphrodite from a different strain that had lost the balancing *sDp3* duplication; in other words, none of these nine mothers should have any maternal wild-type *med-1/2* genes in their germline. Again, contrary to the expectation of Maduro *et al.* (2007), these germline mosaic mothers produce arrested embryos that are only 27% gut granule negative, accepted by the authors as clearly in the low category. According to our model, this result is exactly what would be predicted, simply because the *med* genes show no maternal effect. In contrast, Maduro *et al.* (2007) introduce an unusual *ad hoc* hypothesis, namely that the putative *med* transcripts in the maternal germline are not actually produced in the germline but rather are imported from the anterior intestine [although these cells were not previously reported to express a *med-1*::GFP transgene (Maduro *et al.* 2001)]. In defense of their hypothesis, Maduro *et al.* (2007) point out that such a somatic transport model could resolve a major discrepancy in their results, namely why an *in situ* hybridization signal ascribed to *med* transcripts in the maternal germline is ablated by SKN-1 RNAi when, as the authors point out, there is no evidence that SKN-1 functions in the maternal germline.

In summary, the results of this study completely confirm our previous conclusions (Goszczynski and McGhee 2005): the large majority (>80%) of embryos that lack both copies of *med-1* and *med-2* nonetheless still express markers of endoderm specification. Thus, the original model of Maduro *et al.* (2001) in which *med-1/2* were the sole (or even the major) downstream effectors of SKN-1 in specifying endoderm must be ruled out. Our present results also contradict the revised model of Maduro *et al.* (2007) in which it is claimed that the *med* genes show a maternal effect. However, their claim is based on the assumption that a particular balancing

array causes cosuppression involving multiple transgenic copies of the *med-1* gene. We suggest that their results are more likely to be caused by cosuppression associated with multiple transgenic copies of the *unc-32* gene present on the same balancing array; indeed, we show that expression of gut granules by *med*-deficient embryos appears exquisitely sensitive to *unc-32* RNAi. This suggestion provides a much simpler explanation for several other experiments performed by Maduro *et al.* (2007) that otherwise require the introduction of *ad hoc* hypotheses.

If loss of both *med-1/2* genes causes only a weakly penetrant loss of endoderm and if no maternal *med-1/2* effect exists, what is the major regulatory pathway specifying endoderm? As originally suggested by Zhu *et al.* (1997) and as we had pointed out previously (Goszczynski and McGhee 2005), all evidence points to the SKN-1 transcription factor having the major direct role in specifying endoderm. In particular, Maduro *et al.* (2005) have shown that the activity of the *end-1* promoter (thought to be critically involved in endoderm specification) is severely decreased ("++++" to "+") when an upstream region containing multiple SKN-1 sites is deleted. In contrast, the short proximal *end-1* promoter region containing MED-1/2 sites drives the low ("+") residual level of *end-1* activity.

We certainly do not question the major role that the *med-1/2* genes must play in *C. elegans* development; the severely deranged morphology of *med-2(−); med-1(−)* embryos seen in Figure 1 is convincing evidence of their importance. Nonetheless, the fact remains that >80% of these arrested *med-2(−); med-1(−)* embryos still express endoderm markers and there is no convincing evidence for any *med-1/2* maternal effect.

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LITERATURE CITED

Ahringer, J., 2006 *Reverse Genetics*, edited by The *C.* elegans Research Community. WormBook (doi/10.1895/wormbook.1.47.1, http://www.wormbook.org).

Clokey, G. V., and L. A. Jacobson, 1986 The autofluorescent "lipofuscin granules" in the intestinal cells of *Caenorhabditis elegans* are secondary lysosomes. Mech. Ageing Dev. **35:** 79–94.

Coroian, C., G. Broitman-Maduro and M. F. Maduro, 2006 Med-type GATA factors and the evolution of mesendoderm specification in nematodes. Dev. Biol. **289:** 444–455.

Dernburg, A. F., J. Zalevsky, M. P. Colaiacovo and A. M. Villeneuve, 2000 Transgene-mediated cosuppression in the *C. elegans* germ line. Genes Dev. **14:** 1578–1583.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver *et al.*, 1998 Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391:** 806–811.

Fukushige, T., B. Goszczynski, J. Yan and J. D. McGhee, 2005 Transcriptional control and patterning of the *pho-1* gene, an essential acid phosphatase expressed in the *C. elegans* intestine. Dev. Biol. **279:** 446–461.

Goszczynski, B., and J. D. McGhee, 2005 Reevaluation of the role of the *med-1* and *med-2* genes in specifying the *Caenorhabditis elegans* endoderm. Genetics **171:** 545–555.

Granger, L., E. Martin and L. Segalat, 2004 Mos as a tool for genome-wide insertional mutagenesis in *Caenorhabditis elegans*: results of a pilot study. Nucleic Acids Res. **32:** e117.

Hedgecock, E. M., and R. K. Herman, 1995 The *ncl-1* gene and genetic mosaics of *Caenorhabditis elegans*. Genetics **141:** 989–1006.

Hermann, G. J., L. K. Schroeder, C. A. Hieb, A. M. Kershner, B. M. Rabbitts *et al.*, 2005 Genetic analysis of lysosomal trafficking in *Caenorhabditis elegans*. Mol. Biol. Cell **16:** 3273–3288.

Kamath, R. S., A. G. Fraser, Y. Dong, G. Poulin, R. Durbin *et al.*, 2003 Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature **421:** 231–237.

Maduro, M. F., M. D. Meneghini, B. Bowerman, G. Broitman-Maduro and J. H. Rothman, 2001 Restriction of mesendoderm to a single blastomere by the combined action of SKN-1 and a GSK-3beta homolog is mediated by MED-1 and -2 in *C. elegans*. Mol. Cell **7:** 475–485.

Maduro, M. F., J. J. Kasmir, J. Zhu and J. H. Rothman, 2005 The Wnt effector POP-1 and the PAL-1/caudal homeoprotein collaborate with SKN-1 to activate *C. elegans* endoderm development. Dev. Biol. **285:** 510–523.

Maduro, M. F., G. Broitman-Maduro, I. Mengarelli and J. H. Rothman, 2007 Maternal deployment of the embryonic SKN-1→MED-1,2 cell specification pathway in *C. elegans*. Dev. Biol. **301:** 590–601.

Pujol, N., C. Bonnerot, J. J. Ewbank, Y. Kohara and D. Thierry-Mieg, 2001 The *Caenorhabditis elegans* unc-32 gene encodes alternative forms of a vacuolar ATPase a subunit. J. Biol. Chem. **276:** 11913–11921.

Reinke, V., I. S. Gil, S. Ward and K. Kazmer, 2004 Genome-wide germline-enriched and sex-biased expression profiles in *Caenorhabditis elegans*. Development **131:** 311–323.

Robert, V. J., T. Sijen, J. van Wolfswinkel and R. H. Plasterk, 2005 Chromatin and RNAi factors protect the *C. elegans* germline against repetitive sequences. Genes Dev. **19:** 782–787.

Rosenbluth, R. E., C. Cuddeford and D. L. Baillie, 1985 Mutagenesis in *Caenorhabditis elegans*. II. A spectrum of mutational events induced with 1500 r of gamma-radiation. Genetics **109:** 493–511.

Sonnichsen, B., L. B. Koski, A. Walsh, P. Marschall, B. Neumann *et al.*, 2005 Full-genome RNAi profiling of early embryogenesis in *Caenorhabditis elegans*. Nature **434:** 462–469.

Tavernarakis, N., S. L. Wang, M. Dorovkov, A. Ryazanov and M. Driscoll, 2000 Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. Nat. Genet. **24:** 180–183.

Williams, D. C., T. Boulin, A. F. Ruaud, E. M. Jorgensen and J. L. Bessereau, 2005 Characterization of Mos1-mediated mutagenesis in *Caenorhabditis elegans*: a method for the rapid identification of mutated genes. Genetics **169:** 1779–1785.

Zhu, J., R. J. Hill, P. J. Heid, M. Fukuyama, A. Sugimoto *et al.*, 1997 end-1 encodes an apparent GATA factor that specifies the endoderm precursor in *Caenorhabditis elegans* embryos. Genes Dev. **11:** 2883–2896.

Zipperlen, P., A. G. Fraser, R. S. Kamath, M. Martinez-Campos and J. Ahringer, 2001 Roles for 147 embryonic lethal genes on *C.elegans* chromosome I identified by RNA interference and video microscopy. EMBO J. **20:** 3984–3992.

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