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RESEARCH ARTICLE

Reciprocal signaling by Wnt and Notch specifies a muscle precursor in the *C. elegans* embryo

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ABSTRACT

The MS blastomere produces one-third of the body wall muscles (BWMs) in the *C. elegans* embryo. MS-derived BWMs require two distinct cell-cell interactions, the first inhibitory and the second, two cell cycles later, required to overcome this inhibition. The inductive interaction is not required if the inhibitory signal is absent. Although the Notch receptor GLP-1 was implicated in both interactions, the molecular nature of the two signals was unknown. We now show that zygotically expressed MOM-2 (Wnt) is responsible for both interactions. Both the inhibitory and the activating interactions require precise spatiotemporal expression of zygotic MOM-2, which is dependent upon two distinct Notch signals. In a Notch mutant defective only in the inductive interaction, MS-derived BWMs can be restored by preventing zygotic MOM-2 expression, which removes the inhibitory signal. Our results suggest that the inhibitory interaction ensures the differential lineage specification of MS and its sister blastomere, whereas the inductive interaction promotes the expression of muscle-specifying genes by modulating TCF and β-catenin levels. These results highlight the complexity of cell fate specification by cell-cell interactions in a rapidly dividing embryo.

KEY WORDS: *C. elegans*, *mom-2*, Wnt, *glp-1*, Notch, MS blastomere, Body wall muscles

INTRODUCTION

The mechanism(s) by which a cell becomes committed to a specific developmental fate is one of the central questions in biology, with direct significance for stem cell biology and regenerative medicine. One key insight comes from determining whether the fate of a cell is specified through a cell-autonomous or non-autonomous mechanism. This is often determined experimentally by isolating a cell and comparing the tissue type(s) generated from the isolated cell with those from an *in vivo* fate map (Sulston and White, 1980; Sulston et al., 1983). If a cell can produce in isolation all tissue types that it normally would in an intact embryo, its developmental potential is interpreted as being cell-autonomous. Such analyses in early studies suggested that many cell fates in *C. elegans* embryos are specified cell-autonomously (Sulston and White, 1980; Sulston et al., 1983). However, from follow-on analyses, it became clear that simple cell isolation experiments often overlooked the requirement of cell-cell interactions in cell fate specification, which are now known to be central during *C. elegans* embryogenesis (Priess et al., 1987; Priess and Thomson, 1987; Schierenberg, 1987; Wood, 1991; Bowerman et al., 1992b; Goldstein, 1992, 1993; Hutter and Schnabel, 1994, 1995; Schnabel, 1994, 1995; Priess, 2005; Sawa and Korswagen, 2013).

A good example of dramatic reversal of thinking concerns the ability of an early 8-cell stage blastomere, MS, to produce body wall muscles (BWMs). *C. elegans* embryos produce a total of 81 BWMs, of which 28 derive from MS (Sulston et al., 1983). Upon isolation, or following laser ablation of all other blastomeres, the MS blastomere still generates the wild-type number of 28 BWMs. This result was initially viewed as strong evidence that MS produces BWMs cell-autonomously (Priess and Thomson, 1987; Mello et al., 1992; Schnabel, 1994). However, cell-cell interactions were subsequently found to be required for MS-derived BWM. An inductive interaction was identified that functions by countering an earlier inhibitory interaction, and is dispensable if the inhibitory interaction is absent (Schnabel, 1994). Therefore, an isolated MS blastomere, which receives neither the inhibitory nor the activating interaction, produces the normal number of BWMs (Fig. 1B). The precise nature of the inhibitory or the inductive signal for MS-derived BWM cells has not been identified. However, blastomere ablation experiments have identified probable sources for these two interactions, as well as the developmental stages at which these interactions normally occur (Schnabel, 1994). The mother cell of MS is the EMS blastomere, which, in a 4-cell embryo, contacts the other three blastomeres – P2, ABp and ABa (Fig. 1A). Ablation experiments showed that ABp or its descendants are the sources of the inhibitory interaction, whereas two ABa-derived blastomeres, ABalp and ABara, provide the inductive interaction at the 12- to 14-cell stage (Schnabel, 1994). Interestingly, while MS produces additional tissue types, including pharyngeal muscle cells, somatic gonad and neurons, only BWMs derived from MS require the inductive interaction from the two ABa granddaughters.

Although neither the inhibitory nor the inductive signals had been identified, it was known that the inductive interaction required the expression of a Notch receptor, GLP-1 (Priess et al., 1987; Priess and Thomson, 1987; Yochem and Greenwald, 1989; Schnabel, 1994; Moskowitz and Rothman, 1996). GLP-1 functions in multiple cell-cell interactions in the early *C. elegans* embryo that specify cell fate, including specifying the fate of ABalp and ABara at the 12-cell stage (Priess et al., 1987; Priess and Thomson, 1987; Moskowitz et al., 1994; Priess, 2005). This result suggests that the generation of MS BWMs requires the expression of a protein in ABalp and ABara that is induced by Notch signal. Intriguingly, we now know that the specification of ABp also requires the Notch receptor GLP-1 (Mango et al., 1994; Mello et al., 1994; Moskowitz et al., 1994). This Notch signaling occurs at the 4-cell stage (Fig. 1A), induced by the ligand APX-1 which is expressed in the P2 blastomere (Mickey et al., 1996).

The two Notch signals, one at the 4-cell stage and the other at the 12-cell stage, appear to require different levels of Notch activity. The strong reduction-of-function mutation *e2144* or RNAi of *glp-1*

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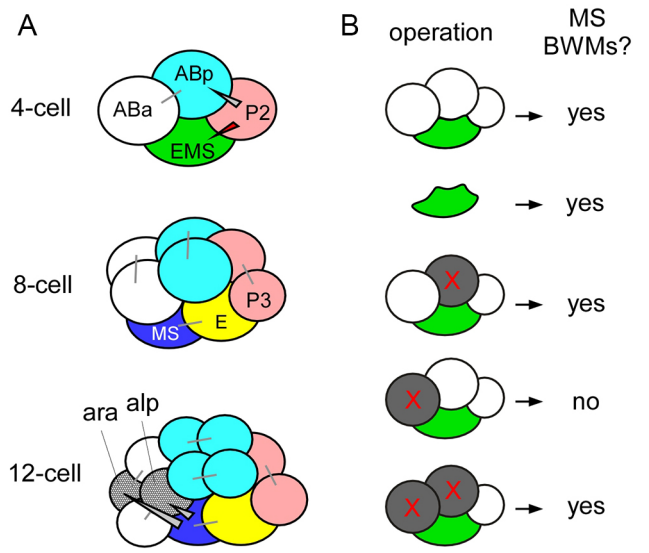

Fig. 1. Schematic of early *C. elegans* embryos. (A) Stages and selected blastomere names are indicated. Blastomeres of the same color are derived from the same predecessor. The ABa granddaughters that contact MS in the 12-cell embryo are stippled. Gray lines connect sister cells. Gray and red triangles denote known Notch- and Wnt-mediated interactions, respectively. (B) Generation of MS BWMs by isolated blastomeres or operated embryos (Priess and Thomson, 1987; Mello et al., 1992; Schnabel, 1994). X denotes laser-ablated blastomere(s).

prevents both signals from occurring (Kodoyianni et al., 1992; Mello et al., 1994), whereas the weak reduction-of-function mutation *e2142* (Kodoyianni et al., 1992) prevents only the 12-cell stage signal (Mello et al., 1994). These two *glp-1* mutations also differentially affect production of MS-derived BWMs. However, counterintuitively, it is only the 'weak' *glp-1* allele *e2142*, and not the 'strong' *glp-1* allele *e2144*, that results in absence of MS-derived BWMs (Schnabel, 1994). The different phenotypes of the two *glp-1* alleles with respect to MS BWM generation could result from Notch signaling regulating both the inhibitory and activating interactions for MS BWMs, either directly or indirectly, by differentially influencing the signaling ability of ABp and ABalp/ABara. In this scenario, the strong allele *e2144* would reduce both the inhibitory and activating signals and therefore, analogous to the isolated MS blastomere, wild-type numbers of BWMs would be generated. The weak allele *e2142* would reduce only the more sensitive activating signal and no BWMs would result. For simplicity, we will refer to the strong and weak *glp-1* alleles as *glp-1(S)* for *e2144* and *glp-1(W)* for *e2142*, respectively.

The *C. elegans* genome contains five Wnt genes, of which only one, *mom-2*, is known to function in the very early embryo (Sawa and Korswagen, 2013). Wnt signaling from P2 to EMS, mediated by translation of maternally supplied *mom-2* mRNA, functions in the specification of the E blastomere as the sole intestinal precursor (Rocheleau et al., 1997; Thorpe et al., 1997; Sawa and Korswagen, 2013). EMS, the mother of MS and E, exhibits the potential to generate all cell types made by MS and E. However, this potential is differentially segregated to the two daughter cells. The P2-derived Wnt/MOM-2 signal is important for E to adopt a fate that is different from the MS blastomere. When either P2 or MOM-2 activity is absent, E does not produce intestinal cells and instead produces tissue types normally made by a wild-type MS blastomere.

Whereas maternally expressed MOM-2 is essential for embryonic viability, it is unclear whether MOM-2 is zygotically expressed and, if so, what function it might have. Genetic results support zygotic expression and that zygotic MOM-2 is dispensable in embryos that express maternally supplied MOM-2 (Rocheleau et al., 1997; Thorpe et al., 1997). However, despite apparent dispensability, zygotically expressed MOM-2 can partially rescue the morphogenesis defect, but not the E specification defect, exhibited by embryos that lack maternally supplied MOM-2. This suggests that maternal and zygotic MOM-2 share overlapping functions during morphogenesis (Thorpe et al., 1997).

We show here that zygotically expressed MOM-2 drives both the inhibitory as well as the inductive interactions regulating MS-derived BWMs. MOM-2 is zygotically expressed in the Notch signal-receiving cells as a result of both the 4-cell and 12-cell Notch-mediated interactions. In addition, we show that Notch/GLP-1 is required for zygotic *mom-2* expression. Absence of zygotic *mom-2* expression restores the generation of MS-derived BWMs in *glp-1(W)* embryos. Furthermore, we show that a defective 4-cell inhibitory Notch interaction leads to loss of lineage-restricted transcription in both the MS and E blastomeres, supporting a model in which the inhibitory interaction serves to repress ectopic (i.e. precocious) activation of both MS and E fates within the EMS blastomere.

RESULTS

Zygotic transcription of *mom-2* in embryos

Using a probe set specific to *mom-2* mRNA in single-molecule fluorescence *in situ* hybridization (smFISH) experiments, we and others have shown that *mom-2* mRNA is present at a high level in oocytes and early embryos (Harterink et al., 2011; Oldenbroek et al., 2013), consistent with maternal contribution. The level of *mom-2* smFISH signal decreases in the embryo, beginning at the 4-cell stage and primarily in the somatic blastomeres, a pattern observed for many maternally supplied mRNAs in early embryos (Seydoux et al., 1996).

We observed, however, that the decrease in *mom-2* smFISH signal was not uniform among AB-derived blastomeres. In the 8-cell embryo, when there are four AB-derived blastomeres (two ABa daughters and two ABp daughters), the smFISH signal was consistently 2- to 4-fold higher in the ABp-derived blastomeres (Fig. 2A-D, Table S1). In the 14- to 15-cell embryo, when there are eight AB-derived blastomeres, elevated smFISH signal was detected primarily in two of the four ABa granddaughters (ABalp and ABara), in addition to all ABp-derived blastomeres (Fig. 2E-H, Table S1). This uneven distribution could reflect either differential degradation of maternal *mom-2* mRNA or zygotic transcription of *mom-2* mRNA in selected somatic blastomeres. We believe the latter to be true for the following reasons. First, we analyzed two other maternal mRNAs, *mex-3* and *spn-4*, both of which have been shown to be preferentially degraded in somatic blastomeres (Draper et al., 1996; Ogura et al., 2003), by smFISH, and we did not consistently detect any uneven distribution of smFISH signal among the AB-derived blastomeres with either probe (Fig. S1). Second, an extrachromosomal transgene in which *gfp::histone h2b* is driven by a 2.9 kb putative promoter region of *mom-2* (*Pmom-2::gfp::h2b*) is expressed in the same subset of somatic blastomeres (ABp derivatives and ABara/ABalp) that exhibit the elevated *mom-2* smFISH signal (Fig. 3A-H). As extrachromosomal transgenes generated by this method are silenced in the gonad (Mello and Fire, 1995; Kelly et al., 1997), transgene expression is likely to be the result of zygotic transcription. Third, and most conclusively, we observed the same blastomeres exhibiting elevated GFP fluorescence in the cross progeny when males carrying the

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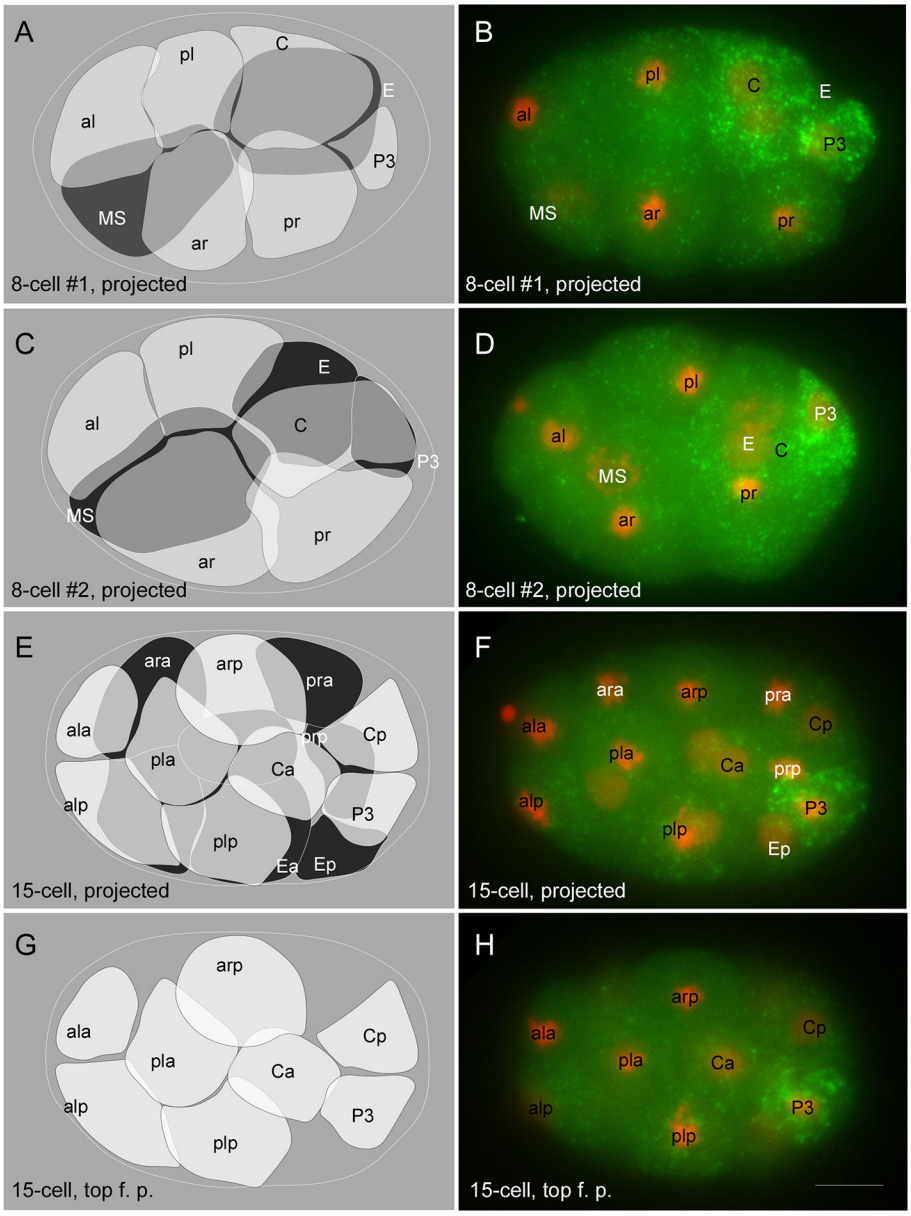

Fig. 2. *mom-2* smFISH. *mom-2* smFISH fluorescence in two representative 8-cell (A-D) and one representative 15-cell (E-H) embryo. (A,C,E,G) Schematic pseudo-3D representation of the corresponding embryo illustrated with two focal planes: lighter shade, top focal plane (partially transparent); dark shade, bottom focal plane. Pertinent blastomeres are marked; AB-derived blastomeres have only their division history notation. (B,D,F,H) Merged image of *mom-2* smFISH (green) and DAPI (red). (B,D,F) Projected images from the 3D *z*-stack of optical slices. (H) Single (top) focal plane from a 15-cell (8AB) embryo. Blastomeres P3 and C (or daughters Ca and Cp) contain maternally supplied *mom-2* mRNA. (B,D) ABp daughters exhibit higher *mom-2* smFISH signals than the ABa daughters. (F,H) ABalp exhibits a higher *mom-2* smFISH signal than ABala. These particular embryo images are unable to demonstrate the ABara versus ABarp difference because these two blastomeres are on different focal planes. Scale bar: 10 µm.

Pmom-2::gfp::h2b reporter were crossed to hermaphrodites lacking the reporter. In this cross, reporter expression must result from zygotic transcription. We conclude that *mom-2* mRNA, in addition to being provided maternally, is transcribed zygotically in a specific subset of blastomeres in early embryos.

Expression of zygotic *mom-2* requires both Notch signaling and the lineage-specific transcription factor SKN-1

The spatial and temporal pattern of *Pmom-2::gfp::h2b* expression resembles that of the six HES-like bHLH transcription factors, namely REF-1, HLH-25, -26, -27, -28 and -29, which we will refer to as the REF-1 family of genes or proteins (Neves and Priess, 2005). Members of the REF-1 gene family are known direct Notch targets (Neves and Priess, 2005). Expression of REF-1 family proteins in ABa- and ABp-derived blastomeres is dependent on Notch signaling and is abolished in embryos lacking *glp-1* activity. Whereas REF-1 family expression in EMS descendants in later embryos is GLP-1 independent, it is dependent on SKN-1, an EMS-specific maternal transcription factor (Neves and Priess, 2005).

We find that expression of *Pmom-2::gfp::h2b* in ABa- and ABp-derived blastomeres is, like that of REF-1 family genes, also dependent on Notch signaling. Reporter expression is greatly reduced or eliminated in *glp-1(S)* embryos raised at a non-permissive temperature (25°C) (Fig. 3I-L). The reduction in reporter expression is less severe in *glp-1(W)* embryos, with GFP detectable at low levels in both ABp- and ABa-derived blastomeres. Like the REF-1 gene family, expression of *Pmom-2::gfp::h2b* in EMS descendants is GLP-1 independent and SKN-1 dependent (Fig. 3K,L,S,T). The ligand(s) for the 12-cell Notch interaction has not been identified, but is believed to require SKN-1 activity (Bowerman et al., 1992a; Shelton and Bowerman, 1996). We observed no *Pmom-2::gfp::h2b* expression in either of the ABa descendants or the EMS lineage in *skn-1(zu67)* embryos (Fig. 3S,T). The ligand for the 4-cell Notch signal is APX-1 (Mickey et al., 1996). In *apx-1(zu183);skn-1(zu67)* embryos, the GFP::H2B signal was completely abolished (Fig. 3O-R, Fig. S2). Together, these results demonstrate that two sequential Notch interactions in the early embryo induce zygotic expression of *Pmom-2 gfp::h2b* in ABp and a subset of ABa descendants.

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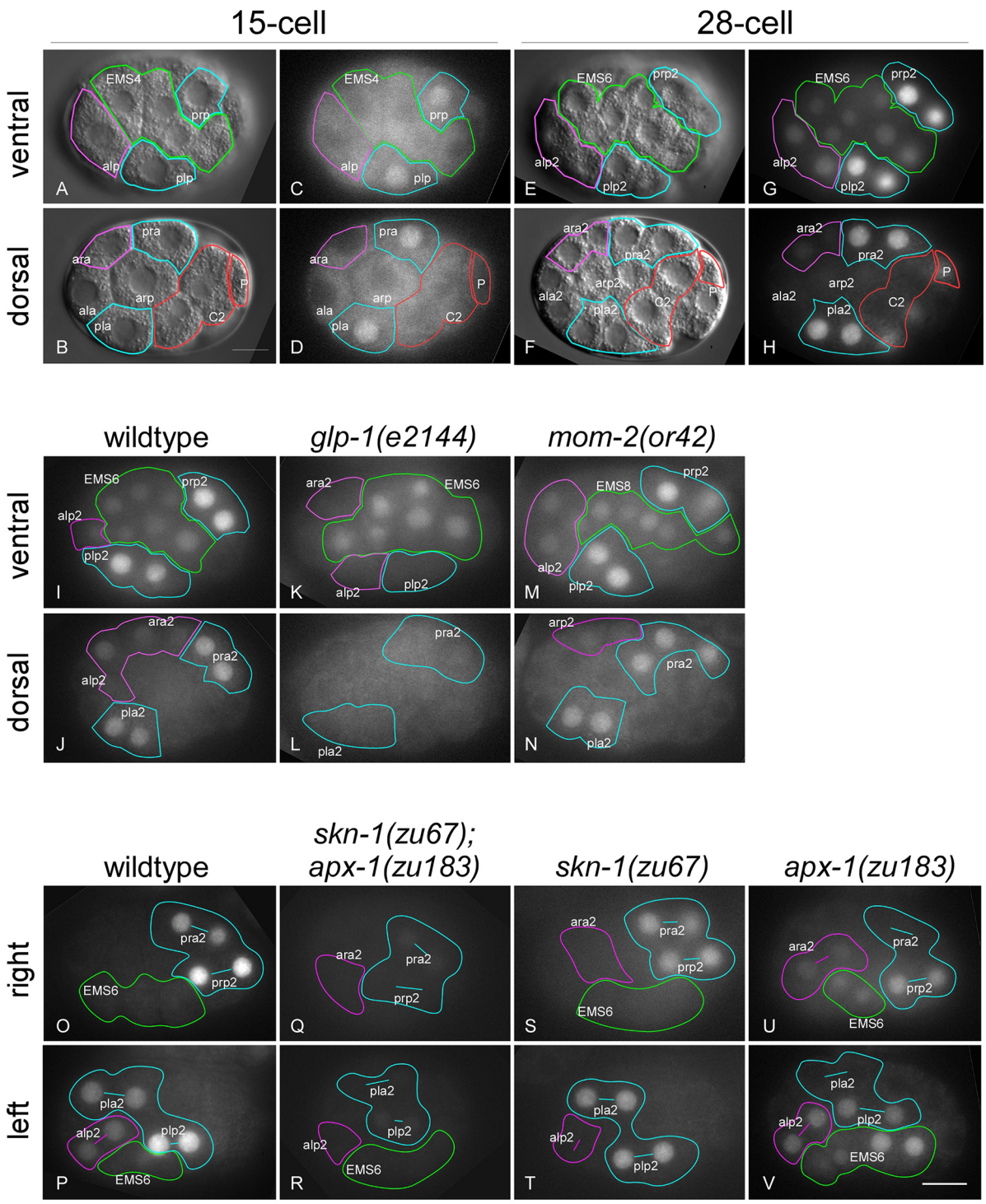

Fig. 3. Expression pattern of *Pmom-2::gfp::h2b* reporter in wild-type and mutant embryos. Two different focal planes of *Pmom-2::gfp::h2b*-expressing embryos from 4D recordings. (A-N) Dorsal versus ventral focal planes; embryos are positioned on their backs. (O-V) Left versus right focal planes; embryos are positioned on their sides. Wild-type 15-cell (A-D) and 28-cell (E-H) embryos showing DIC (A,B,E,F) and corresponding GFP fluorescence image (C,D,G,H). (I-V) GFP fluorescence of 24- to 28-cell embryos of the genotypes indicated. Related blastomeres are outlined by color: light blue, ABp derived; magenta, ABara and ABalp derived; green, EMS derived; and orange, P2 derived. Note that GFP fluorescence is observed in the EMS lineage in wild-type embryos older than ~24 cells. The name of the oldest parent cell within each group is listed, followed by the number of cells in that group. Some sister pairs are connected with an appropriately colored line. Scale bar: 10 µm.

As the P2 blastomere exhibits both Notch and Wnt signaling ability, we also analyzed whether maternally expressed Wnt/MOM-2 regulates the zygotic expression of *Pmom-2::gfp::h2b* in ABp. Although blastomere divisions are slightly altered in *mom-2(or42)* embryos (Rocheleau et al., 1997; Thorpe et al., 1997), we observed expression of GFP in all descendants of ABp and in the two ABa granddaughters that touch MS (Fig. 3M,N). We conclude, therefore, that maternal MOM-2 does not regulate zygotic expression of *Pmom-2::gfp::h2b*.

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Interestingly, our analyses appear to have uncovered a previously unidentified SKN-1-dependent Notch signal from the EMS lineage to the two ABp granddaughter cells, ABprp and ABplp, that contact EMS descendants. In wild-type embryos, ABprp and ABplp exhibit a higher level of GFP::H2B from the *mom-2* reporter than the other two ABp granddaughters (ABpra and ABpla) that do not contact EMS descendants (Fig. 3O,P Fig. S2). This difference in GFP levels was less pronounced in *skn-1(zu67)* embryos (Fig. 3O,P compared with 3S,T, Fig. S2). Furthermore, in *apx-1(zu183)* embryos, GFP::H2B is abolished in ABpra and ABpla but is only reduced in ABprp and ABplp (Fig. 3U,V, Fig. S2). All GFP signal is abolished in *skn-1(zu67);apx-1(zu183)* embryos (Fig. 3Q,R, Fig. S2). The function of this SKN-1-dependent Notch interaction is unclear. Wnt signaling has been proposed to have a direct role in gastrulation. Both ABprp and ABplp daughters surround the two E descendants at the time when gastrulation starts. This Notch interaction would place zygotic MOM-2-expressing cells at the right place and time to regulate this process. Further characterization of this novel SKN-1-dependent Notch interaction is currently under way.

Zygotic *mom-2* mediates the inhibitory interaction for MS BWMs

The expression pattern of *mom-2* suggests zygotic MOM-2 as a candidate ligand for the inhibitory interaction between ABp (or descendants) and EMS, as well as the later activating interaction between certain ABa descendants and MS. If zygotic MOM-2 were the ligand for both interactions, absence of zygotic *mom-2* should remove the inhibitory interaction and thereby restore the formation of MS BWMs in *glp-1(W)* embryos, which are defective in the inducing interaction. However, absence of zygotic *mom-2* would be predicted to have no effect on the ability of MS to generate wild-type numbers of BWMs in *glp-1(S)* embryos, which are already defective in both the inhibitory and the inducing interactions.

To test this possibility, we crossed *glp-1(−) (III);mom-2(ok519) (V)* males with *glp-1(−)* hermaphrodites homozygous for an insertion on LGV, *teIs18(gfp)*, which express a GFP::H2B reporter in intestinal precursors (Shetty et al., 2005). F1 cross progeny would be homozygous for *glp-1(−/−)* and transheterozygous for *mom-2(ok519)* and *teIs18* on LGV. All F2 embryos produced by these F1 hermaphrodites will be *glp-1(−/−)* and express maternal MOM-2 protein [maternal positive, or MOM-2(M+)]. However, ∼25% of F2 embryos would be homozygous for *mom-2(ok519)* and would not express zygotic MOM-2 protein [zygotic negative, or MOM-2(Z−)]. These MOM-2(M+ Z−) embryos would be identifiable by virtue of being negative for intestinal GFP expression, unless an extremely rare recombination event occurred between *mom-2* and *teIs18*. We performed immunofluorescence on these F2 embryos using anti-myosin antibody (Priess and Thomson, 1987), which specifically stains BWMs.

3D image stacks were collected for embryos of various genotypes at 400-450 min post fertilization (Fig. 4A-J), and the numbers of myosin-positive BWMs were counted (Fig. 4K). Wild-type embryos generated on average 81 BWMs (n=10, s.d. 1.5) and *glp-1(S)* embryos generated on average 80 BWMs (n=7, s.d. 2.6). Control *glp-1(W)* embryos generated on average 58 BWMs (n=32, s.d. 4.6), which is less than the wild-type 81, as expected owing to the absence of MS-derived BWMs (Schnabel, 1994). Putative *glp-1(W);mom-2(M+ Z−)* GFP-negative embryos generated on average 80 BWMs (n=9, s.d. 1.9), very close to the number from wild-type embryos. Two additional GFP-negative embryos each generated 62 BWMs, indicating no rescue. A parallel control showed that putative *mom-2(M+ Z−)* embryos generated on average 80.5 BWMs (n=6,

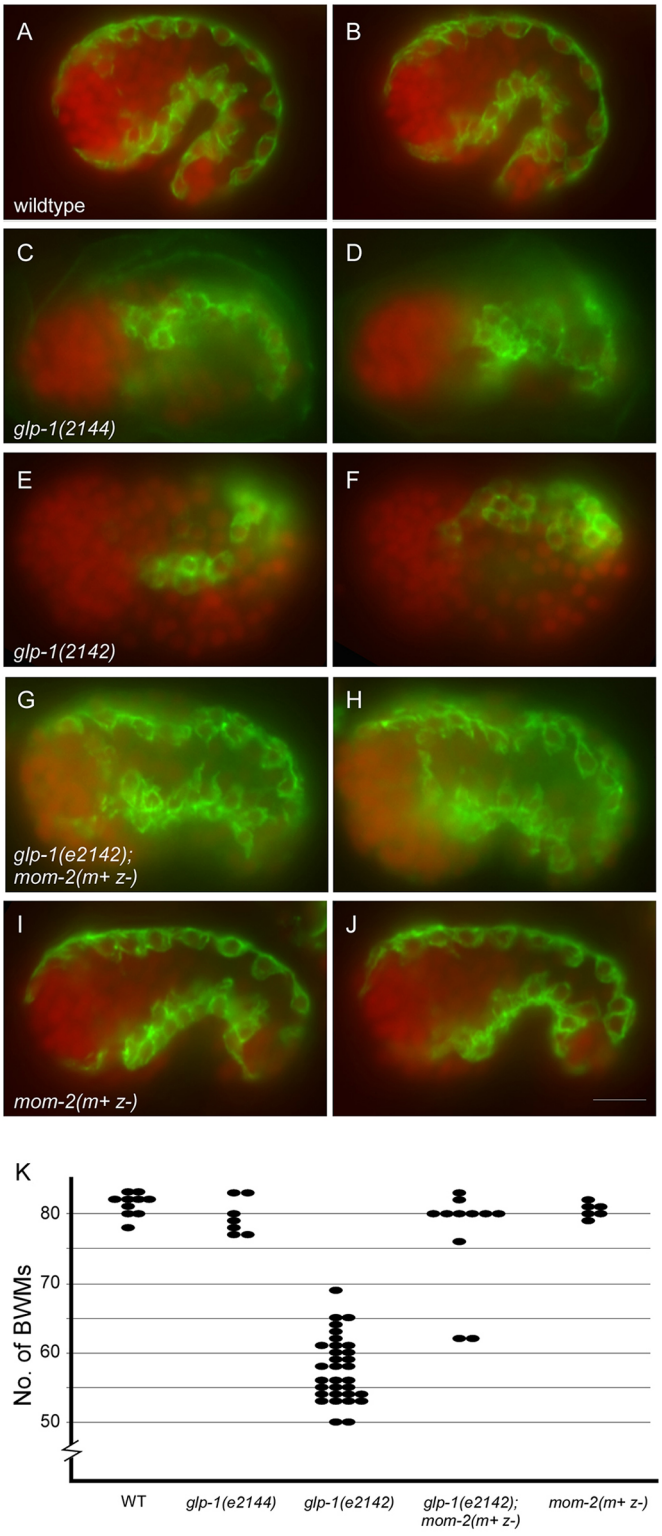

Fig. 4. Zygotic *mom-2* is responsible for the MS BWM inhibitory activity. (A-J) Anti-myosin staining (green) and DAPI staining (pseudocolored red) of wild-type, the 'strong allele' *glp-1(e2144)*, the 'weak allele' *glp-1(e2142)*, *glp-1(e2142); mom-2(m+ z−)* and *mom-2(m+ z−)* embryos at ∼450 min of development. *mom-2(m+ z−)* denotes embryos that are homozygous for *mom-2(ok519)* but express maternally supplied MOM-2. The two columns show images from two focal planes of the same embryo. In wild-type embryos at this stage, muscle cells are arranged in four quadrants along the anterior-posterior axis. Scale bar: 10 µm. (K) Scatter plot of BWM counts from individual embryos of the indicated genotype.

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s.d. 1.0). That removal of zygotically expressed Wnt/MOM-2 suppressed the MS BWM defect in *glp-1(W)* embryos demonstrates that Wnt signaling mediates the inhibitory interaction for MS-derived BWMs.

APX-1 is required for lineage-restricted expression of MS- and E-specific factors

It is curious that MS BWM fate must first be inhibited only to have the inhibition lifted by an activating interaction two cell cycles later. We investigated whether embryos lacking the inhibitory interaction suffer any developmental consequence in the EMS lineage. The MS and E developmental programs are driven by the lineage-restricted expression of two different sets of transcription factors (for a review, see Maduro, 2015; Maduro et al., 2015). The MS blastomere expresses the T-box transcription factor TBX-35, which, along with the homeodomain transcription factor CEH-51, drives both body wall and pharyngeal muscle programs (Broitman-Maduro et al., 2006, 2009). The E blastomere expresses two partially redundant GATA factors, END-1 and END-3, which are together necessary and individually sufficient for the intestinal program (Zhu et al., 1997; Maduro et al., 2005).

To test whether the APX-1-dependent inhibitory interaction between the ABp lineage and the EMS lineage serves to modulate transcription of MS and E lineage-specific genes, we analyzed the expression of a *tbx-35* transgenic reporter or an *end-1* transgenic reporter in *apx-1(zu183)* mutant embryos (Fig. 5). In wild-type embryos expressing the *tbx-35* reporter, GFP is observed exclusively in the MS lineage, starting in the MS daughters (0% embryos with ectopic expression, $n>250$; Fig. 5A, Fig. S3). In *apx-1(zu183)* embryos, approximately one-quarter (21 of 89) exhibited ectopic GFP expression in the E lineage (Fig. 5B, Fig. S3). Similar ectopic lineage expression was also observed for the *end-1* reporter (Fig. 5D,E, Fig. S3). The *end-1* reporter is expressed almost exclusively in the E lineage in wild-type embryos, with rare ectopic expression observed (<1% of embryos, $n>300$). Analogous to the *tbx-35* reporter, ectopic expression of the *end-1* reporter in the MS lineage was observed in almost one-third (19 of 61) of *apx-1(zu183)* embryos. For both transgene reporters, the ectopic GFP levels

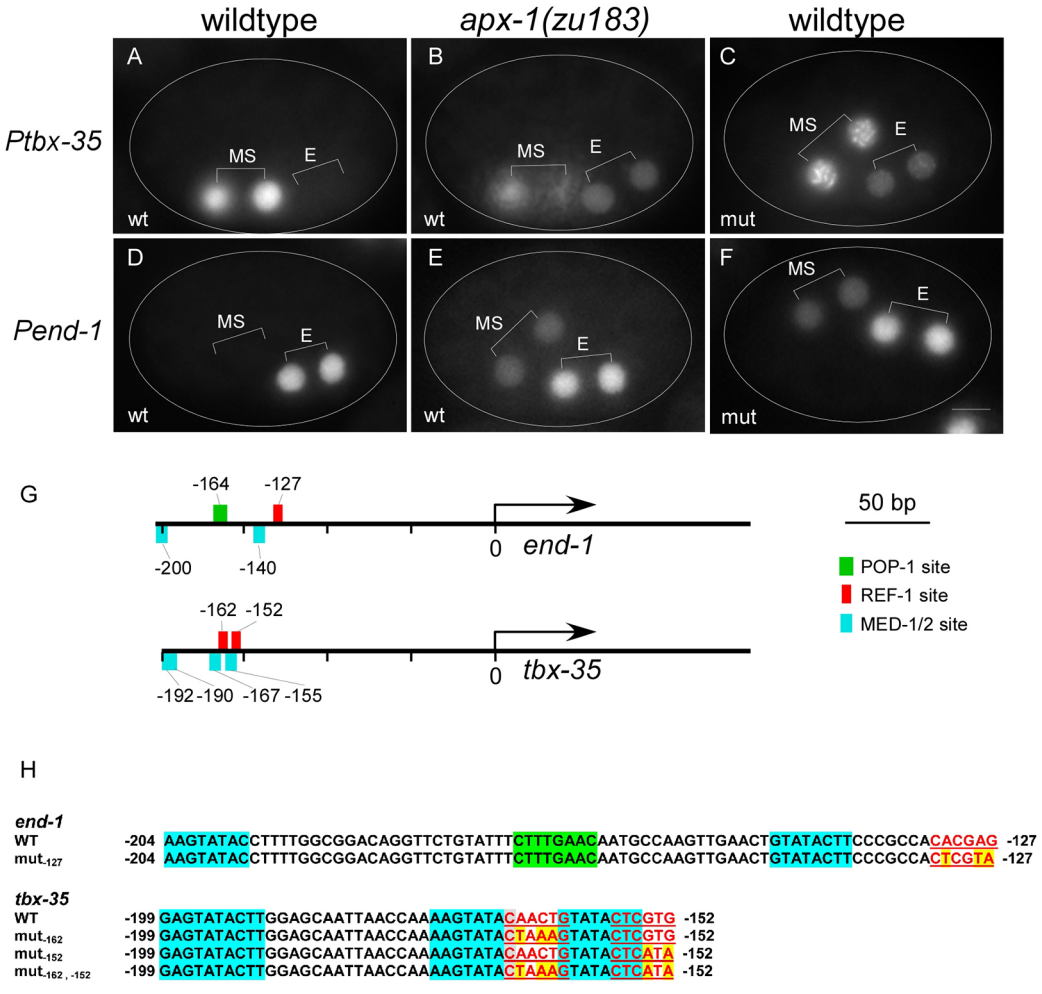

Fig. 5. Lineage-restricted expression of *Ptbx-35* and *Pend-1* reporters requires APX-1 and REF-1 binding sites. (A-F) Nuclear GFP fluorescence of single embryos (outlined by ovals, anterior to the left) expressing *Ptbx-35* (A-C) or *Pend-1* (D-F) transgenes. All embryos shown are at the 15-cell stage with the two MS-derived and two E-derived nuclei indicated. Transgenes contain either wild-type (wt) or mutant (mut) putative REF-1 binding sites (mut-152, mut-162 for *Ptbx-35*, mut-127 for *Pend-1* transgenes). Scale bar: 7 µm. (G) Schematic of the *end-1* and *tbx-35* genes including the proximal 200 bp upstream regulatory regions, with putative POP-1, REF-1 and MED-1/2 binding sites indicated. 0 indicates the predicted translation start sites. (H) Nucleotide sequence of selected regions of *end-1* and *tbx-35* upstream regulatory sequences, with wild-type sequence on top and mutations generated (highlighted in yellow) beneath. Colors indicate putative POP-1, REF-1 and MED-1/2 binding sites as in G.

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detected were almost always weaker than those in the normal lineage of the same embryo. These results suggest that APX-1, and by extension the ABp-derived blastomeres, modulates lineage-restricted transcription in both the MS and E lineages.

Repression of *end-1* and *tbx-35* by REF-1 family bHLH proteins

What might be the nature of the ABp-derived lineage-specific repressive activity? Repression of the MS- and E-specific transcriptional programs must also occur in the EMS blastomere. Two types of DNA-binding proteins, both capable of mediating transcriptional repression, are known to be expressed in the EMS blastomere: the TCF protein POP-1 and the bHLH REF-1 family proteins (Lin et al., 1995; Neves and Priess, 2005; Coroian et al., 2006). We have shown previously that POP-1 is present at a higher nuclear level in MS, where it binds to the *end-1* promoter and represses *end-1* transcription (Shetty et al., 2005). Mutating the POP-1 binding site centered at −164 relative to the first base of the AUG translation start site on an *end-1* transgene resulted in derepression of the *end-1* reporter in the MS lineage (Shetty et al., 2005). Repression of *end-1* transcription in MS by POP-1 requires the co-repressors UNC-37/Groucho and HDA-1/HDAC (Calvo et al., 2001). Depletion of *pop-1*, *unc-37* or *hda-1* resulted in a similar derepression of *end-1* in MS (Calvo et al., 2001; Shetty et al., 2005). The *tbx-35* promoter does not contain a readily identifiable POP-1 binding site. Therefore, MS-restricted transcription of *tbx-35* presumably requires a different mode of suppression specifically in the E lineage. As there are a total of six REF-1 family genes, it is difficult to inactivate REF-1 activity completely (Neves and Priess, 2005). Therefore, to investigate whether the REF-1 family of DNA-binding proteins could contribute to lineage-restricted expression in MS, E, or both blastomeres, we performed mutational analyses on the promoters of the *tbx-35* and *end-1* reporters (Shetty et al., 2005).

The mammalian and fly REF homologs [HES, Hairy and E(spl)] have been shown to bind to DNA sequences CACGNG (Class C sites), CACNAG (N-box) and, to a lesser extent, CANGTG (Class B sites) (Ohsako et al., 1994; Van Doren et al., 1994; Fisher and Caudy, 1998; Jennings et al., 1999; Iso et al., 2003). *C. elegans* REFs have also been shown to bind TGCCACGTGTCCA *in vitro* (Neves and Priess, 2005). Here, we will refer to all three of these DNA sequences as candidate REF-1 sites. Interestingly, both the *tbx-35* and *end-1* promoters include a candidate REF-1 site between the most proximal of multiple MED-1/2 sites and the predicted AUG translation initiation codon (Fig. 5G,H). For *tbx-35*, this candidate REF-1 site (−152) overlaps with the MED-1/2 binding site at −155 (Broitman-Maduro et al., 2005). A second candidate REF-1 site (−162) is located between, and overlaps with, two MED-1/2 sites, one at −155 and another at −167. We considered the possibility that binding by REF-1 family proteins on the *tbx-35* or *end-1* promoter interferes with MED-1/2 binding or activity on the same promoter. We mutated the putative REF-1 site at −127 of the *end-1* reporter (CACGAG to CTCGTA; mut-127), a mutation shown previously to abolish binding by REF-1 (Neves and Priess, 2005). This transgene was derepressed in the MS lineage with no defect in expression in the E lineage (Fig. 5F, Fig. S3). These mutagenesis results suggest that binding of REF protein at the −127 REF-1 site represses transcription of the *end-1* transgene by interfering with MED-1/2 binding or activity at a nearby site.

Mutation of both the −152 (CTCGTG→CTCATA) and −162 (CAACTG→CTAAAG) candidate REF-1 sites on the *tbx-35* transgene (mut-162, mut-152, Fig. 5G,H), without altering the consensus MED-1/2 binding sites, also abolished lineage-restricted expression, resulting in derepressed *tbx-35*::GFP expression in the E lineage (Fig. 5C, Fig. S3). Mutation of either candidate REF-1 site alone had only a minor effect (Figs S3 and S4). Together, these results suggest that REF-1 family proteins repress the transcription of *end-1* in the MS lineage and *tbx-35* in the E lineage and that this is likely to be by interfering with MED-1/2 binding to, or activity at, the same promoter.

Zygotically expressed MOM-2 regulates POP-1 and SYS-1 levels in precursors of MS BWMs

How might zygotic MOM-2 induce MS-derived BWMs? BWMs are generated from two of the four MS granddaughter cells, MSap and MSpp, which are the posterior daughters of MSa and MSp, respectively (Sulston et al., 1983). The anterior daughters of MSa and MSp, however, produce only pharyngeal muscle. A crucial component of the anterior-posterior fate differences in most, if not all, cells in developing *C. elegans* embryos is the nuclear levels of the TCF protein POP-1 and its coactivator, the β-catenin SYS-1 (Lin et al., 1995, 1998; Kidd et al., 2005; Huang et al., 2007). Both proteins exhibit a reciprocal asymmetric distribution between the nuclei of anterior and posterior sisters, with the posterior nuclei exhibiting higher SYS-1 and lower POP-1 levels compared with the corresponding anterior nuclei (Lin et al., 1998; Huang et al., 2007; Phillips et al., 2007). We have shown previously that the ratio of nuclear SYS-1 to POP-1 is the key regulator of the differential anterior-posterior developmental fates in many, and possibly all, pairs of anterior-posterior sisters in the embryo (Huang et al., 2007). In addition, it was shown that an artificially induced high POP-1 level is incompatible with the production of BWM (Fukushige and Krause, 2005). Therefore, it is possible that specification of MS BWMs involves regulation of the levels, or activity, of SYS-1, POP-1, or both, in MSap and MSpp.

Wnt signaling plays an important, but not exclusive, role in regulating the levels of both POP-1 and SYS-1 in early blastomeres (Lin et al., 1998; Meneghini et al., 1999; Rocheleau et al., 1999; Park and Priess, 2003; Lo et al., 2004; Huang et al., 2007; Phillips et al., 2007). We investigated whether zygotically expressed MOM-2 modulates POP-1 or SYS-1 levels in the MSa and MSp daughters. We used a strain carrying an integrated transgene that expresses SYS-1::GFP in embryos, and performed immunofluorescence using anti-POP-1 and anti-GFP antibodies (Huang et al., 2007). In wild-type embryos we observed clear reciprocal asymmetric levels of POP-1 and SYS-1 between daughters of MSa and MSp (Huang et al., 2007). MSaa and MSpa have a higher level of nuclear POP-1 and lower level of SYS-1 than MSap and MSpp, respectively (Fig. 6A-C, Fig. S5). In embryos from hermaphrodites homozygous for *mom-2(or42)*, which lack both maternal and zygotic MOM-2, we observed no asymmetry for SYS-1 and variable asymmetry for POP-1 between MSa and MSp daughters (Fig. 6D-I, Fig. S5). We asked whether providing zygotically expressed MOM-2 in these embryos could rescue the SYS-1 and/or POP-1 defects. We crossed males from a strain expressing *teIs18* into *mom-2(or42)* homozygous hermaphrodites, and performed immunofluorescence on the embryos. Embryos that derived from the cross would express GFP::H2B from *teIs18* and would have one wild-type copy of the *mom-2* gene that can be expressed zygotically [MOM-2(M− Z+)]. Of five 28-cell embryos expressing GFP::H2B in the E lineage, all five exhibit the wild-type pattern of POP-1 asymmetry between daughters of MSa and MSp (Fig. 6J-L, Fig. S5). The effect on SYS-1::GFP asymmetry is difficult to evaluate in this experiment because the level of histone H2B overshadows that of SYS-1::GFP (Fig. 6K). This suggests that zygotic MOM-2 is sufficient to regulate POP-1 asymmetry in the daughters of MSa and MSp.

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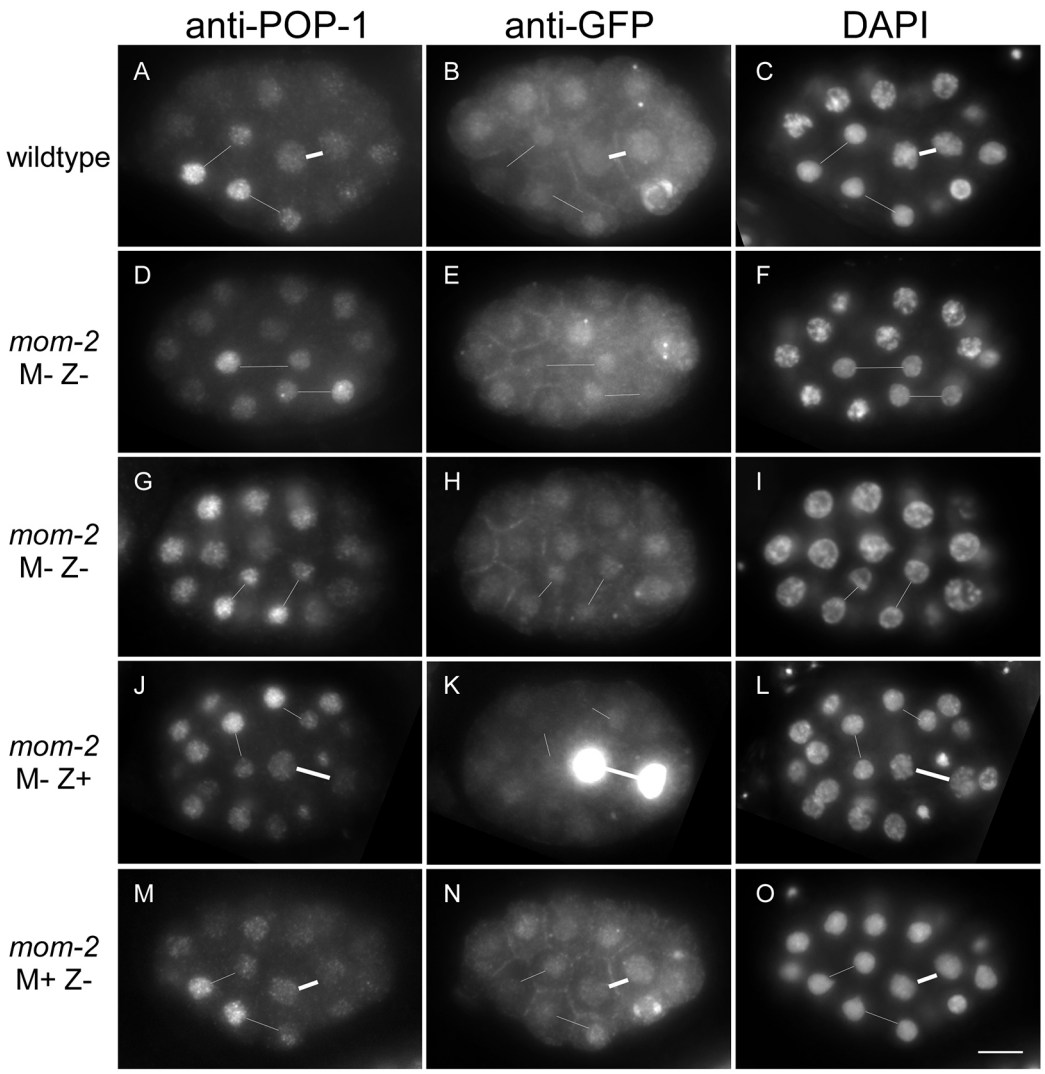

Fig. 6. Zygotic *mom-2* regulates POP-1 and SYS-1 reciprocal asymmetry. Embryos (all 28-cell stage with anterior to the left) expressing GFP::SYS-1 stained with anti-POP-1 (left), anti-GFP (center) and DAPI (right). (A-C) In wild-type embryos, reciprocal asymmetry of nuclear POP-1 and GFP::SYS-1 is evident between daughters of MSa and MSp (connected with thin lines). Thick lines connect E daughters. (D-I) Embryos completely lacking both maternal and zygotic *mom-2* expression (M– Z–). (D-F) Reversed reciprocal asymmetry is observed in MSp daughters. (G-I) Loss of GFP::SYS-1 asymmetry in both pairs and POP-1 asymmetry in daughters of MSa. (J-L) In embryos lacking maternal but expressing zygotic MOM-2 (M– Z+), wild-type reciprocal asymmetry of POP-1 and SYS-1 is observed. (M-O) In embryos expressing maternal but lacking zygotic MOM-2 (M+ Z–), wild-type reciprocal asymmetry is observed. All images shown are *z*-projections from three to four adjacent focal planes to include all four MS granddaughter cells. (K) Note that E daughters express bright GFP::H2B from *teIs18*, precluding analysis of GFP::SYS-1. Scale bar: 10 µm.

We also asked whether zygotic MOM-2 is necessary for SYS-1 or POP-1 asymmetry between daughters of MSa and MSp. We performed immunofluorescence of embryos derived from hermaphrodites transheterozygous for *mom-2(or42)* and *teIs18* [MOM-2(M+ Z–)]. Of the two 28-cell embryos lacking GFP::H2B in the E lineage, which are likely to be homozygous for *mom-2 (or42)*, both have a wild-type pattern of POP-1 and SYS-1 asymmetry (Fig. 6M-O, Fig. S5). This shows that zygotic expression of MOM-2 is not required for POP-1 or SYS-1 asymmetry as long as maternal MOM-2 is present.

Together, our analyses suggest that the levels of POP-1 and SYS-1 in MSap and MSpp can be regulated by either maternally or zygotically expressed MOM-2.

DISCUSSION

We show here that reciprocal signaling by Wnt and Notch specifies the BWM fate of the MS blastomere in *C. elegans* embryos. Two distinct Notch-mediated interactions, separated both spatially and temporally, induce zygotic expression of the Wnt ligand MOM-2 in a subset of descendants from the AB blastomere. Zygotically expressed MOM-2 then mediates two distinct interactions, with the first inhibiting the ability of MS to generate BWMs and the second interaction, two cell cycles later, to lift the repression. Our data suggest a molecular model by which the repression and activation of MS-derived BWM fate can be achieved.

Zygotic Wnt and MS BWMs

Several observations support our conclusion that zygotic *mom-2* serves as a ligand for both the inhibitory interaction between ABp descendants and EMS, and the activating interaction between ABa descendants and MS that regulate MS-derived BWMs (Fig. 7A). First, the temporal and spatial expression patterns of zygotic *mom-2* put it in the right place and at the right time to mediate these interactions. Second, the differences in *mom-2* transgene expression in the *glp-1*

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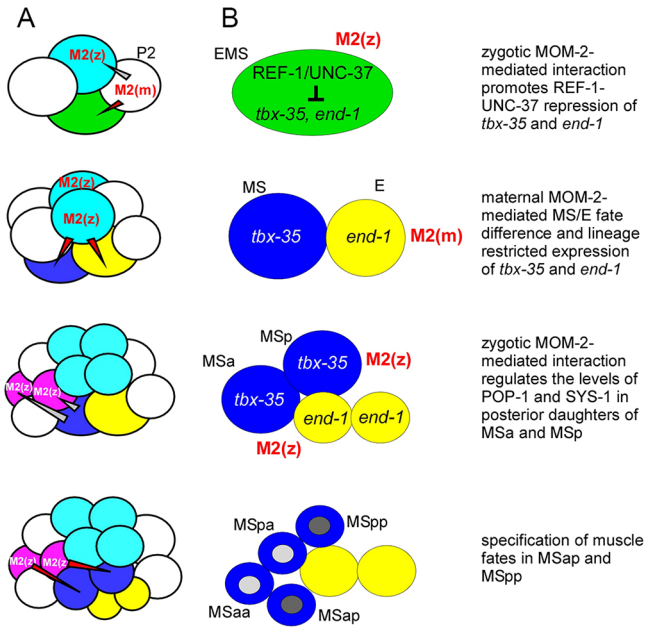

Fig. 7. Model for specification of MS muscle fate by Wnt and Notch signaling in early *C. elegans* embryos. (A) The spatiotemporally distinct expression of zygotic MOM-2 [M2(z)] in ABp (light blue) and a subset of ABa granddaughters (magenta) as a result of two distinct Notch signals (gray triangles), and the proposed interaction mediated by both maternal MOM-2 [M2(m)] and M2(z) (red triangles). (B) Proposed molecular consequences of MOM-2-mediated interactions. M2(z) signal from ABp promotes REF-1 and UNC-37 repression of *tbx-35* and *end-1* in EMS. M2(m) signal from P2 induces lineage-restricted expression of *end-1* and *tbx-35*. M2(z) signal from ABalp and ABara regulates asymmetric POP-1 and SYS-1 levels between MSa and MSp daughters, which is important for MS-derived BWM formation. POP-1 nuclear asymmetry is indicated by gray shading that represents the fluorescence signal as in Fig. 6; light gray, high POP-1; dark gray, low POP-1.

(*e2142*) and *glp-1*(*e2144*) backgrounds can explain the differential MS-derived BWM production in these two mutants (Schnabel, 1994). This supports a model whereby *e2144*, the *glp-1* 'strong' mutant, exhibits defects in both the inhibitory and activating interactions, whereas *e2142*, the *glp-1* 'weak' mutant, exhibits a defect only in the activating interaction. Third, our strongest piece of data supporting this model is the suppression of the MS BWM defect in *glp-1(e2142)* embryos when zygotic expression of MOM-2 is prevented. This clearly demonstrates that the inhibitory interaction requires zygotic *mom-2*. We do not currently have the genetic tools necessary to test directly whether zygotic Wnt also mediates the activating interaction. This idea nevertheless receives strong indirect support from our result that zygotic Wnt can regulate the nuclear levels of both SYS-1 and POP-1 in MSap and MSpp.

Inhibition of lineage-restricted genes in EMS

At face value it appears to be an overly complex strategy for an embryo to specify BWM potential in MS through two sequential interactions, the first an inhibitory interaction that is relieved some minutes later by an activating interaction. Our results here suggest that the function of the initial inhibition is to prevent precocious activation of lineage-restricted genes in MS and E. During the first few hours of *C. elegans* embryogenesis, cell cycle times are short, at ~10-15 min, and a number of cell lineages are specified early during embryogenesis (Sulston et al., 1983). Specification of these precursor blastomeres results from the coordinated action of both maternally supplied factors and the lineage-restricted zygotic expression of transcription factors (see reviews by Schnabel and Priess, 1997; Maduro, 2010). The short cell cycle times demand very precise timing of zygotic expression of lineage-restricted transcription factors in the correct blastomeres. Although MS and E produce mutually exclusive tissue types, they are sister blastomeres. Furthermore, transcriptional activation of both MS and E lineage-restricted genes requires zygotic expression of MED-1 and MED-2, both of which are transcribed in the mother cell, EMS (Maduro et al., 2001; Robertson et al., 2004; Broitman-Maduro et al., 2005, 2006, 2009). This clearly poses the risk of precocious transcriptional activation of MS- and E-restricted transcription factors, with the resultant loss of lineage restriction.

The data presented here suggest a possible mechanism by which such precocious transcriptional activation can be prevented in EMS (Fig. 7B). We propose that *tbx-35* and *end-1* are transcriptionally repressed in EMS and early MS and E cycles by REF-1 family proteins. This repression is a consequence of the arrangement of the respective binding sites on the *end-1* and *tbx-35* promoters, making it incompatible for MED-1/2 to bind or function when the REF-1–UNC-37 complex is bound. The observed ectopic expression of *tbx-35* and *end-1* in *apx-1(zu183)* embryos suggests that this REF-1-mediated repression is regulated by ABp or its descendants. We propose that a zygotic MOM-2-mediated interaction from ABp descendants to EMS regulates REF-1 repression of *tbx-35* and *end-1*. How the ABp interaction regulates the REF-1-mediated repression in EMS remains to be determined.

Activation in MS and E of the respective lineage-restricted genes

This competitive binding model suggests that the repression by REF-1 would be relieved when MED-1 and MED-2 levels increase. However, as MED-1/2 proteins are present in both MS and E, why is *tbx-35* not expressed in E, and *end-1* not expressed in MS? For *end-1*, the repression in MS and expression in E has been explained by the asymmetric levels of POP-1 and SYS-1 between these two cells (Lo et al., 2004; Shetty et al., 2005; Huang et al., 2007). A high SYS-1 and low POP-1 level in E allows POP-1 to function as a transcriptional activator for *end-1*. In the MS blastomere, a high level of nuclear POP-1 binds to UNC-37 and HDC-1, together keeping the *end-1* gene repressed (Calvo et al., 2001).

This same paradigm does not explain the MS-restricted expression of *tbx-35*. We propose that although the *tbx-35* promoter does not contain an obvious POP-1 binding site, the differential nuclear levels of POP-1 still underlie lineage-restricted expression of *tbx-35* between MS and E. Both POP-1 and REF-1 family proteins bind to UNC-37, which is required for transcriptional repression by each complex (Calvo et al., 2001; Neves and Priess, 2005). The levels of UNC-37 and HDC-1 are similar in MS and E nuclei (Calvo et al., 2001). If the level of UNC-37 is limiting compared with POP-1 and REF-1 family proteins, then the repressive activity of REF-1 in MS and E could differ as a consequence of different levels of nuclear POP-1 in these two cells. In the E blastomere where POP-1 nuclear level is low, REF-1 remains effective as a repressor for *tbx-35* even as the level of MED-1/2 increases. In the MS blastomere, where POP-1 nuclear level is high and therefore the availability of unbound UNC-37 for REF-1 binding is low, an increased level of MED-1/2 would overcome the repression by REF-1 family proteins, allowing transcription of *tbx-35*.

Why only MS BWM fate?

Why is it that MS BWM cell fate, but not pharyngeal muscle cell fate, requires an activating interaction? It has been suggested previously that development of BWM fate in *C. elegans* embryos is

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sensitive to the level of POP-1 protein (Fukushige and Krause, 2005). In the MS lineage, BWMs derive from MSap and MSpp, two posterior daughters that in the wild type have low nuclear levels of POP-1 and high levels of SYS-1. We show here that, in MSap and MSpp, higher levels of SYS-1 and lower levels of nuclear POP-1 require either maternal or zygotic MOM-2. Our results supports a model whereby the activating interaction mediated by zygotic MOM-2 expressed in ABalp and ABara, or their daughters, regulates the levels of POP-1 and SYS-1 in MSap and MSpp, leading to the generation of BWMs (Fig. 7B). MS-derived BWMs line the head region of worms, helping to explain the previous observation that zygotic expression of MOM-2 can partially rescue the head morphogenesis defect of *mom-2(or42)* embryos (Thorpe et al., 1997). We show that embryos that express maternal MOM-2 but lack zygotic MOM-2 have normal POP-1 and SYS-1 levels in MSap and MSpp. This observation supports the notion that regulation of SYS-1 and POP-1 levels in MSap and MSpp is only needed when the inhibitory interaction mediated by zygotic MOM-2 occurs earlier in embryogenesis. Further investigation is needed to elucidate the molecular mechanism(s) by which the inhibitory interaction influences the levels of POP-1 and SYS-1.

This study highlights the extent of cell-cell interactions and the tremendous complexity of cell fate specification in an organism once thought to be totally hardwired. Like many other observations in biology, the phenomena often reflect the evolution of the pathways, rather than the simplest solution to a problem.

MATERIALS AND METHODS

Strains

N2 was used as the wild-type strain. Genetic markers utilized in this study: LGIII: *glp-1(e2142)*, *glp-1(e2144)*, *unc-119(ed3)*; LGIV: *skn-1(zu67)*, *DnT1(qIs51)*; LGV: *mom-2(or42)*, *mom-2(ok591)*, *apx-1(zu183)*, *dpy-11(e224)*, *teIs18, DnT1(qIs51).* Strains utilized in this study: TX895 [*unc-119(ed3)III; him-3(e1147)IV; teIs84(Pend-3::gfp::H2B, pDPmm016)X*] (Shetty et al., 2005), TX585 [*unc-119(ed3)III; teIs18(Psdz-23::gfp::H2B, pDPmm016)V*] (Robertson et al., 2004), TX1310 [*unc-119(ed3)III; teEx268(Pend-1::gfp::H2B, pDPmm016)*], TX964 [*teIs98(Ppie-1::gfp::sys-1)*], TX878 [*unc-119(ed3)III; teEx368 (pRL1950 Pend-1$^{(mut-127)}$::gfp::H2B, pDPmm016)*], TX929 [*unc-119(ed3)III; teEx393 (pRL2075 Pend-1$^{(mut-140,-127)}$::gfp::H2B, pDPmm016)*], TX989 [*unc-119(ed3)III; teEx435 (pRL1582 Pmom-2::gfp::H2B, pDPmm016)*], TX1038 [*unc-119(ed3)III; teEx469 (pRL1377 Ptbx-35::gfp::H2B, pDPmm016)*], TX1182 [*unc-119(ed3)III; teEx576 (pPD93.92 Phlh-1::hlh-1::gfp, pDPmm016)*], TX1971 [*unc-119(ed3)III; teEx936 (pRL3467 Ptbx-35$^{(mut-162,-152)}$::gfp::H2B, pDPmm016)*], TX1963 [*unc-119(ed3)III; teEx928 (pRL3467 Ptbx-35$^{(mut-162)}$::gfp::H2B, pDPmm016)*], TX1967 [*unc-119(ed3)III; teEx932 (pRL3467 Ptbx-35$^{(mut-152)}$::gfp::H2B, pDPmm016)*]. All other strains were obtained from the Caenorhabditis Genetics Center (CGC) and were grown at 20°C unless specified. All *teEx* strains were generated by injection and the transgenes are not integrated. For each construct, expression was analyzed and found to be consistent in at least two independent lines.

Imaging

All images were acquired, processed and quantified as described previously (Huang et al., 2007). Expression of reporter GFP in wild-type and mutant embryos was analyzed as described previously (Shetty et al., 2005; Robertson et al., 2014). Embryos were prepared for immunofluorescence using a 1:50 dilution of the anti-myosin heavy chain A monoclonal antibody 5.6 as described (Miller et al., 1983; Priess and Thomson, 1987). smFISH of *mom-2* mRNA was performed as described previously (Harterink et al., 2011; Oldenbroek et al., 2013).

Molecular biology

All plasmids were constructed using Gateway technology as previously described (Guven-Ozkan et al., 2010). A 3.4 kb genomic sequence comprising −2876 to +555 of the *mom-2* gene (promoter, first exon, first intron and part of the second exon) was cloned into pRL1075 upstream of, and in-frame with, *gfp::h2b*. A 54 bp region corresponding to amino acids 2-19 of MOM-2, the putative signal peptide, was then deleted to prevent interference with the nuclear localization of GFP::histone H2B. The two putative LAG1 binding sites (ATGGGAA at −1038 and −992) were mutated to AAGGCAA, with no detectable change in the GFP pattern. All mutations were generated with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene/Agilent Technologies) and confirmed by sequencing. The *end-1* and *tbx-35* promoters used comprise 2.2 kb and 734 bp, respectively, from upstream of the ATG (Robertson et al., 2004). An *end-1* promoter with three other candidate REF sites deleted (−367, −2064, −3100) did not result in derepression of the reporter or enhancement of the derepression when combined with mutation at −127.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.M.R. and R.L. conceived and designed the project, interpreted the data, and wrote the paper. S.M.R. performed all molecular biology experiments. M.O. performed smFISH. J.M. and R.L. performed all other experiments.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.145391.supplemental

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