



The forkhead transcription factor UNC-130/FOXD integrates both BMP and Notch signaling to regulate dorsoventral patterning of the *C. elegans* postembryonic mesoderm

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ABSTRACT

The proper development of a multicellular organism requires precise spatial and temporal coordination of cell intrinsic and cell extrinsic regulatory mechanisms. Both Notch signaling and bone morphogenetic protein (BMP) signaling function to regulate the proper development of the *C. elegans* postembryonic mesoderm. We have identified the *C. elegans* FOXD transcription factor UNC-130 as a major target functioning downstream of both BMP signaling and Notch signaling to regulate dorsoventral patterning of the postembryonic mesoderm. We showed that *unc-130* expression in the postembryonic M lineage is asymmetric: its absence of expression in the dorsal side of the M lineage requires the antagonism of BMP signaling by the zinc finger transcription factor SMA-9, while its expression in the ventral side of the M lineage is activated by LIN-12/Notch signaling. We further showed that the regulation of UNC-130 expression by BMP signaling and Notch signaling is specific to the M lineage, as the ventral expression of UNC-130 in the embryonically-derived bodywall muscles was not affected in either BMP pathway or Notch pathway mutants. Finally, we showed that the function of UNC-130 in the M lineage is independent of UNC-129, a gene previously shown to function downstream of and be repressed by UNC-130 for axon guidance. Our studies uncovered a new function of UNC-130/FOXD in the *C. elegans* postembryonic mesoderm, and identify UNC-130 as a critical factor that integrates two independent spatial cues for the proper patterning and fate specification of the *C. elegans* postembryonic mesoderm.

1. Introduction

The proper development of a multicellular organism requires precise spatial and temporal coordination of cell intrinsic and cell extrinsic regulatory mechanisms. A small number of highly conserved signaling pathways are utilized repeatedly to mediate a variety of developmental processes in metazoa. Understanding how these different signaling pathways are integrated and interpreted to regulate specific cell fate decision events is critical to our ability to dissect the regulatory circuit underlying normal animal development. The *C. elegans* postembryonic mesoderm provides a model to dissect how different signaling pathways are integrated to regulate the diversification and fate specification of multiple cell fates from a single pluripotent precursor cell.

In *C. elegans*, all the non-gonadal postembryonic mesodermal cells are derived from a single precursor cell, the M mesoblast, which is born during embryogenesis (Sulston and Horvitz, 1977). During postembryonic development of a hermaphrodite animal, the M cell first divides dorsoventrally to generate two daughter cells, M.d and M.v.

During subsequent larval development, these two cells further divide to produce distinct dorsal and ventral cell types (Fig. 1A–B). The dorsal cell, M.d, divides three more times to produce six bodywall muscles (BWMs) and two non-muscle coelomocytes (CCs), while the ventral cell, M.v, divides four more times to produce eight BWMs and two multipotent sex myoblasts (SMs), which further migrate and divide to produce eight vulval muscles (VMs) and eight uterine muscles (UMs).

Previous work has shown that the dorsoventral asymmetry in the M lineage is regulated by two different signaling pathways. The LIN-12/Notch pathway is required for specifying the ventral SM fate, while antagonism of the BMP pathway is required for specifying the dorsal CC fate (Foehr et al., 2006; Foehr and Liu, 2008; Greenwald et al., 1983). On the ventral side, three redundant Notch ligands, LAG-2, APX-1 and DSL-1, are expressed in the ventral hypodermal cells directly adjacent to the ventral M lineage cells (Foehr and Liu, 2008). They act together to activate the LIN-12 receptor in the ventral M lineage (Foehr and Liu, 2008; Levitan and Greenwald, 1998; Wilkinson and Greenwald, 1995). Loss of LIN-12/Notch signaling results in a ventral-to-dorsal fate transformation in the M lineage, thus the loss of

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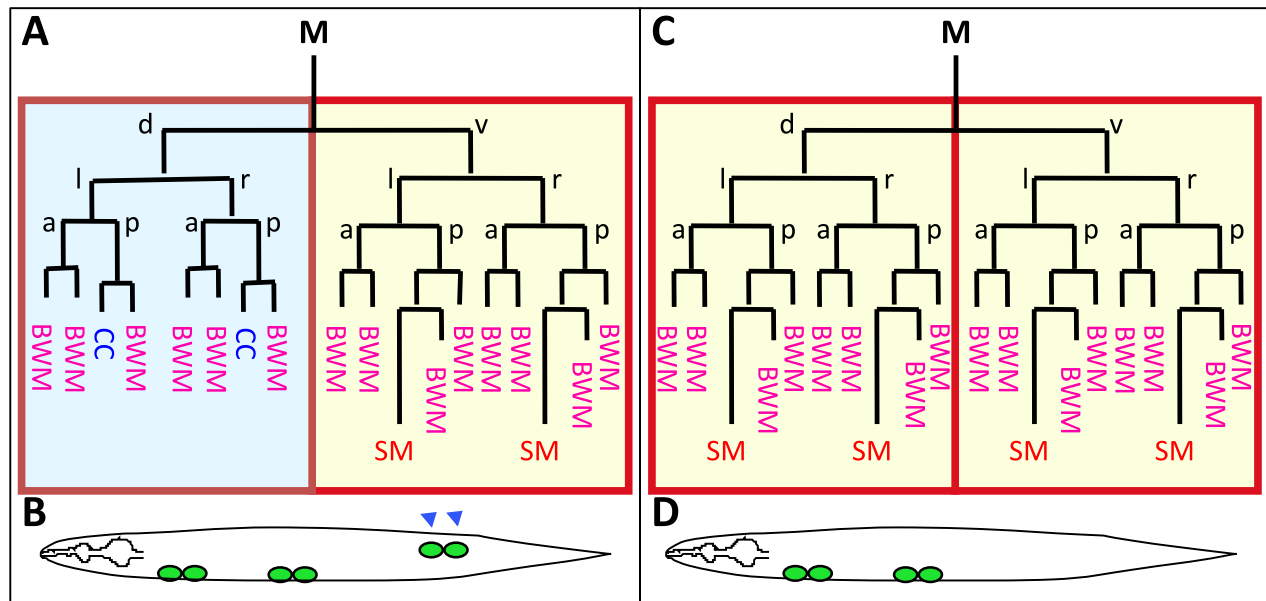
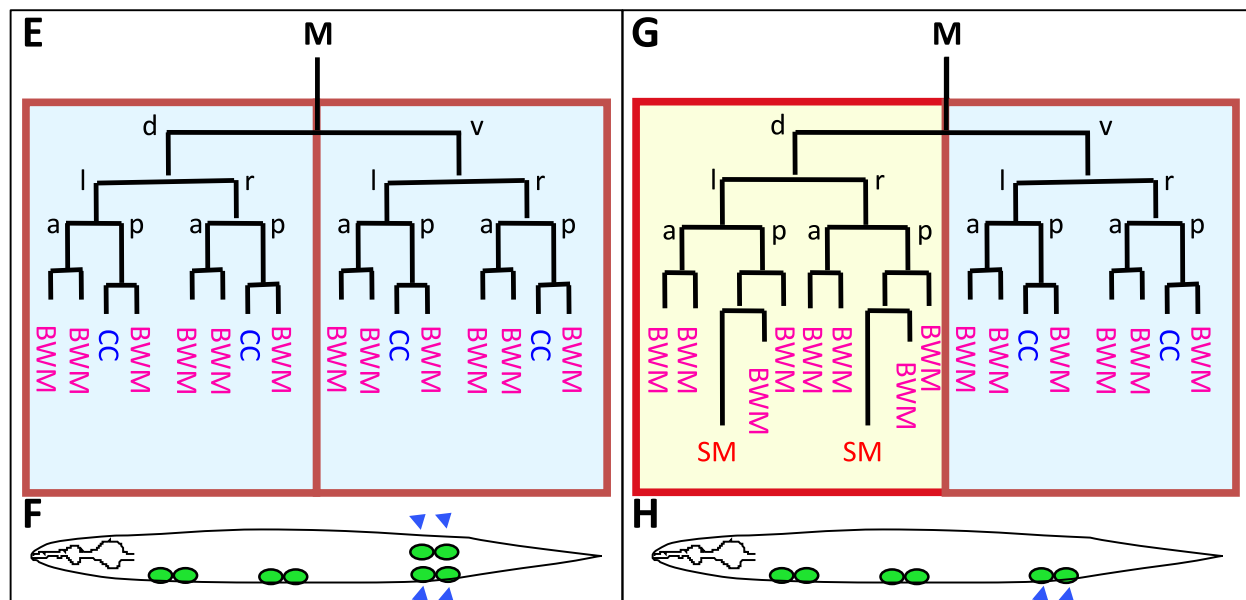
Wild type or *sma-9(0)*; BMP pathway (0)***sma-9(0)******lin-12(0)******lin-12(0); sma-9(0)***

Fig. 1. SMA-9, BMP signaling and LIN-12/Notch signaling regulate M lineage dorsoventral patterning. Diagrams showing the M lineage (A–D) and the locations of CCs (E–H) in wild-type or *sma-9(0)*; *BMP pathway(0)* (A, E), *sma-9(0)* (B, F), *lin-12(0)* (C, G), and *lin-12(0); sma-9(0)* (D, H) animals. Blue arrowheads point to the two M lineage-derived CCs. BWM: body-wall muscle, CC: coelomocyte, SM: sex myoblast. d: dorsal, v: ventral, l: left, r: right, a: anterior, p: posterior. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

the SM fate and gain of the CC fate (Fig. 1E–F) (Foehr and Liu, 2008; Greenwald et al., 1983). Specification of the dorsal M lineage fate requires the zinc finger-containing protein SMA-9, whose *Drosophila* homolog Schnurri is known to act as a transcriptional repressor (Liang et al., 2003; Marty et al., 2000; Pyrowolakis et al., 2004). In *C. elegans*, SMA-9 has also been shown to exhibit transcriptional repressor activity (Liang et al., 2007). In the M lineage, *sma-9(0)* mutants exhibit a dorsal-to-ventral fate transformation, thus the loss of the CC fate and gain of the SM fate (Fig. 1C–D) (Foehr et al., 2006). We have previously obtained genetic evidence showing that SMA-9 specifies the dorsal M lineage fate by antagonizing the DBL-1/BMP-like signaling pathway. Mutations in core components of the BMP pathway specifically suppress the M lineage defect of *sma-9(0)* mutants (Fig. 1A–B)

(Foehr et al., 2006; Liu et al., 2015). Based on double and triple mutant analysis between *sma-9(0)*, *lin-12(0)* and BMP pathway mutations, we proposed a model for how LIN-12/Notch and DBL-1/BMP pathways act independently to regulate dorsoventral patterning in the M lineage (Foehr and Liu, 2008). However, no major target genes have been identified that serve as a node to integrate and interpret these signaling inputs.

In this study, we report our finding that the forkhead transcription factor UNC-130 acts downstream of both SMA-9-BMP signaling and LIN-12/Notch signaling to integrate these spatial cues for the proper patterning of the M lineage. UNC-130 is a conserved transcription factor that belongs to a family of proteins containing a forkhead DNA-binding domain. It specifically falls into the FOXD subclass of forkhead

transcription factors (Hope et al., 2003). FOXD transcription factors play critical roles in embryonic development in vertebrates and invertebrates (Hudson et al., 2016; Imai et al., 2002; Mariani and Harland, 1998; Steiner et al., 2006). In *C. elegans*, UNC-130 was first found to regulate axon guidance and neuronal fate specification (Nash et al., 2000; Sarafi-Reinach and Sengupta, 2000). In particular, UNC-130 represses the expression of a non-canonical TGF β molecule UNC-129 in the ventral BWMs, restricting its expression to the dorsal BWMs (Nash et al., 2000). The resulting dorsal-ventral gradient of UNC-129/TGF β regulates neuronal axon migration (Colavita and Culotti, 1998; Colavita et al., 1998). The ventral BWM-specific expression of *unc-130* appears to be a result of both positive and negative regulatory inputs exerted by multiple transcription factors (Kersey et al., 2016).

We found that UNC-130 is also asymmetrically expressed in the postembryonic mesoderm, and specifically, in the ventral M lineage. We provide evidence that this ventral-specific expression of *unc-130* in the M lineage requires both LIN-12/Notch signaling in the ventral side and antagonism of BMP signaling by SMA-9 in the dorsal side. Our work identifies UNC-130 as a critical factor that integrates two independent spatial cues for the proper patterning and fate specification of the *C. elegans* postembryonic mesoderm.

2. Materials and methods

2.1. *C. elegans* strains

All strains were maintained at 20 °C unless otherwise noted. The following strains were used in this study: *unc-130(ev505)* II, *unc-130(oy10)* II, *sma-6(jj1)* II, *lin-12(n676n930ts)* *unc-32(e189)* III, *unc-36(e251)* *lin-12(n941)* III/*hT2[qIs48]* (I, III), *lon-1(e185)* III, *sma-3(jj3)* III, *unc-129(ev566)* IV, *unc-129(ev554)* IV, *dbl-1(nk3)* V, *lon-2(e678)* X, *sma-9(cc604)* X, *ctIs43[dbl-1p::GFP]* (Suzuki et al., 1999). Additional integrated transgenic lines used are: LW0081: *ccIs4438(intrinsic CC::GFP)* III; *ayIs2(egl-15p::GFP)* IV; *ayIs6(hlh-8p::GFP)* X, *jjIs3900(hlh-8p::NLS::mCherry::lacZ + myo-2p::mCherry)* IV, LW4627: *jjIs4606(hlh-8p::unc-130 cDNA + myo-2p::GFP)*; *ccIs4438(intrinsic CC::GFP)* III; *ayIs2(egl-15p::GFP)* IV; *ayIs6(hlh-8p::GFP)* X, *evIs120[unc-130p::unc-130::GFP + pRF4]* I (gift from Dr. Joe Culotti, Mount Sinai Hospital), KM522: *unc-130(gv45)[unc-130::superGFP]* and KM510 [*unc-129p::GFP*] (gifts from Dr. Mike Krause, NIH/NIDDK).

2.2. Plasmid constructs and transgenic lines

The *unc-130* cDNA clone *yk268f8* (gift from Dr. Yuji Kohara) was used to generate pJKL930.2[*hlh-8p::unc-130 cDNA::unc-54 3'UTR*]. Transgenic lines were generated using the plasmid pCFJ90 (*myo-2p::mCherry::unc-54 3'UTR*) (Frokjaer-Jensen et al., 2008), pJKL449 (*myo-2p::GFP::unc-54 3'UTR*) or pRF4 (*rol-6(d)*) as markers. The integrated transgenic line *jjIs4606(hlh-8p::unc-130cDNA::unc-54 3'UTR)* was generated via gamma-irradiation.

2.3. Body size measurement

Body size measurement was performed by following the protocol described in Tian et al. (2010). Specifically, gravid hermaphrodite adults of different genotypes were collected and treated with hypochlorite. The released embryos were allowed to hatch in M9 buffer at 16 °C to generate synchronized populations of L1 larvae. The larvae were plated onto nematode growth medium (NGM) plates and allowed to develop at 20 °C for 24, 48, 72 or 96 h, respectively, and then collected for body size measurement, as described in Tian et al. (2010). Subsequent statistical analyses were performed using Microsoft Excel.

2.4. Microscopy

Differential interference contrast and epifluorescence microscopy were conducted on a Leica DMRA2 compound microscope equipped with a Hamamatsu Orca-ER camera. Images were captured using the iVision software (Biovision technology, Inc.). Subsequent image processing was performed using Fiji (Schindelin et al., 2012).

3. Results

3.1. *unc-130* is expressed in the ventral cells of the postembryonic M lineage

Previous studies have shown that *unc-130* is expressed in the ventral bodywall muscles (BWMs) to restrict the expression of UNC-129/TGF β to the dorsal BWMs for proper neuronal axon migration (Colavita et al., 1998; Kersey et al., 2016; Nash et al., 2000). The postembryonic mesodermal M lineage also exhibits distinct dorsoventral asymmetry (Fig. 1). We therefore asked whether *unc-129* or *unc-130* or both are also asymmetrically expressed in the M lineage, and if so, whether either plays a role in M lineage patterning.

There are two strains available that express tagged UNC-130::GFP proteins that are fully functional. One of them has a stably integrated transgenic UNC-130::GFP translational reporter *evIs120[unc-130p::unc-130::GFP]* that was generated using an extra chromosomal array (Nash et al., 2000), and will be designated as UNC-130::GFP(OE). Another strain, *unc-130(gv45)[unc-130::superGFP]*, has the endogenous UNC-130 tagged with a superfolder GFP via CRISPR (Kersey et al., 2016), and will be designated as UNC-130::superGFP. We generated strains expressing UNC-130::GFP(OE) or UNC-130::superGFP along with an integrated *hlh-8p::NLS::mCherry::lacZ* reporter that specifically marks the M lineage, and found both strains exhibited the same GFP expression pattern in the M lineage, which is summarized in Fig. 2. UNC-130::GFP expression is not detectable in the early M lineage from the 1-M to the 4-M stage (Fig. 2A–C). Beginning at the 8-M stage and continuing through the 18-M stage, UNC-130::GFP expression is visible in all the ventral, but not in any of the dorsal, M lineage cells (Fig. 2D–F). We noticed that M.vlp and M.vrp and their descendants exhibited relatively higher levels of UNC-130::GFP expression than their anterior counterparts (Fig. 2D–F, see Section 4).

Unlike *unc-130*, a well characterized and stably integrated *unc-129p::GFP* transcriptional reporter *Punc-129(4.2 Kb)::gfp*, which has 4.2 Kb sequences upstream of the translational start site of *unc-129* driving the expression of GFP, shows robust expression in the dorsal embryonically-derived BWMs as previously reported (Kersey et al., 2016; Nash et al., 2000), but is not expressed in the M lineage (Fig. 2H–N). Thus, *unc-130*, but not *unc-129*, is asymmetrically expressed in the ventral M lineage.

3.2. SMA-9 functions to repress *unc-130* expression in the early and dorsal M lineage

Because SMA-9 and LIN-12 regulate dorsoventral patterning of the M lineage (Foebr and Liu, 2008), we asked whether these two factors also regulate *unc-130* expression in the M lineage. We first introduced UNC-130::GFP(OE) and UNC-130::superGFP separately into the null *sma-9(cc604)* mutants. Unlike in wild-type animals where UNC-130::GFP is only detectable in the ventral M lineage starting from the 8-M stage (Fig. 3A–G), in *sma-9(cc604)* mutants UNC-130::GFP(OE) was detectable in all M lineage cells from the 1-M stage through the 18-M stage (Fig. 3H–N). We saw similar pattern of de-repression of UNC-130::superGFP in the dorsal side of the M lineage after the 8-M stage, but not in the early M lineage (1-M to 4-M stage) in *sma-9(cc604)* mutants (data not shown). The lack of UNC-130::superGFP expression in the early M lineage in *sma-9(cc604)*

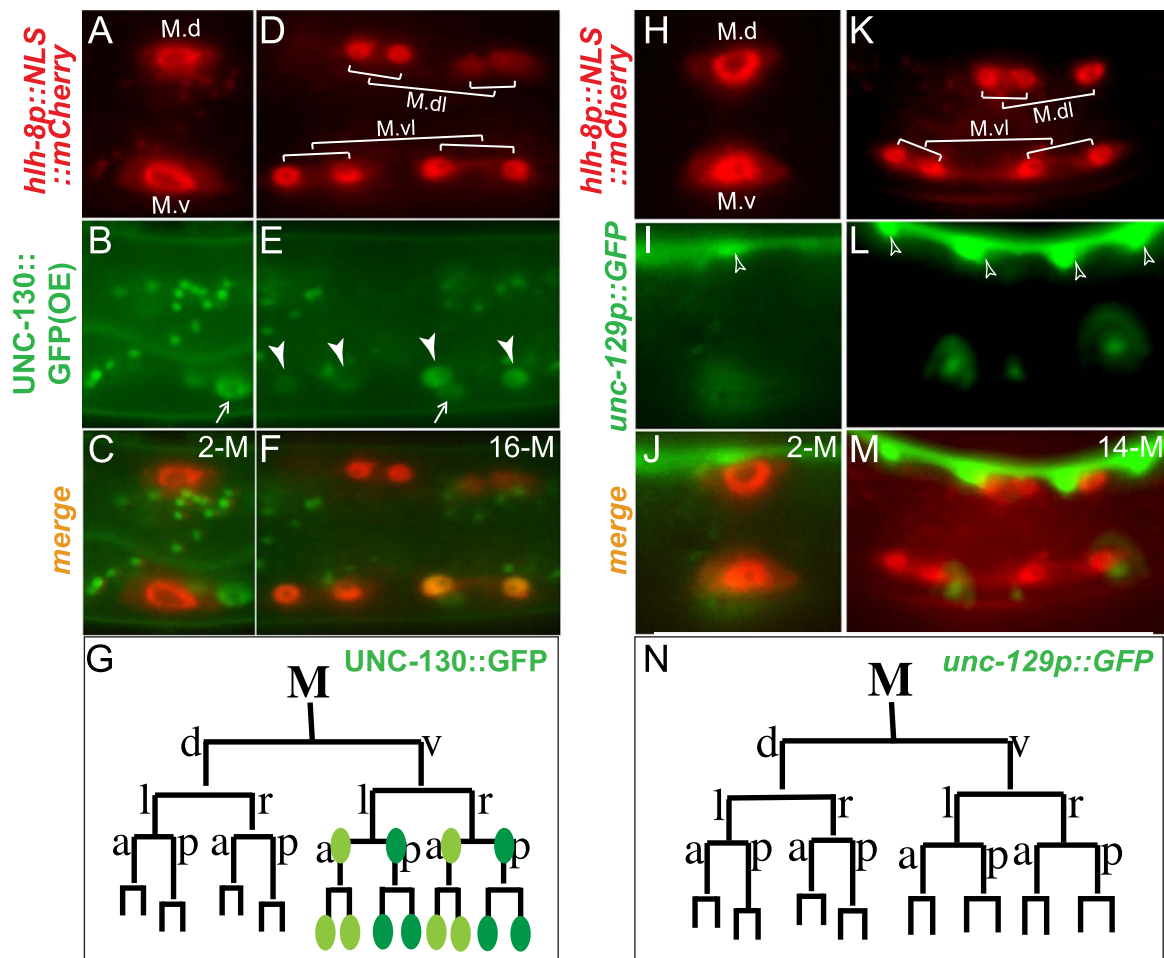


Fig. 2. *unc-130* is asymmetrically expressed in the M lineage on the ventral side. (A–G) UNC-130::GFP(*evIs120*) expression (B, E) in the undifferentiated M lineage cells marked by *hlh-8p::NLS::mCherry::lacZ* (A, D) at the 2-M stage (A–C), and the 16-M stage (D–F). C and F show the corresponding merged images. UNC-130::GFP is not detectable in the M lineage at the 2-M stage (A–C), but detectable in all the ventral M lineage cells (arrowheads in E) at the 16-M stage (D–F). Note that the posterior two cells, M.vlpa and M.vlpp, show stronger GFP signal in panel E. The arrows in panels B and E point to embryonically-derived BWMs that express UNC-130::GFP. (G) Summary of UNC-130::GFP expression in the early M lineage, based on both the integrated UNC-130(*evIs120*) translational reporter and the endogenously tagged and functional UNC-130::GFP generated via CRISPR, *unc-130(gv45)*. UNC-130::GFP-expressing cells are in green. Darker green means stronger UNC-130::GFP expression. UNC-130::GFP expression can be detected in the ventral M lineage starting at the 8-M stage. (H–N) *unc-129p::GFP* (I, L) expression pattern at the 2-M stage (H–J) and the 14-M stage (K–M). The undifferentiated M lineage cells are marked by *hlh-8p::NLS::mCherry::lacZ* (H, K). J and M show the corresponding merged images. *unc-129p::GFP* is not expressed in the M lineage, but is expressed in the dorsal embryonically-derived BWMs (open arrowheads in I and L). (N) Summary of lack of *unc-129p::GFP* expression in the M lineage. All images in this figure and subsequent figures are shown with anterior to the left and dorsal up. Only images of the left side of the animal are shown in panels A–F and H–M. Similar expression pattern is also seen in the equivalent cells on the right side (not shown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

mutants is likely due to the lower expression level of UNC-130::superGFP than that of UNC-130::GFP(OE). Nevertheless, our results indicate that SMA-9 normally functions to repress *unc-130* expression in the early M lineage (1-M to 4-M stage) and in dorsal M lineage cells (8-M stage onward). This function of SMA-9 in regulating *unc-130* expression is specific to the M lineage, because *sma-9(cc604)* mutants retained ventral-specific expression of UNC-130::GFP in the embryonically-derived BWMs, just like in wild-type animals (Fig. 3).

3.3. Repression of *unc-130* expression by SMA-9 is critical for the proper specification of dorsal CCs in the M lineage

To determine if de-repression of *unc-130* expression in the M lineage of *sma-9(0)* mutants has any functional consequences, we tested whether forced expression of *unc-130* throughout the M lineage in wild-type animals can cause a *sma-9(0)*-like M lineage defect. We generated an integrated transgenic line carrying the *hlh-8p::unc-130* cDNA transgene in an otherwise wild-type background. The transgene would lead to forced expression of *unc-130* throughout the M lineage. Indeed, the resulting transgenic animals exhibited a *sma-9(0)*-like

phenotype: 62% of the transgenic worms (N = 120) lack M-derived CCs and had extra M-derived SMs (Table 1). This observation suggests that UNC-130 may be a major player acting downstream of SMA-9 for proper patterning and fate specification of the M lineage. It further supports the notion that the regulation of *unc-130* expression in the M lineage by SMA-9 is at the level of transcription.

To test the above hypothesis, we asked whether depleting *unc-130* in *sma-9(0)* mutants could allow *sma-9(0)* mutant animals to exhibit a wild-type-like pattern of M lineage development. We constructed double mutants between *sma-9(cc604)* and two different alleles of *unc-130*, a null allele *unc-130(ev505)* (Nash et al., 2000) and a partial loss-of-function allele *unc-130(ay10)* (Sarafi-Reinach and Sengupta, 2000). Both alleles suppressed the loss of M-derived CCs phenotype of *sma-9(cc604)* mutants (Table 2). In particular, the null *unc-130(ev505)* allele exhibited a penetrance of 98% (N = 145, Table 2) in its *sma-9* suppression phenotype, which we have previously named the Susm (suppression of the *sma-9(0)* M lineage defect) phenotype (Foehr et al., 2006; Liu et al., 2015). This Susm phenotype is a specific phenotype due to loss of *unc-130* function because introducing the integrated and functional UNC-130::GFP(OE) transgene into

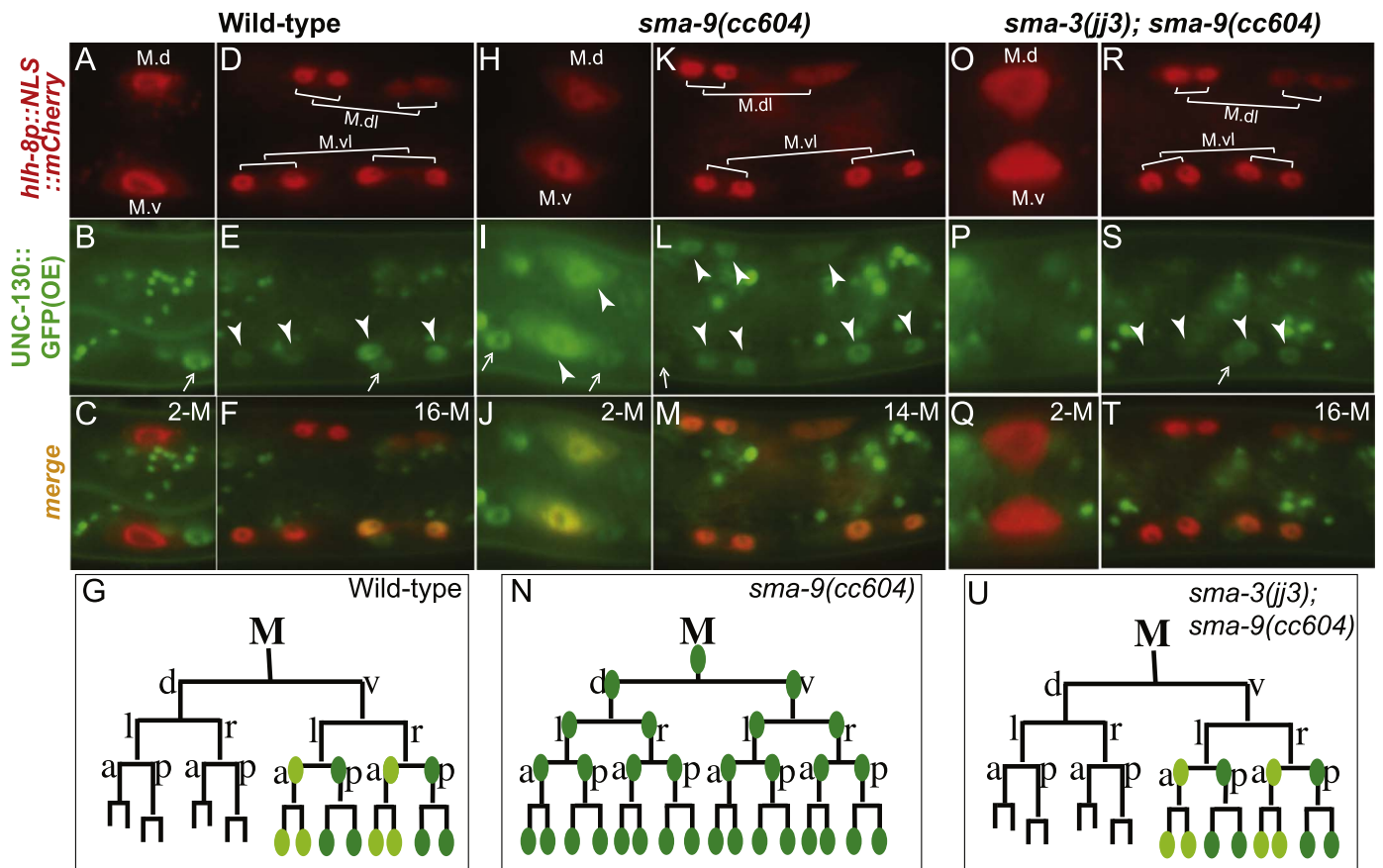


Fig. 3. SMA-9 antagonizes the BMP pathway to repress *unc-130* expression in the early M lineage and in dorsal M lineage cells. UNC-130::GFP(*ev120*) expression (B, E, I, L, P, S) in the undifferentiated M lineage cells marked by *hhlh-8p::NLS::mCherry::lacZ* (A, D, H, K, O, R) in wild-type (A–G), *sma-9(cc604)* (H–N) and *sma-3(jj3); sma-9(cc604)* (O–U) animals at the 2-M stage (A–C, H–J, O–Q), and the 16-M stage (D–F, K–M, R–T). The corresponding merged images are shown in C, F, J, M, Q and T. Panels G, N and U show summaries of the M lineage expression pattern of UNC-130::GFP in the different genetic background. The arrows in panels B, E, I, L, P and S point to embryonically-derived BWMs that expresses UNC-130::GFP. The arrowheads in panels E, I, L and S point to M lineage cells that express UNC-130::GFP. Only images of the left side of the animal are shown in panels D–F, K–M and R–T. Similar expression pattern is also seen in the equivalent cells on the right side (not shown). Note that unlike wild-type or *sma-3(jj3); sma-9(cc604)* animals which only have UNC-130::GFP expression in the ventral M lineage starting at the 8-M stage (G, U), UNC-130::GFP expression can be detected in the early M lineage cells, and both ventral and dorsal M lineage cells in *sma-9(cc604)* mutants (N).

unc-130(ev505); sma-9(cc604) double mutants rescued the Susm phenotype of *unc-130(ev505)* (Table 2). Furthermore, two *unc-129* mutant alleles (Colavita et al., 1998) did not suppress the *sma-9(cc604)* M lineage defect (Table 2). To rule out the possibility that the Susm phenotype of *unc-130(0)* is through the function of *unc-129*, we generated *unc-130(ev505); unc-129(ev554); sma-9(cc604)* triple null mutants. We did not observe any change of penetrance of the Susm phenotype in the triple mutants when compared to *unc-130(ev505); sma-9(cc604)* double mutants (Table 2), suggesting that the function of UNC-130 in the M lineage is independent of UNC-129.

Taken together, our results support the notion that *unc-130* is a major target gene acting downstream of SMA-9 and that repression of *unc-130* expression by SMA-9 in the dorsal and early M lineage is critical for the proper specification of dorsal CCs in the M lineage.

Table 1

Forced expression of *unc-130* in the M lineage leads to a *sma-9(0)*-like M lineage defect.

Genotype	Percent with no M-CCs ^a (# of animals examined)
Wild-type	0% (N = 133)
<i>sma-9(cc604)</i>	97% (N = 128)
<i>jjIs4606[hhlh-8p::unc-130cDNA]</i>	62% (N = 120)

^a The number of CCs was scored using the CC::GFP reporter. Animals with no M-derived CCs also had extra SMs marked by the *hhlh-8p::GFP* reporter and extra VM1s marked by the *egl-15p::GFP* reporter.

Moreover, this M lineage function of UNC-130 is independent of UNC-129.

3.4. De-repression of *unc-130* expression in the M lineage of *sma-9(0)* mutants requires BMP signaling

We have previously shown that mutations in the BMP pathway can suppress the *sma-9(0)* M lineage defect, exhibiting the Susm phenotype (Foehr et al., 2006; Liu et al., 2015). Since *unc-130(0)* mutants exhibited a similar Susm phenotype as BMP pathway mutants, we reasoned that the UNC-130 transcription factor may either function

Table 2

unc-130, but not *unc-129*, mutations suppress the *sma-9(0)* M lineage defects.

Genotype	Susm penetrance ^a (# of animals examined)
<i>sma-9(cc604)</i>	–
<i>unc-130(ev505); sma-9(cc604)</i>	98% (N = 145)
<i>unc-130(oy10); sma-9(cc604)</i>	67% (N = 127)
<i>unc-129(ev554); sma-9(cc604)</i>	0% (N = 100)
<i>unc-129(ev566); sma-9(cc604)</i>	0% (N = 100)
<i>unc-130(ev505); unc-129(ev554); sma-9(cc604)</i>	98% (N = 87)
<i>unc-130(ev505); sma-9(cc604); ev1s120[unc-130p::unc-130::GFP]</i>	3% (N = 179)

^a The Susm penetrance refers to the percent of animals with 1–2M-derived CCs as scored by the CC::GFP reporter.

downstream of BMP signaling to mediate pathway function or function to regulate the expression of one or multiple BMP pathway components. We tested the first hypothesis by asking whether de-repression of UNC-130::GFP in the early and dorsal M lineage of *sma-9(0)* mutants requires BMP signaling. The core members of the BMP pathway include the ligand DBL-1, type I and type II receptors SMA-6 and DAF-4, as well as three Smad proteins SMA-2, SMA-3 and SMA-4 (Gumienny and Savage-Dunn, 2013). When we introduced UNC-130::GFP(OE) into *dbl-1(nk3); sma-9(cc604)*, *sma-6(jj1); sma-9(cc604)* and *sma-3(jj3); sma-9(cc604)* double null mutants, we found that the expression pattern of UNC-130::GFP(OE) in these double mutants returned to the same pattern as that in wild-type animals (Fig. 3O–U, data not shown). Thus the de-repression of *unc-130* expression in the M lineage of *sma-9* mutants requires BMP signaling. Consistent with this, we did not detect any altered expression of a *dbl-1* transcriptional reporter, *ctIs43[dbl-1p::GFP]* (data not shown). Similarly, although *unc-130(ev505)* mutants are slightly smaller than wild-type animals (Fig. 4A), double mutant analysis showed that there is no genetic interaction between *unc-130(ev505)* and mutations in the BMP pathway in body size regulation (Fig. 4B). Although it is unclear why *unc-130(ev505)* mutants are smaller than wild-type animals, our data do not support a role of UNC-130 functioning upstream of the BMP pathway. Instead, they suggest that UNC-130 functions downstream of SMA-9 and the BMP pathway for proper M lineage development.

3.5. UNC-130 functions downstream of LIN-12/Notch signaling to promote the ventral M lineage fate

In addition to SMA-9 antagonism of BMP signaling in specifying the dorsal M lineage fate, LIN-12/Notch signaling is known to promote the ventral M lineage fate (Foehr et al., 2006; Foehr and Liu, 2008; Greenwald et al., 1983). We therefore tested whether LIN-12/Notch signaling plays a role in regulating the expression of *unc-130* in the M lineage. The integrated UNC-130::GFP(OE) transgene *evIs120* appears to map to chromosome I, which prevented us from introducing it into the *lin-12m* null mutant because of the usage of the *hT2[qIs48](I, III)* balancer. We therefore introduced the endogenously tagged UNC-130::superGFP(*gv45*) into the null *lin-12(n941)* mutants, and found that UNC-130::GFP was undetectable throughout the M lineage in *lin-12(n941)* mutants (Fig. 5E–H). Similar results were obtained in the *lin-12(n676n930ts)* mutants at the restrictive temperature 25 °C (data not shown). These results suggest that LIN-12/Notch signaling is required to promote *unc-130* expression in the ventral M lineage.

The absence of UNC-130::GFP expression in *lin-12(n941)* null mutants suggests LIN-12/Notch signaling might act through UNC-130 to specify the ventral SM fate in the M lineage. Indeed, 15% of *unc-130(ev505)* mutant animals (N = 305) exhibit a *lin-12(0)*-like M lineage phenotype: loss of M-derived SMs and gain of M-derived CCs (Table 3). This M lineage phenotype of *unc-130(ev505)* mutants can be partially rescued by forced expression of *unc-130* in the M lineage (Table 3). Similarly, forced expression of *unc-130* in the M lineage in *lin-12(n941)* animals could also partially rescue their M lineage defects (Table 3). The low penetrant (15%) *lin-12(0)*-like M lineage phenotype exhibited by the null *unc-130(ev505)* mutants suggests that LIN-12/Notch signaling promotes the ventral SM fate in the M lineage by acting through not only UNC-130, but also additional factors (see Section 4).

3.6. *lin-12(0); sma-9(0)* double null mutants exhibit reversed expression pattern of UNC-130 in the M lineage

We have previously shown that *lin-12(n941); sma-9(cc604)* double mutants exhibit a dorsoventral reversal phenotype in the M lineage: while both M-derived CCs and SMs are present, they are produced on the opposite side than their wild-type counterparts (Foehr and Liu, 2008). To determine whether this phenotype is correlated with an

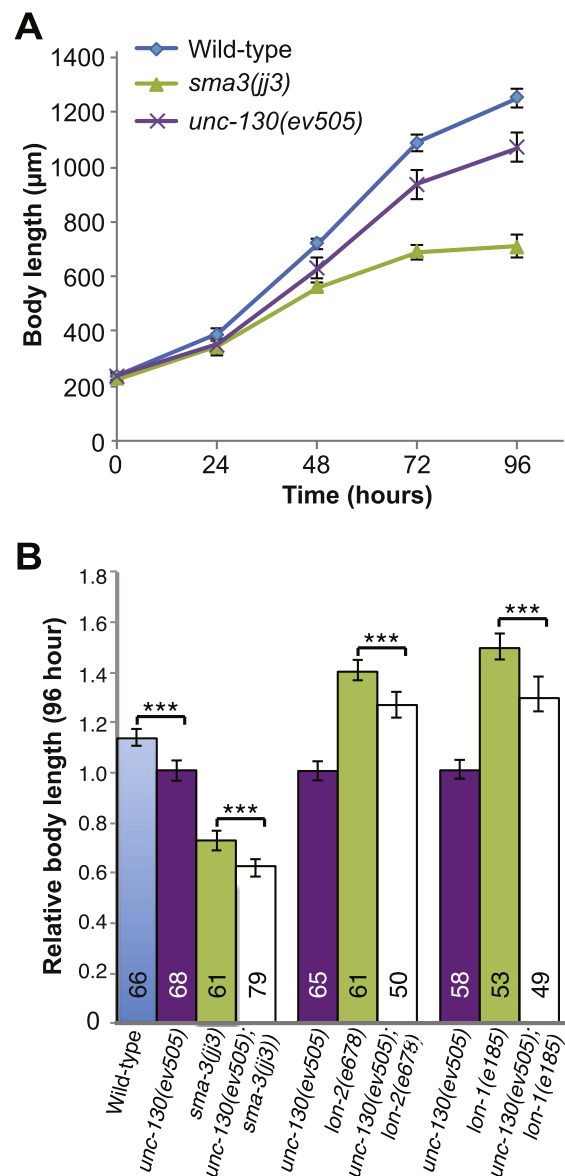


Fig. 4. UNC-130 functions in parallel to the BMP pathway to regulate body size. (A) Growth curves of wild-type (N2), *unc-130(ev505)* and *sma-3(jj3)* worms at 20 °C. Sixty to 120 animals were examined for each genotype at each time point. The body lengths of *unc-130(ev505)* animals are significantly different ($P < 0.0001$, unpaired two-tailed Student's *t*-test) from the body lengths of wild-type and *sma-3(jj3)* animals at 48, 72 and 96 h, respectively. (B) Relative body length of worms with various genotypes at 96 h post-plating (see Section 2). The body length of *unc-130(ev505)* worms was normalized to 1.0. Similar results were obtained using worms at 72 h post-plating (data not shown). *** $P < 0.0001$ (unpaired two-tailed Student's *t*-test). Error bars represent standard deviation.

altered expression pattern of UNC-130 in the M lineage, we examined the expression pattern of UNC-130::superGFP in *lin-12(n941)/+*; *sma-9(cc604)* mutants as well as in *lin-12(n941); sma-9(cc604)* double null mutants. We focused on animals at the 16-M stage for quantification purposes because younger animals prior to this stage did not show robust UNC-130::superGFP expression in the M lineage. As expected, *lin-12(n941)/+*; *sma-9(cc604)* animals (70 of 92 animals examined) showed both dorsal and ventral M lineage expression of UNC-130::superGFP, just like *sma-9(cc604)* single mutants (Fig. 5I–L). In contrast, *lin-12(n941); sma-9(cc604)* double mutants (39 of 41 animals examined) showed only dorsal, but not, ventral M lineage expression of UNC-130::superGFP (Fig. 5M–P), a reversed UNC-

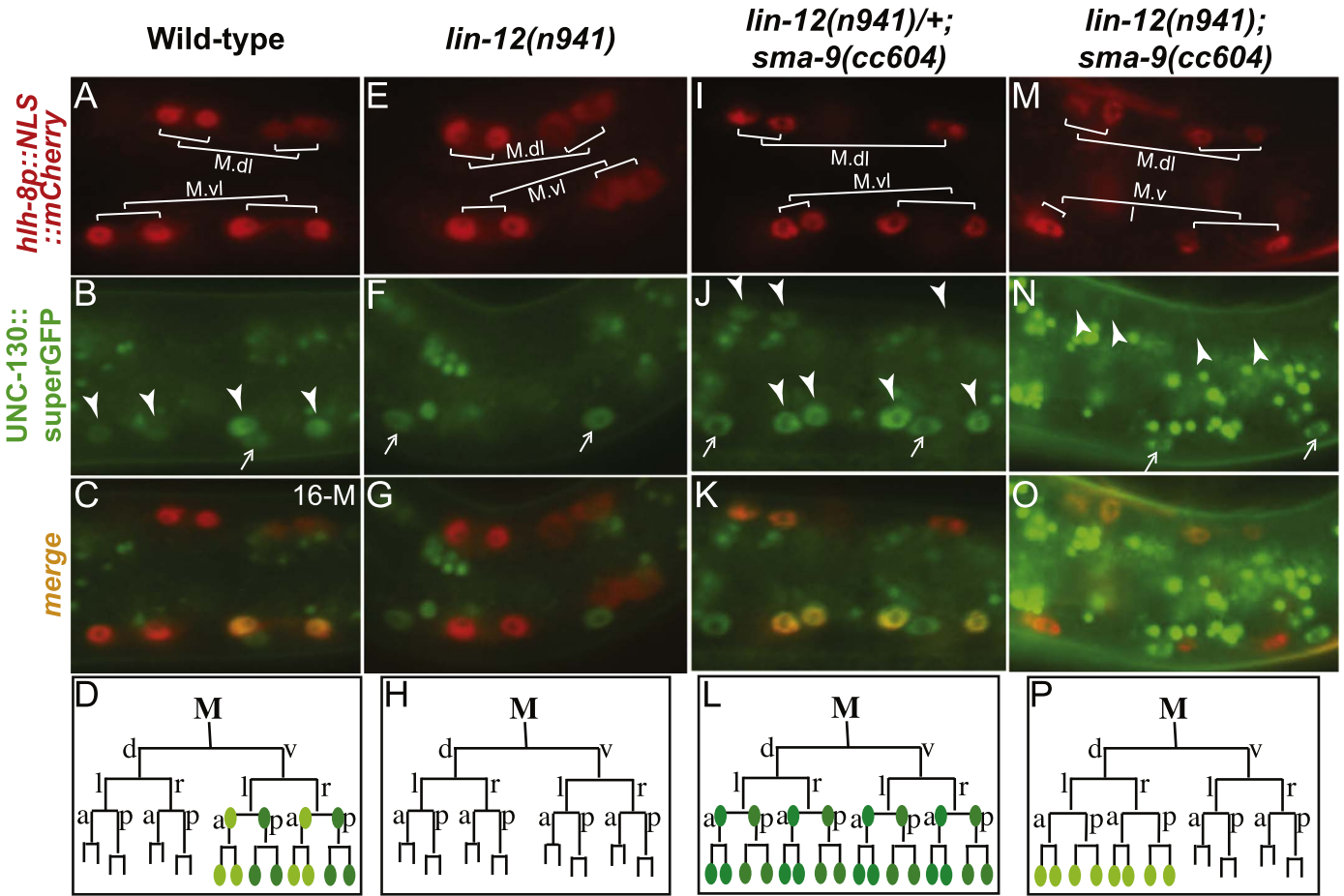


Fig. 5. LIN-12/Notch signaling promotes *unc-130* expression in the ventral M lineage cells. UNC-130::GFP expression (B, F, J, N) in the undifferentiated M lineage cells marked by *hhl-8p::NLS::mCherry::lacZ* (A, E, I, M) in wild-type (A–D), *lin-12(n941)* (E–H), *lin-12(n941)/+; sma-9(cc604)* (I–L) and *lin-12(n941); sma-9(cc604)* (M–P) animals at the 16-M stage. The corresponding merged images are shown in C, G, K and O. Panels D, H, L and P show summaries of the M lineage expression pattern of UNC-130::GFP in the different genetic background. UNC-130::superGFP (*gv45*) was used to assay for UNC-130::GFP expression in *lin-12(n941)* (E–G), *lin-12(n941)/+; sma-9(cc604)* (I–K) and *lin-12(n941); sma-9(cc604)* animals (M–O), because the UNC-130::GFP(OE) transgene *evIs120* (shown in A–C) appeared to map to the same chromosome as *lin-12*. The arrows in panels B, F, J and N point to embryonically-derived BWMs that expresses UNC-130::GFP. The arrowheads in panels B, J and N point to M lineage cells that express UNC-130::GFP. Only images of the left side of the animal are shown. Similar expression pattern is also seen in the equivalent cells on the right side (not shown). Note the lack of M lineage expression of UNC-130::GFP in *lin-12(n941)* animals (F), and the dorsal, but relatively fainter, M lineage expression of UNC-130::GFP in *lin-12(n941); sma-9(cc604)* animals (N).

Table 3
UNC-130 functions downstream of LIN-12/Notch signaling to specify M-derived SM fate.

Genotype	< 2 M-CCs > 2 SMs	2 M-CCs 2 SMs	> 2 M-CCs < 2 SMs	Number of worms examined
Wild-type	0	100	0	133
<i>lin-12(n941)</i>	0	9	91	90
<i>lin-12(n941); jjEx4702[hhl- 8p::unc-130 cDNA]</i>	0	38	62	42
<i>unc-130(oy10)</i>	0	100	0	190
<i>unc-130(ev505)</i>	0	85	15	305
<i>unc-130(ev505); jjEx4535[hhl-8p::unc-130cDNA]</i>	7 ^a	88	5	120
<i>unc-130(ev505); jjEx4541[hhl-8p::unc-130cDNA]</i>	10 ^a	88	2	183

The number of CCs were scored using the *CC::GFP* reporter. Animals with no M-derived CCs also had extra SMs marked by the *hhl-8p::GFP* reporter and extra VM1s marked by the *egl-15p::GFP* reporter.

^a This phenotype is presumably due to overexpression of *unc-130* throughout the M lineage.

130::superGFP expression pattern compared to that in wild-type animals. We noticed that the dorsal M lineage expression of UNC-130::superGFP was relatively faint in *lin-12(n941); sma-9(cc604)* double mutants (Fig. 5N). The underlying reason is currently unknown, and could be simply due to the fact that these double mutants are rather sick. Nevertheless, the above observations are consistent with UNC-130 acting downstream of both SMA-9 and LIN-12/Notch signaling in M lineage development.

4. Discussion

In this study, we identified the *C. elegans* FOXD transcription factor UNC-130 as a major target functioning downstream of both BMP signaling and Notch signaling to regulate dorsoventral patterning of the postembryonic mesoderm. We showed that *unc-130* expression in the postembryonic M lineage is asymmetric: its absence of expression in the dorsal side of the M lineage requires the antagonism of the BMP

pathway by the zinc finger transcription factor SMA-9, while its expression in the ventral side of the M lineage is activated by LIN-12/Notch signaling. We further showed that the regulation of UNC-130 expression by BMP signaling and Notch signaling is specific to the M lineage, because the ventral expression of UNC-130::GFP in the embryonically-derived BWMs was not affected in either BMP pathway or Notch pathway mutants. Finally, we showed that the function of UNC-130 in the M lineage is independent of UNC-129, a gene previously shown to function downstream of and be repressed by UNC-130 for axon guidance (Nash et al., 2000). Our studies uncovered a new function of UNC-130 in proper patterning and fate specification of the *C. elegans* postembryonic mesoderm.

4.1. Regulation of UNC-130/FOXD expression by both BMP and Notch signaling

We have shown that in wild-type animals, UNC-130 expression is restricted to the ventral M lineage cells starting from the 8-M stage (Fig. 2G), and that this ventral expression of UNC-130 requires an active input by LIN-12/Notch signaling (Fig. 5). The onset of UNC-130 expression in the M lineage is consistent with its functioning downstream of LIN-12/Notch signaling; we have previously shown that LIN-12 function is not required for M lineage development prior to the 4-M stage, and that expression of the LIN-12 ligands, LAG-2 and APX-1, is only detectable in cells adjacent to ventral M lineage cells starting from the 4-M stage (Foehr and Liu, 2008). Interestingly, UNC-130::GFP expression in ventral M lineage cells exhibited asymmetry along the anteroposterior axis, stronger in the posterior cells and weaker in the anterior cells (Fig. 2G). This pattern mimics the intensity of LIN-12::GFP expression in the M lineage (Foehr and Liu, 2008), and is likely due to the function of the Wnt/ β -catenin asymmetry pathway in anteroposterior patterning of the M lineage (Amin et al., 2009; Foehr and Liu, 2008; Takeshita and Sawa, 2005).

In addition to LIN-12/Notch signaling, BMP signaling is also capable of activating UNC-130 expression in the M lineage (Fig. 3). However, our data showed that SMA-9 actively represses BMP-dependent UNC-130 expression. The repression of BMP activity by SMA-9 appears to be restricted to the early and dorsal M lineage, because we only observed UNC-130 expression in the dorsal (due to de-repression of BMP signaling by the loss of *sma-9*), but not the ventral, M lineage (due to the loss of *lin-12*) in *lin-12(0); sma-9(0)* double mutants (Fig. 5). Both SMA-9 and some BMP pathway regulators are expressed throughout the M lineage (Foehr et al., 2006; Liu et al.,

2015; Tian et al., 2010). The DBL-1/BMP ligand is produced by the nervous system (Morita et al., 1999). At present, it is not known why SMA-9 and BMP activity are only restricted to the dorsal M lineage. It is unlikely that neuronally-produced DBL-1/BMP can only reach the dorsal M lineage cells. Instead, we propose that there is either an additional factor(s) other than SMA-9 that can repress BMP signaling in the ventral M lineage, or the ventral M lineage lacks a factor(s) that functions together with the BMP pathway to activate UNC-130 expression. A model for how UNC-130 expression is regulated by BMP and Notch signaling is presented in Fig. 6. Future work is needed to determine whether the regulation of UNC-130 expression by BMP and Notch signaling is direct or indirect.

FOXD transcription factors play critical roles in embryonic development in vertebrates and invertebrates (Hudson et al., 2016; Imai et al., 2002; Mariani and Harland, 1998; Steiner et al., 2006). Many of these functions are associated with BMP or Notch signaling. For example, BMP regulates dorsoventral asymmetry and neural tube formation during vertebrate development (see Bier and De Robertis, 2015; Tuazon and Mullins, 2015 for recent reviews). In avian embryos, FoxD3 expression is induced by BMP signaling in the dorsal neural tube during neurulation (Nitzan et al., 2016), while in *Xenopus*, a FOXD factor XBF-2 acts as a transcriptional repressor to down regulate the transcription of BMP4 during neural development (Mariani and Harland, 1998). Also in *Xenopus*, FoxD3 functions to induce mesoderm formation by repressing Nodal expression (Steiner et al., 2006). A regulatory relationship between FOXD and Notch signaling has also been previously reported. In *Ciona*, Notch pathway is known to function downstream of Cs-FoxD in notochord induction (Imai et al., 2002). These studies, together with our findings, highlight the important functions that FOXD transcription factors and different signal pathways, such as the BMP and Notch pathways, play during metazoan development, in particular in dorsoventral patterning. Clearly, the regulatory relationships among these factors and the respective signaling pathways are complex and can differ depending on the specific developmental context.

4.2. Roles of UNC-130/FOXD in postembryonic mesoderm fate specification

How does UNC-130 function within the M lineage for proper fate specification? In *sma-9(0)* mutants where the dorsal M-derived CCs are transformed to SMs, *unc-130* is inappropriately expressed in the early and dorsal M lineage cells (Fig. 3). Removal of *unc-130* in *sma-9(0)*

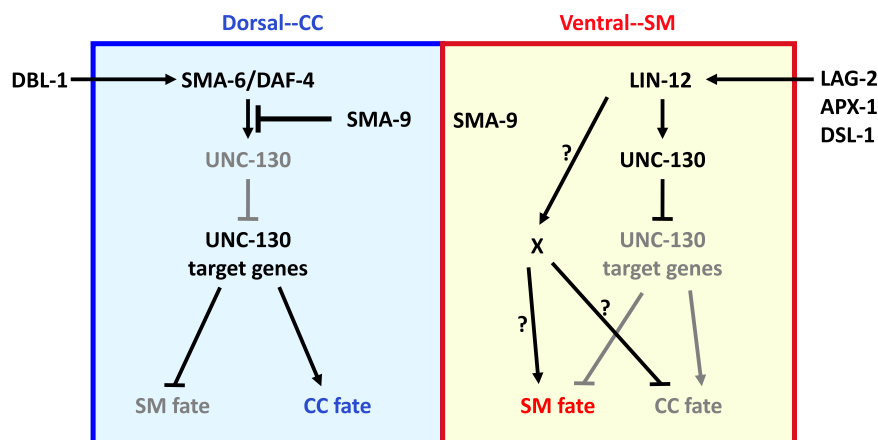


Fig. 6. A model for how UNC-130 integrates both BMP and Notch signaling to regulate dorsoventral patterning of the M lineage. We propose that in the dorsal side of the M lineage, SMA-9 represses DBL-1/BMP-dependent expression of UNC-130, while in the ventral side of the M lineage, UNC-130 expression is activated by LIN-12/Notch signaling. When expressed, UNC-130 acts as a transcriptional repressor to repress the expression of UNC-130 target genes, which promote the CC fate and repress the SM fate. The double negative gate in the dorsal side is sufficient to allow for proper CC specification. In the ventral side of the M lineage, UNC-130 likely functions together with additional LIN-12/Notch target genes to allow for the proper specification of the SMs. Solid lines do not imply direct regulatory relationship. Grey color is used to indicate lack of expression of a specific gene or fate. X refers to additional LIN-12 target(s) involved in SM fate specification.

mutants almost completely suppressed the “loss of M-derived CCs” defect of *sma-9(0)* mutants (Table 2). Furthermore, forced expression of *unc-130* throughout the M lineage caused a *sma-9(0)*-like M lineage phenotype, the loss of M-derived CCs (Table 1). These observations suggest that UNC-130 is a major effector of SMA-9 due to its inhibition of BMP signaling in the dorsal M lineage, and that UNC-130 functions to inhibit CC specification.

UNC-130 and its vertebrate homologs are known to act as transcriptional repressors to regulate distinct developmental processes. In *Xenopus*, XBF-2/FOXD is known to act as a transcriptional repressor to down regulate the transcription of BMP4, promoting neural development (Mariani and Harland, 1998). FoxD3 has also been shown to function as a transcriptional repressor to regulate Nodal expression and mesoderm induction (Steiner et al., 2006; Yaklichkin et al., 2007). In *C. elegans* embryonic body wall muscles, UNC-130 functions to repress the expression of a TGF β -like molecule UNC-129 to regulate axon guidance (Kersey et al., 2016; Nash et al., 2000). UNC-130 can also repress the expression of the Notch ligand LAG-2 in the IL2 neurons during reproductive development (Ouellet et al., 2008). Based on these studies, it is very likely that UNC-130 also acts as a transcriptional repressor to regulate M lineage development.

We propose that UNC-130 inhibits CC specification by repressing genes that promote the CC fate and inhibit the SM fate (Fig. 6). This is consistent with our observation that forced expression of *unc-130* throughout the M lineage led to the loss of M-derived CCs and gain of SMs (Table 1). It is also consistent with the “gain of CCs and loss of SMs” phenotype displayed by *unc-130* null mutants (Table 3). Since only 15% of *unc-130* null mutants exhibited a “gain of CCs and loss of SMs” phenotype in the M lineage (Table 3), we propose that UNC-130 must function with additional factor(s) to promote SM fate specification. We suggest that in the ventral M lineage, these additional factors must function downstream of LIN-12/Notch signaling because *lin-12* null mutants exhibit a near complete loss of SMs and gain of CCs in the M lineage (Table 3). Whether the same factors function in the dorsal M lineage will require further investigation. Future work will be needed to identify the genes regulated by UNC-130 in the M lineage as well as other factors that function together with UNC-130 to allow proper fate specification in the M lineage.

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References

Amin, N.M., Lim, S.E., Shi, H., Chan, T.L., Liu, J., 2009. A conserved Six-Eya cassette acts downstream of Wnt signaling to direct non-myogenic versus myogenic fates in the *C. elegans* postembryonic mesoderm. *Dev. Biol.* 331, 350–360.

Bier, E., De Robertis, E.M., 2015. EMBRYO DEVELOPMENT. BMP gradients: a paradigm for morphogen-mediated developmental patterning. *Science* 348, aas5838.

Colavita, A., Culotti, J.G., 1998. Suppressors of ectopic UNC-5 growth cone steering identify eight genes involved in axon guidance in *Caenorhabditis elegans*. *Dev. Biol.* 194, 72–85.

Colavita, A., Krishna, S., Zheng, H., Padgett, R.W., Culotti, J.G., 1998. Pioneer axon guidance by UNC-129, a *C. elegans* TGF- β . *Science* 281, 706–709.

Foehr, M.L., Liu, J., 2008. Dorsal/ventral patterning of the *C. elegans* postembryonic mesoderm requires both LIN-12/Notch and TGF β signaling. *Dev. Biol.* 313, 256–266.

Foehr, M.L., Lindy, A.S., Fairbank, R.C., Amin, N.M., Xu, M., Yanowitz, J., Fire, A.Z., Liu, J., 2006. An antagonistic role for the *C. elegans* Schnurri homolog SMA-9 in modulating TGF β signaling during mesodermal patterning. *Development* 133, 2887–2896.

Frokjaer-Jensen, C., Davis, M.W., Hopkins, C.E., Newman, B.J., Thummel, J.M., Olesen, S.P., Grunnet, M., Jorgensen, E.M., 2008. Single-copy insertion of transgenes in *Caenorhabditis elegans*. *Nat. Genet.* 40, 1375–1383.

Greenwald, I.S., Sternberg, P.W., Horvitz, H.R., 1983. The *lin-12* locus specifies cell fates in *Caenorhabditis elegans*. *Cell* 34, 435–444.

Gumienny, T.L., Savage-Dunn, C., 2013. TGF- β signaling in *C. elegans*. *WormBook: The Online Review of C.elegans Biology*. pp. 1–34.

Hope, I.A., Mounsey, A., Bauer, P., Aslam, S., 2003. The forkhead gene family of *Caenorhabditis elegans*. *Gene* 304, 43–55.

Hudson, C., Sirour, C., Yasuo, H., 2016. Co-expression of Foxa.a, Foxd and Fgf9/16/20 defines a transient mesoderm regulatory state in ascidian embryos. *eLife*, 5.

Imai, K.S., Satoh, N., Satou, Y., 2002. An essential role of a FoxD gene in notochord induction in *Ciona* embryos. *Development* 129, 3441–3453.

Kersey, R.K., Brodigan, T.M., Fukushige, T., Krause, M.W., 2016. Regulation of UNC-130/FOXD-mediated mesodermal patterning in *C. elegans*. *Dev. Biol.* 416, 300–311.

Levitani, D., Greenwald, I., 1998. LIN-12 protein expression and localization during vulval development in *C. elegans*. *Development* 125, 3101–3109.

Liang, J., Lints, R., Foehr, M.L., Tokarz, R., Yu, L., Emmons, S.W., Liu, J., Savage-Dunn, C., 2003. The *Caenorhabditis elegans* schnurri homolog *sma-9* mediates stage- and cell type-specific responses to DBL-1 BMP-related signaling. *Development* 130, 6453–6464.

Liang, J., Yu, L., Yin, J., Savage-Dunn, C., 2007. Transcriptional repressor and activator activities of SMA-9 contribute differentially to BMP-related signaling outputs. *Dev. Biol.* 305, 714–725.

Liu, Z., Shi, H., Szymczak, L.C., Aydin, T., Yun, S., Constan, K., Schaeffer, A., Ranjan, S., Kubba, S., Alam, E., McMahon, D.E., He, J., Schwartz, N., Tian, C., Plavskin, Y., Lindy, A., Dad, N.A., Sheth, S., Amin, N.M., Zimmerman, S., Liu, D., Schwarz, E.M., Smith, H., Krause, M.W., Liu, J., 2015. Promotion of bone morphogenetic protein signaling by tetraspanins and glycosphingolipids. *PLoS Genet.* 11, e1005221.

Mariani, F.V., Harland, R.M., 1998. XBF-2 is a transcriptional repressor that converts ectoderm into neural tissue. *Development* 125, 5019–5031.

Marty, T., Muller, B., Basler, K., Affolter, M., 2000. Schnurri mediates Dpp-dependent repression of brinker transcription. *Nat. Cell Biol.* 2, 745–749.

Morita, K., Chow, K.L., Ueno, N., 1999. Regulation of body length and male tail ray pattern formation of *Caenorhabditis elegans* by a member of TGF- β family. *Development* 126, 1337–1347.

Nash, B., Colavita, A., Zheng, H., Roy, P.J., Culotti, J.G., 2000. The forkhead transcription factor UNC-130 is required for the graded spatial expression of the UNC-129 TGF- β guidance factor in *C. elegans*. *Genes Dev.* 14, 2486–2500.

Nitzan, E., Avraham, O., Kahane, N., Ofek, S., Kumar, D., Kalcheim, C., 2016. Dynamics of BMP and Hes1/Hairy1 signaling in the dorsal neural tube underlies the transition from neural crest to definitive roof plate. *BMC Biol.* 14, 23.

Ouellet, J., Li, S., Roy, R., 2008. Notch signalling is required for both dauer maintenance and recovery in *C. elegans*. *Development* 135, 2583–2592.

Pyrowolakis, G., Hartmann, B., Muller, B., Basler, K., Affolter, M., 2004. A simple molecular complex mediates widespread BMP-induced repression during *Drosophila* development. *Dev. Cell* 7, 229–240.

Sarafi-Reinach, T.R., Sengupta, P., 2000. The forkhead domain gene *unc-130* generates chemosensory neuron diversity in *C. elegans*. *Genes Dev.* 14, 2472–2485.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682.

Steiner, A.B., Engleka, M.J., Lu, Q., Piwarzyk, E.C., Yaklichkin, S., Lefebvre, J.L., Walters, J.W., Pineda-Salgado, L., Labosky, P.A., Kessler, D.S., 2006. FoxD3 regulation of Nodal in the Spemann organizer is essential for *Xenopus* dorsal mesoderm development. *Development* 133, 4827–4838.

Sulston, J.E., Horvitz, H.R., 1977. Post-embryonic cell lineages of the nematode *Caenorhabditis elegans*. *Dev. Biol.* 56, 110–156.

Suzuki, Y., Yandell, M.D., Roy, P.J., Krishna, S., Savage-Dunn, C., Ross, R.M., Padgett, R.W., Wood, W.B., 1999. A BMP homolog acts as a dose-dependent regulator of body size and male tail patterning in *Caenorhabditis elegans*. *Development* 126, 241–250.

Takeshita, H., Sawa, H., 2005. Asymmetric cortical and nuclear localizations of WRM-1/ β -catenin during asymmetric cell division in *C. elegans*. *Genes Dev.* 19, 1743–1748.

Tian, C., Sen, D., Shi, H., Foehr, M.L., Plavskin, Y., Vatamaniuk, O.K., Liu, J., 2010. The RGM protein DRAG-1 positively regulates a BMP-like signaling pathway in *Caenorhabditis elegans*. *Development* 137, 2375–2384.

Tuazon, F.B., Mullins, M.C., 2015. Temporally coordinated signals progressively pattern the anteroposterior and dorsoventral body axes. *Semin. Cell Dev. Biol.* 42, 118–133.

Wilkinson, H.A., Greenwald, I., 1995. Spatial and temporal patterns of *lin-12* expression during *C. elegans* hermaphrodite development. *Genetics* 141, 513–526.

Yaklichkin, S., Steiner, A.B., Lu, Q., Kessler, D.S., 2007. FoxD3 and Grg4 physically interact to repress transcription and induce mesoderm in *Xenopus*. *J. Biol. Chem.* 282, 2548–2557.