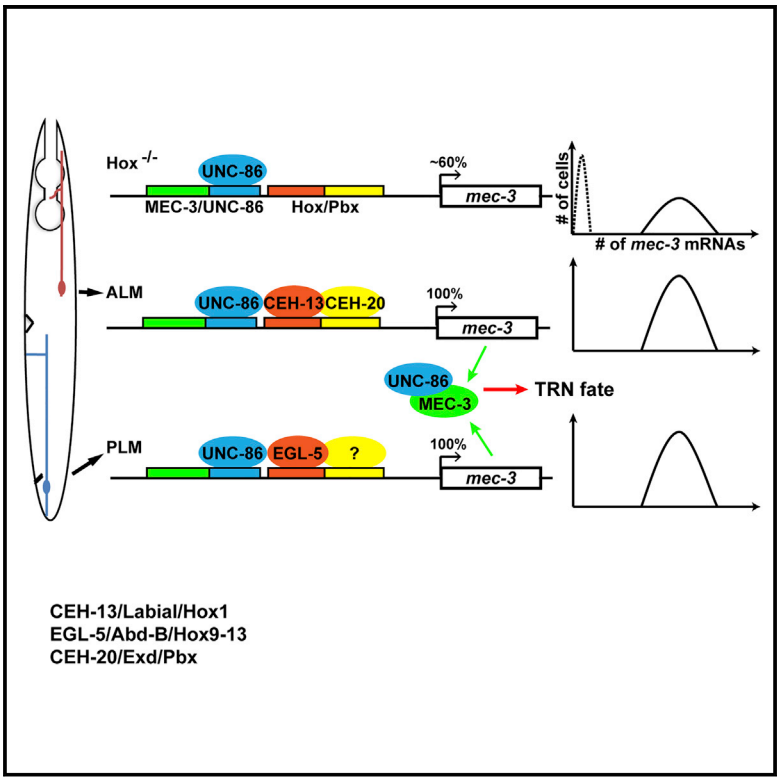
Article

Cell Reports

Hox Proteins Act as Transcriptional Guarantors to Ensure Terminal Differentiation

Graphical Abstract



Authors

Chaogu Zheng, Felix Qiaochu Jin, Martin Chalfie

Correspondence

mc21@columbia.edu

In Brief

Neuronal differentiation usually occurs with high fidelity, but the expression of transcription factors that determine cell fate is variable. Zheng et al. show that Hox proteins overcome this variability by increasing the probability of transcriptional activation of terminal selectors and serve as "guarantors" in order to ensure the robustness of cell differentiation.

Highlights

- Mutations in Hox genes result in an incomplete loss of TRN fate
- Hox proteins regulate terminal selector *mec-3* and TRN program in a binary fashion
- Hox proteins ensure *mec-3* transcriptional activation via a Hox/Pbx binding site
- Different Hox proteins in distinct subtypes promote commitment to the common fate

Zheng et al., 2015, Cell Reports *13*, 1343–1352
November 17, 2015
http://dx.doi.org/10.1016/j.celrep.2015.10.044

CellPress

Cell Reports
Article

Hox Proteins Act as Transcriptional Guarantors to Ensure Terminal Differentiation

Chaogu Zheng,[1] Felix Qiaochu Jin,[1] and Martin Chalfie[1,*]
[1]Department of Biological Sciences, Columbia University, New York, NY 10027, USA
*Correspondence: mc21@columbia.edu
http://dx.doi.org/10.1016/j.celrep.2015.10.044

SUMMARY

Cell differentiation usually occurs with high fidelity, but the expression of many transcription factors is variable. Using the touch receptor neurons (TRNs) in *C. elegans*, we found that the Hox proteins CEH-13/lab and EGL-5/Abd-B overcome this variability by facilitating the activation of the common TRN fate determinant *mec-3* in the anterior and posterior TRNs, respectively. CEH-13 and EGL-5 increase the probability of *mec-3* transcriptional activation by the POU-homeodomain transcription factor UNC-86 using the same Hox/Pbx binding site. Mutation of *ceh-13* and *egl-5* resulted in an incomplete (~40%) loss of the TRN fate in respective TRNs, which correlates with quantitative mRNA measurements showing two distinct modes (all or none) of *mec-3* transcription. Therefore, Hox proteins act as transcriptional "guarantors" in order to ensure reliable and robust gene expression during terminal neuronal differentiation. Guarantors do not activate gene expression by themselves but promote full activation of target genes regulated by other transcription factors.

INTRODUCTION

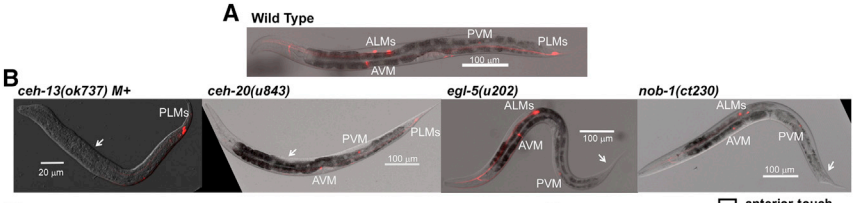
Terminal differentiation allows postmitotic cells to acquire specific cell fates, the specific functions, morphology, and gene expression that distinguish one cell type from another. The process of terminal differentiation requires reliable and robust activation of "terminal selectors" (García-Bellido, 1975; Hobert, 2008), transcription factors that activate a battery of "terminal differentiation genes," whose products define the differentiated properties of a specific cell type (Hobert, 2008).

These considerations about differentiation raise two questions. First, how does the activation of terminal selectors occur reliably so that all cells acquire a given fate? Given that stochastic fluctuation in gene expression is common in prokaryotes and eukaryotes (Ozbudak et al., 2002; Raser and O'Shea, 2004), such variability must be compensated for or regulated so that differentiation occurs with high fidelity. Second, how can cells that differ in position and developmental origin acquire the same cell fate? Here, we report that distinct Hox genes facilitate the commitment to the common neuronal fate in cells along the anterior-posterior (A-P) axis not by acting as terminal selectors but by reducing the expression variability of terminal selectors. Elsewhere, we discuss how Hox genes also induce variations that subdivide similar cells into subtypes (Zheng et al., 2015).

Hox genes encode conserved transcription factors that are expressed along the A-P axis (McGinnis and Krumlauf, 1992). Although one of their most striking effects is the control of regional differences along this axis, Hox genes also appear to determine cellular fate, as seen, for example, in the use of several different Hox proteins to promote the differentiation of motor neurons (MNs) along the mouse spinal cord (Jung et al., 2010; Lacombe et al., 2013; Philippidou et al., 2012; Vermot et al., 2005). The current theory of how Hox proteins regulate terminal neuronal cell fate suggests that Hox proteins activate the expression of terminal selectors, transcription factors essential for cell-fate determination (Dasen et al., 2008; Davenne et al., 1999; Pattyn et al., 2003). However, very few studies have investigated the mechanism of this Hox-mediated regulation. Samad et al. (2004) suggest that *Hoxb1* and *Hoxb2* bind directly to a proximal enhancer of the terminal selector gene *Phox2b* in cranial MNs, but how this binding leads to transcriptional activation remains unclear. In this study, we ask how Hox proteins regulate the expression of terminal selector genes during cell-fate decisions.

One particular aspect of this regulation is the efficiency of Hox-induced cell-fate commitment. For example, only a 37% loss of *FoxP1*$^{+}$ lateral motor column (LMC) neurons was observed in *Hoxa6*/*Hoxc6* double mutants (Lacombe et al., 2013). This incomplete loss of cell fate in Hox mutants is difficult to interpret because of several issues. First, most vertebrates have 39 Hox genes distributed across four clusters (Philippidou and Dasen, 2013). The overlapping expression and redundancy among the Hox paralogs may explain why the mutation of a single Hox gene often results in phenotypic variability and incomplete penetrance (Gaufo et al., 2003; Manley and Capecchi, 1997).

Second, Hox mutations often lead to both programmed cell death and cell-fate loss in terminally differentiated neurons in mice (Tiret et al., 1998; Wu et al., 2008) and *Drosophila* (Baek et al., 2013; Rogulja-Ortmann et al., 2008). Cell death can obscure whether cell-fate changes actually occur. Recent studies blocking cell death found that most of the phrenic MNs deprived of *Hox5* in mice (Philippidou et al., 2012) and most of the leg motor neurons deprived of *Antp* in flies (Baek et al.,



C

	% Animals with ALM			% Animals with PLM			
# of cells	0	1	2	0	1	2	# of animals
Wild Type	0	0	100	0	0	100	100
ceh-13(ok737) M+	15	42	43	0	5	95	86
ceh-13(sw1) M+	13	46	41	0	2	98	56
ceh-20(ok541) M+	27	36	36	0	3	97	67
ceh-20(u843)	23	42	35	0	0	100	100
egl-5(u202)	0	0	100	23	41	36	69
egl-5(tm4746)	0	0	100	16	47	37	100
nob-1(ct230)	0	0	100	61	15	24	82
egl-5(u202) nob-1(ct230)	0	0	100	81	8	11	62
php-3(ok919)	0	0	100	0	0	100	69
egl-5(u202) php-3(ok919)	0	0	100	21	45	34	56

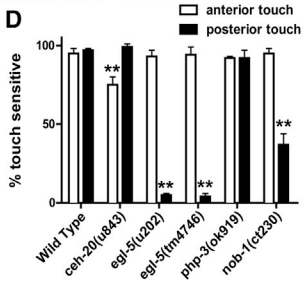


Figure 1. Mutation of Hox genes resulted in the loss of TRN marker expression

(A) TRN marker *uIs115[mec-17p::RFP]* in the six TRNs in a wild-type adult.

(B) *mec-17p::RFP* expression pattern in *ceh-13*, *egl-5*, *nob-1*, and *ceh-20* mutants. White arrows indicated the position of either ALM or PLM cell bodies, which would express the marker in the wild-type. *ceh-13(ok737)* animals arrest at early larvae stages and were obtained from heterozygous mutants (*M+*).

(C) Percentage of TRN subtypes that expressed the *mec-17p::RFP* marker in wild-type, Hox, and cofactor mutants. *ceh-20(ok541)* animals were derived from heterozygous mothers.

(D) Gentle touch sensitivity of wild-type and Hox and cofactor mutant adults. Percentage of animals that responded at least four of five times are shown. *ceh-13(ok737)* animals were arrested at L1 or L2 stage and therefore could not be tested. Error bars represent SEM, and double asterisks indicate $p < 0.01$ in comparison to wild-type.

See also Figure S1.

2013) expressed appropriate cell fate markers, but had innervation defects. These results suggest that Hox activity may not be absolutely required for cell fate adoption but is needed for the position-specific selection of axon trajectory and synaptic targets.

Third, the function of Hox proteins in promoting mouse MN differentiation has usually been tested by counting the number of neurons labeled by specific markers in a cross section of the spinal cord. Each section contains hundreds of nuclei of a given MN subtype, thus the opportunity to track individual neurons and monitor the commitment of neuronal cell fate at single-cell resolution is limited.

We have reexamined the role of Hox genes in the specification of cell fate using the touch receptor neurons (TRNs) of *Caenorhabditis elegans*. *C. elegans* has six Hox genes: an anterior gene (*ceh-13/Lab*), two central genes (*lin-39/Scr* and *mab-5/Antp*), and three *Abd-B*-like posterior genes (*egl-5*, *php-3*, and *nob-1*). The functions of those Hox genes were mainly found in neuroblast migration (Salser and Kenyon, 1992), vulval morphogenesis (Clandinin et al., 1997), and male tail development (Chow and Emmons, 1994).

C. elegans has six TRNs: two embryonic anterior ALM neurons, two embryonic posterior PLM neurons, and postembryonic AVM and PVM neurons. All six share a common fate as mechanosensory neurons that sense gentle touch. In this study, we focus on the ALML/R and PLML/R neurons. Each pair is bilaterally symmetric, but the anterior and posterior pairs differ in many ways from each other. ALM and PLM neurons have different lineage origins and different positions along both A-P and dorsal-ventral (D-V) axes (Sulston, 1983) as well as distinct morphologies and neuronal connections (Chalfie and Sulston, 1981; Chalfie et al., 1985). As a consequence, gentle touch of the ALM and PLM neurons results in backward motion and forward motion, respectively. Despite these differences, ALM and PLM neurons adopt the same TRN fate. This TRN fate is determined by terminal selectors UNC-86 and MEC-3, which form a heterodimer in order to activate a battery of terminal differentiation genes required for TRN function (Way and Chalfie, 1988; Xue et al., 1993). *mec-3* expression is initially activated by UNC-86 and later maintained through autoregulation that requires both the UNC-86/MEC-3 heterodimer and another transcription factor ALR-1 (Topalidou et al., 2011; Xue et al., 1992).

In this study, we report that distinct Hox genes facilitate the commitment of the ALM and PLM neurons to the common TRN cell fate not by switching on the terminal selector gene *mec-3* but by ensuring its robust activation. This activity allows Hox proteins to function as transcriptional "guarantors," by which we mean that they help other transcription factors to ensure reliable activation of target genes by reducing stochastic fluctuation but do not activate genes by themselves.

RESULTS

Distinct Hox Genes Help Determine the Cell Fate of Different TRN Subtypes

To study the role of the six Hox genes in regulating TRN fate, we first examined how mutations in them affected the differentiation of the ALM and PLM neurons. Mutations in *ceh-13* resulted in the loss of expression of a TRN marker (*mec-17p::RFP*) in the ALM neurons, whereas mutations in *egl-5* and *nob-1* led to the absence of the marker in the PLM neurons (Figures 1A–1C; in addition, the loss of the TRN fate in PLM neurons in *egl-5* animals was cold-sensitive [Figure S1A]). Mutations in *lin-39*, *mab-5* (data not shown) or *php-3* did not change the number of TRNs. Furthermore, we confirmed the absence of terminally differentiated ALM or PLM neurons in these Hox mutants by testing the expression of other fluorescent TRN fate markers—namely *mec-3p::RFP* (Table 1), *mec-18p::GFP*, *mec-4p::GFP*, and *mec-7p::GFP*—and staining with antibodies against MEC-18 proteins (data not shown).

A striking feature of the cell-fate loss in *ceh-13*, *egl-5*, and *nob-1* mutants was that it was incomplete in all the marker

Table 1. Hox Proteins Enhanced the Initial Activation of *mec-3* Expression

Genotype	Stage[1]	ALM %	ALM n	PLM %	PLM n
Reporter: *mec-3p::RFP*					
Wild-Type	L1	100	80	100	80
	L4	100	60	100	70
mec-3(e1338)	L1	72	78	79	78
	L4	0	60	0	62
ceh-13(ok737) M+	L1	67	68	99	68
ceh-20(u843)	L1	59	70	100	70
	L4	57	60	100	56
egl-5(u202)	L1	100	58	62	78
	L4	100	62	58	74
Reporter: *mec-3p(mutHP1)::RFP*					
Wild-Type	L1	65	78	70	84
	L4	64	66	68	60
mec-3(e1338)	L1	46	82	53	78
	L4	0	64	0	56

[1]L1 larvae were examined within 30 min of hatching. n = number of cells examined.

strains we examined. The incomplete penetrance in *ceh-13* mutants, which arrested at early larval stages (Brunschwig et al., 1999), did not result from maternal rescue given that *ceh-13* mRNAs were not detectable in early embryos with single-molecule FISH (smFISH; Figure S1B).

Three-amino acid loop extension (TALE) cofactors are homeodomain transcription factors that interact with Hox proteins to enhance their DNA binding specificity (Mann et al., 2009). We found that mutations of the TALE cofactor *ceh-20/Exd/Pbx* also eliminated TRN marker expression in some, but not all, ALM neurons, resulting in a phenotype similar to that of *ceh-13* mutants; PLM neurons were not affected (Figures 1B and 1C). The penetrance of *mec-3p::RFP* and *mec-17p::RFP* expression was similar in ALM neurons in *ceh-20* mutants (compare Figure 1C to Table 1).

Next, we asked whether the loss of TRN marker expression resulted from changes in the lineage, which prevented the generation of the cell, or by the failure of cells to adopt a TRN cell fate. Given that *unc-86* expression begins in the TRN precursors and is maintained throughout the differentiation of these neurons (Finney and Ruvkun, 1990), we used a nuclear-localized *unc-86::EGFP* translational fusion to monitor the presence of the cells that were supposed to become TRNs. We found that *unc-86* expression was maintained in the undifferentiated (non-*mec-17*-expressing) cells in *ceh-13* and *ceh-20* mutants (Figures S1C and S1D), suggesting that Hox gene activity promoted the cell-fate decision and not the generation of the cell. This result is consistent with previous studies that did not detect lineage changes in *ceh-13* embryos (Brunschwig et al., 1999). Similarly, all 20 *egl-5* animals, which failed to express *mec-17p::RFP* in the PLM neurons, had the same number (ten) of *unc-86*-expressing cells at the tail as wild-type (Figure S1E).

In contrast, none of the 40 *nob-1* mutants that lacked *mec-17* expression in the PLM had more than seven *unc-86*-expressing cells in the tail (Figure S2F, top). About 20% of those animals had severely deformed tails that only contained three *unc-86*-expressing neurons (Figure S2F, bottom). These results suggest that, unlike other Hox genes, *nob-1* is required for the generation, and not the differentiation, of the posterior TRN subtype PLM. Consistent with this hypothesis, *egl-5 nob-1* double mutants were not more defective in TRN marker expression than expected from the sum of the phenotypes of the two single mutants (Figure 1C). In addition, *egl-5 php-3* double mutants had very similar penetrance for the TRN marker loss as *egl-5* single mutants (Figure 1C). Thus, neither *nob-1* nor *php-3* acts redundantly with *egl-5* to determine PLM cell fate.

Functionally, 25% of the *ceh-20(u843)* mutants were completely touch-insensitive at the head, and 63% of the *nob-1* mutants were insensitive at the tail (Figure 1D); in both cases, the penetrance was similar to that of the number of animals that completely lack differentiated ALM and PLM neurons, respectively (Figure 1C). In fact, two distinct populations were seen in *ceh-20* and *nob-1* mutants. Animals in the sensitive group responded at least four times out of five stimuli, whereas the insensitive group did not respond (Figures S1G and S1H). Moreover, we confirmed that all of and only the touch-insensitive animals had no *mec-17p::RFP* expression in any ALM or PLM neurons, suggesting that the remaining differentiated TRN cells in *ceh-20* and *nob-1* animals were functional. In contrast, nearly 100% *egl-5* animals were touch insensitive at the tail, even though more than 75% of the mutants expressed *mec-17* in at least one PLM. This result indicates that *egl-5* is needed to enable PLM differentiation as both a TRN and PLM function.

Hox Genes or their Cofactors Are Differentially Expressed in ALM and PLM Neurons

Next, we examined the expression patterns of the Hox genes and their cofactors using translational GFP fusions. The most anterior Hox gene *ceh-13* was expressed in both ALM and PLM neurons, although the expression in PLM was much weaker (Figure 2A). This observation is consistent with previous findings that, although *ceh-13* is homologous to the *Drosophila* anterior Hox gene *labial*, its expression and function is found all along the A-P axis (Tihanyi et al., 2010). The fact that mutations in *ceh-13* only affected the ALM differentiation, but not PLM, could result from the selective expression of the Hox cofactor *ceh-20* in ALM, but not PLM, neurons (Figure 2B). The Meis-class TALE cofactor *unc-62* was also expressed in ALM, but not PLM, neurons and contributed to the differentiation of the anterior TRNs (Figures S2C–S2E). An additional indication that *ceh-13* was not needed in the PLM neurons came from the finding that there were no additional defects in *ceh-13 egl-5* double mutants than *egl-5* animals (Figures S2A and S2B). Therefore, *ceh-13* only affects TRN fate in the ALM neurons.

Unlike the anterior Hox gene *ceh-13*, the posterior Hox genes *egl-5*, *php-3*, and *nob-1* were expressed in PLM but not ALM (Figures 2C and 2D). The middle-body Hox genes *lin-39* and *mab-5* were not detectably expressed in either ALM or PLM and were not derepressed in *egl-5* PLM neurons (data not shown). Therefore, although both ALM and PLM neurons share

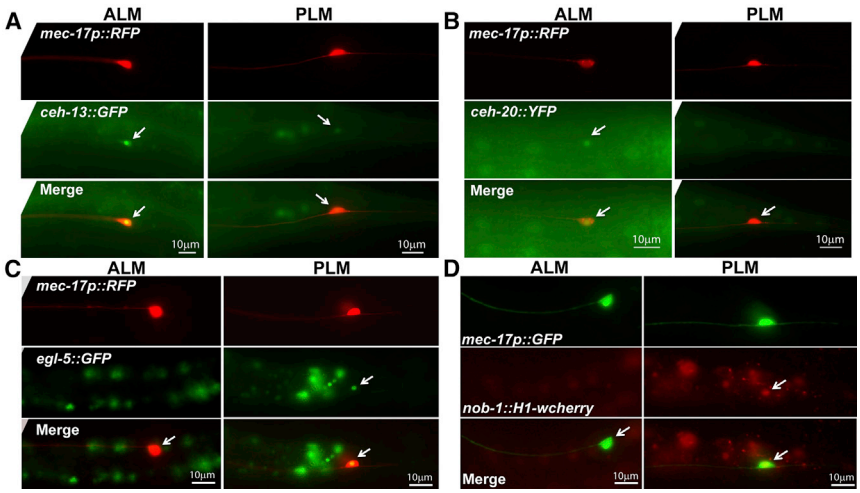


Figure 2. Expression Pattern of Hox Genes and Their Cofactors in ALM and PLM Neurons

(A–D) The expression of translational fusion *uIs221 [ceh-13::GFP]*, *mxIs28[ceh-20p::ceh-20::YFP]*, *uIs116 [egl-5p::egl-5::GFP.]*, and *stIs10808 [nob-1::H1-Wcherry]* in TRNs. *uIs115[mec-17p::RFP]* or *uIs31 [mec-17p::GFP]* were crossed into those reporter strains in order to label the TRN cell bodies. Scale bars represent 10 μm. See also Figure S2.

the same TRN fate and express the same genes associated with that fate, their genetic programs differ by the region-specific expression of Hox and/or TALE cofactor genes.

A Hox/Pbx Binding Site in the *mec-3* Promoter Is Important for ALM and PLM Cell Fate

The TRN cell fate is determined by the terminal selector gene *mec-3*, which activates a battery of genes (e.g., *mec-4*, *mec-7*, and *mec-17*) responsible for various TRN features (Chalfie and Au, 1989; Duggan et al., 1998; Way and Chalfie, 1989; Zhang et al., 2002). The maintenance of *mec-3* expression requires the binding of UNC-86/MEC-3 heteromer to at least two *cis*-regulatory sites in the *mec-3* proximal promoter (Xue et al., 1992, 1993). The fact that Hox genes and Hox cofactors contributed to the activation of *mec-3* suggests that Hox transcription factors may also regulate *mec-3* expression directly. A short (392 bp) promoter upstream of the start codon of *mec-3* produced a normal expression pattern in the six TRNs (*mec-3p392::RFP* in Figure 3A). We crossed this short reporter into Hox mutants and found 38% (n = 42) of ALM neurons in *ceh-13(ok737)* animals and 42% (n = 62) of PLM in *egl-5(u202)* animals failed to express *mec-3p392::RFP*. These results are similar to the observations using the regular reporter, suggesting that the 392 bp *mec-3* proximal promoter contains the DNA elements sufficient for the Hox-mediated regulation.

Consistent with a role for Hox control of *mec-3* expression, we found two conserved Hox/Pbx (HP) binding sites near the UNC-86/MEC-3 binding sites included in *mec-3p392* (Figure 3A). We tested the importance of these sites in the context of the regular full length *mec-3p::RFP* reporter, which contains a 1.9 kb promoter upstream of ATG. Mutation of the HP1 site resulted in the loss of marker expression in ALM and PLM but not the postembryonic AVM and PVM neurons, whereas mutation of the HP2 site had no effect (Figures 3B and 3C). The mutation of TGAT to GACG at position −150 to −153 on the antisense strand in HP1 resulted in normal RFP expression in only 63% (n = 82) of ALM and 68% (n = 80) of PLM neurons (Figure 3A). The rest of the cells had no or significantly diminished RFP expression. This phenotype was slightly different from that seen in the Hox mutants, which have either strong or no RFP expression in the TRNs. We hypothesize that the endogenous *mec-3* expression might drive the residual expression of *mec-3p(mutHP1)::RFP* through autoregulation, but continuous full activation of the *mec-3* promoter requires the HP1 site. Mutation of both HP1 and HP2 resulted in penetrance similar to the mutation of HP1 alone. Moreover, *mec-3p(mutHP1)::RFP* showed similar loss of expression in wild-type and Hox mutant animals (60% [n = 40] of *ceh-13* ALM and 62% [n = 50] of *egl-5* PLM express the RFP]). These results suggest that HP1 is the main *cis*-regulatory element for the modulation of *mec-3* expression by Hox genes.

The initiation of *mec-3* transcription requires the POU homeodomain transcription factor UNC-86, whereas the maintenance of *mec-3* expression requires both UNC-86 and MEC-3 (Xue et al., 1992). As a result, *mec-3p::RFP* expression was visible in about 75% of the newly hatched *mec-3* larvae, but the expression disappeared as the mutant animals matured (Table 1). At the fourth larval stage, virtually no *mec-3* mutants expressed the marker detectably. Within 1 hr after hatching, 33% of *ceh-13* ALM, 41% of *ceh-20* ALM, and 38% of *egl-5* PLM neurons showed no *mec-3* expression; and the percentage of undifferentiated TRNs stayed the same throughout development (Table 1). Moreover, mutation of the HP1 site significantly reduced the number of TRNs expressing the reporter at hatching in both wild-type and *mec-3* backgrounds. Therefore, Hox proteins facilitate the initial activation of *mec-3*. The finding that the HP1 site mutant was still defective in wild-type cells, which should express sufficient UNC-86, MEC-3, and ALR-1 to enable autoregulation, suggests that either Hox proteins are involved in *mec-3* maintenance or they prime the promoter so that autoregulation can occur.

Although the HP1 site partially overlaps with the core of UNC-86/MEC-3 binding site (UM2), the mutation we generated only disrupted HP1 and kept the key component of UM2 intact, as seen by the fact that the HP1 mutation had no effect on AVM and PVM differentiation (Figures 3A and 3B). This result is also consistent with the finding that no mutation in any Hox gene or combination of Hox genes could affect TRN marker expression in these two TRNs, indicating that substantial Hox-mediated regulation of cell fate does not occur in these cells.

The Pbx-class Hox cofactor CEH-20, which was expressed in ALM but not PLM, presumably facilitates the function of CEH-13 through the HP1 site in *mec-3*. However, we were not able to

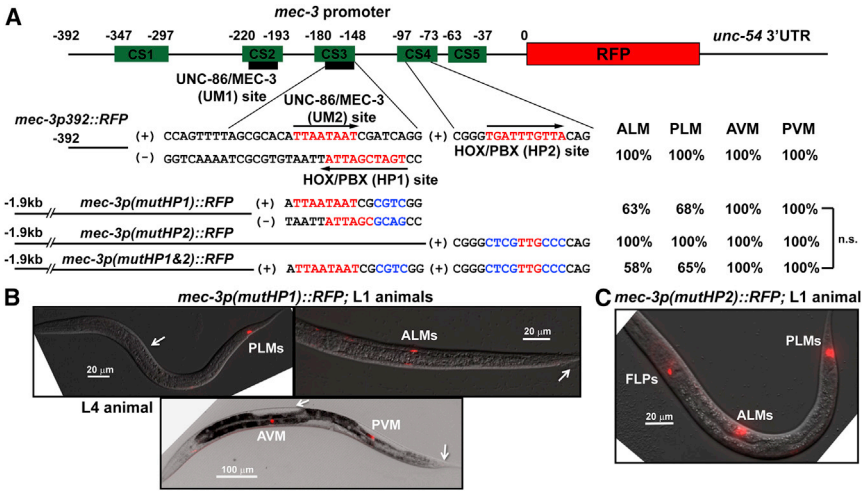


Figure 3. Hox Proteins Facilitate the Initiation of *mec-3* Expression through a Hox/Pbx Binding Site Adjacent to an Essential UNC-86/MEC-3 Binding Site

(A) Effect of mutations in HP1 and HP2 in the *mec-3* proximal promoter. Green blocks in representation of the *mec-3* promoter denote conserved sequence (CS) among nematodes and black blocks denote the two essential UNC-86/MEC-3 binding sites. Sequences of part of CS3 and CS4 are shown, and the two predicted HP binding sites are labeled (the consensus sequence used for prediction is 5′-TGATNNAT[G/T][G/A]-3′). The changes of nucleotide sequences in mutated promoters are shown in blue. The percentages indicate how many of the ALM and PLM neurons express RFP at a wild-type level in the *mec-3* promoter variants.

(B) Variable lack of RFP label from ALM and PLM neurons (top), but not AVM and PVM neurons (bottom) in animals expressing RFP from a *mec-3* promoter with the HP1 site mutated.

(C) Normal ALM and PLM expression in animals carrying *mec-3p::RFP* with the HP2 site mutated.

identify a counterpart of CEH-20 in the PLM for EGL-5. Mutation of the two other known Pbx genes (*ceh-40* and *ceh-60*) alone or together did not change the expression pattern of TRN markers in PLM neurons (data not shown). The only MEIS class cofactor (*unc-62/Hth*) is also not expressed in the PLM neurons, suggesting that EGL-5 may not require a TALE cofactor. Work in *Drosophila* and vertebrates has also suggested that Abd-B-like proteins, like EGL-5, can function independently of TALE cofactors (Rivas et al., 2013; Shen et al., 1997; van Dijk and Murre, 1994). Nevertheless, we cannot rule out the possibility that EGL-5 acts with some unidentified Pbx-like factors in the PLM cells because the HP1 mutation mainly disrupted the Pbx half of the Hox/Pbx bipartite binding site but still affected EGL-5 activity.

Hox Genes Regulate *mec-3* Expression and TRN Fate in a Binary Fashion

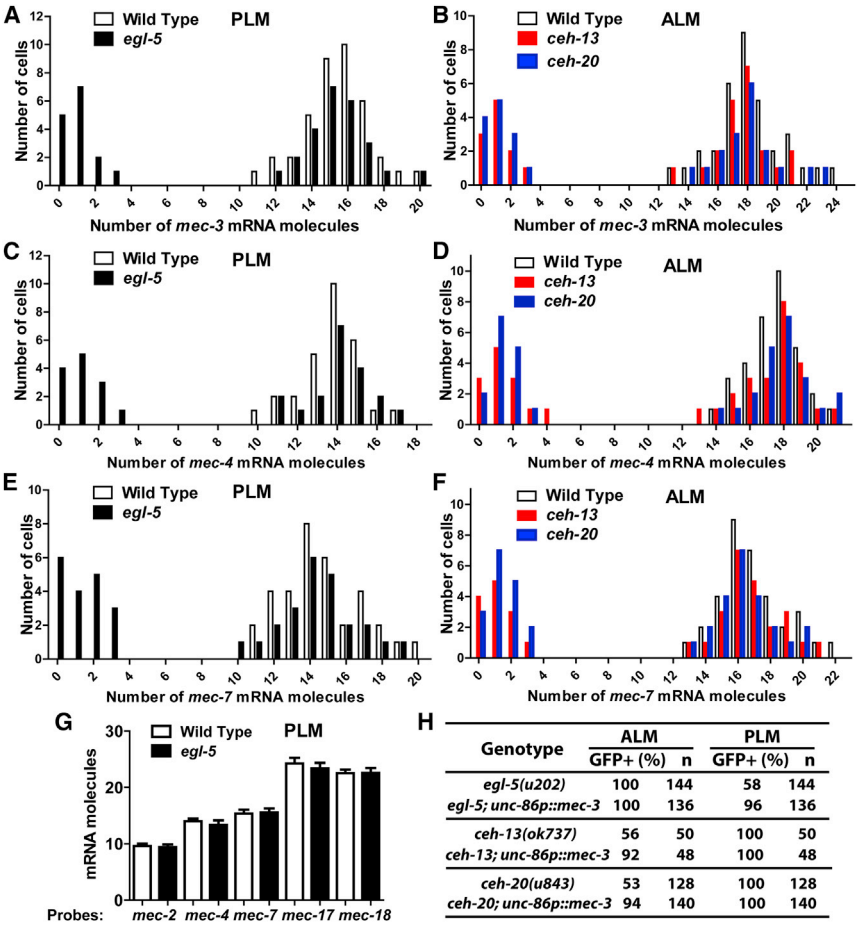
Hox genes appeared to affect TRN cell fate in a binary manner; ALM and PLM cells in Hox mutants expressed TRN markers either at wild-type levels or not at all. This binary phenotype is seen in and may be caused by the expression of endogenous *mec-3* mRNA as measured by smFISH (Raj et al., 2008; Topalidou et al., 2011). For example, the distribution of *mec*-3 mRNA in wild-type PLM neurons had a single peak centered around 16 fluorescent mRNA molecules, but the distribution in *egl-5* mutants had two peaks: 62% (25/40) of the cells had normal levels of *mec-3* mRNA and 38% (15/40) had no more than three labeled molecules (Figure 4A). These percentages are consistent with the penetrance of missing TRN marker expression in *egl-5* mutants (shown in Figure 1), suggesting that Hox proteins affect endogenous *mec-3* transcription. Similar results were obtained for ALM neurons in *ceh-13* and *ceh-20* mutants (Figure 4B). In addition, we were able to confirm these results using probes against the terminal TRN fate markers *mec-4* and *mec-7* (Figures 4C–4G).

These data suggest that a threshold level of *mec-3* mRNA is needed for TRN differentiation and/or to maintain a proper level of *mec-3* expression. If the initial induction of *mec-3* expression exceeds the threshold, then it can be sustained through autoregulation mediated by the UNC-86/MEC-3 heteromer (Xue et al., 1993) and ALR-1 (Topalidou et al., 2011). However, if the initial activation fails to reach the threshold, then *mec-3* expression cannot be maintained and would diminish over time. Hox proteins may help bring *mec-3* expression above the threshold during induction by binding to the HP1 site adjacent to an essential UNC-86 binding site. The finding that *mec-3* mRNA molecules in Hox mutants fit a bimodal distribution with either complete or no expression (Figure S3) supports the hypothesis that Hox proteins enable the cells to express sufficient *mec-3* for TRN differentiation.

If Hox proteins promote the terminal TRN fate by boosting the expression of *mec-3*, then overexpressing *mec-3* from the *unc-86* promoter, which is not regulated by Hox proteins, should restore the loss of TRN characteristics in Hox mutants. As expected, an *unc-86p::mec-3* transgene caused 96% of PLM in *egl-5* mutants, 92% of ALM in *ceh-13* mutants and 94% of ALM in *ceh-20* animals to adopt the TRN fate (Figure 4H). We also noticed that wild-type animals carrying *unc-86p::mec-3* expressed the TRN marker in several additional neurons, including at least FLP, PVD, BDU (the ALM sister cells), and ALN neurons (the PLM sister cells; data not shown). These results suggest that the level of *mec-3* expression critically determines TRN fate, at least in cells closely related to the TRNs.

Hox Genes Promote TRN Fate in the FLP and PVD Neurons

To test the function of Hox genes in ectopically promoting TRN fate in non-TRN cells, we expressed Hox genes in the FLP and PVD neurons using the *mec-3* promoter. FLP and PVD moderately express *mec-3* but do not adopt the TRN fate. Smith et al. (2013) found that the low level of *mec-3* in PVD specifies its elaborate branching pattern, whereas a high level of *mec-3* is correlated with the simple morphology in the TRNs, suggesting that the cell-fate decision between PVD and TRN depends on the dose of MEC-3. In fact, overexpression of *mec-3* from its own promoter (Topalidou and Chalfie, 2011) or from the *unc-86* promoter transforms FLP and PVD into TRN-like cells, which



H

Genotype	ALM GFP+ (%)	ALM n	PLM GFP+ (%)	PLM n
egl-5(u202)	100	144	58	144
egl-5; unc-86p::mec-3	100	136	96	136
ceh-13(ok737)	56	50	100	50
ceh-13; unc-86p::mec-3	92	48	100	48
ceh-20(u843)	53	128	100	128
ceh-20; unc-86p::mec-3	94	140	100	140

Figure 4. Binary Effect of *Hox* Mutations on TRN Cell Fate in a Binary Manner

(A–F) The number of fluorescently labeled *mec-3*, *mec-4*, and *mec-7* transcripts in ALM and PLM neurons from wild-type, *egl-5*, *ceh-13*, and *ceh-20* animals using smFISH. Animals also expressed *mec-17p::RFP* and *unc-86::GFP* in order to identify the TRN cell bodies.

(G) Average number of fluorescently labeled *mec-2*, *mec-4*, *mec-7*, *mec-17*, and *mec-18* transcripts in wild-type PLM neurons and the group of *egl-5*-deficent PLM neurons that expressed the TRN marker *mec-17p::GFP*.

(H) Percentages of ALM and PLM neurons that expressed the TRN marker *mec-17p::GFP* in *egl-5* and *ceh-20* mutants carrying the *unc-86p::mec-3* transgene.

See also Figure S3.

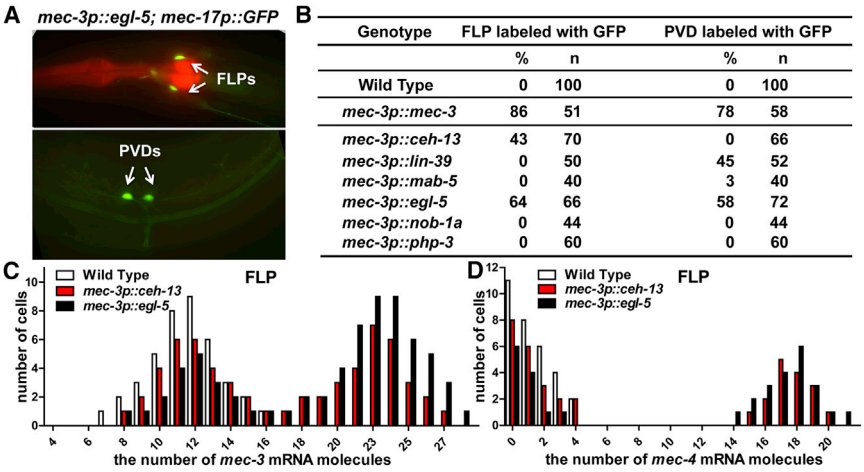
suggests that increasing *mec-3* expression forces those neurons to become TRNs. Overexpression of *egl-5*, but not *nob-1* or *php-3*, induced expression of TRN-specific genes in FLP and PVD (Figures 5A and 5B). In contrast, overexpression of the anterior Hox gene *ceh-13* induced TRN genes in only the FLP neurons, which are located in the head, and misexpression of *lin-39* induced TRN genes in only the PVD neurons, which are positioned just posterior to the middle of the animal. The inability of Hox proteins to induce ectopic TRN fate could not be explained by the absence of Hox cofactors because both *ceh-20*/*Pbx* and *unc-62*/*Meis* are expressed in both FLP and PVD neurons (Figures S4A and S4B). Thus, other constraints may limit the function of those Hox genes. Besides *mec-17p::GFP*, expression of other TRN markers, such as *mec-4p::GFP* and *mec-18p::GFP*, was also observed in the FLP and PVD neurons that had been converted to TRN-like cells (data not shown).

Given that FLP and PVD neurons overexpressing Hox genes showed either strong or no TRN marker expression, these results support the hypothesis that Hox proteins potentiate *mec-3* expression in a binary fashion. smFISH revealed that misexpression of *egl-5* and *ceh-13* increased the *mec-3* transcript level above 16 molecules in only about 65% and 45% of the FLP cells, respectively (Figure 5C). The rest of the cells expressed *mec-3* within the normal range. Moreover, we found that the FLP neurons express the TRN markers *mec-17p::GFP* only when the number of *mec-3* mRNA molecules reached at least 20 (Figure S4C). Two distinct populations of FLP neurons were also seen using smFISH for *mec-4* as a measure of TRN differentiation (Figure 5D).

Hox Proteins Enhance the Activation of *mec-3* Promoter by UNC-86

We used a yeast transcription system (Topalidou et al., 2011) to demonstrate that Hox proteins enhanced UNC-86 activation of *mec-3* expression, thus mimicking the initial phase of *mec-3* expression in the TRNs (Figure 6A). In this system, the transcriptional activation of a single copy of *mec-3p::lacZ* inserted into the yeast genome is measured by the level of β-galactosidase activity. EGL-5 increased the level of *mec-3* activation by UNC-86 by 1.5 fold. CEH-13 by itself did not enhance the activation of *mec-3* promoter by UNC-86. However, co-expression of CEH-20 with CEH-13 significantly increased *mec-3p::lacZ* expression, suggesting that CEH-13 needs CEH-20 for its activity at this promoter. In contrast, CEH-20 did not increase the ability of EGL-5 to enhance *mec-3* expression, a result consistent with the observation that CEH-20 is not expressed in PLM. Furthermore, activation by EGL-5 alone further supports the hypothesis that it functions independently of Hox cofactors to promote TRN fate in the PLM neurons.

This enhancement of *mec-3* transcription depended on the presence of UNC-86. Without UNC-86, neither EGL-5 nor CEH-13/CEH-20 was able to activate the *mec-3* promoter (Figure 6A). Mutation of HP1 site (Figure 2) in the *mec-3* promoter also eliminated EGL-5 and CEH-13/CEH-20 enhancement of *mec-3* transcription but did not affect the activity of UNC-86 (Figure 6A). These results support the model that Hox proteins directly bind to this *cis*-regulatory element in the *mec-3* promoter to facilitate UNC-86-mediated *mec-3* activation.



Genotype	FLP labeled with GFP		PVD labeled with GFP	
	%	n	%	n
Wild Type	0	100	0	100
mec-3p::mec-3	86	51	78	58
mec-3p::ceh-13	43	70	0	66
mec-3p::lin-39	0	50	45	52
mec-3p::mab-5	0	40	3	40
mec-3p::egl-5	64	66	58	72
mec-3p::nob-1a	0	44	0	44
mec-3p::php-3	0	60	0	60

Figure 5. Misexpression of Hox Genes Causes FLP and PVD Neurons to Adopt a TRN-like Cell Fate

(A) FLP and PVD neurons express the terminal TRN fate marker *mec-17p::GFP* upon overexpression of *egl-5* from the *mec-3* promoter.

(B) The percentages of FLP and PVD neurons labeled by *mec-17p::GFP* when various Hox genes were misexpressed from the *mec-3* promoter.

(C and D) *mec-3* (C) and *mec-4* (D) transcripts in FLP neurons in wild-type animals and animals carrying transgenes *mec-3p::egl-5* or *mec-3p::ceh-13*.

See also Figure S4.

In comparison to Hox proteins, MEC-3 was a much stronger co-activator of UNC-86 at the *mec-3* promoter. Co-expression of MEC-3 with UNC-86 in yeast could fully activate the *mec-3p::lacZ* reporter; and adding the Hox proteins either with or without the TALE cofactor did not further enhance *mec-3* activation (Figure 6B). These results support the hypothesis that Hox proteins are not essential for the maintenance of *mec-3* expression. Once Hox proteins help UNC-86 initiate *mec-3* transcription, auto-regulation (with the assistance of ALR-1) is probably sufficient to maintain high levels of *mec-3* expression in the absence of Hox proteins.

DISCUSSION

The six TRNs in *C. elegans* offer a simplified system to study the mechanisms by which conserved Hox genes regulate neuronal differentiation at the level of the single cell. Using these cells, we found that Hox proteins facilitate the adoption of cell fate by increasing the probabilities of transcriptional activation of terminal selectors. Specifically, Hox proteins and cofactors directly act through a Hox/Pbx binding site in the *mec-3* proximal promoter to regulate transcription. As the cells are generated, UNC-86 is recruited to the *mec-3* promoter. UNC-86, which is made in the precursors of the TRNs, and thus before MEC-3 (Finney and Ruvkun, 1990; Way and Chalfie, 1989), is a poor activator of *mec-3* by itself (Xue et al., 1993). Without Hox facilitation, UNC-86 fails to trigger *mec-3* expression in about 40% of the potential TRNs. The binding of Hox proteins to the Hox/Pbx site adjacent to the UNC-86 binding site ensures that *mec-3* transcription always occurs (Figure 6C). Thus, the reliable and robust activation of the *mec-3* promoter requires the Hox proteins.

Hox Proteins Ensure, but Do Not Determine, Neuronal Cell Fate

A striking feature of Hox regulation of TRN cell fate is that Hox proteins are not absolutely required for the TRN fate; 60% of ALM and PLM neurons express TRN fate marker at wild-type levels in the absence of Hox genes. Quantitative measurements using smFISH revealed two groups of cells in Hox mutants: one with normal *mec-3* transcript levels that adopted the TRN fate, and another with very low or no *mec-3* expression that did not. These results suggest that Hox proteins serve as facilitators instead of determinants of cell fate. Consistent with this facilitator role, we found that neither the Hox genes nor the HP1 binding site was needed for TRN identity in the postembryonic TRNs (the AVM and PVM neurons).

Previous studies of the role of Hox genes in neuronal differentiation seemed to indicate that Hox proteins determined cell fate; i.e., they acted as terminal selector genes as seen by the loss of cell-specific markers (Dasen et al., 2005; Lacombe et al., 2013). Confounding this interpretation is the large amount of programmed cell death and cell loss that occurs in *Drosophila* and mammalian Hox mutants (Baek et al., 2013; Wu et al., 2008). Indeed, Philippidou et al. (2012) found that about 80% of surviving mouse spinal cord neurons did express the fate marker *Scip* if apoptosis was blocked in *Hox5* mutants. Thus, Hox proteins in general may not specify cell fate as much as facilitate its acquisition.

Hox Proteins Act as Transcriptional Guarantors

To distinguish this facilitation from the action of selectors, we propose describing Hox proteins and similar proteins as "guarantors." Thus, in the TRNs, UNC-86 and MEC-3 serve as the terminal selectors that specify the TRN fate by directly activating downstream terminal differentiation genes, whereas Hox proteins act as guarantors to secure TRN fate by increasing the chance of successful transcriptional activation of *mec-3*. Null mutations in the selectors lead to a complete loss of TRN fate (Chalfie and Au, 1989), whereas mutations in the guarantors only cause a failure of fate commitment in a proportion of the cells.

Previous work from our laboratory (Topalidou et al., 2011) has identified another transcription factor, the homedomain protein ALR-1/Aristaless, that appears to act as a guarantor as well. The maintained expression of *mec-3* involves autoregulation, but MEC-3/UNC-86 autoregulation is inefficient in the absence of ALR-1; *mec-3* is much more variably expressed in *alr-1* mutants. The presence of ALR-1 restricts the variability of *mec-3* expression to the high end of its range through transcriptional refinement. As with the Hox Proteins, ALR-1 increased *mec-3* expression in our yeast transcription system when UNC-86

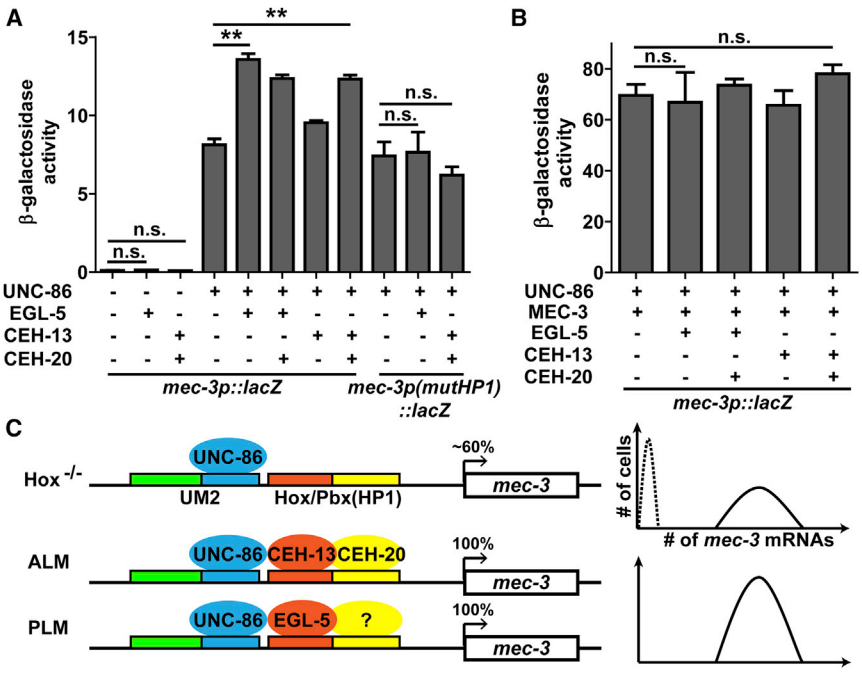


Figure 6. Hox Proteins Increase *mec-3* Expression in a Yeast Transcription System

(A and B) β-galactosidase activities were measured in yeast strains that carry *mec-3p::LacZ* in the genome and express the indicated proteins in the absence (A) or presence (B) of MEC-3.

(C) A model for the guarantor function of Hox proteins during the initial activation of *mec-3*. In the absence of Hox proteins, UNC-86 alone fails to activate *mec-3* in about 40% of embryonic TRNs. CEH-13/Lab with Pbx-class cofactor CEH-20 in ALM neurons and EGL-5/Abd-B with an unidentified cofactor in PLM neurons bind to the HP1 site adjacent to the UNC-86 binding site. Hox proteins ensure that UNC-86 fully activates *mec-3* in all TRNs. The newly expressed MEC-3 is recruited to the MEC-3 binding site (green) in order to help UNC-86 maintain *mec-3* expression.

Error bars represent SEM., and double asterisks indicate $p < 0.01$. n.s., no significant difference.

and MEC-3 were present but not in their absence (Topalidou et al., 2011). In addition, a potential ALR-1 binding site is found near the UNC-86/MEC-3 binding site in the *mec*-3 promoter. We propose that transcriptional guarantors, such as Hox proteins and ALR-1, serve as insurance mechanisms to guarantee the success of cell-fate differentiation.

The Guarantor Function of Hox Proteins Enables Cell Fate Convergence

Different Hox proteins contribute to the acquisition of the same cell fate in distinct subtypes: the anterior Hox protein CEH-13 and its cofactor CEH-20 promote the TRN fate in ALM neurons, whereas the posterior Hox protein EGL-5 contributes to the cell-fate commitment in PLM neurons (Figure 6C). Thus, although a specific cell fate is shared by multiple neurons, the mechanisms of acquiring that fate differ among the subtypes. Because Hox proteins share significant homology in their homeodomains and bind to similar DNA sequences (Gehring et al., 1994), different region-specific Hox proteins are excellent candidates for ensuring robust activation of the same terminal selector in different subtypes. For instance, both Ubx and abd-A in *Drosophila* promote the specialization of ventral-abdominal (Va) neurons by activating the neuropeptidergic terminal selector gene *dim* and its cofactor *dac* (Suska et al., 2011). Similar regulation by the Hox5-Hox8 proteins appears to determine the fate of mouse spinal motor neurons (Lacombe et al., 2013). Moreover, multiple Hox proteins can ectopically induce the same neuronal identity, although they do so with varying efficiencies. For example, misexpression of several Hox gene converted FLP and PVD neurons to TRN-like cells with different efficiencies (Figure 5), and several, but not all, *Hox4-8* paralogs in mice could ectopically induce brachial lateral MN identity (Lacombe et al., 2013). These results support the idea that Hox proteins can promote the convergence to the common cell fate in disparate neurons.

In another study (Zheng et al., 2015), we found that the same Hox proteins also induce variations among the TRNs. Together, that work and this one establish a dual function for Hox proteins in promoting both cell-fate convergence and subtype diversification. A recent study by Crocker et al. (2015) suggests a mechanism for both of these functions. They described a correlation between affinity and specificity of Hox binding sites: conserved high affinity sites bound many different Hox proteins, whereas clusters of low affinity, non-conserved sites bound specific Hox proteins. Here, we have described a single well-conserved Hox/Pbx binding site that mediates the guarantor functions of both CEH-13 and EGL-5. One attractive hypothesis is that high affinity sites allow for cell-fate convergence, whereas clusters of low-affinity sites are needed for the regional specification of neuronal subtypes.

Guarantors Enhance Transcriptional Efficiency Non-redundantly

How could the Hox proteins (and ALR-1) carry out their guarantor function? One possibility is that, by binding to the *mec-3* promoter, they could affect the binding of UNC-86/MEC-3 or its intrinsic transactivation efficiency. Another possibility is that guarantors recruit other proteins that poise promoters to be activated. For example, the binding partner of Hox proteins, TALE cofactors, recruit histone-modifying enzymes in zebrafish in order to promote an active chromatin state and also recruit RNA polymerase II and P-TEFb in order to poise the promoter of target genes for activation (Choe et al., 2014). Efficient transcription is then triggered upon the binding of Hoxb1b to the TALE cofactors. A similar function may explain how CEH-13 facilitates *mec-3* activation. Because EGL-5 most likely functions independently of known TALE cofactors, other proteins may carry out this poising function. One possible candidate is EGL-5 itself, given that Hoxa10, an EGL-5 homolog, directly mediates chromatin hyperacetylation and histone H3K4 trimethylation of its

target genes during bone formation (Hassan et al., 2007). Therefore, Hox proteins may modulate the robustness of transcription through chromatin remodeling and interaction with RNA polymerase II.

Regardless of the mechanism that guarantors ensure differentiation, a striking feature of their action is that they reduce expression variability. Previous studies suggest that a general method whereby cells cope with stochastic variability is redundancy of enhancers, elements within enhancers, or transcription factors that bind to them (reviewed by Lagha et al., 2012). However, guarantors provide an additional mechanism for reducing variability and stabilizing gene expression. Unlike the methods involving redundant components, which are not easily modifiable, guarantors act in a non-redundant fashion and therefore can be modified to enable regulatory processes that shift cells to alternative fates. Moreover, changes in guarantor function may lead to evolutionary diversity.

The Ability to Influence Transcriptional Efficiency of Diverse Genes May Explain the Diversity of Hox Regulation

Hox genes regulate many developmental processes, in addition to neuronal differentiation, along the A-P axis (Pearson et al., 2005). Surprisingly, given the importance of Hox proteins, very few verified Hox targets are known (Pearson et al., 2005). This lack of targets may be explained by the Hox protein acting as guarantors. Unlike selectors, which should cause a qualitative change in gene expression (different genes activated), guarantors would be expected to cause quantitative differences. As in our case, the adjacency of Hox/Pbx binding site may identify other transcription factors that work with the Hox proteins. Because Hox proteins can interacts with many transcription factors (e.g., abd-A binds to 35; Baëza et al., 2015), they may facilitate the activation of target genes by a wide range of transcription factors.

EXPERIMENTAL PROCEDURES

C. elegans wild-type (N2) and mutant strains were maintained as previously described (Brenner, 1974). Most strains were provided by the Caenorhabditis Genetics Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). *ceh-13(ok737) egl-5(u1034)* and *ceh-13(ok737) egl-5(u1035)* doubles were made by creating *egl-5* mutations in *ceh-13*/+ heterozygotes animals using the CRISPR/cas9-mediated genome-editing method (Figure S2A; Dickinson et al., 2013). Constructs were made using the Gateway cloning method by Life Technologies, and *mec-3* promoter mutations were made using site-directed mutagenesis kit (New England Biolabs). Transgenes and fluorescent reporters used in the study can be found in the Supplemental Experimental Procedures.

The expression patterns of TRN markers in Hox mutants were examined at 15°C. smFISH was performed as described previously (Topalidou et al., 2011). Yeast transcription assay was performed using a modified method described previously (Topalidou et al., 2011). *mec-3p::LacZ* was integrated in the URA locus, and proteins expression was induced by 0.05% galactose for 8 hr. Yeast β-Galactosidase Assay Kit (Thermo Scientific) was used to measure LacZ expression. Statistical significance was determined with a Student's t test for comparisons between two sets of data, and the Holm-Bonferroni method was used to correct the p values for multiple comparisons. Single and double asterisks indicate $p < 0.05$ and $p < 0.01$, respectively. Details are presented in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.044.

ACKNOWLEDGMENTS

We thank Bill Wood, Scott Emmons, Tibor Vellai, and Dan Dickinson for sharing materials and reagents. We also thank Oliver Hobert, Richard Mann, Robb Krumlauf, and the members of our laboratory for stimulating discussions and comments. This work was supported by NIH grant GM30997 to M.C.

Received: August 5, 2015
Revised: September 29, 2015
Accepted: October 19, 2015
Published: November 4, 2015

REFERENCES

Baek, M., Enriquez, J., and Mann, R.S. (2013). Dual role for Hox genes and Hox co-factors in conferring leg motoneuron survival and identity in Drosophila. Development *140*, 2027–2038.

Baëza, M., Viala, S., Heim, M., Dard, A., Hudry, B., Duffraisse, M., Rogulja-Ortmann, A., Brun, C., and Merabet, S. (2015). Inhibitory activities of short linear motifs underlie Hox interactome specificity in vivo. eLife *4*, 4.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71–94.

Brunschwig, K., Wittmann, C., Schnabel, R., Bürglin, T.R., Tobler, H., and Müller, F. (1999). Anterior organization of the Caenorhabditis elegans embryo by the labial-like Hox gene ceh-13. Development *126*, 1537–1546.

Chalfie, M., and Au, M. (1989). Genetic control of differentiation of the Caenorhabditis elegans touch receptor neurons. Science *243*, 1027–1033.

Chalfie, M., and Sulston, J. (1981). Developmental genetics of the mechanosensory neurons of Caenorhabditis elegans. Dev. Biol. *82*, 358–370.

Chalfie, M., Sulston, J.E., White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1985). The neural circuit for touch sensitivity in Caenorhabditis elegans. J. Neurosci. *5*, 956–964.

Choe, S.K., Ladam, F., and Sagerström, C.G. (2014). TALE factors poise promoters for activation by Hox proteins. Dev. Cell *28*, 203–211.

Chow, K.L., and Emmons, S.W. (1994). HOM-C/Hox genes and four interacting loci determine the morphogenetic properties of single cells in the nematode male tail. Development *120*, 2579–2592.

Clandinin, T.R., Katz, W.S., and Sternberg, P.W. (1997). Caenorhabditis elegans HOM-C genes regulate the response of vulval precursor cells to inductive signal. Dev. Biol. *182*, 150–161.

Crocker, J., Abe, N., Rinaldi, L., McGregor, A.P., Frankel, N., Wang, S., Alsawadi, A., Valenti, P., Plaza, S., Payre, F., et al. (2015). Low affinity binding site clusters confer hox specificity and regulatory robustness. Cell *160*, 191–203.

Dasen, J.S., Tice, B.C., Brenner-Morton, S., and Jessell, T.M. (2005). A Hox regulatory network establishes motor neuron pool identity and target-muscle connectivity. Cell *123*, 477–491.

Dasen, J.S., De Camilli, A., Wang, B., Tucker, P.W., and Jessell, T.M. (2008). Hox repertoires for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. Cell *134*, 304–316.

Davenne, M., Maconochie, M.K., Neun, R., Pattyn, A., Chambon, P., Krumlauf, R., and Rijli, F.M. (1999). Hoxa2 and Hoxb2 control dorsoventral patterns of neuronal development in the rostral hindbrain. Neuron *22*, 677–691.

Dickinson, D.J., Ward, J.D., Reiner, D.J., and Goldstein, B. (2013). Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination. Nat. Methods *10*, 1028–1034.

Duggan, A., Ma, C., and Chalfie, M. (1998). Regulation of touch receptor differentiation by the Caenorhabditis elegans mec-3 and unc-86 genes. Development *125*, 4107–4119.

Finney, M., and Ruvkun, G. (1990). The unc-86 gene product couples cell lineage and cell identity in C. elegans. Cell *63*, 895–905.

García-Bellido, A. (1975). Genetic control of wing disc development in Drosophila. Ciba Found. Symp. *0*, 161–182.

Gaufo, G.O., Thomas, K.R., and Capecchi, M.R. (2003). Hox3 genes coordinate mechanisms of genetic suppression and activation in the generation of branchial and somatic motoneurons. Development *130*, 5191–5201.

Gehring, W.J., Qian, Y.Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A.F., Resendez-Perez, D., Affolter, M., Otting, G., and Wüthrich, K. (1994). Homeodomain-DNA recognition. Cell *78*, 211–223.

Hassan, M.Q., Tare, R., Lee, S.H., Mandeville, M., Weiner, B., Montecino, M., van Wijnen, A.J., Stein, J.L., Stein, G.S., and Lian, J.B. (2007). HOXA10 controls osteoblastogenesis by directly activating bone regulatory and phenotypic genes. Mol. Cell. Biol. *27*, 3337–3352.

Hobert, O. (2008). Regulatory logic of neuronal diversity: terminal selector genes and selector motifs. Proc. Natl. Acad. Sci. USA *105*, 20067–20071.

Jung, H., Lacombe, J., Mazzoni, E.O., Liem, K.F., Jr., Grinstein, J., Mahony, S., Mukhopadhyay, D., Gifford, D.K., Young, R.A., Anderson, K.V., et al. (2010). Global control of motor neuron topography mediated by the repressive actions of a single hox gene. Neuron *67*, 781–796.

Lacombe, J., Hanley, O., Jung, H., Philippidou, P., Surmeli, G., Grinstein, J., and Dasen, J.S. (2013). Genetic and functional modularity of Hox activities in the specification of limb-innervating motor neurons. PLoS Genet. *9*, e1003184.

Lagha, M., Bothma, J.P., and Levine, M. (2012). Mechanisms of transcriptional precision in animal development. Trends Genet. *28*, 409–416.

Manley, N.R., and Capecchi, M.R. (1997). Hox group 3 paralogous genes act synergistically in the formation of somitic and neural crest-derived structures. Dev. Biol. *192*, 274–288.

Mann, R.S., Lelli, K.M., and Joshi, R. (2009). Hox specificity unique roles for cofactors and collaborators. Curr. Top. Dev. Biol. *88*, 63–101.

McGinnis, W., and Krumlauf, R. (1992). Homeobox genes and axial patterning. Cell *68*, 283–302.

Ozbudak, E.M., Thattai, M., Kurtser, I., Grossman, A.D., and van Oudenaarden, A. (2002). Regulation of noise in the expression of a single gene. Nat. Genet. *31*, 69–73.

Pattyn, A., Vallstedt, A., Dias, J.M., Samad, O.A., Krumlauf, R., Rijli, F.M., Brunet, J.F., and Ericson, J. (2003). Coordinated temporal and spatial control of motor neuron and serotonergic neuron generation from a common pool of CNS progenitors. Genes Dev. *17*, 729–737.

Pearson, J.C., Lemons, D., and McGinnis, W. (2005). Modulating Hox gene functions during animal body patterning. Nat. Rev. Genet. *6*, 893–904.

Philippidou, P., and Dasen, J.S. (2013). Hox genes: choreographers in neural development, architects of circuit organization. Neuron *80*, 12–34.

Philippidou, P., Walsh, C.M., Aubin, J., Jeannotte, L., and Dasen, J.S. (2012). Sustained Hox5 gene activity is required for respiratory motor neuron development. Nat. Neurosci. *15*, 1636–1644.

Raj, A., van den Bogaard, P., Rifkin, S.A., van Oudenaarden, A., and Tyagi, S. (2008). Imaging individual mRNA molecules using multiple singly labeled probes. Nat. Methods *5*, 877–879.

Raser, J.M., and O'Shea, E.K. (2004). Control of stochasticity in eukaryotic gene expression. Science *304*, 1811–1814.

Rivas, M.L., Espinosa-Vázquez, J.M., Sambrani, N., Greig, S., Merabet, S., Graba, Y., and Hombría, J.C. (2013). Antagonism versus cooperativity with TALE cofactors at the base of the functional diversification of Hox protein function. PLoS Genet. *9*, e1003252.

Rogulja-Ortmann, A., Renner, S., and Technau, G.M. (2008). Antagonistic roles for Ultrabithorax and Antennapedia in regulating segment-specific apoptosis of differentiated motoneurons in the Drosophila embryonic central nervous system. Development *135*, 3435–3445.

Salser, S.J., and Kenyon, C. (1992). Activation of a C. elegans Antennapedia homologue in migrating cells controls their direction of migration. Nature *355*, 255–258.

Samad, O.A., Geisen, M.J., Caronia, G., Varlet, I., Zappavigna, V., Ericson, J., Goridis, C., and Rijli, F.M. (2004). Integration of anteroposterior and dorsoventral regulation of Phox2b transcription in cranial motoneuron progenitors by homeodomain proteins. Development *131*, 4071–4083.

Shen, W.F., Rozenfeld, S., Lawrence, H.J., and Largman, C. (1997). The Abd-B-like Hox homeodomain proteins can be subdivided by the ability to form complexes with Pbx1a on a novel DNA target. J. Biol. Chem. *272*, 8198–8206.

Smith, C.J., O'Brien, T., Chatzigeorgiou, M., Spencer, W.C., Feingold-Link, E., Husson, S.J., Hori, S., Mitani, S., Gottschalk, A., Schafer, W.R., and Miller, D.M., 3rd. (2013). Sensory neuron fates are distinguished by a transcriptional switch that regulates dendrite branch stabilization. Neuron *79*, 266–280.

Sulston, J.E. (1983). Neuronal cell lineages in the nematode Caenorhabditis elegans. Cold Spring Harb. Symp. Quant. Biol. *48*, 443–452.

Suska, A., Miguel-Aliaga, I., and Thor, S. (2011). Segment-specific generation of Drosophila Capability neuropeptide neurons by multi-faceted Hox cues. Dev. Biol. *353*, 72–80.

Tihanyi, B., Vellai, T., Regos, A., Ari, E., Müller, F., and Takács-Vellai, K. (2010). The C. elegans Hox gene ceh-13 regulates cell migration and fusion in a non-colinear way. Implications for the early evolution of Hox clusters. BMC Dev. Biol. *10*, 78.

Tiret, L., Le Mouellic, H., Maury, M., and Brûlet, P. (1998). Increased apoptosis of motoneurons and altered somatotopic maps in the brachial spinal cord of Hoxc-8-deficient mice. Development *125*, 279–291.

Topalidou, I., and Chalfie, M. (2011). Shared gene expression in distinct neurons expressing common selector genes. Proc. Natl. Acad. Sci. USA *108*, 19258–19263.

Topalidou, I., van Oudenaarden, A., and Chalfie, M. (2011). Caenorhabditis elegans aristaless/Arx gene alr-1 restricts variable gene expression. Proc. Natl. Acad. Sci. USA *108*, 4063–4068.

van Dijk, M.A., and Murre, C. (1994). extradenticle raises the DNA binding specificity of homeotic selector gene products. Cell *78*, 617–624.

Vermot, J., Schuhbaur, B., Le Mouellic, H., McCaffery, P., Garnier, J.M., Hentsch, D., Brûlet, P., Niederreither, K., Chambon, P., Dollé, P., and Le Roux, I. (2005). Retinaldehyde dehydrogenase 2 and Hoxc8 are required in the murine brachial spinal cord for the specification of Lim1+ motoneurons and the correct distribution of Islet1+ motoneurons. Development *132*, 1611–1621.

Way, J.C., and Chalfie, M. (1988). mec-3, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in C. elegans. Cell *54*, 5–16.

Way, J.C., and Chalfie, M. (1989). The mec-3 gene of Caenorhabditis elegans requires its own product for maintained expression and is expressed in three neuronal cell types. Genes Dev. *3* (*12A*), 1823–1833.

Wu, Y., Wang, G., Scott, S.A., and Capecchi, M.R. (2008). Hoxc10 and Hoxd10 regulate mouse columnar, divisional and motor pool identity of lumbar motoneurons. Development *135*, 171–182.

Xue, D., Finney, M., Ruvkun, G., and Chalfie, M. (1992). Regulation of the mec-3 gene by the C.elegans homeoproteins UNC-86 and MEC-3. EMBO J. *11*, 4969–4979.

Xue, D., Tu, Y., and Chalfie, M. (1993). Cooperative interactions between the Caenorhabditis elegans homeoproteins UNC-86 and MEC-3. Science *261*, 1324–1328.

Zhang, Y., Ma, C., Delohery, T., Nasipak, B., Foat, B.C., Bounoutas, A., Bussemaker, H.J., Kim, S.K., and Chalfie, M. (2002). Identification of genes expressed in C. elegans touch receptor neurons. Nature *418*, 331–335.

Zheng, C., Diaz-Cuadros, M., and Chalfie, M. (2015). Hox genes promote neuronal subtype diversification through posterior induction in Caenorhabditis elegans. Neuron *88*, Published online November 4, 2015. http://dx.doi.org/10.1016/j.neuron.2015.09.049.