

Polycomb Group Regulation of Hox Gene Expression in *C. elegans*

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Summary

Polycomb group (PcG) chromatin proteins regulate homeotic genes in both animals and plants. In *Drosophila* and vertebrates, PcG proteins form complexes and maintain early patterns of Hox gene repression, ensuring fidelity of developmental patterning. PcG proteins in *C. elegans* form a complex and mediate transcriptional silencing in the germline, but no role for the *C. elegans* PcG homologs in somatic Hox gene regulation has been demonstrated. Surprisingly, we find that the PcG homologs MES-2 [E(Z)] and MES-6 (ESC), along with MES-3, a protein without known homologs, do repress Hox expression in *C. elegans*. *mes* mutations cause anteroposterior transformations and disrupt Hox-dependent neuroblast migration. Thus, as in *Drosophila*, vertebrates, and plants, *C. elegans* PcG proteins regulate key developmental patterning genes to establish positional identity.

Introduction

In metazoans, positional identity along the anterior-posterior body axis is defined during development by expression of highly conserved Hox transcription factors. Even subtle alterations in Hox expression can cause transformations of positional identity, leading to abnormal development of a specific region or segment. Hox gene expression is subject to several distinct regulatory mechanisms that have been elucidated largely through genetic studies in *Drosophila*. In *Drosophila*, the initial expression domains of Hox genes are defined embryonically by transiently expressed products of the gap and pair-rule genes (reviewed in Simon, 1995). After the initial Hox domains are established, two classes of chromatin regulators maintain them during later development. The Polycomb group (PcG) proteins function to maintain repression of Hox genes, while the Trithorax group (trxG) proteins maintain active expression (reviewed in Simon and Tamkun, 2002). In animals mutant in either PcG or trxG proteins, initial Hox gene expression domains are established normally. Later in development, however, PcG mutants ectopically express Hox genes in regions where they should be silenced, resulting in homeotic transformations (Struhl and Akam, 1985). In contrast, trxG mutants fail to maintain expression in regions where it should occur (Kennison, 1993). Mutations in trxG genes can suppress the effects of mutating PcG genes, clearly

illustrating the antagonistic activities of the two gene families (Kennison and Tamkun, 1988).

Two complexes containing *Drosophila* PcG proteins have been biochemically defined: the Polycomb repressive complex 1 (PRC1) (Shao et al., 1999) and the ESC-E(Z) complex (Müller et al., 2002; Ng et al., 2000; Tie et al., 2001). Four complexes containing trxG proteins have been identified in *Drosophila* (Papoulas et al., 1998; Petruk et al., 2001). Biochemical activities have been associated with several of the PcG and trxG complexes and are consistent with the functions of the complexes and their antagonistic roles in vivo. trxG complexes are associated with chromatin remodeling and histone acetyltransferase (HAT) activity (Kal et al., 2000; Petruk et al., 2001). In contrast, the PcG PRC1 complex inhibits nucleosome remodeling, at least in vitro (Francis et al., 2001; Shao et al., 1999). The ESC-E(Z) complex has histone methyltransferase activity and may be associated with HDAC1, a histone deacetylase (Cao et al., 2002; Czermin et al., 2002; Muller et al., 2002; Tie et al., 2001). Mammals also have close homologs of the PcG and trxG genes, and these appear to function by mechanisms similar to those of their fly counterparts (Hanson et al., 1999; Kuzmichev et al., 2002; Schumacher and Magnuson, 1997; van der Lugt et al., 1994, 1996).

The *C. elegans* Hox cluster consists of six genes (Abobaker and Blaxter, 2003), all of which are required for normal developmental patterning. In the embryo, the labial homolog *ceh-13* is necessary for anterior patterning (Brunschwig et al., 1999), while the *Abdominal-B* (*AbdB*) homologs *nob-1* and *php-3* are required for posterior patterning (Van Auken et al., 2000). The *Sex combs reduced* (*Scr*) homolog *lin-39* is expressed in the mid-body and is essential for development of the vulva (Clark et al., 1993; Maloof and Kenyon, 1998; Wang et al., 1993). *mab-5*, a *fushi tarazu* (*ftz*) homolog, and *egl-5*, an *AbdB* homolog, are expressed in partially overlapping posterior domains and are required for development of posterior structures in the male (Chisholm, 1991; Ferreira et al., 1999; Kenyon, 1986; Salser and Kenyon, 1996; Salser et al., 1993; Wang et al., 1993). In addition, *mab-5* is required to direct posterior cell migrations in both sexes (Kenyon, 1986).

C. elegans Hox genes, like those of flies and mammals, are subject to negative regulation, which prevents their inappropriate expression. Anterior expression of *mab-5* leads to ectopic differentiation of anterior hypodermal cells into male sense organs called V rays, which normally are restricted to the posterior (Salser and Kenyon, 1996). Two mechanisms that prevent this anterior V ray formation have been identified. First, the bHLH transcription factor LIN-22 blocks anterior hypodermal cells from assuming a posterior fate by repressing Hox gene expression and preventing formation of V rays (Wrischnik and Kenyon, 1997). A second mechanism, involving the Axin homolog PRY-1, blocks a Wnt signaling pathway to prevent ectopic Hox expression and homeotic transformations (Korswagen et al., 2002; Maloof et al., 1999). Although regulation by PRY-1 resembles that of the PcG in some respects, it is likely to act by direct

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modulation of Wnt signaling, rather than by modification of chromatin structure. Thus, it has been unclear whether nematodes have a PcG-like mechanism of Hox regulation and, if so, whether PcG homologs play any role.

C. elegans lacks obvious homologs of the PRC1 complex proteins but does have two proteins homologous to ESC-E(Z) complex components. These are the E(Z) homolog MES-2 (Holdeman et al., 1998) and the ESC homolog MES-6 (Korf et al., 1998). A third MES protein, MES-4, contains a SET domain, a histone methyltransferase motif often found in chromatin regulators (Fong et al., 2002). Like their *Drosophila* homologs, MES-2 and MES-6 have been isolated as part of a complex (Xu et al., 2001a). This complex includes MES-3, a protein with no identified functional domains (Paulsen et al., 1995), but not MES-4. Also, like their *Drosophila* counterparts, the MES proteins have a repressive function. *mes-2*, *-3*, *-4*, and *-6* mutations result in derepression of high-copy transgenes and X chromosome modifications characteristic of active chromatin, suggesting a role in germline silencing of the X chromosome (Fong et al., 2002; Kelly and Fire, 1998). Lack of MES function results in germline degeneration and maternal-effect sterility (Capowski et al., 1991; Paulsen et al., 1995). However, despite the structural and functional similarities to PcG proteins of insects and vertebrates, no role for the MES proteins in somatic patterning and regulation of nematode Hox gene expression has been demonstrated.

Here we have used a genetic screen to identify regulators of male tail neurogenesis. Unexpectedly, we found that MES-2, -3, and -6 do indeed repress Hox gene expression in the *C. elegans* soma. The three *mes* genes act upstream of the Hox genes *mab-5* and *egl-5* during V ray differentiation, and loss of *mes* activity can restore normal ray development and mating ability to males mutant in the *mab-5* activator *pal-1*. Males lacking *mes* activity display anterior expansions of tail structures and ectopic expression of the Hox reporter *egl-5::gfp* and the Hox target *lin-32::gfp*. This regulation is not restricted to the male tail: *mes-2*, *-3*, and *-6* also repress *lin-39::lacZ* expression in the midbody and head and *mab-5* activity in a migrating neuroblast. Consistent with a general somatic regulatory function, MES protein expression is widespread in larvae, particularly males. Our findings suggest that the regulatory relationship between PcG chromatin proteins and the Hox genes has been conserved in nematodes.

Results

A Screen for Regulators of V Ray Development

The *C. elegans* male tail is highly sexually specialized, with many sensory and copulatory structures that function in locating and mating with hermaphrodites. Among these are nine pairs of bilateral sensory rays, each composed of two neurons and a structural cell (Figure 1B). The six pairs of V rays derive from the posterior hypodermal blast cells V5 (ray 1) and V6 (rays 2–6), while the three more-posterior pairs of T rays (rays 7–9) are descendants of the blast cell T (Sulston et al., 1980). Each ray can be identified by its unique shape, position on the body axis, and, in some cases, neurotransmitter

expression (Sulston et al., 1980). Ray development thus provides a model system for identifying genes governing cell fate specification, developmental patterning, and male-specific neural development.

Genetic screens for males that lack V rays have allowed the construction of genetic pathways governing V ray differentiation. In the V6 lineage, the V ray developmental program is initiated by transient expression of the *caudal* homolog *pal-1* during late embryogenesis (Figure 1A) (Hunter et al., 1999; Waring and Kenyon, 1991). PAL-1 activates *mab-5* in V6, and MAB-5 in turn activates *egl-5* during the L2 larval stage (Ferreira et al., 1999; Hunter et al., 1999). Together, these two Hox proteins activate the bHLH gene *lin-32* in the ray precursor cells R1–R6 during the L3 larval stage (Emmons, 1999; Zhao, 1995). The Doublesex-related sexual regulator MAB-3 is expressed in the ray precursors with LIN-32, and both proteins are required for V ray development (Portman and Emmons, 2000; Shen and Hodgkin, 1988; Yi et al., 2000; Zhao and Emmons, 1995). We previously proposed a model in which *mab-3* and *lin-32* act in distinct genetic pathways, with LIN-32 playing a primary role in specifying V ray fate and MAB-3 playing a permissive role (Yi et al., 2000).

To better understand how the *mab-3* and Hox/*lin-32* genetic pathways interact to direct V ray development, we performed a genetic screen for recessive mutations that suppress V ray loss in *mab-3(e1240)* null mutant males. On the basis of the model of V ray development (Figure 1A), we predict that one class of mutation identified in this screen might affect transcriptional targets of MAB-3. A second class of mutations could affect negative regulators of the Hox genes or *lin-32*. Mutations in these negative regulators would result in increased *lin-32* expression, thus bypassing the requirement for *mab-3*. In a screen of 2000 F1 genomes, we identified ten alleles that suppress the ray defects of *mab-3(e1240)*, including three alleles that we propose belong to the second class of mutations. Other *mab-3* suppressor mutations will be described elsewhere.

Mutations in *C. elegans* Polycomb Group Homologs Suppress *mab-3* V Ray Defects

Three of the alleles isolated in the *mab-3* suppressor screen (*ez1*, *ez10*, and *ez12*) displayed a maternal-effect sterile (Mes) phenotype and failed to complement a previously identified mutation in the *mes-3* locus. Sequencing confirmed that these are strong loss-of-function mutations in the *mes-3* gene (Experimental Procedures). All three mutations restore V ray formation in *mab-3* null mutant males to approximately 60% of wild-type levels (Figure 1). As discussed above, *mes-3* is one of a group of genes that are required maternally for *C. elegans* germline development.

MES-2, -3, and -6 have been isolated in a complex that excludes MES-4 (Xu et al., 2001a). To determine whether a similar MES complex might function during V ray development, we tested *mes-2*, *-4*, and *-6* mutations for suppression of *mab-3* V ray defects. The *mes-2(bn11)* and *mes-6(bn66)* mutations partially restore V ray development in *mab-3(e1240)*, while the *mes-4(bn67)* mutation does not (Figure 1E). These results indicate that MES-3 and the PcG homologs MES-2 and

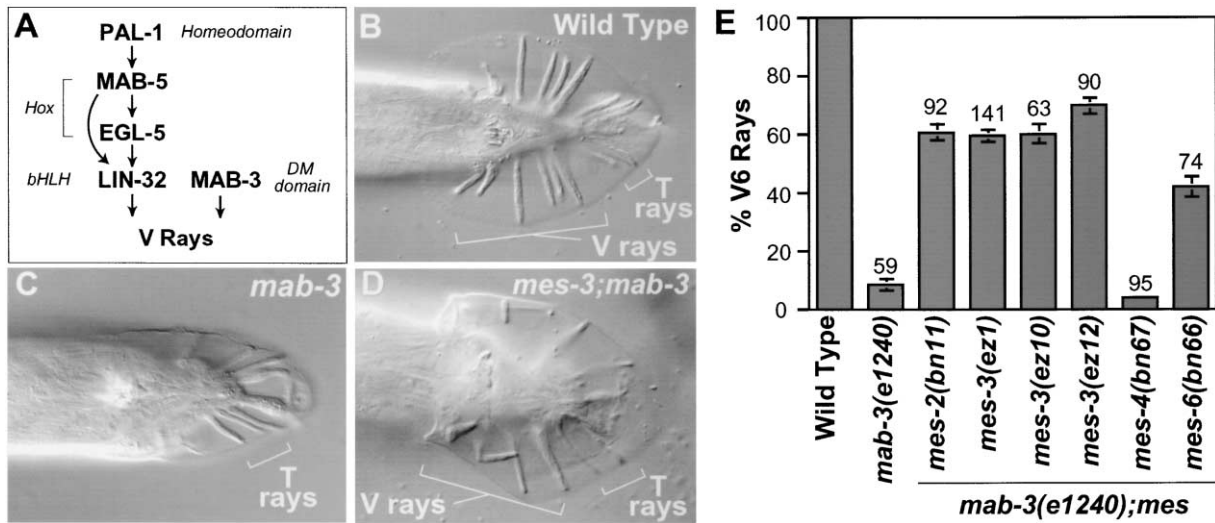


Figure 1. Mutations in *mes* Genes Restore V Ray Development in *mab-3*(null) Males

(A) Regulators of V ray development act in distinct genetic pathways. Mutations that affect either pathway disrupt development of V rays. (B) The wild-type male tail has six bilateral pairs of V rays and three bilateral pairs of T rays. (C) A *mab-3(e1240)* null mutant male tail lacks V rays, but has T rays. (D) A mutation in the *mes-3* gene partially suppresses the V ray defects of the *mab-3(e1240)* mutant male. (E) Mutations in *mes-2*, *mes-3*, and *mes-6*, but not *mes-4*, restore V ray development in *mab-3(e1240)* males. Graph of frequency of V6 rays expressed in percentage of normal number (five per side). V6 rays, rays 2–6, are those derived from the V6 hypodermal seam cell. Ray 1 is occasionally absent in wild-type and thus was not scored. The number of sides scored for each genotype is indicated. *mab-3(e1240)*; *mes* indicates that males scored were doubly mutant for *mab-3(e1240)* and the *mes* gene indicated below the graph. All strains contain the high incidence of male mutation *him-8(e1489)*. Error bars indicate standard error of the mean.

MES-6 are active in the male soma during V ray development and potentially function together in the same complex that is present in the embryo. MES-4 apparently performs a distinct function or, perhaps, does not function in the soma.

mes Genes Act Upstream of the Hox Genes during V Ray Development

Drosophila PcG mutations result in derepression of Hox genes and development of posterior structures in anterior domains. While it is possible that the *mes* genes interact directly with *mab-3* or its transcriptional targets, a simpler model is that the *mes* genes interact directly with the two Hox genes, *mab-5* and *egl-5*, that are required for V ray development. Homology of *mes-2* and *mes-6* to the *Drosophila* PcG suggests that MES-2, -3, and -6 might normally function in the Hox genetic pathway to negatively regulate Hox genes during V ray development. To test this idea, we performed genetic epistasis analysis to determine whether *mes* mutations can suppress V ray defects associated with mutations in the Hox genetic pathway.

The Hox gene *mab-5* is normally required for V ray development (Figure 2A; Table 1). If the *mes* genes act downstream of *mab-5* as negative regulators, one would predict that *mes* mutations could derepress *mab-5* targets sufficiently to restore V ray development in a *mab-5* null mutant. Alternatively, if *mes* genes act as negative regulators upstream of *mab-5*, *mes* mutations would not suppress the *mab-5* mutation because *mab-5* would still be required to activate its downstream targets. To distinguish between these possibilities, we tested the

ability of *mes-3(ez12)* and *mes-6(bn66)* mutations to suppress V ray defects of the *mab-5(e1239)* null allele. Mutations in *mes-3* or *mes-6* fail to restore normal V ray development in *mab-5* mutants, indicating that *mab-5* is required for V ray development, even in the absence of *mes-3* or *mes-6* function (Figure 2B; Table 1). This finding is consistent with the *mes* genes acting upstream of *mab-5* in the genetic pathway that specifies V rays. While V rays do not form normally in *mab-5*; *mes* double mutants, these males do occasionally produce one or two V rays (Table 1). These rays are probably due to *mab-5*-independent *egl-5* expression, as they are not seen in *mab-5(e1239)egl-5(n945)*; *mes-6(bn66)* mutant males (Table 1).

We next tested the ability of *mes* mutations to suppress the ray defects caused by a mutation in the *mab-5* activator *pal-1*. *pal-1(e2091)* mutant males fail to express PAL-1 in V6 because of a regulatory mutation (Hunter et al., 1999; Zhang and Emmons, 2000). As a result, *mab-5* is not activated in the V6 lineage, and these males fail to produce V6-derived rays (Figure 2C). If MES proteins negatively regulate *mab-5*, relieving that repression might circumvent the normal requirement for *pal-1* and thereby restore V6 ray development. Alternatively, *mes* mutations might suppress *pal-1(e2091)* by activating *pal-1* independently of its normal regulation. In either case, suppression of *pal-1(e2091)* by *mes* mutations would provide further evidence that the *mes* genes act genetically upstream of *mab-5*. Mutations in *mes-2*, -3, and -6 do strongly suppress *pal-1(e2091)*, in some cases restoring mating, while a *mes-4* mutation does not (Figure 2D; Table 1). This finding suggests that *mes-2*, -3,

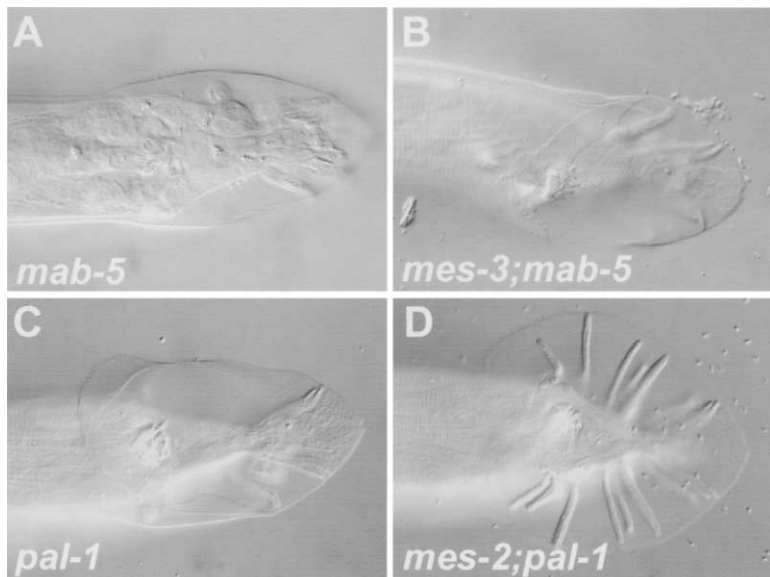


Figure 2. The *mes* Genes Act Upstream of the Hox Gene *mab-5* in V Ray Development (A) A *mab-5(e1239)* null mutant male lacks V rays. (B) A *mes-3(ez12); mab-5(e1239)* double mutant male, showing that a *mes-3* mutation fails to restore V ray development in the *mab-5(e1239)* null mutant. (C) A *pal-1(e2091)* mutant male lacks V6 rays. (D) A *mes-2(bn27); pal-1(e2091)* double mutant male, showing that the *mes-2(bn27)* mutation fully restores V ray development in *pal-1(e2091)* mutant males.

and -6 act together, upstream of *mab-5* during V ray development.

MES Suppression of *pal-1* Does Not Require a Wnt Pathway

Previous work has shown that *mab-5* can be activated in *pal-1(e2091)* mutants via a Wnt pathway (Hunter et al., 1999; Zhang and Emmons, 2000). The Axin homolog PRY-1 is a negative regulator of this Wnt pathway in the V ray lineage (Korswagen et al., 2002). Mutations in *pry-1* cause ectopic *mab-5* expression and *mab-5*-dependent ectopic rays (Korswagen, 2002; Maloof et al., 1999). These *pry-1* phenotypes are suppressed by mutations in the β -catenin homolog *bar-1*, suggesting that the Wnt pathway activates *mab-5* in the anterior seam in the absence of repression by PRY-1 (Maloof et al., 1999). In addition, mutations in transcriptional mediator complex components *sop-1* and *sop-3* suppress *pal-1* by causing inappropriate activation of the Wnt pathway (Boube et al., 2002; Zhang and Emmons, 2000, 2001).

We therefore tested whether suppression of *pal-1(e2091)* by the *mes-3(ez12)* mutation likewise requires

Wnt pathway activity. To do this, we compared V ray development in *mes-3; pal-1* double mutants with V ray development in *mes-3; pal-1; bar-1* triple mutants. *bar-1* normally is not essential for V ray formation, and the null mutation *bar-1(ga80)* does not affect the number of V rays (Maloof et al., 1999). The number of V rays in *mes-3(ez12); pal-1(e2091); bar-1(ga80)* males does not differ significantly from that in *mes-3(ez12); pal-1(e2091)* males, indicating that *bar-1* is not required for suppression of *pal-1(e2091)* by *mes-3(ez12)*. This finding suggests that the mechanism of Hox gene regulation by MES proteins is distinct from that of PRY-1 and the SOP proteins, which act by negatively regulating a Wnt pathway.

MES Proteins Are Required to Specify V Ray Pattern and Position

The Hox genes *mab-5* and *egl-5* are required late in V ray development to specify ray identity and position on the anteroposterior axis. Misregulation of *mab-5* or *egl-5* late in ray development can lead to ray fusions, duplications, altered ray identities, and ectopic rays (Chow and

Table 1. Genetic Interactions between *mes* Genes and V Ray Regulators

Genotype ^a	V6 Rays Present (%)	Sides Scored
Wild-type ^b	100	107
<i>mab-5(e1239)</i> ^c	0	108
<i>mes-3(ez12); mab-5(e1239)</i> ^c	3	140
<i>mes-6(bn66); mab-5(e1239)</i> ^c	6	108
<i>mes-6(bn66); mab-5(e1239) egl-5(n945)</i> ^c	0	170
<i>pal-1(e2091)</i>	8	131
<i>mes-2(bn27); pal-1(e2091)</i>	91	105
<i>mes-3(ez12); pal-1(e2091)</i>	93	112
<i>mes-4(bn67); pal-1(e2091)</i>	3	51
<i>mes-6(bn66); pal-1(e2091)</i>	78	70
<i>mes-3(ez12); pal-1(e2091); bar-1(ga80)</i>	87	22

^aDenotes maternal and zygotic genotype.

^bAll strains harbor the high incidence of male mutation *him-8(e1489)*.

^cV5 and V6 rays could not be distinguished in these strains. As a result, percentage of V rays was calculated on the basis of a wild-type total of six V rays per side.

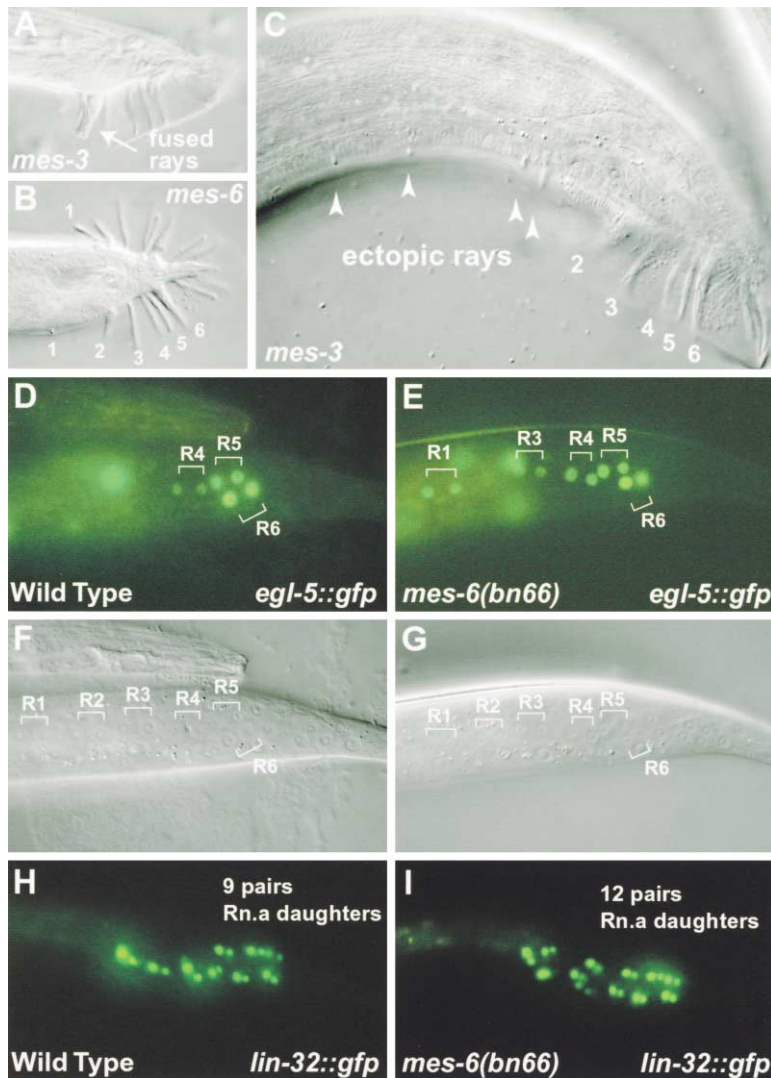


Figure 3. *mes* Genes Are Required to Specify V Ray Pattern, Position, and Hox Gene Expression

(A) A *mes-3(ez12)* mutant male showing a fusion of rays 2 and 3.

(B) A *mes-6(bn66)* mutant male showing anterior displacement of V ray 1. V rays on the affected side are numbered. Ray 1 occupies its normal position on the opposite side.

(C) A *mes-3(ez12)* male with four ectopic rays, indicated by arrowheads, in the lateral hypodermis. V rays 2–6 occupy their normal positions in the posterior.

(D) In a wild-type L3 male, *egl-5::gfp* is expressed in anterior and posterior daughters of V ray precursors R4–R6.

(E) In a *mes-6(bn66)* mutant L3 male, *egl-5::gfp* is expressed outside its normal domain, in daughters of the V ray precursors R1 and R3.

(F and G) Differential interference contrast (DIC) micrographs of tails shown in (D) and (E).

(H) A wild-type L3 male expressing *lin-32::gfp* in nine pairs of descendants of the ray neuroblasts (Rn.a cells).

(I) A *mes-6(bn66)* mutant male expressing *lin-32::gfp* ectopically, in a total of 12 pairs of ray neuroblast descendants.

Emmons, 1994; Salser and Kenyon, 1996). Our genetic evidence indicates that the MES proteins act upstream of the Hox genes *mab-5* and *egl-5* during V ray development. We therefore examined *mes* mutant males for phenotypes associated with Hox gene misregulation. *mes-2(bn27)*, *mes-3(ez12)*, and *mes-6(bn66)* males display ray fusions in 2%–3% of sides scored (Figure 3A; Table 2). In addition, ray 1 is displaced anteriorly in ~60% of *mes-2* males and in ~30% of *mes-3* and *mes-6* males (Figure 3B; Table 2). Most strikingly, a significant proportion of *mes-2*, *-3*, and *-6* mutant males have two to four ectopic rays (Figure 3C; Table 2). The presence

of ectopic rays, ray fusions, and changes in ray position are consistent with Hox misexpression in the V ray lineage of *mes* mutant males.

MES Proteins Regulate Hox Expression in the V Ray Lineage

The ectopic rays and ray fusions observed in *mes* mutant males provide evidence that the MES proteins are required to limit domains of Hox expression in the V ray lineage. If MES proteins negatively regulate Hox genes to specify V ray pattern and position, one would expect ectopic expression of *mab-5* or *egl-5* in the seam cell

Table 2. V Ray Defects Associated with *mes* Mutations

Genotype ^a	Ray 1 Anterior (%)	Multiple Ectopic Rays (%)	Fused Rays (%)	Sides Scored
Wild-type ^b	<1	0	0	107
<i>mes-2(bn27)</i>	59	8	2	106
<i>mes-3(ez12)</i>	32	12	3	122
<i>mes-6(bn66)</i>	31	31	3	118

^a Denotes maternal and zygotic genotype.

^b All strains carry the high incidence of male mutation *him-8(e1489)*.

lineages of *mes* mutant males. To test this, we compared expression of an integrated *egl-5::gfp* reporter gene (Ferreira et al., 1999) in wild-type and *mes-6(bn66)* mutant males. In wild-type males, EGL-5 expression is initiated in a posterior daughter of the V6 lineage during late L2 (Ferreira et al., 1999). EGL-5 expression continues in the descendants of the ray precursors, R4, R5, and R6, that arise from this cell. The R3 lineage also occasionally expresses EGL-5. In wild-type males the *egl-5::gfp* reporter shows similar expression to endogenous EGL-5 in R4, R5, and R6, is rarely expressed in the R3 lineage, and is never expressed in ray precursors anterior to R3 (Figure 3D). In contrast, in *mes-6(bn66)* mutant males, *egl-5::gfp* is ectopically expressed in ray precursor cells anterior to R3 in 17% of males scored (Figure 3E).

The bHLH gene *lin-32* is a target of Hox regulation in the V ray lineage and is required for ray formation. We used a *lin-32::gfp* reporter (Yi et al., 2000) to compare *lin-32::gfp* expression in wild-type and *mes-6* mutant males. *lin-32::gfp* is normally expressed in the V and T ray precursors and in a subset of their descendants (Figure 3H). Consistent with ectopic Hox gene expression and formation of ectopic rays, *lin-32::gfp* is expressed in extra ray lineage cells in *mes-6(bn66)* mutant males (Figure 3I).

MES Proteins Are General Repressors of Hox Gene Expression

We next tested whether the MES proteins repress expression of other Hox genes. MAB-5 expression cannot be assayed directly in the V ray lineage because MAB-5 antibodies are no longer available and *mab-5::gfp* reporters are not expressed in the hypodermis after the L1 stage. Therefore, we instead examined migration of the Q neuroblasts, a process that is dependent on *mab-5* activity (Kenyon, 1986; Salser and Kenyon, 1992). In wild-type L1 larvae, the left neuroblast QL migrates a short distance to the posterior and then begins to express *mab-5*. Some descendants of QL continue to express *mab-5* and migrate posteriorly. In *mab-5* mutants, these cells instead migrate to the anterior. In contrast, the right neuroblast QR and its descendants do not express *mab-5* and normally migrate anteriorly. Ectopic expression of *mab-5* in the QR lineage causes QR descendants to behave like those of QL, that is, to remain in the posterior (Maloo et al., 1999; Salser and Kenyon, 1992). If the MES proteins act as general negative regulators of Hox gene expression, one might expect *mab-5* to be ectopically expressed in QR in *mes* mutants, causing QR to remain in the posterior.

To follow the final positions of Q cell descendants, we used a *mec-7::gfp* reporter, which is expressed in touch neurons, including the QR descendant AVM and the QL descendant PVM (Chalfie et al., 1994). In wild-type worms, AVM is anterior to the touch neuron ALMR in 100% of worms scored (Figure 4A; n = 143). However, in *mes-6(bn66)* mutants, AVM remains posterior to ALMR in 6% of worms scored (Figure 4B; n = 297). This small, but significant, difference in Q cell migration suggests that the MES genes are negative regulators of *mab-5* in the QR lineage. If the posterior displacement of AVM in *mes-6(bn66)* is caused by ectopic *mab-5* expression, it should require a wild-type *mab-5* gene.

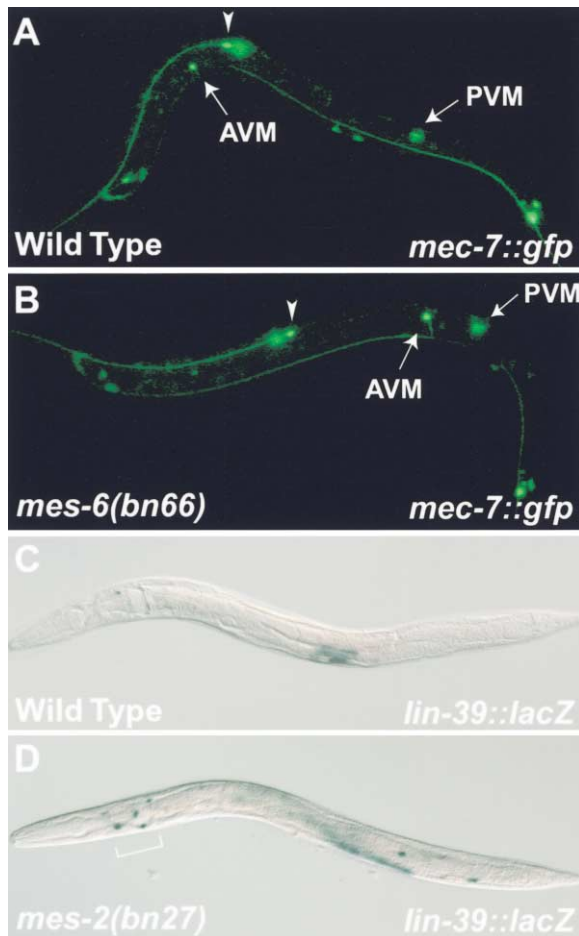


Figure 4. *mes* Genes Restrict Hox Gene Expression outside the V Ray Lineage

(A and B) A *mes-6* mutation disrupts a *mab-5*-dependent cell migration.

(A) Wild-type worm expressing *mec-7::gfp* in touch receptor neurons. The QR neuroblast descendant AVM is located anterior to the touch cell ALMR (arrowhead). The QL neuroblast descendant PVM is located in the posterior of the worm.

(B) A *mes-6(bn66)* mutant expressing *mec-7::gfp* in touch receptor neurons. AVM is located posterior to ALMR (arrowhead). The position of PVM is unaffected by the *mes-6* mutation.

(C and D) *mes-2* is required for normal expression of the Hox reporter *lin-39::lacZ*.

(C) A wild-type hermaphrodite expresses *lin-39::lacZ* in vulval cells and in one head neuron.

(D) A *mes-2(bn27)* mutant hermaphrodite expresses *lin-39::lacZ* in additional head cells (bracket). *lin-39::lacZ* expression in the mid-body is also elevated.

We examined the positions of AVM and PVM in *mab-5(e1239); mes-6(bn66)* mutants and found this to be the case. In 100% of the *mab-5; mes-6* double mutants, AVM was positioned normally, in the anterior (n = 331). This observation confirms that the MES proteins negatively regulate *mab-5* expression in somatic cells. In addition, displacement of AVM occurs in both males and hermaphrodites, indicating that MES regulation of Hox genes is neither male specific nor restricted to the V ray lineage.

The experiments described above provide evidence that the *C. elegans* PcG homologs negatively regulate expression of *egl-5* and *mab-5*. To determine whether MES regulation also affects the midbody Hox gene *lin-39*, we examined expression of a *lin-39::lacZ* reporter (Wang et al., 1993) in wild-type and *mes-2* mutant hermaphrodites. In wild-type worms, *lin-39::lacZ* is expressed in vulval cells, in some neurons of the ventral nerve cord, and, occasionally, in one head neuron (Figure 4C). In *mes-2(bn27)* mutants, expression in the midbody region is both more widespread and stronger, and the reporter is consistently expressed in more than one cell in the head at all stages examined (Figure 4D). This result provides further evidence that the MES proteins act as global regulators of Hox expression in somatic cells. The MES proteins may act in the soma to repress genes in addition to the Hox genes, but this has not been examined.

MES Proteins Are Widely Expressed in the Larval Male Soma

MES proteins have been reported to be expressed in the germline throughout development, in all cells in embryos, and in some somatic cells during hermaphrodite larval development (primarily intestine) (Fong et al., 2002; Holdeman et al., 1998; Korf et al., 1998; Xu et al., 2001b). As described above, *mes* mutations can cause abnormal Hox gene expression in mid to late larval development. If the MES proteins regulate gene expression during larval development, one might expect them to be expressed in the affected lineages close to the time at which the phenotype appears. Alternatively, the MES genes could mediate long-term silencing that is established in the embryo, with consequences not apparent until much later.

We therefore examined MES expression in larvae, with particular attention to the male soma, where MES expression has not been described. We used antibodies to MES-2 and MES-6 to stain larvae in L2 and L3, the period during which male sensory rays are specified (Figure 5). In both sexes the two proteins are expressed in the germline and intestine, as previously reported. In addition, both proteins are expressed in most cells of the larval male tail. As a test of antibody specificity, we confirmed that MES-6 staining is severely reduced by the strong loss-of-function allele *mes-6(bn66)* and that MES-2 staining is absent in the *mes-2(bn27)* null mutant. Our results are consistent with MES protein activity in somatic cells in larvae but do not exclude the possibility that MES-dependent repression is established in the embryo and persists throughout larval development.

Discussion

We have found that the *C. elegans* PcG homologs *mes-2* and *mes-6*, together with *mes-3*, are required for normal anteroposterior patterning during larval development. Phenotypes include shifts in male sensory ray position, number, and identity, as well as abnormal neuroblast migration. In the male tail the *mes* genes act upstream of the Hox genes *mab-5* and *egl-5*, and both the Hox reporter *egl-5::gfp* and the Hox target *lin-32::gfp* are

ectopically expressed in *mes* mutants. The MES proteins appear to repress Hox genes along the entire body axis: in the midbody, *mes* mutants have ectopic *mab-5* activity and *lin-39::lacZ* expression, and, in the head, *mes* mutants have ectopic *lin-39::lacZ* expression. Together these data suggest that the nematode PcG proteins function as global repressors of Hox expression in the soma. Consistent with such a role, MES-2 and MES-6 are widely expressed in the larval soma, with particularly high expression in the male tail.

Somatic versus Germline Functions of the MES Proteins

The *mes-2*, *-3*, *-4*, and *-6* genes were initially identified in *C. elegans* by virtue of their essential role in germline development. Here we have shown that *mes-2*, *-3*, and *-6* also are involved in somatic patterning and that this role does not require *mes-4*. This suggests that *mes-4* is a germline-specific component of the *mes* system and is consistent with the previous identification of a complex in embryos containing MES-2, *-3*, and *-6*, but not MES-4. The germline and somatic functions of the MES proteins are in some respects different. In the germline the *mes* genes are predicted to repress transcription from the X chromosome, whereas the somatic function appears to be selective for specific patterning genes. However, both functions involve repression of gene expression, and it is likely that the germline and somatic functions involve the same underlying mechanism. By analogy to the *Drosophila* PcG complexes, this mechanism is likely to include chromatin modification. Coregulators such as MES-4 in the germline and unknown proteins in the soma may impart target specificity to the complex in different tissues, bringing it to the X chromosome or to Hox genes.

Do Nematode PcG Proteins Function like Those of Other Phyla?

The mechanisms of chromatin-mediated Hox gene regulation are best understood in *Drosophila*, where a combined genetic and biochemical approach has identified several complexes that control Hox expression. The histone methyltransferase and HDAC activities associated with the ESC-E(Z) complex may result in "marking" chromatin in the vicinity of regulated genes with a distinctive histone code (Strahl and Allis, 2000). In one model (Simon and Tamkun, 2002), these marks would then result in the recruitment of the PRC1 complex, which, in vitro, can block chromatin remodeling. PRC1 could then antagonize the HAT activity and chromatin remodeling functions of trxG complexes.

We have shown that homologs of ESC-E(Z) proteins are involved in *C. elegans* Hox repression. Homologs of trxG proteins have been shown to promote expression of *mab-5* and *egl-5* in *C. elegans* (Chamberlin and Thomas, 2000), suggesting that nematodes have conserved chromatin-remodeling activities similar to those of *Drosophila*. It is, however, unclear whether *C. elegans* has an "anti-remodeling" complex equivalent to PRC1, as close homologs of PRC1 components are not apparent in the *C. elegans* genome. Recently, however, a protein with a SAM domain, a motif also present in the

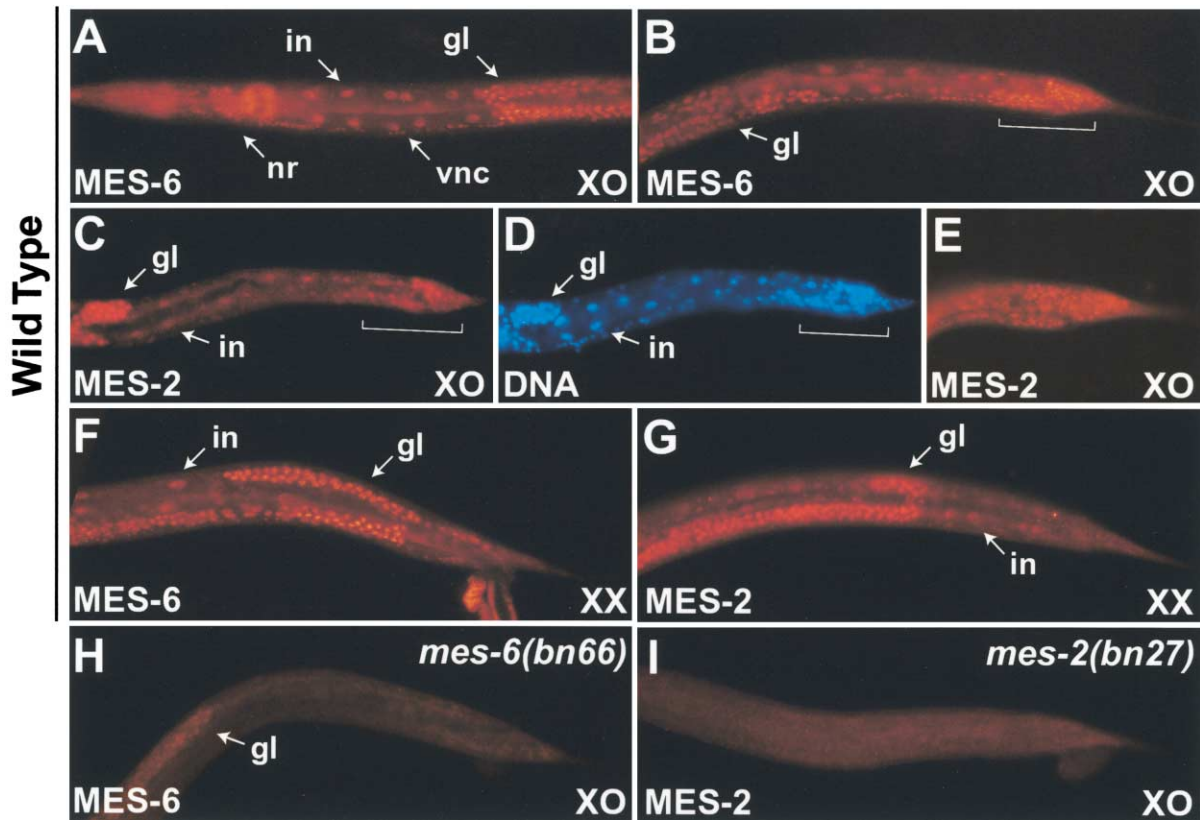


Figure 5. Widespread Larval Expression of MES Proteins

(A and B) Wild-type L3 male stained with MES-6 antiserum. MES-6 is expressed in nuclei of the tail (bracket), germline (gl), intestine (in), nerve ring (nr), and ventral nerve cord (vnc).
 (C) Wild-type early L3 male stained with MES-2 antiserum, showing expression in tail, germline, and intestinal nuclei.
 (D) DAPI nuclear staining of (C), showing that most, but not all, nuclei are MES-2 positive.
 (E) MES-2 expression in the tail of a wild-type late L3 male.
 (F) Wild-type L3 hermaphrodite, showing MES-6 staining in germline and intestine.
 (G) Wild-type L3 hermaphrodite, showing MES-2 staining in germline and intestine.
 (H) MES-6 staining is reduced in a *mes-6(bn66)* mutant L3 male.
 (I) MES-2 staining is absent in a *mes-2(bn27)* mutant L3 male.

PRC1 complex protein Polyhomeotic, has been implicated in *C. elegans* Hox gene repression (SOP-2; Zhang et al., 2003 [this issue of *Developmental Cell*]). An intriguing possibility is that a chromatin-mediated regulatory system similar to that of *Drosophila* has been conserved in *C. elegans* to control Hox expression and axial patterning during somatic development.

The MES-2 and MES-6 proteins are clear homologs of *Drosophila* ESC and E(Z), suggesting the presence of a similar complex in nematodes. The fly ESC-E(Z) complex contains two other core components, the HDAC-associated protein NURF55 and a novel protein, Su(Z)12 (Muller et al., 2002; Tie et al., 2001). *C. elegans* has two NURF55 homologs (*lin-53* and *rba-1*), but it is unknown whether they interact with the MES proteins or affect Hox expression. There is no obvious Su(Z)12 homolog in *C. elegans*. One possibility is that MES-3 assumes some or all of the functions of these components. Obviously, it also is possible that additional *C. elegans* components remain to be identified.

Two catalytic activities have been linked to the *Drosophila* ESC-E(Z) complex. One is an intrinsic histone methyltransferase activity, for which the SET domain of E(Z) is essential. MES-2 has a SET domain, and, thus, the *C. elegans* complex might act by a similar mechanism. The other is a histone deacetylase activity, which may be mediated by association between NURF55 and HDAC1 (Tie et al., 2001). We used feeding RNAi to target six HDACs (*hda-1*, *hda-2*, *hda-3*, F43G6.4, Y51H1A.5, and C10E2.3) and found that depletion of three of them (*hda-1*, F43G6.4, and Y51H1A.5) resulted in an anterior shift of ray 1, reminiscent of the *Mes* phenotype (data not shown). Thus, it is possible that HDACs regulate Hox expression in *C. elegans*, but this possibility needs to be tested further.

In flies the ESC-E(Z) complex is required early in embryos to maintain Hox silencing. The histone methylation mediated by E(Z) is thought to provide a long-term "mark" for silencing of adjacent chromatin. It is unclear whether the *C. elegans* complex acts in a similar fashion.

MES-2, -3, and -6 are expressed in all cells of the *C. elegans* embryo, but our antibody staining demonstrates that at least MES-2 and -6 are also expressed widely in the larval male soma. Thus, we cannot distinguish between the early establishment of a heritable mark that later is required for Hox silencing and axial patterning and a much later function in specific lineages.

Multiple Mechanisms of Global Hox Gene Regulation in *C. elegans*

Our work establishes PcG regulation as a global form of Hox gene repression in *C. elegans*. However, this is not the only such mechanism in the nematode. As described above, at least two other regulatory systems also restrict expression of the Hox genes. One involves the Hairy homolog LIN-22, a transcription factor that prevents anterior hypodermal cells from expressing posterior Hox genes and assuming posterior fates. The other involves the Axin homolog PRY-1, which prevents a Wnt pathway from activating Hox expression inappropriately. The mechanism of MES-mediated Hox gene repression is likely to be distinct from that of PRY-1: we found that the Hox genes can be activated by a *mes* mutation in the absence of functional Wnt signaling (Table 1).

Evolution of Global Hox Gene Repression

PcG proteins regulate Hox expression in several phyla, including nematodes. However, the somatic phenotypes of *C. elegans mes* mutants are subtle and were first apparent only in genetically sensitized backgrounds (*mab-3* or *pal-1* mutants). This contrasts with the more severe phenotypes caused by some *Drosophila* PcG mutations and by mutations in the *C. elegans* SAM domain gene *sop-2* (Zhang et al., 2003), which can include widespread Hox misexpression and lethality.

Why do ESC/E(Z) mutations affect Hox expression less extensively in *C. elegans* than in *Drosophila*? One possibility is functional redundancy between the ESC/E(Z) complex and other regulators. It is unlikely that the *mes* genes are redundant with one another because they are thought to act in the same complex, and elimination of one MES protein can reduce the abundance of the other MES proteins (Holdeman et al., 1998; Korf et al., 1998; Xu et al., 2001b). By analogy to the *Drosophila* PcG system, the *C. elegans* MES proteins would be predicted to recruit a PRC1-like complex, which might include SOP-2. However, the difference in severity between Mes and Sop-2 phenotypes indicates that, in *C. elegans*, additional recruitment factors would have to exist.

Another possibility is that nematodes have retained ESC/E(Z) regulation late in development for the refinement of Hox expression patterns, while recruiting other regulatory mechanisms to maintain silencing in early development. The Mes phenotypes we observe suggest that the primary function of the ESC/E(Z) complex in worms may be to ensure fidelity of Hox patterning within specific lineages or regions. Strong selection for germline viability may have conserved the *mes* genes as a functional system, while allowing them to adopt a more subtle role in the soma of worms than in flies.

Whatever the evolutionary history of the PcG genes

may be, our results make it clear that they do regulate Hox expression in the soma in all metazoan phyla tested.

Experimental Procedures

Strains and Alleles

C. elegans strains were cultured and genetically manipulated as described previously (Sulston and Hodgkin, 1988). Strains were maintained at 20°C, unless otherwise noted. Most strains include a *him-8(e1489)* or *him-5(e1490)* high incidence of male mutation. The following mutations, integrated transgenes, and balancers were used in this study: LGI, *mes-3(ez1, ez10, ez12, bn35)* and *hT2[dpy-18(h662), bli-4(e937)](I, III)*; LGII, *mes-2(bn11, bn27), mab-3(e1240), rol-9(sc148), unc-4(e120), muls16[mab-5::gfp, dpy-20+], muls32[mec-7::gfp]*, and *mnC1[dpy-10(e128) unc-52(e444)]*; LGIII, *pal-1(e2091), mab-5(e1239), egl-5(n945), unc-32(e189)*, and *hT2[dpy-18(h662), bli-4(e937)](I, III)*; LGIV, *mes-6(bn66), him-8(e1489), muls6[lin-39::lacZ, rol-6(su1006d)]*, and *dNT1[unc(n754) letJ (IV, V)]*; LGV, *mes-4(bn67), him-5(e1490)*, and *dNT1[unc(n754) letJ (IV, V)]*; LGX, *bar-1(ga80)* and *bxIs13[egl-5::gfp, lin-15+]*.

The following extrachromosomal transgenic arrays were used in this study: *ezEx161[lin-32::gfp (50 ng/μl)]*, *ceh-22::gfp (10 ng/μl)]*. The *ceh-22::gfp* plasmid pCW2.1 was kindly provided by Peter Okkema.

Genetic Manipulation of *mes* Strains

mes mutants were maintained as balanced heterozygous lines. *mes-2(bn27)* and *mes-2(bn11)* are null alleles (Holdeman et al., 1998). *mes-6(bn66)* and *mes-4(bn67)* are strong loss-of-function alleles (Korf et al., 1998). For phenotypic analysis, *mes^{-/-}* progeny of *mes^{+/-}* hermaphrodites were selected from balanced strains. These hermaphrodites were positive for maternal *mes* contribution and were thus fertile, but they lacked zygotic *mes* activity (*m^{z-}*). Each produced a brood of homozygous *mes^{-/-}* sterile progeny lacking both maternal and zygotic *mes* activity (*m^{z-}*). Analyses of somatic Mes phenotypes were performed on these *m^{z-}* broods.

mab-3 Suppressor Screen

mab-3(e1240); him-8(e1489); rol-9(sc148) hermaphrodites were mutagenized with EMS according to standard protocols (Sulston and Hodgkin, 1988). F2 male progeny from individual self-fertilized F1 hermaphrodites were scored for suppression of the *mab-3(e1240)* phenotype (presence of V rays). Hermaphrodite siblings of affected males were selected, and their progeny were scored in the F3 generation to confirm suppression of *mab-3(e1240)* and, where possible, to identify lines homozygous for *mab-3*-suppressing recessive mutations.

Identification and Sequence Analysis of *mes-3* Alleles

The maternal-effect sterile alleles *ez1, ez10, and ez12* were mapped to LGI by standard genetic linkage analysis. A complementation test versus the *mes-3(bn35)* null mutation determined that *ez1, ez10, and ez12* are alleles of *mes-3*. PCR products containing the exons and exon/intron boundaries of the *mes-3* gene were amplified from genomic DNA from *mes-3(ez1, ez10, and ez12)* mutants. The same primers used for PCR were used to sequence each *mes-3* exon with an ABI Prism automated DNA sequencer.

Sequencing identified mutations in all three *mes-3* alleles, as follows: *mes-3(ez1)*, exon 1, nt T2 → A, aa M1 → K; *mes-3(ez10)*, exon 7, nt G2677 → A, 5' splice site mutation; *mes-3(ez12)*, exon 10, nt C3464 → T, nonsense at R431.

All three lesions appear likely to be strong loss-of-function or null alleles and behave as such genetically (data not shown).

Analysis of V Ray Phenotypes

V rays were identified in adult males on the basis of their shape, their position on the body axis, and the orientation of their sensory openings. Ectopic and anteriorly shifted rays were scored as those rays found outside the normal domain of ray formation by a distance more than four times the average wild-type distance between ray 2 and ray 1. For ectopic ray counts, males were scored only if alae were fully visible along the posterior half of the body.

Antibody Staining

him-8(e1489), *mes-2(bn27)*; *him-8(e1489)*, and *mes-6(bn66)* *him-8(e1489)* L2 and L3 larvae were fixed and stained with MES-2 and MES-6 antisera as described previously (Holdeman et al., 1998; Korf et al., 1998). MES-2 and MES-6 antisera were kind gifts from S. Strome. Secondary antibodies used in this study were Alexa 568-conjugated goat anti-rabbit IgG (Molecular Probes; diluted 1:5000 for MES-6 and 1:2000 for MES-2) or Cy3-conjugated goat anti-rabbit IgG (Jackson Immunochemicals; diluted 1:500 for MES-6). DAPI, at 0.5 µg/ml, was used to stain DNA. The developmental stage of larvae was determined by time of growth at 20°C, by gonad morphology, and, for males, by tail morphology.

Acknowledgments

We thank Julie Ahringer, Scott Emmons, Rik Korswagen, and Susan Strome for strains and reagents and Vivian Bardwell, Weiru Chang, Kara Thoenke, Susan Strome, and members of the Strome lab for critical reading of the manuscript. We thank Susan Strome, Jeffrey Simon, and members of the Zarkower lab and the University of Minnesota Center for Developmental Biology for many helpful discussions and Scott Emmons for sharing results prior to publication. Some strains used in this work were provided by the *C. elegans* Genetics Center, which is funded by the NIH-NCRR. J.M.R. is supported by a University of Minnesota Graduate School Fellowship. This work was supported by a grant from the NIH (GM53099) to D.Z.

Received: February 11, 2003

Revised: March 17, 2003

Accepted: March 20, 2003

Published: June 2, 2003

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