Polycomb-like genes are necessary for specification of dopaminergic and serotonergic neurons in *Caenorhabditis elegans*

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The molecular mechanisms underlying the formation of neurons with defined neurotransmitters are not well understood. In this study, we demonstrate that the *PcG*-like genes in *Caenorhabditis elegans*, *sop-2* and *sor-3*, regulate the formation of dopaminergic and serotonergic neurons and several other neuronal properties. *sor-3* encodes a novel protein containing an MBT repeat, a domain that contains histone-binding activity and is present in PcG proteins SCM and Sfmbt in other organisms. We further show that mutations in *sor-3* lead to ectopic expression of Hox genes and cause homeotic transformations. Specification of certain neuronal identities by these *PcG*-like genes appears to involve regulation of non-Hox gene targets. Our studies revealed that the *PcG*-like genes are crucial for coordinately regulating the expression of discrete aspects of neuronal identities in *C. elegans*.

Hox genes | *PcG* | *sop-2* | *sor-3*

Elucidation of the genetic circuitry that coordinately regulates the generation of the generic and specific phenotypes of neurons with different types and origins is a fundamental issue in understanding the formation of the exquisitely complex nerve circuits in various organisms. One important feature of differentiated neurons is the distinct neurotransmitter produced. Common examples of neurotransmitters include dopamine (DA) and serotonin (5-HT), which control multiple behavior processes (1). The molecular mechanisms by which the neurotransmitters are specified are not well understood.

The development of *Caenorhabditis elegans* male ray neurons provides a simple system for exploring how distinct neurotransmitters are specified and how discrete developmental programs of neurons are coordinately regulated (2). Each finger-like sensory ray consists of the dendritic endings of two distinct neurons, an A-type neuron (RnA) and a B-type neuron (RnB), wrapped in the process of a glial-like structural cell (Rnst) (where n stands for rays 1–9). A subset of A-type neurons, R5A, R7A, and R9A, express the neurotransmitter dopamine, whereas a subset of B-type neurons, R1B, R3B, and R9B, express serotonin. Previous studies show that a TGF-β signaling pathway and Hox gene *egl-5* (ortholog of *Drosophila Abd-B*) play important roles in the specification of dopaminergic and serotonergic ray neurons (3, 4). The identification of additional genes involved in specifying neurotransmitter phenotype may contribute to our understanding of the molecular mechanisms underlying the formation of neurons with defined neurotransmitters in other organisms.

Polycomb group proteins (PcG) are most studied for their roles in specifying positional identity through their transcriptional repression of Hox genes (5). Consistent with their roles in transcriptional repression, PcG proteins mediate the formation of repressive chromatin structures by modifying the histone tails (5). For example, the PRC1 PcG complex functions as an E3 ubiquitin ligase for H2A, and the PhoRC complex contains binding activity for methylated H3K9 and H4K20 (6, 7). *PcG* genes also regulate the expression of non-Hox genes, including factors involved in development and differentiation processes, such as TGF-β and Wnt (8). The roles of *PcG* genes in neuronal specification have yet to be determined.

Global repression of Hox genes in *C. elegans* is mainly mediated by the SOP-2/SOR-1 PcG-like complex (9). Despite a lack of obvious sequence similarity, several conserved properties of the PRC1 complex and the SOP-2/SOR-1 complex, including the presence of the protein–protein interaction SAM domain and RNA binding activity, suggest that they employ conserved mechanisms in Hox gene repression (9).

In this study, we characterize a new *PcG*-like gene in *C. elegans*, *sor-3*, which encodes a novel protein containing an MBT domain. We further demonstrate that the *sop-2* and *sor-3* are involved in specifying neurotransmitter phenotype and several other neuronal properties, including axon pathfinding. Specification of certain neuronal identities by *sop-2* and *sor-3* involves regulation of non-Hox gene targets. Our studies revealed a novel function of *PcG*-like genes in specifying neuronal fate.

Results

Mutations in *sor-3* and *sop-2* Lead to Generation of Ectopic Dopaminergic and Serotonergic Neurons. In WT males, ray neurons express stereotyped neurotransmitter phenotype. Dopaminergic fate is restricted to RnA neurons of rays 5, 7, and 9. Serotonin is synthesized by the RnB neurons of rays 1, 3, and 9. These dopaminergic and serotonergic neurons can be visualized in living animals by *cat-2*::*yfp*, a reporter gene for dopamine biosynthetic enzyme tyrosine hydroxylase, and *tph-1*::*cfp*, a reporter gene for the serotonin biosynthetic enzyme tryptophan hydroxylase, respectively (Fig. 1 *A* and *D*) (3, 10). Two mutations, *bp185* and *bp186*, were isolated in screens to identify mutants with abnormal expression of *cat-2*::*yfp* or *tph-1*::*cfp* in male ray neurons. Subsequent complementation analyses revealed that *bp186* defines an allele of *sop-2*, whereas *bp185* defines a new gene *sor-3* (*sop-2* related -*3*). In *sor-3* and *sop-2* mutants, the dopaminergic and serotonergic ray patterning is disrupted in a similar way: *cat-2*::*yfp* is ectopically expressed in the A-type neurons of rays 4 and 8 (R4A and R8A) (Table 1 and Fig. 1 *B* and *C*), and *tph-1*::*cfp* is ectopically expressed in the B-type neurons of rays 5 and 7, and also in ray 4 at a low frequency (Table 1 and Fig. 1 *E* and *F*).

As in *sop-2*(*bx91*) mutants (9), mutations in *sor-3* also affect the generation of serotonergic CP neurons in the male ventral cord. WT males contain six serotonergic CP neurons, derived from P(3–8).aapp (Fig. 1*G*). However, the average number of CP neurons is 6.97 (n = 186, ranging from 5 to 9) in *sor-3*(*bp185*) mutants and 4.65 (n = 101, ranging from 0 to 8) in *sop-2*(*bp186*)

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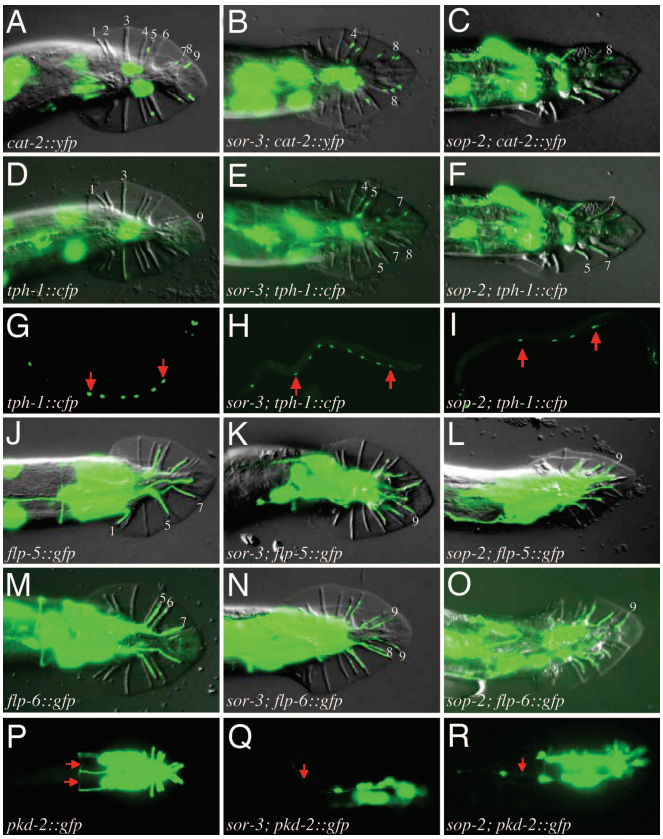

Fig. 1. Expression of neurotransmitter phenotypes in *sor-3* and *sop-2* mutants. (*A*) Expression of *cat-2*::*yfp* in dopaminergic ray neurons. *cat-2*::*yfp* is expressed in rays 5, 7, and 9. (*B* and *C*) Ectopic expression of *cat-2*::*yfp* in a *sor-3* (*B*) and a *sop-2*(*bp186*) (*C*) male tail. The specific rays with ectopic expression of *cat-2*::*yfp* are indicated. (*D*) Expression of *tph-1*::*cfp* in male rays. *tph-1*::*cfp* is expressed in rays 1, 3, and 9. (*E* and *F*) Ectopic expression of *tph-1*::*cfp* in a *sor-3* (*E*) and a *sop-2*(*bp186*) (*F*) male tail. (*G*) Expression of *tph-1*::*cfp* in six serotonergic CP neurons in a WT male ventral cord. (*H* and *I*) Altered number of *tph-1*::*cfp*-positive neurons in a *sor-3* (*H*) and a *sop-2*(*bp186*) (*I*) male. In the *sor-3* male shown here, eight CP neurons are generated (between arrows). In the *sop-2* male shown here, only four CP neurons are generated (between arrows). (*J*) Expression of *flp-5*::*gfp* in male rays. *flp-5*::*gfp* is expressed in rays 1, 5, and 7. (*K* and *L*) Ectopic expression of *flp-5*::*gfp* in a *sor-3* (*K*) and a *sop-2*(*bp186*) (*L*) male. (*M*) Expression of *flp-6*::*gfp* in male rays. *flp-6*::*gfp* is expressed in rays 5, 6, and 7. (*N* and *O*) Ectopic expression of *flp-6*::*gfp* in a *sor-3* (*N*) and a *sop-2*(*bp186*) (*O*) male. (*P*) PKD-2::GFP marks the axons of B-type neurons of all of the rays. R1B has distinct pathfinding route (arrow). (*Q* and *R*) Defects in the axon pathfinding of R1B in a *sor-3* (*Q*) and a *sop-2*(*bp186*) (*R*) male. The R1B axons in both mutants fail to make a turn to the ventral side and continue migrating toward the anterior body region. The color of both *cfp* and *yfp* is shown in green in the figures for easy viewing.

mutants (Fig. 1 *H* and *I*). Thus, the role of *sop-2* and *sor-3* in specification of neurotransmitter phenotype is not restricted in male ray neurons.

In addition to altering neurotransmitter patterning, *sor-3*(*bp185*) and *sop-2*(*bp186*) cause defects in other tissues. *sor-3*(*bp185*) and *sop-2*(*bp186*) animals are long (Lon) and exhibit defects in distal tip cell (DTC) migration, indicating that *sor-3* and *sop-2* play important roles in regulating many biological processes.

***sor-3* and *sop-2* Regulate the Specification of Other Types of Neurotransmitters.** In addition to dopamine and serotonin, *C. elegans* contains a complex profile of FMRFamide-like (FaRP) neuropeptide in RnB neurons (4). We found that the specification of FaRP neuronal fate is also affected in *sor-3* and *sop-2* mutants. In WT males, *flp-5* is expressed in the B-type neurons of rays 1, 5, and 7 (Fig. 1*J*), whereas *flp-6* is expressed in the B-type neurons of rays 5, 6, and 7 (Fig. 1*M*). In *sor-3* and *sop-2* mutants, *flp-5*::*gfp* is ectopically expressed in rays 4, 8, and 9 and *flp-6* is ectopically expressed in rays 2, 8, and 9 (Table 1 and Fig. 1 *K*, *L*, *N*, and *O*).

We also examined the generation of GABAergic neurons in *sor-3* and *sop-2* mutant L1 larvae. In a WT animal, 26 GABAergic neurons can be visualized by *unc-25*::*gfp*, a reporter for the GABA decarboxylase gene (11). In a *sor-3* and *sop-2*(*bp186*) mutant, the number of UNC-25::GFP positive neurons is also 26 ($n > 80$). Therefore, *sor-3* and *sop-2* only regulate the development of a subset of neurotransmitters.

***sor-3* and *sop-2* Are Required for Ray Neuron Axon Guidance.** To determine whether mutations in *sor-3* and *sop-2* affect other aspects of neuronal identities, the selection of axon pathfinding was examined in *bp185* and *bp186* mutants. We used a *pkd-2*::*gfp* transgene to visualize the axon commisure of B-type ray neurons (12). Ray neuron cell bodies, located in the lumbar ganglia, send out axons to the ventrally located preanal ganglion. We determined the trajectory followed by R1B, which has the distinct route from other rays and can be easily identified. The axon of R1B first migrates anteriorly from the lumbar ganglion and then makes an abrupt turn to progress around the body. Upon reaching the ventral side, it turns again toward the posterior and enters the preanal ganglion (Fig. 1*P*). In 33.8% ($n = 136$) of *sor-3*(*bp185*) and 20.3% ($n = 138$) of *sop-2*(*bp186*) male sides, the axons of R1B fail to make a turn toward the ventral side after growing out of the lumbar ganglion (Fig. 1 *Q* and *R*). Taken together, our results indicate that *sor-3* and *sop-2* regulate multiple aspects of neuronal identities, including neurotransmitter choice and axon pathfinding.

***sor-3* Encodes a Novel Protein with an MBT Domain.** *sor-3* was mapped on the linkage group X and cloned by transformation rescue (Fig. 2*A*). The minimal rescuing region, C04E7.2, encodes a protein of 531 aa (Fig. 2*B*). In *sor-3*(*bp185*) mutants, the CAA glutamine codon at position 469 is mutated to TAA ochre stop codon, deleting the C-terminal 62 aa (Fig. 2*B*). *sor-3*(*RNAi*) also leads to expression of ectopic dopaminergic and serotonergic fates among ray neurons, indicating that the defects in *bp185* are due to loss of *sor-3* gene function.

To gain insight into the functional properties of SOR-3, we searched for homologous domain using available databases and found that SOR-3 contains an MBT domain ($E < 10^{-4}$). MBT domain is a newly identified class of methyl-lysine-binding protein module and is present in PcG proteins Sex comb on midleg (SCM) and Sfmbt (Fig. 2*C*). The MBT domains have been shown to be crucial for the role of *Scm* and *dSfmbt* in Hox gene repression (7, 13). The presence of an MBT domain in SOR-3 suggests that it may regulate gene expression through modulating chromatin structure.

***sor-3* Is Ubiquitously Expressed.** To better understand how *sor-3* regulates ray neuron and other cell fates, we constructed a *sor-3*::*gfp* translational fusion gene. The reporter gene contains the entire coding sequence, promoter region, and the 3′ UTR, with *gfp* inserted in the C terminus of *sor-3*, and fully rescues *sor-3* mutant defects. SOR-3::GFP is observed in all cells in the developing embryos, larvae, and adult animals (Fig. 2 *D* and *E*). SOR-3::GFP is evident in both cytoplasm and nucleus (Fig. 2*F*). This wild expression pattern of *sor-3* is consistent with the pleiotropic defects seen in *sor-3* mutants.

***sor-3* Functions as a *PcG*-Like Gene.** The presence of an MBT domain in SOR-3 prompted us to examine whether *sor-3* is involved in Hox gene repression. We examined the expression of Hox gene *egl-5* reporter in *sor-3*(*bp185*) mutants. In a WT larva, *egl-5*::*gfp* expression is confined to cells in the tail (Fig. 3*A*) (14). However, in *sor-3* mutants, *egl-5* is ectopically expressed in many head neurons (Fig. 3*B*). There are an average number of 19.4 ($n = 16$) head neurons

Table 1. Neurotransmitter phenotype in *sor-3* and *sop-2* mutants

Genotype	Ray 1	2	3	4	5	6	7	8	9	*n*
cat-2::*yfp*										
WT	0	0	0	0	100	0	100	0	100	118
sor-3	0	0	1	16	96	1	94	59	100	186
egl-5; *sor-3*	0	0	0	0	0	0	100	33	100	114
dbl-1; *sor-3*	0	0	0	0	4	0	24	54	14	100
sop-2	0	0	0	36	100	0	99	26	100	101
sop-2; *egl-5*	0	0	0	0	0	0	100	26	100	116
sop-2; *sma-4*	0	0	0	0	3	0	7	28	15	69
tph-1::*cfp*										
WT	100	0	100	0	0	0	0	0	100	102
sor-3	96	0	100	0	51	0	24	0	100	152
dbl-1; *sor-3*	94	0	95	0	80	0	0	0	0	87
sop-2	76	0	98	22	88	0	46	0	100	50
sop-2; *dbl-1*	63	0	96	0	36	0	0	0	0	59
flp-5::*gfp*										
WT	100	0	0	0	100	0	100	0	0	128
sor-3	36	2	3	16	99	2	100	11	75	122
sop-2	40	2	7	2	100	0	100	9	94	86
flp-6::*gfp*										
WT	0	0	0	0	100	22	99	0	0	100
sor-3	5	46	4	5	100	94	100	43	83	112
sop-2	2	31	0	14	97	74	97	17	62	106

The frequency of rays ectopically expressing neurotransmitter reporters varies among different temperatures. Animals grown at 20° C were examined for the expression of *cat-2*::*yfp* and *tph-1*::*cfp* and animals grown at 22.5° C were scored for the expression of *flp-5*::*gfp* and *flp-6*::*gfp*. Ray 1 sometimes is missing in *sor-3* and *sop-2* mutants, especially at higher temperatures. Low frequency of expression of *tph-1*::*cfp* in rays 5 in *sop-2*; *dbl-1* animals may be due to the weak expression of this reporter in the double mutants. Mutations in *dbl-1* and *sma-4* result in loss of expression of *cat-2*::*yfp* in rays 5, 7, and 9. In the *egl-5* mutants, expression of *cat-2*::*yfp* and *tph-1*::*cfp* is lost in rays derived from V6 (rays 2 to 6). *sor-3*(*bp185*) and *sop-2*(*bp186*) mutant alleles are used in this study.

expressing *egl-5*::*gfp* in *sor-3* mutants at 21°C. Ectopic expression of Hox genes in *sor-3* leads to homeotic transformations. For example, rays, a fate normally adopted by the posterior seam cells, are generated from the anterior seam cells in *sor-3* mutants (Fig. 3*C*). The ectopic expression of *egl-5* in head neurons was first detected at the threefold stage when the initial expression pattern of *egl-5* has been established, indicating that the expression of *egl-5* is initiated correctly but is later derepressed in *sor-3* mutants. Taken together, *sor-3* functions as a *PcG*-like gene in other systems in maintaining the repressed state of Hox genes.

***sor-3* and *sop-2* Genetically Interact.** The similar roles of *sor-3* and *sop-2* in both specification of neurotransmitter phenotype and Hox gene repression suggest that they may function together in gene regulation. Indeed, mutations in *sop-2* and *sor-3* have synthetic genetic interactions. Because *sop-2*(*bp186*); *sor-3*(*bp185*) double mutants are lethal, *sor-3*(*RNAi*) was used to examine the genetic interactions between *sop-2* and *sor-3*. Both *sop-2*(*bp186*) and *sor-3*(*RNAi*) animals ($n > 500$ for each) are viable at 20°C. However, 52%, 35%, and 13% of *sop-2*(*bp186*); *sor-3*(*RNAi*) animals ($n = 1{,}257$) are arrested at the L1, L2, and L3 stage, respectively. Furthermore, the expression domains of *egl-5* are dramatically expanded in *sop-2*(*bp186*); *sor-3*(*RNAi*) double mutants. There are an average of 6.8 ($n = 18$) and 16.8 ($n = 22$) head neurons expressing *egl-5*::*gfp* in *sor-3*(*RNAi*) and *sop-2*(*bp186*) mutants at 20°C, respectively, whereas an average of 31.4 ($n = 18$) head neurons express *egl-5*::*gfp* in *sop-2*(*bp186*); *sor-3*(*RNAi*) animals (Fig. 3 *D–F*). The synthetic interactions between *sop-2*(*bp186*) and *sor-3*(*RNAi*) suggest that they could function together in gene regulation or, alternatively, that they act in parallel pathways.

***sop-2* Specifies Ectopic Dopaminergic Ray Fate During an Interval Between the Late-L2 and Mid-L3 Stages.** To determine when the *PcG*-like genes act to specify the dopaminergic neuronal fate, we carried out temperature-shift experiments with the temperature-sensitive allele of *sop-2*, *bx91*. *sop-2*(*bx91*) males grown at 15°C for their entire development display the WT number and pattern of dopaminergic ray neurons. By contrast, growth of *sop-2*(*bx91*) males at 21°C results in ectopic expression of dopaminergic fate in rays 4 and 8. In upshift experiments, the embryos or larvae were grown at 15°C for a defined period then were shifted to 21°C for the remainder of their development. Once adults, males were examined for expression of *cat-2*::*yfp*. We found that shifting the animals before the late L2 larval stage results in ectopic expression of dopaminergic fate in rays 4 and 8. The number of rays 4 and 8 expressing *cat-2*::*yfp* is dramatically decreased in the animals shifted after the mid-L3 larval stage (Fig. 4). Consistent with this, in downshift experiments, shifting the animals before the late L2 larval stage dramatically decreases the number of rays 4 and 8 expressing *cat-2*::*yfp* (Fig. 4). The ray neurons are born at the early L4 larval stage (Fig. 4). Thus, *sop-2* needs to be inactivated four to five cell generations before ray neurons are born to cause the generation of ectopic dopaminergic rays 4 and 8.

Deregulation of Hox Gene *egl-5* Is Not Sufficient for Inducing Ectopic Dopaminergic Ray Neurons. Because *egl-5* is misregulated in *sop-2* and *sor-3* mutants, we examined the role of *egl-5* in specification of dopaminergic ray neurons in these mutants. We found that neither ray 4 nor ray 5 expresses dopaminergic fate in *sop-2*; *egl-5* and *egl-5*; *sor-3* mutants (Table 1). *egl-5* could function as a permissive or an instructive role in specifying the dopaminergic rays 4 and 5. To distinguish between these two possibilities, we examined whether overexpression of *egl-5* in animals carrying an *egl-5* cDNA under control of a heat-shock promoter transgene could lead to generation of ectopic dopaminergic ray neurons. Transformation of ray identities and generation of ectopic rays in heat-shocked males

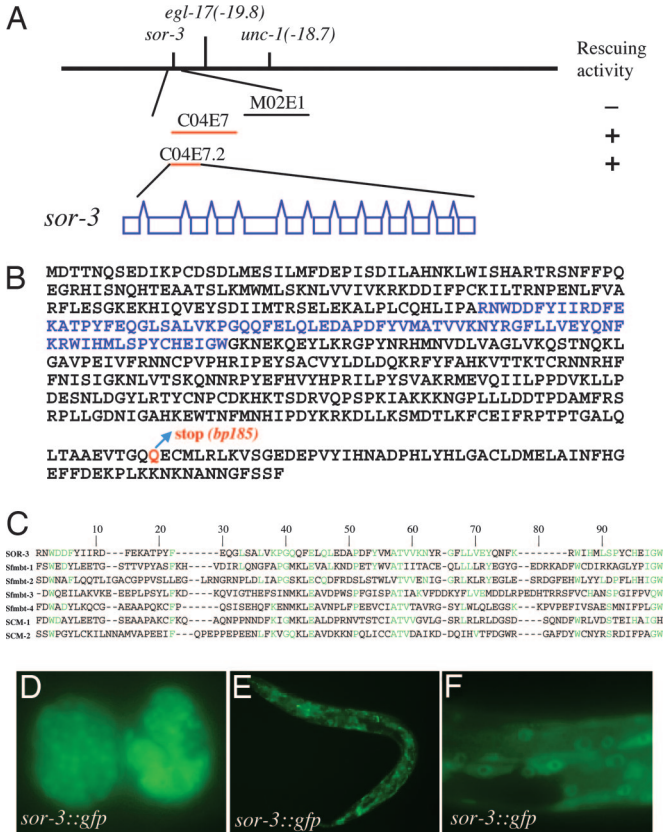

Fig. 2. Molecular structure of *sor-3* and its expression pattern. (*A*) Cosmid C04E7 and the PCR fragment containing C04E7.2 can fully rescue the mutant defects in *sor-3*, whereas the neighboring cosmids F52D1, ZK1193, M02E1, and F38G1 cannot rescue the *sor-3* mutant defects. The cDNA sequence of *sor-3* was confirmed by sequencing RT-PCR products. Sixty-three base pairs in the predicted *sor-3* gene are not present in our cDNAs. The length of the introns and exons is not to scale. (*B*) Protein sequence of SOR-3. *sor-3*(*bp185*) contains a glutamine to stop-codon mutation, deleting the C-terminal 62 aa. The MBT domain is marked in blue. (*C*) Alignment of the MBT domains found in SOR-3, mouse PcG protein Sfmbt, and fly PcG proteins Sex combs on midleg (SCM). The identical amino acids found in the SOR-3 MBT domain and at least in one other MBT domain are highlighted. (*D* and *E*) Expression of *sor-3*::*gfp* in developing embryos (*D*) and larvae (*E*). *sor-3* is expressed in all cells examined. (*F*) SOR-3 is localized in both nucleus and cytoplasm but excluded from nucleolus.

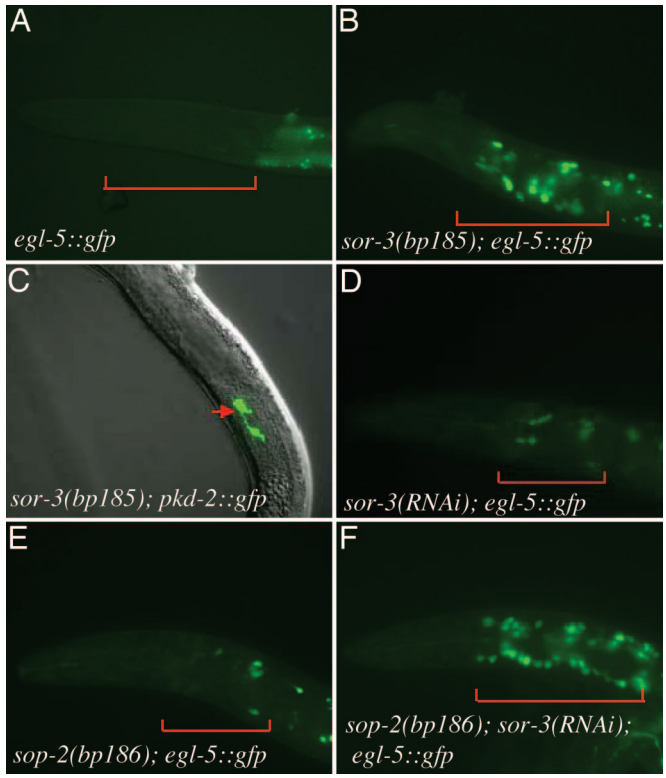

Fig. 3. *sor-3* functions as a *PcG* gene and synergistically interacts with *sop-2*. (*A*) In a WT L2 larva, the expression of *egl-5* is restricted to the tail (not shown) and is absent in the head (marked with bar). (*B*) Ectopic expression of *egl-5* in a *sor-3* mutant. *egl-5* is expressed in many head neurons (marked with bar). (*C*) Instead of being confined to the nine pairs of rays in the tail (not shown), *pkd-2*::*gfp* is ectopically expressed in the middle body region in a *sor-3* male, indicating that ectopic rays are generated. (*D*) Expression of *egl-5*::*gfp* in the head of a *sor-3*(*RNAi*) mutant (marked with bar). (*E*) Expression of *egl-5*::*gfp* in the head of a *sop-2*(*bp186*) mutant (marked with bar). (*F*) Dramatically expanded expression domain of *egl-5*::*gfp* in the head region in a *sop-2*; *sor-3*(*RNAi*) mutant (marked with bar).

confirms ectopic expression of *egl-5* in the tail (Fig. 5*A*). However, ectopic expression of *egl-5* at various developmental stages does not lead to ray 4 expressing *cat-2*::*yfp* (Fig. 5*B*). The expression of dopamine in ray 8, which is derived from seam cell T, remains unaltered in *sop-2*; *egl-5* and *egl-5*; *sor-3* mutants, consistent with the notion that Hox genes are not involved in T ray patterning (2). *cat-2*::*yfp* is also not expressed in ray 8 in heat-shocked animals carrying a *hs*::*egl-5* transgene. Ectopic rays, such as duplication of rays 4, 5, and 6, are generated in the heat-shocked animals carrying a *hs*::*egl-5* transgene, and some of these ectopic rays express *cat-2*::*yfp* (Fig. 5*B*). In conclusion, ectopic expression of *egl-5* is not sufficient to promote ectopic expression of dopaminergic fate in rays 4 and 8. It is possible that mutations in *sor-3* and *sop-2* cause deregulation of multiple factors involved in determining neurotransmitter phenotype.

A TGF-β Signaling Pathway Contributes to Generation of Ectopic Dopaminergic and Serotonergic Ray Neurons in *sop-2* and *sor-3* Mutants. Development of dopaminergic and serotonergic ray neurons is regulated by a TGF-β signaling pathway that includes *dbl-1* (encoding the ligand), *sma-6* and *daf-4* (encoding the type I and type II receptor, respectively), and *sma-2*, *-3*, and *-4* (encoding SMAD) (15). Mutations in pathway component genes result in a dramatic reduction in dopaminergic fate expression in rays 5, 7, and 9. Conversely, elevated expression of the pathway ligand, by means of a heat-shock transgene, induces ectopic dopaminergic fate in ray neurons, notably at the highest frequency in ray 4 (3). We found that removal of the DBL-1 pathway has different effects on different rays in *sop-2* and *sor-3* mutants: ray 4 ectopic dopaminergic fate is abolished, whereas the ray 8 ectopic dopaminergic fate is unaffected (Table 1). TGF-β signaling has no effect on the generation of CP neurons but is required for the expression of serotonin in ray 7 in *sor-3* and *sop-2* mutants. Thus, ectopic dopaminergic fate in ray 4 and serotonergic fate in ray 7 could be due either to changes in intrinsic activity of the pathway components within these rays or exposure to elevated levels of pathway ligand. In this regard, it is interesting that *sop-2* and *sor-3* exhibit a long body phenotype (Lon), which can be induced by elevated expression of the DBL-1 pathway.

Hox genes *egl-5* and *lin-39* Specify the Serotonergic CP Neurons in *sop-2* and *sor-3* Mutants. As mentioned above, *sor-3* males exhibit a variable number of serotonergic CP neurons. In WT males the CP neurons are specified by Hox gene *lin-39* (16). In *lin-39*; *sor-3* mutants ($n > 100$), no *tph-1*::*cfp*-positive CP neurons are present, indicating that *lin-39* activity is still required for generation of CP

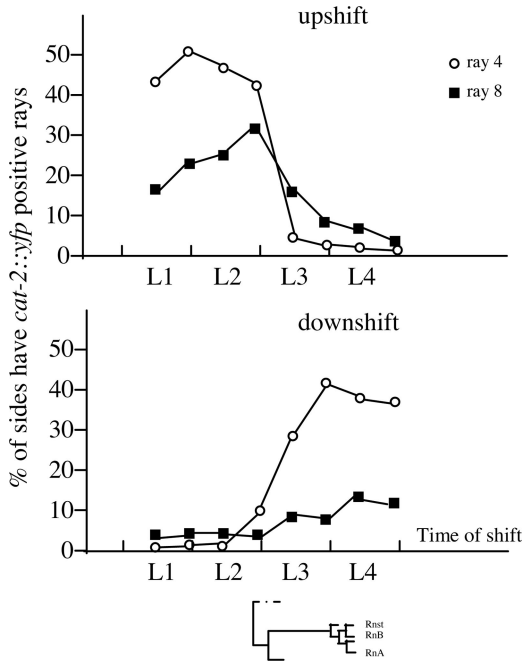

Fig. 4. Timing of action of *sop-2* in specifying dopaminergic rays 4 and 8. *sop-2*(*bx91*) mutants carrying *cat-2*::*yfp* were shifted either from 15°C to 21°C (upshift) or from 21°C to 15°C (downshift) at various developmental stages. Ectopic YFP-positive rays were scored (number of sides scored at each stage for upshift and downshift, respectively: mL1, 62 and 53; lL1, 101 and 59; mL2, 65 and 26; lL2, 54 and 62; mL3, 98 and 51; lL3, 113 and 55; mL4, 61 and 57; lL4, 86 and 34).

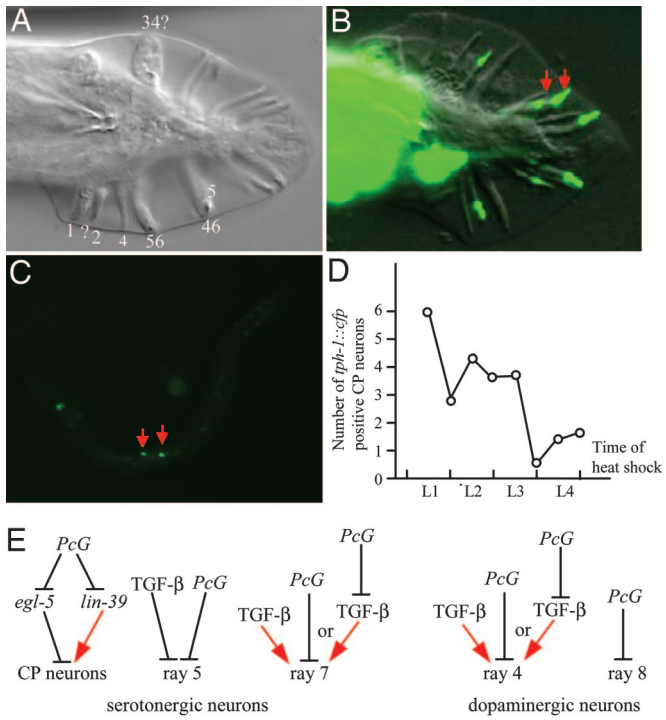

Fig. 5. Role of *egl-5* in specifying the dopaminergic ray neurons and serotonergic CP neurons. (*A*) Formation of abnormal rays in a heat-shocked male carrying a *hs*::*egl-5* transgene. In the upper side of the male tail, rays 3, 4, and an ectopic ray fuse together. On another side, ray 1 fuses with an ectopic ray, and rays 4, 5, and 6 are duplicated, which is characteristic of ectopic expression of *egl-5* in the V6 lineage (21). (*B*) Absence of expression of *cat-2*::*yfp* in ray 4 in a heat-shocked animal carrying a *hs*::*egl-5* transgene. Ectopic rays are generated between rays 6 and 9, and some of them express *cat-2*::*yfp* (arrow). The expression of *tph-1*::*cfp* is very weak in ray neurons and is not followed in this study. (*C*) Overexpression of *egl-5* results in generation of less serotonergic CP neurons. Two CP neurons are generated in the animal shown here (arrow). (*D*) Number of serotonergic CP neurons generated in the animals carrying a *hs*::*egl-5* transgene that were heat shocked at various developmental stages. Each circle represents one stage of worms scored (number of worms scored: mL1, 48; lL1, 28; mL2, 18; lL2, 30; mL3, 31; lL3, 10; mL4, 6; lL4, 18). (*E*) Model for the concerted action of TGF-β signaling and *PcG* in specification of serotonergic and dopaminergic neurons.

neurons in *sor-3* mutants. Because the expression of *lin-39* is restricted in middle body region (16), generation of extra *tph-1*::*cfp*-positive neurons by anterior and posterior P cells in the ventral cord in *sor-3* mutants suggests that the expression domain of *lin-39* is expanded beyond its normal range. We showed previously that mutations in *egl-5* resulted in the increased number of CP neurons in *sop-2* mutants (9), suggesting that ectopic expression of *egl-5* negatively regulates the fate of CP neurons.

To test this hypothesis, we examined whether ectopic expression of *egl-5* using the heat-shock *egl-5* transgenic strain described above has an effect on CP neuron number. Before heat shock, six CP neurons are generated. The average number of CP neurons, however, is reduced when animals carrying the transgene were heat shocked after the mid-L1 larval stages (Fig. 5 *C* and *D*). When the animals are heat-shocked at the late L3 stages, almost no *tph-1*::*yfp*-positive neurons in the ventral cord are generated. Overexpression of *egl-5* at the L4 stage is still effective in blocking the formation of serotonergic CP neurons (Fig. 5*D*). The CP neurons are born at the late L3 stage. Thus, *egl-5* is likely to repress the differentiation of CP neurons. In contrast, we found that ectopic expression of *lin-39* at the L4 stage using the heat-shock *lin-39* transgene increased the number of CP neurons from 6 to an average number of 7.0 ($n = 10$). Taken together, our results indicate that the variable number of serotonergic CP neurons generated in *sor-3* and *sop-2* mutants is due to alteration in the normal boundaries of *egl-5* and *lin-39* in the ventral nerve cord.

Discussion

***sor-3* Functions as a *PcG*-Like Gene.** In this study, we showed that *sor-3* functions as a *PcG*-like gene in maintaining the repressed state of Hox genes outside its normal expression domain. *sor-3*, however, differs from *sop-2* in several ways in regulating the expression of Hox genes. The expression level of Hox genes in *sor-3* mutants, especially *mab-5*, is less severe than the one in *sop-2* mutants. SOP-2 and SOR-3 also display distinct cellular localization pattern. SOP-2 is localized in nucleus and forms distinct nuclear bodies (9). SOR-3 is localized in both nucleus and cytoplasm and does not form nuclear bodies. Thus, SOR-3 is unlikely to be an integral component of the SOP-2/SOR-1 PcG-like complex (17). Mutations in different *PcG* genes in fly and other organisms also have been shown to cause differential misexpression of Hox genes (18). Components in different PcG complex in fly and mammals show unique cellular localization pattern. For example, core components of the PRC1 complex, including PC and PH, form nuclear bodies, whereas components of the ESC/E(Z) complex are homogeneously distributed in the nucleus (19). The presence of an MBT domain in SOR-3 raises the possibility that SOR-3 may constitute a PhoRC-like PcG complex in *C. elegans*. The similar defects in *sop-2* and *sor-3* mutants suggest that, in addition to Hox genes, they have other targets in common. How SOR-3 and the SOP-2/SOR-1 complex function together in gene regulation remains to be investigated.

Role of *PcG*-Like Genes in Specifying the Neurotransmitter Phenotype. We revealed a novel function of *PcG*-like genes in specifying neuronal fate in *C. elegans*. In regulating the neurotransmitter phenotype, *sop-2* and *sor-3* could act directly on a set of genes inextricably linked to specify the neuronal identities, such as *cat-2*.

Alternatively, *sop-2* and *sor-3* could regulate these genes indirectly via other transcription factors such as the Hox genes or the SMADs of the DBL-1 pathway. Hox genes *egl-5* and *lin-39* appear to be one of the factors that mediate the effects of *sop-2* and *sor-3* in specification of serotonergic CP neurons (Fig. 5*E*). In the context of ray neuron patterning, it is likely that the Hox genes are one of many targets of *sop-2* and *sor-3* and that the mutant phenotypes generated in their mutants represent the combined activity of several deregulated targets. Another possibility of the involvement of *sop-2* and *sor-3* in specification of neuronal identities is that they regulate cellular plasticity (20). In *sop-2* and *sor-3* mutants the cells are capable of responding to the preexisting signaling, such as TGF-β, in generation of dopaminergic and serotonergic neurons. Given the similarity between the SOP-2 and the PRC1 complex in other organisms, it is important to investigate the role of *PcG* genes in specifying neurotransmitter and other aspects of neuronal identities in other organisms.

Materials and Methods

Strains. All strains carry the *him-5*(*e1490*) mutation, which gives rise to a high frequency of males in self-progeny. The following strains were used in this study. LGII: *sop-2*(*bx91*), *sop-2*(*bp186*), *juIs76*(*unc-25*::*gfp*), *dpy-10*(*e128*), *unc-4*(*e120*). LGIII: *lin-39*(*n1872*), *egl-5*(*u202*), *sma-2*(*e502*), *sma-4*(*e729*), *ynIs67*(*flp-6*::*gfp*). LGV: *dbl-1*(*wk70*), *bxIs14*(*pkd-2*::*gfp*, *pha-1*(+)); *bxIs16*(*cat-2*::*yfp*, *tph-1*::*cfp*), *egIs1*(*dat-1*::*gfp*), *ynIs24*(*flp-5*::*gfp*). LGX: *bxIs13*(*egl-5*::*gfp*, *lin-15*(+)), *sor-3*(*bp185*); *bpEx5*(*hs*::*egl-5*), *bpEx9*(*hs*::*lin-39*).

Identification, Mapping, and Cloning of *sor-3*. Strain HZ659 [*bxIs16* (*cat-2*::*yfp*, *tph-1*::*cfp*); *him-5*(*e1490*)] was used for mutagenesis, and F_2 males were screened. From 9,000 genomes screened, 10 mutations were identified that cause altered expression pattern of *cat-2* and *tph-1* reporters. Three factor mapping placed *sor-3* between *egl-17* and the left end of the chromosome X. None of the 55 Egl nonUnc recombinants from + *egl-17 unc-1*/*sor-3* + + cross carries *sor-3*(*bp185*). Cosmids from this region were coinjected with pRF4 marker for transformation rescue experiments. The PCR fragment containing C04E7.2 rescues defects in *sor-3*(*bp186*) mutants; 93% (n = 164) of Rol transformants have six CP neurons. Only 0.6% and 3.0% of Rol male sides (n = 120) show expression of *cat-2*::*yfp* in rays 4 and 8, respectively.

Identification of *bp186* as an Allele of *PcG*-Like Gene *sop-2*. *bp186* was mapped on linkage group II in a genetic interval containing *sop-2*, a previously characterized *PcG*-like gene (9). All F_1 progeny from the *bp186* and *sop-2*(*bx91*) cross are arrested at early larval stages at 25°C, suggesting that *bp185* is a new allele of *sop-2*. We sequenced *bp186* and found that it contains a glutamic acid to lysine mutation at amino acid 689.

***sor-3* Reporter Gene.** The *sor-3*::*gfp* reporter contains the DNA derived from C04E7 (nucleotides 16168–24211), which includes a 3-kb promoter region, the entire ORF, and 1.2-kb 3′ UTR of *sor-3*. The *gfp* is inserted at the C terminus of *sor-3*. At least two stable transgenic lines were characterized.

RNAi. Single-stranded RNA (ssRNA) corresponding for *sor-3* was transcribed from the T7 and SP6-flanked PCR templates (C04E7, nucleotides 21082–21882). The ssRNAs were then annealed and injected into *bxIs16*, *bxIs13*, or *sop-2*(*bp186*); *bxIs13* animals. F_1 progenies generated 4 h after injection were scored for phenotypes. In *sor-3*(*RNAi*) animals, 27.6% (n = 29) of males have abnormal number of CP neurons, and 11.4% and 26.4% of male sides (n = 424) showed ectopic expression of *cat-2*::*yfp* in rays 4 and 8, respectively.

Heat-Shock and Temperature-Shift Experiments. For heat-shock experiments, animals carrying a *hs*::*egl-5* transgene were used. Eggs laid in a 1-h period were collected and grown at 20°C. Animals were subjected to heat shock at 33°C for 2 h at various developmental stages, allowed to develop to adulthood at 20°C, and examined for expression of *cat-2*::*yfp* and *tph-1*::*cfp*.

In the temperature-shift experiments, *sop-2*(*bx91*) animals grown at 15°C (for upshift experiments) or 21°C (for downshift experiments) at various developmental stages were shifted at 4-h intervals, and the adults were scored for expression of *cat-2*::*yfp* and *tph-1*::*cfp*.

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