

# Suppressors of the egg-laying defective phenotype of *sel-12* presenilin mutants implicate the CoREST corepressor complex in LIN-12/Notch signaling in *C. elegans*

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Presenilin is an essential component of the LIN-12/Notch signaling pathway and also plays a critical role in the genesis of Alzheimer's disease. Previously, a screen for suppressors of the egg-laying defective phenotype caused by partial loss of presenilin activity in *Caenorhabditis elegans* identified a number of new *spr* genes that are potentially involved in the regulation of LIN-12/Notch signaling or presenilin activity. Here we report the molecular identity of two *spr* genes, *spr-1* and *spr-5*. Our genetic analysis indicates that loss of *spr-1* elevates *lin-12/Notch* gene activity in many different cell fate decisions, suggesting that *spr-1* is a negative regulator of LIN-12/Notch signaling. Sequence analysis revealed that *spr-1* is an ortholog of human CoREST, a known corepressor. SPR-1 is localized to the nucleus and acts in a cell-autonomous manner; furthermore, human CoREST can substitute for SPR-1 in *C. elegans*. We also show that *spr-5* encodes a homolog of p110b, another known member of the CoREST corepressor complex. Our results suggest that the CoREST corepressor complex might be functionally conserved in worms, and we discuss the potential role of SPR-1 and SPR-5 in the repression of transcription of genes involved in, or downstream of, LIN-12/Notch signal transduction.

[*Keywords:* Notch; presenilin; CoREST; *lin-12*; polyamine oxidase; HDAC]

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Many cell-cell interactions that specify cell fate during animal development are mediated by receptors of the LIN-12/Notch family. When activated by transmembrane protein ligands of the Delta/Serrate/LAG-2 family produced by neighboring cells, the extracellular domain of LIN-12/Notch is cleaved near the membrane. This event in turn leads to cleavage within the LIN-12/Notch transmembrane domain, releasing the intracellular domain. Presenilins are essential for transmembrane cleavage. After its release, the intracellular domain translocates to the nucleus, where it forms a complex with the transcription factor known as LAG-1 in *Caenorhabditis elegans*, Suppressor of Hairless in *Drosophila*, or RBP-J or CBF1 (herein referred to generically as CBF1) in mammals. This complex, which also contains other proteins, can activate the expression of target genes (for review, see Weinmaster 2000).

In the absence of LIN-12/Notch signaling, CBF1 has

been shown to function as a transcriptional repressor. Indeed, transcriptional repression of Notch target genes in the absence of Notch signaling is important for correct cell fate patterning in *Drosophila* (Furriols and Bray 2000; Morel and Schweisguth 2000). Two-hybrid experiments showed that CBF1 can interact with different repressor proteins (Kao et al. 1998; Taniguchi et al. 1998; Hsieh et al. 1999), notably SMRT (silencing mediator of retinoid and thyroid hormone receptors) and histone deacetylase 1 (HDAC-1; Kao et al. 1998). CBF1 contains a repressor domain that becomes masked by association with the active intracellular part of LIN-12/Notch (Hsieh and Hayward 1995; Waltzer et al. 1995; Hsieh et al. 1996) and is needed for SMRT-mediated repression (Kao et al. 1998). Kadesch and colleagues have proposed that the intracellular domain of LIN-12/Notch proteins activates transcription by displacing a corepressor complex, thereby preventing it from interacting with CBF1 (Kao et al. 1998). Further work suggested that it also provides a transcriptional activation domain to recruit coactivators to function with CBF1 (Jarriault et al. 1995; Doyle et al. 2000; Petcherski and Kimble 2000a,b; Wu et al. 2000; Zhou et al. 2000; Kitagawa et al. 2001).

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Genetic screens in *C. elegans* and *Drosophila* have been effective in identifying conserved components of the LIN-12/Notch pathway and conserved factors that influence the activity of LIN-12/Notch proteins (for review, see Greenwald 1998). In *C. elegans*, genetic screens, based on suppression or enhancement of *lin-12* (*sel*) mutations, have defined many new *sel* genes (Sundaram and Greenwald 1993a; Levitan and Greenwald 1995; Tax et al. 1997). Isolation of the worm presenilin *sel-12* in such screens provided the first link between LIN-12/Notch signaling and presenilin activity (Levitan and Greenwald 1995); a second presenilin gene in *C. elegans*, called *hop-1*, is functionally redundant with *sel-12* (Li and Greenwald 1997; Westlund et al. 1999). There is intense interest in presenilin as a potential therapeutic target for the amelioration or prevention of Alzheimer's disease, because presenilin mediates transmembrane cleavage of  $\beta$ -amyloid precursor protein, a critical step in the generation of a peptide that can cause Alzheimer's disease (Kopan and Goate 2000).

Presenilin is also required for the transmembrane cleavage of LIN-12/Notch, an essential step for signal transduction by these receptors (De Strooper et al. 1999; Ray et al. 1999; Struhl and Greenwald 1999, 2001; Zhang et al. 2000). Because presenilin is critical for LIN-12/Notch signaling, the absence of both *sel-12* and *hop-1* activity causes phenotypes associated with the loss of the two *C. elegans* LIN-12/Notch genes, *lin-12* and *glp-1* (Li and Greenwald 1997; Westlund et al. 1999). However, because *sel-12* and *hop-1* are functionally redundant, mutants that lack only *sel-12* presenilin activity [*sel-12(-)*] have a relatively mild phenotype. *sel-12(-)* hermaphrodites are unable to lay eggs, a phenotype believed to be due to loss of *lin-12* activity in a subset of gonadal cells (Newman et al. 1995; Cinar et al. 2001).

As the *sel-12(-)* egg-laying defective (Egl) phenotype reflects reduced *lin-12* activity, genetic screens for suppressors of the Egl phenotype of *sel-12(-)* mutants might in principle identify factors that influence *lin-12* activity as well as factors that influence presenilin activity per se. These factors are likely to be negative regulators, as the suppressor mutation will have the net effect of elevating LIN-12/Notch or presenilin function. In a previous study, 14 mutations that act as highly penetrant suppressors of the Egl defect of *sel-12(ar171)* were identified (Wen et al. 2000). These 14 mutations defined at least four genes, called *spr*, for suppressor of presenilin. The analysis of one of these genes, *spr-2*, revealed that it encodes the *C. elegans* ortholog of mammalian SET, a protein that has been implicated in chromatin remodeling, as well as other biochemical processes (Wen et al. 2000).

Here, we report a detailed genetic and molecular characterization of the *spr* gene *spr-1*, and the identification of another *spr* gene, *spr-5*. SPR-1 and SPR-5 are orthologs of human CoREST and p110b, respectively. CoREST was first identified as a corepressor for the neural-restrictive silencing factor REST (Andres et al. 1999); p110b was identified in specific HDAC-containing complexes with CoREST (Humphrey et al. 2001; You et al. 2001; Hakimi

et al. 2002). CoREST and p110b are members of a core corepressor complex, so the observation that their homologs display *spr* activity suggests that the complex activity has been conserved in *C. elegans*. We show that *spr-1* acts in a cell-autonomous manner in specific *lin-12* expressing cells and is a functional ortholog of CoREST. Our genetic analysis indicates that *spr-1* activity negatively influences several different LIN-12/Notch signaling events in *C. elegans* development. Taken together, our results suggest that at least part of the CoREST corepressor complex might be functionally conserved in worms and influence LIN-12/Notch signaling. We discuss the function of SPR-1 and SPR-5 in the context of the important balance of repression and activation in LIN-12/Notch signal transduction.

## Results

After a screen of 27,600 mutagenized haploid genomes for suppressors of the *sel-12* presenilin Egl phenotype, four *spr* mutations were mapped to LG V (Wen et al. 2000). At that time, the name *spr-1* was assigned to *ar200*; the remaining three alleles, *ar201*, *ar205*, and *ar212*, were not assigned by complementation testing to the *spr-1* gene because under some conditions they display semidominance (detailed further in Materials and Methods). The molecular analysis described below has indicated that all four mutations are indeed alleles of *spr-1*, and they will be referred to as such throughout the text. Our analysis also indicates that these alleles reduce *spr-1* activity. Unless otherwise specified, the allele *spr-1(ar200)* was used for the genetic analysis. In all cell fate decisions analyzed, *spr-1(ar200)* displays a wild-type phenotype (see Materials and Methods).

### *The interaction between spr-1 and sel-12 is not allele-specific*

To rule out the possibility that *spr-1* acts as an informational suppressor of *sel-12(ar171)* (W225STOP) we examined its ability to suppress *sel-12(ar131)* (C60S), another *sel-12* loss-of-function allele (Levitan and Greenwald 1995). We found that *spr-1(ar200)* appears to be an efficient suppressor of *sel-12(ar131)* (Table 1), indicating that suppression is not *sel-12* allele-specific. In addition, we examined the ability of *spr-1(ar201)* and *spr-1(ar205)* to suppress the Egl phenotype of *sel-12(ar171)*. The three *spr-1* alleles display a similar degree of suppression and appear to suppress *sel-12(ar171)* at all temperatures (Table 1), suggesting that these *spr-1* alleles have a similar effect on *spr-1* activity.

### *spr-1 suppresses the $\pi$ cell defect caused by sel-12(ar171)*

In the gonad of wild-type hermaphrodites during the L3 larval stage, there are twelve descendants of the ventral uterine precursor cells (VUs) that express LIN-12 (Wilkinson and Greenwald 1995). The anchor cell (AC)

**Table 1.** *spr-1(ar200)* suppresses *Egl* phenotype of different *sel-12* alleles

Relevant genotype	15°C	20°C	25°C
	Egl <sup>+</sup> /total (%)	Egl <sup>+</sup> /total (%)	Egl <sup>+</sup> /total (%)
<i>sel-12(ar171)</i> <sup>a</sup>	0/39 (0%)	0/45 (0%)	2/60 (3.3%)
<i>spr-1(ar200); sel-12(ar171)</i> <sup>a</sup>	43/44 (97.7%)	169/179 (94.4%)	58/64 (90.6%)
<i>sel-12(ar131)</i> <sup>b</sup>	ND	1/44 (2.3%)	ND
<i>spr-1(ar200); sel-12(ar131)</i> <sup>b</sup>	ND	60/71 (84.5%)	ND
<i>spr-1(ar205); sel-12(ar171)</i> <sup>a</sup>	34/40 (85%)	73/73 (100%)	91/92 (98.9%)
<i>spr-1(ar201); sel-12(ar171)</i> <sup>a</sup>	37/38 (97.3%)	98/100 (98%)	ND

Egl<sup>+</sup>, wild-type egg-laying ability (see Materials and Methods). Others are unable to lay eggs (Egl). *spr-1* alone appears wild-type (see Materials and Methods). ND, not determined.

<sup>a</sup>Strains also contained *unc-1(e538)*.

<sup>b</sup>Strains also contained *dpy-11(e224)*.

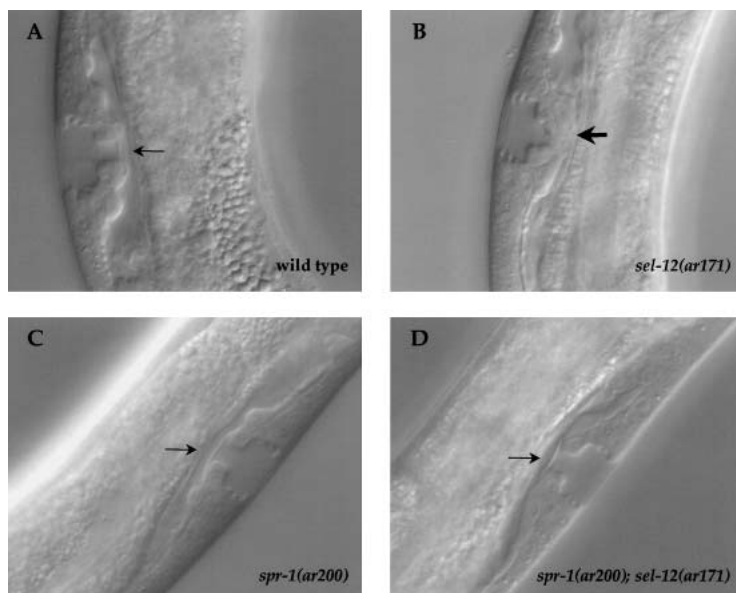
expresses LAG-2 (Newman et al. 2000), which activates LIN-12 in the six neighboring VU descendants to induce them to become  $\pi$  cells; the six VU descendants that do not have contact with the AC adopt the alternative  $\rho$  fate (Newman et al. 1995; Newman and Sternberg 1996). The  $\pi$  cells can be distinguished from the  $\rho$  cells by their morphology and position, by their division pattern, and by cell fate markers. The  $\pi$  cells produce 12 daughters. During the L4 stage, eight of these cells fuse with the AC to form the “utse”, a thin membrane at the junction of the vulva and the uterus that will be broken by the first egg laid (Fig. 1A; Newman et al. 1995, 1996). *lin-12* and *sel-12* loss-of-function mutants lack  $\pi$  cells, whereas more  $\pi$  cells are formed at the expense of the  $\rho$  cells in *lin-12* gain-of-function mutants (Newman et al. 1995; Cinar et al. 2001). The defect in  $\pi$  cell specification appears to be largely accountable for the Egl phenotype of *sel-12* hermaphrodites (Cinar et al. 2001).

Using two criteria, we have determined that *spr-1(ar200)* suppresses the  $\pi$  cell specification defect in *sel-12(ar171)* mutant. First, we analyzed the expression of a  $\pi$  cell fate marker, *lin-11::lacZ*. In wild-type hermaph-

rodites, *lin-11::lacZ* is expressed in the six  $\pi$  cells shortly before they divide, and remains on in their daughters (Newman et al. 1999). The number of cells expressing *lin-11::lacZ* is reduced in *sel-12(ar171)* mutants (Cinar et al. 2001; data not shown), but we found it to be normal in *spr-1(ar200)* and *spr-1(ar200); sel-12(ar171)* hermaphrodites (data not shown). Second, we examined the morphology of the vulval–uterine junction in young adults. In *sel-12* mutants, a thick tissue is observed at the vulva–uterus junction instead of the utse and the AC remains unfused (Fig. 1, cf. A and B; Cinar et al. 2001). As shown in Figure 1C and D, the morphology of the utse appears wild-type in *spr-1(ar200)* and *spr-1(ar200); sel-12(ar171)* mutants.

#### Suppression of the *sel-12* egg-laying defect requires *hop-1* activity

Loss of *hop-1* presenilin activity does not cause an overt phenotype, whereas loss of both *hop-1* and *sel-12* activity causes phenotypes that are similar to those caused by loss of *lin-12* and *glp-1* activity (Li and Greenwald 1997;



**Figure 1.** *spr-1; sel-12* hermaphrodites have a wild-type utse. The utse morphology was examined in (A) wild-type N2; (B) *sel-12(ar171)* (33/35 animals displayed a mutant morphology); (C) *spr-1(ar200)* hermaphrodites (0/24 animals displayed a mutant morphology); (D) *spr-1(ar200); sel-12(ar171)* (0/29 animals displayed a mutant morphology). A normal utse is indicated by a thin arrow; an abnormal utse by a thick arrow. Photomicrographs show a lateral view; (A,B) anterior is to the bottom; (C) anterior is to the left; (D) anterior is to the right. Strains containing *sel-12(ar171)* also contained *unc-1(e538)*.

Westlund et al. 1999). However, the precise double mutant phenotype observed can be influenced by the presence of maternal presenilin activity (Westlund et al. 1999). For example, the *hop-1(0); sel-12(0)* hermaphrodites that segregate from a *hop-1(0); sel-12(0)/+* mother are viable but Egl, and the progeny they produce die as embryos (called a Mel phenotype, for maternal effect lethal). We found that the phenotype of *hop-1(0); spr-1(ar200); sel-12(0)* hermaphrodites segregating from a *hop-1(0); spr-1(ar200); sel-12(0)/+* mother is Egl and Mel (Table 2). These results indicate that *spr-1(ar200)* cannot bypass the need for presenilin activity. Such dependence on presenilin activity is expected if *spr-1* acts on presenilin activity or in a process that is strictly presenilin-dependent.

#### *spr-1 displays genetic interactions with various lin-12 or glp-1 alleles*

As the  $\pi$  cell defect of *sel-12* mutant hermaphrodites that is suppressed by mutations in *spr-1* appears to be due to reduced *lin-12* activity (Newman et al. 1995; Cinar et al. 2001), we wondered whether *spr-1* could act as a modulator of *lin-12/Notch* activity in other cells, a role that might be revealed in sensitized backgrounds. If reduced *spr-1* activity suppresses the *sel-12* Egl phenotype by elevating *lin-12* activity, we might observe suppression of other defects caused by *lin-12* hypomorphic alleles and enhancement of those caused by *lin-12* hypermorphs. Furthermore, if *spr-1* functions generally to influence *lin-12/Notch* activity, we might observe analogous genetic interactions with *glp-1* as well. Therefore, we constructed double mutants between *spr-1* and *lin-12* or *glp-1* alleles and examined the consequences for various cell fate decisions. We observed that *spr-1* mutation displays genetic interactions with various alleles of *lin-12* and *glp-1* in various cell fate decisions, suggesting that *spr-1* may function generally as a negative regulator of *lin-12/Notch* activity.

**Table 2.** *spr-1(ar200)* does not bypass the need for *hop-1* activity

Relevant genotype	Egl/total (%)	Mel/total (%)
Wild type	0% ( <i>n</i> = many)	0% ( <i>n</i> = many)
<i>spr-1(ar200); sel-12(ar171)</i>	3/38 (7.9%)	0/38 (0%)
<i>hop-1(ar179); sel-12(ar171)<sup>a</sup></i>	27/27 (100%)	27/27 (100%)
<i>hop-1(ar179); spr-1(ar200); sel-12(ar171)<sup>b</sup></i>	52/52 (100%)	52/52 (100%)

Maternal *sel-12(+)* activity provided by the duplication (see footnotes) enables *hop-1(-); sel-12(-)* progeny to survive to adulthood and to produce progeny, which arrest as embryos (Mel phenotype). Those *hop-1(-); sel-12(-)* adults are not able to lay eggs (Egl phenotype). *spr-1(ar200)* appears wild-type (see Materials and Methods). All strains contained *unc-1(e538)*.

<sup>a</sup>Segregated from a *hop(ar179); sel-12(ar171) unc-1(e538); mnDp68* mother.

<sup>b</sup>Segregated from a *hop-1(ar179); spr-1(ar200); sel-12(ar171) unc-1(e538); mnDp68* mother.

*The AC/VU decision* Two gonadal cells, Z1.ppp and Z4.aaa, interact with each other so that one becomes the AC and the other a VU (Kimble 1981). Genetic studies have established that a low level of *lin-12* activity is required for the AC fate, whereas high levels of *lin-12* activity lead to the VU fate (Seydoux and Greenwald 1989). Therefore, in *lin-12* null mutants [*lin-12(0)*], both Z1.ppp and Z4.aaa become ACs, whereas in *lin-12* hypermorphic mutants [*lin-12(d)*] both become VUs.

*lin-12(ar170ts)* is a weak *lin-12* hypomorphic allele that at 25°C is highly penetrant for the 2AC phenotype associated with loss of *lin-12* activity. As shown in Figure 2A, *spr-1(ar200)* is able to suppress the 2AC defect of *lin-12(ar170)*, suggesting that *spr-1* may negatively regulate *lin-12* activity during the AC/VU decision.

*Specification of the fates of the vulval precursor cells* Six initially equivalent cells, consecutively numbered P3.p–P8.p, each have the potential to generate vulval cells and therefore are called vulval precursor cells (VPCs; for review, see Greenwald 1998). Cell–cell interactions specify that only the central three VPCs adopt vulval fates: the AC induces P6.p to adopt a vulval fate termed 1°, whereupon it laterally signals its two neighbors, P5.p and P7.p, to adopt a vulval fate termed 2°. The phenotypes of *lin-12* mutants suggest that the receptor for the lateral signal is LIN-12 (Greenwald et al. 1983). When LIN-12 is constitutively active to a high level, as in the strong hypermorph *lin-12(n950)*, most VPCs adopt the 2° fate; the progeny of each 2° VPC organize to form a pseudovulva, so that such hermaphrodites have a distinctive Multivulva phenotype (Muv). However, when LIN-12 is constitutively active to a lesser extent, as in the weaker hypermorph *lin-12(n302)*, the Muv phenotype is not generally evident.

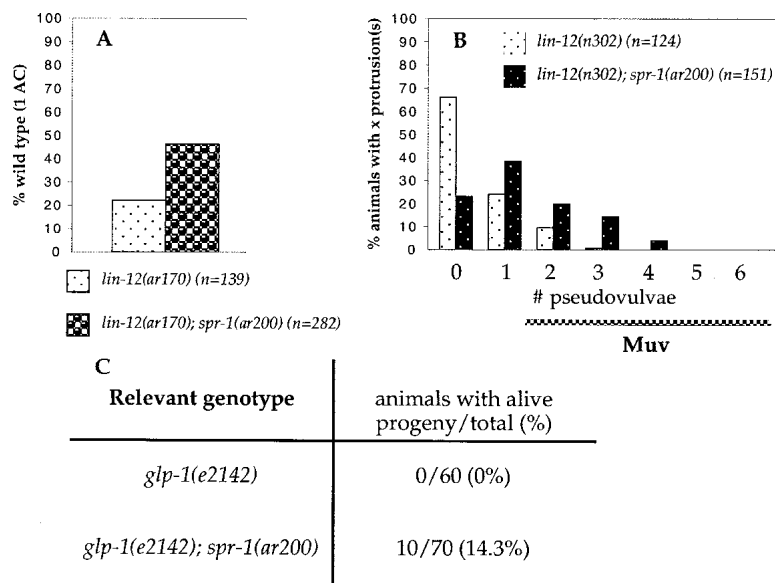
To examine whether *spr-1* negatively regulates *lin-12* activity during VPC specification, we first asked whether *spr-1(ar200)* can enhance the activity of *lin-12(n302)* so as to cause a Muv phenotype. We observed a Muv phenotype in *lin-12(n302); spr-1(ar200)* hermaphrodites, in contrast to the single mutant *lin-12(n302)* (Fig. 2B). These results suggest that *spr-1* negatively regulates *lin-12* activity during VPC specification.

*Early embryonic development* Loss of maternal *glp-1* activity causes embryonic lethality due to abnormal early blastomere specification (Mel), and the *glp-1(e2142)* allele appears to preferentially lack this activity (Priess et al. 1987). As shown in Figure 2C, *spr-1* mutation is able to suppress partially the Mel phenotype of *glp-1(e2142)*, suggesting that *spr-1* is also a negative regulator of *glp-1* activity.

In summary, we have found evidence that *spr-1* appears to act as a negative regulator of LIN-12/Notch activity in the AC/VU decision, VPC specification, and early blastomere specification.

#### *Molecular identification of the spr-1 gene*

*spr-1* had been previously mapped to the *dpy-11-rol-3* interval on LG V (Wen et al. 2000). In order to map *spr-1*



**Figure 2.** *spr-1(ar200)* displays genetic interactions with *lin-12* and *glp-1*. *n*, the total number of animals scored. No cell fate defects were observed in *spr-1(ar200)* hermaphrodites (see Materials and Methods). (A) *spr-1(ar200)* suppresses the 2 AC defect of *lin-12(ar170)*. *lin-12(ar170)* and *lin-12(ar170); spr-1(ar200)* hermaphrodites were scored at 25°C. (B) Percent of animals (*y* axis) with a given pseudovulvae number (*x* axis) are shown. Hermaphrodites with two or more pseudovulvae are considered to have a Multivulva (Muv) phenotype (wild-type hermaphrodites have a single vulva and no pseudovulvae). *spr-1(ar200)* enhances the activity of *lin-12(n302)*, resulting in a Muv phenotype at 14°C. Note that enhancement is very temperature sensitive. The low-penetrance sterility defect of *lin-12(n302)* is also enhanced (data not shown), but the cellular basis for this defect is not known. (C) *spr-1(ar200)* suppresses the maternal embryonic lethality (Mel) defect of *glp-1(e2142)*. All strains were scored at 25°C and contained *unc-32(e189)*.

to a smaller interval, we took advantage of single nucleotide polymorphisms (SNPs) in the *C. elegans* Hawaiian strain CB4856, using SNPs identified by the Genome Project and new ones we identified by sequencing intergenic regions. In this way, we mapped *spr-1* between two SNPs, located on Y97E10AL and D1014. This interval encompassed five cosmids, but none of them showed antisuppression when injected into the double mutant *spr-1(ar200); sel-12(ar171)*. However, injection of three overlapping cosmids [DD11, F58G4, and T23D2 (=D1014)] led to larval lethality, so that antisuppression could not be scored. We sequenced the predicted genes in this region in the *spr-1(ar200)* mutant. One of these genes, *D1014.8*, contained a G→A mutation (as expected for an EMS-induced mutation), changing the AG splice acceptor site in front of predicted exon 4 into AA. Next, we sequenced the three other *spr* mutants on *LG V* and found the same change in *ar201* as well as another G→A mutation introducing a stop codon early in predicted exon 4 of gene *D1014.8* in *ar205* and *ar212* (see Fig. 3A).

The gene *D1014.8* was predicted by GENEFINDER to be in an operon with the 5' gene *D1014.9*. When we analyzed the structure of *spr-1* by PCR screening of three different cDNA libraries, we found that *spr-1* encodes a single mRNA encompassing both *D1014.8* and *D1014.9* predicted genes. The resulting *spr-1* gene structure is shown in Figure 3A.

We confirmed that mutations in the gene *D1014.8* correspond to *spr-1* by injecting a PCR fragment amplified from genomic DNA comprising the *spr-1* coding sequence into *spr-1(ar200); sel-12(ar171)* worms and found that this product caused antisuppression (see Materials and Methods). In addition, we confirmed that *D1014.9* behaved functionally as part of the *spr-1* gene by showing that double-stranded RNA from *D1014.9* as well as *D1014.8* suppressed the Egl phenotype of *sel-12(ar171)*, when delivered by injection (Table 3) or by bacterial feeding (data not shown). These RNA-mediated interference

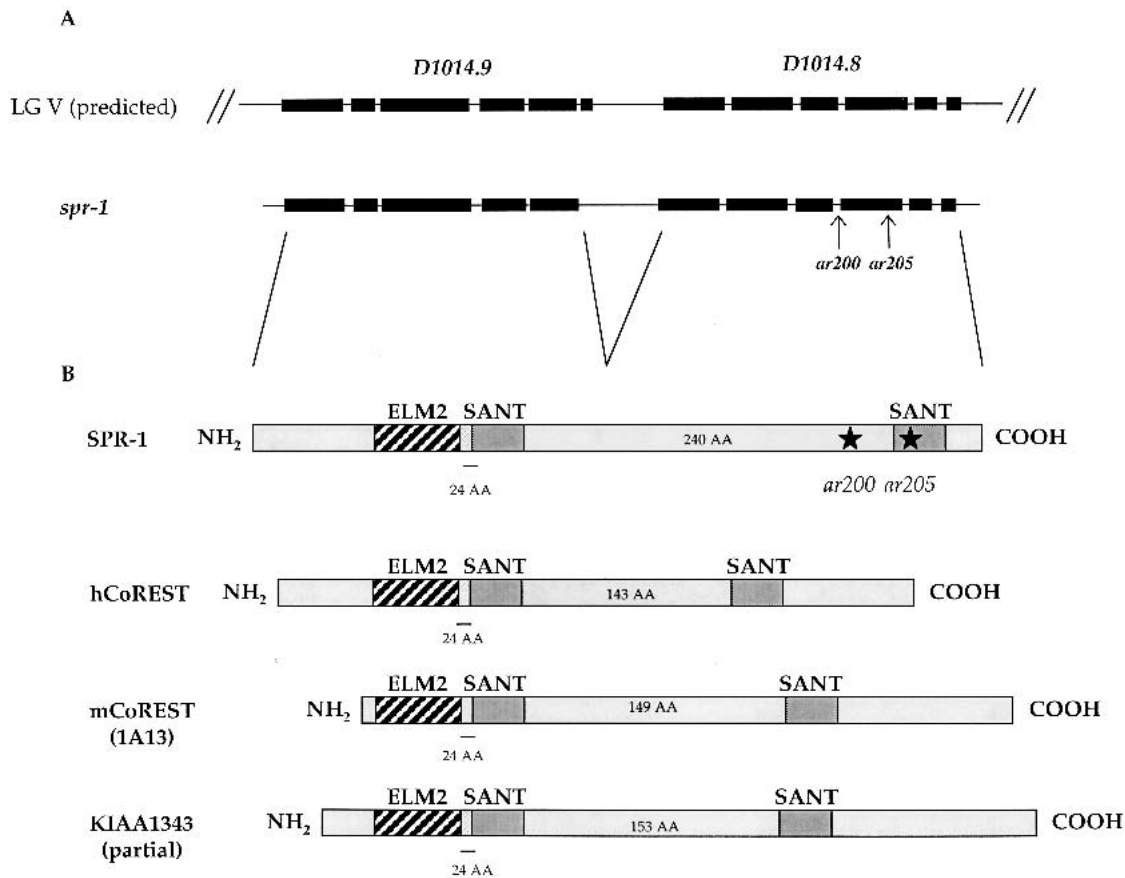
(RNAi) results also suggest that reduced *spr-1* activity can suppress the Egl phenotype of *sel-12(-)* hermaphrodites, implying that *spr-1* alleles reduce or eliminate *spr-1* activity.

We have evidence that in *ar200*, an mRNA is produced that potentially encodes a truncated protein: We cloned a single cDNA corresponding to the mRNA produced in *spr-1(ar200)* animals and showed that the mutation of the AG splice acceptor site led to aberrant splicing to the immediately adjacent AG dinucleotide, leading to a shift in the reading frame and an early stop in exon 9.

The nature of the *spr-1* mutations that were found does not allow us to conclude that *ar200* and *ar205* are null alleles. It may be that the null phenotype of *spr-1* is lethal or sterile, and therefore that null alleles could not be recovered in a genetic screen that requires viability and fertility. However, there is no evidence from RNAi for such phenotypes, and there is no available deficiency for the region to conduct classical tests. It should be noted that RNAi does not always cause complete loss of gene activity, so the lack of a phenotype by RNAi cannot be conclusive. Alternatively, it may be that the null phenotype of *spr-1* is wild-type, and a phenotypic effect can only be revealed when *spr-1(-)* is placed in a sensitized genetic background. A wild-type null phenotype may be evidence that the gene, pathway, or process is redundant (e.g., Greenwald and Horvitz 1980; Thomas 1993).

#### *SPR-1 belongs to a conserved protein family*

Analysis of SPR-1 primary sequence using SMART (<http://smart.embl-heidelberg.de>) suggests that the protein contains an ELM2 (EGL-27 and MTA1 homology domain 2) domain followed by two SANT domains (SWI3, ADA2, NCoR, TFIIB B' subunit). The function of ELM2 domains is unknown, but they have been observed in several transcriptional repressors (Solari et al. 1999). The SANT domain is also found in transcriptional regu-



**Figure 3.** *spr-1* gene structure and protein product. (A) The relationship between the two previously predicted open reading frames, *D1014.8* and *D1014.9*, to the *spr-1* gene structure we determined is shown. *spr-1(ar200)* affects the splice acceptor at the start of exon 9, resulting in a frameshift that leads to a stop codon. *spr-1(ar205)* is a G-to-A transition that results in a stop codon in exon 9. (B) SPR-1/CoREST protein family. The domain structure of SPR-1 and of all the mammalian homologs found in the databases is shown. *spr-1(ar200)* and *spr-1(ar205)* results in truncation of the SPR-1 protein as shown. The spacing between the ELM2 and the first SANT domain is always 24 amino acids; the spacing between the SANT domains is variable, as indicated. Accession nos.: SPR-1, AAA96286; hCoREST, no. AF155595; mCoREST (murine 1A13), no. X83587; KIAA1343, no. BAA92581. Note that KIAA1343 most likely represents a partial cDNA.

lators (Aasland et al. 1996). These observations, plus the homology described further below, suggest that SPR-1 has a role in the modulation of transcription.

BLAST analysis revealed that SPR-1 has been conserved throughout evolution. Proteins with one ELM2 domain followed by two SANT domains (Fig. 3B) appear to represent a small conserved subfamily among proteins with two SANT domains. In particular, the spacing between the ELM2 and the first SANT domain is invariant in the members of this family; the spacing between the two SANT domains appears to vary between 140 and 240 amino acids (see Fig. 3B).

The apparent human ortholog of SPR-1 is known as CoREST, which was isolated on the basis of its ability to bind REST or HDAC-1/-2 (Andres et al. 1999; You et al. 2001). CoREST has been shown to exhibit transcriptional repressor activity when fused to the Gal4 DNA-binding domain and to be necessary for REST-dependent repression of neural-specific gene expression (Andres et al. 1999; You et al. 2001). It copurifies with a limited

number of factors including HDAC-1/-2 and p110b in an equimolar ratio (Humphrey et al. 2001; You et al. 2001; Hakimi et al. 2002) and could represent a new corepressor complex. The HDAC-1/-2 interaction domain in CoREST maps to its SANT domains (Ballas et al. 2001; You et al. 2001), which are also found in other transcrip-

**Table 3.** *spr-1*, but not Y74C9A.4, double stranded RNA suppresses the *Egl* phenotype of *sel-12(ar171)* animals

Injected dsRNA	Po plates with <i>Egl</i> <sup>+</sup> progeny/total (%)
Mock	0/20 (0%)
<i>spr-1 (D1014.8)</i>	12/14 (85%)
<i>spr-1 (former D1014.9)</i>	14/17 (82%)
Y74C9A.4	0/19 (0%)

*sel-12(ar171) unc-1(e538)* adult hermaphrodites were injected. *Egl*<sup>+</sup>, egg-laying ability was restored in their progeny. *D1014.9* and *D1014.8* were originally predicted to be two distinct open reading frames, but are both part of the *spr-1* gene (see text and Fig. 3).

tional corepressors and HDAC interacting partners such as N-CoR, SMRT, MTA1, or MTA2. As described below, human CoREST can functionally replace SPR-1, confirming the orthology predicted by sequence analysis.

We note that another gene encoding a protein with two SANT domains and which shows homology to SPR-1 and CoREST exists in worms, *Y74C9A.4*. However, *Y74C9A.4* appears to lack an ELM2 motif. Nevertheless, we assessed whether *Y74C9A.4* behaves as a *spr* gene by analyzing its ability to suppress the *sel-12* Egl phenotype when its activity is lowered or lost by injection of *Y74C9A.4* double-stranded RNA. We saw no suppression, therefore we provisionally conclude that *Y74C9A.4* is not a *spr* gene (Table 3).

#### Subcellular localization of SPR-1

As expression of SPR-1(+) under its own promoter appears to be highly toxic (see Materials and Methods), we expressed a Myc-tagged SPR-1 protein (SPR-1::MYC) using the *cog-2* promoter (see below) in order to analyze its subcellular localization. As shown in Figure 4, nuclear staining is observed when transgenic animals are stained with an anti-MYC antibody. There also appears to be an intensification of staining within the nucleus, in speckles and a large structure that we presume to be the nucleolus (Fig. 4). Nuclear localization has also been observed for CoREST in mammalian cells (Grimes et al. 2000; Humphrey et al. 2001; S. Jarriault and I. Greenwald, unpubl.) and is consistent with a role for SPR-1 in transcriptional repression.

#### *spr-1* functions cell autonomously

To assess whether *spr-1* activity is required autonomously for  $\pi$  cell induction, we wanted to analyze the ability of *spr-1*; *sel-12* mutants to lay eggs when SPR-1(+) is delivered specifically in the  $\pi$  cell precursors. In the gonad, the *cog-2* promoter has been reported to be active only in the  $\pi$  cell precursors and their daughters, and not in the AC or other gonadal cells (Hanna-Rose and Han 1999). We examined transgenic lines expressing SPR-1::MYC under the *cog-2* promoter in *spr-1*; *sel-12* hermaphrodites and found a high proportion of hermaphrodites unable to lay eggs (Fig. 5). These results suggest that *spr-1* functions autonomously in  $\pi$  cell specification, and by extension, *spr-1* is likely to function autonomously in other *lin-12*-mediated cell fate decisions.

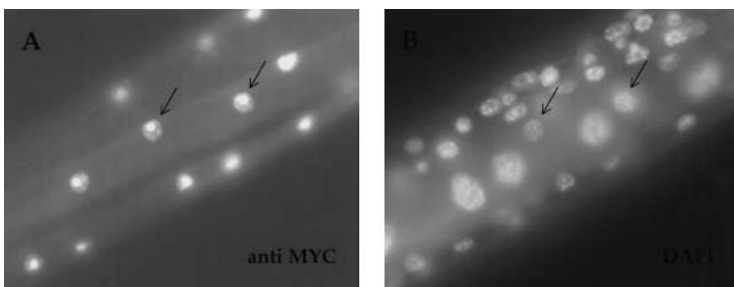
#### Human CoREST can functionally replace SPR-1 in *C. elegans*

Using the *cog-2* promoter to express human CoREST in the  $\pi$  cells, we assayed whether CoREST has SPR-1(+) activity. We found that hCoREST can functionally replace SPR-1, causing *spr-1*; *sel-12* hermaphrodites in which it is expressed to display an increased proportion of Egl hermaphrodites relative to a vector-only control (Fig. 5). These results suggest that SPR-1 and CoREST are true orthologs, and therefore that the information about CoREST function that has been inferred from studies in mammalian cells is likely to be illuminating about SPR-1 function, and vice versa.

#### One homolog of *p110b*, another member of the CoREST corepressor complex, displays *spr* activity in an RNAi assay

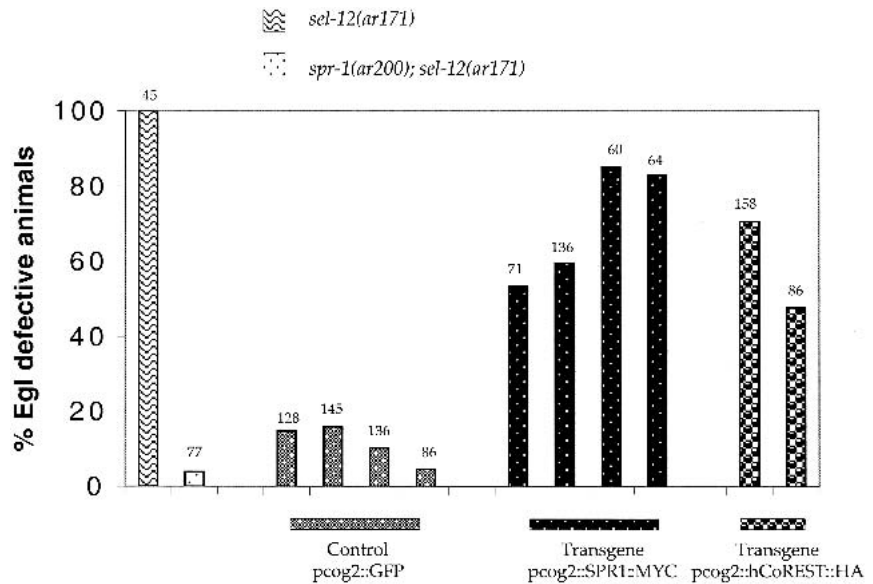
The finding that SPR-1 is the ortholog of CoREST suggested that genes encoding other components of the CoREST complex might have *spr* activity. In addition to the zinc finger protein, REST, a variety of proteins have been recently found to exist in a core complex with CoREST: p110b (a protein with a FAD binding domain and a polyamine oxidase motif, also known as KIAA0601) and histone deacetylases 1 and 2 (HDAC-1/-2; Humphrey et al. 2001; You et al. 2001; Hakimi et al. 2002). Depending on the conditions, additional factors were also found, such as p110a (a zinc finger protein, ZNF217), p40 or BRAF35 (Sox-like putative transcription factors), BCH80 (a CHD3-like molecule), or p80 (a hypothetical, novel protein; Humphrey et al. 2001; You et al. 2001; Hakimi et al. 2002). However, known members of corepressor complexes such as Sin3 (which interacts with REST in HEK-293 cells; Grimes et al. 2000), MTA2, RbAp46 and RbAp48, or MBD2, key components of the Sin3 and NuRD HDAC-1/-2 containing complexes were not coimmunoprecipitated (Humphrey et al. 2001; You et al. 2001).

We did BLAST searches to identify putative *C. elegans* homologs of REST and of the CoREST-interacting partners for which accession numbers were given or readily apparent (ZNF217, p110b, and HDAC-1/-2). Although the zinc finger proteins identified a number of *C. elegans* zinc finger proteins, none is clearly orthologous to REST or ZNF217 (data not shown). However, we identified three proteins that are highly related to class I HDAC-



**Figure 4.** SPR-1 is localized to the nucleus. Photomicrographs of a *spr-1(ar200)*; *sel-12(ar171)* *unc-1* hermaphrodite expressing a SPR-1::MYC protein under the *cog-2* promoter and stained with an antibody directed against MYC (A) or with DAPI (B) are shown. Arrows point to discrete stained muscle nuclei. Anterior is to the right.

**Figure 5.** Expression of SPR-1 or CoREST in  $\pi$  cell precursors complements *spr-1(ar200)*. For transgenic lines, each bar represents an independent array in a *spr-1(ar200); sel-12(ar171)* background. The total number of animals scored is indicated above each bar. All strains containing *sel-12(ar171)* also contain *unc-1(e538)*. (pcog-2::GFP) Control lines, arrays containing plasmid (pWH17). (pcog-2::SPR1::MYC) Expression of SPR-1(+) in  $\pi$  cells complements *spr-1(ar200)*, indicating cell autonomy. (pcog-2::CoREST::HA) Expression of human CoREST in  $\pi$  cells also complements *spr-1(ar200)*, indicating that human CoREST can functionally substitute for the *C. elegans* SPR-1.



1/-2, and three proteins that are highly related to p110b (Fig. 6A).

We assessed their *spr* activity by looking for RNAi-induced suppression of *sel-12(ar171)*. As shown in Figure 6A, all three HDAC homologs, and two of the p110b homologs, failed to show *spr* activity. A negative result by RNAi may not be definitive (see above). Furthermore, it has been shown that both HDAC-1 and HDAC-2 interact with CoREST, raising the possibility of functional redundancy.

In contrast, one of the p110b homologs, *Y40B1B.6*, displayed strong *spr* activity. *Y40B1B.6* is predicted to have a FAD domain partially overlapping the polyamine oxidase domain, and of the three *C. elegans* p110b homologs, has the most similar domain structure to human p110b.

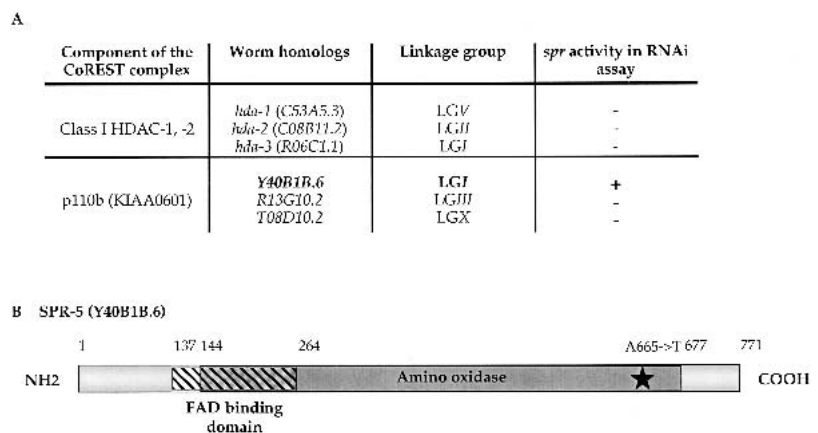
*Y40B1B.6*, the p110b homolog, is *spr-5*

*Y40B1B.6* maps to LG I, where we had previously identified three *spr* mutations: *spr-4(ar208)*, *spr(ar204)*, and

*spr(ar197)*. We sequenced the *Y40B1B.6* coding region from all three mutant strains and identified a sequence change associated with *spr(ar197)*. This gene, which has also been identified by Eimer et al. among their collection of suppressors of *sel-12(ar171)* (S. Eimer, B. Laskowski, and R. Baumeister, pers. comm.) is now designated *spr-5*. *spr-5(ar197)* is a G-to-A change at the end of exon 7, resulting in an A665T missense mutation, which affects a conserved amino acid of the polyamine oxidase domain (Fig. 6B). The sequence and RNAi data suggest that *spr-5(ar197)* results in reduced or absent *Y40B1B.6* function.

Although reducing the activity of *spr-1* and *spr-5* can suppress the *sel-12(ar171)* mutant phenotype, the nature of our alleles does not resolve whether complete absence of the CoREST/p110b complex causes any visible phenotypes. We note that we did not see any effects on viability, fertility, or egg-laying when we performed the following experiments: *spr-1(RNAi)* in *spr-5(ar197)*, *spr-5(RNAi)* in *spr-1(ar200)*, and double RNAi for both *spr-1* and *spr-5* in both wild-type and *spr-1(ar200)* backgrounds

**Figure 6.** Identification of *Y40B1B.6* as *spr-5* by RNAi and sequence analysis. (A) RNAi analysis of *C. elegans* CoREST complex homologs. Double stranded RNA was delivered to *sel-12(ar171) unc-1(e538)* hermaphrodites by either feeding (*hda-1*, *hda-2*, *hda-3*) or injection (*Y40B1B.6*, *R13G10.2*, *T08D10.2*). At least 60 individuals fed dsRNA were examined: (-) no Egl<sup>+</sup> individuals were seen. The progeny of at least 14 injected hermaphrodites were examined: (-) no Egl<sup>+</sup> individuals were seen. For *Y40B1B.6*, 35/37 injected animals gave many Egl<sup>+</sup> progeny. (B) The predicted protein structure of *Y40B1B.6* and the position of the mutation found in *spr-5(ar197)* are shown. The overlapping FAD-binding (hatched box) and polyamine oxidase (gray box) domains are indicated.





(data not shown). Therefore, while it remains possible that molecular null alleles of *spr-1* and/or *spr-5* might cause a visible phenotype, we have not been able to find any evidence that this is so.

## Discussion

In this study, we have described a genetic and molecular characterization of the *spr-1* gene. Through genetic analysis we showed that lowering *spr-1* activity leads to allele-nonspecific suppression of the Egl phenotype of *sel-12* hermaphrodites. We also found that suppression is achieved by restoring  $\pi$  cell specification, the cellular defect that underlies the Egl phenotype of *sel-12* hermaphrodites (Cinar et al. 2001). When SPR-1(+) is expressed in the  $\pi$  cell precursors of *spr-1*; *sel-12* hermaphrodites, suppression is lost, suggesting that *spr-1* function is cell autonomous. Suppression of *sel-12* by *spr-1* depends on the activity of *hop-1* presenilin, suggesting that reduced *spr-1* activity does not bypass the need for presenilin activity. Finally, loss of *spr-1* increases the activity of *lin-12* or *glp-1* in all cell fate decisions examined, suggesting that *spr-1* functions generally as a negative regulator of LIN-12/Notch activity.

Through molecular analysis, we showed that SPR-1 is orthologous to CoREST, which was first identified as a corepressor for the neural-restrictive silencing factor REST (Andres et al. 1999). A functional, epitope-tagged SPR-1 protein is localized to the nucleus in *C. elegans*, consistent with a function in transcriptional repression. Moreover, human CoREST is also localized to the nuclei when expressed in worms (see Materials and Methods) and can partially substitute for SPR-1, indicating that the sequence conservation is likely to extend to functional conservation. Finally, using RNAi, we found that another gene, *Y40B1B.6*, encoding a homolog of a known member of the CoREST complex, also has *spr* activity, and that a previously identified *spr* mutation is in the *Y40B1B.6* gene (now known as *spr-5*). Therefore, at least some of the *spr* genes are likely to function in the same process, and the functional conservation shown between SPR-1 and CoREST might extend to other members of the CoREST corepressor complex.

### How does *spr-1* suppress the Egl phenotype of *sel-12(-)* hermaphrodites?

In considering how mutations in *spr-1* suppress the Egl phenotype of *sel-12* mutants, it must be borne in mind that presenilin is a critical component of the LIN-12/Notch pathway, and that failure of  $\pi$  cell induction is a manifestation of reduced *lin-12* activity (Newman et al. 1995; Cinar et al. 2001). Therefore, loss of *spr-1* activity might affect presenilin level or activity per se, or affect the level or activity of any limiting component of the LIN-12/Notch pathway. Because SPR-1 is a functional ortholog of CoREST, a protein with demonstrated corepressor activity, one straightforward model postulates that SPR-1 and SPR-5 negatively regulate the transcrip-

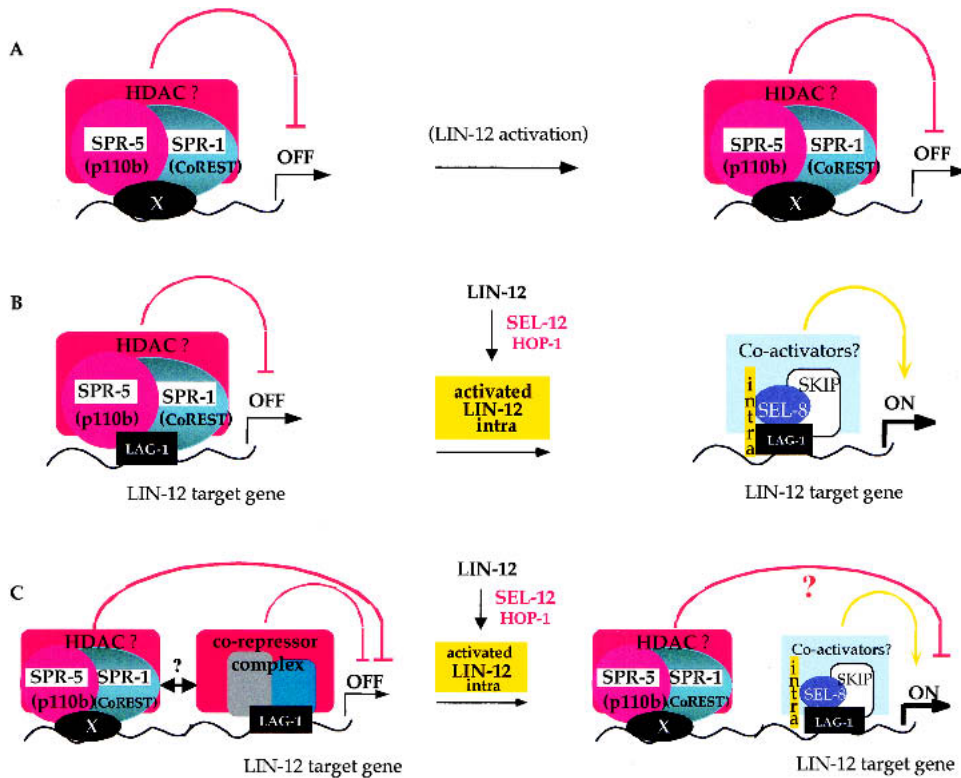
tion of genes encoding members of the LIN-12/Notch pathway or of genes encoding positive regulators of these pathway members (Fig. 7A).

Compatible with this model, a simple hypothesis to account for *hop-1*-dependent suppression of *sel-12* by reduced *spr-1* activity is that *hop-1(+)* is derepressed in the  $\pi$  cells. We attempted to examine the level of *hop-1* transcription in a *spr-1* mutant background. As a detectable *hop-1* transcriptional reporter gene has been elusive (X. Li, pers. comm.), we were unable to address this question with cellular resolution. Instead, we probed a Northern blot containing polyA(+) RNA prepared from hermaphrodites at the L3 and L4 stages (the time of  $\pi$  cell induction and utse formation) for the level of *hop-1(+)* mRNA expression. Hermaphrodites of genotype *sel-12(ar171)*, which are egg-laying defective, appeared to have higher levels of *hop-1(+)* RNA than *spr-1(ar200)*; *sel-12(ar171)* hermaphrodites, which are able to lay eggs, when normalized to an *inf-1* (CeIF-4A) probe (data not shown). These results, although not definitive, suggest that a simple and general derepression of *hop-1* transcription is unlikely to account for the phenotypic suppression observed.

Another simple hypothesis to account for suppression of the Egl phenotype of *sel-12* is that in *spr-1* mutants, *lin-12(+)* is derepressed in the  $\pi$  cells or more generally, or that *lag-2(+)* is derepressed in the anchor cell or more generally. However, we did not detect any ectopic expression, nor any increase in the level of expression, of the transcriptional reporters *lin-12::lacZ* or *lag-2::lacZ* in a *spr-1(-)* background (S. Jarriault and I. Greenwald, unpubl.; see Materials and Methods for more details). In contrast to our results with *spr-1*, strong and ectopic derepression of a *lag-2* reporter gene has been observed in a mutant that lacks one of the histone deacetylases, with concomitant developmental defects (Dufourcq et al. 2002). Although it remains possible that loss of *spr-1* activity leads to derepression of a gene whose activity is limiting for presenilin or LIN-12/Notch signal transducing activity in many different cell fate decisions, there is another class of models for the role of SPR-1 that fits with a large body of work suggesting that the balance between repression and activation of LIN-12/Notch target genes is important. These models will be considered further below.

### Transcriptional repression and LIN-12/Notch signaling

Many pathways appear to activate target gene transcription by promoting a switch from a repressed state to an activated state, displacing a corepressor activity upon signaling (for review, see Barolo and Posakony 2002). This switch appears to be the case for the LIN-12/Notch signaling pathway. Prior to ligand binding, a corepressor complex is associated with the DNA binding protein CBF1 (see Introduction), which is bound to regulatory regions of LIN-12/Notch targets genes (Kao et al. 1998). Several proteins with demonstrated repressor activity have been shown to interact with CBF1 and negatively regulate Notch target genes in cultured cells. These pro-



**Figure 7.** Models for SPR-1 and SPR-5 function. (A) SPR-1 and SPR-5 are part of a corepressor complex affecting the transcription of known members of the LIN-12/Notch pathway. Although no evidence exists, *hop-1* transcription could, for example, be affected in a cell-specific way. Alternatively, the complex could repress the transcription of a subset of crucial genes for  $\pi$  cell fate which would not get activated in *lin-12(-)* or *sel-12(-)* mutants unless this repression is released. (B) SPR-1/CoREST and SPR-5/p110b are part of the LAG-1-associated corepressor complex. After ligand binding leads to the presenilin-dependent release of the intracellular domain of LIN-12 (yellow, noted “intra”), the corepressor complex is released and a coactivator complex formed, leading to activation of the target genes. (C) SPR-1 and SPR-5 form a distinct corepressor complex, which may interact with the LAG-1 repressor complex. The corepressor complex containing SPR-1 and SPR-5 is targeted to the DNA by a factor X, and is distinct from the LAG-1 complex. This complex, which can comprise more factors, may cooperate with the LAG-1 complex to potentiate repression. Association of LAG-1 and LIN-12(INTRA) might or might not release the CoREST corepressor complex as well.

teins include KyoT (Taniguchi et al. 1998), CIR (Hsieh et al. 1999), SMRT, NcoR, and HDAC-1 (Kao et al. 1998). It is not clear, however, what the roles of these different repressors are in vivo, if all of them are recruited simultaneously to Notch target genes, to what extent they could act redundantly, or if spatio-temporal specificities exist.

Ligand binding leads to the presenilin-dependent transmembrane cleavage event that releases the intracellular domain of LIN-12/Notch, which translocates to the nucleus. There, it appears to mask the repressor domain of CBF1 (Waltzer et al. 1995; Hsieh et al. 1996) thereby displacing the CBF1-associated corepressor proteins (Kao et al. 1998). The LIN-12/Notch intracellular domain also appears to recruit coactivator proteins. These coactivators include Mastermind in *Drosophila* and mammals (Petcherski and Kimble 2000b; Wu et al. 2000; Kitagawa et al. 2001), and its likely counterpart, SEL-8 in *C. elegans* (Doyle et al. 2000; Petcherski and Kimble 2000a).

A reduction in presenilin activity leads to decreased release of the intracellular domain of LIN-12/Notch, and therefore to a decrease in signal transduction (De

Strooper et al. 1999; Struhl and Greenwald 1999). In principle, then, it would be more difficult to convert CBF1 from a repressor to an activator. By removing repressor activity, the balance between activator and repressor might be restored. In *sel-12* mutants, reduced presenilin activity results in the *Egl* phenotype; perhaps in *spr* mutants reduced repressor activity makes it easier for target genes to be activated by the reduced level of *lin-12* signal transduction. In the next section, we consider models for how SPR-1 and SPR-5 might be involved in the repression of LIN-12 target genes, based on the available biochemical information about CoREST in mammalian cells as well as our analysis of *spr-1* in *C. elegans*.

*SPR-1/CoREST, SPR-5/p110b, and potential regulation of LIN-12/Notch target genes*

One model for how SPR-1 and SPR-5 might contribute to repression of LIN-12 target genes is that the SPR-1/SPR-5 complex acts as a corepressor of LAG-1 in the absence of LIN-12/Notch activation, and is displaced upon LIN-12/Notch activation (Fig. 7B). This model is analogous to

what has been proposed for CBF1 and SMRT (Kao et al. 1998). Although we have not been able to detect an interaction between CoREST and CBF1 in mammalian cells (S. Jarriault, unpubl.), it may be that the presence of CBF1 in association with the CoREST complex is evident only under certain conditions.

Another model is that SPR-1/CoREST and SPR-5/p110b are instead part of a corepressor complex that binds to LIN-12/Notch target genes through a sequence that is distinct from the LAG-1/CBF1 binding site. Therefore, there may be a specific DNA binding protein (denoted as X in Fig. 7C), which recruits the SPR-1/CoREST complex to LIN-12/Notch target genes. This model allows for potential cooperativity between the corepressor complex associated with LAG-1/CBF1 and the SPR-1/CoREST complex associated with factor X. CoREST has been shown to associate with at least two different zinc finger DNA-binding proteins, REST and ZNF217, suggesting that CoREST may have multiple DNA binding protein partners. In this context, it is interesting to note that Lakowski et al. have found that *spr-3* and *spr-4* encode zinc finger proteins (B. Lakowski, S. Eimer, and R. Baumeister, pers. comm.). Although these do not appear to be orthologous to either REST or ZNF217, they might represent the hypothetical DNA binding factor X that targets SPR-1 and SPR-5 to DNA.

Finally, the SPR-1/CoREST–SPR-5/p110b complex may be involved in more global or long-range repression, perhaps involving dynamic deacetylation of chromatin, rather than being specifically associated with LIN-12/Notch target genes. Perhaps a crucial target gene in the  $\pi$  cells is particularly susceptible to this long-range negative regulation, making the Egl phenotype of *sel-12(-)* hermaphrodites a sensitive background with which to detect subtle relief from a more generalized repression.

We note that the models involving recruitment of the SPR-1/SPR-5 complex to target genes by factor X could be applied either to LIN-12/Notch target genes directly or to intermediate genes in a genetic regulatory cascade. In addition, for all of the models, the nature of the target genes that would be sensitive to a reduction in CoREST/p110b-mediated repression is likely to be influenced by the number of the DNA binding sites allowing recruitment of this complex to regulatory regions and the relative contribution of the CoREST and potential other corepressor complexes to repression.

We also note that these models are not incompatible with the observation that *spr-1(ar200)* does not have *lin-12(d)*-associated cell fate transformations. In a *sel-12(+)* background, activation of a subset of LIN-12/Notch target genes may not be sufficient to cause a phenotype. Furthermore, there is precedent for transcriptional repressors to associate with more than one corepressor complex. Indeed, REST has two domains that recruit corepressor complexes: one recruits CoREST and the other recruits Sin3. When either of these domains is fused to a heterologous DNA-binding domain, or if either one of these domains is deleted, repression by the mutant REST protein is indistinguishable from repression by wild-type REST, suggesting that the corepressor complexes are re-

dundant (Ballas et al. 2001). In addition, loss of the retinoblastoma (Rb) homolog *lin-35* does not result in a visible phenotype in *C. elegans*, apparently because of functional redundancy with a group of novel proteins; roles for *lin-35* Rb-mediated repression are only revealed in sensitized genetic backgrounds (Lu and Horvitz 1998).

#### *All SPR proteins may be part of a single transcriptional repression complex*

Five *spr* genes have been identified to date, all encode nuclear proteins (or proteins that are predicted to be nuclear), indicating that they may be physically as well as functionally associated. As we have discussed here, SPR-1 and SPR-5 correspond to CoREST and p110b, respectively, which have been found in a complex and mediate transcriptional repression. Previously, SPR-2 was shown to be orthologous to SET, a protein that has many biochemical activities attributed to it; intriguingly, much of the evidence points to a role in chromatin structure (summarized in Wen et al. 2000). Finally, Lakowski et al. have shown that SPR-3 and SPR-4 contain multiple zinc fingers (B. Lakowski, S. Eimer, and R. Baumeister, pers. comm.). Therefore, it is possible that a core complex consisting of SPR-1/CoREST, SPR-5/p110b, and possibly a histone deacetylase, is recruited to the regulatory region of target genes through its interaction with zinc finger proteins in worms. These target genes may be LIN-12/Notch target genes, as proposed above, or other genes that influence LIN-12/Notch signaling or cell fate specification. Loss of one of the members of such a complex might allow a weak LIN-12/Notch signal to activate the transcription of a subset of target genes that would otherwise not have been activated in the *sel-12* background.

The *spr* gene screen has not been saturated (Wen et al. 2000), and other genes are likely to be found to have *spr* activity, either through conventional genetic screens or RNAi screens. The key challenges for the future will be to ascertain whether the proteins defined by these genes are part of a single complex and to find the target genes with which this complex (or complexes) is associated. The only potential LIN-12 target gene known to play a role in  $\pi$  cell specification is *lin-11* (Gupta and Sternberg 2002), which appears to be unaffected in a *spr-1* mutant background (S. Jarriault and I. Greenwald, unpubl.). As more potential LIN-12 target genes become available for study, this question may be addressed through biochemical as well as genetic approaches. Finally, it will be interesting to assess whether the SPR-1/CoREST, SPR-5/p110b corepressor complex is also involved in repression of the LIN-12/Notch signaling in other organisms as well.

## Materials and methods

### *Genetic materials and methods*

Standard methods were used for handling, maintenance, and genetic analysis. Experiments were conducted at 20°C unless

otherwise indicated. The wild-type parent for most strains used in this study is *C. elegans* var. Bristol strain N2. The relevant genes and alleles used in this study are:

LG I: *hop-1(ar179)*, *spr-4(ar208)*, *spr(ar204)*, *spr-5(ar197)* (Wen et al. 2000; this study).

LG III: *lin-12(n302, n950)* (Greenwald et al. 1983), *lin-12(ar170ts)* (Hubbard et al. 1996), *glp-1(e2142ts)* (Priess et al. 1987).

LG V: *spr-1(ar200, ar201, ar205, ar212)* (Wen et al. 2000; this study).

LG X: *sel-12(ar131, ar171)* (Leviton and Greenwald 1995).

In addition, the following integrated transgenes have been used: *nIs2* (containing *lin-11::lacZ*; Freyd 1991), *arIs11* (containing *lin-12::lacZ*; Wilkinson et al. 1994), *arIs13* (containing *lag-2::lacZ*; Wilkinson et al. 1994), *arIs41* (containing *LIN-12::GFP*; Levitan and Greenwald 1998), *arIs51* (containing *pcdh-3::gfp*; Pettitt et al. 1996; X. Karp and I. Greenwald, unpubl.).

Additional information about these alleles, as well as about markers used for mapping or for facilitating genetic analysis mentioned in the text, can be found through WormBase (<http://www.wormbase.org>).

#### Mutant analysis and scoring

Strains were grown and scored at 20°C unless otherwise specified and assessed for phenotypes as follows:

Egg-laying ability: L4 larvae were picked to individual plates and scored for 3 consecutive days. An animal was scored as Egl<sup>+</sup> if it showed robust egg-laying and Egl if it laid none or only a few eggs and “bagged” during this period of time.

utse: Late L4 larvae or young adults were examined using Nomarski microscopy.

Anchor cells: L3 larvae were scored using Nomarski microscopy.

Vulval development: The number of pseudovulval protrusions was scored under the dissecting microscope.

Embryonic lethality: L2 larvae grown at 20°C were transferred to individual plates at the nonpermissive temperature for *glp-1(e2142)*, 25°C, and scored for their ability to give live progeny.

#### Genetic characterization of *spr-1* mutants

None of the *spr-1* mutants appeared to display obvious phenotypes. Unmarked *spr-1(ar200)* chromosomes used in this study have been backcrossed two and seven times. In particular, a *spr-1(ar200)* mutant that had been backcrossed seven times did not display any defect in the following scorings: 20/20 had a wild-type vulva and were Egl<sup>+</sup> and fertile (>100 progeny; 15°C), 30/30 were Egl<sup>+</sup> (20°C), 20/20 had one AC and did not appear to display any embryonic lethality (25°C). Therefore, *spr-1(ar200)* and N2 appear indistinguishable for those cell fate decisions.

*spr-1* alleles all display semidominance in their ability to suppress the egg-laying defects of *sel-12(ar171)*. The extent of this semidominance depends on the genotype of the mother, that is, whether the mother is heterozygous or homozygous for *sel-12(ar171)*. More specifically, 9/54 (16.7%) of *dpy-11(e224) spr-1(ar201)/+*, *sel-12(ar171) unc-1(e538)* hermaphrodites were Egl<sup>+</sup> when segregating from a heterozygous *sel-12* mother whereas 2/61 (3.3%) of *dpy-11(e224) spr-1(ar201)/+*, *sel-12(ar171) unc-1(e538)* hermaphrodites were Egl<sup>+</sup> when segregating from a homozygous *sel-12* mother at 20°C.

We note that we did not observe suppression of the egg-laying defect caused by the partial loss-of-function allele *lin-12(n676n930)*. Although we previously used this assay (and oth-

ers) to evaluate *spr-2* (Wen et al. 2000), we now discount a negative result in this assay because the egg-laying defect of this allele reflects the sum of several different cellular defects, so that more than one may need to be corrected for egg-laying ability to be restored (Sundaram and Greenwald 1993b).

#### Detailed genetic mapping of *spr-1*

SNPs from LG V were used for mapping *spr-1(ar200)*. SNPs were identified in *C. elegans* var. Hawaii strain CB4856 by the Genome Sequencing Consortium ([http://genome.wustl.edu/projects/celegans/chrom5\\_layout.html](http://genome.wustl.edu/projects/celegans/chrom5_layout.html); vd50e09.s1 on cosmid T05B11; vr92g12.s1 on C37C3; vd50e05.s1 on F20D6; vm25h11.s1 on K07C11; and vc88c08.s1 on F21F8) or by us (SJ1C13498T, SJ2A13705, SJ3C13798A, SJ4A16984, SJ5A17355C, and SJ6C18005T on cosmid F40A3; SJ7C28575T on F26D11; SJ8ICATTGGCT1363 and SJ9TA3428–29CΔ on Y97E10AL; SJ10T15326C on ZC513; SJ11C7063T, SJ12G19662T, and SJ13T20903 on CO9H5; SJ14A19850–19944 on D1014. Numbers refer to position of the SNPs in the cosmid sequences; Δ indicates deletion and the bold I indicates insertion). The mutant strain *dpy-11(e224) spr-1(ar200) rol-3(e754)*; *sel-12(ar171) unc-1(e538)* was crossed to CB4856 and 109 Dpy non-Rol recombinants were isolated. Analysis of these recombinants allowed us to restrict the *spr-1* interval to the region between SJ9TA3428–29CΔ on Y97E10AL and SJ14A19850–19944 on D1014.

#### Antisuppression experiments and sequencing

Cosmid DNA or PCR amplified genomic DNA spanning the determined *spr-1* region (see above) was injected at 5 or 10 μg/mL each, alone or in pools, together with pRF4 (150 μg/mL) or *pmyo3::gfp* (20 μg/mL) and pBluescript (≤200 μg/mL) into recipient strain *spr-1(ar200)*; *sel-12(ar171) unc-1(e538)*. As a control, each marker was injected with pBluescript (≤200 μg/mL). Transgenic lines were established and their egg-laying ability was checked for antisuppression, indicative of *spr-1(+)* activity. None of the pools that gave viable transgenic lines resulted in detectable antisuppression (data not shown). However, transgenic lines could not be established for cosmids DD11, F58G4, and T23D2 (=D1014); these cosmids appeared to cause larval lethality, so we sequenced candidate genes in the region. *spr-1* was associated with mutations in *D1014.8*, as described in the Results section. A PCR fragment amplified from *C. elegans* genomic DNA with oligos 5'-D1014-8505 (CAGATGACCAGAAAGTGGTC) and 3'-D1014-12185 (GAGAGAGAAAAGA CACGGCC) and extending from the stop codon of predicted *D1014.5* to the ATG of predicted *D1014.3* was then injected at (20 μg/mL) together with *pmyo3::gfp* (20 μg/mL) and pBluescript (≤100 μg/mL) into recipient strain *spr-1(ar200)*; *sel-12(ar171) unc-1(e538)*. Two transgenic lines were obtained that displayed antisuppression (line 1, 22/36 F<sub>2</sub> and 50/73 F<sub>3</sub> were Egl; line 2, 12/16 F<sub>2</sub> and 26/46 F<sub>3</sub> were Egl. All transgenic F<sub>3</sub>s were scored and around 13% in both lines arrested as young larvae). We note that when this PCR fragment was cloned into pCR-XL-TOPO (Invitrogen), and the GFP cDNA was introduced at the ATG or before the TAG of *spr-1*, we were not able to obtain viable lines displaying antisuppression, and no expression of SPR-1::GFP as visualized in live worms or by anti-GFP antibody staining was observed (data not shown). Therefore, overexpression of SPR-1 under the control of its own promoter appears toxic. Nevertheless, fusion proteins similarly tagged at the C terminus, like SPR-1::MYC or hCoREST::HA, are functional when expressed under the control of a heterologous promoter (see text).

*cDNA cloning and correction of the GENEFINDER prediction for D1014.8 and D1014.9*

In order to determine *spr-1* gene structure, and in the absence of available ESTs, three cDNA libraries were screened to clone *spr-1* cDNA. Two rounds of nested PCRs were performed using: (1) *spr-1* primers 3'-race1 (CCAGAACCATCAGACTCGC), 3'-race12 (GAGTAATTTCTGATCTGTCC), and 5'-race9 (GATCAGCGCTCTGTCATCG) and the library-specific primers 5'pPC86 (TATAACGCGTTTGGAACTACT) and 3'pPC86 (GTAAATTTCTGGCAAGGTAGAC) recommended by the manufacturer (ProQuest library from Life Technologies) or T3 and T7 (Stratagene's embryonic and mixed-stage libraries); (2) *spr-1* primers 3'-race2 (5'-CTTCCGGTCCGCTGTGCGG-3'), 3'-race13 (5'-CGTTGTTGAGATGGGCGTGG-3'), 5'-race8 (5'-CAACTGTGGAGAAAAGCTG-3'), and 3'-spr-1 (5'-GTAAATTTGAATCTTCTTCTTCCATATCAGCCAAGCCTTTC TGG-3') or an oligo(dT)(12-18) primer (GIBCO-BRL) and the library-specific primers 5'pPC86, 3'pPC86, or SK. This allowed the cloning of three overlapping fragments of cDNA that were pieced together by PCR (note that no *spr-1* cDNA fragment could be amplified from the Life Technologies library). The resulting sequenced cDNA encompasses previously predicted genes *D1014.9* and *D1014.8* and represented the only mRNA species we could amplify (accession no. AAA96286). We communicated the *spr-1* gene structure to WormBase which was then corrected accordingly. The cDNA corresponding to the mRNA species present in *spr-1(ar200)* mutants was synthesized using an oligo(dT)(12-18) primer (GIBCO-BRL) from total *spr-1(ar200)*; *sel-12(ar171) unc-1(e538)* RNA and further amplified with primers 5'D1014.8/T3 (GCGCGCAATTAACCTCACTAAAGGCTTCTTCCATATCAGCCAGCC) and 3'D1014.8/T7 (CGCGCGTAATACGACTCACTATAGG GCTAACGCGATGAATCGTCCAG). Control experiments were performed similarly on total RNA from *sel-12(ar171)unc-1(e538)* worms. The products were then sequenced and the consequences of the G-to-A mutation for the mutant transcript analyzed.

*RNAi*

Each RNA strand was synthesized according to the manufacturer (Stratagene) using as templates PCR fragments amplified from N2 genomic DNA with the following primers: *spr-1* (corresponding to previously predicted D1014.8), 5'D1014.8/T3 (GCGCGCAATTAACCTCACTAAAGGCTTCTTCCATATCAGCCAGCC) and 3'D1014.8/T7 (CGCGCGTAATACGACTCACTATAGGGCTAACGCGATGAATCGTCCAG); *spr-1* (corresponding to previously predicted D1014.9), 5'D1014.9/T3 (GCGCGCAATTAACCTCACTAAAGGGAATTCCTTCTACTGCTTTGG) and 3'D1014.9/T7 (CGCGCGTAATACGACTCACTATAGGGCGATGGAGAATCTGCTCAATCCG); *Y74C9A.4*, 5'Y74C9A.4/T3 (GCGCGCAATTAACCTCAC TAAAGGCAATATCCCAGCGAACCC) and 3'Y74C9A.4/T7 (CGCGCGTAATACGACTCACTATAGGGCATTCCCAA TTTCTTCCAGC); *Y40B1B.6*, 5'Y40B1B.6E4/T3 (GCGCGCAATTAACCTCACTAAAGGATATCGAAAACGAAAA AGTG) and 3'Y40B1B.6E4/T7 (CGCGCGTAATACGACTCACTATAGGGCCCATTTTCTTAAACTTTTC); *R13G10.2*, 5'R13G10.2E5/T3 (GCGCGCAATTAACCTCACTAAAGGCCAGTTACCAATTTCCGGCAC) and 3'R13G10.2E5/T7 (CGCGCGTAATACGACTCACTATAGGGTCTAACATACC TTTCCAG); *T08D10.2*, 5'T08D10.2E4/T3 (GCGCGCAATTAACCTCACTAAAGGCTCTCGCAGAGGTTGCCCG) and 3'T08D10.2E4/T7 (CGCGCGTAATACGACTCACTATAGG GCTCGAGCAACTTGGCTTTTCAG). Each RNA strand was

purified using QIAGEN RNeasy columns and annealed in 0.5× injection buffer (Mello and Fire 1995). RNAi was performed as in Fire et al. (1998). dsRNA or annealing buffer was microinjected into the pseudocoelomic space of young adults. Injected hermaphrodites were cultured individually and their progeny were scored for their ability to lay eggs. Alternatively, DNA was cloned into the vector pPD129.36 (see below) and transformed into bacterial strain HT115 (Timmons and Fire 1998; Timmons et al. 2001). Eggs or bagging mothers were placed on a lawn of such bacteria, and their ability to lay eggs was scored as adults.

*Plasmid constructions*

All constructs were sequenced.

*RNAi constructs* The *spr-1* cDNA fragment amplified from Stratagene's library with primers 3'-race2 and SK was subcloned into pCR-XL-TOPO (Invitrogen), excised with *EcoRI* and cloned into *EcoRI* digested vector pPD129.36 (Timmons et al. 2001). Exon E3 of C53A5.3 (*hda-1*), exon 2 of C08B11.2 (*hda-2*), and exon 2 of R06C1.1 (*hda-3*) were amplified from genomic DNA using primers 5'hda1-25/RI (CGGAATTCCGACGGTAA GCGCCGTGTCG) and 3'hda1-1340/RI (CGGAATTCCGCT GCTCTCTTCGCATCGC), 5'-C08B11.2E2/RI (CGGAATTCC TTCATCCAATGAAACCTCAAC) and 3'-C08B11.2E2/RI (CG GAATTCCGACAAAAGCAAATTGACC), or 5'-R06C1.1-E2/RI (CGGAATTCCCTTTAAATCTCCAGTTC) and 3'-R06C1.1-E2/RI (CGGAATTCCAGCGTCCCGCCCGTGCCTC), respectively, and cloned into *EcoRI* digested pPD129.36.

*Expression constructs* *pcog-2::SPR1::MYC* was obtained by amplifying *spr-1* cDNA with oligos 5'-Xma/spr-1 (5'-TC CCCC CGGGGGGTGTAGAAAATGGATTGTATGACGAT GATGG-3') and 3'-spr-1MYC/NcoI (5'-CCCATGCCATGGC TACAGATCCTCTTCTGAGATGAGTTTTTTGTTC AATTGA ATCTTCTTCTTCTTCC-3') and cloned into *XmaI-NcoI* digested pWH17 (*pcog-2::GFP*; Hanna-Rose and Han 1999). *pcog-2::CoRest::HA* was obtained by amplifying human CoREST cDNA (You et al. 2001), previously subcloned in pCDNA3.1 with an HA tag added in frame before the stop codon, with oligos 5'-CoREST/Xma (5'-TCCCCCGGGGGGAAAATG GTGGAGAAGGGCCCCGAGGTC-3') and 3'-HA/NcoI (5'- CCCATGCCATGGTCAAGCATAATCAGGAACATC-3') and cloned into *XmaI-NcoI* digested pWH17.

*Expression in  $\pi$  cells and antibody staining*

*pcog-2::SPR1::MYC* or *pcog-2::CoREST::HA* (40  $\mu$ g/mL) were injected together with pWH17 (40  $\mu$ g/mL) and pRF4 (150  $\mu$ g/mL) into the germ line of *spr-1(ar200)*; *sel-12(ar171) unc-1(e538)* (Mello and Fire 1995). As a control, pWH17 (80  $\mu$ g/mL) and pRF4 (150  $\mu$ g/mL) were injected alone. Four, two, and six transgenic lines were obtained, respectively, and their ability to lay eggs was analyzed by scoring all the transgenic animals of the broods segregating from at least two mothers or alternatively all the transgenic animals layed during the two first days of egg-laying. We note that one of the six control lines exhibited comparatively a higher level of Egl animals. Expression of the *pcog-2::gfp* reporter was observed in live transgenic animals. Synchronized transgenic populations were stained according to Bettinger et al. (1996) using antibodies against the MYC tag (146.7  $\mu$ g/mL, monoclonal 9E10, SIGMA) or the HA tag (2  $\mu$ g/mL, monoclonal 12CA5, Roche) and secondary antibodies (Jackson ImmunoResearch Laboratories, nos. 115-165-146 and 115-095-146) together with DAPI, and both SPR1::MYC and CoRest::HA proteins were found to be expressed in the nuclei

of the expected cells. To analyze *lin-11* expression in  $\pi$  cells, synchronized strains containing *nIs2* (see text) were stained according to Bettinger et al. (1996) using antibodies against LacZ protein (Promega, no. Z3781) and secondary antibodies (Jackson ImmunoResearch Laboratories, no. 115-165-146) together with DAPI. As a staining and staging marker, MH27 antibodies against JAM-1 (Francis and Waterston 1991) were also used on a fraction of the fixed animals. Worms were mounted in a drop of antifade (Molecular Probes) and analyzed with a fluorescence microscope (Zeiss Axioplan 2).

#### LacZ activity staining

It has been reported previously that LacZ staining of the *lin-12::lacZ* transcriptional reporter *arIs11* appears faster in *lin-12(d)* compared to *lin-12(+)* worms (Wilkinson et al. 1994). To assess whether *spr-1(ar200)* affected *lin-12* or *lag-2* expression, we analyzed the speed and intensity of LacZ staining of the transcriptional reporters *arIs11* and *arIs13*, respectively (Wilkinson et al. 1994). Synchronized populations were fixed and stained for various periods of time (2.5 h, 4.5 h, 6.5 h, and overnight) according to A. Fire (1992). Staining was analyzed for the following cell fate decisions: AC/VU decision (*lag-2*), VPC specification (*lin-12*), and  $\pi$  cell specification. No difference in staining speed or intensity was observed between *spr-1(ar200)* and control worms.

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