lin-35/Rb and the CoREST ortholog spr-1 coordinately regulate vulval morphogenesis and gonad development in C. elegans

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Abstract

Using a genetic screen to identify genes that carry out redundant functions during development with lin-35/Rb, the C. elegans Retinoblastoma family ortholog, we have identified a mutation in spr-1. spr-1 encodes the C. elegans ortholog of human CoREST, a protein containing Myb-like SANT and ELM2 domains, which functions as part of a transcriptional regulatory complex. CoREST recruits mediators of transcriptional repression, including histone deacetylase, and demethylase, and interacts with the tumor suppression protein REST. spr-1/CoREST was previously shown in C. elegans to suppress defects associated with loss of the presenilin sel-12, which functions in the proteolytic processing of LIN-12/Notch. Here we show that lin-35 and spr-1 coordinately regulate several developmental processes in C. elegans including the ingression of vulval cells as well as germline proliferation. We also show that loss of lin-35 and spr-1 hypersensitizes animals to a reduction in LIN-12/Notch activity, leading to the generation of proximal germline tumors. This defect, which is observed in lin-35; spr-1; lin-12(RNAi) and lin-35; spr-1; hop-1 (RNAi) triple mutants is likely due to a delay in the entry of germ cells into meiosis.

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Introduction

The mammalian pocket proteins, which include the Retinoblastoma protein pRb and its paralogs, p107 and p130, are well-established regulators of the cell cycle (reviewed by Kaelin, 1999; Harbour and Dean, 2000a; Classon and Dyson, 2001). In addition, numerous studies have implicated Rb family members in promoting cell differentiation as well as other processes related to development (reviewed by Harbour and Dean, 2000b; Morris and Dyson, 2001; Wikenheiser-Brokamp, 2006). Although much of the evidence in support of a role for the pocket proteins in development has been obtained from tissue culture-based systems, in vivo results have also provided several notable findings. For example, disruption of Rb family functions in the developing murine lung leads to increased expression of the neuroendocrine cell fate and a concomitant reduction in the specification of other epithelial cell types, suggesting that Rb family proteins control the development of specific cell lineages within the lung (Wikenheiser-Brokamp, 2004). Furthermore, a role for Rb proteins in skin epithelial cell differentiation is supported by findings that ablation of p107 and p130 results in the impaired differentiation of keratinocytes (Ruiz et al., 2003). Interestingly, this study also revealed an apparent role for p107/p130 in the morphogenesis of epidermal hair follicles and incisors, which is independent of differentiation.

The extent to which these observed developmental defects are an indirect consequence of perturbations to the cell cycle is not entirely clear. Nevertheless, a number of recent in vivo findings support the idea that Rb family activities, including those connected to proliferation, differentiation, and apoptosis, may be functionally separable (de Bruin et al., 2003; MacPherson et al., 2003; Wu et al., 2003; Takahashi et al., 2004; Sage et al., 2005). This separation of functions is further supported at the mechanistic level by the recent finding that the RET finger protein (RFP) specifically inhibits gene transcriptional activation by pRb, but does not inhibit the repressive (cell
cycle) functions of pRb (Kruitzfeldt et al., 2005). Furthermore, microarray analyses suggest that the transcriptional targets of Rb family proteins as well as their well-established DNA-binding regulatory partners, the E2Fs, include many non–cell cycle targets such as factors implicated in differentiation, signaling, and cell architecture (e.g., Muller et al., 2001; Markey et al., 2002; Polager et al., 2002; Black et al., 2003; Dimova et al., 2003; Balciunaite et al., 2005).

Further evidence in support of a role for the Rb and E2F family proteins in basic developmental functions includes studies from non-mammalian systems. For example, in Dro sophila, loss of the E2F co-partner dDP results in ventralization of the embryo because of misexpression of the EGF-like ligand Gurkin (Myyster et al., 2000). Conversely in Xenopus, loss of E2F function leads to the elimination of posterior and ventral structures, whereas overexpression inhibits the formation of dorsal and anterior structures (Suzuki and Hemmati-Brivanlou, 2000). A Role for E2F and Dp in establishing proper body axis patterning is also observed in C. elegans, where the loss of these orthologs leads to an early defect in the proper distribution of tissue-specific transcriptional regulators (Page et al., 2001).

Studies on the C. elegans Rb family ortholog, lin-35, have revealed both canonical cell cycle functions as well as many unexpected roles during development that appear in most cases to be unlinked to cell cycle regulation (reviewed by Fay, 2005). Interestingly, the majority of these functions cannot be detected through the analysis of lin-35 single mutants. Rather these functions are revealed only when lin-35 is inactivated in the appropriate mutant backgrounds, indicating that lin-35 functions redundantly with other pathways to regulate both cell cycle and non–cell cycle processes. This precedent for redundancy of lin-35 functions was initiated by the discovery of a role for lin-35 in inhibiting epidermal cells from inappropriately acquiring vulval cell fates (the synthetic multi–vulval [SynMuv] phenotype; Ferguson and Horvitz, 1989; Lu and Horvitz, 1998; reviewed by Fay and Han, 2000), a function that it shares with the C. elegans E2F ortholog, efl-1 (Ceol and Horvitz, 2001). Namely, animals that are mutant for lin-35 (or other members of the so-called Class B group of SynMuv genes) and for genes of either the Class A (Ferguson and Horvitz, 1989) or Class C (Ceol and Horvitz, 2004) groups show a highly penetrant hyperinduction of vulval cells. The basis for this phenotype was recently shown to be the result of ectopic expression of LIN-3, the EGF-like ligand that is the primary inducer of vulval cell fates (Cui et al., 2006). Further studies looking for novel lin-35 synthetic phenotypes have revealed roles for LIN-35 in morphogenesis of the C. elegans pharynx (Fay et al., 2003, 2004), asymmetric cell divisions (Cui et al., 2004), execution of cell lineages within the somatic gonad (Bender et al., 2004), and larval growth (Cui et al., 2004; Cardoso et al., 2005; Chesney et al., 2006; Fay lab unpublished data), as well as a traditional role in cell cycle control (Boxem and van den Heuvel, 2001, Fay et al., 2002). lin-35 also functions non-redundantly to repress the expression of germ-line-associated genes in somatic cells and lin-35 mutants show hypersensitivity to RNAi (Wang et al., 2005). Finally, lin-35 negatively regulates ribosome biogenesis at the level of rRNA expression (Voutev et al., 2006).

In this work, we describe a novel role for lin-35 in the morphogenesis of the C. elegans vulva. Specifically, in double mutants of lin-35 and the C. elegans CoREST transcriptional repressor ortholog, spr-1, cells of the vulval epithelium fail to fully ingress, leading to an abnormally compressed vulval lumen at the L4 larval stage. Furthermore, this phenotype does not appear to result from primary defects in either cell cycle regulation or differentiation. We also show a role for lin-35 and spr-1 in promoting germline proliferation and in inhibiting the formation of proximal germline tumors. Interestingly, human CoREST is a key cofactor of the REST tumor suppressor gene (Andres et al., 1999; Westbrook et al., 2005). Based on these and other findings, we suggest that the non-cell cycle functions of Rb family members may contribute to the tumor-suppressing activities of these proteins.

Materials and methods

C. elegans genetic methods and strains

All C. elegans strains were maintained according to standard methods (Stemmagle, 2005). All experiments were carried out at 20°C. Strains used in these studies include the following: N2 (wild type), CB4856, M1H461 [lin-35 (n745), kuEx119], N2H2246 [ayd(egl-17::GFP)], WY301 [lin-35(n745); egl-17::GFP], SU93 [cisl (ajm-1::GFP)], WY334 [spr-1(fd6); lin-7::GFP], WY258 [spr-1(fd6)], JK2866 [ril56;lag-2::GFP]), MH1317 [kaul929(egl-13::GFP)], GS1214 [sel-12(ar171), unc-1(e358)], WY329 [lin-35(n745); lag-2::GFP], WY298 [lin-35(n745); ajm-1::GFP], WY248 [lin-35(n745); spr-1(fd6); kwa119], WY328 [lin-35(n745); dpy-1(e224), spr-1(fd6), unc-76(e911)], kwa119], WY294 [lin-35(n745); spr-1(fd6); lin-7::GFP, kuEx119], WY300 [lin-35(n745); egl-13::GFP], WY295 [hop-1 (ar179), spr-1(fd6); unc-76(e911)], WY251 [lin-35(n745); spr-1(ar205); kwa119], WY249 [lin-35(n745); spr-1(fd6); ajm-1::GFP, kuEx119], WY296 [lin-35(n745); sel-12(ar171), unc-1(e338)], WY326 [lin-35(n745); spr-1(fd6); sel-12(ar171), unc-1(e338)], DV1575 [nn06(lin-7::GFP)], and WY299 [lin-35(n745); lin-7::GFP].

spr-1/slr-10(fd6) genetic mapping

Based on the failure of spr-1(fd6) to cosegregate with dpy-11 and unc-76, fd6 was assigned to the right arm of LGV. 5979 Dpy Non-Unc and 14/55 Unc Non-Dpy recombinants segregating from lin-35; dpy-11, fd6; unc-76(e911) hermaphrodites retained fd6. The genetic region containing fd6 was further refined by SNP mapping according to standard methods (for details see Fay, 2006). fd6 was mapped to a 425-kb region on LGV between the polymorphisms e911 and egl-13(fd6). A Complementation test

Rescue of fd6 was obtained through the injection of cosmids D1014 and F20D6 together with pRf4 into lin-35; spr-1; kwa119(+) hermaphrodites (for details on methods see Evans, 2005). Rescue was inferred based on the appearance of roller animals that did not require kwa119 for rapid growth or long-term viability. In addition, transgenic rescue was also obtained with YAC Y97E10.

Complementation test

spr-1(ar205) males were crossed to lin-35(n745); dpy-1(e224), spr-1(fd6), unc-76(e911), kwa119 hermaphrodites and Non-Dpy Unc cross progeny containing the kwa119 array were identified in the next generation, clonally transferred, and allowed to self fertilize. 60 non-Dpy Unc kwa119 progeny
were clonally transferred and subsequent generations were analyzed for their dependence on the array. 14/60 (23%; expect 25% based on the expected frequency of lin-35 homozygous mutants) animals were found to require kuEx119 for normal health and viability. Of these 14 animals, nine gave rise to small broods containing Dpy Unc animals, indicating that the parental genotype was lin-35(n745); dpy-11(x224), spr-1(fd6), unc-76(e911); spr-1(ar205); kuEx119. Analysis of one of the nine balanced isolates revealed vulval morphology and gonad defects similar to those observed for lin-35(n745); spr-1 (fd6) mutants (data not shown).

**DAPI staining**

Prior to staining, animals were fixed with ice-cold methanol for 5 min in a glass depression slide. Animals were then washed several times with PBS (pH 7.4) containing 0.1% Tween 20. They were then incubated in 0.01 ng/μl DAPI for 1 h at 4°C, washed several times with PBS, and placed on slides for microscopy.

**Immunohistochemistry**

Animals were collected in M9, placed on glass slides coated with poly-L-lysine, and freeze cracked (for details see Duerr, 2005). Slides were blocked with TBS containing 0.1% Tween 80, and animals were treated with 0.05 U/μl chondroitinase ABC for 1 h at 37°C (Mizuguchi et al., 2003). The slides were then washed with TBS and incubated with the 1-B-5 chondroitin stub mAb (1:2000; Seikagaku) for 1 h at 4°C, washed several times with PBS, and placed on slides for microscopy.

**Western blotting**

Crude extracts were prepared by sonicating worms in 50 mM NaAc (pH 6) with protease inhibitor cocktail (Sigma). The resulting extract (2 μg) was digested with 10 mU chondroitinase ABC (Seikagaku) for 3 h at 37°C and analyzed by SDS-PAGE, as described (Hwang et al., 2003a,b). Chondroitin proteoglycan core proteins were detected by western blotting with the 1-B-5 chondroitin stub mAb (1:1000; Seikagaku) followed by a goat anti-mouse secondary antibody (1:1000; Jackson ImmunoResearch) for 2 h. After additional washing with TBS, slides were coated with mounting solution (Dabco) and visualized by fluorescence microscopy.

**RNAi by feeding or microinjection**

RNAi feeding and microinjection experiments were carried out according to standard methods (Ahringer, 2005). For microinjection, all dsRNAs were injected into the syncytial germ lines of adult hermaphrodites at a concentration of ∼1.0 μg/μl. Injected animals were allowed to recover for 24 h on NGM plates and were then transferred to fresh plates. F1 self-progeny produced from 24–48 h post-injection were scored for the relevant phenotypes. Forward and reverse primer sequences used to amplify products for RNA were as follows: spr-1, ATGGATTTGTATGACGATGATGGA and CTGCATATTTT-CAGCTTTTTTCTCCA; spr-4, ATGTGTCGGAGACCACTTCTT and TGACCTTGTTGAAACAAATTAATTTGTGC; spr-5, GTAAACTCTGAGCAGCAGGAG and CCGGCCATCTTCTTCC; hop-1, GGACTAGTCGTAT- TAGCAAGACGATCAGTGGGAGCCTCTTT and CCGGCTGAGACGGAGCAATTTTGCTC; lin-12, TCAGGGAAAACGGGGAACATT and AATCGGCGTTTTTCCCATCTT; efl-1, ACATGAGGATCCCAGAAGCC and CAATCTTCCTGGAACACATTGC. The T7 promoter sequence used was TAATAAGCTACTATAGGGA. All other primers were as previously described (Fay et al., 2002).

**Results**

**lin-35/Rb and spr-1/slr-10 are genetically redundant**

Using a previously described genetic screen to identify proteins that perform overlapping functions with LIN-35 (Fay et al., 2002), we isolated a single allele (fd6) defining a locus originally designated as slr-10 (for synthetic with lin-35/Rb; hereafter referred to as spr-1). Briefly, lin-35(n745) homozygous animals that carry an extrachromosomal array (kuEx119) expressing wild-type lin-35 along with a GFP reporter were subjected to chemical mutagenesis. Following an F2 clonal screen, strains harboring synthetic mutations were identified based on the expression of visible phenotypes in animals that failed to inherit the array. Whereas both lin-35 and spr-1(fd6) single mutants were largely indistinguishable from wild type, lin-35; spr-1 double mutants were smaller and slower growing and had an abnormally clear or mottled appearance (Figs. 1A, B; data not shown). Also, as described below, lin-35; spr-1 mutants showed defects in vulval morphogenesis, somatic gonad development, and fertility.

**fd6 defines a mutation in spr-1, the C. elegans ortholog of human CoREST**

fd6 was mapped to a small region on the right arm of LGV using classical genetic and SNP mapping techniques (see Materials and methods; Fay, 2006). Two overlapping cosmids in the region, D1014 and F20D6, rescued the synthetic defects
of lin-35; spr-1 animals completely. RNAi of genes contained on these cosmids in the lin-35 mutant background led to the identification of a single clone (corresponding to D1014.8/spr-1) that closely phenocopied the effects of the fd6 mutation (also see below). Sequence analysis of the spr-1 coding region in the fd6 mutant background revealed a C to T transition at nucleotide position 1375 of the spr-1 cDNA. This mutation produces a premature translational stop codon following amino acid 458 of the 558-amino-acid protein. Based on these data, along with the observed synthetic genetic interactions of other spr-1 alleles with lin-35 (see below) and the failure of a previously isolated allele of spr-1 to complement fd6 (see Materials and methods) we conclude that spr-1 is the relevant locus defined by fd6.

spr-1 encodes an ortholog of the human CoREST protein (Jarriault and Greenwald, 2002), which acts within a larger complex that includes the REST/NRSF tumor suppressor (Andres et al., 1999; Westbrook et al., 2005). The REST/CoREST complex is thought to repress the transcription of genes through the recruitment of histone deacetylases (Humphrey et al., 2001; You et al., 2001) and demethylases (Lee et al., 2005) to specific target sites. The fd6 mutation leads to a C-terminal truncation of SPR-1 just preceding the second of two conserved SANT domains. SANT domains are found in a number of chromatin remodeling enzymes and have been shown to function in chromatin binding through interactions with histone tails (Boyer et al., 2002).
Given that the original alleles of spr-1 were identified on the basis of their ability to suppress the egg-laying defective (Egl) phenotype of sel-12 presenilin mutants (Jarriault and Greenwald, 2002), we tested spr-1(fd6) for this capacity and found that it suppressed the Egl phenotype of 10/10 sel-12 (ar171) animals, indicating that spr-1(fd6) can function in its canonical role as a sel-12 suppressor. In contrast, 34/34 lin-35; sel-12 mutants were incapable of laying eggs, indicating that lin-35 does not share this specific suppressive function with spr-1.

lin-35; spr-1 double mutants display defects in vulval morphogenesis

The C. elegans hermaphrodite egg-laying and mating organ, the vulva, is derived from three epithelial precursor cells, which undergo a well-defined series of cell divisions along with morphogenetic events that include migrations, cell shape changes, and cell fusions (Sternberg, 2005). During the mid-L4-stage, the vulva acquires a stereotypical “Christmas tree” morphology at which time it is comprised of seven vertically stacked multinucleate toroid (ring-shaped) cells that contain a large central lumen (Figs. 2A, 3I). A number of mutations have been characterized that specifically affect the morphogenetic steps of vulval development including eight loci that result in a partially collapsed or “squashed” vulval lumen (the Sqv phenotype) at the L4 stage (Fig. 2E; Herman et al., 1999; Sternberg, 2005). Although morphogenesis is severely defective, these sqv mutants display a wild-type pattern of vulval cell divisions along with normal hallmarks of cell differentiation (Herman et al., 1999).

We found that most lin-35; spr-1 double mutants exhibited a phenotype that was similar to that previously reported for the sqv mutants. Namely, the vulval lumen of lin-35; spr-1 mutants was substantially compressed at the mid-L4 stage relative to wild type or the large majority of lin-35 and spr-1 single mutants (Figs. 2B, C; Table 1; data not shown). This defect in vulval morphogenesis was also observed in lin-35; spr-1(RNAi) and spr-1; lin-35(RNAi) animals (Fig. 2D; Table 1) including (in the latter case) multiple independently isolated alleles of spr-1 (Table 1). The Sqv-like phenotype was also manifest in double mutants at time points preceding the mid-L4 stage (Figs. 2A, B insets, 3C, 4I), similar to previously described sqv mutants (Herman et al., 1999). This indicates that underlying defect in lin-35; spr-1 double mutants somehow impinges on the ability of the vulval cells to undergo normal ingression.

![Fig. 3. lin-35; spr-1 mutants undergo normal vulval cell divisions, differentiation patterns, and cell fusion events. DIC (A, C, E, G) and corresponding GFP fluorescence (B, D, F, H) images of wild-type (E, F) and lin-35; spr-1(fd6) (A–D, G, H) developing vulvae. Panel I shows the vulval cell lineage during the L3 stage and a schematic of vulval morphology at the mid-L4 stage in wild-type animals. The fates of nuclei within the L3 lineage and the L4 vulva are indicated by color; wild-type egl-17::GFP expression is indicated by the green line circumscribing cells in the lineage diagram (A, B) L3- and (C, D) L4-stage lin-35; spr-1 mutants showing normal expression of egl-17::GFP in L3-stage P6.p derivatives (A, B; white arrowheads) and in the L4-stage N-cells of P5.p and P7. p (C, D; white arrows; the T-cells are not visible in this focal plane). (E–H) ajm-1::GFP, which marks the adherens junctions between the vulval toroids in wild-type (E, F) and lin-35; spr-1 L4 animals (G, H). Black arrowheads indicate the GFP-marked junctions between the vulval toroids. Although expression of this marker is fainter in the lin-35; spr-1 mutants (most likely due to the silencing effect of lin-35 mutations on transgenic arrays; Hsieh et al., 1999), seven toroids can reproducibly be identified in vulvae from both animals. Scale bar in panel A, 10 μm, for panels A–H.](image-url)
Vulval and germline phenotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sqv (%)</th>
<th>Oocytes/arm</th>
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<tbody>
<tr>
<td>N2</td>
<td>0 (n=31)</td>
<td>7.0 (±1.4) (range, 4–10) (n=52)</td>
</tr>
<tr>
<td>lin-35(n745)</td>
<td>11 (n=27)</td>
<td>6.7 (±1.7) (range, 4–12) (n=27)</td>
</tr>
<tr>
<td>spr-1(fd6)</td>
<td>3 (n=30)</td>
<td>7.8 (±1.9) (range, 5–11) (n=28)</td>
</tr>
<tr>
<td>spr-1(ar205)</td>
<td>0 (n=26)</td>
<td>ND</td>
</tr>
<tr>
<td>spr-1(ar200)</td>
<td>7 (n=28)</td>
<td>ND</td>
</tr>
<tr>
<td>lin-35; spr-1(fd6)</td>
<td>63 (n=88)</td>
<td>2.9 (±1.2) (range, 1–5) (n=28)</td>
</tr>
<tr>
<td>lin-35; spr-1(ar205)</td>
<td>53 (n=30)</td>
<td>2.6 (±0.9) (range, 1–4) (n=38)</td>
</tr>
<tr>
<td>spr-1(fd6); lin-35(RNAi)</td>
<td>48 (n=21)</td>
<td>ND</td>
</tr>
<tr>
<td>spr-1(ar200); lin-35(RNAi)</td>
<td>26 (n=19)</td>
<td>ND</td>
</tr>
<tr>
<td>spr-1(ar205); lin-35(RNAi)</td>
<td>27 (n=22)</td>
<td>ND</td>
</tr>
<tr>
<td>lin-35; spr-1(RNAi)</td>
<td>64 (n=36)</td>
<td>4.5 (±1.7) (n=36)</td>
</tr>
<tr>
<td>spr-4(RNAi)</td>
<td>8 (n=35)</td>
<td>ND</td>
</tr>
<tr>
<td>lin-35; spr-4(RNAi)</td>
<td>53 (n=15)</td>
<td>ND</td>
</tr>
<tr>
<td>spr-5(RNAi)</td>
<td>10 (n=20)</td>
<td>ND</td>
</tr>
<tr>
<td>lin-35; spr-5(RNAi)</td>
<td>67 (n=24)</td>
<td>ND</td>
</tr>
<tr>
<td>lin-35; spr-1(fd6); hop-1(RNAi)</td>
<td>54 (n=26)</td>
<td>2.5 (±1.0) (range, 1–5) (n=29)</td>
</tr>
<tr>
<td>lin-35; spr-1(fd6); hop-1(RNAi)</td>
<td>77 (n=22)</td>
<td>2.9 (±1.0) (range, 1–5) (n=30)</td>
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<tr>
<td>lin-12(RNAi)</td>
<td>7 (n=27)</td>
<td>6.4 (±0.9) (range, 5–8) (n=27)</td>
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<tr>
<td>hop-1(RNAi)</td>
<td>0 (n=21)</td>
<td>6.5 (±1.0) (range, 5–9) (n=21)</td>
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</tbody>
</table>

All RNAi experiments were carried out using injection methods. n values for oocyte counts were based on individual gonad arms.

Our analysis of vulval development in the double mutants based on the cell lineage did not reveal any obvious defects. Cells divisions occurred in their normal characteristic planes and with the appropriate relative timing to consistently produce 22 nuclei (data not shown). To more thoroughly assess vulval cell fate specification and differentiation in lin-35; spr-1 animals, we examined expression of the egl-17::GFP marker in the double mutants (gift of M. Stern). In wild-type animals, egl-17::GFP is initially expressed in dividing vulval cells that are derivatives of P6.p. Following the terminal divisions, this expression shifts to the N and T lineages of P5.p and P7.p (Olson et al., 2006). Although there may have been a slight reduction in the initiation and elongation of heparan sulfate (Kitagawa et al., 1999; Herman et al., 2000; Hawgn and Horvitz, 2002a,b; Hwang et al., 2003a). Furthermore, the identification of a glycosyltransferase (SQV-5) that is specific for the biosynthesis of chondroitin has implicated chondroitin deficiencies as the underlying cause of the Sqv phenotype (Hwang et al., 2003b). In support of this, we found that rib-2, which encodes an enzyme required specifically for the initiation and elongation of heparan sulfate (Kitagawa et al., 2001; Morio et al., 2003), was not required for the formation of a normal vulval lumen (n=40; Fig. 2F). Interestingly, we did observe a low frequency (14%, n=29) of vulval induction defects in rib-2(gk318) mutants (Fig. 2F), suggesting that as in other systems, heparan sulfate may modulate paracrine signaling through the conjugation of peptide growth factors in C. elegans (Powell et al., 2004; Iwamoto and Mekada, 2006).

In order to test whether chondroitin biosynthesis is impaired in lin-35; spr-1 double mutants, lysates were prepared from synchronized wild-type, lin-35, and spr-1 single-mutant animals as well as lin-35 mutants subjected to spr-1 RNAi feeding. Following treatment with chondroitinase ABC, samples were separated by electrophoresis, transferred to a membrane, and probed with a mAb directed against the remaining stub oligosaccharides on chondroitin proteoglycans (Olson et al., 2006). Although there may have been a slight reduction in the total levels of chondroitin proteoglycans in lin-35 single mutants versus wild type, the levels in lin-35; spr-1 (RNAi) double mutants were equivalent to those in lin-35 single mutants (based on two independent experiments; Fig. 5A and data not shown). These results indicate that lin-35 and spr-1 do not function redundantly to promote chondroitin biosynthesis, and that in contrast to the sqv mutants, the Sqv phenotype in double mutants is not correlated with a strong reduction in chondroitin biosynthesis.

To determine whether lin-35; spr-1 mutants might exhibit localized changes in chondroitin levels, we carried out immunocytochemistry to assay chondroitin expression in whole animals. Although this staining procedure showed some inherent variability within controlled sample sets, we did observe clear vulval staining in the majority of L4-stage wild-type (24/27) and lin-35; spr-1 (17/24) double mutants. Furthermore, staining levels of the two genotypes were observed to be roughly equivalent (Figs. 5B–E; data not shown). As a control, no staining was observed in sqv-2(n2826) mutants, which are defective at chondroitin biosynthesis (Figs.
Based on these findings, along with several qualitative differences in the outward appearance of the vulval phenotype (see above), we conclude that unlike previously identified sqv mutants, the synthetic Sqv phenotype of \textit{lin-35; spr-1} double mutants is not attributable to either substantive global or local alterations in chondroitin biosynthesis or distribution. This conclusion is further supported by our observation that simultaneous loss of \textit{lin-35}, \textit{spr-1} and either \textit{sqv-2} or \textit{sqv-7} leads to vulval phenotypes that are qualitatively additive and also typically more severe than either \textit{lin-35; spr-1} or the \textit{sqv} mutants alone (data not shown).

\textit{lin-35; spr-1} double mutants display fertility and germline defects

\textit{lin-35; spr-1} animals that failed to inherit the rescuing array displayed a substantially reduced brood size compared with wild type and single mutants (Table 2). This effect on brood size...
was greatly exacerbated in subsequent generations of double-mutant animals derived from parents that lacked the *kuEx119* extrachromosomal array (Table 2). Similar fertility defects were also observed in *lin-35; spr-1* mutant animals, further demonstrating that other alleles of *spr-1* can synergize with *lin-35* loss of function (LOF; Table 2). Consistent with the observed fertility defects, DIC and DAPI staining revealed that double mutants have substantially smaller gonad arms relative to those of wild type or single mutants and contain fewer germ cells (the Glp phenotype) at all stages and fewer oocytes in adults (Figs. 4A, B, 6A–D; Table 1; data not shown). The gonadal and germline defects observed in the double mutants were robustly rescued by expression of either *lin-35* or *spr-1* via a repetitive extrachromosomal array (Fig. 1; data not shown). Given that such arrays are typically silenced in the germline, this suggests that the underlying defects may be due to a somatic requirement for these genes.

The impaired fertility and diminutive gonads of *lin-35; spr-1* double mutants are similar to previous findings for *xnp-1 lin-35* double mutants (Bender et al., 2004). In the case of *xnp-1 lin-35* animals, this defect could be attributed to an absence or significant reduction in the number of gonadal sheath cells, which are required for proper expansion of the germline stem cell population during larval development (McCarter et al., 1997; Killian and Hubbard, 2005). To examine the status of sheath cells in double mutants, we used the *lim-7::GFP* reporter, which is expressed consistently in the cytoplasm (and more variably in the nuclei) of 8 of the 10 sheath cell pairs (1–4) that encapsulate each gonad arm (Hall et al., 1999). We observed an average of 7.0 sheath cell nuclei (n = 24; range, 6–8) per gonad arm in wild-type animals (Figs. 4C, D). Similarly, *lin-35; spr-1* double mutants had 6.5 sheath cell nuclei (n = 29; range, 5–8) per gonad arm (Figs. 4E, F). From this we conclude that there is not a gross deficiency in the number of sheath cell nuclei in *lin-35; spr-1* double mutants, although it is possible that these cells are nevertheless defective in their ability to promote germ cell proliferation. We note that although sheath cell number did not differ appreciably from that of wild type, we

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Brood size</th>
<th>Sterility (%)</th>
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<tbody>
<tr>
<td>N2</td>
<td>264 (±18)</td>
<td>0 (n = 50)</td>
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<tr>
<td><em>lin-35</em>(<em>n745</em>)</td>
<td>98 (±43)</td>
<td>3 (n = 100)</td>
</tr>
<tr>
<td><em>spr-1</em>(<em>jd6</em>)</td>
<td>215 (±41)</td>
<td>0 (n = 20)</td>
</tr>
<tr>
<td><em>lin-35; spr-1</em>(<em>jd6</em>)</td>
<td>20 (±18)</td>
<td>10 (n = 48)</td>
</tr>
<tr>
<td><em>lin-35; spr-1</em>(<em>ar205</em>)</td>
<td>5 (±13)</td>
<td>86 (n = 70)</td>
</tr>
<tr>
<td><em>lin-35; spr-1</em>(<em>ar205</em>)</td>
<td>19 (±13)</td>
<td>4 (n = 25)</td>
</tr>
<tr>
<td><em>lin-35; spr-1</em>(<em>ar205</em>)</td>
<td>12 (±13)</td>
<td>57 (n = 30)</td>
</tr>
</tbody>
</table>

* Brood size results taken from Fay et al., 2002.
* Mat− indicates that the double-mutant animals were derived from parents that lacked the *kuEx119* extrachromosomal array, which may contribute maternal *lin-35*.

The impaired fertility and diminutive gonads of *lin-35; spr-1* double mutants are similar to previous findings for *xnp-1 lin-35* double mutants (Bender et al., 2004). In the case of *xnp-1 lin-35* animals, this defect could be attributed to an absence or significant reduction in the number of gonadal sheath cells, which are required for proper expansion of the germline stem cell population during larval development (McCarter et al., 1997; Killian and Hubbard, 2005). To examine the status of sheath cells in double mutants, we used the *lim-7::GFP* reporter, which is expressed consistently in the cytoplasm (and more variably in the nuclei) of 8 of the 10 sheath cell pairs (1–4) that encapsulate each gonad arm (Hall et al., 1999). We observed an average of 7.0 sheath cell nuclei (n = 24; range, 6–8) per gonad arm in wild-type animals (Figs. 4C, D). Similarly, *lin-35; spr-1* double mutants had 6.5 sheath cell nuclei (n = 29; range, 5–8) per gonad arm (Figs. 4E, F). From this we conclude that there is not a gross deficiency in the number of sheath cell nuclei in *lin-35; spr-1* double mutants, although it is possible that these cells are nevertheless defective in their ability to promote germ cell proliferation. We note that although sheath cell number did not differ appreciably from that of wild type, we

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*Fig. 5. Analysis of chondroitin expression in wild type and mutants. (A) Western blot indicating levels of chondroitin in wild-type, *lin-35* single-mutant, and *lin-35; spr-1*(RNAi) animals. The multiple bands indicate several species of chondroitin proteoglycan core proteins. DIC (B, D, F) and fluorescence (C, E, G) images of wild-type (B, C), *lin-35; spr-1* (D, E), and *spr-2*(n2826) mutants early- or mid-L4 vulvae stained with a mAb specific for the chondroitin stub epitope. The majority of both wild-type and *lin-35; spr-1* L4 animals showed similar vulval-specific staining patterns that were never observed in *spr-2* (n2826) mutants, which cannot synthesize chondroitin. Also see Results. Scale bar in panel B, 10 μm, for panels B–G.*
did observe a strong shift in the positioning of the sheath cells in lin-35; spr-1 animals toward the distal half of the gonad arm (Figs. 4E, F, K), a phenomenon that could be a secondary consequence of germline proliferation defects. We also examined cells of the ventral uterine lineage using the cog-2:GFP reporter (Hanna-Rose and Han, 1999). We observed an average of 12 cells in both wild-type (n=24; range, 11–13) and lin-35; spr-1(RNAi) (n=24; range, 10–13) animals, indicating that cells are also correctly specified in the double mutant. However, whereas 79% of wild-type animals consistently had 6 cells per lateral side at the mid-L4 stage (n=24), only 25% (n=24) of lin-35; spr-1(RNAi) animals displayed an equal lateral distribution of these cells (Figs. 4G–J). In contrast, single mutants did not display cell distribution defects (data not shown). These data suggest that although lin-35 and spr-1 are not required for cell-type specification or differentiation per se, they may redundantly regulate subtle aspects of somatic gonad development such as organization, morphology, and function.

spr-1 is synthetic with other Class B SynMuv genes

To determine whether the vulval and germline defects observed in lin-35; spr-1 mutants could be phenocopied by
reducing the activities of other SynMuv genes (see Introduction), we tested several class B as well as one class A and one class C genes for genetic interactions with spr-1 (Table 3; reviewed by Sternberg, 2005; Fay and Han, 2000). Of the class B genes tested, lin-37 along with the E2F ortholog efl-1 produced strong and moderate effects, respectively. In contrast, lin-36 failed to show an interaction, indicating that some, but not all, class B genes function with lin-35 in its redundant role with spr-1. Furthermore, the class A (lin-15a) and class C (trr-1) genes showed only weak or no effects, similar to previous slr mutants studied (Fay et al., 2002, 2003, 2004; Bender et al., 2004).

We also note that inactivation of several other components of the C. elegans CoREST complex in the lin-35 mutant background led to similar synthetic defects as those observed with spr-1. These included spr-4, which encodes a C2H2-type finger protein (Lakowski et al., 2003), and spr-5, a putative histone demethylase (Table 1; Eimer et al., 2002; Jarriault and Sternberg, 2005). Taken together, these results support the model that both lin-35 and spr-1 carry out their redundant functions within the context of multisubunit transcriptional regulatory complexes.

The lin-35; spr-1 phenotype is not likely to be attributable to LIN-12/Notch hypersignaling

As described above, spr-1 was originally identified for its ability to suppress the Egl phenotype of sel-12 mutants (Jarriault and Greenwald, 2002), and sel-12 is itself a suppressor of gain-of-function (GOF) mutations in lin-12/Notch (Levitan and Greenwald, 1995). sel-12 encodes a presenilin-like protease that is required for the (site 3) cleavage and signaling capacity of Notch receptors (Greenwald, 2005). A second presenilin gene in C. elegans, hop-1, functions redundantly with sel-12, as hop-1; sel-12 double mutants are synthetically lethal (Li and Greenwald, 1997; Westlund et al., 1999). It has been suggested that the C. elegans CoREST complex may act directly on the hop-1 locus to mediate transcriptional repression, as hop-1 mRNA is upregulated in several CoREST complex mutant backgrounds (Eimer et al., 2002; Lakowski et al., 2003). Thus, it was possible that the synthetic phenotype of lin-35; spr-1 mutants is due, at least in part, to an increase in hop-1 abundance and, by extension, LIN-12/Notch signaling activity.

To test this model, we inactivated lin-12 and hop-1 in lin-35; spr-1 mutants using RNAi injection methods and scored for suppression of the synthetic Sqv and germline defects. The efficacy of these RNAi treatments was first verified by showing that lin-12(RNAi) induced a highly penetrant Egl phenotype in wild-type animals and that hop-1(RNAi) led to synthetic lethality in the sel-12 mutant background (data not shown; Sundaram and Greenwald, 1993; Li and Greenwald, 1997; Westlund et al., 1999). We found that neither treatment was capable of mitigating either the vulval or germline defects of the double mutants (Table 1). These results indicate that increased levels of HOP-1 activity or LIN-12 signaling are unlikely to be a cause of the double mutant phenotype. Consistent with this, other phenotypes associated with lin-12 hypermorphic alleles, such as the ectopic induction of secondary vulval cell fates or loss of the gonadal anchor cell, were not observed in the double mutants (data not shown; Sternberg, 2005; Greenwald, 2005).

lin-35; spr-1 double-mutant germlines are hypersensitive to reductions in lin-12/Notch signaling

Although we failed to detect any suppressive effects of hop-1 or lin-12 RNAi on the vulval and germline defects of lin-35; spr-1 mutants, we did observe proximal germline tumors (the Pro phenotype) in 21 and 24% of individual gonad arms of lin-35; spr-1; hop-1(RNAi) and lin-35; spr-1; lin-12(RNAi) animals, respectively (referred to as triple mutants; Figs. 6E, G, H; Table 4). In contrast, lin-35; spr-1 double mutants as well as all other possible binary combinations of double mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Pro (%)</th>
<th>Adults containing residual bodies (%)</th>
</tr>
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<tbody>
<tr>
<td>N2</td>
<td>0 (n=69)</td>
<td>6 (n=69)</td>
</tr>
<tr>
<td>lin-35; spr-1</td>
<td>2 (n=98)</td>
<td>11 (n=37)</td>
</tr>
<tr>
<td>hop-1; spr-1</td>
<td>2 (n=126)</td>
<td>13 (n=31)</td>
</tr>
<tr>
<td>lin-35; hop-1(RNAi)</td>
<td>0 (n=58)</td>
<td>ND</td>
</tr>
<tr>
<td>lin-35; spr-1; hop-1(RNAi)</td>
<td>21 (n=154)</td>
<td>44 (n=52)</td>
</tr>
<tr>
<td>lin-35; sel-12</td>
<td>0 (n=40)</td>
<td>ND</td>
</tr>
<tr>
<td>lin-35; sel-12; spr-1b,c</td>
<td>10 (n=61)</td>
<td>23 (n=61)</td>
</tr>
<tr>
<td>spr-1; lin-12(RNAi)</td>
<td>0 (n=37)</td>
<td>11 (n=37)</td>
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<tr>
<td>lin-35; lin-12(RNAi)</td>
<td>4 (n=72)</td>
<td>4 (n=72)</td>
</tr>
<tr>
<td>lin-35; spr-1; lin-12(RNAi)</td>
<td>24 (n=45)</td>
<td>62 (n=45)</td>
</tr>
</tbody>
</table>

All RNAi experiments were carried out using injection methods. The fd6 allele of spr-1 was used in all experiments.

- Phenotypes were scored per gonad arm.
- spr-1(fd6) was linked to unc-76(e611).
- sel-12(ar171) was linked to unc-1(e538).
- Actual strain was of genotype lin-35; spr-1; lin-12(RNAi); kuEx119, which is rescued for the lin-35 mutant defect.
showed little or no expression of the Pro phenotype (Table 4). In addition, adult triple mutants often harbored residual bodies (from spermatocytes) in the proximal gonad region (Fig. 6F; Table 4). Residual bodies are a relatively short-lived bi-product of spermatogenesis and are typically absent in adults. Residual bodies were not observed in wild-type adults, although they were observed at somewhat elevated frequencies in most double-mutant combinations (Table 4).

The Pro phenotype is defined by abnormal cell divisions in the proximal region of the adult germline and is discernible as a mass of densely packed mitotic nuclei (Seydoux et al., 1990). This phenotype has been reported to arise by several distinct mechanisms including the dedifferentiation of developing (meiotic) spermatocytes into mitotically dividing cells (Subramaniam and Seydoux, 2003). In addition, careful analysis has shown the Pro phenotype to result indirectly from delays in the initial entry of proximal germ cells into meiosis (Pepper et al., 2003a,b; Killian and Hubbard, 2004, 2005). This delay can occur when the germline fails to proliferate sufficiently during larval development, such that the proximal germ cells remain under the mitosis-promoting influence of the distal tip cell (via LAG-2/Delta and GLP-1/Notch signaling). This failure of the proximal germ cells to enter meiosis in a timely manner renders them susceptible to the mitosis-promoting influence of the proximal sheath cells (Sh2–5) that come into contact with the proximal germ cells during the mid-L4 stage (Killian and Hubbard, 2005). Therefore, it is critical that germ cells enter meiosis prior to the birth of Sh2–5, as subtle delays can lead to maintenance of a mitotic state and the generation of proximal tumors.

Several features of the meiotic entry delay mechanism were consistent with our observations of the triple mutants including a highly penetrant reduction in the proliferation capacity of the triple-mutant germline. In addition, it has been shown that lin-12 LOF results in delays in the timing of initial meiosis (Killian and Hubbard, 2005; Killian and Hubbard, 2004) and that lin-12 null mutants form proximal tumors and contain adult-stage spermatocytes (Seydoux et al., 1990). In contrast, puf-8(RNAi), which leads to highly penetrant proximal tumors via the spermatocyte dedifferentiation pathway, does not typically affect germline proliferation (Subramaniam and Seydoux, 2003). Furthermore, other phenotypes associated with puf-8 LOF that occur as a consequence of meiotic defects were not observed in the double or triple mutants examined (data not shown; Subramaniam and Seydoux, 2003). Finally, the presence of residual bodies in triple mutant adults is consistent with a delay in the initiation of meiosis and in the subsequent induction of spermatogenesis.

To test for delays in the timing of initial meiosis in lin-35; spr-1; hop-1(RNAi) and lin-35; spr-1; lin-12(RNAi) triple-mutant animals, we examined gonads by DAPI staining at the mid-L4 stage, at which time a substantial proportion of wild-type germ cells have normally entered meiosis (Hubbard and Greenstein, 2005). We found that in contrast to wild type as well as all possible double-mutant combinations, triple mutants were specifically defective in meiotic entry (Table 5; Figs. 6I, J). These results correlate precisely with the expression of the Pro phenotype in the triple-mutant, but not the double-mutant, combinations (Table 4). Furthermore, these findings are highly consistent with previous studies linking delays in the timing of initial meiosis to the Pro phenotype (Pepper et al., 2003a,b; Killian and Hubbard, 2004, 2005). In particular, we note that lin-35; lin-12(RNAi) animals showed neither a pronounced meiotic entry delay nor a Pro phenotype, indicating that our level of lin-12 inactivation was not sufficient on its own to induce obvious germline defects (Fig. 6I; Table 4); lin-35; lin-12(RNAi) animals did display the Egl phenotype, and 2/15 animals assayed contained two anchor cells (data not shown), consistent with a partial loss of lin-12 function. Thus, lin-35; spr-1 mutants are highly sensitized to the effects of lin-12 LOF on germline development. This result is reminiscent of previous findings showing that ablation of sheath cell pair 1 (Sh1), which leads to both a meiotic entry delay and germline under-proliferation, sensitizes the animals to slight increases in GLP-1/Notch activity, effectively resulting in a synthetic Pro phenotype.

### Discussion

**lin-35/Rb and spr-1/CoREST function redundantly during C. elegans development**

We found that lin-35 and spr-1 redundantly control several aspects of somatic gonad and germline development. With respect to development of the vulva, our results demonstrate that lin-35 and spr-1 coordinate promote the normal progression of vulval cells during the late L3 and L4 stages of development (Table 1; Figs. 2, 3). This phenotype is superficially similar to that of the sqv mutants, which act in a common pathway to promote chondroitin biosynthesis (reviewed by Bulik and Robbins, 2002). Although our findings

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**Table 5**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mitotic</th>
<th>Transition</th>
<th>Pachytene</th>
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<tr>
<td>N2</td>
<td>148±24</td>
<td>19±4</td>
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<td>lin-35; hop-1(RNAi)</td>
<td>95±13</td>
<td>12±5</td>
<td>38±5</td>
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<tr>
<td>lin-35; lin-12(RNAi)</td>
<td>85±15</td>
<td>10±2</td>
<td>46±7</td>
</tr>
<tr>
<td>spr-1; hop-1(RNAi)</td>
<td>93±11</td>
<td>25±5</td>
<td>38±6</td>
</tr>
<tr>
<td>spr-1; lin-12(RNAi)</td>
<td>85±16</td>
<td>20±6</td>
<td>29±5</td>
</tr>
<tr>
<td>lin-35; spr-1</td>
<td>37±6</td>
<td>8±2</td>
<td>31±9</td>
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<td>5±3</td>
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<tr>
<td>lin-35; spr-1; lin-12(RNAi)</td>
<td>47±14</td>
<td>5±3</td>
<td>16±13</td>
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</table>

All RNAi experiments were carried out using injection methods. The fdd6 allele of spr-1 was used in all experiments. n indicates the number of gonad arms scored.

a Scored animals contained the lin-35(n745) mutation together with the lin-35 rescuing array kuEx119.

b 3/8 gonad arms contained no transition-stage nuclei. p-value based on t test (relative to lin-35; spr-1) was <0.01.

c 2/8 gonad arms contained no pachytene-stage nuclei. p-value based on t test (relative to lin-35; spr-1) was <0.01.

d 8/17 gonad arms contained no transition-stage nuclei. p-value based on t test (relative to lin-35; spr-1) was <0.001.

e 8/17 gonad arms contained no pachytene-stage nuclei. p-value based on t test (relative to lin-35; spr-1) was <0.001.

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**Figures**: 6I. DAPI staining of adult hermaphrodites for gonads from wild type, lin-35; spr-1, and lin-35; spr-1; hop-1(RNAi).
do not support a model whereby chondroitin biosynthesis or localization is grossly altered in the double mutants (as is the case for the sqv mutants; Fig. 5), we cannot rule out the possibility that very subtle differences in chondroitin levels, processing, or conjugation to specific proteoglycan core components may play a role in the observed phenotype. Nevertheless, clear qualitative differences in the vulval morphologies of lin-35; spr-1 and the sqv mutants, along with the normal localization pattern of chondroitin observed in lin-35; spr-1 vulvae, argue against chondroitin-related defects as being causative to the lin-35; spr-1 vulval phenotype.

We have also shown a role for lin-35 and spr-1 in germline proliferation and in the prevention of proximal germline tumors when LIN-12/Notch signaling is compromised (Figs. 4, 6; Tables 4, 5). Based on previous published studies (Killian and Hubbard, 2004, 2005), we hypothesize that lin-35 and spr-1 contribute to the Pro phenotype through their roles in redundantly promoting germline proliferation (also see results). At present, our data do not indicate whether lin-35 and spr-1 promote germline autonomy or via the soma. Nevertheless, our studies strongly imply that the Pro phenotype of lin-35; spr-1; lin-12(RNAi) and lin-35; spr-1; hop-1(RNAi) triple mutants results from a delay in the timing of initial meiosis, consistent with previous studies (Pepper et al., 2003a,b; Killian and Hubbard, 2004, 2005). Curiously, loss of lin-35 can also suppress the Pro phenotype of pro-1 mutants by partially alleviating defects due to rRNA processing deficiencies (Voutev et al., 2006). Thus, depending on developmental context and strain background, lin-35 can act as either a suppressor or enhancer of proximal germline proliferation.

Redundancy among transcriptional regulators

Genetic redundancy, which implies an overlap in the functions of the encoded protein products, can occur through a myriad of mechanisms. For example, the synthetic hyperproliferative phenotype of lin-35; fzb-1 mutants results from a failure to repress the expression of G1 cyclins adequately (Fay et al., 2002). In this case, LIN-35, acting as a transcriptional repressor, and FZR-1, functioning as a component of the APC ubiquitin ligase complex, act at distinct points (transcriptional versus post-translational) to control the protein levels of a mutual target. In contrast, as described in the Introduction, LIN-35 acting together with other SynMuV genes specifically represses lin-3 at the level of transcription (Cui et al., 2006). Although the precise molecular functions of the Class A SynMuV genes are not known, these proteins localize to the nucleus and are likely to function as transcriptional regulators (Clark et al., 1994; Huang et al., 1994; Davison et al., 2005).

It is of interest to note that of the seven genes identified through our lin-35 synthetic screen, four function as regulators of transcription (spr-1; Bender et al., 2004; Cui et al., 2004; D.S.F. and J.D. McEnerney, unpublished data). Furthermore, mutations in gon-14 are also synthetic with lin-35, and GON-14 contains a putative THAP DNA-binding domain and localizes to the nucleus (Chesney et al., 2006). Thus, although transcriptional regulators account for less than 8% of encoded genes in C. elegans, they are strongly over-represented among the group of known lin-35 synthetic interactors. This observation is consistent with findings from a large-scale synthetic screen in S. cerevisiae showing that genes encoding proteins with similar molecular functions (based on gene ontologies) are more likely to display genetic redundancy than are genes with dissimilar functions (Tong et al., 2004). Thus, a useful (though imperfect) predictor for functional redundancy in metazoans may be based on proteins that have similar molecular functions.

The role of pocket proteins in tumor suppression

In this study, we have identified an additional role for lin-35 in epithelial cell movement and ingression. Although we have yet to identify the underlying basis for this phenotype, straightforward possibilities include reduced or altered adhesion properties of the migrating vulval cells or defects in cell polarity that may lead to a diminution of cell movements. Together with our previous observations demonstrating a role for lin-35 in controlling cell orientation and polarity during pharyngeal morphogenesis (Fay et al., 2003, 2004), these results suggest that lin-35 controls several properties of cells that are known to be specifically derailed in mammalian metastatic cancers (reviewed by Christofori, 2006).

It is possible that similar functions for Rb family members also exist in mammals but have not been observed to date because of functional overlap between the Rb family members, or as is the case in C. elegans, because of genetic redundancy with independent pathways. Additional tumor-suppressive functions may further explain why the Rb pathway is functionally inactivated in most or all human cancers (Sherr, 1996, 2004; Sherr and McCormick, 2002). Furthermore, a role for Rb in tumor suppression beyond cell cycle control is supported by the finding that loss of cell cycle control alone is not sufficient to predispose cells of the retina to form Retinoblastomas (Nakayama et al., 1996; Levine et al., 2000; Dyer and Cepko, 2001; Cunningham et al., 2002; reviewed by Bremner et al., 2004). We speculate that genes identified as showing synthetic interactions with lin-35 may function in certain contexts as tumor suppressors in mammals. Our identification of spr-1/CoREST, which functions in mammals as the co-partner of the REST tumor suppressor protein, lends credence to this possibility. Another gene identified by our screen, fzb-1/Cdh1, is strongly downregulated in murine lymphoma cells, and transgenic expression of murine fzb-1 in these cells reverses their tumorous growth properties (Wang et al., 2000). Finally, the recent finding that LIN-35 redundantly represses expression of the EGF family member LIN-3 suggests that suppression of paracrine growth factor signaling may be another mechanism by which Rb family members inhibit cell growth and thereby function to prevent tumorogenesis (Cui et al., 2006). Thus, the analysis of lin-35 functions during development may provide valuable insights into novel mechanisms of tumor suppression by the Rb family.
Acknowledgments

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References


