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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.

Keywords: *lin-35*; Retinoblastoma; *spr-1*; CoREST; *C. elegans*; Development

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

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cycle) functions of pRb (Krutzfeldt 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 *Drosophila*, loss of the E2F co-partner dDP results in ventralization of the embryo because of misexpression of the EGF-like ligand Gurkin (Myster 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 multivulval [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 germline-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 (Stiernagle, 2005). All experiments were carried out at 20°C. Strains used in these studies include the following: N2 (wild type), CB4856, MH1461 [*lin-35*(*n745*), *kuEx119*], NH2246 [*ayIs4*(*egl-17*::GFP)], WY301 [*lin-35*(*n745*); *egl-17*::GFP], SU93 [*jcis1* (*ajm-1*::GFP)], WY334 [*spr-1*(*fd6*); *lim-7*::GFP], WY258 [*spr-1*(*fd6*)], JK2868 [*qIs56*(*lag-2*::GFP)], MH1317 [*kuIs29*(*egl-13*::GFP)], GS1214 [*sel-12*(*ar171*), *unc-1*(*e538*)], WY329 [*lin-35*(*n745*); *lag-2*::GFP], WY298 [*lin-35*(*n745*); *ajm-1*::GFP], WY248 [*lin-35*(*n745*); *spr-1* (*fd6*); *kuEx119*], WY328 [*lin-35*(*n745*); *dpy-11*(*e224*), *spr-1*(*fd6*), *unc-76*(*e911*); *kuEx119*], WY294 [*lin-35*(*n745*); *spr-1*(*fd6*); *lim-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*); *kuEx119*], WY249 [*lin-35*(*n745*); *spr-1* (*fd6*); *ajm-1*::GFP; *kuEx119*], WY296 [*lin-35*(*n745*); *sel-12*(*ar171*), *unc-1* (*e538*)], WY326 [*lin-35*(*n745*); *spr-1*(*fd6*); *sel-12*(*ar171*), *unc-1*(*e538*)], DG1575 [*tnIs6*(*lim-7*::GFP)], and WY299 [*lin-35*(*n745*); *lim-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. 59/79 Dpy non-Unc and 14/55 Unc non-Dpy recombinants segregating from *lin-35; dpy-11, fd6, unc-76*/+ 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 vm23b06.s1@42,a,42 on cosmid F40A3 and eam66g04.s1@467,t,51 on cosmid F21F8.

Transgene rescue

Rescue of *fd6* was obtained through the injection of cosmids D1014 and F20D6 together with pRF4 into *lin-35; spr-1; kuEx119*(+) hermaphrodites (for details on methods see Evans, 2005). Rescue was inferred based on the appearance of roller animals that did not require *kuEx119* 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*); *dpy11*(*e224*), *spr-1*(*fd6*), *unc-76*(*e911*); *kuEx119* hermaphrodites and Non-Dpy Unc cross progeny containing the *kuEx119* array were identified in the next generation, clonally transferred, and allowed to self fertilize. 60 non-Dpy Unc *kuEx119*+ progeny