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Cell cycle-dependent sequencing of cell fate decisions in *Caenorhabditis elegans* vulva precursor cells

Victor Ambros

Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA
Email: victor.ambros@dartmouth.edu

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SUMMARY

In *Caenorhabditis elegans*, the fates of the six multipotent vulva precursor cells (VPCs) are specified by extracellular signals. One VPC expresses the primary (1°) fate in response to a Ras-mediated inductive signal from the gonad. The two VPCs flanking the 1° cell each express secondary (2°) fates in response to *lin-12*-mediated lateral signaling. The remaining three VPCs each adopt the non-vulval tertiary (3°) fate. Here I describe experiments examining how the selection of these vulval fates is affected by cell cycle arrest and cell cycle-restricted *lin-12* activity. The results suggest that *lin-12* participates in two developmental decisions separable by cell cycle phase: *lin-12* must act prior to the end of VPC S phase to influence a 1° versus 2° cell fate choice, but must act after VPC S phase to influence a 3° versus 2° cell fate choice. Coupling developmental decisions to cell cycle transitions may provide a mechanism for prioritizing or ordering choices of cell fates for multipotential cells.

Key words: *Caenorhabditis elegans*, Vulva, Cell cycle, LIN-12/NOTCH, Hydroxyurea

INTRODUCTION

Cell-cell signaling is a common mode of cell fate specification in animal development. In many cases, signals must act at defined developmental stages or steps in a cell lineage, so a cell's ability to select a specific fate in response to particular extracellular signals can be influenced by timing mechanisms linked to developmental stage (Servetnick and Grainger, 1991; Slack, 1991) or the cell cycle (Gomer and Firtel, 1987; McConnell and Kaznowski, 1991; Thomas et al., 1994; Weigmann and Lehner, 1995). Temporal or cell cycle control of developmental decisions may be particularly important in situations where a cell is sensitive to multiple signals that specify distinct outcomes and specific cell fate choices need to be executed with a certain priority or temporal sequence.

Situations where developmental events are linked to cell cycle progression have been identified by the ability of cell cycle inhibitors to prevent the expression of molecular or phenotypic developmental markers, as is the case for a dependence of *even-skipped* expression on completion of S phase in the *Drosophila* nervous system (Weigmann and Lehner, 1995). In other situations, developmental decisions, and even overt differentiation, can occur independently of cell cycle progression, for example, in the case of neural development in *Xenopus* (Harris and Hartenstein, 1991). In such cases, the relative timing of events may still be critical to normal development, but are linked to temporal cues other than cell cycle progression.

Vulva development in *Caenorhabditis elegans* offers a convenient experimental system for exploring the temporal and spatial regulation of multiple developmental decisions in response to multiple extracellular signals (Kenyon, 1995). The six multipotent vulva precursor cells (VPCs) are born in the L1 and, regardless of the fate that they adopt, they all divide approximately 20 hours later in the mid-L3 (Fig. 1A; Sulston and Horvitz, 1977). The VPCs each have the potential to adopt one of three fates, 1°, 2° and 3° (Fig. 1). These different fates are distinguished by patterns of cell division and terminal differentiation. P6.p normally adopts the 1° fate and generates eight vulval cells. P5.p and P7.p normally adopt the 2° fate and generate seven vulval cells each. The remaining three VPCs (P3.p, P4.p and P8.p) each divide once to produce two non-vulval hypodermal cells (constituting the 3° fate).

The fates of the VPCs are specified by extracellular patterning signals that include the LIN-3 EGF-like inductive signal from the gonad that acts via a receptor tyrosine kinase (RTK) and Ras-mediated signaling cascade (Hill and Sternberg, 1992; Han and Sternberg, 1990), an inhibitory 'synMuv' signal from the surrounding hypodermis (Herman and Hedgecock, 1990; Clark et al., 1994; Huang et al., 1994) and a lateral signal among the VPCs that acts via the LIN-12 receptor (Greenwald et al., 1983; Greenwald, 1985; Ferguson et al., 1987; Sternberg, 1988; Sternberg and Horvitz, 1989). Laser ablation and temperature-shift experiments indicate that the fates of the VPCs become determined during a period beginning shortly after the L2 molt (Kimble, 1981; Greenwald et al., 1983; Sternberg and Horvitz, 1986; Ferguson et al., 1987), indicating that the final few hours of the 20 hour VPC cell cycle contain a critical period for VPC competence. Wild-

type VPCs have a long G_1 phase of the cell cycle, extending from the L1 throughout the L2; VPC S phase occurs in the early L3, approximately 1-2 hours after the L2 molt (Euling and Ambros, 1996). A regulatory pathway of heterochronic genes, including *lin-14* (Fig. 1), controls when VPCs transit through G_1/S of the cell cycle and when they are competent to respond to extracellular signals that determine their fates (Ambros and Horvitz, 1984; Euling and Ambros, 1996).

The normal pattern of precisely one 1° VPC flanked by two 2° VPCs is critical for normal vulval morphogenesis (Kimble, 1981; Greenwald et al., 1983; Sternberg and Horvitz, 1986). Since the 1° VPC is a likely source of the lateral signal that specifies the 2° fates for its neighbors (Sternberg, 1988; Simske and Kim, 1995; Koga and Ohshima, 1995), the 1° cell can be thought of as an organizer of the fates of surrounding cells. This organizing role of the 1° cell sets a high priority on the selection of the 1° fate relative to 2° and 3° fates and suggests that Ras-mediated inductive signaling and LIN-12-mediated lateral signaling may be correspondingly prioritized for the VPCs. In this paper, I describe the results of experiments that suggest that the prioritization of the 1° vulval fate in part reflects the fact that the 1° versus 2° decision occurs earlier in the VPC cell cycle than does the 2° versus 3° decision. *lin-12* must act prior to the completion of VPC S phase in order to overcome selection of the 1° fate and thereby restrict the 1° fate to a single induced VPC. In contrast, *lin-12* cannot influence the 2° versus 3° decision until after completion of VPC S phase. The sequencing of cell fate choices by cell cycle phases may provide a mechanism for temporally organizing the selection of different fates by multipotential cells.

MATERIALS AND METHODS

C. elegans methods

C. elegans strains were grown and maintained as described (Brenner, 1974; Wood, 1988). All experiments were performed at 20°C unless otherwise noted.

C. elegans strains

The wild-type strain used was *C. elegans* var. Bristol strain N2 (Brenner, 1974). Other strains used were: VT796 *lin-12 (n137gfn460ts); maIs103[rnr::GFP]* (Hong et al., 1998), GS2061 *arIs1[lin-12(intra)]* (Struhl et al., 1993), GS2461 *arIs41[lin-12::GFP]* (Levitan and Greenwald, 1998), NH2466 *ayIs4[egl-17::GFP]; dpy-20(e1282)* (Burdine et al., 1998), NH2539 *ayIs4[egl-17::GFP]; dpy-20(e1282)*; *lin-15(n309)*, VT832 *lin-12(n137gfn460ts); ayIs4[egl-17::GFP]*, MT1035 *lin-12(n137gfn460ts)*.

Hydroxyurea treatment

Animals in L2 lethargus were obtained from mixed populations or from populations of animals synchronized at hatching (Wood, 1988), and were identified by size and molting behavior (Singh and Sulston, 1978). They were either shifted immediately to plates containing 40 mM hydroxyurea (HU) and a small spot of *E. coli* or they were monitored periodically using the dissecting microscope to identify the time of ecdysis (evidenced by resumption of pharyngeal pumping), allowed to grow for a period of time, then transferred to HU plates. After a period of time in HU, animals were either examined using Nomarski optics, or transferred to plates without HU and examined after a period of further development.

Microscopy and photography

Images of living animals anesthetized with 1 mM levamisole were captured with an Optronics integrating color CCD video camera and a Scion CG-7 RGB video capture board on a Power MacIntosh 8500AV. GFP fluorescence images were taken with exposure ranging from 1/8 second to 4 second and DIC images were taken with an automatic exposure. The digital images were processed using Adobe Photoshop.

Antibody staining

Antibody staining with anti-GFP antiserum was performed using described methods (Bettinger et al., 1996). Worms were synchronized by feeding starved hatchlings (Wood et al., 1988), grown to the L2 lethargus, treated with hydroxyurea for 8 hours, fixed and stained with polyclonal rabbit anti-gfp (Clonetech) followed by Cy3-conjugated goat anti-rabbit secondary antisera (Jackson ImmunoResearch).

Temperature-shift experiments

MT1035 *lin-12(n137gfn460ts)*, VT832 *lin-12(n137gfn460ts); ayIs4*, VT796 *lin-12(n137gfn460ts); maIs103*, or GS2061 *lin-12(intra)* animals were shifted between 15°C and 25°C at various times after the L2 lethargus, and were examined at the adult stage for the number of psuedovulvae. *lin-12(n137gfn460ts); ayIs4* animals were scored for expression of *egl-17::GFP* in the early L3, and in the early L4 stage, and were scored at the adult stage for the Muv phenotype. Shifted animals were transferred to plates that had been equilibrated to the second temperature. Times were corrected according to the following: the length of the *lin-12(n137gfn460ts)* L3 was measured to be approximately 16 hours at 15°C, 9 hours at 20°C and 7.5 hours at 25°C.

RESULTS

Induction of the 1° fate prior to VPC S phase

To test whether induction of the 1° fate in VPCs correlates with cell cycle phase, cell cycle expression of an early marker for the 1° fate, *egl-17*, was monitored. *egl-17* functions to guide the migration of sex muscle precursors to the region of the developing vulva and *egl-17* is expressed in the presumptive 1° VPC (Burdine et al., 1998; Fig. 1). Expression of a transgene (*ayIs4*) consisting of the *egl-17* promoter fused to sequences encoding the green fluorescent protein (GFP) depends on the inductive signal from the gonad and is first detected in the early L3 (Burdine et al., 1998). In animals treated with hydroxyurea beginning at the L2 molt to arrest the VPC cell cycle in S phase, *egl-17::GFP* expression was observed in P6.p (Table 1; Fig. 2). This observation indicates that the first steps in execution of the 1° fate (as evidenced by *egl-17::GFP* expression) occur prior to the completion of VPC S phase.

A second early marker for the 1° fate is the downregulation of LIN-12 protein accumulation in a VPC that will adopt the 1° fate. This downregulation of LIN-12 is normally evident in P6.p prior to cell division using *arIs41*, a transgene encoding a functional LIN-12::GFP reporter protein (Levitan and Greenwald, 1998). To test whether LIN-12::GFP accumulation can decrease in P6.p prior to completion of S phase, animals were treated with hydroxyurea for 9 hours beginning at the L2 molt to arrest the VPC cell cycle in S phase, and then were fixed and stained with fluorescent anti-GFP antiserum. Of 92 *lin-12::GFP* animals with hydroxyurea-arrested VPCs, 36 displayed markedly reduced anti-GFP fluorescence in P6.p compared to the other VPCs (Fig. 3). These results indicate that by two criteria, upregulation of *egl-17::GFP* and downregulation of LIN-12::GFP, induction of the 1° fate can

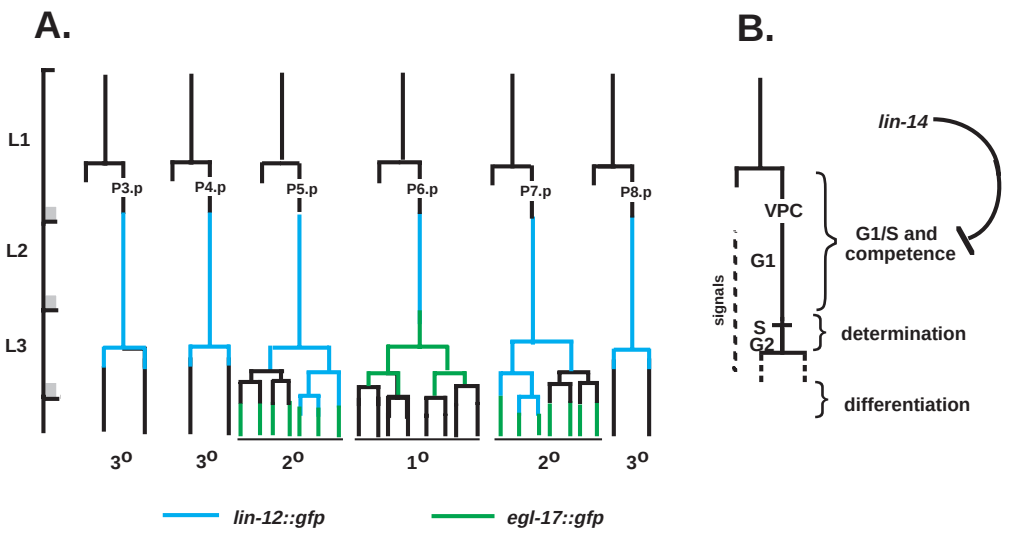

Fig. 1. Wild-type vulval development. (A) The six vulva precursor cells (VPCs) are born in the L1 stage as the posterior daughters of P3-P8 (P3.p-P8.p); the anterior sisters of P3.p-P8.p become non-vulval neuroblasts (Sulston and Horvitz, 1977). The VPCs divide in the mid L3, to produce three sets of differentiated progeny: cells corresponding to the 1° and 2° VPC fates participate in vulva morphogenesis; 3° fates produce two non-vulval cells that fuse with the hypodermal syncytium (Sulston and Horvitz, 1977). Blue signifies the expression of LIN-12::GFP (Levitan and Greenwald, 1998); green signifies the expression of *egl-17::GFP* (Burdine et al., 1998). *egl-17::GFP* is expressed strongly in the 1° cell lineage until the L3 molt, after which it decreases; *egl-17::GFP* expression appears in the 2° cell lineage in the late L4 (Burdine et al., 1998). (B) Summary of vulval development: a generic VPC cell undergoes S phase shortly after the L3 molt (Euling and Ambros, 1996) and becomes determined in response to extracellular signals in association with late G_1 and G_2. Signals and signal transduction machinery influencing VPC fates are proposed to be pre-existing before the normal time of vulval determination, but the time of G_1/S, and the time of acquisition of competence to respond to the signals, is regulated by a pathway of heterochronic genes, including *lin-14* (Euling and Ambros, 1996). Dashed lines indicate that further divisions occur (in the case of 1° or 2° fates).

occur prior to completion of VPC S phase. The fact that upregulation of *egl-17::GFP* seemed to be more efficient than downregulation of LIN-12::GFP in hydroxyurea-treated animals suggests that extension of S phase by hydroxyurea treatment may favor accumulation of gene product expressed from the *egl-17* promoter, but may impede the process of reducing LIN-12::GFP protein. In non-arrested VPCs, LIN-12::GFP accumulation from *arIs41* was observed to be more efficiently downregulated than was observed here for hydroxyurea-arrested VPCs (Levitan and Greenwald, 1998). This suggests that, although downregulation of LIN-12 can evidently begin before completion of S phase, hydroxyurea treatment may impede LIN-12 reduction.

Lateral signaling prior to VPC S phase

The fact that P6.p was essentially the only VPC observed to express *egl-17::GFP*, or to downregulate LIN-12::GFP during hydroxyurea arrest, suggests that a P6.p cell can signal laterally prior to the completion of S phase to prevent expression of 1° fates in neighboring VPCs. To further test for whether lateral signaling occurs prior to the end of S phase, *egl-17::GFP* expression was monitored in *lin-15(n309)* animals treated with hydroxyurea. *lin-15(n309)* is a mutation impairing the inhibitory 'synMuv' pathways and hence releases VPCs from dependence on the gonadal signal and causes all six VPCs to express 1° or 2° fates (Ferguson et al., 1987; Sternberg, 1988). Lateral signaling results in an alternating pattern of 1° and 2° fates in *lin-15* animals (Sternberg, 1988), a pattern reflected by the pattern of *egl-17::GFP* expression (Burdine et al., 1998). *ayIs4; lin-15(n309)* animals were treated with hydroxyurea to block VPCs in S phase and *egl-17::GFP* expression was monitored. A statistically significant fraction of the *ayIs4; lin-15(n309)* animals examined displayed an alternating on/off pattern of *egl-17::GFP* expression consistent with lateral signaling (Table 1; Fig. 4).

The observations described above indicate that lateral signaling occurs among VPCs prior to the completion of S phase. In wild-type *C. elegans* vulva development, *lin-12* mediates lateral signaling among the VPCs (Greenwald et al., 1983; Sternberg, 1988), preventing the expression of 1° fates in adjacent VPCs, and specifying the expression of 2° fates in the VPCs flanking a 1° VPC (Fig. 1). The *lin-12* gene product is a member of the LIN-12/Notch family of transmembrane receptors (Greenwald, 1998). Consistent with a key early role for *lin-12* in lateral signaling among VPCs, a *lin-12* gain-of-function (gf) allele blocks the expression of *egl-17::GFP* in hydroxyurea-arrested VPCs of *lin-15(n309)* animals (Table 2). This result indicates that *lin-12(gf)* can act prior to the completion of S phase to block the initiation of 1° fates. Since *lin-15(n309)* permits the expression of 1° fates independently of the gonadal inductive signal, the block to *egl-17::GFP* expression in *lin-12(gf); lin-15(n309)* animals cannot be due to absence of an anchor cell and more likely reflects the activity of the LIN-12 receptor protein in VPCs.

Time of action of *lin-12* for the 1° versus 2° cell fate choice

The above results, which show that *lin-12(gf)* activity can inhibit the expression of primary fates prior to the completion of VPC S phase, raise the question of whether *lin-12* can also act later in the cell cycle to inhibit primary fates. To control the cell cycle timing of *lin-12* activity, a temperature-dependent *lin-12(gf)* mutation was employed in temperature-shift experiments.

lin-12(n137n460) is a cold-sensitive Multivulva (Muv) mutation (Greenwald et al., 1983). It is a double mutant containing the *lin-12(gf)* lesion, *n137*, which causes a ligand-independent activation of LIN-12, and *n460*, which apparently makes the protein less stable at 25°C. Thus, at 15°C, *lin-*

Table 1. Expression of *egl-17::GFP* in hydroxyurea-arrested VPCs of L3 larvae at 20°C

Genotype	Cell(s) expressing *egl-17::GFP*						No. of animals
	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	
ayIs4							
	–	–	–	–	–	–	3
	–	–	–	+	–	–	17
ayIs4; lin-15(n309)							
class I	–	–	–	+	–	–	2
	–	–	–	–	+	–	1
class II*	–	–	–	+	–	+	5
	–	–	+	–	+	–	1
	+	–	–	–	+	–	1
	–	–	+	–	–	+	1
	–	+	–	+	–	–	3
	+	–	–	+	–	–	1
class III‡	–	+	–	+	–	+	8
	+	–	–	+	–	+	5
	+	–	+	–	+	–	1
	–	+	–	+	–	+	2
	–	+	+	+	–	–	1
	–	–	+	+	+	–	1
class IV§	+	–	+	+	–	+	4
	+	–	+	+	+	–	3
	+	+	–	+	–	+	1
	+	–	+	+	+	+	1
	–	–	+	+	+	+	1
	–	+	+	+	–	+	1

Animals at or shortly after L2 lethargus were placed on plates containing 40 mM hydroxyurea (HU) in the presence of bacteria and maintained at 20°C for 7-9 hours. Animals were then immobilized using levamisole, and examined by DIC and epifluorescence microscopy. In all cases, VPCs were undivided (control animals of the same age that had not been treated with HU exhibited one or more rounds of VPC cell division).

*If VPCs were expressing *egl-17::GFP* randomly, then 1/3 of animals with exactly two expressing cells would be expected to contain a pair of adjacent expressing cells; the observed 0/12 is statistically different than the expectation (χ^2=6; P=0.015).

‡If VPCs were expressing *egl-17::GFP* randomly, then 4/5 of animals with exactly three expressing cells would be expected to contain a pair of adjacent expressing cells; the observed 2/18 is statistically different than the expectation (χ^2=46; P<0.005).

§Often the fluorescence of adjacent cells differed significantly in intensity. The proportion of animals with greater than three expressing cells (11/44) is as expected, based on the average number of expressing cells per animal and a Poisson distribution.

12(n137n460) hermaphrodites are Muv, since all the VPCs (including those that would normally express the 3° fate) adopt the 2° fate associated with LIN-12 activation. These animals also lack an anchor cell (AC), another phenotype associated with LIN-12 activation. In contrast, at 25°C, *lin-12(n137n460)* hermaphrodites have an AC and display normal vulval development, and 3° fates are expressed normally. Here, this allele is referred to as *lin-12(n137gfn460ts)* to emphasize this dual character. The temperature-sensitive period (TSP) for the *lin-12(n137gfn460ts)* Muv phenotype was previously

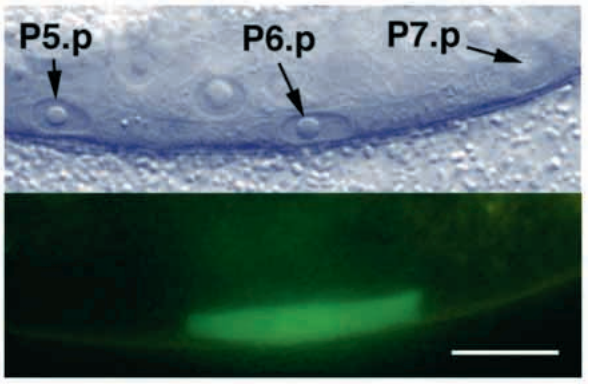

Fig. 2. Expression of *egl-17::GFP* in hydroxyurea-arrested P6.p. Animals were treated with hydroxyurea as described in Materials and Methods for 8 hours beginning at the L2 molt, and were examined by DIC microscopy to visualize nuclei (top panel) and by fluorescence to detect *egl-17::GFP* expression (bottom panel). Scale bar, 5 μm.

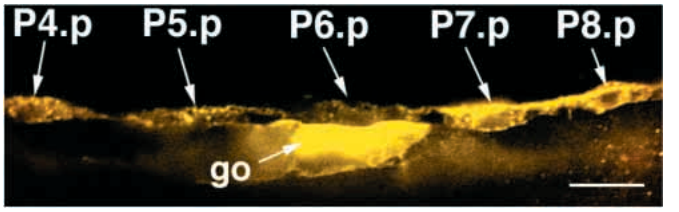

Fig. 3. LIN-12::GFP accumulation in hydroxyurea-arrested VPCs. *arIs41[lin-12::GFP]* animals were treated with hydroxyurea for 8 hours beginning at the L2 molt, and then were fixed and stained with anti-gfp antisera as described in Materials and Methods. The animal shown here expressed LIN-12::GFP at relatively high levels (evidenced by the bright, punctate fluorescence) in all the VPCs except P6.p, where the fluorescence level was relatively reduced. The somatic gonad (go) underlies P6.p and expresses LIN-12::GFP very strongly (Levitan and Greenwald, 1998). Scale bar, 5 μm.

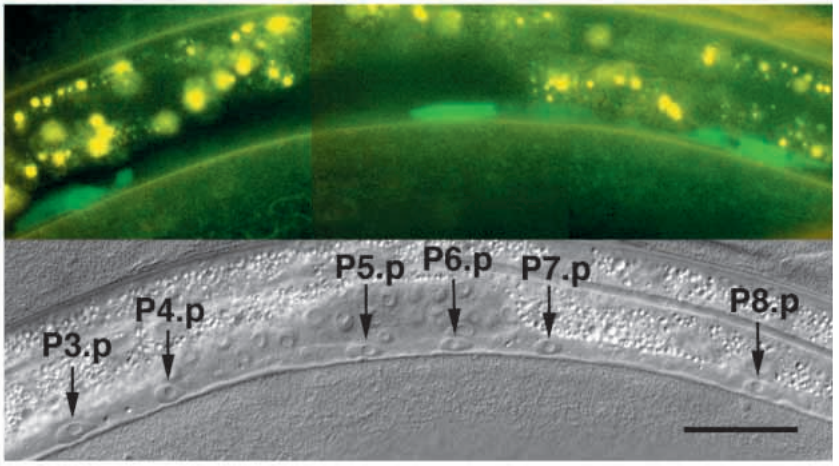

Fig. 4. Expression of *egl-17::GFP* in hydroxyurea-arrested VPCs of an *ayIs4; lin-15(n309)* animal. The expression pattern is variable in this strain (see Table 1), and in this animal, GFP fluorescence is detected in P3.p, P6.p and P8.p. Animals were treated with hydroxyurea, and examined by DIC (top panel) and fluorescence (bottom panel) microscopy. Scale bar, 10 μm.

determined to correspond to approximately the early L3 stage, towards the end of the VPC cell cycle and prior to VPC mitosis (Greenwald et al., 1983).

To more precisely delineate the time of action of *lin-12(gf)* with respect to S phase of the VPC cell cycle, it was first necessary to confirm that VPC S phase in *lin-12(n137gfn460ts)* animals, as in the wild type, occurs in early L3. *lin-12(n137gfn460ts)* animals were treated with hydroxyurea at various times during the L3 and VPC mitosis was monitored using DIC microscopy. By this measure, S phase of *lin-12(n137gfn460ts)* VPCs is completed by approximately 2.5 hour (at 20°C) after the L2 molt (Table 3), which is similar to, but slightly later than, in the wild type (Euling and Ambros, 1996). Second, the occurrence of S phase was assayed using a reporter transgene (*maIs103*) consisting of *GFP* expressed from a ribonucleotide reductase (*rnr*) promoter (Hong et al., 1998). This *rnr::GFP* transgene becomes activated in cells undergoing S phase, and hence provides a fluorescent signature for VPC cells in S or G_2. *lin-12(n137gfn460ts)*; *maIs103* animals expressed GFP beginning in the early L3,

Table 2. Expression of *egl-17::GFP* in hydroxyurea-arrested VPCs of L3 larvae at 15°C

	Cell(s) expressing *egl-17::GFP*						
Genotype	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	No. of animals
*ayIs4; lin-15(n309)**	–	–	–	+	–	–	2
	–	–	–	–	+	–	1
	–	–	–	+	–	+	5
	–	–	+	–	+	–	1
	+	–	–	–	+	–	1
	–	+	–	+	–	–	2
	–	–	–	+	–	+	2
	+	–	–	+	–	+	3
	+	–	+	–	+	–	1
	–	+	–	+	–	+	2
	–	+	+	+	–	–	1
	–	+	+	+	–	–	1
	–	+	+	+	–	+	1
	+	–	+	+	+	–	1
	+	+	+	+	+	–	1
ayIs4; lin-12(n137n460)	–	–	–	–	–	–	25
	–	–	–	+	–	–	1
ayIs4; lin-12(n137n460); lin-15(n309)	–	–	–	–	–	–	11
	–	–	–	+	–	–	5
	–	–	–	–	+	–	2
	+	–	–	–	–	–	1
	–	+	–	–	–	+	1
	+	–	–	+	–	+	1

Animals were grown from hatching at 15°C, were transferred to plates containing 40 mM hydroxyurea (HU) in the presence of bacteria and maintained at 15°C for 10-11 hours. Animals were then immobilized using levamisole, and examined by DIC and epifluorescence microscopy. In all cases, VPCs were undivided.

*If VPCs of *ayIs4; lin-15(n309)* animals were expressing *egl-17::GFP* randomly, then 1/3 of animals with exactly two expressing cells would be expected to contain a pair of adjacent expressing cells; the observed 0/11 is statistically different than the expectation (χ^2=5.5; P=0.02). Similarly, 4/5 of animals with exactly three expressing cells would be expected to contain a pair of adjacent expressing cells; the observed 2/8 is statistically different than the expectation (χ^2=15; P<0.005). The proportion of *ayIs4; lin-15(n309)* animals with greater than three expressing cells (3/25) is approximately that expected (5/25), based on the average number of expressing cells per *ayIs4; lin-15(n309)* animal and a Poisson distribution.

Table 3. Hydroxyurea sensitivity of Vulva Precursor Cells (VPCs) in *lin-12(n137gfn460ts)*

Time after ecdysis*	No. of animals	% VPCs divided*
L2 + 0:50	1	0
L2 + 1:20	3	0
L2 + 2:30	4	75

*Animals in L1 or L2 lethargus and at various times (hours:minutes) after ecdysis (identified by resumption of pharyngeal pumping) were shifted to hydroxyurea-containing plates and examined 8-10 hours later for the presence of progeny from P3.p-P8.p.

approximately 1 or 2 hours after the L2 molt, as in the wild type.

To examine the effects of *lin-12(gf)* on the 1° versus 2° cell fate choice, temperature shifts were performed using VT832 *lin-12(n137gfn460ts); ayIs4(egl-17::GFP)* animals, and the expression of the primary fate was monitored by *egl-17::GFP* expression (Fig. 5). VT832 larvae were raised at 25°C until the L2 molt (to permit production of an anchor cell; Greenwald et al., 1983) and were then given a transient (3-4 hour) treatment with hydroxyurea to delay the completion of S phase. Animals were shifted to 15°C to activate *lin-12(gf)* either at the beginning of the HU treatment (in late G_1; Fig. 5C) or after removal of hydroxyurea (in late S phase or in G_2; Fig. 5D). All animals were examined by fluorescence microscopy just prior to release from HU and the expression of *egl-17::GFP* in P6.p was verified for all the animals (data not shown), confirming that expression of the 1° fate had been initiated. Essentially all the downshifted animals were Muv (Fig. 5C,D), indicating that 3° fates were efficiently converted to 2° fates by *lin-12(gf)*. In contrast, relatively few of the animals exhibited loss of their primary fates. *lin-12(gf)* activity starting during late G_1 (Fig. 5C) was somewhat more effective at reversal of commitment to primary fates than was *lin-12(gf)* activity starting in S phase (Fig. 5D). It should be noted that, although an animal was scored as Muv in Fig. 5 if it expressed one or more ectopic pseudovulvae, the Muv animals reported in Fig. 5B-D were of similar general appearance and most displayed two or three ectopic pseudovulvae. These results indicate that from the late G_1 through G_2 of the VPC cell cycle, the 1° fate is relatively stable to LIN-12 activation, while the 3° cell fate is easily reversible to the 2° fate, even after S phase. It should be noted that the 11% of animals that lost expression of the 1° fates after the shift at S phase could reflect some asynchrony in the population of worms, which were selected at the L2 lethargus by visual criteria (see Materials and Methods). The VPCs of some of these shifted animals may have been still in G_1 at the time of the shift and hence were more sensitive to LIN-12 activation. Thus, the 1° fate of S phase VPCs may be more stable than these data suggest.

Time of action of *lin-12* for the 2° versus 3° cell fate choice

The above results indicated that 1° fates and 3° fates may differ in how they are affected by *lin-12* activity. By the time of VPC S phase, the 1° fate appears to be relatively stable to *lin-12(gf)* activity, while the 3° fate can be efficiently converted to the 2° fate. To test whether the apparent ability of LIN-12 signaling to impose the 2° fate on otherwise 3° VPCs after the onset of S phase might be an artifact of hydroxyurea treatment, temperature-shift experiments were performed with *lin-12(n137gfn460ts); maIs103* animals. In these experiments, hydroxyurea was not employed, and S phase was monitored by GFP fluorescence. *lin-12(n137gfn460ts); maIs103* animals in the early L3 stage were selected using the dissecting microscope from cultures growing at either 15°C or 25°C, and were further selected by fluorescent microscopy for the presence of *rnr::GFP* expression in undivided VPCs (and hence to confirm that the VPCs were in S or G_2). The animals were then immediately shifted to the opposite temperature. In

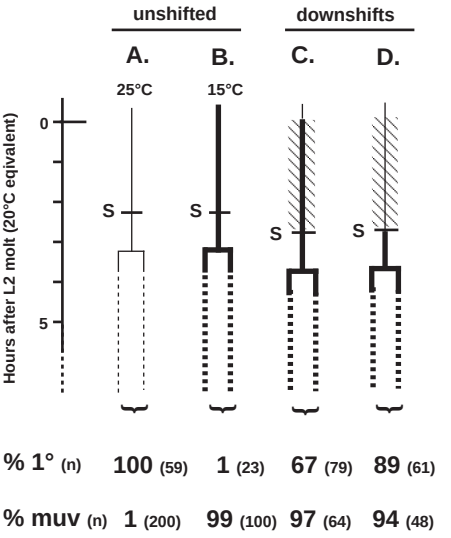

Fig. 5. Differential sensitivity of 1° and 3° fates to *lin-12(gf)* activity. VT832 *lin-12(n137gfn460ts); ayIs4(egl-17::GFP)* hermaphrodites were maintained at 25°C (thin lines) or 15°C (thick lines) for various periods of time during the L3 stage and then analyzed later using DIC and fluorescence optics. Time (hours after the L2 molt) is corrected to 20°C-equivalent developmental time (see Materials and Methods). The approximate time of S phase in *lin-12(n137gfn460ts)* was determined by hydroxyurea sensitivity (Table 3) and expression of *rnr::GFP* (Hong et al., 1998). Some animals were placed on plates containing 40 mM hydroxyurea for the time indicated by the hatched bars. S phase for hydroxyurea-treated animals is arbitrarily placed after release from hydroxyurea. The delay of VPC division by hydroxyurea was confirmed by direct observation of animals treated in parallel. Control animals raised continuously at 25°C (A) or 15°C (B) displayed opposite phenotypes. Most animals raised at 15°C lack an anchor cell and hence rarely express the 1° fate (Greenwald et al., 1983). Experimental larvae (C,D) were raised at 25°C until the L2 molt (to permit production of an anchor cell) and were then given a transient (4 hour) treatment with hydroxyurea to delay S phase. Animals were shifted to 15°C either at the beginning of the HU treatment (C) or after removal from hydroxyurea (D). All animals were examined by fluorescence microscopy at the time of release from HU, and the expression of *egl-17::GFP* in P6.p was verified for all the animals. % 1°, percent animals that showed *egl-17::GFP* expression in P6.p progeny in the late L3 (Burdine et al., 1998). For some animals, expression of the primary fate was corroborated by vulval morphology in the L4 (Sternberg and Horvitz, 1986). % Muv, per cent animals with one or more ectopic pseudovulvae at the adult stage. Dashed lines indicate either that further divisions occurred but were not monitored directly (in the case of 2° or 1° fate; see Fig. 1) or no further divisions occurred (in the case of the 3° fate). The results from animals treated in parallel are pooled; the number of animals scored is in parentheses.

Table 4. Temperature shift experiments using *lin-12(n137gfn460ts); maIs103* hermaphrodites

Temperature	Time of shift*	Percent induced‡ P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	(*n*)§
15°C	unshifted	78	98	100	100¶	93	93	(40)
25°C - 15°C	S or G2	39	82	98	100**	100	66	(44)
25°C	unshifted	0	7	100	100‖	100	7	(28)
15°C - 25°C	S or G2	3	10	47	87¶	33	20	(30)

*VPC cell cycle stage was determined by monitoring *rnr::GFP* expression. *lin-12(n137gfn460ts); maIs103* animals in the early L3 stage were selected using the dissecting microscope from cultures growing at either 15°C or 25°C, and were further selected by fluorescent microscopy for the presence of *rnr::GFP* expression in undivided VPCs. The animals were then immediately shifted to the opposite temperature. Shifted and unshifted control animals were examined as L4 larvae using DIC microscopy and the fates expressed by each of the six VPCs was determined by morphological criteria.

‡The percent of VPCs of each (for example, P3.p) that expressed either the 1° or 2° fate. 1° vs 2° fates could not be unambiguously distinguished in all cases, but all induced fates appeared to be 2°, except as noted below.

§The number of animals (and hence the number of VPCs of each lineal designation) screened.

¶Since these animals experienced *lin-12(gf)* activity during the L2, they often lacked an anchor cell, and hence variably expressed either the 1°or 2° fate by P6.p.

**Predominantly 1° fates.

‖All 1° fates.

these experiments, activating the ligand-independent LIN-12 receptor (by downshifting) after the beginning of S phase resulted in a Muv phenotype, based on the expression of ectopic 2° fates by P3.p, P4.p and P8.p (Table 4). In contrast, inactivating the receptor (by upshifting) resulted in a nonMuv phenotype, based on the relative absence of ectopic 2° fates (Table 4). In other words, the temperature-shifted animals expressed a phenotype corresponding to the state of *lin-12* activity after the beginning of S phase. These results confirm that *lin-12* can act after S phase to specify the 2° fate, as opposed to the 3° fate, in P3.p, P4.p and P8.p.

To test whether specification of 2° versus 3° fates via *lin-12(gf)* can occur only after completion of S phase, hydroxyurea treatment was employed to delay the completion of S phase, and reciprocal temperature shifts were performed. S phase was delayed in VPCs of *lin-12(n137gfn460ts)* animals (Fig. 6) by placing larvae in hydroxyurea for a period of time beginning at the L2 molt. The animals were later removed from hydroxyurea at the same time that they were either upshifted or downshifted in temperature. Control animals were treated in parallel, but without hydroxyurea, or without the temperature shift. In these experiments, VPCs expressed fates corresponding to the temperature (and hence the state of *lin-12* activity) after S phase. For example, *lin-12(n137gfn460ts)* animals in which VPC S phase was delayed before *lin-12(gf)* was activated by a downshift displayed the Muv phenotype corresponding to *lin-12(gf)* phenotype (Fig. 6C), while control animals shifted after VPC mitosis were non-Muv (Fig. 6D). (Hydroxyurea treatment alone, without a shift in temperature, had no detectable effect on the phenotype at 15°C or 25°C; data not shown.) Similarly, animals that were upshifted to inactivate *lin-12(gf)* after an S phase delay did not express the Muv phenotype (Fig. 6C) while the corresponding control animals (upshifted after VPC mitosis) were Muv (Fig. 6D). Similar results (Fig. 6) were obtained using animals expressing a

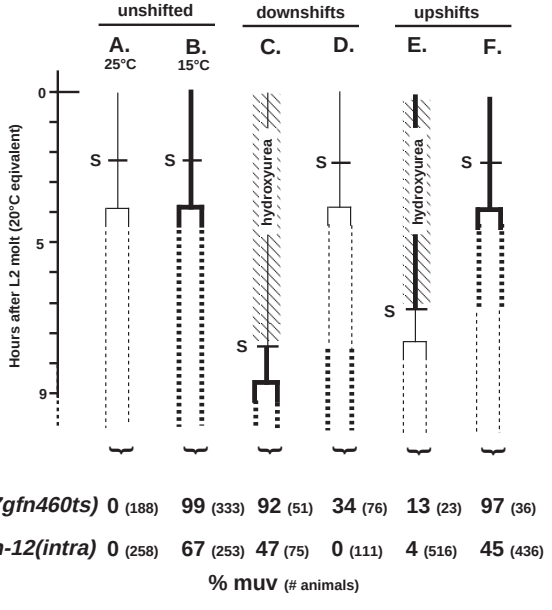

Fig. 6. Cell cycle dependence of *lin-12* activity in *lin-12(n137gfn460ts)* and *lin-12(intra)* animals. Temperature shifts were performed in combination with hydroxyurea treatment, and data are presented, as in Fig. 5. The percent of cells expressing ectopic 2° fates (the Muv phenotype), is shown below each panel.

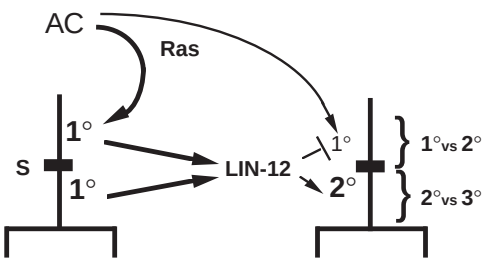

Fig. 7. A model for how LIN-12 controls two cell cycle-gated choices of cell fate for VPCs. Initially, LIN-12 signaling is relatively low in all VPCs. The VPC closest to the anchor cell (AC) receives a higher inductive signal (transduced by the Ras signal transduction pathway) than a neighboring VPC and initiates expression of the 1° fate. The presumptive primary signals laterally to its neighbor via LIN-12 to repress the 1° fate and bias it towards 2°. After S phase, the high levels of LIN-12 signaling in the neighbor commit it to the 2° fate, while VPCs more distant from the 1° cell (not shown) receive no LIN-12 signal and adopt the 3° fate.

temperature-sensitive intracellular domain of LIN-12 (*lin-12(intra)*; Struhl et al., 1993). The *lin-12(intra)* temperature-sensitive period had been shown previously to correspond to approximately the same stage of VPC development as the *lin-12(n137gfn460ts)* TSP (Struhl et al., 1993). It is important to note that the Muv phenotype scored in these particular temperature-shift experiments reflects ectopic pseudovulvae resulting from expression of ectopic 2° fates by VPCs that normally express 3° fates (P3.p, P4.p and P8.p); the expression of a 1° fate by P6.p was not monitored. Thus, these results show that *lin-12* must act at or after the completion of S phase to control the 2° versus 3° cell fate choice.

DISCUSSION

These findings suggest two cell cycle-gated stages of LIN-12 action in VPCs that correspond to separate cell fate choices. The first stage occurs in late G_1 or early S phase when LIN-12 signaling can act in opposition to RTK/Ras inductive signaling to inhibit the 1° fate and permit the expression of 2° fates. At this step, the levels of both Ras and LIN-12 signals are assessed and the 1° fate is prevented if LIN-12 signaling is relatively high or is selected if Ras signaling predominates (Beitel et al., 1995). One key consequence of this LIN-12/Ras interaction would be that any moderate level of inductive signal that may occur in cells flanking a presumptive 1° cell would be prevented from causing ectopic 1° fates. The second stage of LIN-12 action occurs upon completion of VPC S phase when VPCs with high LIN-12 signal commit to 2° fates and VPCs with low LIN-12 activity commit to 3° fates. This latter step may not necessarily involve an interaction of LIN-12 signaling with the Ras pathway, since specification of the 2° fate by LIN-12 does not seem to require LET-23 RTK activity (Simske and Kim, 1995; Koga and Ohshima, 1995).

lin-12 acts in a 1° versus 2° fate choice before completion of VPC S phase

Based on the expression of 1°-specific reporters in the presence of hydroxyurea, it appears that a VPC can be induced to begin expression of the 1° fate before completion of S phase. *egl-17::GFP* expression, which had been previously shown to depend on the anchor cell inductive signal (Burdine et al., 1998), and downregulation of LIN-12::GFP (Levitan and Greenwald, 1998) can occur in the presence of hydroxyurea (Figs 2, 3). Because hydroxyurea probably blocks cells at a point after the initiation of S phase, the experiments here do not distinguish whether induction of the 1° fate begins during G_1 or during S phase. Levitan and Greenwald (1998) observed a more efficient downregulation of LIN-12::GFP in VPCs of untreated animals than I observed in hydroxyurea-arrested VPCs. This difference could result from a side effect of hydroxyurea unrelated to cell cycle arrest. Alternatively, perhaps completion of S phase is required for complete downregulation of LIN-12::GFP.

During normal development, 1° cells signal laterally to their neighboring VPCs by a pathway involving the LIN-12 receptor and prevent these adjacent VPCs from also adopting the 1° fate (Sternberg, 1988; Sternberg and Horvitz, 1989). This lateral inhibition occurs in hydroxyurea-arrested VPCs, suggesting that lateral signaling from 1° cells does not require completion of S phase. This conclusion is based on two observations: (1) only P6.p, and not its neighbors P5.p or P7.p, express *egl-17::GFP* and downregulate LIN-12::GFP in hydroxyurea-treated L3 larvae, and (2) in hydroxyurea-treated *lin-15(lf)* animals, *egl-17::GFP* is expressed predominantly in an alternating on/off pattern consistent with inhibition of adjacent 1° fates. In these *lin-15* experiments, some cases were observed of adjacent *egl-17::GFP*-expressing cells (Tables 1, 2), indicating that prior to completion of S phase, lateral inhibition of 1° fates (as assayed by *egl-17::GFP* expression) is, although efficient, not absolutely complete. Using a different assay for the primary fate, downregulation of LIN-12::GFP, Levitan and Greenwald (1998) also observed a less-than-complete lateral inhibition in VPCs of *lin-15* animals. Together, these results suggest that lateral signaling occurs prior to completion of S phase, but may be less efficient at that stage in the absence of *lin-15* activity than in the wild type.

The lateral inhibition of adjacent 1° fates before completion of S phase is likely to be mediated by the LIN-12 receptor. This conclusion is based on the observation that a *lin-12(gf)* mutation causes a potent inhibition of *egl-17::GFP* expression in hydroxyurea-arrested VPCs of *lin-15* animals. Temperature-shift experiments using a temperature-sensitive *lin-12(gf)* mutation indicate that *lin-12* must act well before S phase to inhibit the induced 1° fate. Specifically, inhibition of the 1° fates by *lin-12(gf)* was only moderately efficient if the period of *lin-12(gf)* activity began in late G_1, and even less efficient if it began in S phase (Fig. 5). In these *lin-12* animals shifted in G_1 or S phase, 3° fates were efficiently switched to 2°, indicating that the temperature shift led to the activation of ligand-independent LIN-12 activity, yet the expression of the 1° fates was relatively resistant to its action (Fig. 5). This result could be interpreted to indicate that LIN-12 may effectively inhibit the 1° fate only if LIN-12 signaling precedes high levels of Ras signaling.

Although the results reported here indicate that expression of the 1° fate in response to Ras signaling *can* begin before completion of VPC S phase, these experiments did not directly test for whether induction of the 1° fate *must* begin then. However, M. Wang and P. Sternberg (personal communication) found that the response of VPCs to heat-shock-induced LIN-3 expression is consistent with the idea that induction of the 1° fate may be G_1 or S specific. The experiments reported here also did not address when commitment to the 2° fate finally occurs. Activation of the LIN-3 inductive signal in VPC daughters can reverse the 2° fate to 1°, suggesting that the 2° fate is uncommitted with respect to the 1° fate for as much as one full VPC cell cycle (M. Wang and P. Sternberg, personal communication).

There are numerous possible mechanisms for how the 1° versus 2° cell fate decision could occur prior to completion of S phase. One simple model involves RTK/Ras inductive signaling late in G_1 of the VPC cell cycle, and a decisive role for the downregulation of LIN-12 in the presumptive 1° cell prior to completion of S phase (Levitan and Greenwald, 1998). According to this model, a VPC with strong Ras activity (relative to LIN-12 activity) towards the end of G_1 would rapidly downregulate LIN-12 protein and begin to produce LIN-12 ligand (Levitan and Greenwald, 1998). The reduction in LIN-12 receptor would be a pivotal commitment step, since it would preclude subsequent activity of LIN-12 signaling in

the induced VPC and thus eliminate the 2° fate option for that cell. Further, LIN-12 ligand production by the 1° cell would activate LIN-12 in neighboring VPCs and thereby bias them towards 2° and prevent them from becoming 1°. Downregulation of LIN-12 at the end of G_1 could occur through translational repression and/or by protein degradation (Levitan and Greenwald, 1998). One candidate for a factor that may target LIN-12 for degradation is SEL-10, a member of the CDC4 family of proteins, some of which can act in G_1 progression by directing the degradation of cell cycle components (Hubbard et al., 1997).

Lateral and inductive signals are integrated to affect the 1° versus 2° cell fate choice at a point in the Ras signaling pathway after the action of the LIN-1 transcription factor (Beitel et al., 1995). This suggests that LIN-12 signaling may oppose Ras signaling on the level of gene expression, and could act in concert with other negative regulators of vulval gene expression, such as LIN-1 or gene products of the synMuv pathways (Herman and Hedgecock, 1990; Lu and Horvitz, 1999). The fact that components of the synMuv pathways include LIN-35, a protein related to the tumor suppresser Rb, and LIN-53, an Rb-associated protein, raises the intriguing possibility that a reduction of Rb activity associated with G_1/S progression (reviewed by Weinberg, 1995) could contribute to the timing of cell fate commitment for VPCs.

lin-12 acts in a 2° versus 3° fate choice after VPC S phase

Temperature-shift experiments indicated that VPCs do not execute a 2° versus 3° cell fate choice in response to LIN-12 activity until after S phase. The key result leading to this conclusion is that the fates of VPCs were affected by an activated LIN-12 receptor only if it was active after initiation of S phase (as defined by *rnr::gfp* expression, Table 4, or by release from hydroxyurea treatment, Figs 5, 6). The fact that similar results were obtained whether the temperature-shift experiments were conducted with or without hydroxyurea treatment argues against this result being significantly influenced by any artifacts of hydroxyurea treatment. Although there are alternative explanations for how experimental activation of *lin-12* could be less effective in G_1 than G_2, the conclusion that the response of VPCs to *lin-12* signaling is cell cycle gated seems like the simplest one consistent with these data.

Previous studies with *lin-12(n137gfn460ts)* found that VPCs temperature shifted after mitosis were unchanged by the shift (Greenwald et al., 1983), and in this study, animals that were temperature-shifted when their VPCs were at the G_2/M transition (as determined using DIC microscopy) exhibited the phenotype corresponding to unshifted animals (data not shown). These results imply that *lin-12(gf)* signaling is ineffective after VPC mitosis and suggests that the 2° versus 3° cell fate choice occurs in VPC G_2. However, since it was not possible to know how rapidly the temperature-sensitive LIN-12 protein was inactivated or activated after a temperature shift, it is conceivable that LIN-12 may control the 2° versus 3° decision shortly after VPC mitosis – during early G_1 of VPC daughters, when 3° cell differentiation likely occurs (M. Wang and P. Sternberg (personal communication).

How is the influence of LIN-12 on the 2° versus 3° decision restricted to a time after VPC S phase? Among the various possible models, one simple proposal would involve a change in the mode of LIN-12 regulation between G_1 and G_2 of the VPC cell cycle. Perhaps the expression of LIN-12 in VPCs prior to the end of S phase depends on general factors and then, after S phase, becomes dependent on a positive feedback from LIN-12 signaling activity (Wilkinson and Greenwald, 1995). Thus, LIN-12 levels would be maintained (and hence 2°-specific gene expression would occur) only in the progeny of those VPCs that had sufficient lateral signal after S phase (Wilkinson and Greenwald, 1995; Levitan and Greenwald, 1998). This proposed change in the mode of LIN-12 regulation after VPC S phase could reflect an activity of the cell cycle machinery at or after completion of S phase that modifies the LIN-12 intracellular domain or downstream factors.

It should be noted that the experiments described here utilize a *lin-12(gf)* mutation and a *lin-12(intra)* construct (Struhl et al., 1993) that produce mutant LIN-12 proteins with ligand-independent activity. It is formally possible that these mutant LIN-12 proteins signal to the cell nucleus by a pathway different from normal, ligand-dependent signaling. However, in the absence of data to that effect, one can assume that the cell cycle-dependent behavior of *lin-12(gf)* and *lin-12(intra)* reflects the wild-type situation. *lin-12(intra)* produces a truncated cytoplasmic form of the LIN-12 protein, so the cell cycle restriction on the consequences of *lin-12(intra)* signaling probably reflects a mechanism acting downstream of membrane-bound LIN-12 receptor, perhaps in the nucleus (Struhl and Adachi, 1998).

Cell cycle and cell fate decisions

The linkage of steps in cell fate determination to specific cell cycle phases could be a strategy for temporally coordinating cell fate choices among equivalent cells, or for sequencing and prioritizing the selection of different developmental programs within a single cell lineage. In VPCs, commitment to the 1° fate, which can begin prior to completion of S phase, may occur prior to commitment to the 2° or 3° fates, which requires S phase. This sequencing would help set a priority on expression of the primary fate in the anchor cell- proximal VPC. The key role of the 1° cell in then organizing the selection of 2° fates in G_2 of neighboring VPCs would help consolidate the normal pattern of one 1° VPC flanked by two 2° VPCs. Sequencing of cell fate choices by cell cycle phase may be particularly significant for multipotential cells such as the VPCs, where one signaling pathway (in this case, LIN-12) can influence multiple cell fate choices.

In more complex multicellular environments, linking signal transduction to cell cycle could provide a means to regulate the number of cells in a population that respond to various signals. In the developing mammalian cerebral cortex, the fates of asynchronously dividing cortical progenitor cells become specified in association with completion of their respective S phases. This would allow only a subset of the population of progenitors to be specified by any particular temporally restricted signal, thus ensuring an ample supply of responsive cells over time (McConnell and Kaznowski, 1991). Similarly, in *Dictyostelium*, the response of cells to a differentiation signal is cell cycle restricted, so the number of cells selecting the prestalk and prespore fates is controlled by the cell cycle distribution of cells at the time of induction (Gomer and Firtel, 1987).

Developmental decisions, or even cellular differentiation, can proceed independently of cell cycle progression (Hartenstein and Posakony, 1990; Edgar and O'Farrell, 1990; Harris and Hartenstein, 1991). Even in cases where the proper sequencing of developmental events is crucial, cell cycle progression need not be a source of temporal signals. A given signal transduction pathway may be engaged with the cell cycle in certain developmental contexts, but not in others. For example, no temporal constraints on LIN-12/NOTCH signaling events would be required in situations where the relative timing of cell fate choices is not important. A challenge for the future is to understand the precise molecular mechanisms by which the output of a signaling pathway such as LIN-12/NOTCH can be modulated by cell cycle in certain contexts, and independent of cell cycle in other situations.

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REFERENCES

Ambros, V. R. and Horvitz, H. R. (1984). Heterochronic mutants of the nematode Caenorhabditis elegans. *Science* **226**, 409-416.

Beitel, G. J., Tuck, S., Greenwald, I. and Horvitz, H. R. (1995). The Caenorhabditis elegans gene LIN-1 encodes an ETS-domain protein and defines a branch of the vulval induction pathway. *Genes Dev.* **9**, 3149-3162.

Bettinger, J. C., Euling, S. and Rougvie, A. E. (1996). Stage-specific accumulation of the terminal differentiation factor LIN-29 during *C. elegans* development. *Development* **122**, 2517-27.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics* **77**, 71-94.

Burdine, R. D., Branda, C. S. and Stern, M. J. (1998). EGL-17 (FGF) expression coordinates the attraction of the migrating sex myoblasts with vulval induction in *C. elegans*. *Development* **125**, 1083-1093.

Clark, S. G., Lu, X. and Horvitz, H. R. (1994). The Caenorhabditis elegans locus *lin-15*, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. *Genetics* **137**, 987-997.

Edgar, B. A. and O'Farrell, P. H. (1990). The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G_2 by *string*. *Cell* **62**, 469-480.

Euling, S. and Ambros, V. (1996) Heterochronic genes control cell cycle progress and developmental competence of Caenorhabditis elegans vulva precursor cells. *Cell* **84**, 667-676.

Ferguson, E. L., Sternberg, P. W. and Horvitz, H. R. (1987). A genetic pathway for the specification of vulval cell lineages of Caenorhabditis elegans. *Nature* **326**, 259-267.

Gomer, R. H. and Firtel, R. A. (1987). Cell-autonomous determination of cell-type choice in *Dictyostelium* development by cell-cycle phase. *Science* **237**, 758-762.

Greenwald, I., Sternberg, P. W. and Horvitz, H. R. (1983). The *lin-12* locus specifies cell fates in Caenorhabditis elegans. *Cell* **34**, 435-444.

Greenwald, I. (1985). *lin-12*, a nematode homeotic gene, is homologous to a set of mammalian proteins that includes epidermal growth factor. *Cell* **43**, 583-590.

Greenwald, I. (1998) LIN-12/Notch signaling: lessons from worms and flies. *Genes Dev.* **12**, 1751-1762.

Han, M. and Sternberg, P.W. (1990). *let-60*, a gene that specifies cell fates during *C. elegans* vulval induction, encodes a Ras protein. *Cell* **63**, 921-931.

Harris, W. A. and Hartenstein, V. (1991). Neuronal induction without cell division in Xenopus embryos. *Neuron* **6**, 499-515.

Hartenstein, V. and Posakony, J. W. (1990). Sensillum development in the absence of cell division: the sensillum phenotype of the Drosophila mutant *string*. *Dev. Biol.* **138**, 147-158.

Herman, R. K. and Hedgecock, E. M. (1990). Limitation of the size of the vulval primordium of Caenorhabditis elegans by *lin-15* expression in surrounding hypodermis. *Nature* **348**, 169-171.

Hill, R. J. and Sternberg, P. W. (1992). The gene *lin-3* encodes an inductive signal for vulval development in C. elegans. *Nature* **358**, 470-476.

Hong, Y., Roy, R. and Ambros, V. (1998). Developmental regulation of a cyclin-dependent kinase inhibitor controls postembryonic cell cycle progression in C. elegans. *Development* **125**, 3585-3597.

Huang, L. S., Tzou, P. and Sternberg, P. W. (1994). The *lin-15* locus encodes two negative regulators of Caenorhabditis elegans vulval development. *Molecular Biology of the Cell* **4**, 395-411.

Hubbard, E. J. A., Wu, G., Kitajeewski, J. and Greenwald, I. (1997). *sel-10*, a negative regulator of *lin-12* activity in Caenorhabditis elegans, encodes a member of the CDC4 family of proteins. *Genes Dev*. **11**, 3182-3193.

Kenyon, C. (1995) A perfect vulva every time: gradients and signaling cascades in C. elegans. *Cell* **82**, 171-174.

Kimble, J. (1981). Lineage alterations after ablation of cells in the somatic gonad of Caenorhabditis elegans. *Dev. Biol.* **87**, 386-300.

Koga, M. and Ohshima, Y. (1995). Mosaic analysis of the *let-23* gene function in vulval induction of *Caenorhabditis elegans*. *Development* **121**, 2655-2666.

Levitan, D. and Greenwald, I. (1998). LIN-12 protein expression and localization during vulval development in C. elegans. *Development* **125**, (in press).

Lu, X. and Horvitz, H. R. (1999). *lin-35* and *lin-53*, two genes that antagonize a *C. elegans* Ras pathway, encode proteins similar to Rb and its binding protein RbAp48. *Cell* (In press).

McConnell, S. K and Kaznowski, C. E. (1991). Cell cycle dependence of laminar determination in developing cerebral cortex. *Science* **254**, 282-285.

Servetnick, M. and Grainger, R. M. (1991). Changes in neural and lens competence in *Xenopus* ectoderm: evidence for an autonomous developmental timer. *Development* **112**, 177-188.

Simske, J. S. and Kim, S. K. (1995). Sequential signaling during Caenorhabditis elegans vulval induction. *Nature* **375**,142-146.

Singh, R. N. and Sulston, J. E. (1978). Some observations on molting in Caenorhabditis elegans. *Nematologica* **24**,63-71.

Slack, J. M. W. (1991). *From Egg to Embryo*. Oxford, England: Oxford University Press.

Sternberg, P. W. and Horvitz, H.R. (1986). Pattern formation during vulval induction in C. elegans. *Cell* **44**, 761-772.

Sternberg, P. W. (1988). Lateral inhibition during vulval induction in Caenorhabditis elegans. *Nature* **325**, 551-554.

Sternberg P. W. and Horvitz, H. R. (1989). The combined action of two intercellular signaling pathways specifies three cell fates during vulval induction in C. elegans. *Cell* **58**, 679-693.

Struhl, G., Fitzgerald, K. and Greenwald, I. (1993). Intrinsic activity of the LIN-12 and notch intracellular domains in vivo. *Cell* **71**, 331-345.

Struhl, G and Adachi, A. (1998). Nuclear access and action of notch in vivo. *Cell* **93**, 649-660.

Sulston, J. E. and Horvitz, H. R. (1977). Postembryonic cell lineages of the nematode Caenorhabditis elegans. *Dev. Biol.* **56**, 110-156.

Thomas, B. J., Gunning, D. A., Cho, J. and Zipursky, S. L. (1994). Cell cycle progression in the developing Drosophila eye, *roughex* encodes a novel protein required for the establishment of G_1. *Cell* **77**, 1003-1014.

Weigmann, K. and Lehner, C. F. (1995). Cell fate specification by *even-skipped* expression in the *Drosophila* nervous system is coupled to cell cycle progression. *Development* **121**, 3713-3721.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. *Cell* **81**, 323-330.

Wilkinson, H. A. and Greenwald, I. (1995). Spatial and temporal patterns of *lin-12* expression during C. elegans hermaphrodite development. *Genetics* **141** 573-526.

Wood, W. B. (ed.). (1988). *The Nematode Caenorhabditis elegans*. New York: Cold Spring Harbor Laboratory Press