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Organ-specific cell division abnormalities caused by mutation in a general cell cycle regulator in *C. elegans*

Ivana Kostić and Richard Roy*

Department of Biology, McGill University, Montreal, Quebec, Canada
*Author for correspondence (e-mail: richard.roy@mcgill.ca)

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SUMMARY

The precise control of cell division during development is pivotal for morphogenesis and the correct formation of tissues and organs. One important gene family involved in such control is the p21/p27/p57 class of negative cell cycle regulators. Loss of function of the *C. elegans* p27 homolog, *cki-1*, causes extra cell divisions in numerous tissues including the hypodermis, the vulva, and the intestine. We have sought to better understand how cell divisions are controlled upstream or in parallel to *cki-1* in specific organs during *C. elegans* development. By taking advantage of the invariant cell lineage of *C. elegans*, we used an intestinal-specific GFP reporter in a screen to identify mutants that undergo cell division abnormalities in the intestinal lineage. We have isolated a mutant with twice the wild-type complement of intestinal cells, all of which arise during mid-embryogenesis. This mutant, called *rr31*, is a fully dominant, maternal-effect, gain-of-function mutation in the *cdc-25.1* cell cycle phosphatase that sensitizes the intestinal lineage to an extra cell division. We showed that *cdc-25.1* acts at the G1/S transition, as ectopic expression of CDC-25.1 caused entry into S phase in intestinal cells. In addition, we showed that the *cdc-25.1(gf)* requires cyclin E. The extra cell division defect was shown to be restricted to the E lineage and the E fate is necessary and sufficient to sensitize cells to this mutation.

Key words: *cki-1*, CDC25, E lineage, Endoderm, Cell cycle, *C. elegans*

INTRODUCTION

Cell proliferation is essential for many key processes that occur during development including organogenesis, tissue renewal and germline formation. (Bartkova et al., 1997; Clurman and Roberts, 1995; Pines, 1995; Sandhu and Slingerland, 2000). Therefore, the timing of cell division and differentiation must be precisely coordinated with signals that specify morphogenesis, patterning and growth in a temporal, positional and cell type-specific manner (reviewed by Vidwans and Su, 2001). This coordination is executed through regulating both positive and negative regulatory components of the basal cell cycle machinery.

The cell cycle machinery is well conserved among eukaryotes and complex mechanisms ensure that cell cycle progression occurs in a timely and precise sequence. Cyclin-dependent kinases (Cdks) drive progression through the different cell cycle phases (reviewed by Nigg, 2001). In yeasts, these catalytic subunits are regulated through their association with stage-specific cyclin regulatory subunits (Wittenberg et al., 1990; Forsburg and Nurse, 1991). However, in more complex multicellular organisms, larger families of Cdks and cyclins exist, and their elaborate regulation provides cell-type and functional diversity.

These individual Cdks are activated in a cell cycle stage-specific manner (reviewed by Sherr, 1994; Sherr, 1996; Tsai et al., 1993; Draetta and Beach, 1988). The activity of these cyclin/Cdk complexes is required to phosphorylate substrates necessary to drive cell cycle progression and are regulated by activating and/or inhibitory kinases, or phosphatases, such as those of the *cdc25* family (Nilsson and Hoffmann, 2000; Nigg, 2001). Cdks can also be negatively regulated by cyclin-dependent kinase inhibitors (CKIs); small polypeptides that bind to and inhibit the catalytic activity of these kinases (Sherr and Roberts, 1999).

Among the various cell cycle transitions, the G_1/S transition represents an important regulatory milestone where extracellular signals are integrated resulting in the progression of cell division or, alternatively, cell cycle arrest in G_1 or G_0 (Pardee, 1989; Sherr, 1994). Coordination of cell cycle progression and arrest may depend on the function of the CKI $p27^{KIP1}$, while final growth arrest and differentiation may require the downregulation of positive cell cycle regulators (Koff and Polyak, 1995; Casaccia-Bonnefil et al., 1999).

In a multicellular organism, cell divisions must be coordinated with the developmental program to ensure the cellular integrity in all tissues of the organism. These developmental signals converge on many of the same key cell cycle components described above. Studies performed in *Drosophila* have shown that developmental signals impinge on the positive cell cycle regulator String, a homolog of the G_2/M-specific Cdc25 phosphatase, at several points during development (Foe, 1989; Edgar et al., 1994a; Edgar et al., 1994b; Edgar and O'Farrell, 1989). The G_1/S transition is also

developmentally regulated in flies through the activity of CKIs and cyclin E and cyclin D levels (Cayirlioglu and Duronio, 2001; Moberg et al., 2001; de Nooij et al., 1996; Lane et al., 1996).

In addition to cell cycle regulators that act globally, the activity of some regulators is important for the proper proliferation of cells in tissues at specific times during development. For example, in *Drosophila*, Roughex (Rux), acts specifically in the eye and in the male germ line to arrest cells in G_1 phase (Thomas et al., 1994; Gonczy et al., 1994; Avedisov et al., 2000). Decapentaplegic, a TGFβ family member, is required for the establishment of G1 arrest before differentiation during *Drosophila* eye development (Horsfield et al., 1998), while it is also essential for proliferation in the wing and in the germline (Burke and Basler, 1998; Xie and Spradling, 1998). Therefore, the complexity of tissues and the regulated development of many multicellular organisms make it difficult to characterize precisely how cell divisions are controlled in a specific developmental context.

The invariant cell lineage of *C. elegans* provides an invaluable tool to study cell division abnormalities at single cell resolution (Brenner, 1974). As the timing and fate of every cell division has been documented in a lineage map, the analysis of the effects of various developmental regulators on the cell cycle at specific developmental points and/or in specific cell lineages is possible (Sulston and Horvitz, 1977; Sulston et al., 1983).

Several conserved developmental regulatory genes have been shown to control embryonic and postembryonic cell division, and often, the resulting daughter cell fates in *C. elegans* (Kimble and Simpson, 1997; Euling and Ambros, 1996; Rougvie and Ambros, 1995). Mutations of conserved negative regulators have also been described, where the number of cell divisions and exit to G_0 has been shown to be regulated through the degradation of G_1 cyclins (Kipreos et al., 1996). The *C. elegans* $p27^{KIP1}$ homolog, *cki-1*, has been shown to confer developmental G_1 cell cycle arrest and to be one of the downstream effectors of many developmental pathways (Hong et al., 1998). Loss of *cki-1* results in extra cell divisions in numerous lineages causing abnormalities in the organogenesis of the vulva, the somatic gonad, the hypodermis, and intestine (Hong et al., 1998).

To understand the nature of the developmental signaling pathways that regulate cell division in specific lineages and during organogenesis, we designed a screen to isolate mutants that had altered cell division in specific organs without affecting overall cell division. To do this, we focused on mutants that phenocopy the loss of *cki-1* in the intestinal lineage using a lineage-specific GFP reporter. The study of mutants with organ-specific cell cycle aberrations could serve to elucidate the important role of *cki-1* or other upstream regulators in linking developmental signals with normal cell type-specific cell cycle dynamics, while providing further tools to identify factors that confer tissue specificity.

We report the identification and the characterization of a maternal-effect, gain-of-function allele of the proto-oncogene *cdc-25.1*, one of the four *C. elegans cdc25* homologs, which has a conserved role in positively regulating the G_1/S transition (Galaktionov et al., 1995b; Ashcroft et al., 1998). This allele causes tissue-specific embryonic cell cycle abnormalities, which occur in the cells that form the *C. elegans* intestine.

MATERIALS AND METHODS

Strains and genetics

In this study, we used the following strains and chromosome rearrangements: N2 wild-type Bristol, RW7000 wild-type Bergerac, CB4856 wild-type Hawaiian, VT765 (*maIs103* [*(rnr::GFP unc-36(+)]X*) (Hong et al., 1998), KM32 (*gvEx32* [*cye-1::GFP; rol-6D*]; a gift from M. Krause, KR1142 (*hDf8/szT1(lon-2(e678)) I; +/szT1 X*), JK1726 (*qDf16/dpy-5(e61) unc-15 (e1402)*), EU384 [*dpy-11(e1180) mom-2(or42) V/ nT1 (let-?(m435)] (IV;V)*, MR136 (*rrEx04* [*elt-2::GFP*]; a gift from J. McGhee), MR156 (*rrIs01* [*elt-2::GFP; unc-119(+)*]), *rrEx12* [*hs::cdc-25.1(+); ttx-3::GFP*], *rrEx13* [*hs::cdc-25.1(gf); ttx-3::GFP*], MR142 (*rr31; rrIs01*), MR178 (*maIs103; rrEx12*), MR180 (*maIs103; rrEx13*), MR196 (*rrEx12; gvEx32*), and MR197 (*rrEx13; gvEx32*). Strains were cultured using standard techniques (Brenner, 1974).

Screening for mutants which phenocopy *cki-1(RNAi)*

rrls01 animals were mutagenized with 40 mM ethylmethanesulfonate (EMS) (Brenner, 1974). Mutagenized L4 hermaphrodites were picked to plates (25-30 per plate) and allowed to produce progeny at 25°C. F_1 animals in the L4 stage were transferred to 60 mm plates, five per plate, and the F_2 progeny were screened for mutants that have extra numbers of intestinal nuclei, a phenocopy of *cki-1(RNAi)* animals, scoring with a fluorescent dissecting microscope. Candidate mutants were recovered and transferred to separate plates, and their progeny were examined for the presence of extra intestinal nuclei. 10,320 haploid genomes were screened.

Cloning of *cdc-25.1*

rr31 was mapped to the right arm of chromosome I using RW7000 and STS markers (Williams et al., 1992), SNIP-SNP mapping using CB4856 (Wicks et al., 2001), followed by three factor mapping to the *dpy-5 unc-13* interval.

Plasmid constructions

pMR405 and pMR409 were generated by inserting 2098 bp of the *cdc-25.1* sequence amplified from *rr31* [*cdc-25.1(gf)*] and wild-type animals, respectively, into the pGEM-T vector (Promega). pMR407 and pMR408 were generated by inserting a 7495 bp PCR product, including 5035 bp of upstream sequence and 366 bp 3′ to the translational stop site corresponding to the mutant (*rr31*) or the wild-type *cdc-25.1(gf)* gene, respectively, into pGEM-T (Promega). pMR410 and pMR411 were generated by inserting the wild-type *cdc-25.1* genomic sequence into the *Nco*I/*Sac*I sites of pPD49.78 and pPD49.83, respectively. pMR412 and pMR413 were generated by inserting the mutant *cdc-25.1* genomic sequence into the *Nco*I/*Sac*I sites of pPD49.78 and pPD49.83, respectively. For sequencing of the mutant or wild-type cDNA, polyA RNA was isolated from *cdc-25.1(gf)* or wild-type animals, and mutant and wild-type cDNA was amplified after reverse transcription. The corresponding PCR products were placed into pGEM-T to yield pMR421 and pMR418.

Microinjection and transformation

Worms were transformed by microinjection as previously described (Mello et al., 1991). A 7495 bp PCR product corresponding the *cdc-25.1* gene was amplified from *cdc-25.1(gf)* or wild-type N2 genomic DNA, and injected *rrls01* at the concentration of 17 ng/μl with the co-transformation marker pRF4 (*rol-6D*) at the concentration of 128 ng/μl. MR178 (*maIs103; rrEx12*) was constructed by injection of 20 ng/μl pMR410 and pMR411 into the *rnr::GFP* strain with 100 ng/μl *ttx-3::GFP* (Hobert et al., 1997). MR180 (*maIs103; rrEx13*) was constructed by injection of 20 ng/μl pMR412 and pMR413 into *rnr::GFP* with 100 ng/μl *ttx-3::GFP*.

Sequencing

pMR405, pMR409, pMR421 and pMR418 were sequenced and the sequences were compared with each other and with published genomic sequences available from Wormbase (www.wormbase.org).

RNA interference

The *cki-1* dsRNA was produced and injected according to Hong et al. (Hong et al., 1998). *cyd-1* and *cye-1* dsRNA was produced according to Park and Krause (Park and Krause, 1999) and Fay and Han (Fay and Han, 2000), respectively. *cdc-25.1* dsRNA was produced by restriction enzyme digestion of pMR409 with *Nde*I or *Sac*I for the sense and antisense *cdc-25.1* RNA. Gel-purified template (1 μg) was used for in vitro transcription reactions according to Fire et al. (Fire et al., 1998). Double stranded *cdc-25.1* RNA was injected into *rrls01* or *rr31; rrls01* animals at a concentration of 1 mg/ml, and the injected animals were transferred daily to new plates, where the intestinal cell number of the F_1 progeny was scored.

Lineage analysis

Embryos dissected from gravid *rrls01* or *rr31; rrls01* hermaphrodites were placed on NGM pads and cell divisions were observed from the zygote stage onwards. For the *cki-1(RNAi)* lineage, F_1 embryos of *cki-1* dsRNA-injected hermaphrodites were mounted on NGM pads and cell division timing was recorded by following E cell divisions using the *elt-2::GFP* reporter.

Heat-shock experiments

Animals carrying the mutant or wild type *cdc-25.1* transgenes (MR178, MR179, MR181, MR196, MR197) driven by the *hsp16-2* and *hsp16-41* promoters, or the heat-shock constructs alone were placed in the *cye-1::GFP* and *rnr::GFP* background in order to assay the entry into S phase. Adult transformed and non-transformed hermaphrodites were placed at 33°C for 3 hours and then allowed to recover for 2 hours at room temperature. The hermaphrodites were then mounted on 2% agarose pads in 2 mM levamisole, and *cye-1::GFP*, or *rnr::GFP* expression was observed.

Immunostaining

Antibody staining of embryos with anti-PHA-4 antibody or anti-CDC-25.1 antibody was performed according to Boxem et al. (Boxem et al., 1999) and Ashcroft et al. (Ashcroft et al., 1999), respectively. For immunostaining of larvae, animals were fixed in 3% formaldehyde and antibody staining was performed according to standard procedures (Finney and Ruvkun, 1990).

Image capture and processing

Images of live embryos, or animals anesthesized with 1 mM levamisole, were captured using the Leica DMR compound microscope equipped with a Hamamatsu C4742-95 digital camera. Image analysis, computational deconvolution and pseudocoloring were performed using Openlab 3.01 software from Improvision. Images were merged using Adobe Photoshop.

RESULTS

cki-1(RNAi) and *rr31* animals display defects in intestinal cell number

The phenotype associated with the loss of *cki-1* activity through dsRNA-mediated interference (RNAi) has demonstrated a role of this CKI in the regulation of cell division timing (Hong et al., 1998). Embryos homozygous for a deficiency that uncovers *cki-1* arrest with substantially more endodermal precursors implicating a gene mapping within this genetic interval in the embryonic control of cell divisions in the E lineage (M. Fukuyama, S. Gendreau and J Rothman, personal communication). Intestinal cell numbers are increased in adult *cki-1(RNAi)* animals which possess an average of 50 as compared to 30 intestinal nuclei in wild-type animals (Table 1). To isolate mutants that may regulate *cki-1* expression or function in the gut, we screened for mutants that would phenocopy this extra intestinal cell phenotype, using the intestinal-specific reporter *elt-2::GFP* (Fukushige et al., 1998). 10,320 haploid genomes were screened and although several mutants with fewer intestinal cells were isolated, we have identified only one mutant with extra intestinal cells. This mutant, *rr31*, has 57±4 intestinal nuclei at the adult stage, or approximately twice the wild-type complement (Fig. 1; Table 1).

Other than the intestinal cell defect, *rr31* mutants appear phenotypically normal. To test whether other cell types were affected by the *rr31* mutation, we examined cell numbers in *rr31* by DIC, DAPI staining and with anti-PHA-4 antibody, which marks mesodermal pharyngeal precursors (Horner et al., 1998). *rr31* mutants and wild-type controls showed no differences in these cell lineages examined; however, the number of intestinal cells was markedly elevated judged by the increased number of *elt-2::GFP* expressing nuclei (data not shown). However, we cannot rule out that there may be other less obvious lineage defects that were not apparent from our examination of *rr31* mutants.

Table 1. *cki-1(RNAi)* and *rr31* cause increases in the number of intestinal nuclei during development

	Time after hatching (hours)		
Genotype	0	48	72
rr31	38±3 (*n*=22)	54±5 (*n*=19)	57±4 (*n*=25)
cki-1 (RNAi)	29±3 (*n*=26)	43±6 (*n*=18)	50±7 (*n*=21)
rr31; cki-1 (RNAi)	45±7 (*n*=27)	50±9 (*n*=21)	58±7 (*n*=25)
N2	20 (*n*=20)	30±2 (*n*=15)	30±2 (*n*=21)

Strains were maintained at 15°C before and after RNA injection. The animals are at the L1 stage at 0 hours, L3 stage at 48 hours and adult stage at 72 hours after hatching.

dsRNA was injected and the P0s were transferred to new plates after 24 hours. The animals were allowed to recover for 24 hours and the F_1 progeny laid after these 24 hours were scored for intestinal cell number by counting the *elt-2::GFP*-expressing nuclei.

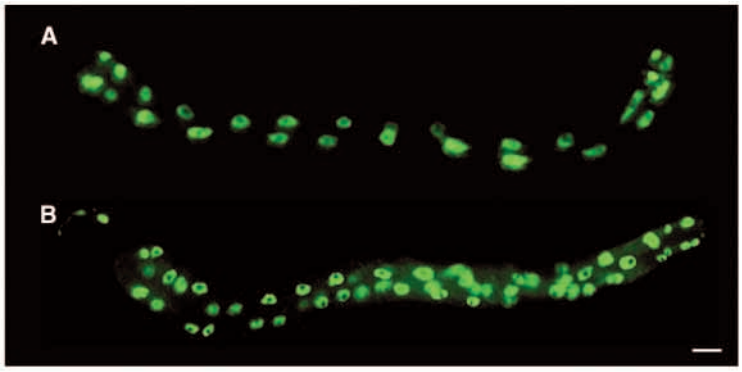

Fig. 1. *cdc-25.1(gf)* mutants have increased numbers of intestinal nuclei. (A) Wild-type adult animals expressing *elt-2::GFP*, which marks intestinal nuclei. (B) *rr31* adult showing an increased number of *elt-2::GFP* expressing nuclei. Scale bar: 25 μm. Anterior is towards the left.

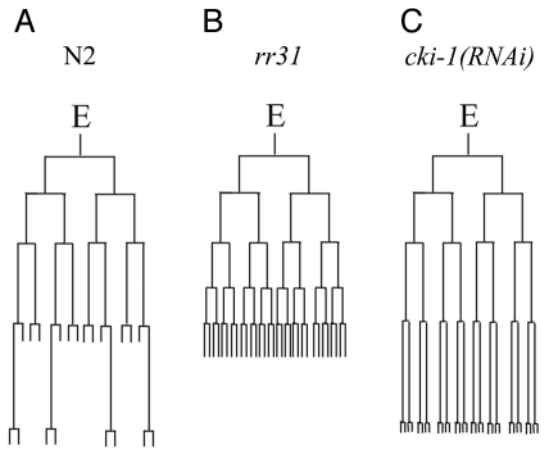

Fig. 2. Lineage analysis of the E blastomere in *rr31* and *cki-1(RNAi)* animals. (A) Lineage map of the wild-type intestinal cell divisions during embryogenesis. Schematic representation of a characteristic lineage map of the (B) *rr31* mutant and (C) *cki-1(RNAi)* intestinal cell divisions during embryogenesis. The vertical distances represent approximate time of development.

During normal development, after a series of mitotic divisions that occur during embryogenesis, the posterior intestinal cells undergo a single nuclear division at the end of the L1 stage, producing binucleate intestinal cells. Therefore, the extra intestinal nuclei in *rr31* mutants could be the result of additional mitotic divisions during embryogenesis, or alternatively, extra postembryonic nuclear divisions. To address this, we scored the number of intestinal cells in newly hatched wild-type, *rr31* and *cki-1(RNAi)* L1 larvae. *rr31* and *cki-1(RNAi)* L1s possess an average of 38 (±3) and 29 (±3) intestinal cells, respectively, compared with 20 in wild type. Therefore, we conclude that in *rr31* mutants, like *cki-1(RNAi)*, the extra cells in the intestine arise at a point during embryogenesis before hatching. Furthermore, *rr31* and *cki-1(RNAi)* animals stained with the MH27 antibody, which stains the cell junctions of all epithelial cells (Priess and Hirsh, 1986; Waterston, 1988), display numerous extra cell borders in the intestine, indicating that there is an increase in the number of cells, rather than extra nuclear divisions (data not shown).

rr31 and *cki-1(RNAi)* affect different embryonic cell divisions

The extra cells in both *rr31* and *cki-1(RNAi)* backgrounds could arise from additional divisions of intestinal cells during embryogenesis, or from a mis-specification of another cell type into intestinal cells. To further understand when and how the defects occur in these mutant backgrounds, we performed lineage analysis on *rr31* animals and *cki-1(RNAi)* animals. In wild-type animals, the intestine is formed from the E (endoderm) blastomere. During embryogenesis, this founder cell divides four times to give rise to 16E cells, while four of these cells undergo a fifth division, giving rise to the 20 intestinal cells present at hatching (Sulston et al., 1983) (Fig. 2). At the end of the L1 stage, 14 of these cells undergo a nuclear division leading to the formation of binucleate intestinal cells, followed by endocycles that coincide with each larval molt (Sulston and Horvitz, 1977) (Table 1). *rr31* mutants display an additional cell division after the 8E stage during embryogenesis, giving rise to 16 intestinal cells at this time instead of the wild-type 8E cells (Fig. 2). All 16 of these cells divide afterwards, as in wild-type animals, giving rise to 32 cells. The final number of intestinal cells at hatching (38±3) suggests that, as in wild type, only a subset of intestinal cells undergo a final mitotic division (in wild type, this results in 20 cells being formed from 16, while in *rr31* mutants, the number increases from 32 to 38±3). The increase in the number of intestinal nuclei during postembryonic development in *rr31* mutants (from 38±3 to 57±4) indicates that the L1-specific nuclear divisions also occur in *rr31* mutants. Finally, the series of endocycles that occur following each larval molt also seem to be unaffected in *rr31* mutants.

In *cki-1(RNAi)* animals, a similar supernumerary intestinal cell division occurs, but instead it occurs later, after four rounds of division, in cells that should normally have ceased dividing (Fig. 2). The difference in the timing of the lineage defect observed in *rr31* and *cki-1(RNAi)* suggests that these two genes do not act in the same pathway that controls embryonic intestinal cell divisions.

To further strengthen this, *cki-1 (RNAi)* was performed in the *rr31* genetic background. If these genes function in a common pathway, one would expect to observe some epistasis; however, if they act in parallel pathways, some enhancement should be apparent. Although both of the mutants had increased intestinal cell numbers at hatching [38±3 for *rr31* mutants, and 29±3 for *cki-1(RNAi)* animals], the double mutant *rr31; cki-1(RNAi)* showed an increase in the number of intestinal nuclei at hatching compared with the single mutants (45±7), suggesting that the *rr31* and *cki-1* function in parallel pathways. Interestingly, the total number of intestinal cells at the adult stage was not significantly different in the single and double mutants [58±7 in *rr31; cki-1(RNAi)* animals and 57±4 in *rr31* mutants] (Table 1), implying the presence of downstream components limiting the proliferative capacity of intestinal cells, which are common to both *cki-1* and *rr31*.

rr31 is a dominant maternal-effect, gain-of-function allele of the *cdc-25.1* dual-specificity phosphatase

To understand how *rr31* functions at the molecular level, we mapped the mutant and then used a novel positional cloning strategy to characterize the *rr31* allele molecularly. Genetic analysis showed that the *rr31* mutation segregated in a dominant, maternal-effect manner. All the F_1 progeny of a hermaphrodite heterozygous for the *rr31* mutation displayed the extra intestinal cell phenotype, including the homozygous +/+ larvae (Table 2), whereas when homozygous *rr31* males were crossed into N2 hermaphrodites, none of the F_1 progeny had extra intestinal cells. To determine whether the dominant *rr31* mutation was due to a gain-of-function mutation, or a loss of function in a haploinsufficient gene, we analyzed the effects of *rr31* when hemizygous with either of two deficiencies that uncover this region (*hDf8* and *qDf16*). Progeny of +/Df hemizygotes showed no evidence of extra intestinal cell divisions, whereas the progeny of *rr31*/+ heterozygotes were all affected, indicating that *rr31* is not a loss-of-function mutation in a haploinsufficient gene. Furthermore, in the progeny of animals hemizygous for *rr31* and *qDf16* or *hDf8*, the extra intestinal cell phenotype was still present and fully penetrant. From these results, we conclude that the *rr31* mutation is a dominant, gain-of-function mutation.

Table 2. *rr31* is a gain-of-function mutation that causes increased numbers of intestinal cells

Genotype of P0 animals	Percentage of F_1 animals with extra intestinal cells (%)
rr31/rr31	100 (*n*=88)
rr31/+	100 (*n*=60)
hDf8/+	0 (*n*=50)
hDf8/rr31 unc-13	98 (*n*=96)
qDf16/+	0 (*n*=145)
qDf16/rr31 unc-13	100 (*n*=67)

Strains were maintained at 20°C. The number of intestinal cells was scored in adult animals by counting the number of *elt-2::GFP*-expressing nuclei. According to mapping data, it was concluded that the deficiencies *hDf8* and *qDf16* uncover the region to which the *rr31* mutation is mapped. The genotype is indicated as the genotype of the P0 hermaphrodite.

Table 3. The injection of *cdc-25.1* phosphatase gene results in an extra intestinal cell phenotype

	T23H2.5 Ras-related	K06A5.7a *cdc-25.1* phosphatase (mutant)	K06A5.7a *cdc-25.1* (WT)
Number of F_1 animals with extra intestinal cells	0 (*n*=67)	7 (*n*=93)	0 (*n*=96)

F_1 animals that were transformed with the PCR fragments containing potential candidates along with the *rol-6D* cotransformation marker were examined for the presence of extra intestinal nuclei by counting the number of *elt-2::GFP*-expressing cells.

Table 4. The *cdc-25.1* gene product is required for extra cell divisions in *rr31* mutants

Genotype	24 hours	48 hours
cdc-25.1 (RNAi)	100 (*n*=29)	96 (*n*=25)
rr31; cdc-25.1 (RNAi)	50 (*n*=24)	88 (*n*=17)
rr31	0 (*n*>50)	0 (*n*>50)
N2	100 (*n*>50)	100 (*n*>50)

dsRNA was injected into *rr31* or N2 animals, which were transferred after every 24 hour period thereafter. Results are expressed as a percentage of animals that showed a wild-type intestinal cell number in the adult stage.

As the *rr31* mutation is a dominant gain-of-function mutation, it was impossible to clone the gene using standard transformation rescue techniques. To circumvent this problem, we attempted to phenocopy the extra intestinal cell phenotype by injecting wild-type animals with PCR-amplified genomic regions from the *rr31* mutant that corresponded to the predicted genes within the genetic interval where *rr31* mapped. The injection of a 7.4 kb fragment corresponding to the *cdc-25.1* gene resulted in the formation of extra intestinal cells in the transformed F_1 progeny, while other candidates had no effect (Table 3). This phenotype was incompletely penetrant and did not persist in subsequent generations, probably owing to a requirement for transgene expression in the germ line to provide maternally expressed products (Kelly et al., 1997).

RNAi-mediated removal of *cdc-25.1* activity suppresses the *cdc-25.1(gf)* phenotype

Considering that the injection of the 7.4 kb PCR product amplified from *rr31* mutant genomic DNA encoding the *cdc-25.1* gene phenocopied the *rr31* gain-of-function phenotype, we predicted that a gain-of-function mutation in *cdc-25.1* could be responsible for the intestinal phenotype in the *rr31* mutant. We performed *cdc-25.1(RNAi)* to test whether the *rr31* phenotype could be suppressed by removing all *cdc-25.1* gene activity (Fire et al., 1998). The injection of *cdc-25.1* dsRNA into wild-type animals carrying the *elt-2::GFP* intestinal specific promoter, produced a variably penetrant embryonic lethal phenotype as previously reported, as well as 'escapers', which were later sterile or not affected (Ashcroft et al., 1999). Most of the adult F_1 *cdc-25.1(RNAi)* progeny possessed a wild-type number of intestinal nuclei. Alternatively, when *cdc-25.1* dsRNA was injected into *rr31* mutant animals, the resulting F_1 progeny showed a marked reduction in the number of intestinal nuclei and the final intestinal cell count approached the wild-type complement of intestinal cells (Table 4). Progeny of uninjected *rr31* animals showed no decrease in the number of intestinal nuclei. This indicated that *cdc-25.1* is absolutely required for the extra intestinal cell divisions characteristic of the *rr31* phenotype.

To verify whether the extra intestinal cell phenotype was indeed due to a mutation in *cdc-25.1,* we analyzed the genomic and cDNA sequence of the mutant and wild-type *cdc-25.1* genes (Fig. 3). A single GC to AT transition was detected at the first nucleotide position of exon 2 in both *rr31* genomic DNA and in the mutant cDNA, resulting in a G to D substitution at amino acid 47 in the N-terminal region of the protein (Fig. 3C). Initial structural predictions of the mutant CDC-25.1 protein imply that this substitution imparts a more flexible loop domain adjacent to a region of the polypeptide chain, which is strongly predicted to form a buried alpha helix.

cdc-25.1(gf) requires *cye-1* function to promote the extra intestinal cell division

To further understand the mechanism of action of the *cdc-25.1(gf)*, we examined the RNAi phenotypes of potential candidate cell cycle regulators that may play an important role in the generation of extra intestinal cells during embryogenesis in *cdc-25.1(gf)* mutants. As the mammalian homolog of *cdc-25.1*, Cdc25A, is presumed to accelerate G_1/S by dephosphorylating CDK2, effectors that modulate CDK2 would be good candidates to investigate (Blomberg and Hoffmann, 1999). The mammalian cyclin E plays such a role through its association with CDK2, which, when activated through this association, triggers the initiation of S-phase (Tsai et al., 1993). Removal of the *C. elegans* cyclin E homolog by RNAi of the *cye-1* gene causes embryonic lethality at the 100-cell stage in *C. elegans* (Fay and Han, 2000). When we performed RNAi with *cye-1* dsRNA, 15-50% embryonic lethality was observed, while most of the other animals 'escaped' but arrested shortly after hatching. Because the *cdc-25.1(gf)* cell cycle defect occurs after the time of the terminal embryonic phenotype of *cye-1(RNAi)*, we examined these escapers for suppression of the extra intestinal cell defects following *cye-1(RNAi). cye-1(RNAi)* animals had 20 intestinal cells on average at hatching, while when *cye-1* function was removed in *cdc-25.1(gf)* animals, the extra intestinal cell phenotype was suppressed from 38 (±3) in *cdc-25.1(gf)* mutants alone, to 20 (±5) in *cdc-25.1(gf); cye-1(RNAi)* animals

Fig. 3. *cdc-25.1* mutant sequence. (A) *cdc-25.1* mutant DNA sequence including the translation start to up to and including exon 2. Exon sequence is in blue, intron sequence in red and the mutated nucleotide in yellow. (B) *cdc-25.1* mutant cDNA sequence including exons 1 and 2. (C) CDC-25.1 mutant amino acid sequence (N-terminal region). The yellow amino acid indicates a G to D substitution at amino acid 47. The residues marked with red dots indicate a higher probability of being phosphorylated in the mutant while those marked with blue, indicate a decreased probability of being phosphorylated (PredictProtein software).

(Table 5). No effect on the suppression of the extra cell division could be detected following removal of cyclin D by RNAi of the *cyd-1* gene in *cdc-25.1(gf)* mutants, despite a larval arrest phenotype and the inability to undergo postembryonic intestinal cell divisions (Table 5) (Park and Krause, 1999). This indicates that the positive cell cycle regulator *cye-1* is required for the formation of extra intestinal cells in *cdc-25.1(gf)* mutants, while *cyd-1* is not.

The mutant *cdc-25.1* specifically affects the E lineage

Although *cdc25* genes have been shown to be important general cell cycle regulators important for the G_1/S or G_2/M transition, the *cdc-25.1(gf)* seems to only confer the ability to undergo an additional round of division to the intestinal cell lineage. This lineage restriction could be due to the presence of a factor in the E lineage that predisposes these cells to *cdc-25.1(gf)*, or perhaps the lack of an activity present in other cells, which blocks such an effect.

The entire *C. elegans* endoderm is derived from one single blastomere, E, at the eight-cell stage of embryogenesis. The E-cell fate is specified through maternally provided factors, which are asymmetrically localized within the early embryo. These factors induce the E-cell fate through cell-cell interactions that are mediated mainly by the Wnt signaling pathway (Thorpe et al., 1997; Rocheleau et al., 1997).

To ascertain whether the *cdc-25.1(gf)* effect on the E lineage is dependent on Wnt signaling and/or subsequent E specification, or whether it may be due to other signals from surrounding blastomeres, we blocked Wnt signaling using a *mom-2* background, which undergoes an E to MS cell fate transformation. (Thorpe et al., 1997; Rocheleau et al., 1997). If the E-to-MS transformed cell still overproliferates in *mom-2;cdc-25.1(gf)*, then the *cdc-25.1(gf)* defect could be considered independent of E-cell fate specification by Wnt signaling and as such, more mesodermal cells should be present in *mom-2;cdc-25.1(gf)* compared with *mom-2* single mutants. If this defect depends on Wnt signals and/or E specification, then the *mom-2;cdc-25.1(gf)* mutant should show the same number of (MS) mesodermal precursors as the *mom-2* mutant alone. We found that the *mom-2;cdc-25.1(gf)* mutants did not form endoderm and produced the same number of mesodermal precursor cells as the *mom-2* single mutants (Fig. 4). This suggests that a cell must be specified as endodermal (E) through Wnt-signaling to be sensitive to *cdc-25.1(gf)*.

It is therefore plausible that the extra cell division in the E lineage is exclusively due to a cell-autonomous effect in cells of the E lineage. To confirm this prediction, we performed a reciprocal experiment with *pop-1(RNAi)* in the *cdc-25.1(gf)* mutant. *pop-1* mutants have an MS to E transformation and produce extra intestinal cells at the expense of mesoderm (Lin et al., 1995). *cdc-25.1(gf);pop-1(RNAi)* embryos demonstrated a twofold increase in the number of intestinal cells compared with *pop-1(RNAi)* embryos alone (Fig. 5). This indicates that the MS blastomere, which was transformed to 'E' in *pop-1 (RNAi)* animals, is now also predisposed to *cdc-25.1(gf)* and, as a result, undergoes an additional round of division similar to its neighboring endogenous E blastomere.

Abnormal cell division timing can cause apparent cell fate transformations, and other blastomere fate transformations can occur under specific genetic circumstances giving rise to 'ectopic' E cells (Ambros, 1999; Maduro et al., 2001; Mello et al., 1992). To further confirm the cell-autonomous effect of *cdc-25.1(gf)*, we ablated the E blastomere in five embryos immediately following its formation after division of EMS in *cdc-25.1(gf)* mutants. In wild-type animals, the ablation of E results in embryos that arrest embryonic development with no endoderm. In Fig. 5E we show that early E blastomere ablation in *cdc-25.1(gf)* animals results in the complete absence of intestinal cells, indicating that the extra intestinal cells in *cdc-25.1(gf)* animals result exclusively from the additional cell divisions of the E cell descendants.

Table 5. The number of intestinal nuclei in various genetic backgrounds at specific times during development

Genotype	Number of intestinal nuclei at L1 stage
N2	20 (*n*=20)
rr31	38±3 (*n*=22)
cye-1(RNAi)	20±1 (*n*=20)
*cyd-1(RNAi)**	23±5 (*n*=11)
rr31; cye-1(RNAi)	20±5 (*n*=24)
*rr31; cyd-1(RNAi)**	47±10 (*n*=11)

Strains were maintained at 20°C before and after RNA injection. *cye-1* or *cyd-1* dsRNA was injected and the P0s were transferred to new plates after each 24 hour period. The second window plates were scored for extra intestinal cells by counting the *elt-2::GFP*-expressing nuclei.

*The number of intestinal nuclei in the *cyd-1(RNAi)* animals was scored in arrested larvae to ascertain that only the RNAi-affected animals were scored.

cdc-25.1 acts at the G_1/S transition

The mammalian Cdc25 homologs function as dual-specificity

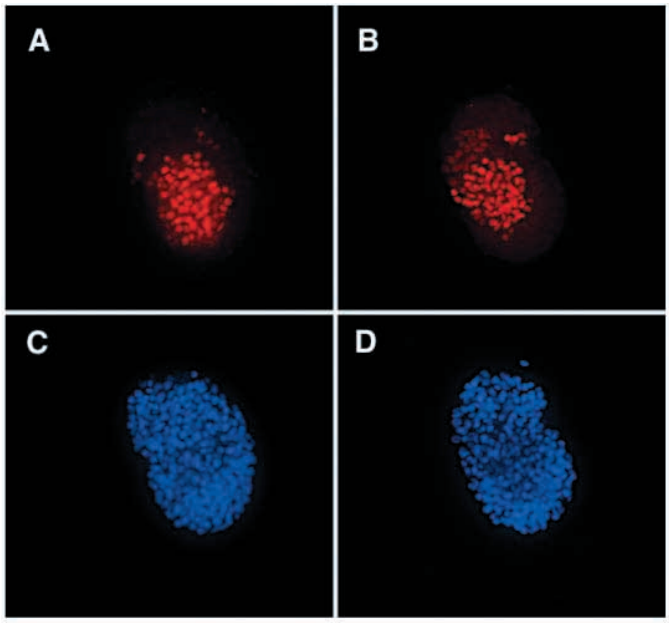

Fig. 4. The *cdc-25.1(gf)* defect is specific to the E lineage. (A) *mom-2* embryos produce extra mesoderm at the expense of endoderm, as seen by anti-PHA-4 staining, which marks pharyngeal precursors (descendants of the MS blastomere). (B) *cdc-25.1(gf); mom-2* embryos have similar amounts of mesoderm as *mom-2* mutants alone. (C,D) The embryos in A,B have similar cell numbers measured by counting DAPI stained nuclei: (C) *mom-2* embryo in A; (D) the *cdc-25.1(gf); mom-2* embryos in B. *C. elegans* embryos are approximately 50 μm in length.

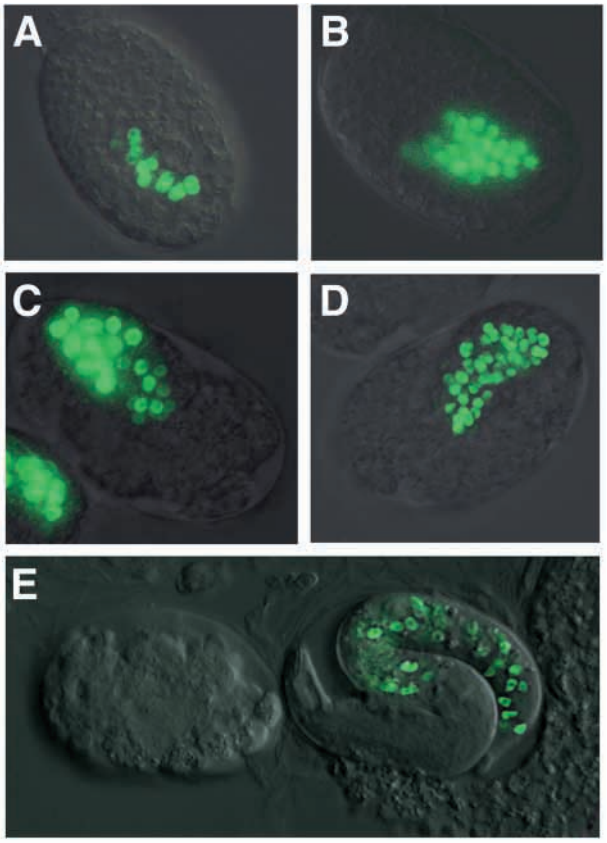

Fig. 5. *cdc-25.1(gf)* enhances the *pop-1* phenotype. (A) Wild-type number of intestinal nuclei in 300 minute embryo visualized with *elt-2::GFP.* (B) *cdc-25.1(gf)* embryo at 300 minutes showing extra intestinal nuclei. (C) *pop-1(RNAi)* embryos have extra intestinal nuclei due to a MS to E transformation. (D) *cdc-25.1(gf); pop-1(RNAi)* embryos have twice as many intestinal nuclei as *pop-1(RNAi)* embryos alone. (E) Laser-mediated cell ablation of the E blastomere in *cdc-25.1(gf)* animals results in embryos arrested without any intestine as seen by the absence of *elt-2::GFP* expression. The embryo to the right is an unablated *cdc-25.1(gf)* mutant embryo, allowed to develop to late embryogenesis.

phosphatases at different points in the cell cycle. Cdc25A plays a role at the G1/S transition, whereas, Cdc25B and Cdc25C promote the G2/M transition (Nilsson and Hoffmann, 2000). To determine whether *cdc-25.1* acts at G_1/S or G_2/M, we ectopically expressed mutant or wild-type *cdc-25.1* under the control of the heat-shock promoter in adult worms carrying the *rnr::GFP* or *cye-1::GFP* reporter constructs. Both *rnr::GFP* and *cye-1::GFP* are expressed strongly in cells which are entering S phase (Hong et al., 1998) (M. Krause, personal communication). In these animals, we assayed the reporter gene expression in order to see whether *cdc-25.1* was able to induce S-phase entry in cells that should have normally ceased division. Overexpression of mutant or wild-type *cdc-25.1* caused adult intestinal cells to enter S phase, but did not cause any apparent lineage or morphological abnormalities in other tissues when animals were heat-shocked during larval or adult stages. Heat-shock alone had no effect on reporter expression (Fig. 6). We conclude that *cdc-25.1* can induce S phase in intestinal cells and thus acts as a positive regulator of the G_1/S transition. No divisions were observed in these cells.

CDC-25.1(gf) perdures longer than the wild-type CDC-25.1 protein

To test whether there are any differences in localization of the CDC-25.1 wild-type or gain-of-function protein, which could provide insight into the mutant phenotype, we performed anti-CDC-25.1 antibody staining in wild-type or *cdc-25.1(gf)* embryos (a kind gift from Andy Golden and Neville Ashcroft). The wild-type CDC-25.1 protein product localized to oocytes, cortical membranes and ubiquitously in all nuclei of embryos up to the 28-cell stage (2E), as previously described (Ashcroft et al., 1999). After the 28-cell stage, there is no detectable staining in wild-type animals, as can be observed in Fig. 7A. In *cdc-25.1(gf)* embryos antibody staining was identical to wild type up to the 28-cell embryonic stage. After this point, we were able to detect nuclear CDC-25.1 staining up until the 100-cell stage (Fig. 7B,C). This suggests that the CDC-25.1(gf) protein perdures abnormally and may not be properly degraded in *cdc-25.1(gf)* mutants.

DISCUSSION

Identification and characterization of a novel gain-of-function mutation of the *C. elegans cdc-25.1* cell cycle phosphatase

We have identified and characterized a mutant (*rr31*) that has increased numbers of intestinal cells, similar to *cki-1(RNAi)*

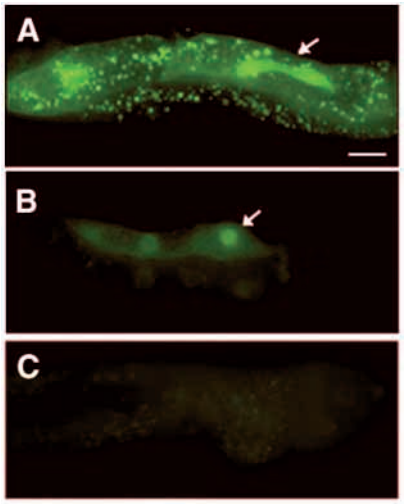

Fig. 6. Heat-shock ectopic expression of *cdc-25.1* causes entry into S phase in the intestinal cells. Posterior intestinal cells of adult hermaphrodites expressing the S-phase reporter (A) *cye-1::GFP* or (B) *rnr::GFP* after heat shock-induced expression of mutant *cdc-25.1*. (C) Posterior intestinal cells of adult hermaphrodites after heat shock. In C, animals harbor the S-phase reporter transgene *cye-1::GFP* and the empty heat-shock vector. Arrows indicate intestinal nuclei expressing the S-phase reporters. Scale bar: 10 μm. Anterior is leftwards.

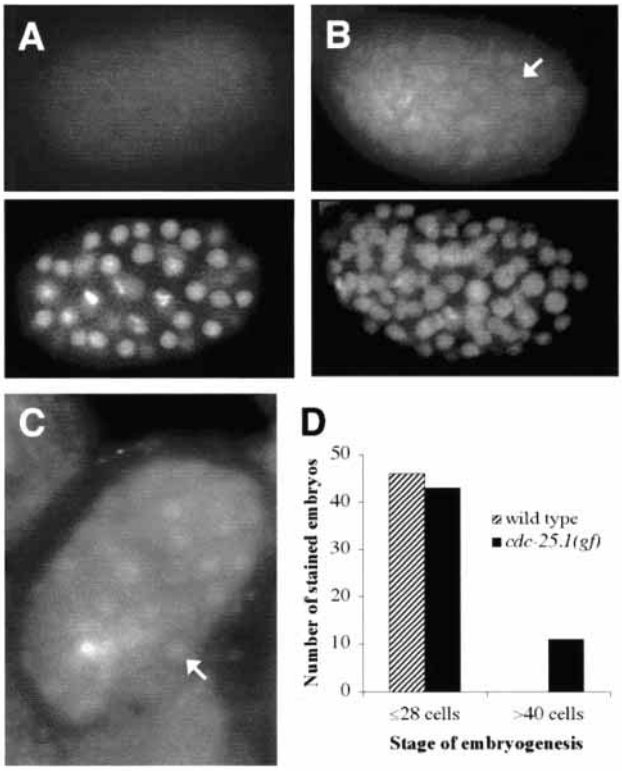

Fig. 7. The CDC-25.1(gf) protein perdures after the 28-cell stage. (A) Wild-type embryo at the 64-cell stage stained with anti-CDC-25.1 antibody (top) or DAPI (bottom), showing no apparent CDC-25.1 staining. (B) *cdc-25.1(gf)* embryo at the 100-cell stage stained with anti-CDC-25.1 antibody (top) or DAPI (bottom). (C) Enlarged *cdc-25.1(gf)* embryo stained with anti-CDC-25.1 showing nuclear staining as indicated by arrows (B,C). (D) The proportion of wild-type (hatched bar) or *cdc-25.1(gf)* (black bar) embryos that stain with anti-CDC-25.1 antibody up to the 28-cell stage, and after the 40 cell stage. *n*=48 and *n*=54 for wild type and *cdc-25.1(gf)*, respectively.

animals. We mapped the mutation and through three independent methods (phenocopy, RNAi, sequence analysis) we demonstrated that the mutation that causes this defect occurs in *cdc-25.1*. From genetic analysis, we conclude that *rr31* is a novel maternal effect, dominant, gain-of-function allele of this gene. The Cdc25 phosphatases are important regulators of the cell cycle and act as potential oncogenes that act downstream of the Ras and Myc oncogenes, particularly because of their role in activating Cdks (Galaktionov et al., 1995a; Galaktionov et al., 1995b; Galaktionov et al., 1996). In addition, Cdc25 phosphatases are principal players in the DNA damage and DNA replication checkpoints (Lopez-Girona et al., 1999; Mailand et al., 2000; Falck et al., 2001).

The *C. elegans* homolog of Cdc25A, *cdc-25.1*, belongs to a family of four *cdc25* homologs in *C. elegans*, and plays an important role in the proper progression of meiosis prior to embryogenesis (Ashcroft et al., 1998; Ashcroft et al., 1999). Both mice and humans have three homologues Cdc25A, Cdc25B and Cdc25C, each of which show different spatial and temporal expression patterns (Wu and Wolgemuth, 1995; Hernandez et al., 2000; Hernandez et al., 2001). This may also be true for the *cdc25* genes in *C. elegans*, suggesting a tissue-specific function for each of these cell cycle regulators (Ashcroft et al., 1998; Ashcroft et al., 1999).

The *cdc-25.1(gf)* mutation seems to cause cell division defects uniquely in the intestinal cell lineage, without an apparent effect on any other cell types examined, unlike *cki-1(RNAi)* animals, which display a diverse array of postembryonic cell division defects (Hong et al., 1998). Because *cdc-25.1(gf)* and *cki-1(RNAi)* display their respective defects at different stages of embryogenesis, we believe that they do not function in the same pathway.

CDC-25.1 is a maternally provided protein and its proper regulation may be important for the correct number of intestinal cell divisions

The *cdc-25.1(gf)* allele segregates in a manner consistent with it being a maternal-effect, dominant mutation. As previously mentioned, the CDC-25.1 protein product is localized to all nuclei of embryos up to the 28-cell stage (Ashcroft et al., 1999). The finding that the CDC-25.1(gf) protein is present in nuclei of *cdc-25.1(gf)* embryos at later stages of development than in wild-type embryos, suggests that the mutant protein is able to perdure for a longer time. This would explain how the extra intestinal cell defect in *cdc-25.1(gf)* can occur much later in embryogenesis than when the wild-type protein is normally expressed. It is therefore possible that the point mutation in CDC-25.1 affects the stability of the protein.

Our genetic data supports the hypothesis that the gain-of-function mutation in *cdc-25.1* probably does not give rise to a dominant negative product by antagonizing wild-type CDC-25.1 function. The highly conserved catalytic region of CDC-25.1 is located at the C terminus, whereas the less-conserved N-terminal domain plays a regulatory function, although little is known about how it imparts such control (Fauman et al., 1998). It has been shown that the phosphatase activity of the

CDC25 family of proteins is regulated by extensive phosphorylation in this domain of the protein (Strausfeld et al., 1994; Hoffmann et al., 1994; Kumagai and Dunphy, 1992). The G47D substitution in the N-terminal region could therefore confer a more favorable site for phosphorylation on surrounding residues in the region of the mutation. Alternatively, the G47D substitution might itself mimic or impede a regulatory phosphorylation event that normally occurs on residues in this vicinity, through the increased charge that is due to the novel acidic residue. Therefore, the gain-of-function phosphatase could potentially escape normal negative controls permitting it to perdure, thereby conferring an extended period of activity to dephosphorylate typical or atypical substrates (such as a different Cdks), to promote the extra round of embryonic cell division.

The analysis of the interaction with the G_1/S-positive cell cycle regulator cyclin E, *cye-1* supports these possibilities. CDK2 is normally inactivated by phosphorylation on highly conserved threonine and tyrosine residues (Gu et al., 1992). At the G_1/S transition, the Cdc25A phosphatase dephosphorylates these conserved residues, thus activating CDK2. Cdc25A can also act as a target of the CDK2/Cyclin E complex at the G_1/S transition, creating a positive autoregulatory feedback loop (Hoffmann et al., 1994; Blomberg and Hoffmann, 1999). The reduction of *cye-1* activity in *cdc-25.1(gf)* mutants suppressed the extra intestinal cell phenotype, suggesting that in *cdc-25.1(gf)* mutants, *cye-1* is required for the extra cell division in the intestinal lineage and that *cdc-25.1(gf)* could act through positive regulators of the G_1/S transition.

Ectopic expression of Cdc25A accelerates the G_1/S transition and prematurely activates Cdk2 (Blomberg and Hoffmann, 1999). Consistent with this function, we have shown using the S-phase-specific reporters *rnr::GFP* and *cye-1::GFP*, that when overexpressed in adults, *C. elegans cdc-25.1* is capable of inducing S-phase entry in intestinal cells, and therefore resembles the Cdc25A family of phosphatases. Extra intestinal (or other) cell divisions (mitoses) were not observed after overexpression of CDC-25.1, despite S-phase entry, suggesting that these cells are G_2/M blocked by the limited activity of positive regulators, such as CDK1, B-type cyclins or Cdc25 phosphatases (reviewed by Nigg, 2001).

Why is the E lineage uniquely affected in *cdc-25.1(gf)* mutants?

Why the mutant CDC-25.1 protein is capable of causing additional cell divisions in the intestinal cell lineage, despite the fact that it should indiscriminately dephosphorylate and activate CDK2 in all cells of the embryo is still unclear. What makes endodermal cells competent to respond to this gain-of-function phosphatase, or what negative cell cycle regulator is not expressed specifically in the intestine? These are major questions that may be answered through genetic modifier screens that are currently under way in our laboratory.

Noteworthy of mention, the expression of the *wee-1.1* kinase, which inhibits the activity of the G_2/M cyclin-dependent kinase CDK1, is specifically restricted to the E blastomere and AB progeny early in the embryo, and its expression is downregulated after the first division of E (Lundgren et al., 1991; Wilson et al., 1999). However, the removal of *wee-1.1* kinase activity through RNAi does not result in aberrant divisions of the endodermal cells, probably due to redundancy, leaving its E-specific expression and function unclear (Wilson et al., 1999) (I. K., unpublished).

It does appear, however, that E specification through Wnt signaling makes cells susceptible to the *cdc-25.1(gf)* mutation, although at present we cannot discern whether this is a direct or indirect effect. It has been shown that in other systems Wnt does affect cell division through effects on Cdc25 (Johnston and Edgar, 1998; Rimerman et al., 2000).

We suggest that the early embryo contains a pool of maternally supplied CyclinE/CDK2 that is non-limiting for most of the early divisions; however, much of it may be inactive because of inhibitory phosphorylations on CDK2. In the *cdc-25.1(gf)* mutant, the continued presence of the mutant protein might render a small portion of this maternal Cyclin E/Cdk2 pool active at a specific window during the formation of the intestine, thereby causing an extra round of cell division. For example, such a window might reflect a maternal to zygotic transition for a negative Cdk regulator (such as *wee-1*). The divisions of other cell types, as well as further divisions of the E lineage might be dependent on zygotic expression of positive regulators, which could later become controlled by *cki-1*. This would explain why the early divisions of the E lineage are unaffected by the loss of *cki-1* activity, while the later divisions are.

The proper control of E lineage divisions might be especially important as the cell division of endodermal precursors are blocked by the onset of morphogenetic movements typical of gastrulation, which begins at the 28-cell stage. In *Drosophila*, CDC25/String proteolysis has been shown to be important for the proper coordination of gastrulation and ingression of the mesoderm anlage (Mata et al., 2000; Grosshans and Wieschaus, 2000). A similar mechanism might be acting in the coordination of *C. elegans* endodermal divisions, whereby correct division timing, with specification and function, is essential for gastrulation and ensuing embryogenesis.

Unlike the early embryonic cell cycles in *Drosophila*, which are synchronous, the *C. elegans* early blastomeres demonstrate distinct and invariant cell division timing. These divisions are coordinated by maternally supplied factors, and zygotic transcription is not required for cell cycling until the 100 cell stage (Powell-Coffman et al., 1996; Edgar et al., 1994c). Little is known about these maternally controlled early embryonic cell divisions, nor have the important regulators that drive these divisions been identified, but our work stresses the importance of the proper control of these regulators to ensure the correct execution of cell divisions characteristic of each lineage.

The important finding that a mutation in a general cell cycle regulator can cause overproliferation in a specific tissue is not unique. The intestine in *C. elegans* and in other organisms seems very sensitive to changes in cell cycle regulators and their upstream regulators (Boxem et al., 2001; Smits et al., 1999). Understanding what sensitizes tissues to changes in cell cycle regulators will help us gain insight into how different cell types alter their cell cycle programs independently to impart increased tissue diversity and corresponding developmental potential.

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