Regulation of embryonic and postembryonic cell divisions in *Caenorhabditis elegans*

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Abstract

The formation of a complex multicellular organism from the one cell stage to the reproductive adult is a result of the precise sequencing of cell division, morphogenesis, cell fate specification and differentiation events in distinct cell types during development. To understand the molecular basis of developmental control of cell division during *C. elegans* organogenesis, two different approaches were taken. First, a screen was performed to identify mutants with altered numbers of intestinal nuclei using a reporter transgene specific to the intestinal nuclei. The intestine displays three different cell division patterns; mitosis, karyokinesis and endoreplication, therefore, in this screen we could potentially isolate mutants in genes affecting any of these different cell cycles. An F2 semi-clonal screen was performed and mutants with fewer or supernumerary numbers of intestinal nuclei were isolated. One mutant, *rr31,* with twice the wild type complement of intestinal nuclei was mapped and the defect was subsequently shown to be due to a gain-of-function mutation in the cell cycle phosphatase *cdc-25.1.* Further characterization of the *cdc-25.1(gf)* mutant, showed that the extra intestinal cells arise from an additional division of the intestinal cell precursors during embryogenesis, and that this phenotype is unique to the intestinal lineage. The mutant CDC-25.1 protein was shown to perdure for a longer time during embryogenesis in all nuclei, as compared to the wild type protein.

The importance of the proper coordination of cell cycle during organogenesis was further investigated by characterizing the relationship between the timing of asymmetric cell divisions and appropriate cell fate specification in the somatic gonadal lineage. The removal of the p27 CIP/KIP homologue, *cki-1,* using RNA interference resulted in the formation of extra somatic gonadal cells, including the distal tip cells and anchor cells, which were shown to arise from precursors of other somatic gonadal cell types. These results suggest that *cki-1* acts not only to restrict the number of cells in a specific lineage, but also to link the proper timing of asymmetric cell divisions and the adoption of distinct cell fates in the resulting daughter cells.

The correct regulation of a positive cell cycle regulator, *cdc-25.1* during early embryogenesis, as well as the timely activity of the negative cell cycle regulator, *cki-1,* during the formation of the somatic gonad, underscore the importance of negative regulation of cell division during development for the proper formation and/or function of tissues and organs in *C. elegans.*

Résumé

La formation de tout organisme pluricellulaire - d'une cellule unique à l'adulte fertile- est le résultat d'une succession précise d'événements coordonnés durant le développement. Ceux-ci comprennent la division cellulaire, la morphogenèse ainsi que la spécification et la différenciation cellulaire dans les différents types de cellules composant un organisme. Pour comprendre les fondements moléculaires des mécanismes développementaux régulant le cycle cellulaire durant l'organogenèse du nématode *C. elegans*, deux approches ont été utilisées. La première fut d'effectuer un criblage sur une souche de vers exprimant un transgène GFP spécifique aux noyaux des cellules intestinales dans le but d'isoler des mutants ayant des variations dans le nombre de ces noyaux, comparé à la souche sauvage. Trois types caractéristiques de cycles cellulaires ont lieu dans les cellules intestinales : on y trouve la mitose, la caryocinèse et l'endoréplication. Avec cette approche, il est donc potentiellement possible d'isoler des mutations dans des gènes affectant n'importe lequel de ces cycles cellulaires. Un criblage semi-clonal en F2 a permis d'isoler des mutants ayant un nombre inférieur ou supérieur de noyaux intestinaux. *rr31*, un mutant possédant deux fois plus de noyaux intestinaux que le type sauvage a été découvert. Le gène défectueux a été cartographié. La lésion responsable de ce phénotype est due à une mutation "gain-of-function" située dans le gène *cdc-25.1*, une phosphatase activatrice du cycle cellulaire.

Dans une deuxième approche, la caractérisation détaillée du mutant *cdc-25.1(gf)* permit de démontrer que les cellules intestinales supplémentaires provenaient d'une division additionnelle ayant lieu dans les cellules précurseurs de l'intestin durant l'embryogenèse. De plus, ce phénotype est spécifique à la lignée intestinale. Lorsque l'on compare la protéine mutante CDC-25.1 à la protéine naturelle, on remarque qu'elle subsiste plus longtemps dans tous les noyaux durant l'embryogenèse. L'importance de la bonne coordination du cycle cellulaire durant l'organogenèse a été examinée en caractérisant, dans la lignée somatique des gonades, le timing entre le moment des divisions asymétriques et la spécification appropriée de ces cellules. La suppression par ARN interférence de *cki-1*, la

protéine homologue à p27 CIP/KIP, provoque l'augmentation du nombre de cellules somatiques dans les gonades, incluant les cellules distales et les "anchor cells" qui proviennent elles-mêmes d'autres cellules précurseurs de cette lignée. Ces résultats suggèrent que *cki-1* agit d'une part pour restreindre le nombre de cellules dans une lignée particulière, mais établit également le bon timing entre les divisions asymétriques et la bonne spécification des cellules-filles qui en résultent.

La régulation appropriée de *cdc-25.1*, un régulateur positif de la division cellulaire durant l'embryogenèse, ainsi que l'activation correcte de *cki-1*, un régulateur négatif de la division cellulaire durant la formation de la partie somatique des gonades, soulignent l'importance de la régulation négative du cycle cellulaire durant le développement en ce qui concerne l'établissement exact et/ou fonctionnel des tissus et organes chez *C. elegans.*

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Chapter I

Literature Review

General Introduction

The regulation of cell cycle, morphogenesis and differentiation are essential for the proper growth and patterning of metazoan organisms. Cell division timing must be particularly tightly controlled during early embryogenesis for the formation and specification of blastomeres. Once the fates of these cells are specified, they are capable of undergoing lineage-specific patterns of cell division, and quiescence, coordinated with morphogenetic movements and differentiation events to form the reproductive adult.

Developmental regulators are the spatial and temporal links between the developmental program and cell cycle effectors. Understanding why cells of a certain lineage divide, differentiate or die at a certain time during development and how these events relate to cell cycle timing is the major goal of my research.

Main Events in the Cell Cycle:

The eukaryotic cell cycle is divided into four phases: G1 (first gap) phase, S (DNA synthesis) phase, G2 (second gap) phase and M (mitotic) phase (Murray and Hunt, 1993). The phases between mitoses are known as interphase. Non-dividing cells that have exited the cell cycle arrest in the G0 state. The events that occur during cell division are common to most cells albeit some modifications exist, but essentially daughter cells must receive an integral copy of the genome and this must be transmitted with high fidelity.

The G1 phase is a stage where the cell integrates extracellular mitogenic signals that determine whether it will progress through the cell cycle. The G1/S transition, or restriction point, as it is called in mammalian cells, is the crucial step of cell cycle entry, and once this point is bypassed, cells are committed to complete mitosis (Pardee, 1989; Sherr, 1996). In addition, cells accumulate mass during this time, and synthesize cellular proteins and RNA required for S phase. During S phase, the chromosomes are replicated, and after the replication of chromosomes, the two sister chromatids remain attached to each

other while the spindle is established (Murray and Hunt, 1993). The second gap phase is a preparative phase for mitosis, or M phase, where the sister chromatids separate into two daughter cells through a complex sequence of events (Nasmyth et al., 2000). All the steps of the cell cycle are extensively regulated ensuring the formation of two daughter cells with a full genome complement. In addition to these processes, the cell continuously grows during the cell cycle. This growth includes the synthesis of ribosomes, mitochondria, the endoplasmic reticula, cellular proteins and membranes (Murray and Hunt, 1993).

In many cells, the major point of control of the cell cycle is maintained through extracellular inputs such as diverse signaling pathways mediated by small molecules and growth factors. This culminates in the G1/S transition, and when this transition is bypassed, the cell becomes largely unresponsive to extracellular inputs and completes mitosis (Pardee, 1989).

The G1/S Transition: The Commitment to progress through the Cell Cycle

The expanding picture we have today of the basic components of the cell cycle machinery and the mechanisms that regulate different aspects of cell division, was pieced together through extensive studies in yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe,* higher eukaryotes such as sea urchins, *Xenopus laevis, Drosophila melanogaster,* and *Caenorhabditis elegans,* as well as using mammalian cell culture experiments. Recent directions are leading towards understanding the function of cell cycle regulators in the context of developing organisms, tying in the basic cell cycle machinery with a network of developmental regulators (Nurse, 2000). The link between pathways, which act as environmental cues to control cell cycle and the execution of cell division, lies predominantly at the G1/S transition for most cells in most organisms, and the activities of cyclin-dependent protein kinases, and their partners, cyclins.

START In Budding Yeast

In *Saccharomyces cerevisiae,* the G1/S transition, or START, is the major point in the cell cycle where the cell depends on environmental inputs such as signals from other cells or the availability of nutrients to continue cell division (Murray and Hunt, 1993). A single cyclin-dependent kinase, Cdc28, directs different cell cycle transitions by assembling into active complexes with G1, S or mitotic cyclins (Mendenhall and Hodge, 1998; Rudner et al., 2000). When nutrients are available, cells in the G1 phase grow to reach a critical size, which is followed by budding, the duplication of spindle bodies and the initiation of DNA replication. This is mediated by the association of Cdc28 with G1 cyclins: Cln1, Cln2 and Cln3. The overexpression of Cdc28 has little effect on the cell cycle, which is normally not limiting, unlike the overexpression of rate-limiting G1 cyclins, which results in a shortening of the G1 phase and a decrease in cell size (Richardson et al., 1989; Mendenhall and Hodge, 1998).

Cln1 and Cln2 encode highly unstable proteins, which reach the highest level of expression in late G1 phase (Wittenberg et al., 1990). Cln3 is the first cyclin to act in the order of events that promote the G1/S transition (Tyers et al., 1992). In a complex with Cdc28, it has a role in increasing the transcription of Cln1 and Cln2, which subsequently acts to trigger S phase events (Stuart and Wittenberg, 1995). The transcription of Cln1 and Cln2 is controlled by a heterodimeric transcription factor, SBF, which is composed of the Swi4 and Swi6 proteins. SBF activity is, in turn, regulated by a positive feedback loop of phosphorylation by Cln/Cdc28 complexes (Nasmyth and Dirick, 1991; Dirick and Nasmyth, 1991; Cross and Tinkelenberg, 1991).

The subsequent progression of S phase is dependent on a complex that consists of Cdc28 and the cyclins Clb5 and Clb6, which directly activate DNA replication (Schwob and Nasmyth, 1993). Clb5 and Clb6 complexes with Cdc28 are present from the G1 phase, however, their activities are inhibited by the cyclin-dependent kinase inhibitor, Sic1, which prevents premature entry into S phase (Sherr, 1994; Schwob et al, 1994). At the G1/S transition, Sic1 is phosphorylated at multiple sites by the Cln1/Cln2/Cln3/Cdc28 complexes and targeted for degradation by the cellular protein-degrading machinery through the activity of SCF-like E3 complexes (Schwob et al, 1994; Nash et al., 2001). Once Sic1 is degraded, the Clb5/Clb6/Cdc28 complexes are no longer blocked.

In addition to a budding cell division program, haploid yeast cells can arrest in G1 and mate by cell and nuclear fusion to form diploids. This decision is induced by pheromones, which initiate a mating factor signal transduction pathway through a seven transmembrane receptor and a heterotrimeric G protein to activate a MAP kinase cascade (Leberer et al., 1997). One of the downstream targets of the MAP kinases is Far1, an inhibitory protein required for cell cycle arrest through the inhibition of the kinase activities of Cln/Cdc28 complexes (Chang and Herskowitz, 1990). Once cells pass START, they become unresponsive to environmental signals such as pheromone- and nutrient control- mediated arrest of cell division.

The G1/S transition in multicellular organisms

The regulation of START in yeast, and the regulation of the G1/S transition in higher eukaryotes show a remarkable degree of conservation (Pardee, 1989). As in yeast, the G1/S transition in higher eukaryotes is carried out by the activities of cyclin-dependent kinases (Cdks) in a complex with stage-specific cyclins: cyclin D, E and A, and their activities are regulated by activating and inhibitory phosphorylations and binding of cyclin dependent kinase inhibitors (Sherr, 1994).

G1 Cyclins and Cyclin-dependent Kinases

The effect of extracellular inputs is mediated through the expression of D-type cyclins, which act as growth factor sensors and form active complexes with Cdk4 and Cdk6 early in the G1 stage (Sherr, 1994). In a response to mitogens, the D-type cyclins are synthesized, while when mitogens are withdrawn, they are rapidly degraded (Sherr, 1994). The transcription of D-type cyclins has been shown to be induced by transcription factors such as c-Myc, AP-1 and NF-κB (Perez-Roger et al., 1999; Bouchard et al., 1999; Herber et al., 1994; Guttridge et al., 1999). The formation of the cyclin D/Cdk4/6 complexes is also dependent on stimulation by mitogens. The synthesis of Cdk4 has also been shown to be downregulated by the presence of transforming growth factor beta, a growth factor that blocks cell division in many tissue culture models (Ewen et al., 1993). In mammalian cells, three D-type cyclins are expressed in a cell type-specific manner, and their overexpression can decrease the length of time cells spent in G1 phase *in vitro* (Baldin et al., 1993). Interestingly, increased expression of D-type cyclins has been observed in human and mouse cancers (Sherr, 1996.)

One of the main functions of the cyclin D/Cdk4/6 complex is the phosphorylation of the retinoblastoma tumor suppressor, Rb (Kato et al., 1993). In its hypophosphorylated form, Rb, and similar proteins such as p130 and p107, are able to bind a group of transcription factors, E2Fs (Weintraub et al., 1992).

When Rb is phosphorylated, some of these transcription factors are converted to activators of expression of genes required for S phase entry, such as cyclin E, p107, cyclin A, cdc2, b-myb and dihydrofolate reductase (DHFR) (Sherr, 1996). In addition, Rb is associated with histone deacetylase (HDAC) and SWI/SNF in a complex that blocks transcription of cyclin E and cyclin A during the G1 phase (Zhang et al., 2000). When Rb is phosphorylated by cyclin D/Cdk4/6, HDAC is released from this complex, and the transcription of cyclin E is initiated. The further phosphorylation of Rb by the newly formed cyclin E/Cdk2 complex allows the transcription of cyclin A which in association with Cdk2 has a role in S phase progression (Zhang et al., 2000). Cyclin D/Cdk4/6 complexes also promote the G1/S phase transition by binding and sequestering the p27 cyclin-dependent kinase inhibitor, which is responsible for blocking cyclin E/Cdk2 activity (Perez-Roger et al., 1999; Bouchard et al., 1999). The activity of cyclin D/Cdk4/6 complexes thus results in the timely activation of cyclin E/Cdk2 to the levels required for S phase entry.

Studies in mice, *Drosophila* and *Caenorhabditis elegans,* have shown that cyclin D/Cdk4/6 complexes in these organisms might have a more prominent function in driving cell growth (Rane et al., 1999; Robles et al., 1996; Park and Krause, 1999; Boxem and van den Heuvel, 2000). Mice which lack the function of CycD1 or Cdk4 are smaller in size than their wild type counterparts, yet viable, while when overexpressed in mice, CycD1 causes hyperplasia in multiple tissues and an increased body size (Rane et al., 1999; Robles et al., 1996). In *C. elegans* and *Drosophila,* there is a single cyclin D and Cdk4 present, which also seem to display somewhat different roles than expected. As in mice, both *Drosophila* and *C. elegans* Cdk4 mutants have a small body size, and have slower growth rates, respectively (Meyer et al., 2000; Boxem and van den Heuvel, 2000). However, neither the *C. elegans* cyclin D (*cyd-1)* nor Cdk4 (*cdk-4)* homologues are required for the embryonic cell proliferation which involves cell division in the absence of growth, but are required for the cell divisions which occur postembryonically, which do require growth. When ectopically expressed, the *C. elegans cyd-1* and *cdk-4* are, however, capable of inducing S phase entry

as observed by the expression of the *mr::GFP* reporter transgene (Park and Krause., 1999). The *Drosophila* Cdk4 mutants, as previously mentioned, do display a smaller body size due to a defect in the growth of the cells and organism, but are able to progress through cell division, while the removal of cyclin D/Cdk4 activity has few effects on embryonic cell cycle progression (Meyer et al., 2000; Meyer et al., 2002). Although there seems to be only a single Cdk4 homologue in flies, it is not an essential gene and the overexpression of cyclin D/Cdk4 complexes has little effect on G1 arrest, or the levels of cyclin E (Meyer et al., 2002).

Cyclin D/Cdk4/6 complexes appear to play a role in activating the G1/S transition through the activation of cyclin E/Cdk2 at least in some cell types. Interestingly, the human cyclin E can substitute for the loss of cyclin D1 function in mice, strongly suggesting that the major role of cyclin D in the system is the activation of cyclin E expression (Geng et al., 1999). The expression of cyclin E peaks at the G1/S transition, and the activation of the cyclin E/Cdk2 complex creates a positive feedback loop of Rb phosphorylation and activation of S phase genes (Sherr, 1994). In multicellular organisms, cyclin E is the rate-limiting factor of G1/S progression, as it is rapidly turned over by the ubiquitin-mediated proteolytic machinery (Clurman et al, 1996, Won and Reed, 1996). High levels of cyclin E/Cdk2 complexes accelerate G1 progression and can initiate DNA replication even in fibroblasts which lack mitogenic factors (Leone et al., 1999; Connell-Crowley et al, 1998).

During *Drosophila* embryogenesis, a majority of epidermal cells stop proliferating after mitosis 16 following a series of synchronous divisions regulated by non-limiting maternal stores of positive regulators (Edgar and O'Farrell., 1989). Cells then enter G1 arrest due to the elimination of cyclin E/Cdk2 activity (Knoblich et al., 1994). When cyclin E is overexpressed, these cells are unable to maintain G1 arrest, demonstrating its role in promoting the G1/S phase transition. The *C. elegans* cyclin E homogoue, *cye-1* also has a role in promoting S phase entry (Fay and Han, 2000). RNA interference of *cye-1* results in

embryonic cell cycle arrest, at the 100-cell stage, demonstrating its requirement for embryonic cell proliferation (Fay and Han, 2000). When maternal stores of *cye-1* are intact, as in *cye-1* mutant animals derived from heterozygous mothers, zygotic *cye-1* promotes cell division during postembryonic development, and the vulval precursor cells in *cye-1* mutants spend a prolonged length of time in the G1 phase of the cell cycle (Fay and Han, 2000).

Regulation of Cdks by Phosphorylation

The phosphorylation state of cyclin-dependent kinases has been shown to correlate with their catalytic activity, and the role of kinases and phosphatases which act on G2/M Cdks has been extensively characterized in yeast (Murray and Hunt, 1993). Full Cdk activity is achieved by cyclin binding and phosphorylation of a threonine residue adjacent to the active site of the kinase by a Cdk-activating kinase (CAK) (Solomon et al, 1992). In higher eukaryotes, Cdks are phosphorylated by CAK only after the binding of cyclins, which are the rate-limiting subunits of these kinases, thus achieving a highly regulated order of events necessary to drive cell division (Kaldis, 1999).

Two inhibitory phosphorylations also play an important role in the regulation of Cdk activity. Phosphorylation on the conserved tyrosine 15 (Y15) residue that lies in the active site is found on most Cdks, while Cdks of higher eukaryotes are also phosphorylated on an adjacent threonine 14 (T14) residue (Gu et al., 1992; Berry and Gould, 1996). Phosphorylation of these residues blocks Cdk activity, and the kinase responsible for Y15 phosphorylation is Wee1p, which has homologues in all eukaryotes (Lee and Yang, 2001). A second protein kinase, Myt1, is capable of phosphorylating both T14 and Y15 residues (Fattaey and Booher, 1997). The removal of these inhibitory phosphates is achieved by the Cdc25 family of dual-specificity phosphatases, thus reactivating the Cdk, and both Wee1 and Cdc25 are responsive to factors such as cell growth and DNA damage (Nilsson and Hoffmann, 2000; Mailand, et al., 2000). While the fission yeast *Schizosaccharomyces pombe,* has only one member of the Cdc25 phosphatases, which removes the inhibitory phosphate

groups from Cdc2 (Cdc28) at the G1/S and G2/M transitions, humans and mice have three members of this family, which act at different cell cycle stages (Nilsson and Hoffmann, 2000). In *Drosophila,* there are two members of the Cdc25 family of phosphatases, *string* and *twine*, which have roles in promoting mitotic and meiotic cell cycles, respectively (Courtot et al., 1992; Alphey et al., 1992; Edgar and O'Farrell, 1989). The *C. elegans* Cdc25 family has four members, which appear to have distinct roles during development (Ashcroft et al., 1998; Ashcroft et al., 1999).

Negative Regulation of Cdks: Cyclin-dependent Kinase Inhibitors

In addition to the complex regulatory mechanisms in which different positive and negative components of the cell cycle machinery are expressed at different times, as well as the stage-specific proteolysis of cyclins and positive and negative phosphorylation events, the G1/S cyclin-dependent kinases are also regulated by binding of cyclin-dependent kinase inhibitors (Ckis) (Sherr and Roberts, 1999). There are two families of the G1/S cyclin-dependent kinase inhibitors: the INK4 family, and the Cip/Kip family of Ckis. The members of the INK4 family of Ckis include p15, p16, p18 and p19, which specifically bind to Cdk4 and Cdk6 (Sherr and Roberts, 1999). The binding of these Ckis to their target kinases cause a conformational change which renders the kinase inactive and unable to bind its cyclin partner (Russo et al., 1998., Brotherton et al., 1998.) The other negative regulators of the G1/S transition include the Cip/Kip family of Ckis: p21CIP1, p27KIP1, and p57KIP2 family of cyclin-dependent kinase inhibitors (Sherr and Roberts, 1999). These Ckis have a conserved amino-terminal Cdk-inhibitory domain containing cyclin and Cdk binding sites and can inhibit both cyclin D/Cdk4/6 kinases and cyclin E/A/Cdk2 kinases (Sherr and Roberts, 1999; Russo et al., 1996). Unlike the INK4 family, the Cip/Kip cyclin-dependent kinase inhibitors bind to Cdk/cyclin complexes, and block the active site of the Cdks (Russo et al.,1996).

The levels of Ckis are essential in determining whether a cell divides or enters quiescence in response to developmental signals or genotoxic stress. The

members of the mammalian Cip/Kip family are upregulated during cell differentiation, suggesting a role in cell cycle exit, whereas their overexpression in cultured cells causes a G1 phase arrest (Halevy et al., 1995; Parker et al., 1995; Toyoshima and Hunter, 1994). Mice nullizygous for the p27KIP1 gene display increased numbers of cells in multiple organs, likely due to a cell-autonomous failure to exit cell division, however, the differentiation program in these cells is unaffected (Casaccia-Bonnefil et al., 1999; Kiyokawa et al., 1996). p27KIP1 is rapidly degraded via the ubiquitin/proteasome pathway at the G1/S transition and the SCF ubiquitin ligase specifically ubiquitinates p27 that has been phosphorylated by cyclin E/Cdk2 (Carrano et al., 1999; Montagnoli et al., 1999).

Drosophila has one Cip/Kip-like cyclin-dependent kinase inhibitor, *dacapo,* which can bind and inhibit the activity of cyclin E/Cdk2 complexes at the G1/S transition (de Nooij et al., 1996; Lane et al., 1996). The expression of *dacapo* has been observed in cells that are exiting cell division during embryogenesis as well as larval development. Consistent with the expression pattern, the loss of function of *dacapo* causes an extra round of division in the embryonic epidermis, and aberrant mitotic divisions are observed in optic lobes of third instar larvae, while the ectopic expression of *dacapo* causes inappropriate G1 arrest (de Nooij et al., 1996; Lane et al., 1996; Wallace et al., 2000).

The *C. elegans* cyclin-dependent kinase inhibitor, *cki-1,* has been similarly shown to act as a *bona fide* p27KIP1-like Cki (Hong et al., 1998). *cki-1* has been shown to have a role in promoting developmental G1 phase arrest of precursor blast cells during postembryonic development and the removal of *cki-1* through RNA-mediated interference causes ectopic cell divisions in multiple lineages. In addition, the overexpression of CKI-1 during embryonic development causes G1 arrest (Hong et al., 1998). Interestingly, *cki-1* expression has been shown to be regulated, at least in part, at the transcriptional level, and functions downstream of multiple developmental signals (discussed later).

The levels of CKI-1 protein in *C. elegans* are regulated by a member of the cullin family of ubiquitin ligases, *cul-2* (Feng et al., 1999). Consistent with its

function to promote the G1/S transition, CUL-2 is expressed in dividing cells, and when its function is removed, the levels of CKI-1 are increased, and there is consequently reduced proliferation in the germline. Additional components of the SCF complex regulating the G1/S transition have been identified in *C. elegans,* such as the cullin *cul-1* and the F-box protein *lin-23*, which are responsible for targeting proteins for degradation (Kipreos et al., 1996; Kipreos et al., 2000). In *cul-1* and *lin-23* mutants, multiple lineages undergo an additional round of cell division, probably due to the inability to degrade G1 cyclins.

Developmental control of cell division

In a developing organism, cell proliferation occurs in a precise spatial, stage- and sex-specific pattern regulated by a variety of developmental regulators. During development there are a variety of different cell cycles: early embryonic cell cycles which are often rapid and occur in the absence of cell growth, cell cycles which are regulated at the G1 or G2 stage of cell division by environmental, nutritional or developmental cues, asymmetric cell divisions, as well as endoreplicative cell cycles. Accordingly, the developmental inputs regulating these cell cycles show distinct temporal and spatial expression patterns. Studies in *Drosophila,* and more recently, *C. elegans,* have provided insight into how cell division is regulated by these inputs.

Cell cycle control during *Drosophila* development

During *Drosophila* embryogenesis, after a period of 13 rapid syncitial cell cycles without gap phases, which are driven by maternal stores of cell cycle regulators, the cell cycle in the cellular blastoderm is controlled by the transcription of the Cdc25 phosphatase, *string,* which regulates the embryonic cell cycles 14-16 (Edgar et al., 1994a; Edgar et al., 1994b). At this point, *string* is the rate-limiting factor for these divisions as the required Cdks 1 and 2 and cyclins E, A and B show constitutive expression, likely due to maternal stores

(Edgar and Lehner., 1996). The distinction between the cell cycle patterns of different lineages also lies on the timing of *string* expression in these cells (Foe 1989; Edgar et al., 2001). Interestingly, the *cis*-regulatory elements of *string* were shown to direct expression in a variety of different cell types at different times of development, suggesting that it is transcriptionally regulated (Lehman et al., 1999). After mitosis 16, the cells withdraw from the mitotic cycle and enter G1 arrest since cyclin E/Cdk2 stores become rate-limiting, and the expression of the CIP/KIP inhibitor *dacapo* is induced in the G2 phase of the final mitotic cycle (Knoblich et al 1994; de Nooij et al., 1996; Lane et al, 1996). *Dacapo* mutants show an inability to arrest in the G1 phase of the cell cycle following mitosis 16. Consistent with this, when cyclin E/Cdk2 activity is enhanced, cells are capable of undergoing an additional round of cell division.

The importance of the proper developmental regulation of the levels of G1 cyclins is further supported by studies in the developing *Drosophila* eye. Functional characterization of *roughex,* a gene which when mutated causes a rough eye phenotype due to the inability of cells to enter G1 phase, an arrest required for the proper patterning of the ommatidia, has revealed that the inactivation of cyclin A is essential for G1 arrest (Thomas et al., 1994; Gonczy et al., 1994).

During *Drosophila* larval development, growth occurs by endoreplication cycles where S phases occur without intervening cytokineses. The switch to endoreplication cycles is triggered by the loss of cyclin A and cyclin B activities, and the continued cyclic expression of cyclin E (Sauer et al., 1995; Follette et al., 1998).

Other cells in the developing fly, namely cells in the imaginal discs, which produce adult appendages, and cells of the CNS, or neuroblasts, undergo cell divisions that require growth, and can be regulated at both the G1 and G2 phases by a variety of developmental signals. The cells of the imaginal discs adopt different identities depending on their position along the anterior/posterior or dorsal/ventral axis (Brook et al., 1996). Secreted morphogens, such as Decapentaplegic (*dpp*) or Wingless (*wg*), which play a role in patterning different

cell types along these axes, also have been shown to have a role in the regulation of proliferation of these cells. The loss of function of *dpp* or *wg* causes a reduction in cell numbers in specific cells of the wing imaginal disc, while the ectopic expression of DPP, its activated receptor, or WG, causes overproliferation in many cells of the anterior/posterior, or dorsal/ventral boundary, respectively (Burke and Basler, 1996; Neumann and Cohen, 1996).

Interestingly, these factors play an opposite role later in development where both *wg* and *dpp* have been shown to block cell cycle progression. *Wg* acts at the wing margin in a region termed the zone of non-proliferating cells (ZNC), and the removal of its function by using a temperature-sensitive allele of *wg* prevents the arrest of cell division in these cells (Johnston and Edgar, 1996). This arrest is mediated by the *wg*-dependent induction of the transcription factors *achaete* and *scute* in neighbouring cells, which act directly to repress the transcription of *string,* causing a G2 arrest (Johnston and Edgar, 1996). Cells in the ZNC that do not express the proneural genes *achaete* and *scute* arrest in G1. *Dpp* has a similar role in halting cell cycle progression in the differentiating cells of the morphogenetic furrow of the developing eye (Horsfield et al., 1998). The cells within the morphogenetic furrow arrest in G1, an event requiring the function of the *roughex* gene, and the downregulation of cycin E and cyclin A (Thomas et al, 1994). Cells which lack the function of *dpp* receptors fail to undergo G1 arrest and divide due to the continued expression of cyclin E and cyclin B, while the overexpression of *dpp* can reduce the number of cells which are capable of entering S phase (Horsfield et al., 1998).

While the cells within the morphogenetic furrow arrest in G1, the cells that are located posterior to the furrow are capable of exiting G1 and synchronously entering S phase (Wolff and Ready, 1993). The overexpression of the *Drosophila* retinoblastoma homologue, *Rbf,* inhibits this second mitotic wave (Duman-Scheel et al., 2002; Xin et al., 2002). The ability of these cells to normally enter S phase, has been shown to be an effect of the signaling molecule, Hedgehog (*Hh*) (Duman-Scheel et al., 2002). Overexpression of downstream components of the *Hh* signaling pathway, the transcription factor, *Ci,*

in the cells within the morphogenetic furrow allows these cells to enter S phase through the induction of cyclin D and cyclin E expression.

Cell cycle control during *C. elegans* development

Unlike synchronous cell divisions that occur during early embryogenesis of some multicellular organisms, many of the early embryonic cell cycles in *C. elegans* are asymmetric. This results in the formation of embryonic founder cells, which adopt different fates and eventually follow different cell cycle programs. The inherent difference between these founder cells is mediated by the activity of *par* genes (for *par*titioning defective), which are responsible for the asymmetric segregation of maternally provided factors among the daughter cells (Bowerman, 1998). Once formed, the founder cells undergo cell divisions at different rates, possibly due to differences in the duration of S phase (Edgar and McGhee, 1988). Although the timing of cell division varies between different lineages during the first seven hours of embryogenesis, after this initial proliferative period, there is a general block in cell division, replaced by morphogenetic movements and differentiation of tissues in the worm (Sulston and Horvitz, 1977).

During postembryonic development, a period of growth coordinated with cell division occurs, where 55 of the 558 cells of the newly hatched larva must undergo a series of divisions to form the seam cells, a functional nervous system, somatic gonad, intestine, and vulva, while the germ cells also undergo continuous mitotic divisions (Sulston and Horvitz, 1977). The initial divisions that occur during the first larval stage (L1) are dependent on the presence of nutrients. In the absence of food, all cell division is blocked and a global developmental arrest occurs (Hong et al., 1998). This cell cycle arrest is mediated by the action of the cyclin-dependent kinase inhibitor *cki-1,* since the removal of *cki-1* function through RNA-mediated interference causes cells to enter S phase, as observed by the expression *rnr::GFP.* Similarly, the overexpression of *cyd-1* and *cdk-4* in the starved L1 animals can cause S phase entry in multiple cells (Park and Krause, 1999). One of the cell types that divides in the absence of food in *cki-1(RNAi)* animals are the germ cells, a phenotype

also observed in *nos-1;nos-2* double mutants, suggesting that *cki-1* might be regulated by these *nanos*-related proteins which are normally required for the development of the primordial germ cells (Subramaniam and Seydoux, 1999).

Another example of a global cell cycle arrest which occurs in *C. elegans* in response to environmental influences is the formation of dauer, which occurs in response to nutrient availability, temperature and the presence of dauer-inducing pheromone (overcrowding) (Riddle et al.,1997). The decision to enter dauer as a response to these stimuli is regulated by three different pathways, a TGF-β, insulin- and cGMP signaling pathway. As in starved L1 larvae, there is no expression of the *rnr::GFP* S phase marker during dauer, while a *cki-1::GFP* transcriptional fusion is expressed (Hong et al., 1998). The removal of *cki-1* function in a dauer-constitutive mutant, *daf-7,* encoding a TGF-β homologue, enables cells to enter S phase (Hong et al., 1998). This suggests that *cki-1* might be one of the downstream cell cycle effectors of pathways that lead to, or block dauer formation.

Similar to the global effect of the pathways controlling dauer formation on cell division, a series of genes has been identified which determine the stage-specific timing of cell division, cell fate specification and other developmental events of a subset of postembryonic cell types. They have been named the heterochronic genes and include *lin-4, lin-14, lin-28, lin-29, let-7, daf-12, lin-41* and *lin-42.* Mutations in these genes cause cell division patterns that are either precocious or retarded resembling those typical of a different developmental stage (Ambros and Horvitz, 1984; Ambros, 2000). *lin-14* is a nuclear protein, expressed during the early L1, and is responsible for the execution of L1-specific fates, and is downregulated by the small RNA, *lin-4* (Olsen and Ambros, 1999). *lin-28,* an RNA-binding protein specifically promotes L2-specific fates, while *lin-29* has been shown to regulate the larva/adult switch in hypodermal cells (Ambros, 2000). *cki-1* may play a role as a downstream effector of many heterochronic genes, conferring developmental cell cycle arrest until the appropriate larval stage (Hong et al., 1998). In *lin-14* and *lin-28* mutants, the vulval precursor cells (VPCs), which normally maintain a G1 arrest until the mid-

L3 stage, undergo precocious divisions in the L2 (Euling and Ambros, 1996). Similarly, in *cki-1(RNAi)* animals, the VPCs divide early, during the L2 stage. Consistent with it being a downstream effector of *lin-14,* the expression of the *cki-1::GFP* reporter transgene is reduced in *lin-14* mutants, while in *lin-4* mutants, which fail to downregulate *lin-14* activity, *cki-1::GFP* expression was not decreased (Hong et al., 1998).

In *lin-29* mutants that do not undergo the larval to adult switch, the hypodermal seam cells fail to terminally differentiate, and instead progress through additional divisions (Rougvie and Ambros, 1995). In wild type animals, at the L4 moult the hypodermal seam cells express the *cki-1::GFP* reporter transgene strongly, while *lin-29* mutant cells fail to do so, implicating *cki-1* as a downstream target of *lin-29* (Hong et al., 1998).

One of the cell lineages whose divisions are not regulated by heterochronic genes is the somatic gonad (Ambros and Horvitz, 1984). The somatic gonad precursors, Z1 and Z4 undergo a series of asymmetric divisions, migrations and cell fate specifications to give rise to 143 cells of the somatic gonad, including the distal tip cells, uterine, sheath, spermathecal cells, and the anchor cell (Kimble and Hirsch, 1979). The initial divisions of the somatic gonadal precursors have been shown to be controlled by the gene *gon-2,* a member of the TRP family of cation channels (West et al., 2001). *gon-2* mutants fail to undergo divisions of the somatic gonadal precursor cells, or do so in a delayed manner. Interestingly, in wild type animals, *cki-1::GFP* is expressed strongly in the Z1 and Z4 somatic gonad precursors, and is downregulated when they divide (Hong et al., 1998). *gon-2* might, therefore, be an upstream developmental regulator of *cki-1* since *cki-1::GFP* expression remains high in the undivided Z1 and Z4 cells of *gon-2* mutant larvae.

Although developmental regulation has only been demonstrated for several cell cycle regulators in *C. elegans,* an emerging view that is also becoming apparent in *Drosophila,* is that lineage-specific cell division patterns are a result of an intricate network of gene activities that coordinate with their functions as important regulators of development.

Objective

Caenorhabditis elegans is a free-living nematode, which has been extensively used to study a variety of biological processes (Wood, 1988). Due to its transparency and a small number of cells, the invariant cell lineage has been characterized thus facilitating the study of mutants that display alterations in cell lineage patterns (Sulston and Horvitz, 1977). This has allowed the analysis of mutants that affect cell division in multiple lineages, and, as mentioned above, several conserved general cell cycle regulators also have homologues in the worm. In order to identify and characterize developmental regulators of the cell cycle, or genes which act in specific organs to restrict or promote cell division, my goal was to identify and characterize mutants which display cell division abnormalities in specific lineages that give rise to organs, namely the intestine. In addition, by studying animals lacking the function of the Cdk inhibitor, *cki-1,* we aimed to study the consequences of how altered cell division timing could not only affect cell numbers but also the assumption of appropriate cell fates during development, thereby providing more insight to link these two essential processes.

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Chapter II

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

Kostic, I. And Roy, R., (2002). Organ-specific cell division abnormalities caused by mutation in a general cell cycle regulator in *C. elegans. Development* **129**: 2155-2165.

Abstract

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 homologue, *cki-1,* causes extra cell divisions in numerous tissues including the hypodermis, the vulva, and the intestine. We 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, since 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 germ-line 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, 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; reviewed by 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 G1/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 G1 or G0

(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 homologue of the G2/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 G1/S transition is also developmentally regulated in flies through the activity of CKIs and cyclin E and 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 G1 phase (Thomas et al., 1994; Gonczy et al., 1994; Avedisov et al., 2000). Decapentaplegic, a TGF-β family member, is required for the establishment of G1 arrest prior to differentiation during *Drosophila* eye development (Horsfield et al., 1998), while it is also essential for proliferation in the wing and in the germ-line (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 precisely characterize 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). Since 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 G0 has been shown to be regulated through the degradation of G1 cyclins (Kipreos et al., 1996). The *C. elegans* $p27^{KIP1}$ homologue, *cki-1*, has been shown to confer developmental G1 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 signalling 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.

Here 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* homologues, which has a conserved role in positively regulating the G1/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 (*mals103* [*(rnr::GFP unc-36(+)]X*) (Hong et al., 1998), KM32 (*gvEx32* [*cye-1::GFP; rol-6D*]) 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*]) 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 *(mals103; rrEx12)*, MR180 *(mals103; rrEx13)*, MR199 *(rrEx16* [*lin-31::cdc25(gf); unc-119*]), MR196 (*rrEx12; gvEx32)*, MR197 (*rrEx13; gvEx32).* Strains were cultured using standard techniques (Brenner, 1974).

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

elt-2::GFP animals were mutagenized with 40mM ethylmethanesulfonate (EMS) (Brenner, 1974). Mutagenized L4 hermaphrodites were picked to plates, 25-30 per plate and allowed to produce progeny at 25°C. F1 animals in the L4 stage were transferred to 60 mm plates, five per plate, and the F2 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. 10320 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))* or wild type animals, respectively, into the pGEM-T vector (Promega). pMR407 and pMR408 were generated by inserting a 7495bp PCR product corresponding to the *cdc-25.1* gene and including 5035bp of upstream sequence and 366bp of sequence 3' to the translational stop site from *cdc-25.1(gf)* and wild type genomic DNA, respectively into pGEM-T (Promega). pMR410 and pMR411 were generated by inserting the wild type *cdc-25.1* genomic sequence into the *NcoI/ SacI* sites of pPD49.78 and pPD49.83, respectively. pMR412 and pMR413 were generated by inserting the mutant *cdc-25.1* genomic sequence into the *NcoI/ SacI* 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, and 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 into the *elt-2::GFP* strain at the concentration of 17 ng/µl with the cotransformation marker pRF4 (*rol-6D)* at the concentration of 128 ng/µl. MR178 *(mals103; rrEx12)* was constructed by injection of 20 ng/µl pMR410 and pMR411 into the *mr::GFP* strain with 100 ng/µl *ttx-3::GFP* (Hobert et al., 1997). MR180 *(mals103; rrEx13),* was constructed by injection of 20 ng/µl pMR412 and pMR413 into *mr::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. (1998). *cyd-1* and *cye-1* dsRNA was produced according to Park and Krause (1999) and Fay and Han., (2000), respectively. *cdc-25.1* dsRNA was produced by restriction enzyme digestion of pMR409 with *NdeI* or *SacI* for the sense and antisense *cdc-25.1* RNA. 1 µg of the gel-purified template was used for *in vitro* transcription reactions according to Fire et al. (1998). Double stranded *cdc-25.1* RNA was injected into *elt-2::GFP* or *rr31; elt-2::GFP* 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 F1 progeny was scored.

Lineage analysis

Embryos dissected from gravid *elt-2::GFP* or *rr31; elt-2::GFP* hermaphrodites were placed on NGM pads and cell divisions were observed from the zygote stage onwards. For the *cki-1(RNAi)* lineage, F1 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., (1999), and Ashcroft et al., (1999), respectively. For immunostaining of larvae, animals were fixed in 3% formaldehyde and antibody staining was performed according to standard procedure (Finney and Ruvkun,1990).

Image Capture and Processing

Images of live embryos, or animals anaesthesized with 1mM levamisole, were captured using the Leica DMR compound microscope equipped with a Hamamatsu C4742-95 digital camera. Image analysis, computational deconvolution and pseudocolouring were performed using Openlab 3.01 software from Improvision Ltd. 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 (Joel 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). 10320 haploid genomes were screened and while several mutants with fewer intestinal cells were isolated, we have identified only one mutant with extra intestinal cells. This mutant, *rr31*, has 57+/- 4

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

Genotype	Time after hatching (hours)		
	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 prior to and following 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 F1 progeny laid after these 24 hours were scored for intestinal cell number by counting the *elt-2::GFP* expressing nuclei.

intestinal nuclei at the adult stage, or approximately twice the wild type complement (Figure 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). We, however, cannot rule out that there may be other less obvious lineage defects that were not apparent from our examination of *rr31* mutants.

During normal development, after a series of mitotic divisions which 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 to 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 prior to 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).

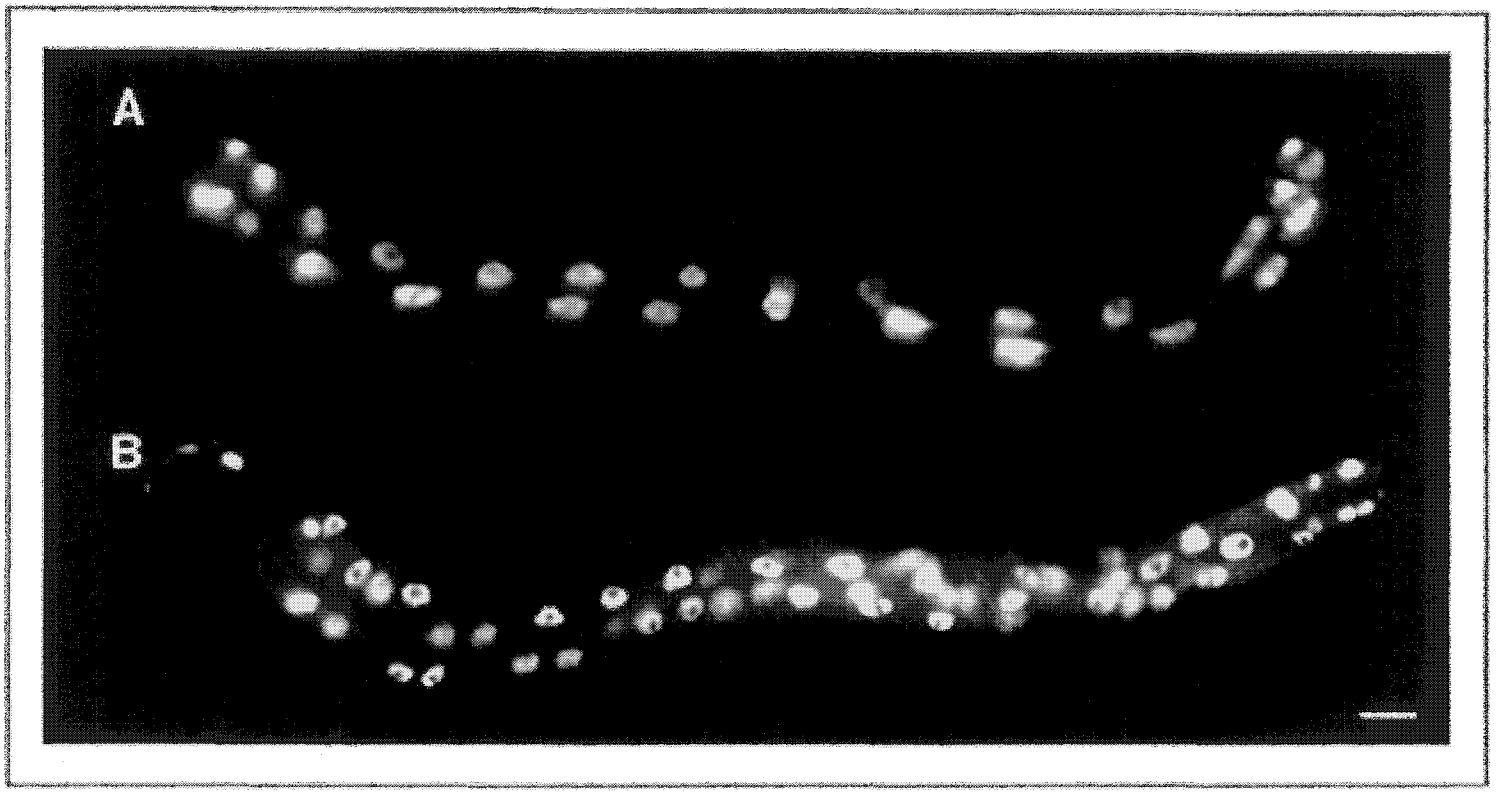

Figure 1. *cdc-25.1(gf)* mutants have increased numbers of intestinal nuclei. A) Wild type adult animals expressing *elt-2::GFP* which marks intestinal nuclei. **B)** *rr31* adult showing an increased number of *elt-2::GFP* expressing nuclei. Scale bar is 25 μm. Anterior is left.

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 misspecification 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 by a fifth division, giving rise to the 20 intestinal cells present at hatching (Sulston et al., 1983; Figure 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 (Figure 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 moult 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 (Figure 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 controlling 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

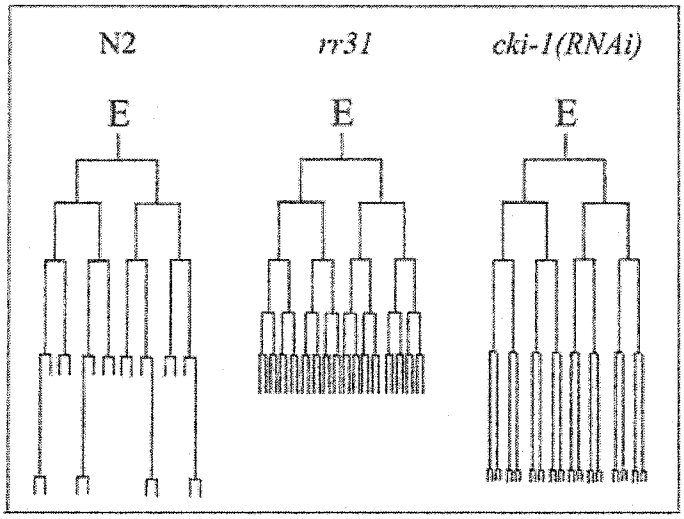

Figure 2. Lineage analysis of the E blastomere in *rr31* and *cki-1(RNAi)* animals. **A)** Lineage map of the wild type intestinal cell divisions during embryogenesis. Schematic representation of a characteristic lineage map of the **B)** *rr31* mutant and **C)** *cki-1(RNAi)* intestinal cell divisions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

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 between 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 molecularly characterize the *rr31* allele. Genetic analysis showed that the *rr31* mutation segregated in a dominant, maternal-effect manner. All the F1 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 F1 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 F1 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 where the *rr31* mutation mapped to. The genotype is indicated as the genotype of the P0 hermaphrodite.

Since 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 F1 progeny, while other candidates had no effect (Table 3). This phenotype was incompletely penetrant and did not persist in subsequent generations, probably due 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 7.4kb 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 F1 *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 F1 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.

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 F1s with extra intestinal cells	0 (n=67)	7 (n=93)	0 (n=96)

F1 animals which 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 which showed a wild type intestinal cell number in the adult stage.

To verify whether the extra intestinal cell phenotype was indeed due to a mutation in *cdc-25.1,* we analysed the genomic and cDNA sequence of the mutant and wild type *cdc-25.1* genes (Figure 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 (Figure 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 which may play an important role in the generation of extra intestinal cells during embryogenesis in *cdc-25.1(gf)* mutants. Since the mammalian homologue of *cdc-25.1*, Cdc25A, is presumed to accelerate G1/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 homologue 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. Since 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 (Table

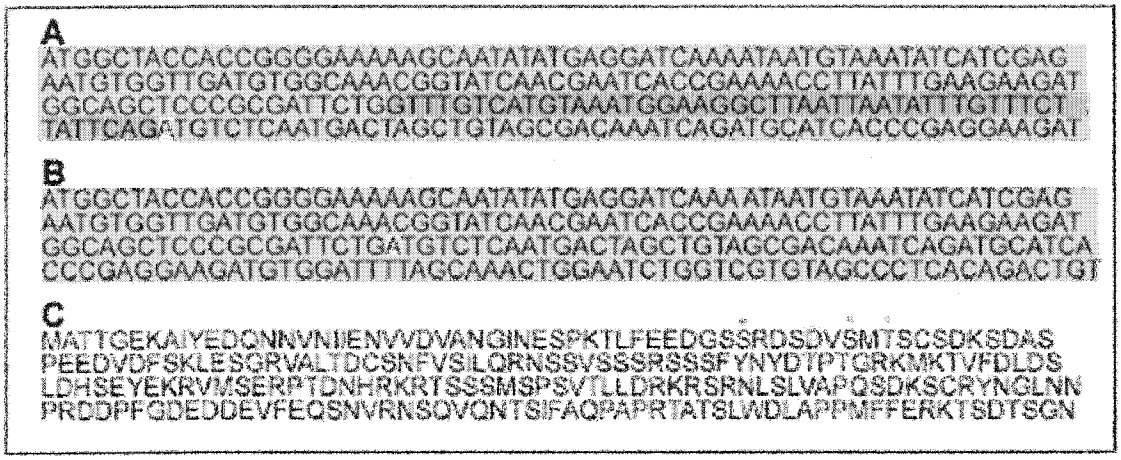

Figure 3. *cdc-25.1* mutant sequence. A) *cdc25.1* mutant DNA sequence including the translation start to up to and including exon 2. Exon sequence is in blue, intron sequence in red, and the mutated nucleotide in yellow. **B)** *cdc25.1* mutant cDNA sequence including exons 1 and 2. **C)** CDC-25.1 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 in the mutant sequence (PredictProtein software).

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 G1/S or G2/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, that blocks such an effect.

The entire *C. elegans* endoderm is derived from one single blastomere, E, at the eight-cell stage of embryogenesis (Wood et al., 1988). 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 signalling 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 signalling and/or subsequent E specification, or whether it may be due to other signals from surrounding blastomeres, we blocked Wnt signalling using a *mom-2* background, which undergo 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 signalling and as such, more mesodermal cells should be present in *mom-2;cdc-25.1(gf)* compared to *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

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 prior to and following RNA injection. *cye-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.

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. (Figure 4.) This suggested that a cell must be specified as endodermal (E) through Wnt-signalling 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 to *pop-1(RNAi)* embryos alone (Figure 5). This indicated that the MS blastomere, which had been transformed to "E" in *pop-1 (RNAi)* animals, was now also predisposed to *cdc-25.1(gf)* and, as a result, underwent an additional round of division similar to its neighbouring 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 Figure 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.

cdc-25.1 acts at the G1/S transition

The mammalian Cdc25 homologues function as dual-specificity 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

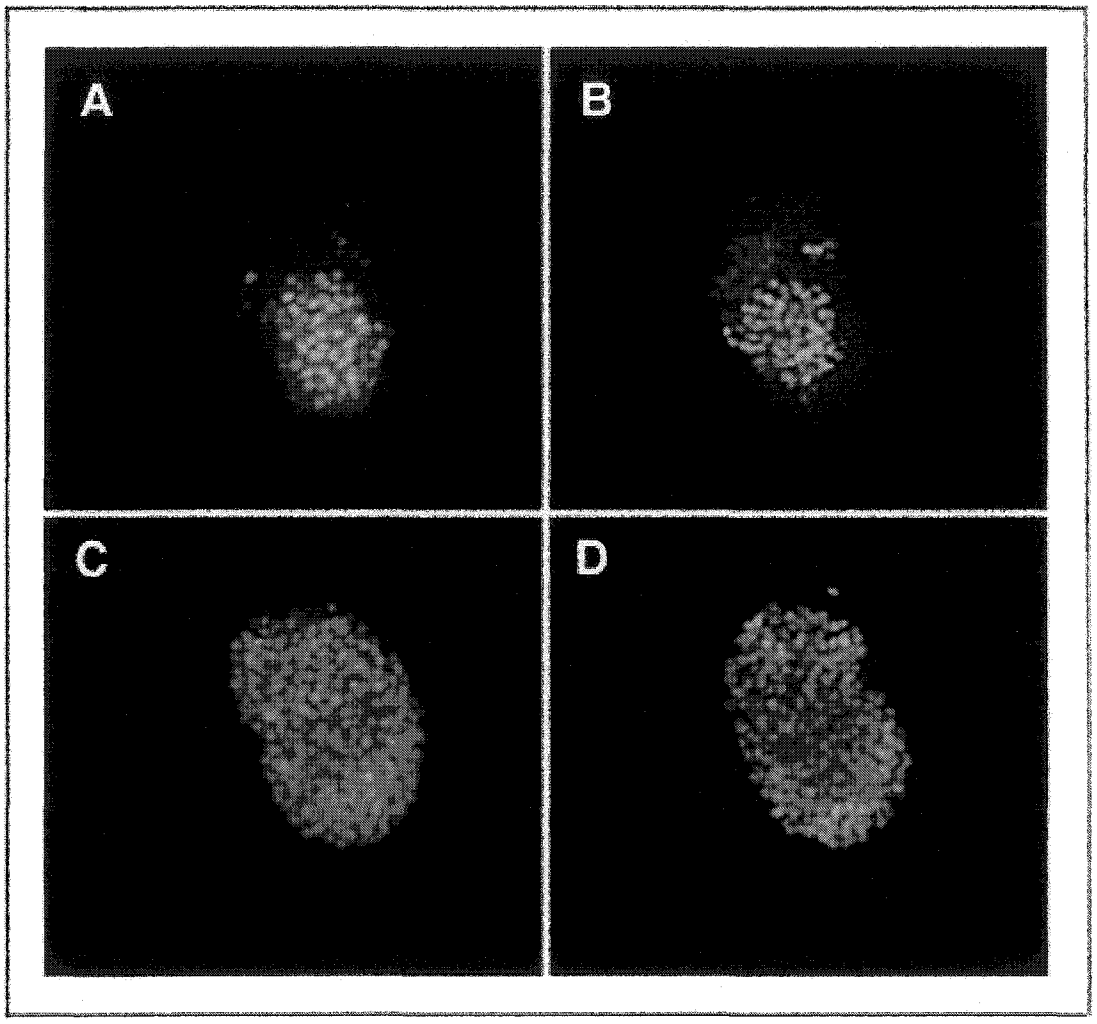

Figure 4. The *cdc-25.1(gf)* defect is specific to the E lineage. A) *mom-2* embryos produce extra mesoderm at the expense of endoderm as seen by anti-PHA-4 staining, which marks pharyngeal precursors (descendants of the MS blastomere). **B)** *cdc-25.1(gf); mom-2* embryos have similar amounts of mesoderm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

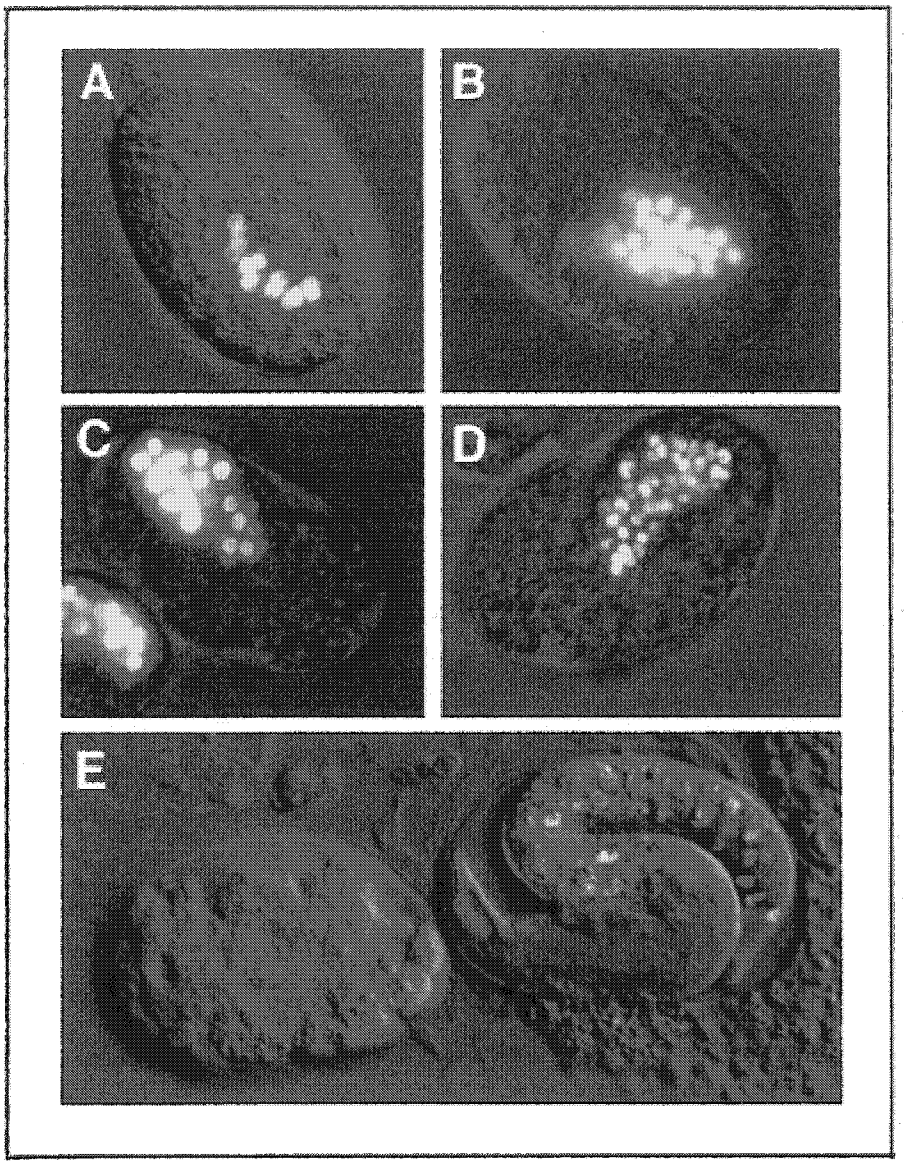

Figure 5. *cdc-25.1(gf)* enhances the *pop-1* phenotype. **A)** Wild type number of intestinal nuclei in 300 minute embryo visualized with *elt-2::GFP.* **B)** *cdc-25.1(gf)* embryo at 300 minutes showing extra intestinal nuclei. **C)** *pop-1(RNAi)* embryos have extra intestinal nuclei due to a MS to E transformation. **D)** *cdc-25.1(gf); pop-1(RNAi)* embryos have a two-fold greater number of intestinal nuclei as compared to *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.

(Nilsson and Hoffmann, 2000). To determine whether *cdc-25.1* acts at G1/S or G2/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 (Figure 6). We conclude that *cdc-25.1* can induce S phase in intestinal cells and thus acts as a positive regulator of the G1/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 have performed anti-CDC-25.1 antibody staining in wild type or *cdc-25.1(gf)* embryos (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 Figure 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 (Figure 7B,C). This suggests that the CDC-25.1(gf) protein predures abnormally and may not be properly degraded in *cdc-25.1(gf)* mutants.

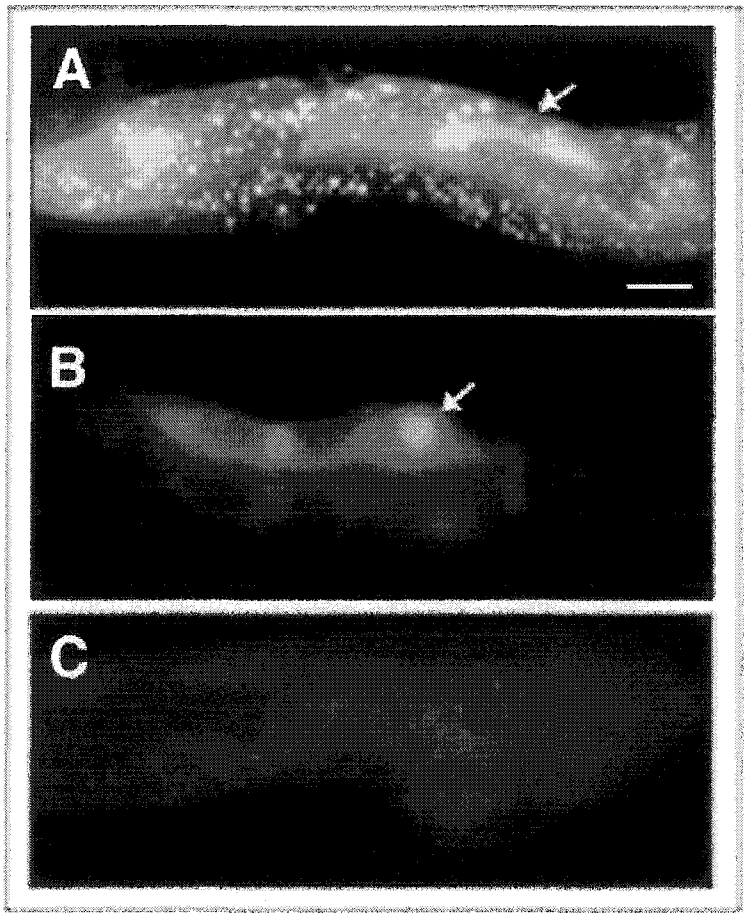

Figure 6. Heat-shock ectopic expression of *cdc-25.1* causes entry into S phase in the intestinal cells. Posterior intestinal cells of adult hermaphrodites expressing the S-phase reporter **A)** *cye-1::GFP* or **B)** *mr::GFP* after heat shock-induced expression of mutant *cdc-25.1*. **C)** Posterior intestinal cells of adult hermaphrodites after heat shock. In C), animals harbour 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 represents approximately 10 µm. Anterior is left.

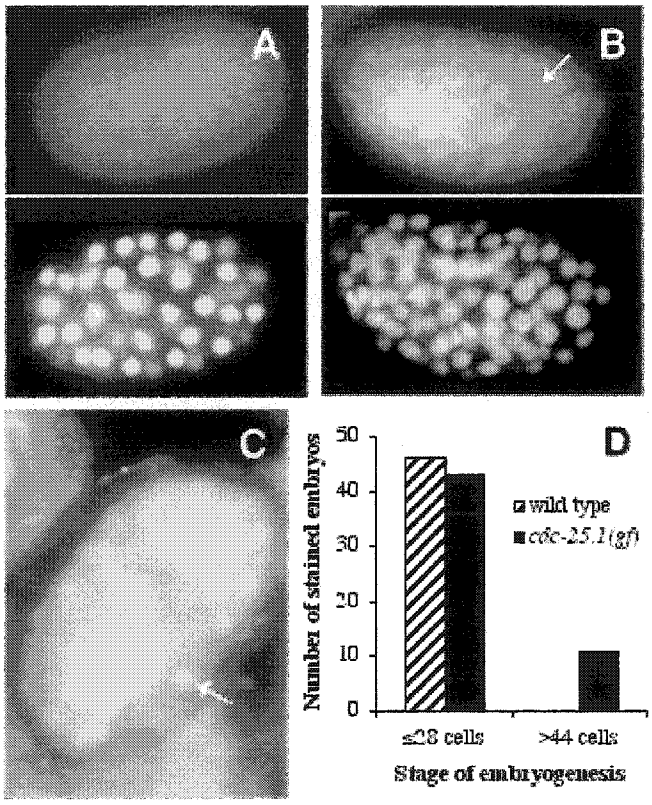

Figure 7. The CDC-25.1(gf) protein perdures after the 28-cell stage. A) Wild type embryo at the 64-cell stage stained with anti-CDC-25.1 antibody (upper panel), or DAPI (lower panel), showing no apparent CDC-25.1 staining **B)** *cdc-25.1(gf)* embryo at the 100-cell stage stained with anti-CDC-25.1 antibody (upper panel) or DAPI (lower pannel). **C)** *cdc-25.1(gf)* embryo stained with anti-CDC-25.1 showing nuclear staining as indicated by arrow. **D)** The proportion of wild type (dashed bar) or *cdc-25.1(gf)* (solid 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 54 for wild type and *cdc-25.1(gf)* respectively.

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)* animals. We mapped the mutation and through three independent methods (phenocopy, RNAi, sequence analysis) we have 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 due to 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* homologue of Cdc25A, *cdc-25.1,* belongs to a family of four *cdc25* homologues 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 mouse and humans have three homologues Cdc25A, B, and C, 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) (rr31)* 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 extra 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 favourable 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 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 G1/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 G1/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 G1/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 G1/S transition.

Ectopic expression of Cdc25A accelerates the G1/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 following overexpression of CDC-25.1, despite S-phase entry suggesting that these cells are G2/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 underway in our laboratory.

Noteworthy of mention, the expression of the *wee-1.1* kinase, which inhibits the activity of the G2/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 (Russell and Nurse, 1987; 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, likely due to redundancy, leaving its E-specific expression and function unclear (Wilson et al., 1999; IK, data not shown).

It does appear, however, that E specification through Wnt signalling 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 due to 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 since 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 (Foe et al. 1993; 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, along 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 which drive these divisions been identified, but our work stresses the importance of the control of these regulators to ensure the proper 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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Chapter III

cki-1 Coordinates Cell Fate Specification with Cell Division in the Somatic Gonad of *C. elegans*

Introduction

The formation of a complex multicellular organism requires the precise specification of many different cell types at the correct time and position. Signals specifying various cell fates must act at defined periods of competence during development, therefore it is essential for signalling to be coordinated with competence so that specification can occur appropriately. Coordination of these two processes can occur through many possible mechanisms and is extremely important if a cell is competent to respond to more than one extracellular signals specifying mutually exclusive cell fates. (Ambros, 2001).

The acquisition of a particular fate by a cell in response to various extracellular signals can be temporally linked to the developmental stage of an organism, or alternatively, by using intracellular cues such as progression through a cell cycle (Servetnick and Grainger, 1991; McConnell and Kaznowski, 1991; Weigmann and Lehner, 1995; Lehner and Lane, 1997). Some of the molecular mechanisms that coordinate cell cycle progression and cell fate specification have been analyzed in higher organisms (Ambros, 1999; Wang and Sternberg, 1999; Weigmann and Lehner, 1995; Cui and Doe, 1995). During the development of the central nervous system in *Drosophila,* the correct specification of neurons in the central nervous system is dependent on the expression of *even-skipped* (Weigmann and Lehner, 1995). After the first asymmetric division of the neuroblast NB1-1, the expression of *even-skipped* begins in one of its daughters, the ganglion mother cell. This expression is proposed to be activated by a positive regulator produced in the neuroblast during the G2 phase of the cell cycle, before its first asymmetric cell division. *Even-skipped* expression, however, only occurs after the ganglion mother cell undergoes S phase, probably when the positive regulator is able to gain access to the regulatory region of the *even-skipped* gene and activate its expression (Wiegmann and Lehner, 1995).

In *C. elegans,* a similar sequencing of cell fate decisions has been demonstrated for the vulval precursor cells (Ambros, 1999; Wang and Sternberg,

1999). Only one of the six equipotential cells may adopt a primary cell fate, with its neighbours adopting the secondary, and the remaining three cells adopting the tertiary cell fate (Kenyon, 1995; Sternberg and Horvitz, 1989). This sequencing of cell fate choices is accomplished by linking these steps in cell fate determination to various cell cycle phases. Here, the commitment to the primary cell fate is determined prior to S phase progression of the vulval precursor cells (Ambros, 1999). Once the primary cell is determined, and after S phase progression, its neighbours are competent to adopt the secondary cell fate.

Similarly, the *C. elegans* cyclin E homologue *cye-1* regulates the proper timing of differentiation of vulval precursor cells (Fay and Han, 2000). The analysis of the development of the vulva in *cye-1* mutants has shown that there is an increased number of vulval precursor cells that adopt the vulval cell fate as a result of an extended G1 phase. This suggests that a timing mechanism controlling cell cycle length may, in addition, control the number of vulval precursor cells that adopt the final vulval cell fate.

The formation of the *C. elegans* somatic gonad also requires precise specification of many different cell types at the correct time and position. The development of the somatic gonad is a result of a series of asymmetric cell divisions of the somatic gonad founder cells, Z1 and Z4, in a lineage that is highly invariant (Kimble and Hirsch, 1979). These cells divide to give rise to the distal tip cells, which lead the outgrowth of the gonad arms and promote germline mitosis, the gonadal sheath cells, which maintain the correct spatial arrangement of oocytes in the proximal gonad arm and are required for normal meiotic maturation and ovulation, as well as spermathecal cells, uterine cells, and the anchor cell which plays an important role in the specification of the vulval cell fates (Hall et al., 1999; Hubbard and Greenstein, 2000; McCarter et al., 1997; Newman et al., 1996).

The *C. elegans* p27KIP1 homologue, *cki-1*, has recently been shown to be one of the downstream effectors of many developmental pathways, which confer developmental G1 arrest (Hong et al., 1998). The *cki-1::GFP* reporter is expressed in blast cells which are in the G1 phase of the cell cycle as well as in

differentiating cells, and is developmentally regulated (Hong et al., 1998). The removal of *cki-1* activity through RNA-mediated interference (RNAi) causes extra larval cell divisions in multiple lineages, many of which are a consequence of early divisions of blast cells. Here we show that although most of the hyperproliferative defects resulting from the removal of *cki-1* activity are a result of early duplications of blast cell divisions, the hyperplasia and the appearance of extra cells in the somatic gonad lineage is the result of an apparent cell fate transformation as opposed to cell duplications more typical of *cki-1* loss of function. Using laser microsurgery experiments, we show that extra distal tip cells and anchor cells can arise from other somatic gonadal precursors, eventually resulting in abnormalities in the numbers of several cell types critical for correct gonadogenesis and consequently gametogenesis. Our results underscore a unique stem-cell like property of sheath cells to recapitulate mother cell fates when cell division timing is misregulated. The role of *cki-1* in properly regulating the G1/S transition may be important for the somatic gonadal cells to remain in the G1 phase of the cell cycle therefore emphasizing the importance of developmental quiescence in order to acquire or retain their proper cell fates.

Materials and Methods

Strains and Genetics

C.elegans strains were derived from the wild type N2 Bristol strain and cultured using standard techniques by Brenner (1974). In this study we used the following strains: N2, JK2868 [*unc-119(ed3); qIs56 (unc-119(+); lag-2::GFP)*], CB4037 *glp-1(e2141)*, PS1269 [*unc-31(e169); sylS3 (lin-3::lacZ)*], PS3352 [*dpy-20(e1282) sylS50 (dpy-20(+); cdh-3::GFP)*], and VT825 [*dpy-20(e1282);maIS113(dpy-20(+); cki-1::GFP*].

RNA interference

cki-1 double stranded RNA (dsRNA) was produced and injected according to Hong et al., (1998). 1μg of the gel-purified template was used for in vitro transcription reactions and the RNA was then phenol/chloroform extracted, ethanol precipitated and annealed. (Fire et al., 1998). *cki-1* dsRNA was injected into JK2868, CB4037, PS1269 or PS3352 animals at a concentration of 1mg/ml and the F1 progeny of the injected animals was transferred daily to new plates, and the distal tip cell, germ cell, or anchor cell number was scored, by observing *lag-2::GFP, cdh-3::GFP* or *lin-3::lacZ* expression or staining, respectively.

Laser microsurgery

For the laser microsurgery of the distal tip cells in L2 animals, the F1 L2 stage progeny of JK2868 *cki-1(RNAi)* hermaphrodites, or uninjected JK2868 L2 animals were placed on 2% agarose pads coated with 2% sodium azide and anaesthetized. After laser microsurgery, the animals were removed from pads and placed in a drop of M9 buffer to recover. 2-3 hours after ablation, the ablated animals were inspected for *lag-2::GFP* expression using a fluorescent dissecting microscope to assess the success of the laser ablations. The animals were then allowed to develop until the young adult stage at 20°C and the number of distal tip cells was scored in both JK2868 and JK2868 *cki-1(RNAi)* animals. To confirm

the RNAi effect, L2 *cki-1(RNAi)* F1 animals were left unablated and the percentage of animals with extra distal tip cells was scored in each case.

For the ablations of Z1 and Z4 in JK2868 and JK2868 *cki-1(RNAi)* animals, a similar procedure was employed as above, except the F1 progeny of *cki-1(RNAi)* animals or JK2868 controls were allowed to hatch in the absence of food, and the undivided Z1 and Z4 somatic gonad precursors were ablated in L1-arrested animals. Similarly, for the ablations of Z1.a Z4.p or Z1.p Z4.a, in JK2868 *cki-1(RNAi)* and JK2868 controls, the L1 animals were hatched in the absence of food, and then transferred to food and allowed to develop for approximately 5 to 6 hours at 25°C, or until the Z1 and Z4 divisions were complete as assessed by DIC microscopy. In all cases, L1 *cki-1(RNAi)* F1 animals were left unoperated and the percentage of animals with extra distal tip cells was scored in order to confirm the RNAi effect. For the assessment of the anchor cell formation in JK2868, or JK2868 *cki-1(RNAi)* animals, Z1.p Z4.a were similarly ablated and the number of animals which formed vulvae at the adult stage were scored.

Immunostaining and DAPI staining

Antibody staining of wild type and *cki-1(RNAi)* extruded gonads was performed by fixing the gonads in 3% formaldehyde and antibody staining was performed similar to standard procedure (Rose et al., 1997). DAPI staining was performed by the final addition of 100µg/ml of DAPI.

Hydroxyurea treatment

JK2868, or JK2869 *cki-1(RNAi)* animals at the L2 or L3 stage were obtained from mixed populations and identified both by size and lineage characteristics, and shifted to plates containing 40mM hydroxyurea (HU) and *E. coli.* After 7 hours on hydroxyurea plates, the animals were transferred to NGM plates and allowed to develop to adulthood. The number of distal tip cells in each case was assessed at early adulthood, and the efficiency of hydroxyurea treatment was observed by checking the sterility of the wild type controls.

Image Capture and Processing

Images of animals anaesthetized with 1mM levamisole, or antibody-stained gonads were captured using the Leica DMR compound microscope equipped with a Hamamatsu C4742-95 digital camera. Image analysis, computational deconvolution and pseudocolouring were performed using Openlab 3.01 software (Improvision). Images were merged using Adobe Photoshop.

Results

cki-1(RNAi) animals have extra distal tip cells

The removal of the *C. elegans* p27KIP homologue *cki-1* through RNA interference (RNAi) demonstrated a role in maintaining postembryonic blast cells in G1 during development (Hong et al., 1998). *cki-1(RNAi)* animals display multiple postembryonic cell division abnormalities including extra lateral hypodermal cell divisions, precocious divisions of the vulva precursor cells and precocious entry into S phase during dauer stage (Hong et al., 1998). Consistent with this role, *cki-1(RNAi)* animals display germline hyperplasia and rarely produce progeny (Figure 1A). The *C. elegans* germline consists of both mitotic and meiotically arrested germ cell nuclei enclosed within the gonadal syncytium (Kimble and Ward, 1988). Since CKI-1 protein is present in the gonad, the germline hyperplasia of *cki-1(RNAi)* animals could therefore be a result of additional mitoses of the germline nuclei through a cell-autonomous mechanism, where the germ cells are unable to arrest in the G1 phase of the cell cycle (Hong et al., 1998; Feng et al., 1999). Alternatively, the extra germ cell divisions in *cki-1(RNAi)* animals could be a result of the formation of observed extra distal tip cells (DTCs) which would induce additional germline mitosis and proximal proliferation through a cell non-autonomous mechanism (Hong et al., 1998; Henderson et al., 1994; Crittenden, 1994). In order to discern between these two mechanisms of *cki-1* activity, we analysed the gonads of *cki-1(RNAi)* animals and quantitated distal tip cell numbers in animals carrying the *lag-2::GFP* reporter

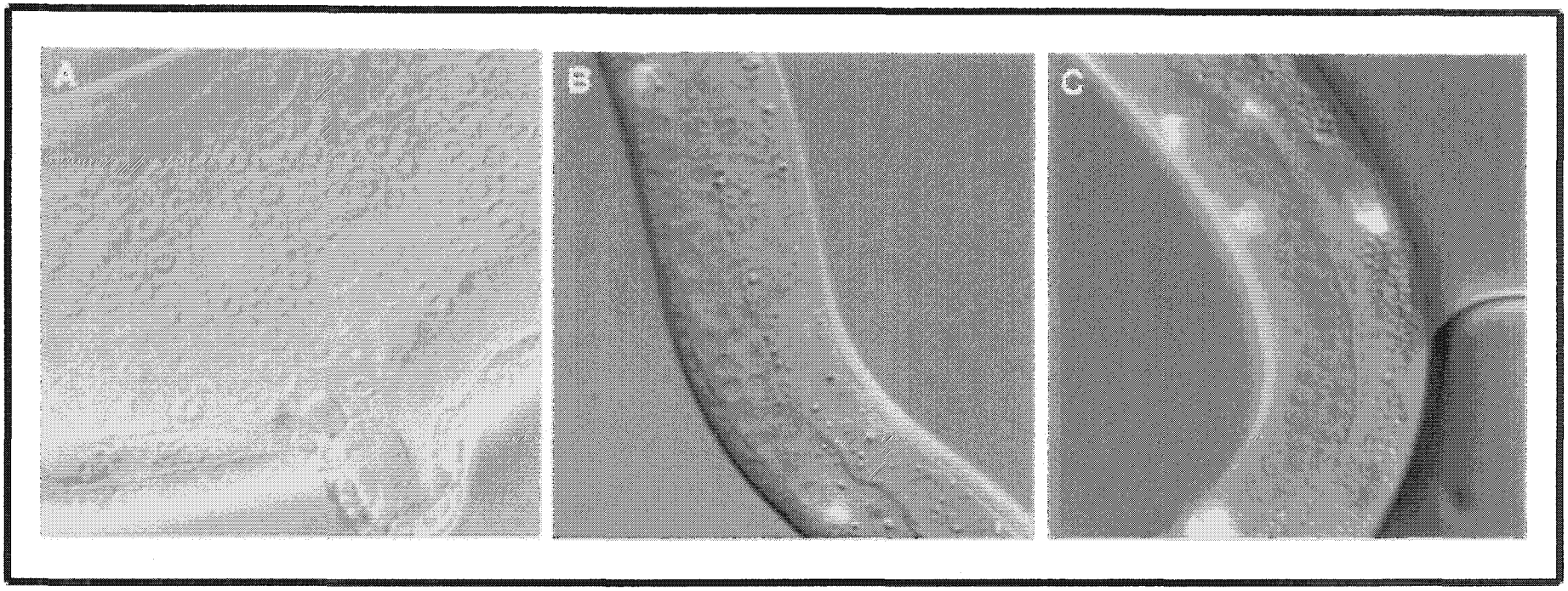

Figure 1. *cki-1(RNAi)* animals exhibit germline hyperplasia and extra gonad arms caused by extra distal tip cell formation. A) *cki-1(RNAi)* animals lack oocytes and sperm, and show proximal proliferation and germline hyperplasia in the proximal arms of the gonad. **B)** Wild type hermaphrodite showing two distal tip cells expressing the distal tip cell-specific reporter *lag-2::GFP*. **C)** *cki-1(RNAi)* hermaphrodite showing extra *lag-2::GFP* expressing distal tip cells.

transgene. *lag-2* encodes a Delta-like Notch ligand and is expressed in the distal tip cells throughout postembryonic development to maintain distal germ cell proliferation (Tax et al., 1994; Henderson et al., 1994). Wild type animals have two *lag-2::GFP* expressing cells indicative of two distal tip cells (Figure 1B). However, in *cki-1(RNAi)* animals, more than two *lag-2::GFP* expressing cells were observed, indicating that more than two distal tip cells were formed (Figure 1C). In addition, the extra DTCs in *cki-1(RNAi)* animals are capable of leading the outgrowth of gonad arms, since extra gonadal arms are often observed in RNAi-affected animals (Figure 1C). However, occasionally, animals with one or no distal tip cells were also observed in *cki-1(RNAi)* animals. These animals showed no migration of the distal tip nor mitotic proliferation of the germline, suggesting that the absence of *lag-2::GFP* expression in these cells is due to the loss of distal tip cell identity (data not shown).

To confirm that the germline hyperplasia in *cki-1(RNAi)* animals is exclusively due to the presence of extra distal tip cells rather than a cell autonomous hyperproliferation of germ cells, we removed the function of *cki-1* in *glp-1* mutant animals. *glp-1* is a Notch-like receptor that transduces the *lag-2* signal emanating from the distal tip cell to maintain the most distal germ cells in mitosis, while the more proximal cells execute the default meiotic program (Kimble and Ward, 1988; Crittenberg et al., 1994). If *cki-1* acts to block germ cell division autonomously in the germline, loss of *cki-1* in a *glp-1* background may lead to the formation of more germ cells than *glp-1* alone. When *glp-1* function is removed, the germ cells present at hatching are capable of undergoing several mitoses and subsequently enter meiosis and differentiate as sperm, therefore developing into sterile animals lacking germ cells (Austin and Kimble, 1987). When *cki-1* function is removed in *glp-1* animals, the animals also develop into sterile adults without an apparent population of mitotic germ cells, suggesting that *glp-1* function is required for the germline hyperplasia observed in *cki-1(RNAi)* animals (data not shown). This indicates that the germ cell hyperplasia in the *cki-1(RNAi)* germline is unlikely to be exclusively due to an autonomous role of *cki-1*

in the germ cells, but rather upstream of GLP-1, probably in controlling distal tip cell numbers.

Extra distal tip cells in *cki-1(RNAi)* animals do not arise from preexisting distal tip cells.

In order to determine when the extra distal tip cells arise in *cki-1(RNAi)* animals, *cki-1(RNAi)* animals were examined during various stages of development for the presence of extra *lag-2::GFP* expressing cells. Extra distal tip cells were never observed prior to the L3 stage of development (data not shown). To confirm this, we performed laser microsurgery on wild type and *cki-1(RNAi)* L2 animals. If the extra distal tip cells in *cki-1(RNAi)* animals arise from a division of preexisting DTCs, the ablation of these DTCs during the L2 stage of development would eliminate the formation of any DTCs at later stages. However, if the extra DTCs arise from an earlier cell division followed by delayed expression of *lag-2::GFP* or another cell type in *cki-1(RNAi)* animals, then the ablation of DTCs during the L2 stage would have no effect on the later formation the extra DTCs. Therefore, the two distal tip cells of wild type and *cki-1(RNAi)* L2 stage animals were ablated. When the DTCs were ablated in wild type L2 stage animals, none of the ablated animals possessed DTCs as adults (data not shown). Surprisingly, the early ablation of DTCs in *cki-1(RNAi)* animals did not eliminate the formation of DTCs later in development and, contrary to their wild type counterparts, the ablated *cki-1(RNAi)* animals possessed between one and four *lag-2::GFP* expressing DTCs as adults (data not shown). This indicated that the extra DTCs produced in *cki-1(RNAi)* animals do not arise from preexisting DTCs, but from the transformation of another cell type into a DTC or a DTC precursor.

In order to test whether other somatic gonadal cell types might undergo a similar transformation in *cki-1(RNAi)* animals, we monitored the number of anchor cells present in *cki-1(RNAi)* animals. During *C. elegans* vulval development the vulva precursor cells choose between two vulval cell fates (primary and secondary) and a non-vulval epidermal fate (tertiary fates) in response to

intercellular signals (Wang and Sternberg, 2001). An EGF-like-inductive signal (*lin-3*) produced by the anchor cell induces the vulval precursors to adopt the primary and secondary vulval fates (Hill and Sternberg, 1992). Wild type hermaphrodites have one anchor cell, that can be detected by *lin-3::lacZ* staining, or *cdh-3::GFP* expression (Figure 2A; Hill and Sternberg, 1992; Pettitt et al., 1996). *cki-1(RNAi)* animals display more than one anchor cell as observed through multiple foci of *lin-3::lacZ* staining and *cdh-3::GFP* expression (Figure 2B). These anchor cells are most probably functional since close proximity of ectopic anchor cells can induce ectopic vulvae in *cki-1(RNAi)* animals (data not shown).

Extra DTCs in *cki-1(RNAi)* animals arise from the somatic gonadal cells

Given that we identified more somatic gonadal cell types than expected in *cki-1(RNAi)* animals, and considering our results that suggested that extra DTCs arose from a cell fate transformation, we sought to identify the cell type that gives rise to the extra DTCs in *cki-1(RNAi)* animals. To identify whether it was a somatic gonadal precursor that could be transformed into a DTC, or an alternative cell type, we ablated the somatic gonad precursor cells, Z1 and Z4 in the L1 stage of development. Z1 and Z4 give rise to the entire somatic gonad, whereas two other cells positioned between them, Z2 and Z3, are the germline precursors (Kimble and Hirsch, 1979).

Although unlikely, it is formally possible that a germ cell could transform to a somatic gonadal cell and thus give rise to ectopic DTCs under our conditions. Therefore, as a control, we then ablated the two germline founder cells, Z2 and Z3, the cells that will give rise to the entire germline. In wild type animals we saw the formation of sterile animals due to the ablation of these germline precursors, but the two DTCs were still formed (Table 1.). In *cki-1(RNAi)* early L1s we often saw an early division of the Z2 and Z3 germline precursors, therefore we had ablated the four germline precursors, leaving the somatic gonadal precursors unoperated. As in wild type animals, in *cki-1(RNAi)* animals, more than two DTCs were often present in ablated adults, but no germline was detectable.

Table 1. Extra DTCs in *cki-1(RNAi)* animals arise from the somatic gonad and not from the germline.

	Number of DTCs after ablation	
Cells ablated	**Wild type**	***cki-1(RNAi)***
Z1/Z4	0 (6/6)	0 (17/18)
Z2/Z3	2	>2 (11/15)

The second day F1 progeny of *cki-1* dsRNA-injected animals or wild type, uninjected animals, at the L1 stage of development were anaesthetized and the Z1/Z4 somatic gonad founder cells were ablated, or alternatively, the Z2/Z3 germline founder cells were ablated. A group of *cki-1(RNAi)* L1s were left unoperated to confirm the penetrance *cki-1(RNAi)* effect (data not shown). Both the ablated wild type and *cki-1(RNAi)* animals were transferred to plates and cultured at 20°C. When the animals reached young adult stage, the total number of *lag-2::GFP* expressing cells were scored. The numbers in brackets indicate the number of animals with the indicated DTC number over the total number of animals scored.

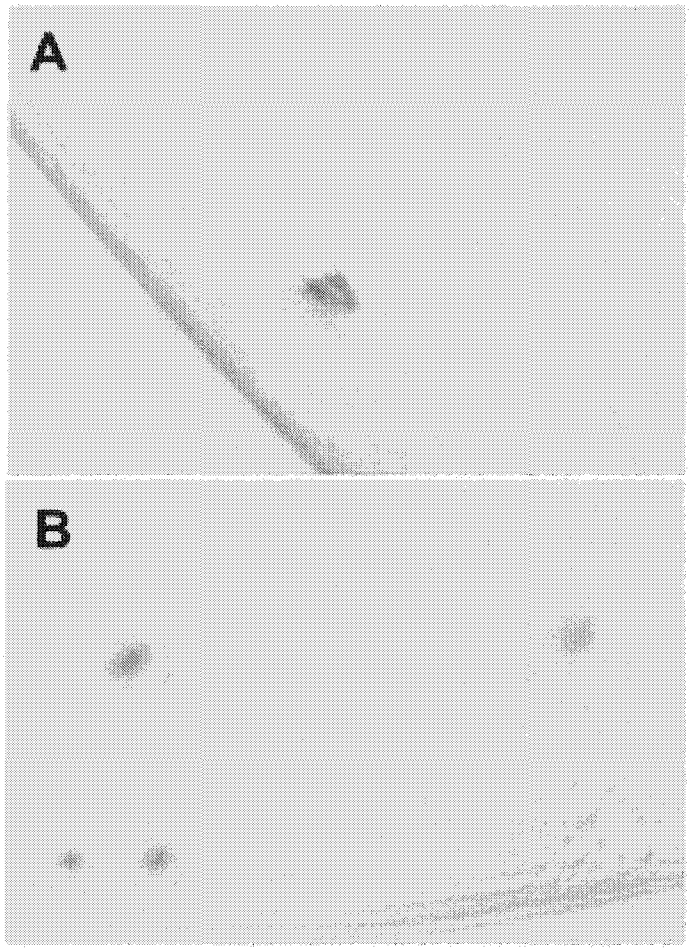

Figure 2. *cki-1(RNAi)* animals have extra anchor cells. A) Wild type hermaphrodites have one anchor cell as observed by *lin-3::lacZ* staining. **B)** *cki-1(RNAi)* animals have more than one anchor cell that are distributed throughout the gonad as observed by *lin-3::lacZ* staining.

Therefore, the extra DTCs do not arise from the transformation of a germ cell into a DTC and the *cki-1(RNAi)*-induced ectopic DTCs do not require any signal from the germline for their formation.

During the first two larval stages, Z1 and Z4 generate twelve cells that form a symmetric somatic gonad primordium. Among these, only the distal tip cells and the anchor cell do not divide further. The other nine cells divide to give rise to the ten sheath cells, 24 spermatheca cells, 6 spermathecal-uterine junction cells per arm, and 60 uterine cells. If the Z1 and Z4 somatic gonad precursors are ablated in wild type animals, no somatic gonadal cells are formed, including the DTCs. (Table 1.) *cki-1(RNAi)* animals in which the Z1/Z4 precursors were untouched, still showed the formation of extra distal tip cells, showing the RNAi effect. However, when these founder cells were ablated in *cki-1(RNAi)* animals, only 1/18 animals had more than 0 DTCs, indicating that the extra DTCs arise from the somatic gonad lineage.

Extra DTCs in *cki-1(RNAi)* animals can arise from both the Z1.a Z4.p or Z1.p Z4.a lineage

Z1 and Z4 divide in an invariant fashion to generate 143 cells of the somatic gonad (Figure 3). The first division of Z1 and Z4 occurs in the mid-L1 (Kimble and Hirsch, 1979). This division is asymmetric, giving rise to Z1.a and Z1.p, and Z4.a and Z4.p, respectively. In order to further delineate the cellular origin of the extra DTCs in *cki-1(RNAi)* animals, we ablated the Z1.a and Z4.p, or Z1.p and Z4.a cells of the somatic gonad. The Z1.a/Z4.p lineage normally gives rise to the DTCs, sheath and spermathecal cells, whereas the Z1.p Z4.a lineage gives rise to the ventral and dorsal uterine cells, the anchor cell, and sheath and spermathecal cells. When Z1.a and Z4.p were ablated in wild type animals, no DTCs were seen, since DTCs normally arise from this lineage (Table 2). However, in *cki-1(RNAi)* animals, DTCs were observed, indicating that the extra DTCs must arise from the Z1.p and Z4.a lineage which had remained unablated in this experiment.

Table 2. Extra DTCs and anchor cells in *cki-1(RNAi)* animals can arise from both the Z1.a/Z4.p and Z1.p/Z4.a lineage

	Number of DTCs after ablation	
Cells ablated	Wild type	*cki-1(RNAi)*
Z1.a/Z4.p	0 (6/6)	>0(6/16)
Z1.p/Z4.a	2 (3/3)	>2 (5/8)
Cells ablated	**Number of animals with vulva induction**	
Z1.p/Z4.a	0 (n=4)	6 (n=11)

The second day F1 progeny of *cki-1(RNAi)* or wild type, uninjected animals, at the L1 stage of development were anaesthetized and both the Z1.a/Z4.p cells were ablated, or alternatively, the Z1.p/Z4.a cells were ablated with a laser microbeam. A group of *cki-1(RNAi)* L1s were left unoperated to confirm the *cki-1(RNAi)* effect (data not shown). Both the ablated and the unoperated wild type and *cki-1(RNAi)* animals were transferred to plates and cultured at 20°C. When the animals reached the young adult stage, the total number of *lag-2::GFP* expressing cells were scored, or the number of animals with vulval induction as indicated by the presence of a true vulva, or blips, were scored. The numbers in brackets indicate the number of animals with the indicated DTC number over the total number of animals scored.

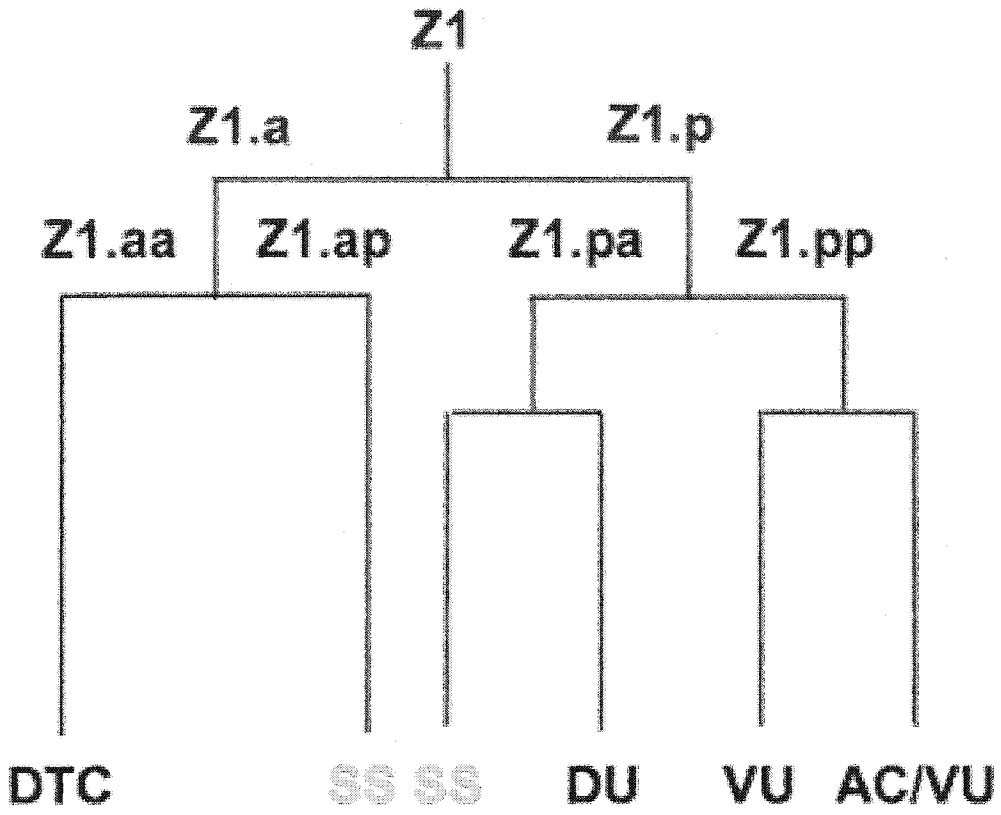

Figure 3. The lineage of the somatic gonad precursor Z1. The Z1 lineage is a mirror image of the Z4 lineage. Z1 (and Z4) divide in the mid-L1 stage to give rise to Z1.a and Z1.p (the equivalent of Z4.p and Z4.a, respectively). Z1.a (and Z4.p) continue to divide and give rise to the distal tip cells and the sheath and spermathecal cells. Z1.p (and Z4.a) divide to give rise to the sheath, spermathecal, dorsal uterine, ventral uterine and anchor cells. The common cell type which is derived from both the Z1.a (Z4.p) and Z1.p (Z4.a) lineage, the lineages capable of giving rise to extra distal tip cells is the sheath/spermathecal lineage (outlined in red).

The converse experiment involved the ablation of Z1.p and Z4.a in wild type or *cki-1(RNAi)* animals (Table 2). As mentioned above, these cells give rise to the ventral and dorsal uterine, sheath, spermathecal and anchor cells, but not the distal tip cells. When the Z1.p and Z4.a cells were ablated in wild type animals, two distal tip cells were formed. In *cki-1(RNAi)* animals, extra DTCs were still seen after the ablation of this lineage, indicating that the Z1.a and Z4.p lineage, which had remained unoperated and never produces DTCs under normal conditions, can also give rise to the extra distal tip cells in *cki-1(RNAi)* animals.

In order to determine whether a similar mechanism was giving rise to the extra anchor cells in *cki-1(RNAi)* animals, we ablated the two cells which normally give rise to the anchor cell precursors, Z1.p and Z4.a, in wild type and *cki-1(RNAi)* animals. In order to evaluate anchor cell formation, we assessed whether vulva formation was induced in these animals (Table 2.) Whereas wild type animals in which Z1.p/Z4.a were ablated were consistently vulvaless, *cki-1(RNAi)* animals occasionally did show vulva formation, suggesting that the anchor cells in *cki-1(RNAi)* animals can potentially be formed from another cell type, outside the Z1.p/Z4.a lineage, similar to the situation observed for the distal tip cells.

The sheath/spermatheca precursors could be transformed into distal tip cells in *cki-1(RNAi)* animals

To further pinpoint the origin of the extra distal tip cells in *cki-1(RNAi)* animals, the resulting cell types of both the Z1.a Z4.p and Z1.p Z4.a lineages were compared to assess whether there is a common cell type which may give rise to the extra DTCs. Interestingly, the common somatic gonad cell type that is formed from both the Z1.a/Z4.p and Z1.p/Z4.a lineages is the sheath/spermathecal lineage (Figure 3). The sheath/spermathecal lineage, may therefore be the lineage capable of undergoing a transformation into a distal tip cell fate.

The gonadal sheath cells appear to play several roles important for the structure, integrity and reproductive function of the gonad (McCarter et al., 1997). This is achieved mainly due to their role in maintaining the correct spatial arrangement of oocytes in the proximal gonad arm as is required for normal meiotic maturation and ovulation. These cells arise from four SS (sheath/spermatheca precursor) cells following asymmetric divisions during the first and second larval stage (Kimble and Hrisch., 1979). Cell ablation of one SS precursor has been shown to cause defective ovulation, due to the inability of the mature oocytes to enter the spermatheca and become fertilized, leading to the formation of endomitotic oocytes (McCarter et al., 1997). These oocytes leave diakinesis arrest and undergo multiple cycles of DNA synthesis lacking karyokinesis and cytokinesis leading to the formation of highly polyploid oocytes.

Since the sheath/spermathecal precursor lineage may aberrantly give rise to extra DTCs in *cki-1(RNAi)* animals, we performed immunostaining of wild type and *cki-1(RNAi)* gonad arms with anti-CEH-18 antibody to see whether the sheath cell number or arrangement were affected as a result of these lineage abnormalities. CEH-18, a POU-class homeoprotein, is a marker of sheath cell differentiation, and in wild type adult animals there are 10 thin gonadal sheath cells per arm which can be subdivided into 5 pairs with each pair having a distinct position along the proximal-distal axis of each gonad arm (Rose et al., 1997; Hall et al., 1999). *cki-1(RNAi)* animals, however, display aberrant numbers of sheath cells, having highly variant numbers, with either less than the appropriate number, or more sheath cells per gonad arm (Table 3). The aberrant numbers of sheath cells in *cki-1(RNAi)* animals indicates that the sheath cell lineage is affected by the removal of *cki-1* function, and that *cki-1* might play a role in the correct formation and specification of the gonadal sheath.

As mentioned above, the ablation of one SS precursor has shown to cause defective ovulation and leads to the formation of highly polyploid endomitotic oocytes. In mutants in *ceh-18*, the shape and arrangement of sheath cells is not correct resulting in endomitosis of oocytes, or an Emo phenotype (Greenstein et al., 1994). Similarly, in *lin-26* mutants the lineage leading to the

Table 3. *cki-1(RNAi)* animals have an aberrant number of sheath cells as observed by anti-CEH-18 staining.

	Percentage of gonad arms with the indicated number of CEH-18 expressing cells		
Genotype	**<10 CEH-18 expressing cells**	**10 CEH-18 expressing cells**	**>10 CEH-18 expressing cells**
N2	0	100	0
cki-1(RNAi)	43	35	22

Gonad arms were dissected from wild type N2 and *cki-1(RNAi)* animals, and antibody staining was performed with the sheath cell specific antibody anti-CEH-18. The number of CEH-18 positive cells were counted in N2 and *cki-1(RNAi)* animals and the results represent the percentage of stained gonad arms that possessed greater numbers of gonad arms with greater or less than the normal complement of sheath cells (10). The total number of stained gonad arms was n=18.

formation of SS precursors is aberrant and leads to the formation of only four sheath cells per arm, which again results in the formation of highly polyploid endomitotic oocytes (den Boer et al., 1998). Therefore, any alteration in the sheath cell number or arrangement will result in polyploidization of oocytes. Since *cki-1(RNAi)* animals showed a defect in the proper number and arrangement of sheath cells, it is expected that this would also lead to the formation of endomitotic oocytes. As can be seen in DAPI staining of wild type and *cki-1(RNAi)* gonad arms, *cki-1(RNAi)* arms display highly polyploid endomitotic oocytes (Figure 4). This suggests that loss of *cki-1* causes lineage abnormalities in the somatic gonad that results in defects in the formation and/or correct specification of gonadal sheath cell lineage.

Our analysis of somatic gonadal cell types using laser ablation, specific reporters, and an anti-CEH-18 antibody, strongly suggests that *cki-1* is required to ensure the proper formation and/or specification of the gonadal sheath cells. Interestingly, upon dissection of *cki-1(RNAi)* gonad arms, branched arms were occasionally observed, where an ectopic distal tip cell is present in a position that is proximal to a normal DTC presumably arising from a position normally occupied by a sheath cell (Figure 5). This newly formed DTC is apparently capable of inducing germline mitoses, and can lead the outgrowth of a new arm, leading to the formation of a branched arm. Since ablation experiments have shown that the extra DTCs in *cki-1(RNAi)* animals do not arise from pre-existing DTCs, the branching of the gonad arms could be explained by the fact that the sheath cells themselves could transform into DTCs.

Hydroxyurea treatment of *cki-1(RNAi)* animals

The early cell divisions which lead to the formation of the somatic gonadal primordium occur by the L2 stage, followed by a period of mitotic quiescence, then a series of short-range migrations of these cells (Kimble and Hirsch, 1979). Following this period of cellular quiescence, divisions resume in these cells at the mid-L3 stage, with the exception of the terminally differentiated distal tip and anchor cells. To determine whether the extra distal tip cells in *cki-1(RNAi)*

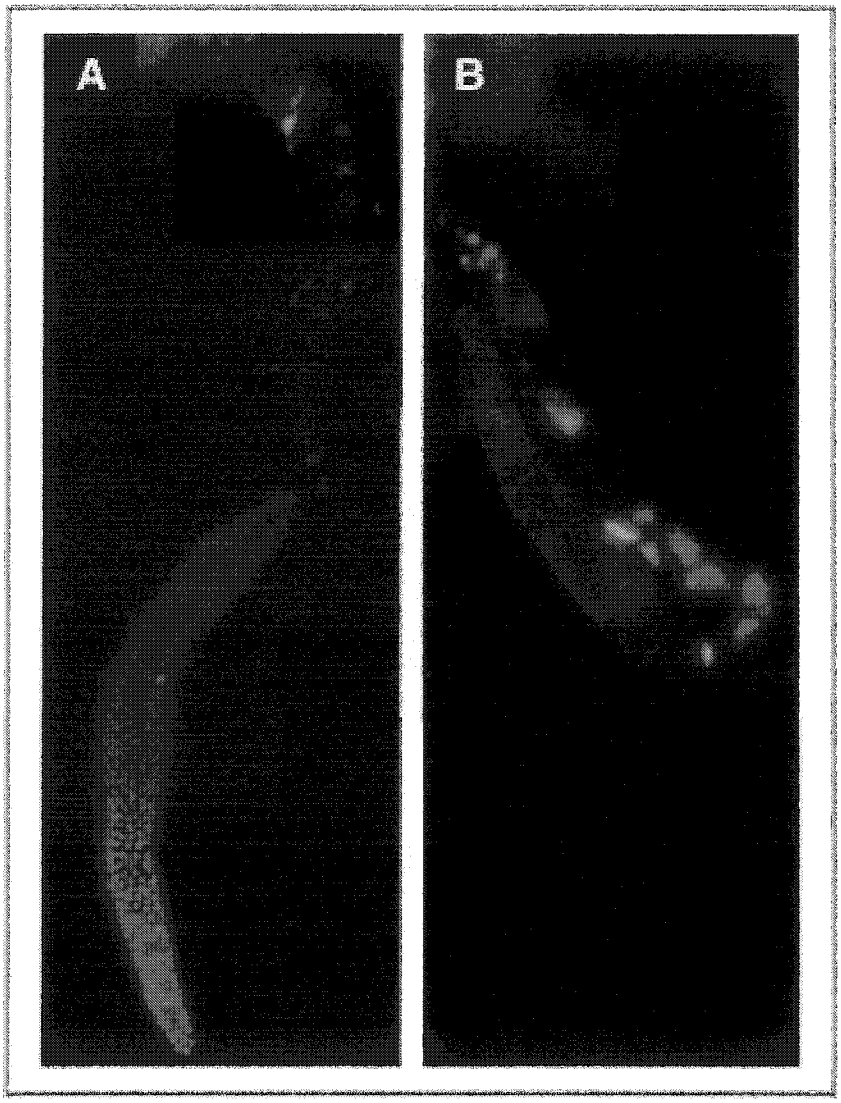

Figure 4. *cki-1(RNAi)* animals form highly polyploid endomitotic oocytes. **A)** Wild type gonad arm stained with DAPI for DNA, shows haploid oocytes which are arranged in the proximal arm of the gonad (arrowhead indicates diakinetic chromosomes of oocytes). **B)** *cki-1(RNAi)* animals show highly polyploid oocytes as seen by intense DAPI staining, with no evidence of chromosomes in diakinesis arrest typical of wild type oocytes.

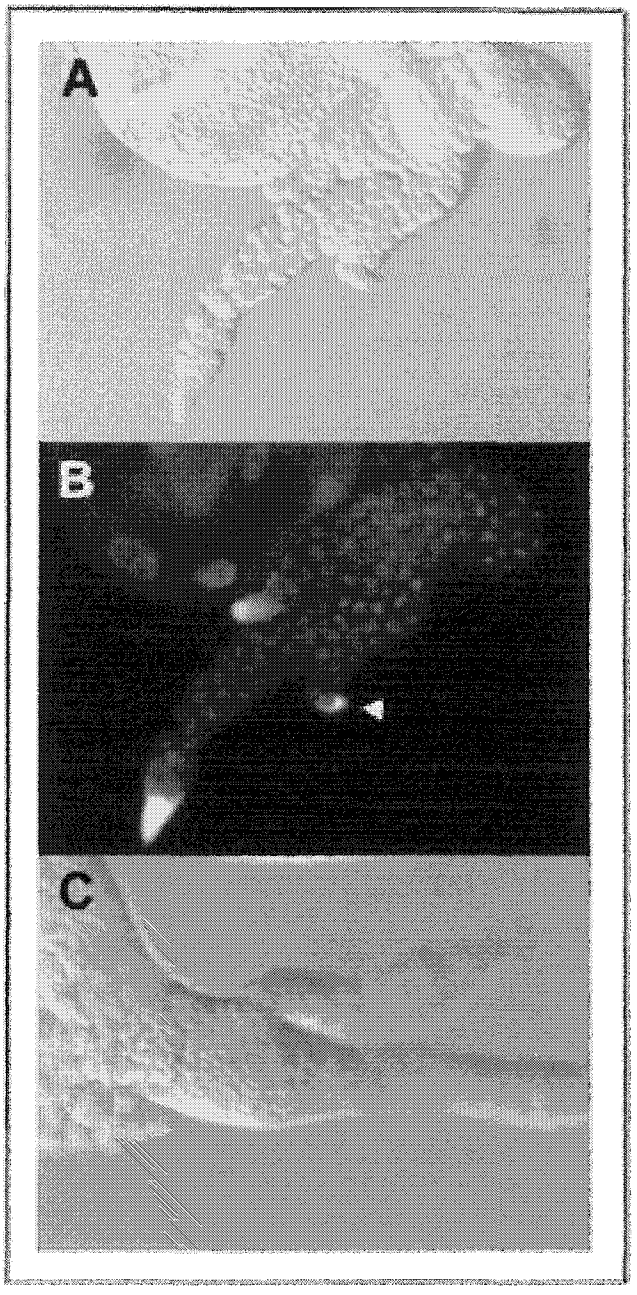

Figure 5. *cki-1(RNAi)* animals form "branched" gonad arms. Gonad arms dissected from *cki-1(RNAi)* young adult hermaphrodites show distinct fork-like appearance typical of the initiation of a new outgrowth from an existing gonad arm. **A)** A GFP image overlayed on a DIC image showing a "branched" arm. **B)** DAPI staining of the image in A. Here the DTC (arrowhead), which can be seen by leading the outgrowth of a branch in the gonad arm, shows *lag-2::GFP* expresion. **C)** Additional example of "branched" arms in *cki-1(RNAi)* animals.

animals are produced from extra cell divisions caused by the inability to maintain this period of quiescence or whether the loss of *cki-1* function in the somatic gonadal precursors causes these cells to adopt a distal tip cell fate without supernumerary divisions, we placed L2 and L3 animals on hydroxyurea (HU) containing plates. Hydroxyurea is a potent inhibitor of DNA replication, blocking all cell divisions in these animals. If the loss of *cki-1* causes extra cell divisions in the somatic gonad, placing the *cki-1(RNAi)*-affected animals on hydroxyurea-containing plates, would block these divisions, and no extra distal tip cells would be observed. However, if *cki-1* has an independent role in specifying cell fates in the somatic gonad, the animals treated with hydroxyurea would still produce ectopic distal tip cells. When wild type animals expressing the *lag-2::GFP* transgene were placed on HU-containing plates for 7 hours at the L2, or L3 stage of development, and subsequently removed to NGM plates and allowed to develop to adulthood, two distal tip cells were formed (Figure 6, data not shown for L3 stage). Therefore, HU has no effect on the production or maintenance of this cell type. However, when early L2 *cki-1(RNAi)* animals were placed on HU-containing plates, extra distal tip cells were still formed, albeit at a lower frequency, suggesting that the extra distal tip cells in these animals are not a result of extra cell divisions that occur after the L2 stage, but another, cell cycle-independent mechanism.

Discussion

Extra DTCs in *cki-1(RNAi)* animals do not arise from preexisting DTCs

We and others have previously shown that a prominent germline hyperplasia is associated with the loss of *cki-1* function (Hong et al., 1998; Feng et al., 1999). Our recent results indicate that this is not due to an autonomous role of *cki-1* within the germ cells themselves, but rather an indirect role of *cki-1* in limiting cell divisions in the somatic gonadal precursors and thereby ensuring the integrity of the fates of the resulting daughter cells. Using a *lag-2::GFP* reporter, we show that extra DTCs are produced following *cki-1(RNAi)* and each of these seem to adopt the proper DTC fate as they are able to induce germline mitosis,

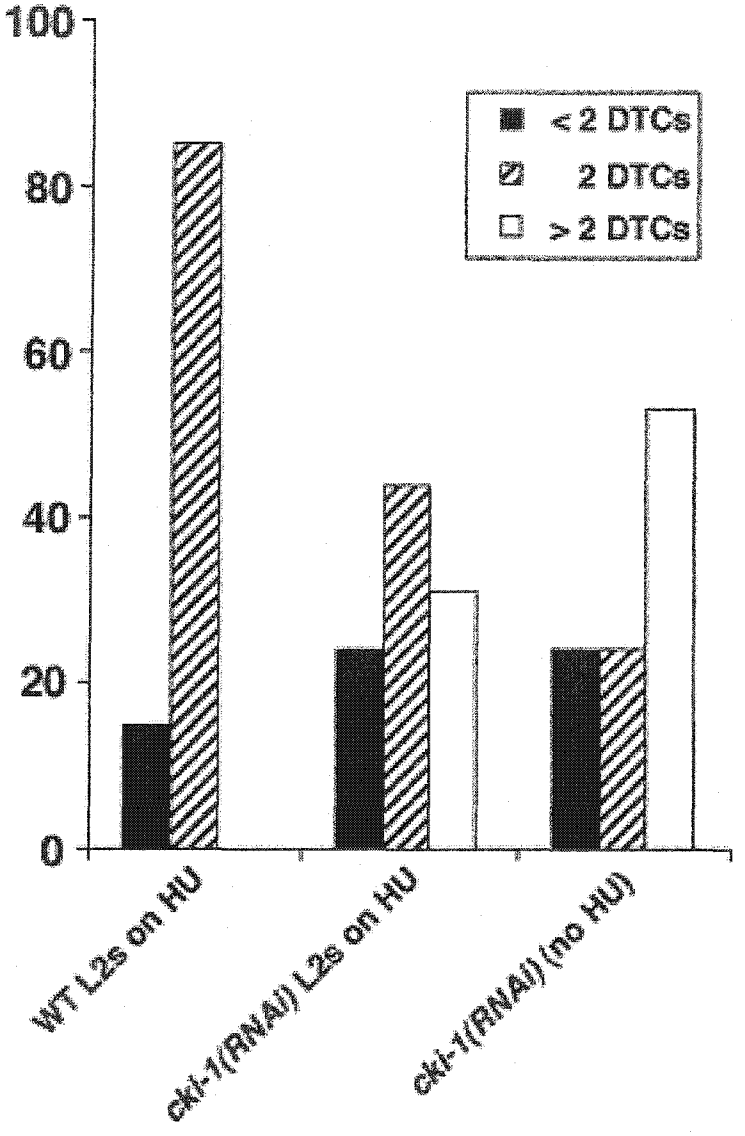

Figure 6. The block of cell division in *cki-1(RNAi)* animals does not suppress the formation of extra DTCs. Animals were placed on 40mM HU-containing plates, or NGM plates at either the L2 stage of development and after 7 hours were switched to NGM plates until the adult stage. All adult animals were sterile due to the HU treatment. The number of distal tip cells was determined by scoring *lag-2::GFP* expressing cells in wild type or *cki-1(RNAi)* animals and is representative of two independent experiments.

form gonad arms and lead their outgrowth, indicating that *cki-1* does not block this, and, other cells' ability to differentiate. In addition, we show that extra anchor cells can also appear in *cki-1(RNAi)* animals.

Most hyperproliferative phenotypes which appear due to the loss of the p27KIP homologues are due to precocious or additional divisions of blast cells, and the expansion of the initial population of blast cells (Nakayama et al., 1996; Hong et al., 1998). The examination of the early somatic gonad in *cki-1(RNAi)* L1 animals did not reveal any supernumerary divisions among the somatic gonad blast cells (data not shown). In addition, the appearance of *cki-1(RNAi)*-induced ectopic *lag-2::GFP* expressing cells does not occur until the L3 stage of development, a time when *lag-2::GFP* becomes strongly expressed in the DTC in wild type animals. By performing laser ablations of the DTCs in wild type or *cki-1(RNAi)* animals at the L2 stage of development we confirmed that the ablation of the existing distal tip cells in L2 wild type animals eliminated the formation of DTCs and a functional gonad at adulthood. However, the ablation of the two existing DTCs in *cki-1(RNAi)* L2s did not suppress the later formation of DTCs. This indicated to us that the extra DTCs in *cki-1(RNAi)* animals are formed from a cell type other than the DTC.

Extra DTCs in *cki-1(RNAi)* animals might arise from the transformation of a sheath/spermatheca precursor into a DTC.

The origin of the extra DTCs in *cki-1(RNAi)* animals was further delineated by showing that the ablation of the somatic gonad precursors Z1 and Z4, but not the germ cells Z2 and Z3, in wild type and *cki-1(RNAi)* L1s eliminated the formation of distal tip cells later during development. The first division of the Z1 and Z4 somatic gonad precursors is asymmetric, giving rise to two lineages, the Z1.a Z4.p and the Z1.p Z4.a lineage. The ablation of either the Z1.a Z4.p or the Z1.p Z4.a lineage in *cki-1(RNAi)* animals, did not suppress the formation of the extra DTCs later during development. The observation that the extra anchor cells in *cki-1(RNAi)* animals also may arise from a lineage which does not normally produce anchor cells, suggests that the somatic gonadal precursors display an

inherent plasticity in the cell fates they can adopt, when cell division timing, or S phase progression is altered.

Our interpretation of these data was that there must be a cell type which arises from the later divisions of both the Z1.a Z4.p and Z1.p Z4.a lineage which is capable of being transformed into the extra DTCs in *cki-1(RNAi)* animals. Since the sheath and spermatheca lineage which arises from the SS cell of the somatic gonad primordium meets this requirement, we investigated whether *cki-1(RNAi)* animals demonstrate abnormalities in this lineage by staining with a sheath cell-specific antibody. Our anti-CEH-18 antibody staining results confirmed that the sheath cell lineage was abnormal since *cki-1(RNAi)* gonads exhibit aberrant numbers and arrangements of sheath cells. Furthermore, like other mutants that disrupt sheath cell function or numbers, such as *ceh-18* or *lin-26*, *cki-1(RNAi)* animals also have endomitotic oocytes.

The presence of branched arms in *cki-1(RNAi)* animals also provides indirect evidence that a sheath cell might be transformed into a distal tip cell. The only somatic gonadal cell type to be positioned along the gonad arm are the sheath cells. Although rarely, we did observe gonad arms in which a DTC was present proximally to the already present distal tip cell, and formed a "bud", or in some cases, a branched arm, suggesting that the sheath cells themselves could be adopting the distal tip cell fate.

How does the loss of function in a cell cycle inhibitor cause cell fate transformations?

There are many possibilities which could only be tested by lineage analysis of somatic gonadal precursors in *cki-1(RNAi)* animals, a task complicated by variable penetrance and the effects of *cki-1(RNAi)* on positioning of the various gonadal cells. Firstly, there may be a direct transformation of a sheath/spermatheca cell precursor (SS) into a DTC. According to this scenario, if an SS precursor were transformed into a DTC the resulting two arms would have a smaller complement of sheath and spermatheca cells per arm than wild type. In addition, an SS cell may also reiterate its mother cell fate, leading to the

formation of a DTC and an SS cell. This would also result in an extra DTC and less sheath/spermatheca per gonad arm. The first possibility is supported by the experiments where blocking cell division using hydroxyurea did not suppress the formation of distal tip cells in *cki-1(RNAi)* animals during the L3 stage. Therefore, the possibility of a direct transformation of a somatic gonadal precursor into a DTC or vice versa, at the expense of one another, could be the favoured hypothesis. This is supported by the fact that occasionally *cki-1(RNAi)* animals form only one, or no distal tip cells, and the variations in CEH-18 staining cells. If the extra distal tip cells arose from extra divisions of this cell type, the complete loss of DTC should never be observed.

Although Ckis have been mostly implicated in cell division control, there is some evidence that these molecules may have direct roles in cell fate specification or maintenance of a differentiated state. Studies of cell fate specification in the *Xenopus* retina have revealed a distinct role of a cyclin-dependent kinase inhibitor, p27Xic1, in cell fate specification of Muller glia from retinoblasts in addition to its well-characterized role as a Cdk inhibitor (Ohnuma et al., 1999). The birth order of the cells in the retinal cells has been determined, leading to the formation of several distinct cell types, the last of which are the Muller glia (Cepko, 1999). The expression of p27Xic1 increases in the developing retina, leading to the specification of the Muller glial cell fate (Ohnuma et al., 1999). Interestingly, the misexpression of p27Xic1 induces the Muller glia identity, a function independent of the Cdk inhibiting domain.

We observed that the extra distal tip cells in *cki-1(RNAi)* arise around the time of the first divisions of the 12 cells of the somatic gonad primordium, in the mid-L3 stage. The twelve cells of the somatic gonad primordium are formed in the early L2, and give rise to distinct cell types, but remain undivided until the mid-L3. CKI-1 could therefore play a role in the cell fate acquisition of these somatic gonadal cell types after they resume their divisions, similar to the *Xenopus* retina model, where increasing levels of CKI-1 might promote later cell fates. Interestingly, as previously reported, *cki-1::GFP* expression was only observed in the Z1 and Z4 somatic gonadal precursors, after which expression is

downregulated, and then showed a gradual increase by the early L4 stage, where it is expressed in multiple cells of the somatic gonad, including the proximal sheath, and uterine cells (Hong et al., 1998). CKI-1 levels might be too low for detection during early gonadogenesis, but increase later where it may be required in locking cells into a fixed determined, or possibly in some cases, a differentiated state (Hong et al., 1998; Zhang et al., 1998). If CKI-1 plays a similar role in the *C. elegans* somatic gonad, then the removal of its activity may be responsible for other cells in the somatic gonad acquiring alternate fates, in the case of the sheath cell precursors, perhaps by recapitulating the fate of its mother cell to act in a stem cell like manner and generate DTCs.

In summary, we have determined that extra distal tip cells in *cki-1(RNAi)* animals do not result from the duplication of preexisting DTCs, but likely from a transformation in the cell lineage that normally gives rise to sheath and spermatheca cells. The competence of the SS precursor cells to acquire their proper fates may be dependent on the *cki-1* cyclin dependent kinase inhibitor, and the loss of *cki-1* in these cells may uncover their inherent ability to revert to earlier fates typical of stem cells. This further underscores the importance of cellular quiescence during the specification of cell fates during development and the important role of this negative cell cycle regulator in achieving this state.

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Chapter IV

Summary and Discussion

The results described above indicate the importance of the proper regulation of the cell cycle during *C. elegans* development. As in other systems, cell division must occur in a timely manner, and any aberrations in its control may lead to diseases such as cancer. *C. elegans* undergoes an invariant pattern of cell divisions characteristic of each lineage, suggesting that different developmental regulators may act in specific lineages, and/or that the same regulators are controlled differently among different cell types.

In order to identify such regulators, a screen using the intestinal-specific GFP reporter, *elt-2::GFP,* was designed and proved to be successful in the isolation of mutants that display cell cycle abnormalities specifically in the intestine. Since the intestine undergoes three different types of cell division, further screens could potentially be useful in the isolation of mutants that affect endoreplication, karyokinesis, or the mitotic divisions in the intestine. Not much is known how these cell cycles occur at precise points during development.

Using this screen, I have isolated and characterized the *cdc-25.1(gf)* mutant, which shows intestinal-specific cell cycle abnormalities. This was one of the first instances where it was shown that mutations in general cell cycle regulators can lead to lineage-specific cell cycle abnormalities. Therefore, cell cycle control in different organs may be inherently different, possibly due to different expression patterns of important developmental regulators. The gain-of-function mutation in *cdc-25.1* has confirmed the importance of the N-terminal region of this phosphatase in its proper regulation, and is likely important for the timely degradation of the protein during development. Whereas CDC-25.1(gf) protein is ubiquitously expressed during early embryonic development, it only affects the intestinal lineage, and the intestinal cell fate is necessary and sufficient for the additional round of cell division occurring during embryogenesis. What remains to be addressed is why only this cell type is affected by the gain-of-function phosphatase. Yeast two hybrid experiments using the N-terminal domain of *cdc-25.1* could potentially identify the factors involved in the timely degradation and regulation of the activity of this phosphatase. The isolation and

characterization of suppressors of the *cdc-25.1(gf)* mutant could also provide insight into the regulation of *cdc-25.1*.

The importance of accurate cell cycle regulation during organogenesis was further examined by analyzing the formation of the somatic gonad during *C. elegans* postembryonic development in *cki-1(RNAi)* animals. The removal of *cki-1* function results in the formation of supernumerary somatic gonadal cells, such as the distal tip and anchor cells through an apparent cell fate transformation. The presence of these extra cells results in improper gonadogenesis and, ultimately, infertility of the animal. The results point to a cell fate transformation of sheath cell precursors, or sheath cells themselves, into distal tip cells, independent of cell cycle progression, suggesting that *cki-1* may play an important role in the specification and maintenance of cell fate of somatic gonadal precursors. It would, therefore, be interesting to determine whether other cell types are similarly affected in *cki-1(RNAi)* animals. This work highlights a novel role of *cki-1* in the coordination of cell cycle and cell fate specification during development.

Appendix I

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

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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)*

rrIs01 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 *NcoI/SacI* sites of pPD49.78 and pPD49.83, respectively. pMR412 and pMR413 were generated by inserting the mutant *cdc-25.1* genomic sequence into the *NcoI*/*SacI* 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 *rrIs01* 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.

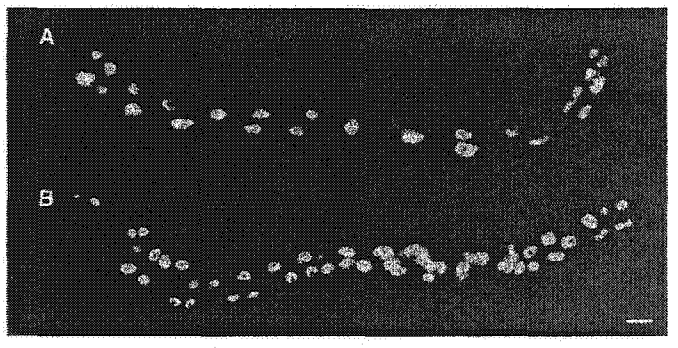

Fig. 1. *cdc-25.1(gf)* mutants have increased numbers of intestinal nuclei. (A) Wild-type adult animals expressing *elt-2::GFP*, which marks intestinal nuclei. (B) *rr31* adult showing an increased number of *elt-2::GFP* expressing nuclei. Scale bar: 25 μm. Anterior is towards the left.

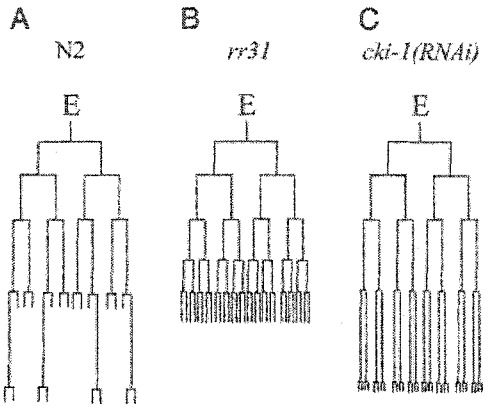

Fig. 2. Lineage analysis of the E blastomere in *rr31* and *cki-1(RNAi)* animals. (A) Lineage map of the wild-type intestinal cell divisions during embryogenesis. Schematic representation of a characteristic lineage map of the (B) *rr31* mutant and (C) *cki-1(RNAi)* intestinal cell divisions during embryogenesis.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

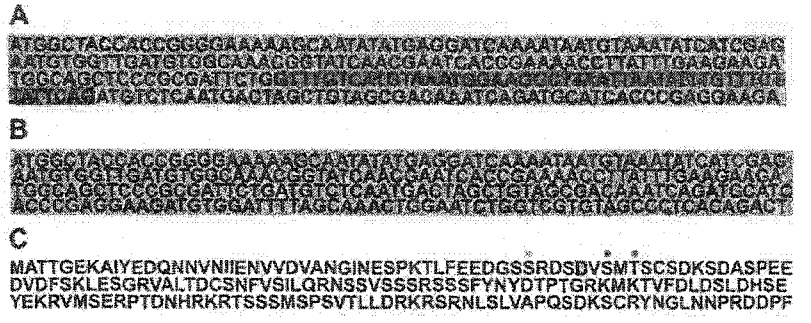

Fig. 3. *cdc-25.1* mutant sequence. (A) *cdc-25.1* mutant DNA sequence including the translation start to up to and including exon 2. Exon sequence is in blue, intron sequence in red and the mutated nucleotide in yellow. (B) *cdc-25.1* mutant cDNA sequence including exons 1 and 2. (C) CDC-25.1 mutant 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

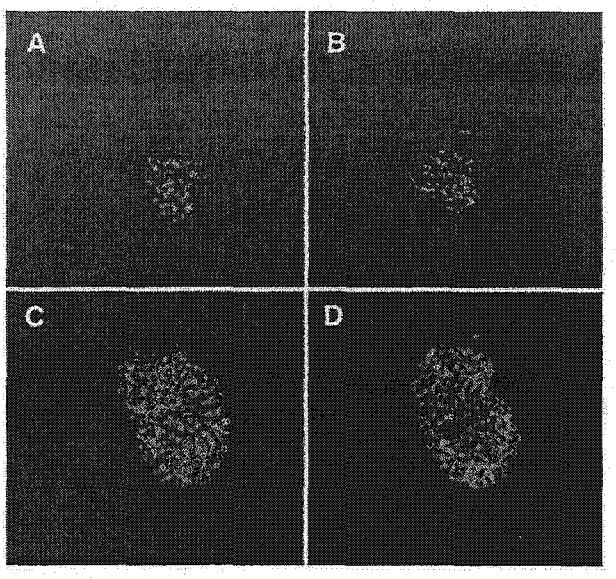

Fig. 4. The *cdc-25.1(gf)* defect is specific to the E lineage. (A) *mom-2* embryos produce extra mesoderm at the expense of endoderm, as seen by anti-PHA-4 staining, which marks pharyngeal precursors (descendants of the MS blastomere). (B) *cdc-25.1(gf); mom-2* embryos have similar amounts of mesoderm 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.

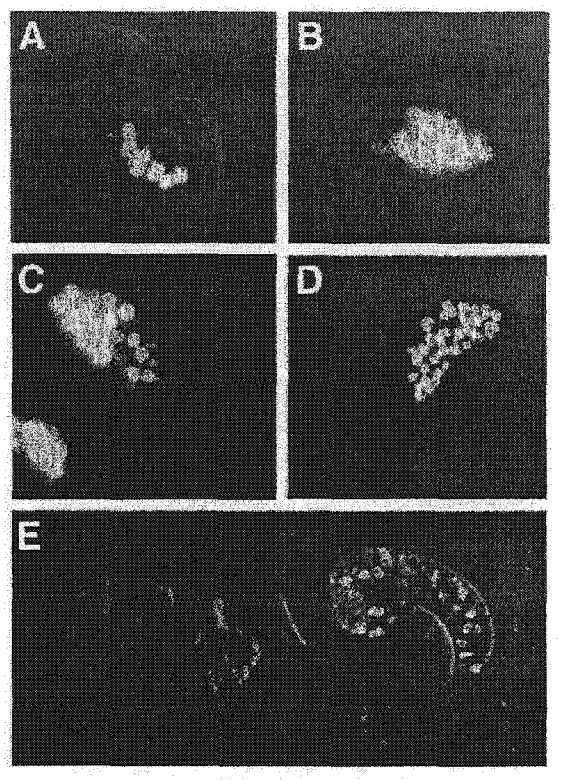

Fig. 5. *cdc-25.1(gf)* enhances the *pop-1* phenotype. (A) Wild-type number of intestinal nuclei in 300 minute embryo visualized with *elt-2::GFP.* (B) *cdc-25.1(gf)* embryo at 300 minutes showing extra intestinal nuclei. (C) *pop-1(RNAi)* embryos have extra intestinal nuclei due to a MS to E transformation. (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)*

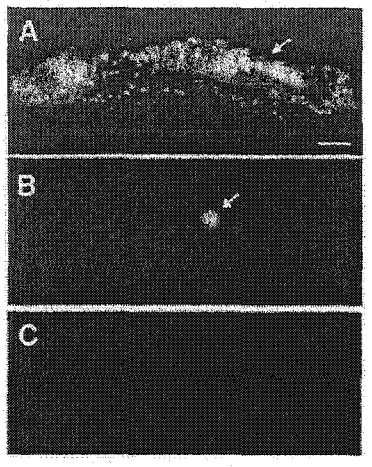

Fig. 6. Heat-shock ectopic expression of *cdc-25.1* causes entry into S phase in the intestinal cells. Posterior intestinal cells of adult hermaphrodites expressing the S-phase reporter (A) *cye-1::GFP* or (B) *rnr::GFP* after heat shock-induced expression of mutant *cdc-25.1*. (C) Posterior intestinal 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.

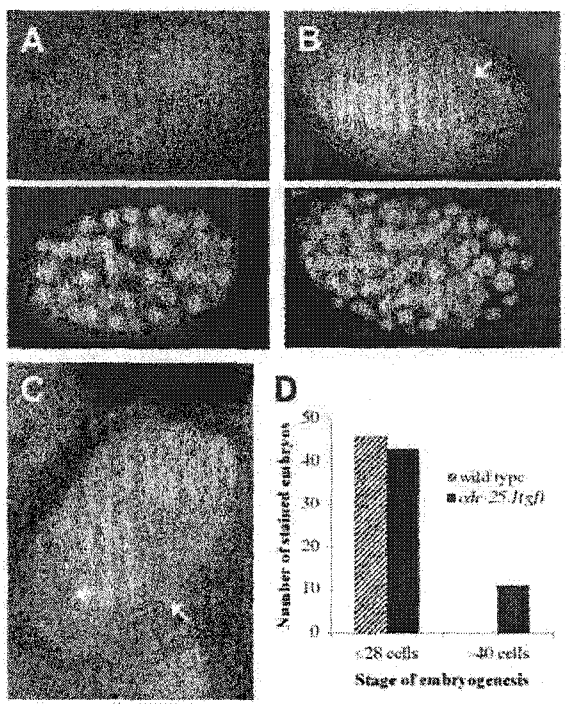

Fig. 7. The CDC-25.1(gf) protein perdures after the 28-cell stage. (A) Wild-type embryo at the 64-cell stage stained with anti-CDC-25.1 antibody (top) or DAPI (bottom), showing no apparent CDC-25.1 staining. (B) *cdc-25.1(gf)* embryo at the 100-cell stage stained with anti-CDC-25.1 antibody (top) or 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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