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cki-1 links cell division and cell fate acquisition in the *C. elegans* somatic gonad

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Abstract

The formation of a complex multicellular organism requires the precise specification of many diverse cell types at the correct time and position throughout development. This may be achieved by coordinating cell fate specification processes with progression through the cell cycle. Here, we show that the extra distal tip cells (DTCs) associated with the loss of *cki-1*, a *Caenorhabditis elegans* homologue of the cyclin-dependent kinase inhibitor p27, do not arise from duplications of pre-existing DTCs, but that they are formed from another cell type within the somatic gonad. Results from our laser microsurgery experiments suggest that the extra DTCs are caused by aberrant somatic gonadal precursor cell divisions in the absence of *cki-1*, resulting in abnormal daughter cell fates. *cki-1(RNAi)* animals also possess extra anchor cells and ectopic gonad arms with variable sheath cell numbers and positioning. In addition, *cki-1(RNAi)* animals display an endomitotic oocyte (Emo) phenotype. Our results uncover a novel role of this CKI in cell fate acquisition, either by directly influencing specification, or through a more conventional role in appropriately linking cell cycle phase with this process.

Keywords: *cki-1*; Somatic gonad; Distal tip cell; Cell cycle; *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 during development. This is predominantly controlled by signals that specify various cell fates that act at defined periods of competence. Therefore, it is essential that these two processes, signalling and competence, be coordinated so that fates can be established appropriately, and is particularly critical if a cell is responsive to multiple signals that specify mutually exclusive cell fates (Ambros, 2001).

The acquisition of cell fates can be temporally linked to the developmental stage of an organism by birth order, or alternatively, by using intracellular cues such as progression through the cell cycle (Isshiki et al., 2001; Servetnick and Grainger, 1991; McConnell and Kaznowski, 1991; Weigmann and Lehner, 1995; Lehner and Lane, 1997). Despite our understanding of the functions of various developmental regulators and most of the core cell cycle components, our understanding of how progression through the cell cycle can affect developmental outcomes is limited. Some of the molecular mechanisms that coordinate cell cycle progression and cell fate specification have been analyzed in genetic model systems (Ambros, 1999; Wang and Sternberg, 1999; Weigmann and Lehner, 1995; Cui and Doe, 1995). These studies indicate that such relationships are cell-type-specific and the competence to respond to developmental cues can be associated with defined phases of the cell cycle.

An example of this occurs during the development of the central nervous system in *Drosophila*, where the correct specification of neurons is dependent on the expression of *even-skipped*. *Even-skipped* expression is initiated only after the ganglion mother cell undergoes S-phase, probably when a regulatory factor can access the *even-skipped* gene promoter and activate its expression (Weigmann and Lehner, 1995).

In *C. elegans*, a similar sequence of cell fate decisions has been demonstrated for the vulval precursor cells (Am-

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bros, 1999; Wang and Sternberg, 1999). Only one of the six equipotent cells will 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 sequence of cell fate choices is accomplished by linking these steps in cell fate determination to various cell cycle phases (Ambros, 1999).

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 contribute to this process.

The *C. elegans* $p27^{KIP1}$ homologue *cki-1* has been shown to be one of the downstream effectors of many developmental pathways that confer developmental G1 arrest (Hong et al., 1998; Boxem and van den Heuvel, 2001). 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 the gonadal/germ line hyperplasia associated with *cki-1(RNAi)* is the result of an apparent cell fate transformation, as opposed to the cell duplications more typical of *cki-1* reduction. Using laser microsurgery, we show that extra DTCs and anchor cells can arise from other somatic gonadal precursors, eventually resulting in abnormalities in the numbers of several cell types critical for normal gonadogenesis and consequently gametogenesis. This further demonstrates the flexibility of some somatic gonadal cell types to adopt atypical fates and/or to recapitulate mother cell fates in the absence of *cki-1*.

Materials and methods

Strains and genetics

C. elegans strains were derived from the wild-type N2 Bristol strain and cultured using standard techniques (Brenner, 1974). The following strains were used in this study: N2 (Bristol), JK2868 [*unc-119(ed3); qIs56 (unc-119(+); lag-2::GFP)*], CB4037 *glp-1(e2141)*, PS1269 [*unc-31(e169); syIs3 (unc-31(+); lin-3::lacZ)*], PS3352 [*dpy-20(e1282) syIs50 (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 into JK2868, CB4037, PS1269, or PS3352 animals at a concentration of 1 mg/ml (Hong et al., 1998). The DTC, germ cell, or anchor cell numbers were 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 DTCs, Z1/Z4, Z1.a/Z4.p, or Z1.p/Z4.a, the F_1 progeny of JK2868 *cki-1(RNAi)* hermaphrodites at the appropriate stage (as determined by DIC microscopy and synchronization), or uninjected JK2868 were placed on 2% agarose pads coated with 2% sodium azide and anaesthetized. Following ablations, the animals were removed from pads and placed in a drop of M9 buffer to recover. Two to three hours later, animals were inspected for *lag-2::GFP* expression to confirm the cell kills. Animals were then allowed to develop until the young adult stage when DTC number was scored in both JK2868 and JK2868 *cki-1(RNAi)* animals. To assess anchor cell formation in JK2868, or JK2868 *cki-1(RNAi)* animals, Z1.p/Z4.a were similarly ablated, and the number of animals that formed vulvae were scored at the adult stage (Miskowski et al., 2001). To confirm the RNAi effect, *cki-1(RNAi)* F_1 animals were left unablated, and the percentage of animals with extra DTCs was scored in each case.

Lineage analysis

Lineage analysis on wild-type and *cki-1(RNAi)* animals was performed according to standard methods (Kimble and Hirsh, 1979).

Immunostaining and DAP1 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 as described (Rose et al., 1997). DNA counterstaining was performed by the final addition of 100 μg/ml of DAPI. DIC and fluorescence images of animals anaesthetized with 1 mM levamisole, or of antibody-stained gonads, were captured and processed as described elsewhere (Kostic and Roy, 2002).

Germ cell counts

Wild-type and affected *cki-1(RNAi)* L2 animals were mounted on 2% agarose pads, anaesthetized with 1 mM levamisole, and the germ cell nuclei were counted. In *cki-1(RNAi)* animals, the germ cell nuclei were scored only in animals which had two DTCs, but were clearly affected by *cki-1(RNAi)*, as seen by the presence of extraintestinal cells and/or precocious vulval precursor cell (VPC) divisions (Hong et al., 1998; Kostic and Roy, 2002).

Hydroxyurea treatment

JK2868 or JK2868 *cki-1(RNAi)* animals in L1 and L2 lethargus were collected and transferred to seeded plates

containing 40 mM hydroxyurea (HU). After 12 h on HU, the animals were transferred to seeded NGM plates and allowed to develop to the adult stage. The number of DTCs was scored in young adults, and the efficiency of the HU treatment was evaluated by scoring sterility and VPC divisions in the treated animals.

Results

cki-1(RNAi) animals have multiple defects in the somatic gonad

The removal of the *C. elegans* $p27^{KIP}$ 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 commonly show germ line hyperplasia and rarely produce progeny (Fig. 1A). Since CKI-1 protein is present in the gonad, the germ line hyperplasia of *cki-1(RNAi)* animals could therefore be a result of additional mitoses of the germ line nuclei through a cell-autonomous mechanism, or alternatively, a result of the formation of extra distal tip cells (DTCs) which would induce additional mitotic foci in the germ line through a cell non-autonomous mechanism (Hong et al., 1998; Feng et al., 1999; Henderson et al., 1994; Crittenden, 1994). In order to discern between these two mechanisms of *cki-1* activity, we quantitated DTC numbers in *cki-1(RNAi)* animals carrying the *lag-2::GFP* reporter transgene. *lag-2* encodes a Delta-like Notch ligand and is expressed strongly in Z1/Z4 and the DTC-lineage throughout postembryonic development to maintain proliferation of the distal germ cell nuclei (Tax et al., 1994; Henderson et al., 1994). Wild-type animals have two *lag-2::GFP*-expressing cells indicative of two DTCs (Fig. 1B). However, in *cki-1(RNAi)* animals, more than two and up to eight *lag-2::GFP*-expressing cells were observed (Fig. 1C). These extra DTCs are capable of leading the outgrowth of new gonad arms (Fig. 1C). Interestingly, the ectopic DTCs often do not obey normal migration cues and thereby can direct the gonad arm along unconventional migration paths (Fig. 1C).

In addition, no difference in the number of germ cell nuclei in *cki-1(RNAi)* and wild-type animals was found in the L1 and L2 stage before the extra DTCs were formed, indicating that the early divisions of Z2 and Z3 typical of *cki-1(RNAi)* are unlikely to contribute to the observed germ line hyperplasia (Table 1; Subramaniam and Seydoux, 1999).

To further confirm the previous findings, we removed the function of *cki-1* in *glp-1(e2141)* mutant animals. *glp-1* is a Notch-like receptor that transduces the *lag-2* signal emanating from the DTC to maintain proliferation of the distal germ cell nuclei (Kimble and Ward, 1988; Crittenden et al., 1994). If *cki-1* acts to block germ cell mitosis autonomously in the germ line, loss of *cki-1* in a *glp-1* background may lead to the formation of more germ cell nuclei than in *glp-1* alone. We found that *glp-1;cki-1(RNAi)* animals develop into sterile adults without any apparent differences in the population of mitotic germ cell nuclei, suggesting that the germ cell hyperplasia in *cki-1(RNAi)* animals occurs upstream of GLP-1, probably in controlling DTC numbers (data not shown).

In addition to extra DTCs, frequently *cki-1(RNAi)*-affected animals had one or no DTCs based on the absence of *lag-2::GFP* expression in the gonad arms. The DTC-lacking gonad arms of these animals showed no elongation or mitotic proliferation of the germ cell nuclei, suggesting that the absence of *lag-2::GFP* expression in these cells faithfully reflects the loss of DTC identity (Fig. 1D).

In order to test whether other somatic gonadal cell types might be affected by *cki-1(RNAi)*, we monitored the number of anchor cells present in *cki-1(RNAi)* animals. Wild-type hermaphrodites have one anchor cell that can be detected using a *lin-3::lacZ* or *cdh-3::GFP* reporter transgene (Hill and Sternberg, 1992; Pettitt et al., 1996). *cki-1(RNAi)* animals display more than one anchor cell since multiple cells stain for *lin-3::lacZ* and show *cdh-3::GFP* expression (Fig. 1E; data not shown).

In addition to the above-described gonad abnormalities, we also observed the formation of an ectopic gonad in *cki-1(RNAi)* animals (Fig. 1F and G). However, this occurs at low frequency and the gonad is unlikely to be functional, as all cells within this structure express the *lag-2::GFP* transgene, indicating that they are somatic in nature.

Extra distal tip cells in cki-1(RNAi) animals do not arise from pre-existing distal tip cells

Since the pleiotropic effects of the removal of *cki-1* function (such as the appearance of extra, or one, or no DTCs) indicate that these somatic gonadal phenotypes do not result exclusively from supernumerary divisions or duplications of progenitor cells within the gonad, we examined *cki-1(RNAi)* animals during various stages of development for the appearance of extra *lag-2::GFP*-expressing cells. As previously reported, extra DTCs were never observed prior to the L2 stage of development (Hong et al., 1998). If the extra DTCs in *cki-1(RNAi)* animals arise from a division of pre-existing 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, then the ablation of DTCs during the L2 stage would have no effect on the later formation of the extra DTCs. When the DTCs were ablated in wild-type L2-stage animals, none of the operated animals possessed DTCs as adults (data not shown; Hong et al., 1998). Surprisingly, the early ablation of DTCs in *cki-*

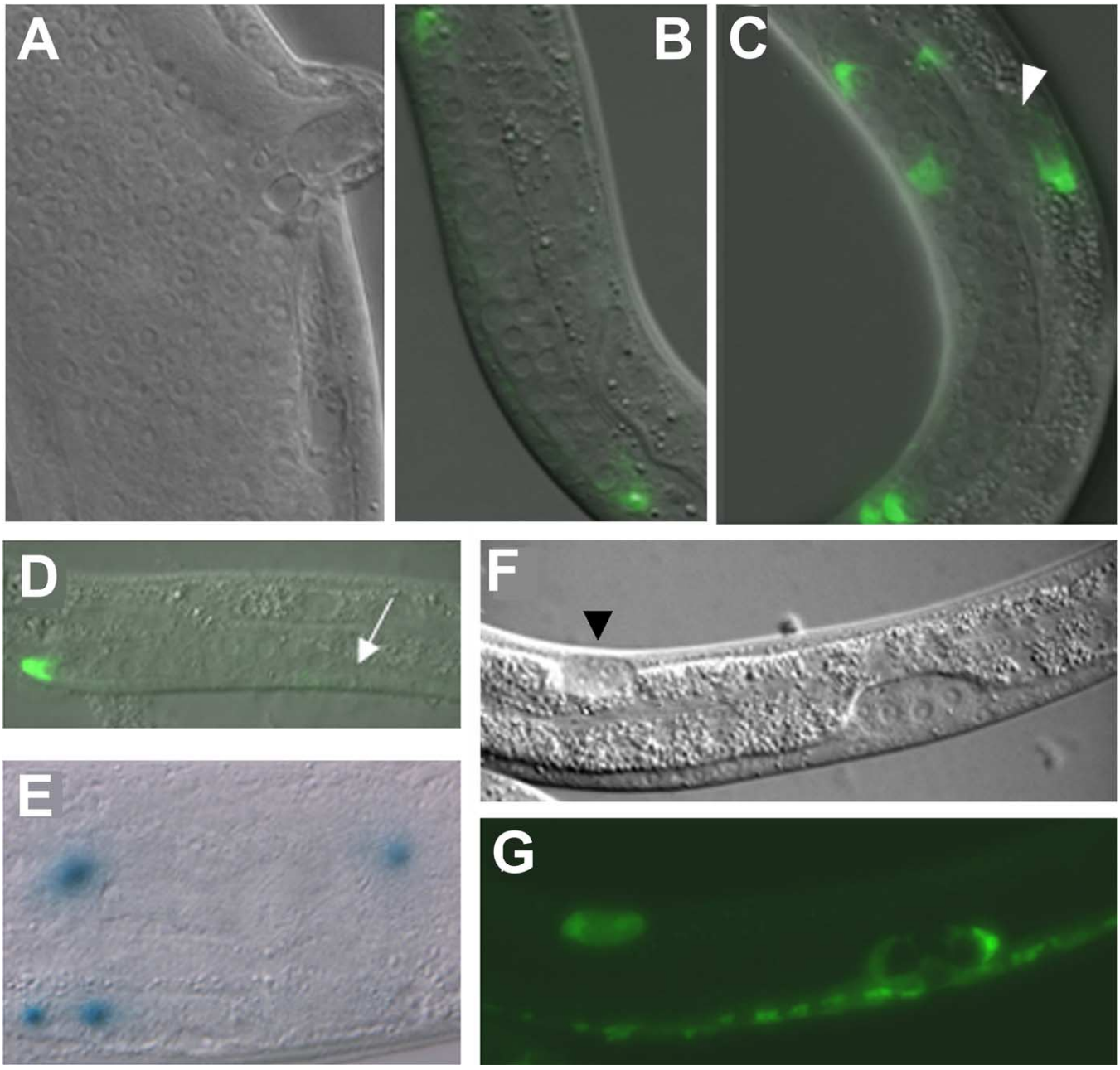

Fig. 1. *cki-1(RNAi)* animals exhibit multiple somatic gonad abnormalities. (A) Adult *cki-1(RNAi)* animals often lack oocytes and sperm, and show proximal proliferation and germ line hyperplasia in the proximal arms of the gonad (dorsal is left and anterior is up). (B) Wild-type L3 stage hermaphrodite showing two DTCs expressing the DTC-specific reporter *lag-2::GFP*. (C) *cki-1(RNAi)* L3-stage hermaphrodite showing extra DTCs expressing *lag-2::GFP*. The white arrowhead indicates the outgrowth of a new gonad arm lead by an extra DTC. (D) *cki-1(RNAi)* L3 hermaphrodite with only one *lag-2::GFP*-expressing cell, and only one elongating gonad arm. The white arrow delineates the distal extremity of the posterior gonad arm (anterior is left, dorsal is up). (E) *cki-1 (RNAi)* animals have more than one anchor cell that are distributed throughout the gonad as observed by *lin-3::lacZ* staining. (F) DIC image of an early L2-stage *cki-1(RNAi)* animal with two developing gonads (black arrowhead points to the ectopic gonad). (G) *lag-2::GFP* expression in the same animal. Note that all the cells in the ectopic gonad express the GFP reporter (anterior is left and dorsal is up).

1(RNAi) animals did not eliminate the formation of DTCs later in development (data not shown; Hong et al., 1998). This indicated that the extra DTCs produced in *cki-1(RNAi)* animals do not arise from pre-existing DTCs, but from the transformation of another cell type into a DTC, or a DTC precursor, before the L2 stage.

To identify whether a somatic gonadal precursor, or an alternative cell type could be transformed into a DTC, we ablated the somatic gonadal precursor cells, Z1 and Z4, or the germ line precursor cells, Z2 and Z3, during the early L1 stage (Table 2). When we eliminated the two germ line founder cells, Z2 and Z3, the operated wild-type animals were sterile, but the two DTCs were still formed (Table 2). In *cki-1(RNAi)*, Z2 and Z3 often undergo a premature divi-

Table 1
cki-1(RNAi) L2 animals have the same number of germ cells as wild-type

	Number of germ cells
N2	27.4 ± 4 (n = 20)
cki-1(RNAi)	28.9 ± 4 (n = 20)

Note. L2 stage animals with two distal tip cells were collected and their germ lines were examined by DIC. To be sure that *cki-1(RNAi)* animals were indeed affected, only L2 stage animals with extra intestinal cells [a visible indicator of *cki-1(RNAi)*] were scored. In each *cki-1(RNAi)* brood, approximately 50% of the F_1 progeny isolated 24 h after injection possessed extra DTCs at the adult stage.

sion (Subramaniam and Seydoux, 1999); therefore, we ablated the two (or four) germ line precursors, while leaving the somatic gonadal precursors (*lag-2::GFP*-expressing cells) unoperated. Most of *cki-1(RNAi)* animals (11/15) generated two or more DTCs after operation, but lacked a germ line. The remainder of the ablated *cki-1(RNAi)* animals had two or less DTCs [less than two DTCs is also a *cki-1(RNAi)*-related phenotype (Fig. 1D)]. 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 germ line for their formation.

If the Z1 and Z4 somatic gonad precursors are ablated in wild-type animals, no somatic gonadal cells are formed, including the DTCs (Table 2). This is also the case in *cki-1(RNAi)* animals, indicating that the extra DTCs must 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

The somatic gonad progenitor cells Z1 and Z4 divide in an invariant fashion to generate the 143 cells of the adult somatic gonad (Fig. 2A). The first division of Z1 and Z4 occurs in the mid-L1 and is asymmetric, giving rise to Z1.a and Z1.p, and Z4.a and Z4.p, respectively (Kimble and Hirsh, 1979). In order to further delineate the 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 (Fig. 2B and C, respectively). When Z1.a and Z4.p were ablated in wild-type animals, no DTCs were observed in operated adults (Fig. 2B; Table 3). However, in operated *cki-1(RNAi)* animals, DTCs were detected, indicating that the extra DTCs must arise from the Z1.p and Z4.a lineage, which never produces DTCs under normal conditions (Table 3).

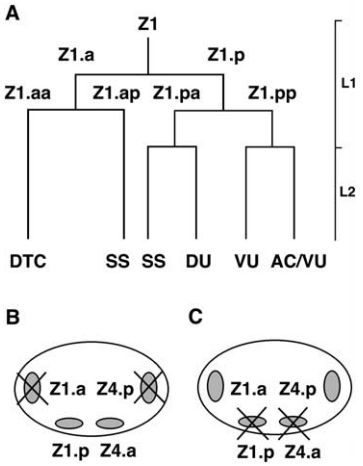

Fig. 2. The lineage of the somatic gonad precursor Z1. (A) The Z1 lineage (anterior) is a mirror image of the Z4 lineage (posterior). Z1 (and Z4) divide in the mid-L1 stage to give rise to Z1.a and Z1.p (the equivalent of Z4.p and Z4.a, respectively). Z1.a (and Z4.p) progresses through another division to give rise to the DTCs and the sheath and spermathecal cells. Z1.p (and Z4.a) divides to give rise to the sheath (SS) and spermathecal (SS), dorsal uterine (DU), ventral uterine (VU), and the anchor cell (AC). The 12 cells that make up the L2-stage somatic gonad primordium will then remain undivided until the mid-L3 stage. (B) Schematic diagram for the ablation of the Z1.a/Z4.p lineage and (C) the Z1.p/Z4.a lineage.

To confirm this, the reciprocal experiment was performed where Z1.p and Z4.a in wild-type or *cki-1(RNAi)* animals were ablated (Fig. 2C; Table 2). When the Z1.p and Z4.a cells were ablated in wild-type animals, two DTCs were formed. In the majority of operated *cki-1(RNAi)* animals, extra DTCs were still observed, indicating that the Z1.a and Z4.p lineage, which had remained unoperated, can also give rise to the extra DTCs in *cki-1(RNAi)* animals (Table 3).

In order to determine whether a similar mechanism could give rise to the extra anchor cells in *cki-1(RNAi)* animals, we ablated Z1.p and Z4.a, the two cells which normally produce the anchor cell precursors, and we assessed whether vulval fates were induced in wild-type and *cki-1(RNAi)* animals (Table 3) (Miskowski and Kimble, 2001). Whereas wild-type animals in which Z1.p/Z4.a were ablated were consistently vulvaless, due to the absence of a functional anchor cell, *cki-1(RNAi)* animals occasionally executed vulva formation, suggesting that the anchor cells in *cki-*

Table 2
Extra DTCs in *cki-1(RNAi)* animals arise from the somatic gonad and not from the germ line precursors Z2/Z3

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

Note. The second day F_1 progeny of *cki-1(RNAi)* animals or wild-type, uninjected animals, at the L1 stage of development were anesthetized and the Z1/Z4 somatic gonad precursor cells were ablated, or alternatively, the Z2/Z3 germ line founder cells were ablated. A group of *cki-1(RNAi)* L1 larvae were left unoperated to confirm the penetrance of the *cki-1(RNAi)* effect. Both the ablated 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. The numbers in brackets indicate the number of animals with the indicated DTC number over the total number of animals scored.

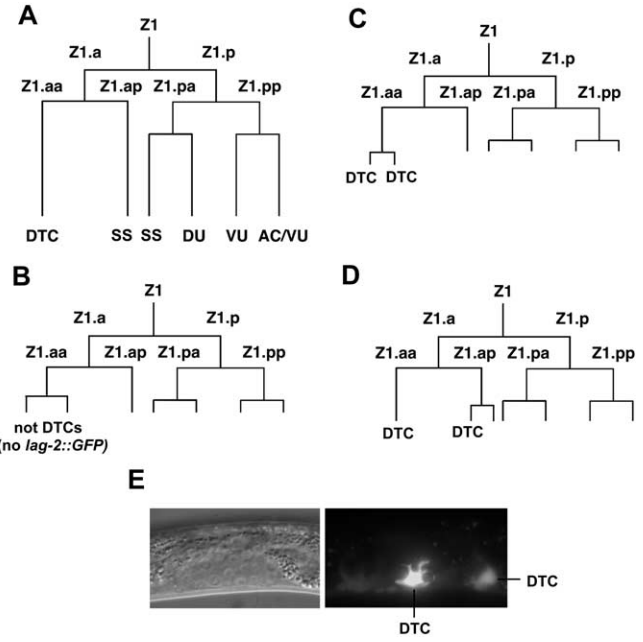

Fig. 3. Loss of *cki-1* disrupts timing and integrity of the somatic gonadal precursor divisions. (A) Wild-type somatic gonadal lineage. (B) In one class of *cki-1(RNAi)* animals, the presumptive DTC (Z1.aa) underwent an additional division, giving rise to two cells, none of which became DTCs ($n = 3$). (C) Alternatively, we observed DTC precursors that divided to give rise to two DTCs ($n = 2$). (D) In this case, the sister cell of the DTC (SS precursor) divided once more to give rise to a DTC, which was not distally located ($n = 1$). (E) We also observed L2-stage *cki-1(RNAi)* animals where strong *lag-2::GFP* expression (more typical of a late L3-stage DTC) was seen in several cells of the somatic gonad, which did not arise from Z1.aa or Z4.pp, and eventually formed up to nine DTCs ($n = 2$).

Table 3
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		Number of animals with vulva induction	
Cells ablated	Wild-type	*cki-1(RNAi)*	Wild-type	*cki-1(RNAi)*
Z1.a/Z4.p	0 (6/6)	0 (10/16) 1 (1/16) 2 (3/16) 3 (2/16)	N/D	N/D
Z1.p/Z4.a	2 (3/3)	6 (1/8) 3 (4/8) 2 (1/8) 1 (1/8) 0 (1/8)	0/4	6/11

Note. The second day F_1 progeny of *cki-1(RNAi)* or wild-type, uninjected animals, at the L1 stage of development were anesthetized 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)* L1 larvae 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 vulva (confirmed by cell division pattern), or ectopic pseudovulvae, were scored. The numbers in brackets indicate the number of animals with the indicated DTC number over the total number of animals scored.

Table 4
The formation of ectopic distal tip cells is not dependent on cell division during the L2 and L3 stage

cki-1 (RNAi) without HU	WT + HU (40 mM)		*cki-1 (RNAi)* + *HU (40 mM)*	
	L2	L3	L2	L3
47% ($n = 67$)	0% ($n = 76$)	0% ($n = 68$)	38% ($n = 87$)	46% ($n = 60$)

Note. Animals in L2 or L3 lethargus were isolated and placed on NGM plates containing 40 mM hydroxyurea (HU) for 12 h and then transferred back to regular seeded plates and allowed to develop to the adult stage. The effect of the drug was verified by evaluating stage-specific cell divisions in the vulval lineage. All animals treated with HU became sterile. Extra DTCs were scored in adult animals by monitoring the *lag-2::GFP* expression in the DTCs: The values indicated are representative of two independent experiments.

1(RNAi) animals can be formed from a cell outside the Z1.p/Z4.a lineage, similar to the situation observed for the DTCs.

Ectopic DTCs arise from the generation of a DTC precursor prior to the L2 stage and does not require further cell divisions

The early cell divisions that lead to the formation of the somatic gonadal primordium occur by the L2 stage and are followed by a period of mitotic quiescence, and then by a series of short-range migrations of these cells (Kimble and Hirsh, 1979). To determine the time period when the extra DTCs arise in *cki-1(RNAi)* animals, and whether they are produced from supernumerary cell divisions caused by the inability to maintain the L2 quiescence, we placed L2- and L3-stage *cki-1(RNAi)* animals on plates that contained hydroxyurea (HU) (Euling and Ambros, 1996; Ambros, 1999). If the loss of *cki-1* causes extra cell divisions in the somatic gonad during the period of quiescence during the L2, thereby giving rise to the ectopic DTCs, placing the *cki-1(RNAi)*-affected animals on HU plates would block these divisions, and no extra DTCs would be observed. However, if *cki-1* has an early role in the coordination of the initial somatic gonadal divisions and the proper specification of cell fates in the somatic gonad, the animals treated with HU would still produce ectopic DTCs.

When wild-type animals expressing the *lag-2::GFP* transgene were grown 12 h on HU beginning at the L2, or L3 stage, and subsequently removed to plates without the drug and allowed to develop to adulthood, two DTCs were formed, although all stage-specific cell divisions were blocked by HU. Therefore, this HU treatment has no effect on the production or maintenance of this cell type. However, when early L2- or L3-stage *cki-1(RNAi)* larvae were treated with HU, extra DTCs were still generated (Table 4). Taken together, our data suggest that the extra DTCs in these animals are not the result of additional cell divisions

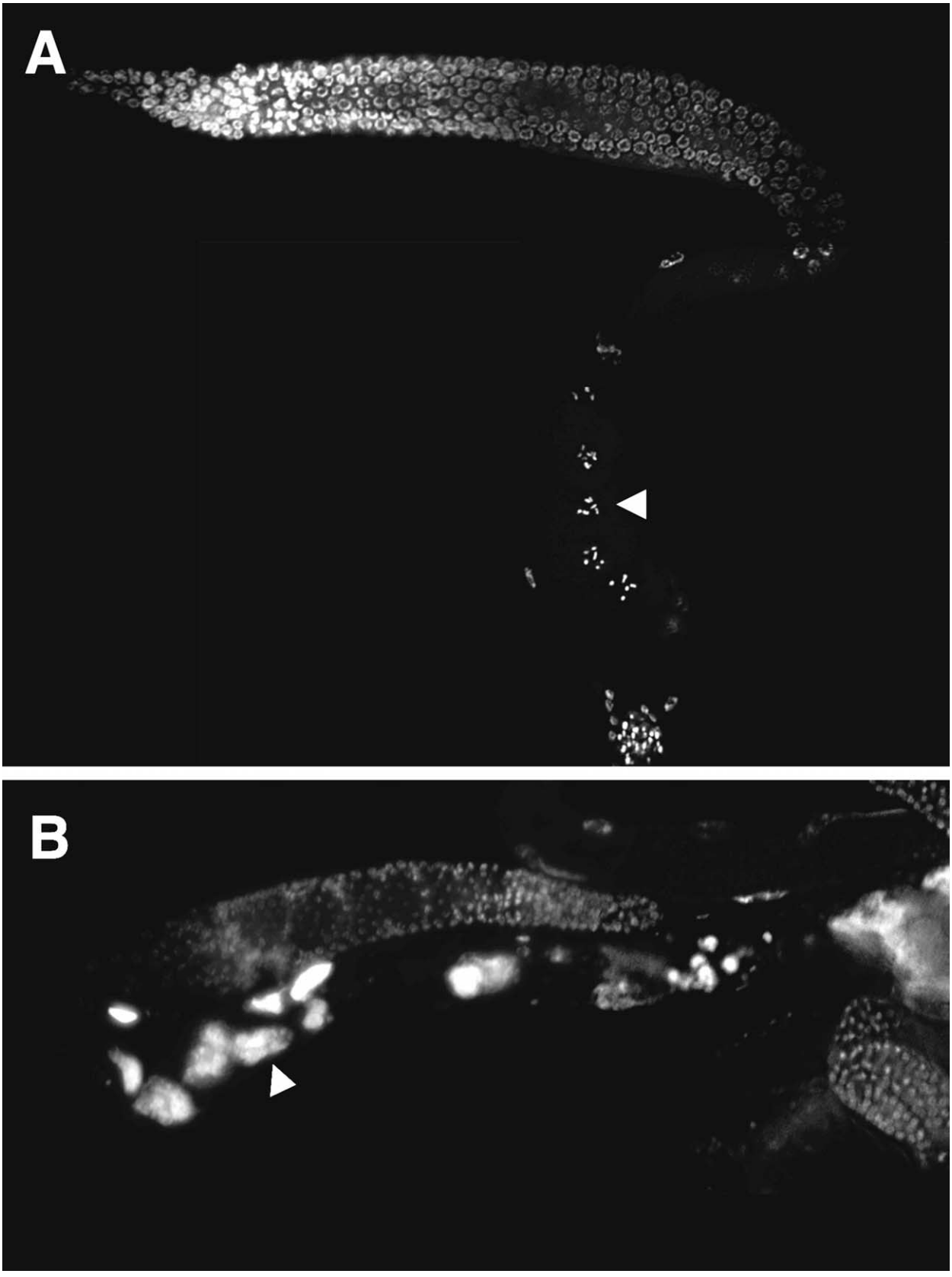

Fig. 4. *cki-1(RNAi)* animals form highly polyploid endomitotic oocytes. (A) Wild-type gonad arm stained with DAPI shows haploid oocytes which are arranged in the proximal arm of the gonad (arrowhead indicates diakinetic chromosomes of oocytes). (B) *cki-1(RNAi)* animals show highly polyploid oocytes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

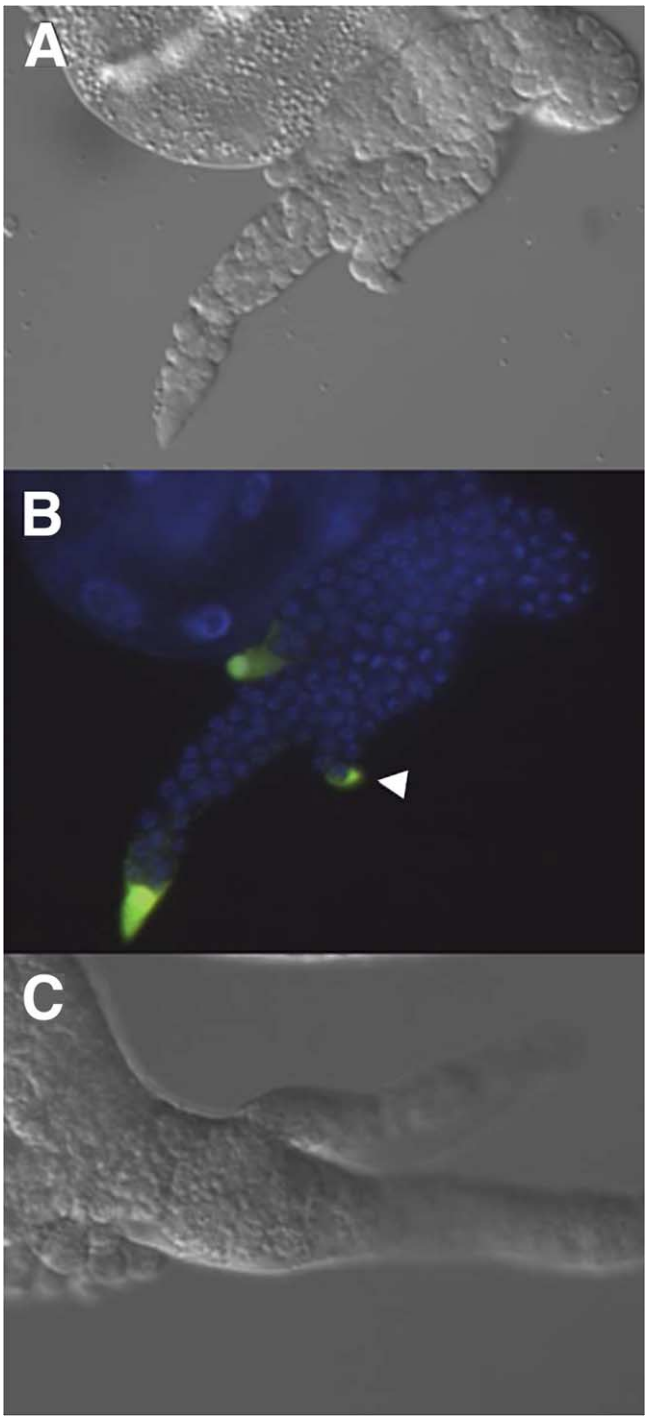

Fig. 5. *cki-1(RNAi)* animals form "branched" gonad arms. Gonad arms dissected from *cki-1(RNAi)* young adult hermaphrodites show a distinct fork-like appearance typical of the initiation of a new outgrowth from an existing gonad arm. (A) A DIC image showing a "branched" arm. (B) DAPI stained gonad overlaid onto the GFP image. Here, the DTC (arrowhead), which can be seen leading the outgrowth of a branch from the main gonad arm, shows *lag-2::GFP* expression. (C) A DIC image of a second "branched" gonad arm in a *cki-1(RNAi)* animal.

that occur after the L2 stage in the absence of *cki-1*, but rather by abnormalities in the somatic gonadal divisions that occur during, or prior to, the L2 stage.

Lineage analysis indicates that cki-1 ensures division integrity in the early somatic gonadal divisions

As the HU experiments distinguished that extra DTCs occurred primarily before the L2 stage, we performed lineage analysis to characterise how the loss of *cki-1* affects cell division in this lineage. We recorded the cell divisions in the somatic gonad from several *cki-1(RNAi)* animals during the L1 and L2 stages of development. Animals were then recovered and the final number of DTCs observed in the adults was noted. The divisions and appearance of the somatic gonad were highly variable among the observed animals likely due to RNAi penetrance effects, and the lineages reported can therefore not be considered representative. However, we identified four classes of lineage abnormality that correlated with the *cki-1(RNAi)*-related variability in DTC number.

Fig. 3A shows a wild-type somatic gonadal lineage (Z1). In one class of *cki-1(RNAi)* animals, the presumptive DTC, Z1.aa (before *lag-2::GFP* became strongly expressed) underwent an additional division, giving rise to two cells, none of which became DTCs, nor expressed the *lag-2::GFP* marker afterward (Fig. 3B). Alternatively, in a second class, we observed DTC precursors that divided to give rise to two DTCs, both of which expressed the *lag-2::GFP* reporter strongly (Fig. 3C). Finally, we observed a sister cell of the DTC (SS precursor) that executed an additional division to give rise to a DTC that was not positioned distally, and expressed *lag-2::GFP* strongly (Fig. 3D). We also observed *cki-1(RNAi)* animals where strong *lag-2::GFP* expression was apparent in several cells of the L2-stage somatic gonad, which did not arise from the typical DTC precursors (Fig. 3E); and eventually formed up to nine DTCs. In the pool of animals that were lineaged, there were four animals that had a highly disorganized gonad due to mispositioned cells as early as the L1 stage, where it was difficult to determine the identity of the early somatic gonadal cells. Our lineage analysis suggests that loss of *cki-1* does not cause premature cell division of Z1/Z4 in this lineage, but results in alterations in cell division timing and daughter cell fates beginning at the initial asymmetric division of Z1/Z4. We therefore conclude that *cki-1* plays a role in regulating the timing of the early somatic gonadal cell divisions (after the initial Z1/Z4 division) and/or the correct specification of the resultant daughter cell fates.

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

From our lineage analysis, we found that the sheath cell precursors were capable of forming DTCs. The sheath cells arise from four SS (sheath/spermatheca precursor) cells following asymmetric divisions during the first and second larval stage (Kimble and Hirsh, 1979). Cell ablation of a single SS precursor has been shown to cause defective ovulation due to the inability of the mature oocytes to enter

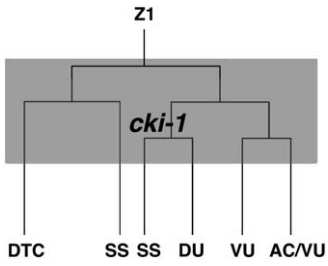

Fig. 6. Model for *cki-1(RNAi)*-induced extra somatic gonadal cells. *cki-1* plays a role in coordinating early somatic gonadal divisions and cell fate specification in wild-type. The typical quiescence of Z1 and Z4 is not exclusively under *cki-1* control, but the timing and appropriate fate of their daughters are regulated by *cki-1* in a direct or indirect manner by coordinating cell cycle phase with signals that specify cell fate.

the spermatheca and become fertilized. This causes oocytes to exit their typical diakinesis stage and undergo multiple cycles of DNA synthesis to form polyploid endomitotic (Emo) oocytes (McCarter et al., 1997).

Anti-CEH-18 staining in *cki-1(RNAi)* animals showed that the sheath cell number and arrangement was affected. CEH-18 is a marker of sheath cell differentiation, and in wild-type adult animals, there are 10 thin gonadal sheath cells per arm (Rose et al., 1997; Hall et al., 1999). *cki-1(RNAi)* animals, however, display highly variable numbers of sheath cells, with either less or more sheath cells per gonad arm (Table 5). DAPI staining revealed that the few oocytes that do form in the gonad arms of *cki-1(RNAi)* animals are indeed highly polyploid, consistent with the Emo phenotype caused by sheath cell defects (Fig. 4) (McCarter et al., 1997).

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 cell precursors. Interestingly, upon dissection of *cki-1(RNAi)* gonad arms, branched arms were occasionally observed, where an ectopic DTC is present proximal to a "normal" DTC in a position that would typically be occupied by a sheath cell (Fig. 5). This newly formed DTC is apparently capable of inducing germ line mitoses, and can direct the outgrowth of a new arm, leading to the formation of a branched gonad arm. This observation could be explained either by a transformation of a sheath cell into a DTC later during development, or by the migration of extra DTCs and their breaking through the layer of sheath cells to form a branched arm by directing outgrowth from the basal lamina surrounding the gonad arm.

Discussion

Extra DTCs in cki-1(RNAi) animals do not arise from pre-existing DTCs

We have shown that the prominent germ line hyperplasia associated with *cki-1(RNAi)* is not due to an autonomous role of *cki-1* within the germ cells themselves, but rather an indirect role of *cki-1* in coordinating cell divisions and cell fate specification in the somatic gonadal precursors. Using a *lag-2::GFP* reporter, we show that extra DTCs are produced following *cki-1(RNAi)* and that each of these seem to adopt the proper DTC fate as they are able to induce germ line mitosis, form gonad arms, and lead their outgrowth, indicating that *cki-1* does not block the ability of this cell type, nor the ability of many other cells to terminally differentiate, although a role in this process should not be ruled out. In addition, we show that extra anchor cells and other somatic gonadal defects can also arise in *cki-1(RNAi)* animals.

Most hyperplasia associated with the loss of the $p27^{KIP1}$ homologues is due to precocious or additional divisions of blast cells, and the corresponding expansion of the initial population of these cell types (Nakayama et al., 1996; Hong et al., 1998). In contrast to what is observed in the germ cell precursors Z2 and Z3, our examination of the early somatic gonad in *cki-1(RNAi)* animals did not reveal any premature divisions in Z1 and Z4, despite the strong expression of *cki-1::GFP* in these cells during this period (Hong et al., 1998). This suggests that *cki-1* may not be acting alone to inhibit cell division in these cells, or that it may have some additional, cell cycle-independent function.

By performing laser ablations of the DTCs in wild-type or *cki-1(RNAi)* animals at the L2 stage showed that the extra DTCs in *cki-1(RNAi)* animals are formed from a cell type other than the existing DTCs, thereby ruling out the possibility that DTCs, or their immediate precursors, simply duplicate in the absence of *cki-1*. Moreover, this would also explain the production of other somatic gonadal cells, such as the anchor cells, which do not normally express *cki-1::GFP* and probably do not arrest their divisions in a *cki-1*-dependent manner (Hong et al., 1998).

Extra DTCs in cki-1(RNAi) animals arise from the transformation of other somatic gonad progenitors

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 forma-

Table 5
cki-1(RNAi) animals demonstrate variability in sheath cell number

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

Note. 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 than, or less than, the normal complement of sheath cells (10).

tion of the extra DTCs later during development. The observation that the extra anchor cells in *cki-1(RNAi)* animals may also arise from a lineage which does not normally produce this cell type suggests that the somatic gonadal precursors display an inherent flexibility in the fate that these cells can adopt when division timing, or more precisely, S-phase progression is altered by removing *cki-1*. Interestingly, similar fate alterations have been reported in *sys-1/pop-1* mutants which have irregularities in the proximal/distal integrity of these early divisions (Siegfried and Kimble, 2002). Therefore, loss of *cki-1* could affect appropriate segregation of POP-1 to daughter cells, consequently disrupting its role in cell fate specification.

We conclude that a cell type which arises from the divisions of both the Z1.a/Z4.p and Z1.p/Z4.a lineage is transformed into the extra DTCs in *cki-1(RNAi)* animals. Lineage analysis of *cki-1(RNAi)* animals showed that cells within the somatic gonad are capable of undergoing additional early divisions that disrupt the normal specification of daughter cell fates. This suggests that *cki-1* blocks the further divisions of the DTC precursor so that it can acquire or fix the DTC cell fate. Additionally, other cells can reiterate their mother cell divisions (such as the SS precursor cells), and give rise to DTCs, thus confirming the previous finding. This supports the notion that *cki-1* plays an important role in allowing cells to acquire and/or maintain cell fates, presumably by blocking cell division to coordinate cell cycle phase with appropriate signals necessary for cell fate specification.

We then investigated whether *cki-1(RNAi)* animals demonstrate abnormalities in other somatic gonadal cell types. Anti-CEH-18 staining results confirmed that the sheath cell lineage was abnormal. Furthermore, like other mutants that disrupt sheath cell function or number, such as *ceh-18* or *lin-26, cki-1(RNAi)* animals also have endomitotic oocytes. Although loss of a negative cell cycle regulator could cause this Emo phenotype, it is improbable since the loss of *cki-1* in other lineages does not cause inappropriate or increased endoreplication (data not shown; Kostic et al. 2002). *cki-1(RNAi)*-induced extra gonadal arms also show a dramatic reduction in the number of germ cell nuclei per arm, which might also be indicative of the incorrect number and arrangement of sheath cells in these animals (McCarter et al., 1997; data not shown).

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

In the presence of *cki-1*, somatic gonadal cell divisions proceed as shown in Fig. 6. Two different possibilities outline the possible mechanisms of *cki-1(RNAi)*-induced cell fate transformations, as observed by lineaging *cki-1(RNAi)* animals. The first involves additional early divisions in the somatic gonad, so that the somatic gonadal founder cells may undergo an additional division where the daughter cells reiterate their mother cell fates. However, *cki-1(RNAi)* animals often lack DTCs; therefore, another scenario where the integrity of the initial divisions is aberrant might account for this phenotype. Thus, there may be a direct transformation of one somatic gonadal cell type into another as a result of these deregulated divisions. Our lineage analysis demonstrated that precursors, or the ectopic DTCs themselves, are formed early, consistent with both of these possibilities.

Although CKIs have been predominantly described in cell division control, evidence that these molecules may have direct roles in cell fate specification or maintenance and/or timing of differentiation has also been demonstrated (Durand et al., 1998; Ohnuma et al., 1999). 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 retina 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.

The 12 cells of the somatic gonad primordium are formed during the early L2 stage and give rise to distinct cell types, but remain undivided until the mid-L3. CKI-1 could therefore play a direct, or indirect, role in the initial cell fate acquisition of these somatic gonadal cell types, until 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 to peak in 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). If CKI-1 plays a similar role as p27Xic in the *C. elegans* somatic gonad, then the removal of its activity may be responsible for the alternative fates that these cells acquire in its absence.

In summary, we have determined that extra DTCs in *cki-1(RNAi)* animals do not result from the duplication of pre-existing DTCs, but from cell fate alterations likely caused by aberrant division patterns of somatic gonadal precursors that occur during postembryonic development. The loss of *cki-1* in these cells uncovers their inherent ability to recapitulate earlier fates and acquire abnormal ones, thereby generating extra DTCs, anchor cells, and sheath cells, all of which have critical roles during gonadogenesis. This further emphasises the importance of this CKI during the specification of cell fates in this organ and potentially in many others throughout the course of development.

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