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Essential embryonic roles of the CKI-1 cyclin-dependent kinase inhibitor in cell-cycle exit and morphogenesis in *C. elegans*

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Abstract

Following a phase of rapid proliferation, cells in developing embryos must decide when to cease division and then whether to survive and differentiate or instead undergo programmed death. In screens for genes that regulate embryonic patterning of the endoderm in *Caenorhabditis elegans*, we identified overlapping chromosomal deletions that define a gene required for these decisions. These deletions result in embryonic hyperplasia in multiple somatic tissues, excessive numbers of cell corpses, and profound defects in morphogenesis and differentiation. However, cell-cycle arrest of the germline is unaffected. Cell lineage analysis of these mutants revealed that cells that normally stop dividing earlier than their close relatives instead undergo an extra round of division. These deletions define a genomic region that includes *cki-1* and *cki-2*, adjacent genes encoding members of the Cip/Kip family of cyclin-dependent kinase inhibitors. *cki-1* alone can rescue the cell proliferation, programmed cell death, and differentiation and morphogenesis defects observed in these mutants. In contrast, *cki-2* is not capable of significantly rescuing these phenotypes. RNA interference of *cki-1* leads to embryonic lethality with phenotypes similar to, or more severe than, the deletion mutants. *cki-1* and *-2* gene reporters show distinct expression patterns; while both are expressed at around the time that embryonic cells exit the cell cycle, *cki-2* also shows marked expression starting early in embryogenesis, when rapid cell division occurs. Our findings demonstrate that *cki-1* activity plays an essential role in embryonic cell cycle arrest, differentiation and morphogenesis, and suggest that it may be required to suppress programmed cell death or engulfment of cell corpses.

Keywords: *Caenorhabditis elegans*; Cell cycle; Programmed cell death; Organogenesis; Cyclin-dependent kinase inhibitor

Introduction

During early embryogenesis cells initially undergo rapid and continuous cell proliferation. After many rounds of division, they subsequently receive cues that arrest their division at specific stages and cause them to differentiate or instead to undergo programmed cell death. Although many pathways have been identified that control cell fate specification and tissue patterning, little is known about how these developmental cues regulate cellular processes such as the cell cycle, cell migration, cell shape change and programmed cell death.

The nematode *C. elegans* is a useful system with which to study this interplay between developmental signals and regulation of cellular behavior. *C. elegans* embryos develop within the protective confines of a durable eggshell that separates the developing embryo from its external environment, obviating cell growth between successive cell divisions. Maternal products are stockpiled in the unfertilized oocyte, whose cytoplasm is partitioned into 558 cells by the completion of embryogenesis. Different tissues, and even different sub-lineages within a single tissue, contain cells that become post-mitotic at distinct times during embryogenesis (Sulston et al., 1983). Thus, the regulation of cell cycle events, such as periodicity of division and time of mitotic exit, is intrinsic to each cell lineage and must respond to cell fate specification signals to arrest the cells at the appropriate time. While major advances have been made in our understanding of how early blastomeres in *C. elegans* are specified (reviewed in Bowerman, 1998; Newman-

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Smith and Rothman, 1998; Rose and Kemphues, 1998; Labouesse and Mango, 1999), the factors that respond to specification cues and cause cells to divide and arrest division at the appropriate time have been largely unexplored.

Here we report the embryonic function of the *C. elegans cki-1* and *cki-2* genes, which are members of the conserved Cip/Kip cyclin-dependent kinase inhibitors (CKI) family (Hong et al., 1998; Feng et al., 1999; Boxem and van den Heuvel 2001), and demonstrate that these genes mediate cell cycle arrest, morphogenesis and differentiation, and suppression of programmed cell death. The vertebrate Cip/Kip CKIs have been shown to directly inhibit the activity of cyclin/cyclin-dependent kinase (cdk) complexes that control G1/S progression, leading to G1 arrest (Sherr and Roberts, 1995). The *Drosophila dacapo* gene, also a Cip/Kip inhibitor, is required to arrest embryonic epidermal cell proliferation at the correct developmental stage (de Nooij et al., 1996; Lane et al., 1996). *dacapo* is transiently expressed in the late G2 phase preceding the terminal division. Premature *dacapo* expression results in G1 arrest in both embryonic and postembryonic tissues.

We find that mutants in which both of the adjacent *cki-1* and *cki-2* genes are deleted show widespread embryonic hyperplasia in many tissue types, accumulation of excessive numbers of programmed cell death corpses, and profound defects in morphogenesis of the body and the feeding organ, the pharynx. In particular, cells that normally exit the cell cycle before their close relatives undergo an additional round of division, implying that cells of the same tissue type and lineage employ distinct molecular mechanisms to arrest the cell cycle. We found that *cki-1* alone can efficiently rescue the hyperplasia, morphogenesis, and cell death defects in the deletion mutants. Moreover, RNA interference of *cki-1*, but not *cki-2*, phenocopies the deletion mutants. The *cki-1* reporter gene is expressed when embryonic cells become post-mitotic. Unexpectedly, we found that *cki-2* is expressed continuously in many cells starting from early embryogenesis, when cells are rapidly dividing. Our results demonstrate that *cki-1* acts in some, but not other closely related lineages to promote timely exit from the cell cycle and also regulates morphogenesis and differentiation in response to developmental cues during embryogenesis.

Materials and methods

Strains and alleles

Nematode strains were maintained according to Brenner (1974). Experiments were conducted at 20°C, except when noted. The following strains were provided by the *C. elegans* Genetics Center (CGC) unless otherwise noted. SP784: *unc-4(e120) mnDf100/mnC1[dpy-10(e128) unc-52(e444)] II.* (Sigurdson et al., 1984). SP790: *unc-4(e120) mnDf103/mnC1[dpy-10(e128) unc-52(e444)] II.* (Sigurdson et al., 1984). ML335: *dpy-2(e489) mcDf1 unc-4(e120)/mnC1[dpy-10(e128) unc-52(e444)] II.* (Labouesse, 1997). MT2551: *ced-4(n1162) dpy-17(e164) III.* (Ellis and Horvitz 1986). MT4770: *ced-9(n1950) III.* (Hengartner et al, 1992). MT1522: *ced-3 (n717) IV* (Ellis and Horvitz, 1986).

The following transgenic lines, which contain integrated arrays, were used: JR368: *wIs21*: expresses *lacZ::gfp* driven by the *ges-1* promoter in the embryonic gut. JR1120: N2; *wIs84*: expresses *lacZ::gfp* driven by the *elt-2* promoter in the embryonic gut.

Plasmid constructions

pMF27 contains the *cki-1* genomic fragment including 6 kb upstream from the putative start codon and 0.6 kb downstream of the poly A addition site in pCR2.1 (Invitrogen). pMF14 contains the *cki-2* genomic fragment including 4.4 kb upstream from the putative start codon and 0.2 kb downstream from the poly A addition site subcloned in pCR2.1. *cki-1* or *-2*::GFP reporters: pSG18 and pMF80.3 contain 2 kb and 6kb of *cki-1* upstream sequence subcloned into pPD96.04, respectively (Fire et al., 1990). pSG22 contains 4 kb *cki-2* upstream sequence subcloned into pPD96.04. Heat-shock constructs: pSG13.16 and pSG14.16 include the open reading frame of *cki-1* in pPD49.78 and pPD49.83, respectively. pMF28.2 and pMF29.1 contain the open reading frame of *cki-2* in pPD49.78 and pPD49.83, respectively.

Genomic fragments were amplified by PCR, using purified T05A6 with high-fidelity LA taq (TAKARA) and primers containing an appropriate restriction site. Primers used were: pMF27: ATGCGGCCGCCGGTTTGAAGGACGCAATT and ATGGGCCCTCCCGTTCCCTCTTCAA, pMF14: TGCCACCCAAATAGACATTGCGTGAAGA and ATAATTCAGCAAATTCCTCATCAGT, pSG18: AACTGCAGAATATGGGCGGAGCGAAG and CGCGGATCCCGACGAGCAGCCGAAATTTG, pMF80.3: AGTCGACATTGAAAAGAGTGAGGAAACCTGGA and TGCATGCCGGTTTGAAGGTACGCAATTTA, pSG22: CCCAAGCTTAGGGAACGGGAGGGATATTA and CGGGATCCTTGCCCCGATTTGTGTTA, pSG13.16 and pSG14.16: CTAGCTAGCATGTCTTCTGCTCGTCT and GGGGTACCCTAGTATGGAGAGCATGAA, pMF28.2 and pMF29.1: CTAGCTAGCATGGCGGCAACAACAGCCGGCGC and ATGGGCCCGTATCAAACATGGATCTGGAAAAAGCCA.

Mutagenesis

Young adults of strain JR368 were treated with 50 mM ethylmethanesulfonate (Brenner, 1974). ~7,000 F1 progeny (14,000 mutagenized haploid genomes) from mutagenized hermaphrodites were cloned to individual plates and scored for F2 embryonic lethality. Over 400 independent lines were examined by Nomarski microscopy and two independent lines were identified that displayed the hyperplastic embryonic phenotype characteristic of the *mnDf100* phenotypes. One line was lost, the other is defined by the defi-

ciency *wDf5 II. wDf5* is a zygotic embryonic lethal. The strain is propagated as a heterozygote balanced by *mnC1*.

RNAi, germline transformation, and rescue experiments

The *cki-1* and *cki-2* cDNAs (yk390e10 and yk374e4, respectively) were provided by Yuji Kohara. Using PCR-amplified cDNAs as templates, double-stranded(ds) RNAs were synthesized in vitro with the Megascript kit (Ambion). 1 to 2 mg/ml of annealed dsRNA was injected into the gonad of young adult worms. As has been reported for other genes (e.g., the *med* genes; Maduro et al., 2001), we noticed that injection of *cki-1* dsRNA into the gonad causes greater embryonic lethality than is observed when injected into the body cavity. Moreover, we found that the embryonic lethality induced by *cki-1(RNAi)* peaks within 15 hours of injection. Thus, embryos laid between 0 and 15 hours after injection were collected for phenotypic analysis.

DNA transformation was performed as described by Mello et al. (1991). To determine whether transgenes can rescue the phenotypes of homozygous *mnDf100* embryos, we used embryonic elongation and pharynx differentiation/morphogenesis as evidence for rescue. The F1 progeny of SP784 [*unc-4(e120) mnDf100/mnC1 [dpy-10(e128) unc-52(e444)]II*; Sigurdson et al., 1984] mothers show 26% (n = 911) embryonic lethality with a uniform terminal arrest phenotype, indicating that *mnDf100* causes 100% embryonic arrest. SP784 mothers carrying an extrachromosomal array with cosmid T05A6 and pRF4 [*rol-6(d)*] produce 27% (n = 422) arrested F1 embryos, 60% of which contain a well-developed pharynx. These percentages imply that the arrested embryos with a well-developed pharynx are homozygous *mnDf100* embryos rescued by the array and not their heterozygous *mnDf100* or homozygous balancer (*mnC1[dpy-10(e128) unc-52(e444)]*) siblings. To test if the *cki-1* transgene alone can rescue the *mnDf100* phenotypes, pMF27, pRF4, and pTG96.2 (which carries the *sur-5::gfp* marker; Yochem et al., 1998) were co-transformed into SP784 mothers. The resultant five lines each produced arrested embryos with a well-developed pharynx. All such embryos carried the array based on expression of the *sur-5::gfp* marker, thus confirming rescue. For subsequent analysis of nuclear numbers and cell corpses, we used one of the lines, JR1420, which gave high transmission of the array. JR1420 mothers produce 33% (n = 242) arrested F1 embryos, indicating that approximately one-quarter of the arrested embryos are not *mnDf100* homozygotes. However, among unhatched F1 progeny, 46% (n = 359) showed a well-developed pharynx, indicating that the *cki-1* transgenic array can rescue the defects in pharynx formation in homozygous *mnDf100* embryos. Although we cannot exclude the possibility that up to half of the "rescued" embryos are not *mnDf100* homozygotes, comparison with the average number of nuclei and cell corpses in WT, rescued, and unrescued embryos indicate that the *cki-1* transgenic array has rescuing activity for the extra nuclei and cell corpse phenotypes.

Nuclear counts

To estimate the number of nuclei in embryos from each genetic background, embryos were squashed between a slide and a coverslip, and subsequently fixed and mounted with the glycerol-based media containing 2 μg/ml of diamidinophenolindole (DAPI) as described in Miller and Shakes, 1995. The number of nuclei was scored in well-squashed embryos by obtaining images with a CCD camera attached to a Nikon Microphot SA microscope. The ages of the embryos were as follows: N2: 5 hours post-laying at 25°C. Homozygous *mnDf100*, rescued *mDf100* and *cki-1(RNAi)* embryos: 15 hours post-laying at 20°C or 12 hours post-laying at 25°C.

Analysis of cell death phenotypes

Developmental profiles of cell death corpses were analyzed as described by Sugimoto et al. (2001). JR1420 was used for the deficiency mutant rescue experiments. Embryos were observed continuously from the early bean stage, and the genotypes deduced from the terminal phenotypes, as well as the presence of GFP expression derived from the array. Arrested animals were scored as homozygotes if they showed the *mnDf100* phenotypes (see Results), and were considered rescued if they showed GFP expression, evidence of elongation, and pharynx differentiation (e.g., see Fig. 6). *ced; mnDf100* double mutants were generated by crossing *mnDf100*/+ males into either of the *ced* mutant hermaphrodites described above.

Results

Identification of a genomic region required for normal embryonic cell cycle arrest

From a genome-wide screen for point mutants defective in patterning of the endoderm, we identified a mutation that leads to supernumerary gut nuclei and zygotic embryonic lethality (see Materials and methods. data not shown). Genetic and physical mapping revealed that this mutation is a chromosomal deficiency (deletion), henceforth called *wDf5*, that removes at least 200 kb of DNA in the middle of linkage group II (Fig. 1). Several previously identified overlapping deficiencies that delete this region were found to result in similar phenotypes (Figs. 2A and 2B). The phenotype of the smallest of these, *mnDf100*, was studied further. Embryos homozygous for *mnDf100* display penetrant pleiotropic defects. As apparent by Nomarski microscopy, these embryos fail to undergo the normal anteroposterior body elongation; in addition, morphogenesis and differentiation of the internal organs, in particular the pharynx and intes-

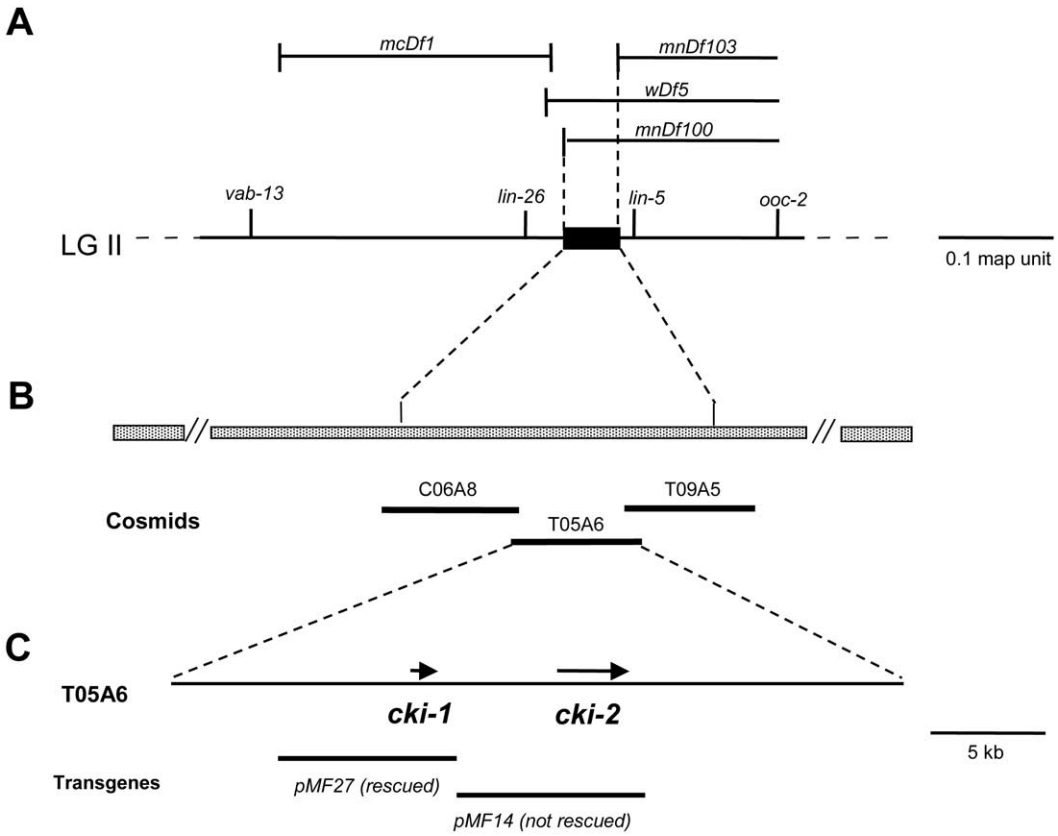

Fig. 1. Genetic and molecular identification of the *cki* genes. (A) Genetic map of the middle of linkage group II. The approximate position of deficiency endpoints was delimited by PCR as described (Williams, 1995). *mnDf103* does not cause a conspicuous cell cycle defect while *mnDf100* does. The endpoints of *mnDf100* and *mnDf103* reside in the region of cosmids CO6A8 and T09A5, respectively, thereby defining the region of interest covered by C06A8, T05A6 and T09A5 (black bar on the genetic map). (B) Physical map defined by cosmids. Cosmid T05A6 rescues the hyperplastic phenotype of *mnDf100* embryos. (C) T05A6 contains two apparent cyclin-dependent kinase inhibitors, *cki-1* and *cki-2*. The *cki* genes are transcribed in the same direction (indicated by arrows), and separated by ~5 kb. Subclones pMF14 and pMF27 are shown.

tine, were incomplete (Figs. 2C and 2D). The arrested embryos appeared to contain more numerous, and generally smaller, nuclei compared to wild-type. We quantified total nuclei in squashes of terminal homozygous deficiency mutant embryos and found that *mnDf100* homozygotes contained over 800 embryonic nuclei, compared with ~500 counted in late-stage wild-type animals (Fig. 3).

Several cell types were examined to determine whether the hyperplastic phenotype extends to multiple tissue types. *mnDf100* embryos were found to contain an average of 32 gut nuclei (n = 48) compared to the wild-type 20 nuclei (Figs. 2A and 2B). Moreover, an adherens junction-reactive antibody revealed an apparent excess of smaller-than-normal epidermal cells on the surface of *mnDf100* embryos (Figs. 2E and 2F). In contrast, we found that cell division in the germline appears to be unaffected in *mnDf100* embryos: the normal number of two germline progenitors was always observed (Figs. 2G and 2H).

To determine the source of the extra cells, we followed individual cell division patterns in the endoderm and mesoderm in developing *mnDf100* embryos. Cell lineage analysis revealed that every cell that normally exits the mitotic cycle after four rounds of cell division from the intestinal precursor, or E cell, undergoes a single additional mitosis (Fig. 2I). No sixth round divisions were observed in any intestinal lineage. Cell lineage analysis of a muscle precursor, the D cell, revealed a similar defect: cells that normally stop dividing after four rounds of division from D undergo a single additional mitosis, while all cells that normally exit the mitotic cycle five rounds from D were unaffected (data not shown). The high density of very small cells in the mutant embryos precluded analysis of other cell lineages. These observations imply that cells of the same tissue type and lineage arrest the cell cycle by genetically distinct mechanisms.

Excessive accumulation of programmed cell death corpses in mutant embryos

In addition to the hyperplastic phenotype, we also observed an accumulation of cell corpses in *mnDf100* embryos (Fig. 4). Terminal *mnDf100* embryos (~12 hours post-fertilization) contain numerous cell corpses (11.4 ± 1.2; n = 7; Fig. 4), in contrast to wild-type embryos at the same stage, which contain very few (0.11 ± 0.1; n = 28; Fig. 4). The extra corpses seen late in embryogenesis may not be caused simply by a general failure of *mnDf100* embryos to

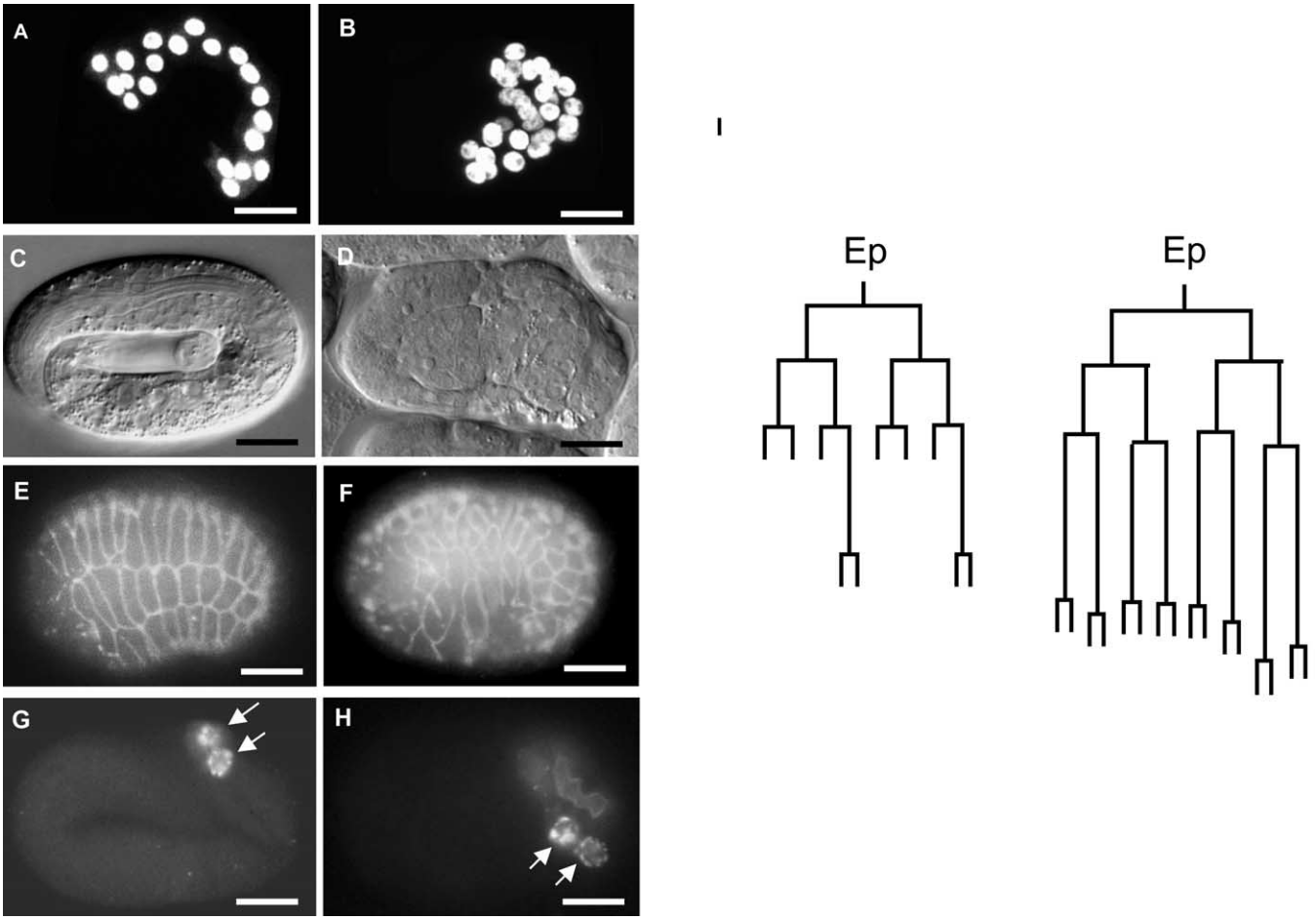

Fig. 2. Phenotypes of *mnDf100* embryos. Fluorescence (A, B, E–H) and Nomarski (C, D) images of wild-type (A, C, E, G) and *mnDf100* homozygous (B, D, F, H) embryos are shown with anterior at left in this and all subsequent figures. A *C. elegans* embryo is about 50 μ long. (A, B) 12 hour-old wild-type and 15 hour-old *mnDf100* embryos expressing nuclear localized GFP in the gut. GFP was driven by the *elt-2* promoter (Fukushige et al., 1999). While wild-type intestine contains 20 gut cells as seen here, there are ~30 visible in the *mnDf100* embryo. (C, D) Developing 12 hour-old wild-type and terminal, 15 hour-old *mnDf100* embryos viewed with Nomarski optics. Both embryonic elongation and pharynx morphogenesis are obvious in the wild-type and are entirely defective in the *mnDf100* embryo. (E, F) Embryos stained with antibody MH27, which outlines hypodermal cells (Francis and Waterston, 1985). Embryos were allowed to develop for 5 hours post-laying at 20°C, and fixed. Note the greater number and smaller size of hypodermal cells in the *mnDf100* embryo. (G, H) Embryos stained with a P-granule specific antibody. 12 hour wild-type and ~15 hour *mnDf100* embryos were stained with either OIC1D4 or anti-PGL-1 (Strome and Wood, 1983; Kawasaki et al., 1998). In the >100 *mnDf100* embryos examined, all displayed only two P-granule positive cells, as seen in wild-type embryos. (I) Cell lineages of Ep cells. Embryonic lineage analysis (Sulston et al., 1983) was performed by 4-D time-lapse analysis (e.g., Moskowitz et al., 1994). E descendants that normally stop dividing after only four division rounds in wild-type embryos (left lineage) undergo an additional cell division in *mnDf100* (right lineage). Similar lineage defects were observed in the D lineage (data not shown). Scale bars represent 10 μm.

undergo cell-corpse engulfment, since the number of cell corpses observed in *mnDf100* embryos was found to be similar to that of wild-type throughout embryogenesis (Fig. 4), during the stages at which nearly all programmed cell death normally occurs. The magnitude of the increase in cell corpse number (approximately eleven-fold) far exceeded that of the increase in nuclear number (less than two-fold) observed in the mutant. Thus, these extra corpses are not likely to be the result of duplication of cells that are normally fated to undergo programmed cell death.

We asked whether the extra cell corpses seen at the end of embryogenesis are produced by the normal genetically programmed cell death pathway by examining the requirements for known components of the pathway. Loss-of-function mutations in *ced-3* and *ced-4*, and a gain-of-function mutant in *ced-9* prevent normal programmed cell death (Ellis and Horvitz, 1986; Hengartner et al., 1992). We found that all three mutations eliminated all cell corpses in *mnDf100* embryos (Figs. 5A–D), indicating that the extra cell corpses are indeed dependent on proper CED-3, CED-4, and CED-9 function.

The cki-1 gene alone rescues the cell proliferation, cell death, and morphogenesis defects in mnDf100 embryos

The findings described above suggest that the gene(s) deleted in the deficiency mutants are required for exit from the cell cycle at the appropriate time, suppression of programmed cell death or corpse engulfment, and embryonic morphogenesis.

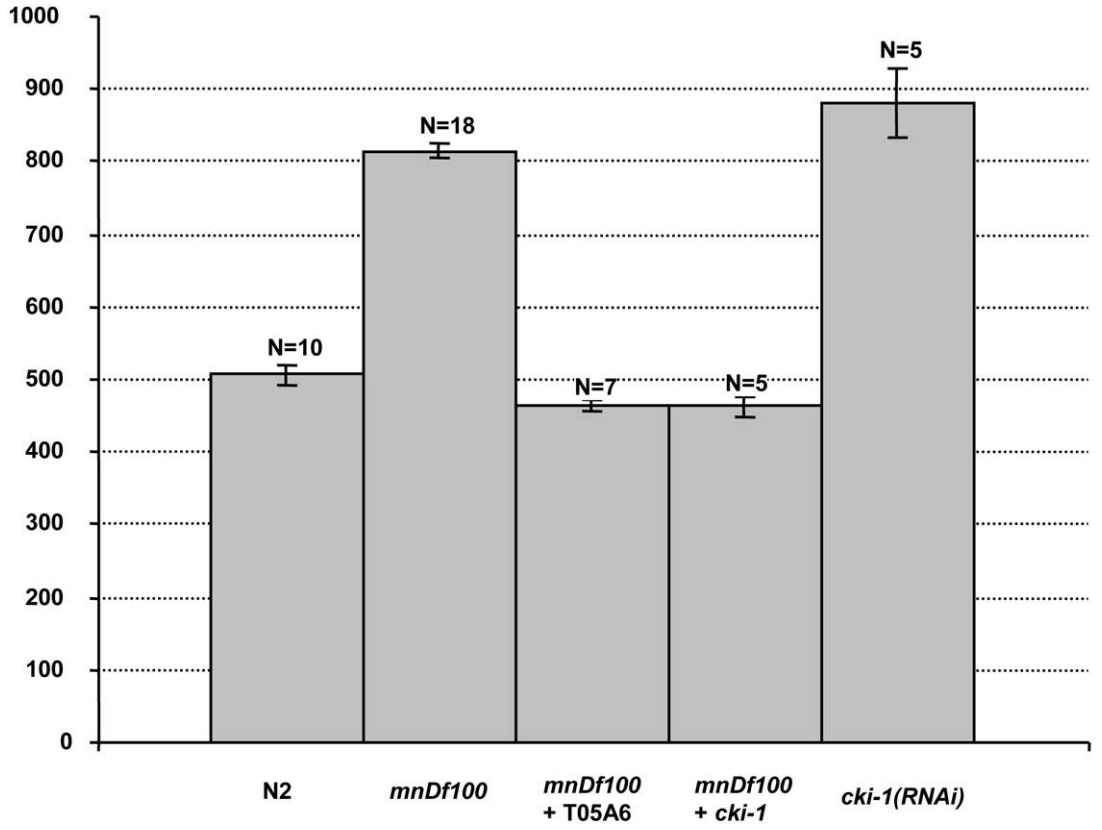

Fig. 3. Loss of *cki-1* activity results in excess nuclei. Nuclear numbers were scored on embryos of the indicated genotype as described in Materials and methods. Error bars represent standard error of the mean.

In an effort to identify the gene(s) responsible for these defects in *mnDf100* mutant animals, cosmids spanning a small genomic region, defined by mapping multiple overlapping deletions that do and do not lead to the cell cycle phenotype, were injected into deficiency heterozygotes (Fig. 1B). The cosmid T05A6 rescued the hyperplastic and pharynx malformation phenotypes in *mnDf100* embryos (Fig. 6B). The sequence of this cosmid predicts the presence of two adjacent genes with similarity to the Cip/Kip family of cyclin-dependent kinase inhibitors (CKIs; Fig. 1C). These two genes have been designated *cki-1* (*c*yclin-dependent *k*inase *i*nhibitor) and *cki-2* (in agreement with the laboratories of V. Ambros and E. Kipreos. Hong et al., 1998; Feng et al., 1999). We found that introduction of the *cki-1*, but not the *cki-2*, transgene alone can rescue the defects in body elongation and pharynx formation and differentiation in *mnDf100* embryos (Figs. 6A, 6C and 6D). Co-transformation of both *cki-1* and *cki-2* was no more effective at rescuing than was *cki-1* alone. Surprisingly, many structural features of the pharynx were restored, including its elongation and the appearance of terminally differentiated structures: i.e., the buccal cavity, lumen, and grinder (Fig. 6D). These observations suggest that *cki-1* may perform dual roles in promoting terminal differentiation and arresting the cell cycle (see Discussion). Nuclear counts revealed that terminal rescued *mnDf100* embryos contain nearly wild-type number of nuclei (Fig. 3). In addition, analysis of the developmental cell corpse profile shows that these rescued animals contain significantly fewer numbers of cell corpses, compared to unrescued animals (Fig. 4). These observations suggest that the hyperplasia, accumulation of excess cell corpses, and failure of body elongation and pharynx morphogenesis seen in the deficiency mutants results from the zygotic loss of the *cki-1* gene.

cki-1 is specifically required for embryonic cell cycle exit and morphogenesis

The capacity of *cki-1*, but not *cki-2*, to rescue *mnDf100* suggests that it alone may be the relevant gene whose removal results in the cell proliferation, cell death, morphogenesis, and differentiation defects of *mnDf100* homozygotes. We used RNA interference (RNAi; Fire et al., 1998) to test this hypothesis. We found that RNAi of *cki-1* results in extra larval cell division (Gendreau and Rothman, unpublished data), similar to observations reported elsewhere (Hong et al., 1998; Feng et al., 1999). However, by optimizing conditions for RNAi, we also found that *cki-1(RNAi)* results in reproducible embryonic lethality (32% embryonic lethality among F1 progeny laid within 15 hours after injection at 20°C; n = 289. See Materials and Methods). Interestingly, the degree of embryonic lethality caused by

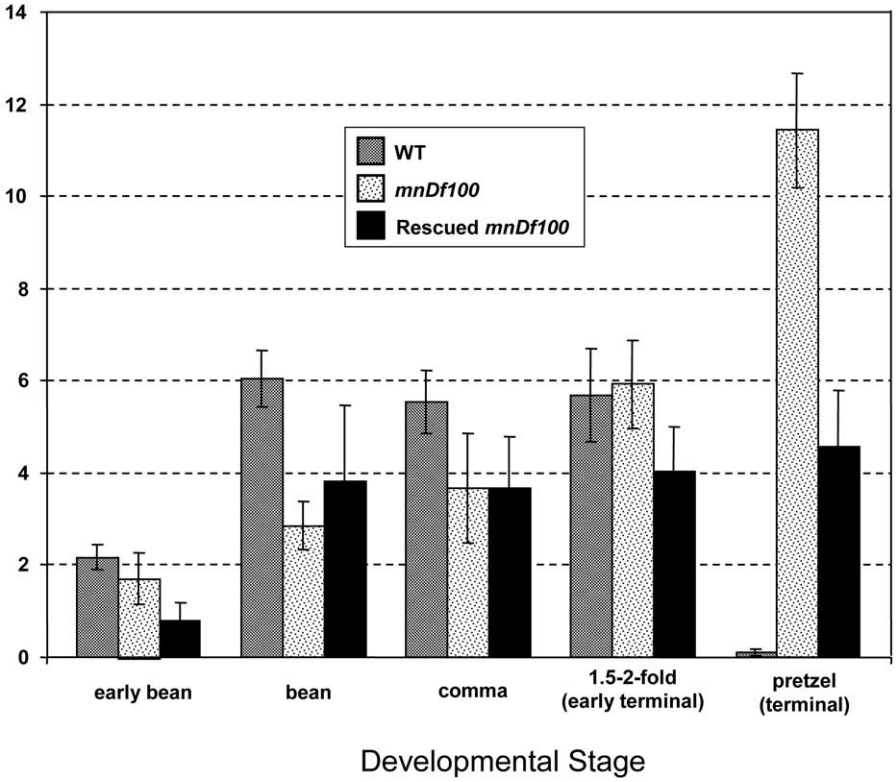

Fig. 4. *cki-1(+)* can suppress the appearance of excess cell corpses in *mnDf100* embryos. Developmental profiles of cell corpse number in wild-type (*unc-4, mnDf100/mnC1* or *mnC1*), homozygous *mnDf100* embryos, and homozygous *mnDf100* embryos carrying the *cki-1* transgene were analyzed as described in Materials and Methods. Cell corpses were scored from the "bean" stage. Rescued and unrescued *mnDf100* embryos were staged as "early terminal" and "terminal" at approximately 3 and 9 hours after bean stage, respectively. At least five embryos were scored at each embryonic stage for each of the indicated genotypes. Error bars represent standard error of mean.

cki-1 (RNAi) progressively reduces over time after injection of dsRNA: we did not see any embryonic lethality among the progeny laid later than 24 hours after injection (n = 239). Affected embryos arrest at various stages ranging from unelongated, *mnDf100*-like embryos, to 3-fold elongated embryos (Figs. 7A–C). These observations suggest that the embryonic activity of *cki-1* is highly resistant to RNAi compared to its postembryonic activity. However, we found that the embryonic lethality becomes nearly fully penetrant when *cki-1* RNAi is performed in an *rrf-3(pk1426)* mutant (not shown), which is hypersensitive to RNAi (Simmer et al., 2002). Some unelongated, *cki-1(RNAi)* embryos are morphologically quite similar to the *mnDf100* embryos: such embryos contain small cells with granules on the surface and many cell corpses (up to 13) at the anterior. The pharynx is under-differentiated and the only discernible characteristic of a normal pharynx is a basement membrane (Fig. 7A), indicating that *cki-1* plays an important role in pharynx morphogenesis. The arrested *cki-1(RNAi)* embryos contained as many as 987 nuclei (Fig. 4), suggesting that most embryonic cells require *cki-1* activity to arrest the cell cycle. This contrasts with *mnDf100* embryos, in which a smaller number of nuclei were present in arrested embryos. One possible explanation for this difference might be that *mnDf100* deletes a gene encoding a positive cell cycle regulator, whose elimination is partially epistatic to removal of *cki-1*. Consistent with such a notion, we found that homozygous *mnDf100* embryos rescued by a *cki-1* transgene arrest with somewhat fewer nuclei than fully developed wild type embryos (Fig. 3). A second explanation for this discrepancy might be that maternally provided CKI-1 protein also contributes to timely cell cycle arrest in some cells. Unlike *mnDf100*, which removes only the zygotic *cki-1* product, RNAi is expected to eliminate both zygotic and maternal products of the gene. Feng et al. (1999) reported that CKI-1 protein is present in the germline; therefore, the expression and/or activity of maternal CKI-1 might be upregulated later in embryogenesis.

Similar to *mnDf100* embryos, *cki-1(RNAi)* embryos contain on average 33 gut nuclei (n = 9; Fig. 7D). We also found that *cki-1(RNAi)* embryos show disorganized hypodermal cells (data not shown) and some clearly contain extra lateral epidermal (seam) cells (Figs. 1E and 7E). As with *mnDf100* embryos, the number of germ precursor cells (the Z2 and Z3 cells) is not affected in the *cki-1(RNAi)* embryos, suggesting that these germline cells halt proliferation in a *cki-1*-independent manner.

In contrast, we found that *cki-2(RNAi)* animals did not show any conspicuous phenotypes. In addition, double RNAi of both *cki* genes did not enhance or alter the embryonic phenotypes of

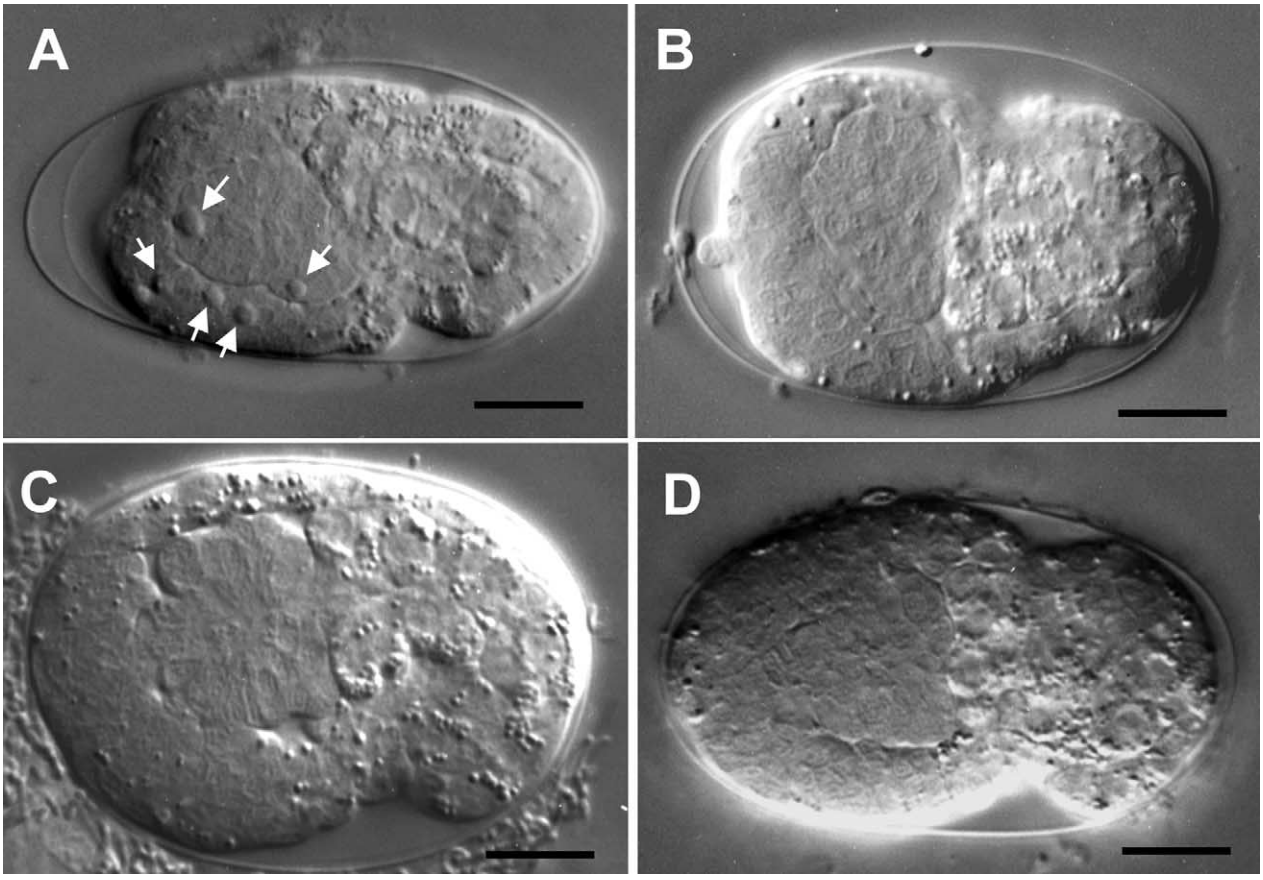

Fig. 5. The excess cell corpse phenotype of *mnDf100* embryos is dependent on CED-3 and CED-4 function. Nomarski micrographs of terminal (at least 12 hour post-laying at 20°C) embryos are shown. (A) *mnDf100.* Arrows point to cell corpses. (B) *ced-3(n717); mnDf100.* (C) *ced-4(e1752); mnDf100.* (D) *ced-9(n1250gf); mnDf100.* No cell corpses were visible in any of the *ced* double mutant strains. Scale bars represent 10 μm.

cki-1(RNAi) alone. Although it is possible that the *cki-2* gene is resistant to RNAi, the data presented here supports the hypothesis that *cki-1* alone is required for timely exit from the cell cycle and morphogenesis and differentiation during embryogenesis. Further, they suggest that the gene may be involved in suppressing programmed cell death, as has been suggested for its mammalian homologues (Polyak et al., 1996; Poluha et al., 1996; Wang and Walsh, 1996; Gorospe et al., 1997; Levkau et al., 1998; Yu et al., 1998; Asada et al., 1999). However, additional analyses are required to address whether the excess corpses caused by loss of *cki-1* activity arise from a failure in suppression of programmed cell death or in cell corpse engulfment.

While both cki genes are expressed at the time of cell cycle exit, cki-2 is also continuously present during early cell proliferation

Expression of *cki-1* and *cki-2* was examined by generating transcriptional fusions of the presumed promoter regions to a green fluorescent protein (GFP) reporter (see Materials and methods). *cki-1* and *cki-2* reporter constructs showed different spatiotemporal expression patterns in the embryo. *cki-1* reporter expression is first detectable in a group of cells of the pharynx and nervous system at about six hours post-fertilization (Fig. 8A,B; Hong et al., 1998). This represents the time at which most cells in the embryo become post-mitotic. Following this initial burst of expression, reporter expression expands to include most of the anteriorly located cells, consistent with our observation that the *cki-1* transgene can rescue the morphogenetic defects in the pharynx of *mnDf100* embryos. However, expression of reporters containing 2 or 6 kb of sequences upstream of the gene was not observed in the embryonic gut lineage. It has been shown that cell- or tissue-specific *cis*-regulatory elements of the *Drosophila string/cdc25* gene, a developmentally controlled cell cycle regulator, extend over a 30 kb interval (Lehman et al., 1999). Thus, it is conceivable that the spatiotemporal expression of *cki-1* is also controlled by an extended stretch of *cis*-regulatory elements. The 20 kb region upstream of the *cki-1* gene does not contain any predicted protein coding sequences (Hong et al., 1998) and it is possible that a *cis*-acting element required for embryonic gut expression may reside $>$ 6kb upstream of the gene.

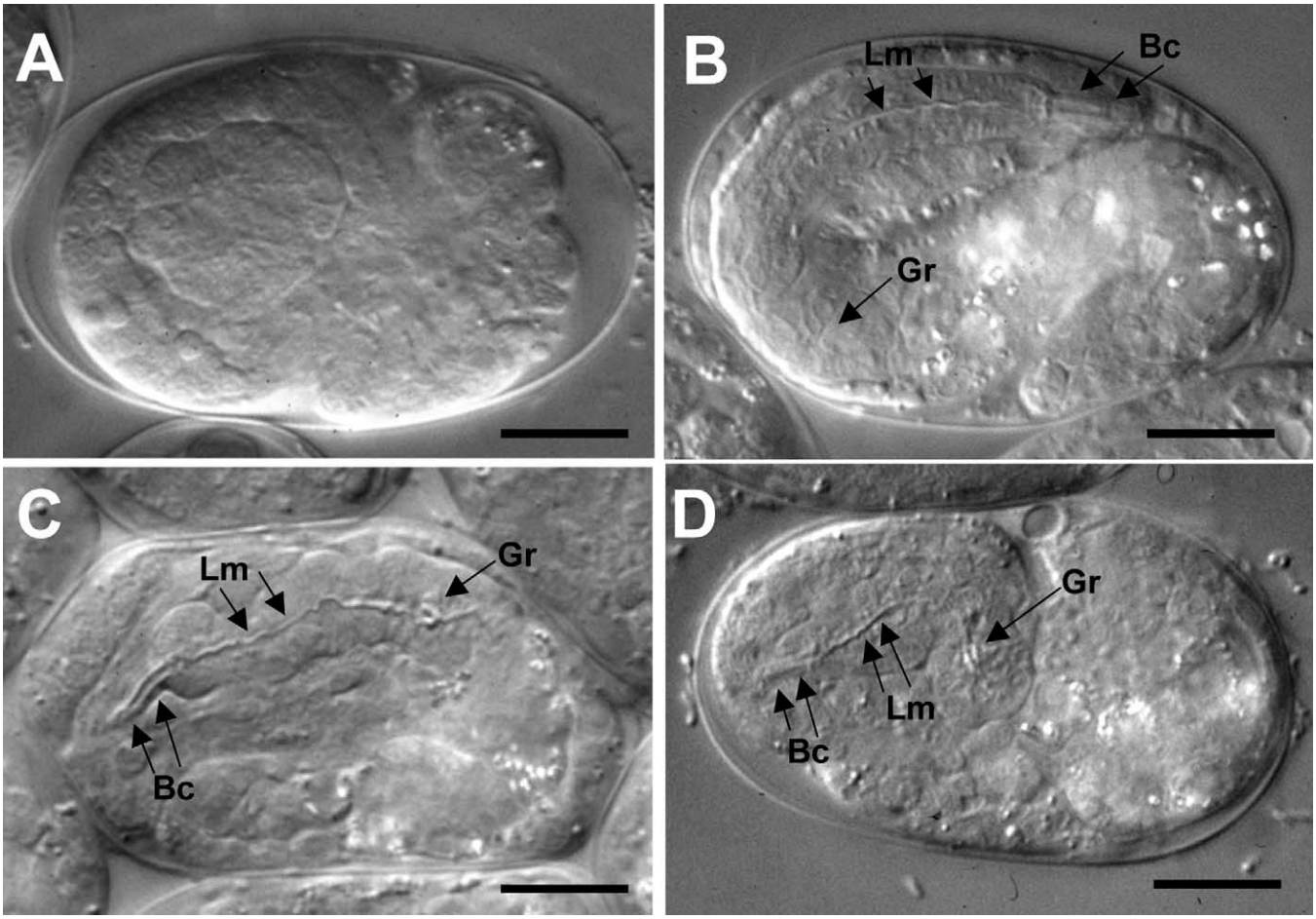

Fig. 6. Introduction of the *cki-1* transgene restores partial elongation and pharynx morphogenesis in *mnDf100* embryos. Nomarski micrographs of terminal embryos are shown. Embryos were allowed to develop at least 15 hours post-laying at 20°C. (A) *mnDf100* embryo carrying the *cki-2* transgene. Such embryos are indistinguishable from *mnDf100* embryos (compare with Fig. 2D). (B) *mnDf100* embryos rescued with cosmid T05A6. ~33% of the rescued embryos (n = 157) were elongated. (C, D) *mnDf100* embryos rescued by the *cki-1* and *cki-2* genes (C) and the *cki-1* gene only (D). Unlike the *mnDf100* embryos rescued by T05A6, >90% of the *mnDf100* embryos rescued by the *cki* transgenes remained unelongated. Arrows point to pharyngeal structures. Bc: buccal cavity. Lm: pharyngeal lumen. Gr: grinder. Scale bars represent 10μm.

Indeed, we found that when a *cki-1* reporter gene was co-transformed with two cosmids containing >30 kb of sequence upstream of the gene, the resultant extrachromosomal array, which is presumably generated by homologous recombination of the introduced DNA, expresses GFP in the gut cells at around the comma stage (not shown). However, the level of expression is too low and variable to identify in which gut cells GFP is expressed. Moreover, the cosmid T05A6, which contains about 11 kb upstream sequence of the *cki-1* gene, can rescue the gut hyperplasia in *mnDf100* embryos. Blastomere isolation experiments suggested that the endoderm precursor, the E cell, contains a cell-intrinsic program that arrests the cell cycle on schedule, resulting in the normal 20 embryonic gut cells (Leung et al., 1999). Since loss of *cki-1* activity results in hyperplasia in the gut (Fig. 7D), and *cki-1* is expressed in the endodermal lineage, it is likely to function cell-autonomously to control cell cycle exit.

In contrast, embryonic expression of the *cki-2* reporter starts much earlier, at about the 64-cell stage in anteriorly located blastomeres (Figs. 8C and 8D). This expression pattern apparently continues throughout embryogenesis with a gradual increase in fluorescence intensity. The *Drosophila* Cip/Kip CKI, Dacapo, is also expressed in rapidly proliferating cells during embryogenesis; its expression disappears before cellularization except in germ cells and reappears when cells become post-mitotic (Lane et al., 1996; de Nooij et al., 1996). Unlike *Dacapo*, the *cki-2* reporter is continuously expressed in many cells throughout embryogenesis. The physiological role of this continuous expression remains to be revealed.

Overexpression of the CKI proteins can induce embryonic cell cycle arrest

To ask whether CKI-1 and CKI-2 are sufficient to arrest embryonic cell cycles in vivo, each was overexpressed in early embryos using heat shock promoters (Stringham et al.,

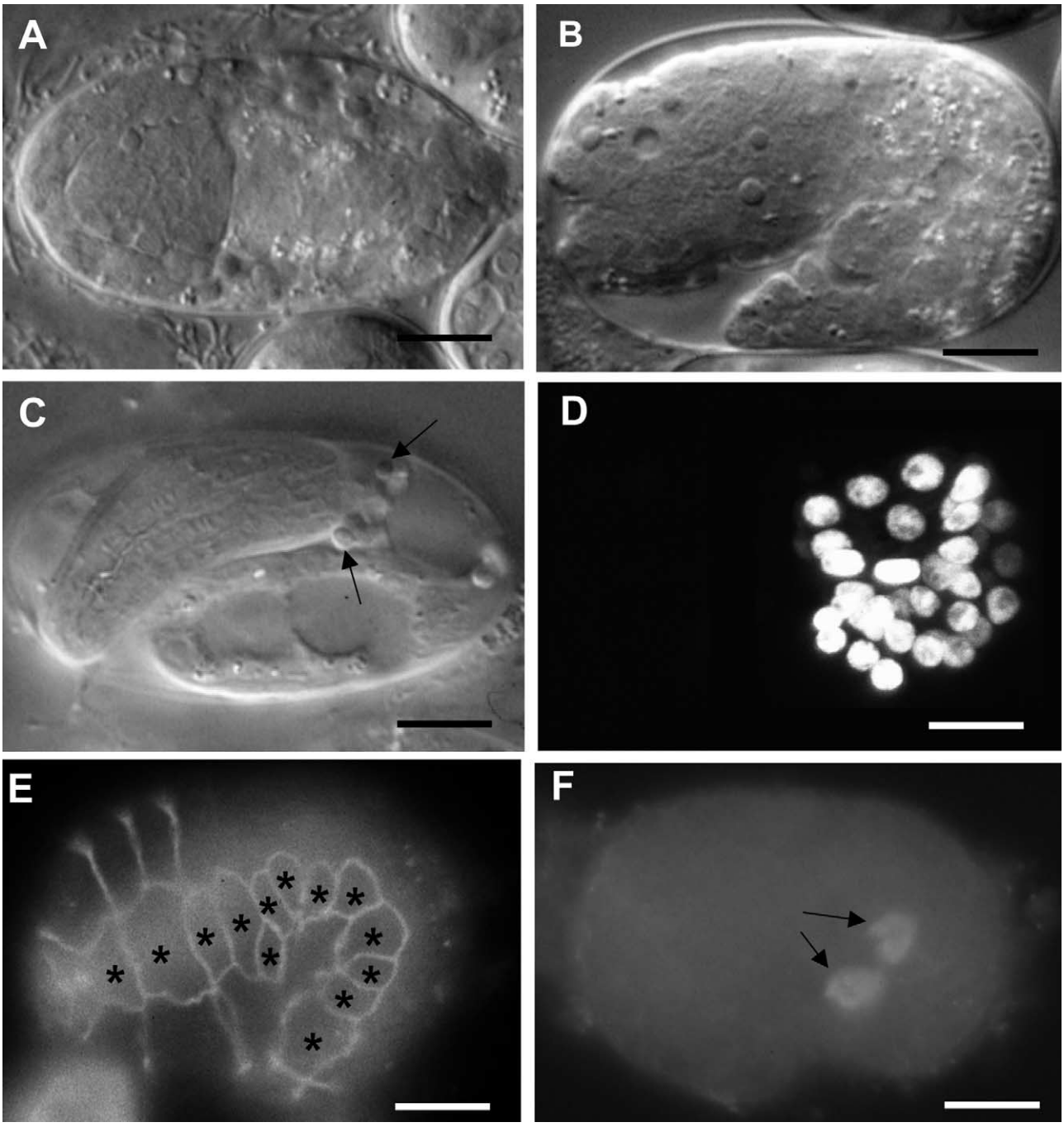

Fig. 7. *cki-1(RNAi)* results in embryonic hyperplasia. Nomarski (A–C) and fluorescent (D–F) micrographs of *cki-1(RNAi)* embryos are shown. (A–C) Terminal *cki-1(RNAi)* embryos. Reminiscent of *mnDf100* embryos (see Fig. 2D), most severely affected embryos arrest with many cell corpses and without elongating or undergoing pharynx morphogenesis or differentiation. Other embryos arrest with varying degrees of elongation (B, C); these often contain vacuoles and dissociating cells (arrows). (D) *cki-1(RNAi)* in embryos carrying the *elt-2*::GFP reporter (Fukushige et al., 1999). The embryo shown contains 37 nuclei expressing GFP. (E) *cki-1(RNAi)* embryo stained with MH27 (Francis and Waterston, 1985). Note that this embryo contains ~13 lateral seam cells (asterisks) on the left side, compared with 10 seam cells in wild-type embryos (compare with Fig. 1E). This embryo was fixed 6 hours post-laying at 20°C. (F) Terminal *cki-1(RNAi)* embryo stained for P-granules (Kawasaki et al., 1998). All 39 *cki-1(RNAi)* embryos examined showed only two immunoreactive cells (arrows), as in wild-type embryos. Scale bars represent 10 μm.

1992). Overexpression of CKI-1 disturbed embryogenesis, leading to arrest with unusually large blastomeres, indicative of premature cell division arrest (Figs. 9A and 9B). Moreover, we found that overexpression of CKI-2 can also produce similar effects on embryos (Figs 9A and 9C). Thus, we conclude that, while *cki-1* alone appears to be necessary for developmental exit from the cell cycle, both CKI-1 and CKI-2 are sufficient to do so.

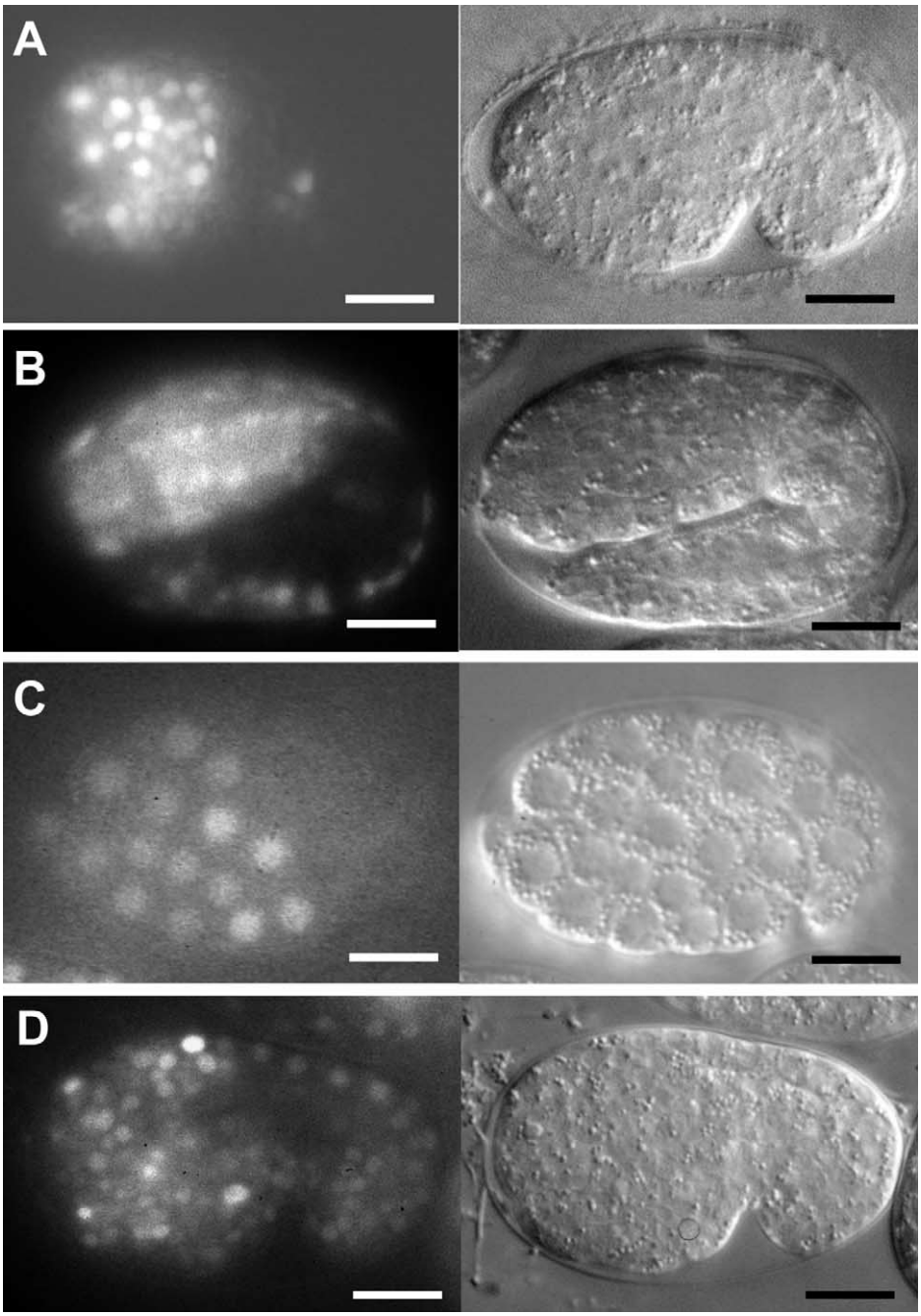

Fig. 8. Embryonic expression of *cki-1*::GFP and *cki-2*::GFP. (A–D) Fluorescence (left panels) and Nomarski (right panels) photographs of embryos carrying GFP reporters. (A, B) *cki-1*::GFP expression is first seen around the comma stage (A). Extensive expression around the head continues while the embryo is elongating (B). (C, D) *cki-2*::GFP expression begins in the anterior blastomeres, including AB descendants at around the 50-cell stage (C). This expression pattern apparently continues throughout embryogenesis, resulting in many cells expressing GFP around the head, tail and body surface (D). Scale bars represent 10μm.

Discussion

Requirement for CKI-1 function in somatic cells

We report that CKI-1 is essential for normal embryonic cell cycle exit in many lineages, demonstrating that it may be used by all somatic lineages to control the time at which cells stop proliferating and differentiate. In particular, CKI-1 is used in those cells that exit the cell cycle one division earlier than their close relatives. These findings indicate that activation of CKI function as an inhibitor of the cell cycle is a crucial step in specification of the proper number of cells produced by each lineage. Moreover, they reveal that the decision to exit the cell-cycle is controlled by genetically distinct mechanisms (CKI-1-dependent and -independent) in closely related cells.

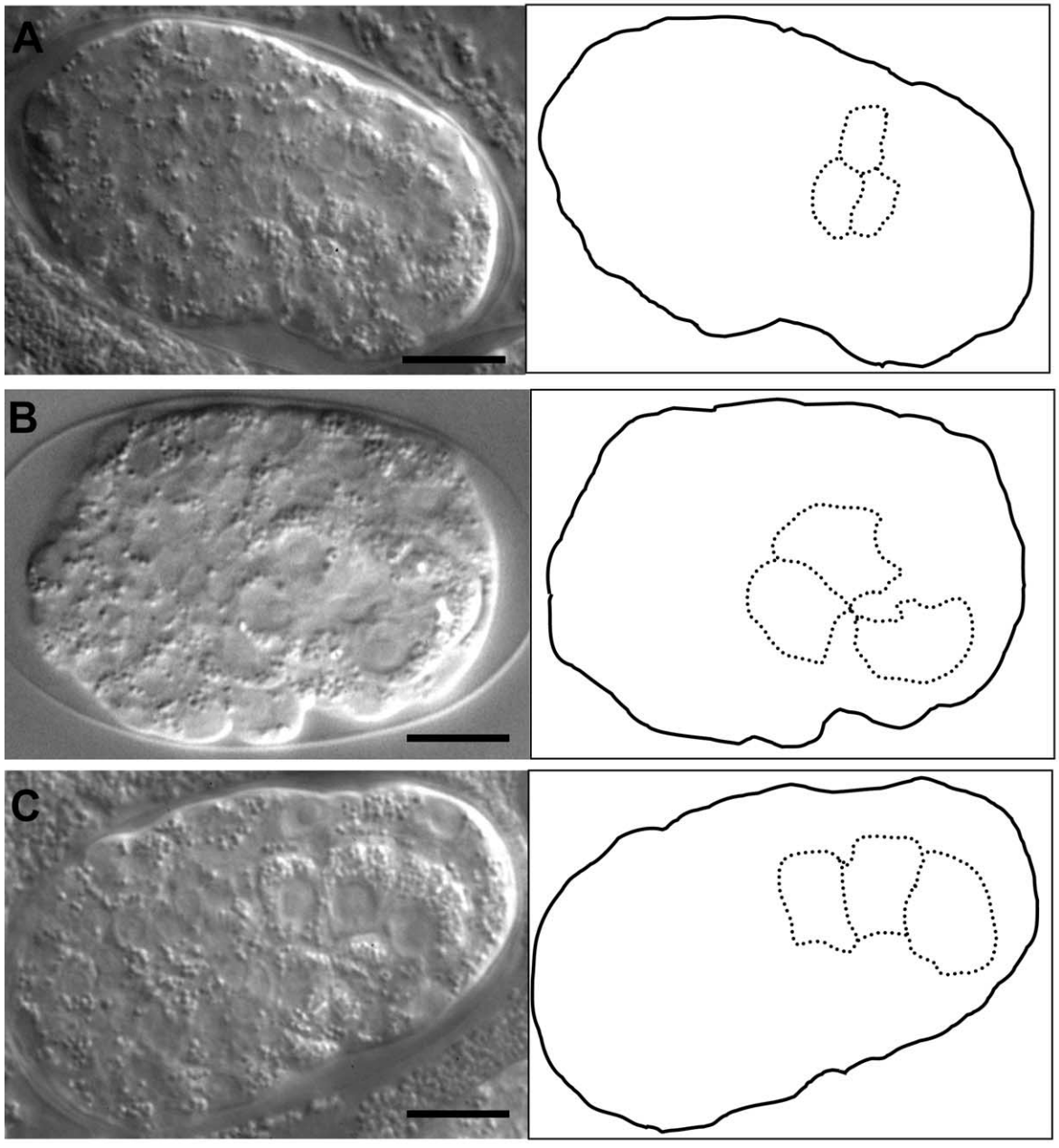

Fig. 9. Overexpression of both *cki-1* and *cki-2* can arrest the embryonic cell cycle. Wild type embryos (A) and embryos carrying a hs-*cki-1* (B) or hs-*cki-2* (C) transgene (in which the *cki* gene is fused to a heat shock promoter; Stringham et al. 1992) were given a 33°C heat-shock at the 28 to 100-cell stage for 30 minutes, and subsequently allowed to develop at 20°C for 6 hours. DIC images are shown in the left panels. Outlines of embryos (solid line) and three selected gut cells (dotted lines) from the DIC images are drawn in the right panels. Notice the significant difference in size of gut cells between wild type and transformed embryos. The heat treatment *per se* did not appear to interfere with embryonic cell cycle in wild-type embryos. Scale bars represent 10 μm.

Our observations also indicate that the transient arrest of germline cells during embryogenesis is independent of the CKIs. This may reflect a fundamental difference between the regulatory mechanisms that control the cell-cycle of germline vs. somatic cells. While somatic cells in *C. elegans* undergo a limited and determinate number of cell divisions, the germline develops by an indeterminate number of divisions. In addition, unlike somatic cells, germline cells are subject to checkpoint control that leads to their division arrest and p53-dependent programmed cell death in response to genotoxic damage (Derry et al., 2001; Schumacher et al., 2001)

Role of cki-1 in terminal differentiation and morphogenesis

In addition to the involvement of *cki-1* in cell cycle arrest and programmed cell death, we found that introduction of

the *cki-1* transgene alone can restore the differentiated features of the pharynx and partial body elongation in *mnDf100* embryos, indicating that proper cell migration and cell-shape changes are rescued. Conversely, RNAi-mediated reduction in *cki-1* activity severely perturbs embryonic morphogenesis, including body elongation and formation of the pharynx. These defects contrast with the effects of extra cell divisions on postembryonic development of the vulva and uterus, in which case hyperplasia or duplication of affected tissues occurs with relatively normal differentiation and morphogenesis (Hong et al., 1998; Feng et al., 1999). Since, as we have shown, *cki-1(RNAi)* results in embryonic lethality, the *cki-1(RNAi)* larvae possess some residual activity of the gene which, though insufficient to mediate larval cell cycle arrest, may nonetheless be capable of promoting differentiation and morphogenesis.

Why is an apparent cell cycle regulator so critical for morphogenesis of the body and organogenesis of the pharynx? These processes require that cell divisions and migrations are tightly coordinated. Extra cell divisions caused by loss of *cki-1* might be expected to perturb the coordination of such cellular events during embryonic morphogenesis. However, it is also reasonable to suppose that *cki-1* may play a direct role in promoting differentiation in the embryo. Ectopic expression of vertebrate Cip/Kip CKIs has been shown to affect cell fate determination and differentiation in vertebrate cells (Di Cunto et al., 1998; Ohnuma et al., 1999). Moreover, genetic studies using knockout mice have suggested that $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ control both the cell cycle and differentiation (Zhang et al., 1998; Zhang et al., 1999; Dyer and Cepko, 2000). In contrast to mammalian development, which is highly dependent on intercellular signals mediated by growth factors and cytokines, most cell types differentiate by cell-autonomous mechanisms in *C. elegans* embryos (Sulston et al., 1983). Thus, *cki-1* may promote differentiation directly.

Cancer is characterized not only by dysregulation of the cell cycle, but also by a loss of differentiation in transformed cells. In this respect, it should be noted that down-regulation of $p27^{Kip1}$ protein is highly correlated with a poor prognosis in many human cancers (reviewed in Tsihlias et al., 1999). Thus, further study of the *cki-1* gene will help not only to elucidate the multifaceted cellular roles of Cip/Kip CKIs in cell cycle arrest, programmed cell death, and terminal differentiation, but may also assist in delineating the molecular mechanism of tumorigenesis.

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