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The cyclin-dependent kinase inhibitors, *cki-1* and *cki-2*, act in overlapping but distinct pathways to control cell-cycle quiescence during *C. elegans* development

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Abstract

Cyclin-dependent kinase inhibitors (CKIs) are major contributors to the decision to enter or exit the cell cycle. The *Caenorhabditis elegans* genome encodes two CKIs belonging to the Cip/Kip family, *cki-1* and *cki-2*. *cki-1* has been shown to act as a canonical negative regulator of cell-cycle entry, while the role of *cki-2* remains unclear. We identified *cki-2* in a genome-wide RNAi screen to reveal genes essential for developmental cell-cycle quiescence. Examination of *cki-2* knockout animals revealed extra rounds of cell divisions, verifying a role in establishing or maintaining the temporary cell-cycle arrest. Despite the overlapping defects, the pathways mediated by *cki-1* and *cki-2* are discrete since the extra cell phenotype conferred by a putative *cki-2(null)* mutation is enhanced upon additional loss of *cki-1* activity. Moreover, the extra cell division defect of *cki-2* is not increased with the additional loss of *lin-35* Rb, as is seen with *cki-1*. Thus, both *cki-1* and *cki-2* mediate cell-cycle quiescence, but our genetic and phenotypic analyses demonstrate that they act within distinct pathways to exert control over the cell-cycle machinery.

Keywords

C. elegans; vulva development; cyclin-dependent kinase inhibitor; cell-cycle quiescence

Introduction

The developmental decision of cells to divide or arrest is generally determined during the G1 phase of the cell cycle. Studies in yeasts and cultured mammalian cells have identified a period, called START and the restriction point respectively,[1–3] during G1 in which an irreversible commitment to the cell cycle is made. For example, animal cells that have progressed past the restriction point are refractory to serum withdrawal and can complete the cell cycle in the absence of further stimulation. Thus an elaborate system of checks and balances during G1 ensures that cell divisions are initiated appropriately during development. An important regulator of G1 progression, pRb, is encoded by the retinoblastoma tumor suppressor gene.[4–8] One mechanism by which pRb inhibits G1/S progression is through transcriptional inhibition of genes required for S phase, such as cyclin E.[9] In parallel, members of the Cip/Kip family of CKIs can interact with and inhibit the kinase activity of the Cdk2-cyclin E complex.[10] By inactivating existing Cdk2-cyclin E

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complexes and inhibiting the production of new cyclin E, Cip/Kip and pRb act in concert to potentiate a G1 arrest.

We use the development of the *C. elegans* vulva as a model system to examine the regulatory mechanisms governing cell-cycle quiescence. The adult vulva originates from six vulva precursor cells (VPCs) that arise during the first larval stage (L1) and remain temporarily arrested in the G1 phase of the cell cycle until L3.[11, 12] Since the vulva is not an organ that is required for viability of the animal, even defects that severely perturb its development or function can be studied. Using a *lin-12* Notch gain-of-function mutation to direct the six VPCs to adopt inappropriate cell fates,[13] we mark the VPCs as protrusions on the ventral surface of the adult known as pseudovulvae. Thus, in the *lin-12(gf)* genetic background, defects producing extra VPCs will result in easily observed extra pseudovulvae. We refer to the generation of greater than six pseudovulvae as the enhancer of *lin-12(gf)* multivulva (Elm) phenotype. *elm* genes identified in this manner act to limit the number of VPCs generated to six by controlling either cell-fate adoption or cell-cycle progression.[14]

A forward genetic screen for the Elm phenotype identified genes important in the regulation of cell cycles, such as *cdc-14*, *lin-1*, *lin-31* and *mdt-13*.[14, 15] We continued the Elm phenotype screen using RNAi feeding libraries to inhibit gene activities. Here, we describe the analysis of *cki-2*, a locus identified as an *elm* gene in the RNAi screen. The extra cell division defect of a putative null mutant indicates that *cki-2* activity is required for the temporary cell-cycle arrest of the VPCs. In contrast to the related *cki-1* locus, loss of *cki-2* activity does not result in defects of multiple tissues nor are the defects enhanced by loss of *lin-35* Rb function. These data suggest that both *cki-1* and *cki-2* perform functions during cell-cycle quiescence, but these functions are performed independently of each other.

Results

A genome-wide RNAi screen to discover genes necessary for normal VPC development identified the cyclin-dependent kinase inhibitor, *cki-2*

We previously performed a forward genetic screen for the enhancer of *lin-12(gf)* mutivulva (Elm) phenotype which identified genes required to direct a developmental cell-cycle arrest.[14, 15] In ongoing screens for the Elm phenotype using RNAi, we identified a clone targeting *cki-2*, one of two *C. elegans* members of the Cip/Kip family of cyclin-dependent kinase inhibitors (CKIs).[16–18] Unlike control-fed *lin-12(gf)* animals that generate six VPCs which in turn produce a maximum of six adult pseudovulvae, *lin-12(gf)* animals that ingested bacteria targeting *cki-2* for RNAi-mediated inhibition (*cki-2(RNAi)*) can display greater than six pseudovulvae (Fig. 1A–B). The *cki-2(RNAi); lin-12(gf)* animals displayed an average of 5.1 pseudovulvae compared to 4.5 for control treated animals (Fig. 1C). The emergence of additional pseudovulvae following inhibition of *cki-2* function by RNAi indicates the important role of *cki-2* in restricting VPC production.

cki-2 mutation results in extra VPCs through disruption of cell-cycle quiescence

To confirm the RNAi phenotype and to perform a careful genetic characterization of *cki-2* function during development, we examined two deletion alleles of *cki-2, tm3496* and *ok2105* (Fig. 2A and Materials and Methods). The *cki-2(tm3496)* mutation deletes 362 base pairs to eliminate the weakly conserved C-terminus. In contrast, *cki-2(ok2105)* substitutes 1,606 base pairs, including the majority of the *cki-2* coding sequence, with a duplication of the 9 base pair sequence immediately preceding the breakpoint. If expressed, the CKI-2(ok2105) truncated protein is predicted to be comprised of only 27 amino acids. Therefore, the *cki-2(ok2105)* mutation likely results in a null allele.

Since *cki-2(RNAi)* results in the production of extra pseudovulvae by the tester strain and the Cip/Kip family of CKIs control G1/S progression, we next examined the VPCs of the *cki-2* mutant animals for extra rounds of cell divisions which would indicate defects in cell-cycle quiescence. The VPCs of both *cki-2(tm3496)* and *cki-2(ok2105)* mutant animals undergo extra cell divisions prior to the L2/L3 molt (Figs. 2E and 4), verifying a requirement for *cki-2* activity in VPC cell-cycle quiescence. The *cki-2(tm3496)* extra VPC defect is less penetrant than *cki-2(ok2105)*, supporting the notion that *cki-2(ok2105)* is a null allele. We confirmed the requirement for *cki-2* by introducing a *cki-2(wt)* transgene into the *cki-2(ok2105)* mutant strain and observing rescue of the extra cell division defect (Fig. 4). Therefore, *cki-2* activity contributes to the establishment or maintenance the L1-to-L3 cell-cycle quiescent period during VPC development.

For subsequent genetic analyses of *cki-2* function, we focused on the probable null allele, *cki-2(ok2105)*. The *cki-2(ok2105)* animals appear superficially wild type despite the extra VPCs. We specifically note a lack of embryonic lethality and adult sterility, two phenotypes that can result from impaired cell cycles.[19] The average brood size for *cki-2(ok2105)* hermaphrodites was reduced (195.1±101.1, 9 broods) compared to wild type (280.5±28.3, 11 broods). However, we determined that this decrease is the result of a partially penetrant egg-laying defect (Egl) that drastically reduced the number of offspring produced by the afflicted individual. Since non-Egl *cki-2(ok2105)* animals are otherwise capable of producing over 300 progeny (see Materials and Methods), *cki-2* is not required for production of normal-sized broods. Importantly, the *cki-2(ok2105)* embryos exhibited neither defects in centrosome content (Fig. 2C) nor lethality (0.5%, n=1756) compared to wild-type embryos (0.7%, n=1411). Therefore, the *cki-2(ok2105)* mutation yields superficially normal animals that do not display cell cycle associated phenotypes, such as centrosome amplification or embryonic and larval lethality.

The *cki-2* promoter is generally expressed during development

In order to determine the spatiotemporal expression pattern of *cki-2*, we generated transgenic worms expressing green fluorescent protein (GFP) under the control of the *cki-2* promoter (*Pcki-2::gfp*). As previously reported,[17] GFP is widely observed during embryogenesis. Similarly, GFP is also generally expressed during larval development, being observed within both dividing and terminally differentiated cells. For example, cells within the seam (Fig. 3A) and intestinal (Fig. 3B) lineages strongly express the *Pcki-2::gfp* reporter. The VPCs (Fig. 3C) and their descendent cells (Fig. 3D) also express GFP, further supporting a role for *cki-2* in vulva development. While *cki-2* activity may be regulated by additional post-transcriptional mechanisms that would not be discernible by the transcriptional reporter, the broad expression of GFP nevertheless indicates that *cki-2* may act in the cell-cycle quiescence of a wide range of cell types.

cki-2 mutation does not result in a general disruption of cell-cycle quiescence

Since *cki-2* is widely expressed and loss of *cki-1* activity disrupts the cell-cycle quiescence of multiple developing tissues, we examined cell types other than the VPCs in the *cki-2(ok2105)* animals for similar extra cell division defects. In prior studies, *cki-1(RNAi)* resulted in the production of extra intestinal nuclei, seam cells and distal tip cells.[16, 20] In addition, loss of *cdc-14*, a regulator of *cki-1*, produced extra cells within the M-lineage at a low frequency.[15] Accordingly, we examined intestinal nuclei, distal tip cells, seam cells and cells within the M-lineage of *cki-2(ok2105)* mutant animals and found no evidence for defects in control of cell cycles (Table 1). Therefore, for these non-vulval cell types that express the *cki-2* promoter during development, *cki-2* activity is not required for normal cell-cycle quiescence.

cki-2 controls cell-cycle quiescence through a cyclin E-dependent pathway

To establish a requirement for *cki-2* activity within the regulatory hierarchy controlling cell-cycle quiescence, we examined the *cki-2(ok2105)* phenotype in combination with mutations of *cye-1* and *lin-35*, the sole *C. elegans* homologs of cyclin E and Rb, respectively.[21, 22] As predicted for a cyclin-dependent kinase inhibitor, the extra VPC divisions in *cki-2(ok2105)* animals were strongly suppressed in combination with even heterozygous mutation of the *cye-1* locus (Fig. 4). Intriguingly, the *cki-2(ok2105)* extra VPC phenotype was not enhanced in the *lin-35(n745); cki-2(ok2105)* double mutant animals (Fig 4), in contrast to the strong synergy displayed between *cki-1* and *lin-35* mutations.[20] These data indicate that both *cki-1* and *cki-2* depend on *cye-1* activity to promote the ectopic cell divisions but only the *cki-1* activity is functionally redundant with the role played by *lin-35* Rb. Thus, *cki-1* and *cki-2* perform partially overlapping roles in controlling VPC cell-cycle quiescence that are genetically distinguishable.

Genetic interactions suggest that *cki-1* and *cki-2* act in parallel pathways

Since *cki-1* and *cki-2* both regulate cell-cycle quiescence of the VPCs, we examined the genetic relationship between the two loci. We examined *cki-1(gk132)*, the only identified mutation believed to specifically disrupt *cki-1* activity. Since *cki-1(gk132)* homozygous animals die during embryogenesis, our analyses were limited to evaluation of *cki-1(gk132)* heterozygotes. Unlike the *cki-1(gk132)* mutation that displays weak haploinsufficiency for control of VPC cell-cycle quiescence,[15] the *cki-2(ok2105)* mutation appears completely recessive (Fig. 4). To assess the effect of decreasing both *cki-1* and *cki-2* activities simultaneously, we first examined *mnDf100*, a chromosome II deficiency that deletes both *cki-1* and *cki-2* loci.[17, 23] Animals heterozygous for the *mnDf100* mutation present an extra VPC defect that is indistinguishable from *cki-1(gk132)* heterozygotes. Unexpectedly, upon analysis of the *cki-1(gk132)*/*cki-2(ok2105)* double-heterozygous combination, VPC cell-cycle quiescence was strongly disrupted as demonstrated by the highly penetrant extra VPC divisions (Fig. 4). The enhanced extra cell division phenotype is surprising since both the *mnDf100*/+ and the *cki-1(gk132)*/*cki-2(ok2105)* animals are predicted to be hemizygous for both *cki-1* and *cki-2* loci. The most likely explanation for the greater than expected extra cell division defect of *cki-1(gk132)*/*cki-2(ok2105)* animals is that the *cki-1(gk132)* mutation also disrupts the activity of the adjacent *cki-2* locus. The equally strong extra VPC defect seen in *cki-2(ok2105)*/*mnDf100* animals is consistent with the hypothesis that the *gk132* mutation, like *mnDf100*, disrupts both *cki-1* and *cki-2* functions. Overall, these data indicate that *cki-1* and *cki-2* likely act in parallel pathways to control cell-cycle quiescence since a decrease in *cki-1* activity enhances the extra VPC defect of the putative *cki-2* null allele.

Given the strong enhancement of the *cki-2* cell-cycle defect in combination with loss of *cki-1* in the VPCs, we re-investigated a role for *cki-2* in regulation of cell cycles outside the VPCs. As described above, intestines of *cki-2(ok2105)* mutants develop with the wild type nuclear complement, therefore we examined if loss of *cki-2* function in combination with mutations of known cell-cycle regulators, such as *cki-1*, could reveal a weak role. The *cki-1(gk132)*/*cki-2(ok2105)* trans-heterozygous combination that results in a highly penetrant extra VPC defect yields an average nuclei number that is identical to heterozygous *cki-1(gk132)* alone (Fig. 5). Similarly, inhibition of *cki-1* by RNAi in either wild-type or *cki-2(ok2105)* mutant animals results in an indistinguishable extra intestinal nuclei phenotype. Moreover, we examined the combination of *cki-2(RNAi)* and *fzr-1(ku298)*, a mutation of a *C. elegans* Cdc20/Cdh1 homolog that functions redundantly with *lin-35*.[24] Again, loss of *cki-2* activity did not enhance the extra intestinal nuclei defect of *fzr-1(ku298)* mutant animals (Fig. 5). Thus, the inability of *cki-2* loss of function to enhance the extra intestinal nuclei phenotype of either *cki-1* or *fzr-1* supports that *cki-2* plays no role in regulation of cell-cycle quiescence during development of the intestine.

cki-2 expression is impaired by the *cki-1(gk132)* mutation

In order to test the hypothesis that the *cki-1(gk132)* mutation inhibits expression of the *cki-2* locus, we used quantitative real-time PCR to measure *cki-2* mRNA levels. Since homozygous *cki-1(gk132)* mutation results in embryonic lethality, measurement of *cki-2* expression in the *cki-1(gk132)* homozygotes during larval development is not possible. Thus, we examined *cki-1(gk132)/cki-2(ok2105)* trans-heterozygotes. This mutant combination ensures that only mRNA expressed from the *cki-2* locus located on the *cki-1(gk132)* chromosome is measured since the *cki-2(ok2105)* deletion eliminates sequences necessary for PCR amplification (Fig. 2B). mRNA expression of the *cki-2* locus adjacent to the *cki-1(gk132)* mutation is decreased 8-fold when compared to expression from *cki-2* with a *cki-1(wt)* neighbor (Fig. 6). These data demonstrate that the *cki-1(gk132)* mutation inhibits expression of the *cki-2* locus that is approximately 5 kb downstream, thus providing a molecular explanation for the unexpected genetic interaction.

Discussion

Our studies demonstrate that *cki-2* regulates cell-cycle quiescence, likely through the control of the core components of the cell-cycle machinery. While our conclusions are based upon the characterization of phenotypes resulting from a putative *cki-2* null allele, our attention to *cki-2* was initially directed by RNAi data. RNAi and the related transgene-mediated co-suppression techniques provide convenient methods to inhibit gene activity; however, their expedience is counter-balanced by the potential for inconsistent or indirect effects.[25–27] For example, a previous report using co-suppression to inhibit *cki-2* activity (*cki-2cs* [28]) found that *cki-2cs* germ lines were unable to properly expel centrioles, resulting in multipolar mitotic spindles and embryonic lethality. While there is little doubt that centrosome amplification and death was observed, the *cki-2(ok2105)* mutant animals clearly demonstrate that these previously reported defects were not the direct result of *cki-2* loss of activity. We conclude that *cki-2* is not required for centriolar expulsion during oogenesis since *cki-2(ok2105)* animals are deficient in *cki-2* activity but display no evidence of impaired centrosome function, fertility nor viability.

The data presented here reveal a rate-limiting role for *cki-2* as a canonical CKI, similar to the previously characterized role of *cki-1*. Several prior studies determined that *cki-1(RNAi)* causes defects in cell-cycle quiescence and results in obvious developmental disruptions.[16–18] In contrast, *cki-2(RNAi)* did not yield an obvious phenotype[16–18] and only upon closer experimental scrutiny was a subtle effect previously observed.[20] Interestingly, overexpression of either CKI-1 or CKI-2 are sufficient to induce cell-cycle arrest[16, 17]; although only transgenes expressing *cki-1(wt)*, and not *cki-2(wt)*, were able to provide limited rescuing activity to *mnDf100* homozygous embryos.[17] In the present study, we examined the putative null allele, *cki-2(ok2105)* and conclude that *cki-2* plays an important role in cell-cycle quiescence of the VPCs. As observed in our RNAi screen, *cki-2* loss of function results in the production of extra VPCs through additional rounds of cell divisions. The cell-cycle quiescence defect conferred by *cki-2(ok2105)* is sensitive to the expression of *cye-1*, supporting that *cki-2* functions as a CKI to regulate the core cell-cycle machinery. Since the *cki-2(ok2105)* mutation disrupts quiescence without affecting *cki-1* expression (data not shown), *cki-1* does not provide sufficient activity to compensate for loss of *cki-2* activity. Therefore, *cki-1* and *cki-2* provide independent functions during cell-cycle quiescence.

Our data reveal two important distinctions between the roles played by *cki-1* and *cki-2*. First, despite the general expression of the *cki-2* reporter, the *cki-2(ok2105)* extra cell phenotype is cell-type restricted. In contrast, *cki-1* loss-of-function produces defects in multiple tissues. The phenotypic differences between *cki-1* and *cki-2* mutant animals likely reflect differences

in post-transcriptional regulation, cell-type specific dependence or protein function between the two genes. Although overexpression of either CKI-1 or CKI-2 is sufficient to arrest cell divisions, it is currently not possible to unequivocally demonstrate that the proteins perform unique functions, such as initiation *vs.* maintenance of quiescence. Second, we observe that *cki-2* activity, unlike *cki-1*, is not functionally redundant with *lin-35*. A prior study determined that the *lin-35*-mediated process acted in parallel to *cki-1* and *cki-2* based on increased cell divisions in *lin-35* mutant animals following concurrent RNAi inactivation of *cki-1* and *cki-2*.[20] In fact, our *cki-2* data suggest that the previously observed *lin-35* enhancement was due solely to the inactivation of *cki-1*. These data suggest that either *cki-1*, *cki-2* and *lin-35* act within three partially overlapping pathways or that *cki-2* and *lin-35* act together within with a pathway that is parallel to a *cki-1*-mediated process. Recent data obtained from replacing the p57 locus with p27 indicate that these two related mammalian CKIs perform both specific and shared roles.[29] As technologies emerge that would allow high efficiency homologous recombination in *C. elegans*,[30] it will be possible to uncover the specific molecular functions that differentiate *cki-1* and *cki-2*.

Materials and Methods

Strains

All strains are N2 Bristol derived and grown under standard conditions at 20°C.[31] The following mutant alleles were used in these studies: *cye-1(eh10), lin-35(n745), mnDf100 unc-4(e120), cki-1(gk132), cki-2(ok2105), cki-2(tm3496), rrf-3(pk1426), lin-12(n950), lag-2(sa37), ayIs7[hlh-8::GFP], wIs78[ajm-1::gfp; scm-1::gfp; unc-119(+); F58E10(+)], rtIs14[elt-2::GFP]* and *qIs19[Plag-2::GFP]*.

RNAi screen

The Elm phenotype screen[14] was modified to accommodate the reverse genetic approach. The *rrf-3(pk1426)* mutation[32, 33] was incorporated into the *lin-12(n950); lag-2(sa37)* tester strain to enhance sensitivity to RNAi. The screening of the genome-wide library[34] was performed essentially as described previously,[35] with the exception that our screen searched only for the Elm phenotype using the triple-mutant tester strain. The feeding-RNAi clone, II-5H10, was found to induce the Elm phenotype. The clone was sequenced and verified as targeting T05A6.2, also known as *cki-2*.

Analysis of *cki-2*

The FX3496 strain was obtained from the National BioResource Project. RB1692, containing the *cki-2(ok2105)* deletion allele, was obtained from the *C. elegans* Genetics Center. The strain was back-crossed at least four generations before phenotypic analysis. PCR was used to localize the gene disruption and to generate a product that was sequenced to reveal the molecular lesion. Approximate positions of oligonucleotide primers used in reactions are shown in Figure 2A. Primer combination A+C amplified a 1.9 kb product from wild-type genomic DNA template, while a 0.3 kb product was generated using the *cki-2(ok2105)* deletion template. Primer combination A+B amplified 0.26 kb product from wild-type genomic DNA and was used to confirm the absence of full-length *cki-2* sequences in the homozygous mutant animals. Oligonucleotide primer sequences are as follows:

Primer A: 5′-CTCGAGGATCATGGCGGCAACAACAGCCG-3′

Primer B: 5′-TCGGAAGCAGAAATCGACTC-3′

Primer C: 5′-AAACAAGAGCGAAGGTCGAA-3′

The sequence of the *cki-2(ok2105)* deletion/insertion mutation was determined to be:

…ATCTGATATTCATGATATTCATGCAAATGGT…

The underlined bases indicate the nine base pair duplication replacing the 1,606 bp normally found in wild type.

The *Pcki-2::gfp* transcriptional reporter was created by insertion of the 3.1 kb region upstream of the *cki-2* coding sequence into pPD114.108, a kind gift from A. Fire. The reporter was co-injected with the dominant Rol-marker, pRF4, and four stable transgenic lines were isolated. Mosaic maintenance of the extrachromosomal transgenic array allowed visualization of GFP expression within specific tissues.

cDNA production and Real-time PCR analysis

cki-1(gk132)/mIn1 hermaphrodites and *cki-2(ok2105)* homozygous males were mated and cross-progeny were collected based on GFP expression. Approximately 100 L2-aged GFP-negative and GFP-positive individuals corresponding to *cki-1(gk132)/cki-2(ok2105)* and *mIn1/(cki-2(ok2105)*, respectively, were pooled and their RNA isolated with the RNeasy kit (Qiagen) and reverse transcribed using Superscript III (Invitrogen). Relative expression levels were determined using the ΔΔCt method [36] with data from triplicate multiplexed reactions normalized to *gpd-2* mRNA. Real-time PCR was performed with SYBR Green PCR Mix (Applied Biosystems) on a 7900HT machine using the 9600 emulation setting (Applied Biosystems). *cki-2* was amplified using Primers A and B (above). Amplification of *gpd-2* used the following primers: 5′-ACCGGAGTCTTCACCACCATC-3′ and 5′-TTCCTGATGGTCCGTCAACAG-3′.

Microscopy

To assay cell-cycle quiescence during development, synchronously developing populations were examined for extra cell divisions at 0, 18, 24 or 48 hours post-feeding. VPCs were visualized using Nomarski optics. Seam cells, distal tip cells, cells of the M-lineage and intestinal nuclei were visualized with the aid of the *scm*[37], *lag-2*[38], *hlh-8*[39] and *elt-2*[40] GFP markers, respectively. Embryos were processed for immunohistochemistry using standard methanol/formaldehyde fixation protocols.[41] Anti-α-tubulin monoclonal antibody (DM1A; Sigma-Aldrich) was used at 1:2000 dilution. Alexa Fluor 488 labeled goat anti-mouse secondary antibody (A11017; Invitrogen) was used at 1:500 dilution. Nomarski and epifluorescent images were collected using a Zeiss AxioImager microscope, AxioCam camera and Axiovision software. Image cropping and annotations were performed using Adobe Photoshop and Illustrator software.

Determination of viability and brood size

L4-aged hermaphrodites were individually placed on fresh plates. The parental animals were transferred at least once daily for at least three days. After two days, larvae and unhatched eggs were counted. Eleven N2 animals produced broods of 252, 252, 254, 260, 261, 278, 282, 286, 313, 317 and 330 total progeny. A subset of plates was analyzed to determine embryonic lethality, ten unhatched eggs were found among 1,411 viable siblings. Nine *cki-2(ok2105)* broods were enumerated as described. Egg-laying defective *cki-2(ok2105)* mutants produced broods of 34, 86,127 and 152 progeny, while *cki-2(ok2105)* mutants able to lay eggs for the duration of the experiment produced broods of 203, 249, 286, 309 and 310 offspring. Embryonic lethality was determined to be 0.51% in these broods with nine unhatched eggs found among the 1,756 total viable siblings.

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Abreviations

CKI	cyclin-dependent kinase inhibitor
Elm	enhancer of *lin-12(gf)* multivulva
RNAi	RNA interference
VPC	vulva precursor cell

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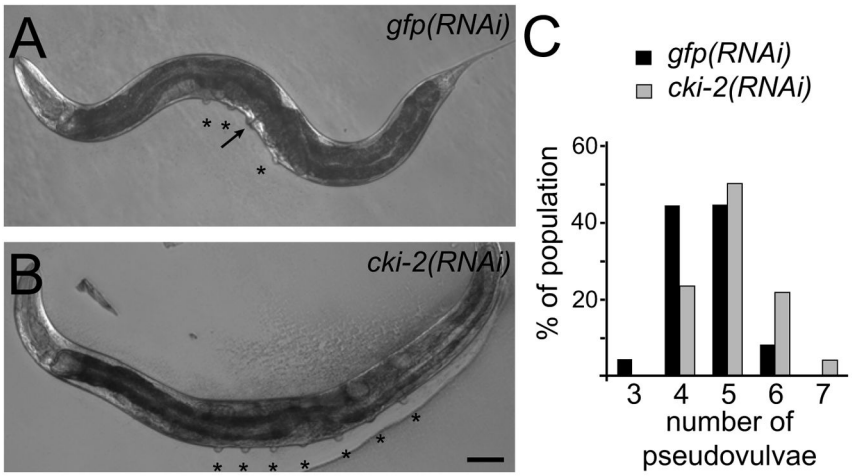

Figure 1.

The Elm phenotype of *cki-2(RNAi)*. (A) An adult *rrf-3(pk1426); lin-12(n950gf); lag-2(sa37)* triple mutant tester animal treated with a control RNAi clone targeting *gfp* displays a maximum of six pseudovulvae (asterisks). Arrow indicates pseudovulva at normal vulva location. (B) Inactivation of *cki-2* by RNAi results in the production of greater than six pseudovulvae on the tester strain. Scale bar indicates 50 μm. (C) Quantification of pseudovulvae displayed by the populations (n=50 each) represented in (A) and (B). *cki-2(RNAi)* significantly increased the average pseudovulvae number compared to the control population ($P < 0.01$).

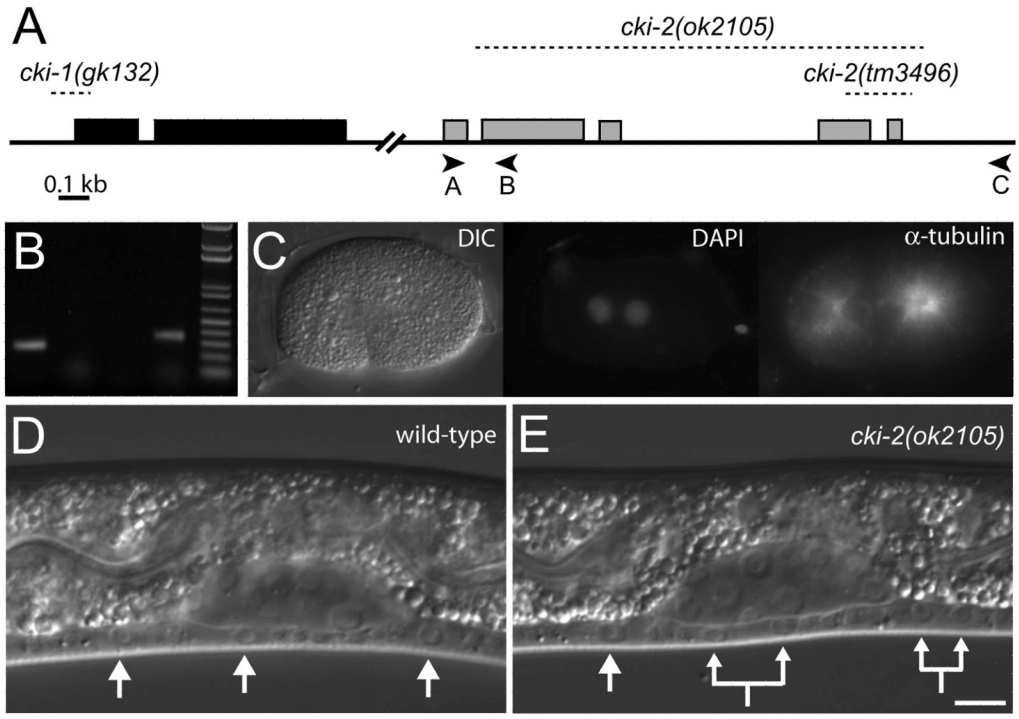

Figure 2.

cki-2(ok2105) mutant animals display cell-cycle quiescence defects. (A) Genomic organization of the *cki-1* (black boxes) and *cki-2* (grey boxes) coding sequences. Dashed lines designate regions deleted by the indicated mutations. Oligonucleotides used in PCR-based analyses are indicated as arrowheads. Break indicates approximately 4.8 kb separating *cki-1* and *cki-2*. (B) PCR using genomic DNA template confirms *cki-2(ok2105)* deletion. Using conditions optimized for short products, PCR of wild-type DNA readily amplifies the 0.26 kb product of the A+B primer set (lane 1) while the 1.9 kb A+C product (lane 3) is not appreciable produced. Conversely, the *cki-2(ok2105)* mutant DNA does not support amplification using the A+B primer set, rather in combination with A+C generates a 0.3 kb product that spans the breakpoint of the mutation (lanes 2 and 4, respectively). (C) DAPI and anti-α-tubulin antibody staining of *cki-2(ok2105)* homozygous embryos visualizes the normal centrosome number, cells with greater than two centrosomes were not observed (n=30 embryos). (D) Nomarski image of wild-type L2 larvae shows three of six VPCs (arrows) temporarily arrested. (E) VPCs of *cki-2(ok2105)* L2 larva undergo extra cell divisions resulting from defective cell-cycle quiescence. Split arrows indicate sibling cells generated by extra rounds of cell divisions. Scale bars indicate 10 μm.

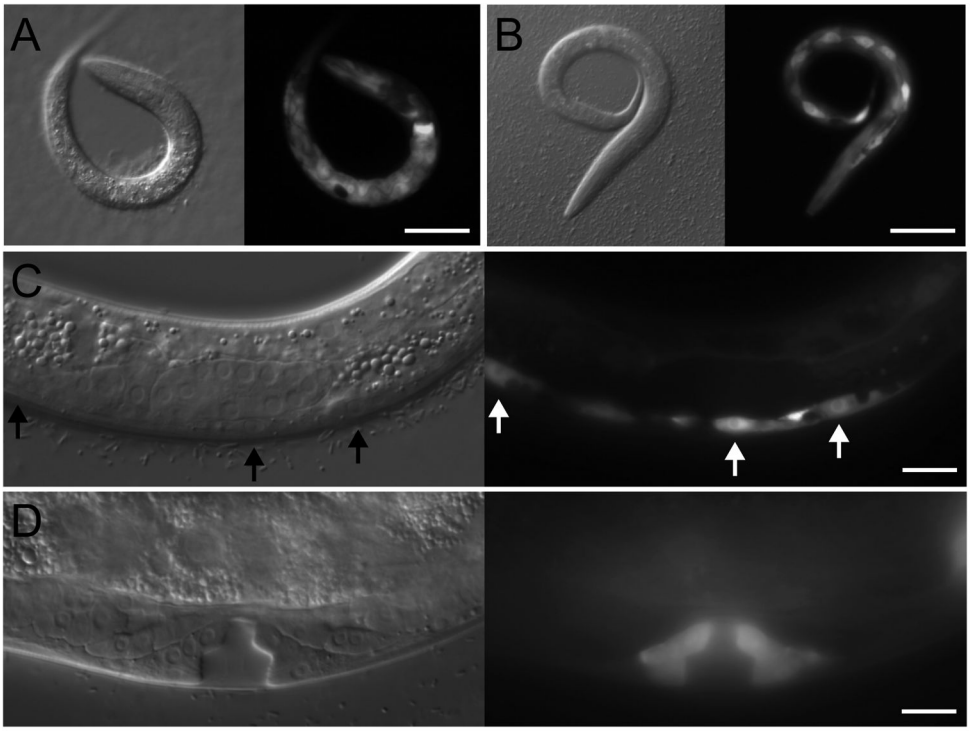

Figure 3.
A *Pcki-2::gfp* reporter is widely expressed during larval development. During L1, GFP expression can be observed within cells of the intestine (A) and seam (B) lineages. Cells of the vulva lineage strongly express GFP at both the L2 (C) and L4 (D) stages. All images depict Nomarski (left panels) and GFP expression (right panels) of larvae harboring a *Pcki-2::gfp* extrachromosomal array. Arrows indicate VPCs and scale bars represent 10 μm.

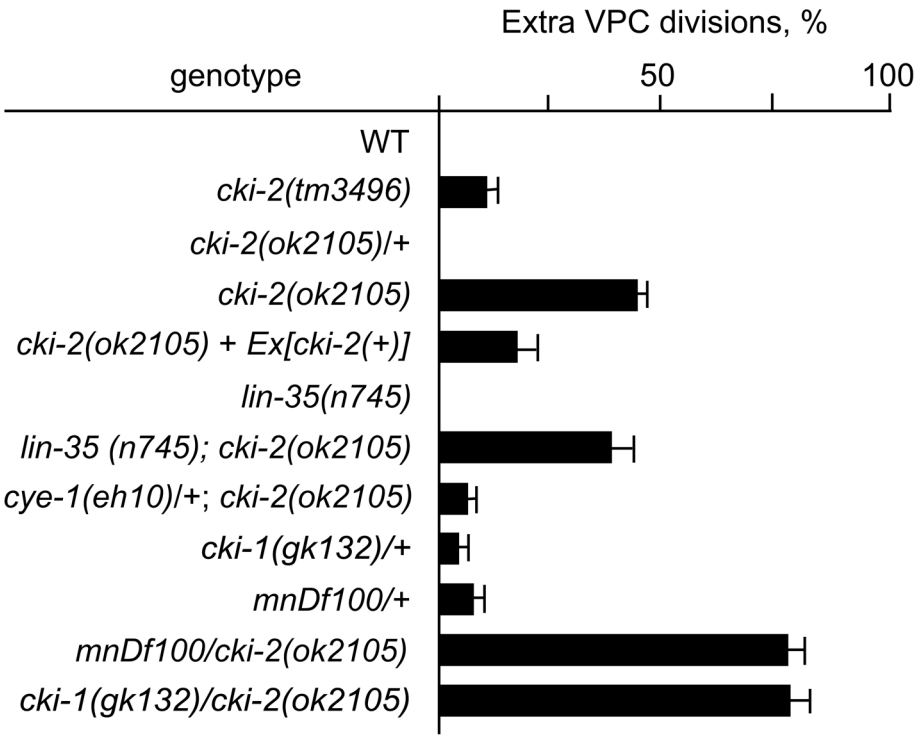

Figure 4.

cki-2 is an important regulator of VPC cell-cycle quiescence. The percentage of VPCs observed to have undergone an extra round of cell division within the indicated genotype is presented. Except for the rescue experiment (n=7), at least 15 animals were examined just prior to the L2-to-L3 molt. Mean ± standard error values are indicated.

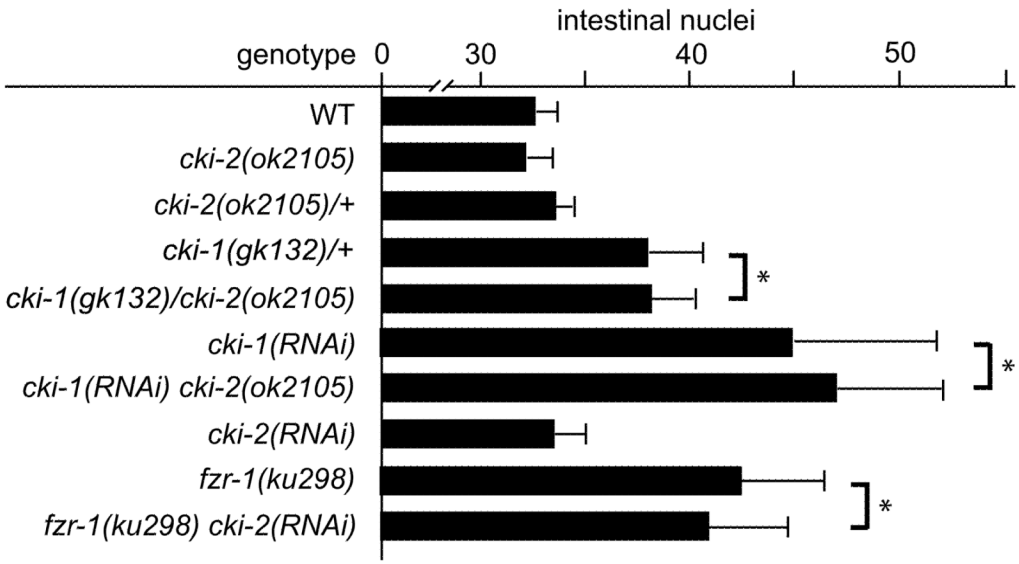

Figure 5.

cki-2 is not required for control of intestinal divisions. Young adult animals were analyzed for each of the indicated genotypes to determine the average number of intestinal nuclei (n≥20). Bars indicate mean ± standard deviation. * indicates that $P > 0.2$.

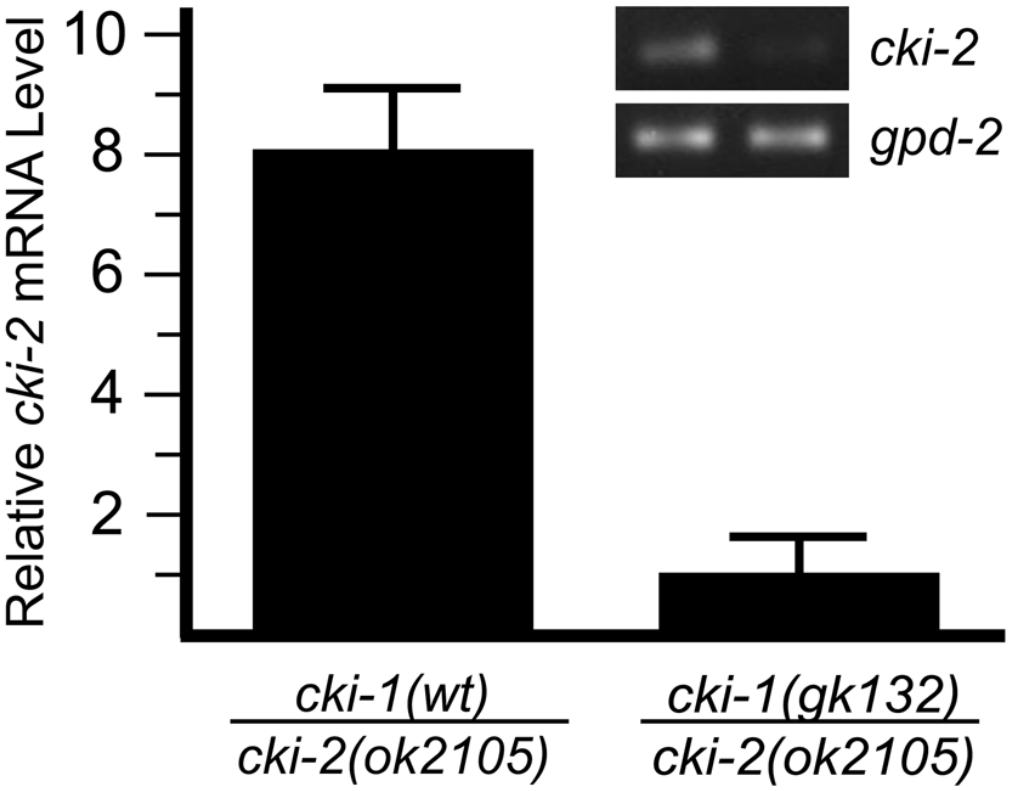

Figure 6.
The *cki-1(gk132)* mutation disrupts expression of the adjacent *cki-2* gene. Comparison of quantitative real-time PCR analysis of steady-state mRNA indicates that *cki-2* expression from the *cki-1(gk132)* chromosome is decreased approximately 8-fold compared to the wild-type control. Inset image illustrates a representative result of a semi-quantitative, reverse-transcription PCR to visualize *cki-2* mRNA from wild-type (left lane) and *cki-1(gk132)* (right lane) animals. GPDH (*gpd-2*) is used as the normalization control for both experiments.

Table 1

Tissue-restricted defects of *cki-2(ok2105)* mutant animals

tissue[1], age	**wild-type**	**n**	***cki-2(ok2105)***	**n**
intestinal nuclei, hatching	19.8±0.5	20	20.0±0.2	20
intestinal nuclei, L4	32.6±1.0	20	32.2±1.2	20
M-Lineage, hatching	1±0	20	1±0	20
M-Lineage, L2	16.2±0.2	19	16.4±0.2	20
Distal Tip cell, L4	2±0	50	2±0	50
seam cell, young adult	16±0.4	20	15.9±0.5	20
VPC, L2	6±0	30	8.6 ±0.8	30

[1]Examination of intestine, M-lineage, Distal Tip and seam cells was aided using the *elt-2*, *hlh-8*, *lag-2* and *scm* tissue-specific GFP markers, respectively.