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lin-35 Rb and *cki-1* Cip/Kip cooperate in developmental regulation of G1 progression in *C. elegans*

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SUMMARY

We have investigated the regulation of cell-cycle entry in *C. elegans*, taking advantage of its largely invariant and completely described pattern of somatic cell divisions. In a genetic screen, we identified mutations in *cyd-1* cyclin D and *cdk-4* Cdk4/6. Recent results indicated that during *Drosophila* development, cyclin D-dependent kinases regulate cell growth rather than cell division. However, our data indicate that *C. elegans cyd-1* primarily controls G1 progression. To investigate whether *cyd-1* and *cdk-4* solely act to overcome G1 inhibition by retinoblastoma family members, we constructed double mutants that completely eliminate the function of the retinoblastoma family and cyclin D-Cdk4/6 kinases. Inactivation of *lin-35* Rb, the single Rb-related gene in *C. elegans*, substantially reduced the DNA replication and cell-division defects in *cyd-1* and *cdk-4* mutant animals. These results demonstrate that *lin-35* Rb is an important negative regulator of G1/S progression and probably a downstream target for *cyd-1* and *cdk-4*. However, as the suppression by *lin-35* Rb is not complete, *cyd-1* and *cdk-4* probably have additional targets. An additional level of control over G1 progression is provided by Cip/Kip kinase inhibitors. We demonstrate that *lin-35* Rb and *cki-1* Cip/Kip contribute non-overlapping levels of G1/S inhibition in *C. elegans*. Surprisingly, loss of *cki-1*, but not *lin-35*, results in precocious entry into S phase. We suggest that a rate limiting role for *cki-1* Cip/Kip rather than *lin-35* Rb explains the lack of cell-cycle phenotype of *lin-35* mutant animals.

Key words: *C. elegans*, Cyclin, CDK, pRb, Cip/Kip, Cell cycle

INTRODUCTION

The development of a multicellular organism requires the careful coordination of cell division with growth and differentiation. In part, this coordination is achieved through integration of extracellular signals during the G1 phase, to which cells respond by either advancing into or withdrawing from another division cycle (Pardee, 1989). Ultimately, mitogenic and antiproliferative signals affect the cell-intrinsic cell-cycle machinery, of which the cyclin-dependent kinases (CDKs) are key components. The importance of the G1 control mechanisms is underscored by the finding that most, if not all, tumor cells have defects in one or more genes involved in G1 progression (Sherr, 1996). Despite extensive research, the in vivo functions of such genes have been poorly characterized.

Significant insights have been gained from studying mammalian cells in tissue culture and gene alterations in human cancer. Members of the retinoblastoma (Rb) tumor-suppressor family (pRb, p107 and p130 in mammals) have been found to inhibit progression through the G1 phase (Sherr, 1996). This negative-regulatory function of the pRb protein is constrained by phosphorylation at multiple CDK phosphorylation sites. Sequential phosphorylations disrupt the binding between pRb and transcription factors such as E2F, thereby allowing these transcription factors to activate genes required for DNA synthesis and removing active transcriptional repression by pRb (Dyson, 1998). One of the critical targets of the E2F transcription factor is cyclin E (Dyson, 1998), which together with its partner CDK2 is required for the initiation of DNA replication (Duronio and O'Farrell, 1995; Knoblich et al., 1994; Ohtsubo et al., 1995; Tsai et al., 1993; van den Heuvel and Harlow, 1993).

CDKs appear to act at multiple levels of this G1 control pathway. The first CDKs to become active in the cell cycle consist of a CDK4 or CDK6 catalytic subunit and a D-type cyclin regulatory subunit (Sherr, 1993). It is generally thought that cyclin D-dependent kinases initiate pRb phosphorylation in mid G1, while the subsequent activation of cyclin E-CDK2 kinases leads to completion of this process (Mittnacht, 1998). However, the presence of multiple D-type cyclins, CDK4/6 kinases and pRb-related proteins has hampered a direct demonstration of their functions in vivo. For example, it is not clear to what extent Cyclin D-CDK4/6 kinases are essential for pRb inactivation and cell-cycle progression in vivo. Moreover, it remains unknown whether Cyclin D has critical targets other than proteins of the pRb family. Potential targets include cyclin-dependent kinase inhibitors (CKIs) of the Cip/Kip family (Sherr and Roberts, 1999). $p21^{CIP1}$ and $p27^{KIP1}$ associate with cyclin E-CDK2 complexes and prevent their kinase activity. The same inhibitors also bind cyclin D and

CDK4/6, but apparently mediate their assembly rather than inhibiting kinase activity (Cheng et al., 1999; LaBaer et al., 1997). This association may result in sequestering the Cip/Kip inhibitors from cyclin E-CDK2 complexes, which further promotes progression through the G1/S transition.

Studies in genetic model systems should contribute further insights in the in vivo functions of G1 regulatory genes. Such analyses have already provided surprising results. For example, recent studies in *Drosophila* revealed a requirement for CDK4 in cell growth, rather than cell division (Datar et al., 2000; Meyer et al., 2000). The nematode *C. elegans* provides an attractive animal model for cell-cycle studies. The somatic cell-lineage of *C. elegans* is largely invariant and has been completely described; thus, the timing of division is known for every cell (Sulston and Horvitz, 1977; Sulston et al., 1983). In addition, most of the cell-cycle regulators mentioned above are represented by single genes in *C. elegans*, which simplifies their functional analysis. For example, a single D-type cyclin (*cyd-1*), CDK4/6-related kinase (*cdk-4*), cyclin E (*cye-1*) and pRb family member (*lin-35* Rb) are present (The *C. elegans* Sequencing Consortium, 1998; Fay and Han, 2000; Lu and Horvitz, 1998; Park and Krause, 1999). In addition, two genes, *cki-1* and *cki-2*, encode cyclin-dependent kinase inhibitors of the Cip/Kip family (The *C. elegans* Sequencing Consortium, 1998; Feng et al., 1999; Hong et al., 1998). Inhibition of gene function by RNA interference has revealed roles for *cyd-1/cdk-4* and *cki-1* as positive and negative regulators of the G1/S transition, respectively (Hong et al., 1998; Park and Krause, 1999). However, the network of gene activities that regulates G1 progression in *C. elegans* remains entirely unknown. In fact, *lin-35* Rb was identified as a member of the 'synthetic multivulva' (synMuv) genes that inhibit the expression of vulval cell fates (Ferguson and Horvitz, 1989), and a function in cell-cycle regulation has not been reported for *lin-35* Rb.

To identify G1 regulators in *C. elegans*, we performed a screen for mutants that arrest cell-division in G1 phase. In this screen we isolated mutations in *cyd-1* cyclin D and *cdk-4* CDK4/6. We used these alleles to address the interaction between cyclin D-dependent kinases and pRb family members, by creating double mutant animals that lack activity of *cyd-1/cdk-4* and *lin-35*. Our results demonstrate that *lin-35* Rb is an important negative regulator of cell division and probably a major downstream target of *cyd-1*/*cdk-4*. However, *lin-35* Rb did not appear to be the only target of *cyd-1*/*cdk-4*. In addition, we provide evidence that the Cip/Kip family members *cki-1* and *cki-2* cooperate with *lin-35* Rb in controlling cell-cycle entry, and that these two pathways provide non-overlapping levels of cell-cycle control.

MATERIALS AND METHODS

Culture conditions and strains

We used the wild-type strains N2 and RW7000 and the following mutations, descriptions of which can be found elsewhere (Riddle et al., 1997).

LGI: *dpy-5(e61)*, *lin-35(n745* and *n2239)* (Lu and Horvitz, 1998), *unc-29(e403)*.

LGII: *dpy-10(e128)*, *rol-1(e91)*, *cyd-1(he112* and *he116)* (this study).

LGX: *lon-2(e678)*, *unc-9(e101)*, *cdk-4(he109*, *he110*, *he111* and *gv3)* (this study) (Park and Krause, 1999).

Deficiency: *heDf1*.

Rearrangements: *mnC1(II)* (Wood, 1988).

Integrated arrays: *maIs103[rnr::GFP unc-36(+)]X* (Hong et al., 1998), *rtIs14[elt-2::GFP; osm-10::HT150Q]IV* (a gift from P. W. Faber and A. Hart).

Screen for positive regulators of G1/S progression

Animals of genotype *unc-36(e251);maIs103[rnr::GFP unc-36(+)]* (Hong et al., 1998), expressing GFP controlled by *ribonucleotide reductase* (*rnr*) promoter sequences, were mutagenized with 25 mM ethylmethanesulfonate as described (Brenner, 1974). Individual mutagenized F1 animals were picked to plates and their progeny examined for the presence of 1/4 sterile uncoordinated animals. Such mutants were further examined for absence of postembryonic cell divisions and GFP expression. Candidate mutations identified from ~10,000 haploid genomes were recovered from heterozygous siblings and mapped to chromosomes by PCR, making use of primers based on polymorphic Sequence-Tagged Sites in the RW7000 Bergerac strain (Williams et al., 1992). Two mutations (*he112* and *he116*) were placed on chromosome II and three mutations (*he109*, *he110* and *he111*), as well as the deletion *heDf1*, were placed on the X chromosome. Standard two- and three-factor mapping with *dpy-10(e128)* and *rol-1(e91)* or *lon-2(e678)* and *unc-9(e101)* was performed for further mapping. DNA sequence analysis revealed molecular lesions in *cyd-1* (*he112*, Q^{292}->stop) and *cdk-4* (*he110*, E^{85}->K; *he111*: W^{208}-> STOP; *he109*: Q^{292}-> STOP). PCR and Southern blotting experiments revealed that *he116* and *heDf1* are deletions.

Quantitation of DNA and cell division

Quantitative determination of DNA content was performed essentially as described before (Boxem et al., 1999), by measuring the pixel intensity of serial *z*-sections taken on a confocal microscope (Zeiss) of animals stained with propidium iodide.

The number of cell divisions was determined by counting nuclei in fixed specimens. The daughter cells of P2 to P10 as well as the intestinal nuclei could be recognized unambiguously and are therefore used in the figures. Owing to partial rescue of cell division, *cyd-1* and *cdk-4* mutants were recognizable and formed 1/4 of the total offspring. Strains carrying the *elt-2::GFP* reporter were used to recognize intestinal nuclei in the experiments shown in Figs 6 and 7.

Two-hybrid assays

A full-length *cki-1* cDNA was obtained from *C. elegans* cDNA by PCR using primers 5′-ggggaccactttgtacaagaaagctgggtgtatggagag-catgaagatcg and 5′-ggggacaagtttgtacaaaaaagcaggcttgtcttctgctcgtcg-ttgc. Sequence analysis confirmed the obtained PCR product corresponded to the wild-type *cki-1* sequence. The *cki-1* cDNA was cloned into pPC97 and used as bait in a two-hybrid experiment as described (Vidal, 1997). CYE-1 and CYD-1 were each isolated independently five times as a CKI-1 interacting protein, out of a total of 2×10^6 yeast colonies transformed with the *cki-1* bait construct.

Timing of DNA replication

N2 animals were injected with *lin-35* dsRNA or *cki-1* dsRNA, and allowed to produce progeny for 24 hours. Eggs produced in the next 24 hours at 15°C were hatched in the absence of food, which results in a developmental arrest immediately after hatching. Subsequently, synchronous development was induced by transferring the arrested larvae to fresh agar plates containing *E. coli* bacteria. Developing larvae were fixed in Carnoys fixative at 1 hour intervals and stained with the DNA stain propidium iodide. DNA contents of 10 In cells were determined as described above.

Growth rate of *cyd-1* mutants

Embryos were collected by hypochlorite treatment of gravid *cyd-*

1(he112)/mnC1 adults and allowed to hatch in S-medium without food (Wood, 1988). Synchronous L1 development was initiated 24 hours later by transferring the starved L1 animals to agar plates containing *E. coli* bacteria. We determined the total body size of five *cyd-1* mutants and five heterozygous siblings at various times, using the size measurement function of the Openlab software package. The software was calibrated using a size standard slide.

RESULTS

Identification of a D-type cyclin and Cdk4/6 related kinase in a screen for positive regulators of G1 progression

To identify positive regulators of G1 progression, we used a reporter construct with S phase-specific transcription; the green fluorescent protein (GFP) expressed under the control of *ribonucleotide reductase* promoter sequences (*rnr::GFP*, Hong et al., 1998). The F2 progeny from mutagenized animals carrying the *rnr::GFP* transgene were examined for the presence of mutants that lack postembryonic cell division and expression of the *rnr::GFP* marker (Fig. 1A,B). We identified six independent mutations that fulfill these criteria. Initial mapping placed two mutations (*he112* and *he116*) on chromosome II and four mutations (*he109*, *he110*, *he111* and *heDf1*) on the X chromosome.

he112 and *he116* failed to complement each other, indicating they may affect the same locus. Standard three-factor mapping placed both mutations in the proximity of the cell-cycle regulatory gene *cyd-1*, which encodes the sole D-type cyclin in *C. elegans* (Park and Krause, 1999). Molecular lesions that affect the *cyd-1* gene were identified by DNA sequence analysis (*he112*) and by PCR and Southern blotting experiments (*he116*). *cyd-1*(*he112)* contains a nonsense mutation predicted to truncate the C-terminal 114 amino acids (Fig. 2). The *cyd-1(he116)* mutation deletes the *cyd-1* promoter region as well as the first two exons (Fig. 2).

The predicted partner for CYD-1 is CDK-4, a CDK4- and CDK6-related kinase encoded by the *cdk-4* gene located on the X-chromosome (Park and Krause, 1999). Introduction of a wild-type *cdk-4* transgene in germline transformation experiments completely suppressed the defects caused by three X-linked mutations, *he109*, *he110* and *he111*. Sequence

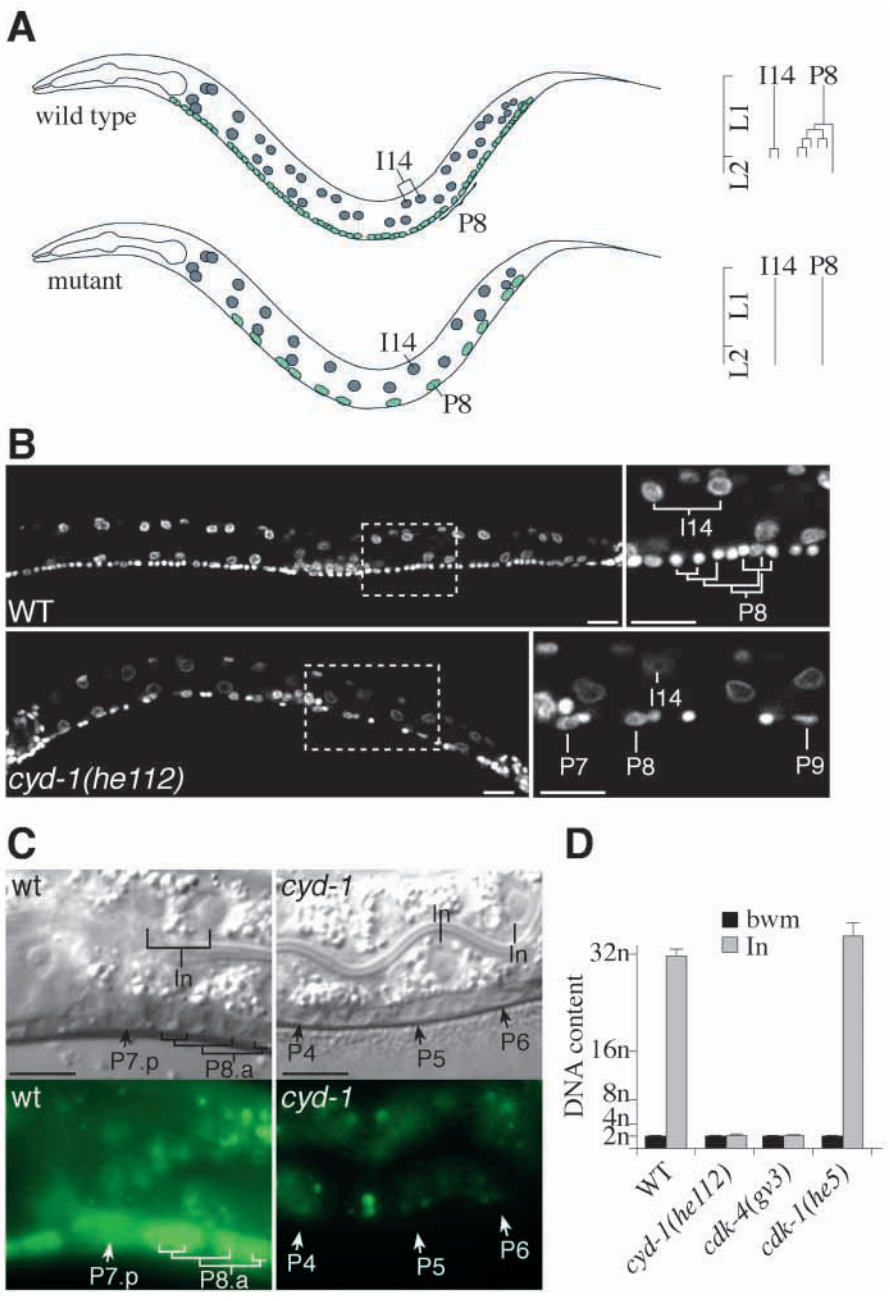

Fig. 1. Identification and characterization of *cyd-1* and *cdk-4* mutant animals. (A) Positions of cells in the ventral cord precursor (P, green) and intestinal (I, gray) lineages in late L1 wild-type (top) and cell-cycle mutant (bottom) larvae. The lineages of an individual P and I cell are indicated for each genotype (right). (B) Postembryonic blast cells remain undivided in *cyd-1* and *cdk-4* mutants, as indicated for intestinal and P precursor cells in the enlarged sections (right). The panels show a late L1 wild-type larva (top) and similar stage *cyd-1(he112)* mutant (bottom) after fixation and DNA staining with propidium iodide. (C) Expression of the *rnr::GFP* S-phase marker in wild-type animal (left) and *cyd-1(he112)* mutant (right). Nomarski images (top) and corresponding epifluorescent images (bottom) show several cells of the P and intestinal lineages. In the *cyd-1* animal, only autofluorescence of the intestinal cells is detectable. (D) Quantitative measurements of DNA content in the intestinal nuclei (gray bars) of wild-type animals and mutant strains of indicated genotype. Body wall muscle cells (black bars) serve as 2n DNA standards. Scale bars: 10 μm. Values indicated are mean±s.e.m.

Fig. 2. Molecular lesions in *cyd-1* and *cdk-4* mutant alleles, illustrated with respect to the genomic structure. Exons are shown as boxes, introns as lines.

analysis identified point mutations within the *cdk-4*-coding sequences in each of the three mutant strains. Alleles *he109* and *he111* contain nonsense mutations that should terminate translation after 207 and 291 amino acids, respectively (Fig. 2). The *he110* allele contains a missense mutation that converts a glutamate at position 85, which is conserved in protein kinases, to lysine (Fig. 2).

The mutant phenotype associated with *heDf1*, the fourth X-linked mutation, was not rescued by a wild-type *cdk-4* transgene. However, this mutation did map in the proximity of *cdk-4*, and failed to complement the *cdk-4(gv3)* allele. PCR and Southern blotting experiments revealed that *heDf1* is a deletion that removes the entire *cdk-4* gene (Fig. 2). In addition to the cell-cycle arrest, *heDf1* mutant animals displayed defects in growth and morphology that were not observed in animals homozygous for any of the other *cdk-4* alleles, including animals homozygous for the *cdk-4(gv3)* deletion. Because of the lack of rescue and pleiotropic defects, we conclude that *heDf1* deletes *cdk-4* and probably another gene required for larval development. *heDf1* was not analyzed further.

All mutations isolated were recessive and conferred fully penetrant cell-cycle defects (Table 1). Moreover, the defects observed in both *cdk-4* and *cyd-1* mutants were at least as severe as those caused by RNA-mediated interference (RNAi) of these genes (Park and Krause, 1999). Based on these genetic and molecular characterizations, the *cyd-1* and *cdk-4* alleles confer strong loss-of-function or null phenotypes.

cyd-1 and *cdk-4* are required for G1/S progression in postembryonic cell divisions

Following a reverse genetics approach, Park and Krause (Park and Krause, 1999) previously identified essential roles for *cyd-1* and *cdk-4* in cell division. We compared the *cyd-1* and *cdk-4* mutant phenotype to the reported defects caused by *cyd-1* RNAi and the *cdk-4(gv3)* deletion allele. Animals homozygous for the *cyd-1* alleles *he112* and *he116* or *cdk-4* alleles *he109, he110,* and *he111* completed embryogenesis. The only embryonic defect we observed was a failure of *cyd-1* mutants, but not *cdk-4* mutants, to complete the final few embryonic intestinal divisions. Consequently, *cyd-1(he112)* and *cyd-1(he116)* larvae hatched with 16 intestinal cells (16.0, *n*=10), rather than the 20 cells present in wild-type animals. Although wild-type maternal product could mask an embryonic role of *cyd-1* and *cdk-4* in homozygous mutants, RNAi of *cyd-1* or *cdk-4* also did not cause embryonic lethality and prevented cell division only during larval development (Park and Krause, 1999). As RNAi usually impedes both maternal and zygotic gene function, *cyd-1* and *cdk-4* appear to be predominantly required for postembryonic somatic cell cycles.

Table 1. Characterization of *cyd-1* and *cdk-4* alleles

Parental genotype	% larval arrest of progeny (*n*)*	Postembryonic blast cells that initiate division during the L1 stage	
		P2-P10	I
Wild type	0.0 (>1000)	9/9	14/20
cyd-1(he112)/mnC1	25.5 (697)	0/9	0/16
cyd-1(he116)/+	24.14 (994)	0/9	0/16
cdk-4(he109)/+	25.16 (930)	0/9	0/20
cdk-4(he110)/+	24.73 (1314)	0/9	0/20
cdk-4(he111)/+	23.65 (985)	0/9	0/20

*The percentage larval arrest was determined by counting the total progeny from three to five heterozygous animals of the indicated genotype.

Divisions of P and I cells were scored in L3 stage mutants fixed and stained with the DNA stain PI. Divisions of P1, P11 and P12 could not be unambiguously determined and, therefore, are excluded.

Cell divisions are represented as number of cells divided/number of cells present in individual animals.

More than 10 animals of each genotype were examined.

Two observations indicate that *cyd-1* and *cdk-4* mutant larvae arrest cell divisions prior to S phase. As first observed in the screen, each *cyd-1* or *cdk-4* mutant lacked detectable expression of the *rnr::GFP* S-phase reporter in the postembryonic lineages (Fig. 1C; Z1 and Z4, the somatic gonad precursor cells, were the only exception). In addition, we found no evidence of DNA replication in postembryonic cell lineages. Specifically, we determined the DNA content of cells in two postembryonic lineages: precursor cells of the ventral nerve cord (P), which undergo four rounds of cell division during the first larval stage, and intestinal nuclei, 14 of which divide once after hatching (Sulston and Horvitz, 1977). After this division, all intestinal nuclei go through a round of endoreduplication during each larval stage, resulting in a 32n DNA content (Hedgecock and White, 1985). The P and intestinal cells arrested with a 2n DNA content in both *cyd-1* and *cdk-4* mutants (Fig. 1D and not shown). By contrast, 4n and 32n DNA contents were found in the P and intestinal lineages, respectively, of *ncc-1/cdk-1* mutants whose cells arrest after DNA synthesis in the G2 phase (Boxem et al., 1999; Fig. 1D). Expression of *ribonucleotide reductase* normally coincides with DNA replication, but does not depend on it (Duronio and O'Farrell, 1994). The fact that cells arrest with 2n DNA amounts and lack *rnr::GFP* expression shows that the cell-cycle arrest occurs before S phase.

Although our results largely correspond to those described by Park and Krause (Park and Krause, 1999), the *cyd-1* mutant phenotype was slightly more severe than the reported *cyd-1(RNAi)* phenotype. The *cyd-1(RNAi)* animals hatched with 20 intestinal cells that arrested with a 4n DNA content (Park and Krause, 1999), while *cyd-1(he112)* and *cyd-1(he116)* mutant larvae have 16 intestinal cells that arrest with a 2n DNA content. The simplest explanation for the difference is that RNAi did not completely inactivate *cyd-1* function. Our data further support the conclusion that *cyd-1* and *cdk-4* are essential for G1/S progression in all postembryonic cell divisions.

cyd-1 and *cdk-4* are primarily required for cell division and not cell growth

An essential role for *cyd-1* and *cdk-4* in G1 progression

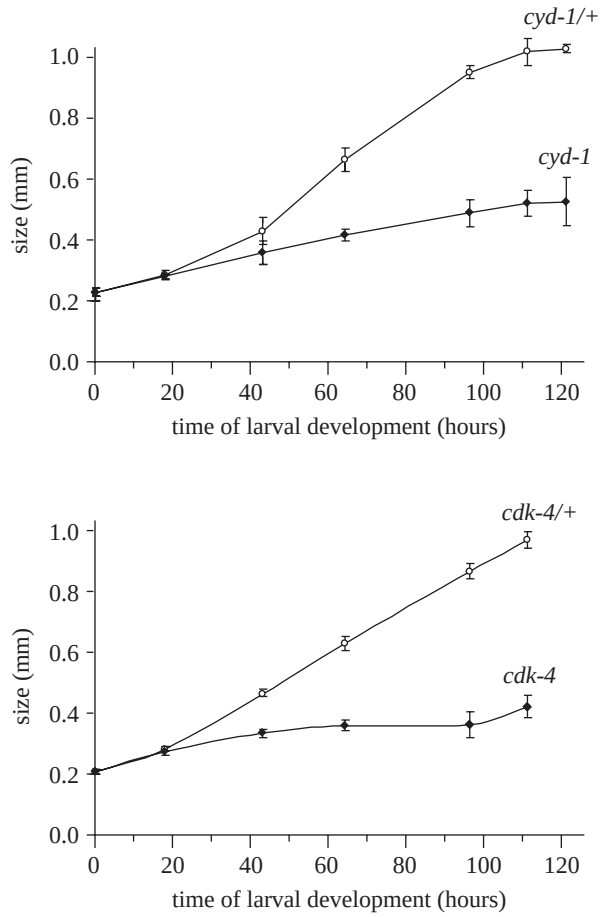

Fig. 3. *cyd-1* and *cdk-4* cell division defects precede growth defects. Size of *cyd-1(he112)* and *cdk-4(gv3)* homozygous mutants and wild-type siblings (genotypes: *cyd-1(he112)/mnC1* and *cdk-4/+* or *+/+)* is plotted as a function of time of postembryonic development at 15°C. The growth retardation of *cyd-1* and *cdk-4* mutants becomes first apparent in the L2 stage, subsequent to failure of some late embryonic and all postembryonic L1 divisions. Points indicate mean of five measured animals±s.d.

contrasts with results obtained in the fruit fly. *Drosophila cdk4* is not essential for most divisions; rather the Cdk4/Cyclin D complex has been implicated in regulation of cell growth (Datar et al., 2000; Meyer et al., 2000). To examine the possibility that the cell-division defects observed in *cyd-1* and *cdk-4* mutants are a secondary consequence of a cell-growth defect, we compared the growth rates of *cyd-1(he112)* and *cdk-4(gv3)* mutants with wild-type larvae. Until approximately 20 hours of larval growth at 15°C, *cyd-1(he112)* and *cdk-4(gv3)* mutants and wild-type siblings were indistinguishable in size (Fig. 3). After 20 hours, the growth rate of the wild-type siblings increased, whereas *cyd-1(he112)* and *cdk-4(gv3)* mutants continued to grow at a slow rate. The first larval (L1) stage ends with a molt at approximately 16 hours of postembryonic development at 15°C. Cells in a variety of cell lineages divide during this stage in the wild-type, starting with the Q neuroblasts at approximately 5 hours of postembryonic development at 15°C. As none of these divisions occur in *cyd-1* and *cdk-4* mutants, the cell-division defects precede the growth-retardation phenotype. Moreover, the embryonic divisions take place in the absence of growth, yet the final intestinal divisions failed to occur during embryogenesis in *cyd-1* mutants. Together, our results indicate that *cdk-4* and *cyd-1* primarily promote cell-cycle entry in *C. elegans*, in agreement with the view derived from mammalian tissue culture experiments.

lin-35 Rb is an important negative regulator of G1/S progression and a major downstream target of *cyd-1* and *cdk-4*

Mammalian D-type cyclins in association with the CDK4 and CDK6 kinases phosphorylate members of the pRb protein family in vitro (Kato et al., 1993; Meyerson and Harlow, 1994). However, it has been difficult to test whether this phosphorylation is crucial in vivo for inactivation of the G1/S inhibitory function of the pRb protein, as multiple genes of each type (D-type cyclins, CDK4/6 kinases and Rb-family members) are present in mammals. By contrast, these different regulators are encoded by single genes in *C. elegans* (The *C. elegans* Sequencing Consortium, 1998), making this organism an ideal system in which to address whether cyclin D and CDK4/6 are solely required to overcome G1/S inhibition by pRb family members.

The single *Rb*-related gene in *C. elegans*, *lin-35*, was previously identified as a regulator of vulval cell-fate specification (Lu and Horvitz, 1998). Surprisingly, animals homozygous for *lin-35* presumed null mutations are viable and show no cell-division defects. If *cyd-1* and *cdk-4* only act to inhibit *lin-35* Rb, then the cell-cycle arrest of *cyd-1* and *cdk-4* mutants should be fully overcome by *lin-35* Rb inactivation. To test this hypothesis, we used several assays. First, we examined whether inactivation of *lin-35* in a *cyd-1* mutant background restores expression of the *rnr::GFP* S-phase marker. Mutations in *lin-35* had previously been shown to result in general suppression of transgene expression (Hsieh et al., 1999). However, the *rnr::GFP* transgene was not silenced in the F1 progeny of animals injected with *lin-35* dsRNA (M. B. and S. v.d.H., unpublished). Importantly, inactivation of *lin-35* by RNAi efficiently restored *rnr::GFP* expression in *cyd-1* and *cdk-4* homozygous mutants (Fig. 4A).

Next we tested whether inactivation of *lin-35* could restore DNA replication in the intestinal nuclei. We constructed *lin-35;cyd-1* and *lin-35;cdk-4* double mutant strains, using two different alleles of *lin-35*, *n745* and *n2239*, which contain early nonsense mutations that probably completely eliminate *lin-35* function (Lu and Horvitz, 1998). Quantitative DNA measurements of intestinal nuclei showed that *lin-35(n745* or *n2239);cyd-1(he112)* and *lin-35(n745);cdk-4(gv3)* double mutant larvae were able to undergo multiple rounds of DNA replication, giving rise to a level of polyploidy in the double mutants that is similar to wild-type animals (Fig. 4B). In addition to DNA replication, *lin-35;cyd-1* and *lin-35;cdk-4* double mutants reached near wild-type body and gonad size (not shown) and *lin-35(n745);cdk-4(gv3)* double mutants occasionally produced viable progeny. Thus, loss of function of *lin-35* Rb overcomes the G1 arrest of cells in *cyd-1* and *cdk-4* mutants. These results demonstrate that *lin-35* Rb is an important inhibitor of the G1/S transition and, by analogy with other systems, probably a major target of *cdk-4* and *cyd-1*.

The *C. elegans* genome contains single members of the D and E subfamilies of G1 cyclins. Inactivation of *cye-1* cyclin

E by RNAi causes embryonic arrest at approximately the 100-cell stage (Fay and Han, 2000). Homozygous *cye-1* mutant larvae derived from heterozygous mothers display late larval defects and complete sterility (Fay and Han, 2000). RNAi for *lin*-35 did not affect the *cye-1(eh10)* mutant phenotype. Similarly, the embryonic arrest caused by *cye-1* RNAi was equally severe in a wild-type or *lin-35(n745)* mutant background (data not shown). Thus, *lin-35* inactivation specifically suppresses *cyd-1* and not *cye-1* loss of function. Although other interpretations are possible, these results are consistent with a model in which *lin-35* Rb acts downstream of *cyd-1*/*cdk-4* and upstream of *cye-1*.

lin-35 is probably not the only target of *cyd-1* and *cdk-4*

Although we observed substantial rescue of DNA replication, the number of cell divisions in the postembryonic lineages was not restored to wild-type levels in *lin-35;cyd-1* or *lin-35;cdk-4* double mutants (Fig. 4C). The double mutant animals also remained largely sterile. It appears unlikely that this lack of complete rescue resulted from incomplete inactivation of *lin-35* Rb, as probable null mutations were introduced (alleles *n745* and *n2239*; Lu and Horvitz, 1998), and identical effects were observed following *lin-35* RNAi. Incomplete rescue is also unlikely to be caused by inactivation of a positive cell-cycle function of *lin-35* Rb, as *lin-35* single mutants do not display apparent cell division defects. These results strongly suggest that *cdk-4* and *cyd-1* do not act exclusively upstream of *lin-35* Rb but most probably activate or inactivate additional targets.

lin-35 Rb is not rate limiting for S-phase entry

Because our results demonstrate that *lin-35* Rb acts as a negative regulator of cell division, we examined homozygous *lin-35* mutants in more detail for the presence of any defects in cell division. Using both Nomarski microscopy of live *lin-35* mutant larvae and fluorescence microscopy of fixed animals stained with propidium iodide (PI), we did not observe premature or additional cell divisions (data not shown). Alternatively, loss of *lin-35* might cause premature entry into S phase, which could be compensated for by expanding later cell-cycle phases. We compared the timing of DNA replication in wild-type and *lin-35(n745)* mutant animals to examine this possibility. Using quantitative DNA measurements at different times of L1 development, we determined that the first round of DNA synthesis in the intestinal cells occurs between 6 and 8 hours of postembryonic development in wild-type animals. This timing was identical in *lin-35(n745)* mutants (Fig. 5). Thus, in contrast to mouse embryo fibroblasts that lack Rb family members, inactivation of *lin-35* Rb is not rate limiting in the normal regulation of S-phase initiation in vivo, and additional regulatory pathways probably control the timing of DNA replication in the absence of *lin-35*.

The Cip/Kip family members *cki-1* and *cki-2* cooperate with *lin-35* in G1 regulation

Two observations suggest that *lin-35* Rb cooperates with other regulatory pathways in controlling progression through G1 phase. First, loss of *lin-35* did not fully overcome the cell division defects of *cyd-1* and *cdk-4* mutants. Second, the timing of S-phase entry and cell division remained intact in mutants lacking *lin-35* function.

Several levels of control may converge at the level of Cyclin

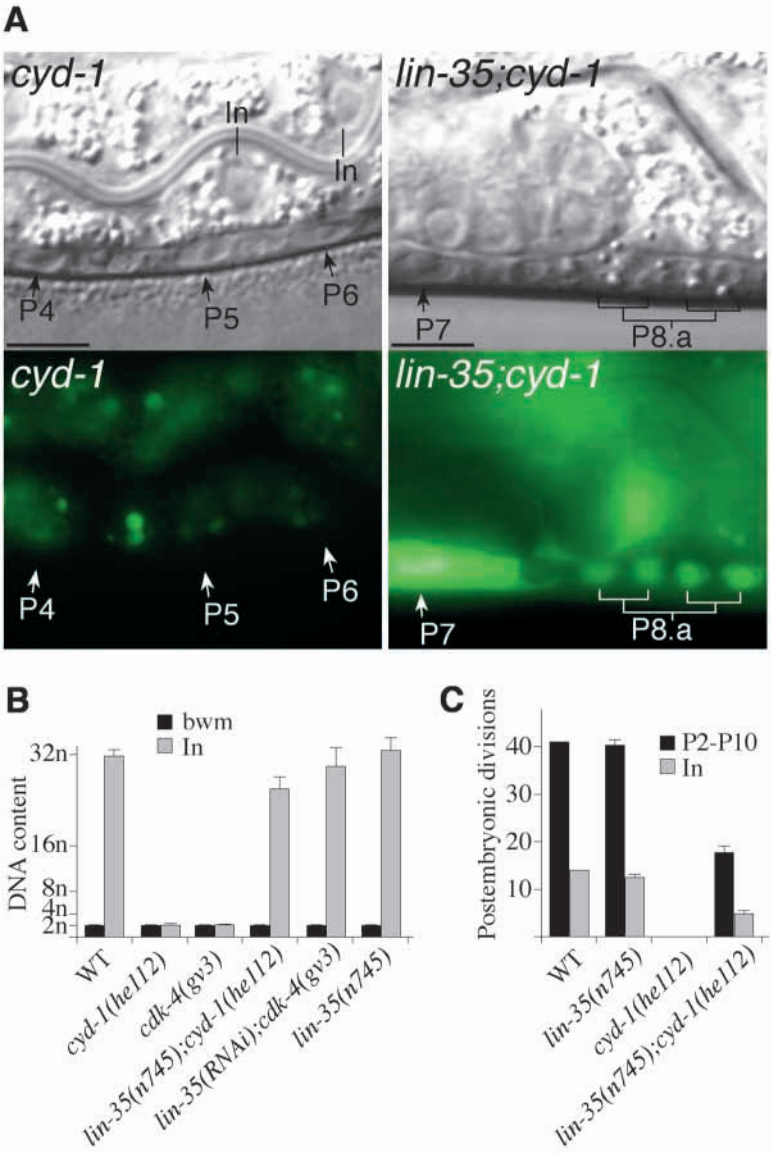

Fig. 4. *lin-35* acts as a negative regulator of cell-cycle progression. (A) Expression of the *rnr::GFP* S-phase marker in *cyd-1(he112)* (left) and *lin-35(RNAi); cyd-1(he112)* (right). Nomarski images (top) and corresponding epifluorescent images (bottom) show several cells of the P and intestinal lineages. In the *cyd-1* animal, only autofluorescence of the intestinal cells is detectable, whereas *lin-35* RNAi resulted in expression of *rnr::GFP* in the P cells and other lineages. (B) Quantitative measurements of DNA content in the intestinal nuclei (gray bars) of wild-type (WT) and mutant animals of indicated genotype. Body wall muscles (black bars) serve as 2n DNA standard. (C) Rescue of postembryonic cell divisions by *lin-35*. The cell number in the P2-P10 and intestinal lineages were counted in animals of indicated genetic backgrounds. Scale bars: 10 μm. Values indicated are mean±s.e.m.

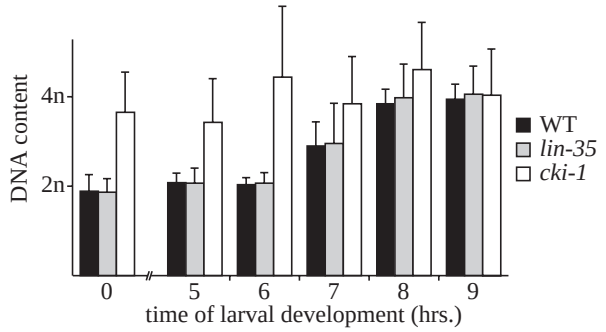

Fig. 5. *lin-35* is not rate limiting for S-phase entry. DNA content of intestinal cells of wild-type, *lin-35(n745)* and *cki-1(RNAi)* animals was determined at 1 hour intervals from the start of postembryonic development. Bars indicate mean of 10 intestinal nuclei±s.d.

E/CDK2, as Cyclin E-CDK2 kinase activity is necessary and sufficient to induce S-phase in a variety of systems (Duronio and O'Farrell, 1995; Knoblich et al., 1994; Ohtsubo et al., 1995; Tsai et al., 1993; van den Heuvel and Harlow, 1993). In addition to transcriptional suppression by pRb, cyclin E/CDK2 kinases are also regulated by CDK inhibitors of the Cip/Kip family (Ekholm and Reed, 2000; Sherr and Roberts, 1999). The *C. elegans* genome contains two Cip/Kip family members, *cki-1* and *cki-2* (Feng et al., 1999; Hong et al., 1998). Several observations suggested that *cki-1* and possibly *cki-2* have conserved functions as CDK inhibitors. Ectopic expression of *cki-1* has been shown to arrest the cell cycle in G1 phase (Hong et al., 1998). Moreover, inactivation of *cki-1*, but not *cki-2*, by RNAi resulted in supernumerary divisions in various cell lineages (Feng et al., 1999; Hong et al., 1998). We confirmed the *cki-1* RNAi phenotype and observed low penetrant postembryonic cell divisions in *cyd-1* mutant animals after *cki-2* RNAi (see below and data not shown). Finally, CKI-1 was found to interact with CYD-1 and CYE-1 in two-hybrid assays (Materials and Methods). Together, these observations suggested that *cki-1* and *cki-2* (collectively referred to as *cki-1,2*) are *C. elegans* Cip/Kip family members with conserved functions in the regulation of G1/S progression.

We examined if *cki-1,2* Cip/Kip activity is sufficient to control cell-cycle progression in the absence of *lin-35* Rb function. In contrast to *lin-35* inactivation, *cki-1* RNAi caused premature entry into S phase (Fig. 5). A significant number of intestinal nuclei obtained 4n DNA amounts even without stimulation of L1 development (Fig. 5, 0 hour). Interestingly, RNAi for *cki-1* alone or *cki-1* and *cki-2* together resulted in only a single round of DNA replication in *cyd-1(he112)* mutant animals (Fig. 6A). Thus, inactivation of the *cki-1,2* inhibitors appears rate limiting for S-phase entry and allows one round of DNA duplication even in the absence of *cyd-1*/*cdk-4* function. However, subsequent rounds of DNA synthesis require the activity of *cyd-1* and *cdk-4* or inactivation of *lin-35* Rb. These results demonstrate that *cki-1,2* Cip/Kip and *lin-35* Rb contribute non-overlapping levels of control over the G1/S transition.

Next we determined whether *lin-35* and *cki-1,2* cooperate in negatively regulating cell division. As shown above, inactivation of *lin-35* partly restored postembryonic cell division in *cyd-1* and *cdk-4* mutant animals (Figs 4C, 7B). Similarly, *cki-1,2* RNAi caused rescue of cell division in *cyd-1*

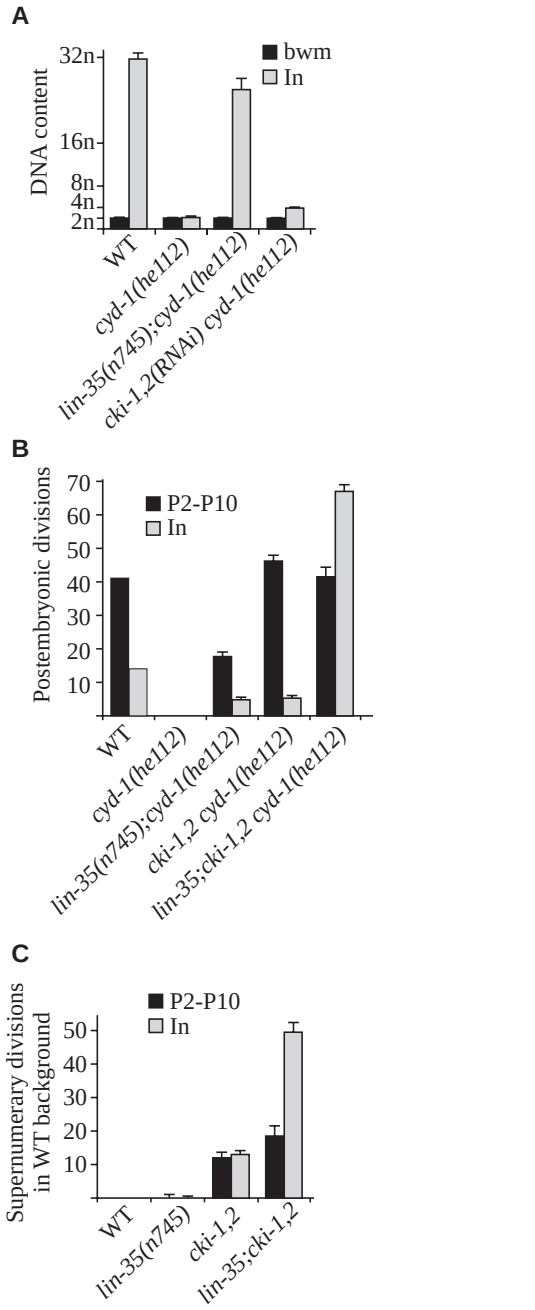

Fig. 6. *cki-1* and *cki-2* cooperate with *lin-35* Rb in regulating G1 progression. (A) Quantitative measurements of DNA content in the intestinal nuclei (gray bars) of wild-type and mutant animals of indicated genotype. Body wall muscles (black bars) serve as 2n DNA standard. (B) Rescue of postembryonic cell divisions. The cell numbers in the P2-P10 and intestinal lineages were counted in strains of indicated genotype. Genes that do not carry allele designations were inhibited by RNAi. (C) Additional cell divisions in a wild-type background caused by inactivation of the indicated genes. The wild-type is defined as zero supernumerary divisions. In all three panels, bars indicate mean±s.e.m.

mutants, resulting in an approximately normal number of P-cell divisions and a limited number of intestinal divisions (Figs 6B, 7C). Importantly, we observed dramatically increased numbers of cell divisions when inactivation of *lin-35* and *cki-*

1,2 were combined. Even in a *cyd-1* mutant background, this resulted in a total number of intestinal nuclei that far exceeded the number in wild-type animals (Figs 6B, 7D). Double inactivation of *lin-35* Rb and *cki-1,2* Cip/Kip also caused a synergistic increase in the number of supernumerary divisions in a wild-type background (Fig. 6C). Although the absolute effects vary between different cell lineages, *cki-1,2* Cip/Kip and *lin-35* Rb cooperate in regulating G1/S phase progression.

DISCUSSION

Control of G1 progression is crucial to the development of all eukaryotes. We followed a genetic approach in the nematode *C. elegans* to learn more about the pathways that regulate G1 progression in vivo. A screen for positive regulators of G1 progression identified a D-type cyclin and a CDK4/6 related kinase. Characterization of the mutant phenotypes confirmed the previous conclusion by Park and Krause (Park and Krause, 1999) that *cyd-1* and *cdk-4* are essential for entry into S-phase during postembryonic development. We used the mutant alleles of *cyd-1* and *cdk-4* to directly address whether cyclin D and/or CDK4/6 act in a linear pathway with Rb family members in vivo. We found that although *lin-35* Rb is an important negative regulator of G1 progression and probably acts downstream of *cyd-1* and *cdk-4*, *lin-35* does not provide the only level of regulation of S-phase entry. An additional level of control is contributed by the CDK inhibitors *cki-1* and *cki-2*, which were found to act in parallel to *lin-35*, and to cooperate with *lin-35* in controlling the G1/S transition. Below we discuss our results in light of the existing knowledge about G1 control in other animal systems.

Considering the nature of the screen, it is somewhat surprising that we identified only a D-type cyclin and CDK4/6 related kinase as essential positive regulators. Although few genes may be essential for both *rnr::GFP* expression and cell division, there are several reasons why some regulators may have been missed. First, although multiple alleles of *cyd-1* and *cdk-4* were identified, it is unlikely that the screen was saturating. Moreover, mutation of some positive regulators may not have resulted in a prominent phenotype, owing to functional redundancy with other genes. In addition, a mutation may not result in a cell-cycle specific phenotype if the gene affected has additional essential functions. Finally, mutations will have been missed that cause a cell-cycle arrest before or after the L1 stage. In the presence of wild-type maternal product, the stage at which a mutant phenotype first becomes apparent is determined by the requirement for zygotic gene function. Although many *C. elegans* cell-cycle regulators display their mutant phenotype in the first larval stage (e.g. *ncc-1, lin-5, cul-1;* Boxem et al., 1999; Kipreos et al., 1996; Lorson et al., 2000), zygotic expression of other genes is required during embryonic development or after the first larval stage. For example, mutations in *cye-1*, the *C. elegans* cyclin E homolog, result in late larval defects, although RNAi experiments revealed an essential function during embryogenesis (Fay and Han, 2000; M. B. and S. v.d.H., unpublished). We did not identify mutations in candidate positive regulators of the E2F/DP transcription factor families. Accordingly, the recent analysis of *efl-1* E2F and *dpl-1* DP mutations revealed that these genes are not generally needed for cell division in *C. elegans* (Ceol and Horvitz, 2001; Page et al., 2001).

The cyclin D kinase is essential for G1 progression

A large number of observations have implicated cyclin D-CDK4/6 kinases in G1 control (Sherr, 1996; Sherr and Roberts, 1999). Most studies have documented the effects of 'gain of function' of kinase activity. For example, increased activity of D-type kinases is found in tumor cells, can shorten G1 phase of cells in tissue culture and can overcome the G1 arrest induced by ectopic pRb expression. Such observations do not establish whether or not these kinases are essential for cell-cycle progression. Indirect evidence in support of an essential role has been provided by overexpressing the CDK4/6 inhibitor $p16^{INK4A}$ (Bruce et al., 2000; Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995). This has been shown to arrest G1 progression of pRb-positive cells, but not of cells lacking either pRb or p107 and p130. Mice nullizygous for cyclin $D1^{-/-}$ or $CDK4^{-/-}$ develop to adults with growth defects (Fantl et al., 1995; Rane et al., 1999; Sicinski et al., 1995; Tsutsui et al., 1999); however, the effects have yet to be described of gene knockout of all three D-type cyclins, or of CDK4 and CDK6. The most complete inactivation of cyclin D kinase activity may have been achieved in mice double null for p21 and p27 Cip/Kip, which act as assembly factors for CDK4/6-cyclin D kinases (Cheng et al., 1999; LaBaer et al., 1997). Double inactivation of p21 and p27 has been found to reduce CDK4/6 kinase activity below the level of detection, yet these double mutant animals do not show cell-division defects (Cheng et al., 1999). Such results have challenged the prevalent view about the requirement for cyclin D kinase activity.

C. elegans and *Drosophila* are thus far the only organisms in which the effects of complete loss of cyclin D-dependent kinase activity has been studied. Inactivation of the sole CDK4/6-related kinase in *Drosophila* did not affect cell division in a general way (Datar et al., 2000; Meyer et al., 2000). Homozygous *Cdk4* mutant flies develop into small adults with reduced fertility (Meyer et al., 2000). The small size did not appear to be caused by a decrease in cell numbers. Rather, based upon the analysis of *Cdk4* mutants and ectopic expression of CycD-Cdk4, the primary role of CycD-Cdk4 appears to be stimulation of cell growth in *Drosophila* (Datar et al., 2000; Meyer et al., 2000). By contrast, the cell-division defects in the *C. elegans cyd-1* and *cdk-4* mutants precede a detectable growth defect and first appear in late embryogenesis before growth takes place. Thus, the rate limiting function of cyclin D-CDK4/6 kinases may vary between species, demonstrating the value of using multiple model organisms in studying gene function.

lin-35 Rb probably acts downstream of *cdk-4/cyd-1* in cell-cycle control

Upon inactivation of the retinoblastoma family member *lin-35* Rb, cells were able to complete multiple rounds of S phase in the apparent absence of CYD-1/CDK-4 activity. This clearly establishes *lin-35* Rb as a negative regulator of S phase which acts downstream of or in parallel to *cyd-1/cdk-4.* Previously, *lin-35* was identified as a member of a set of genes that negatively regulate vulval cell fate (Ferguson and Horvitz, 1989; Lu and Horvitz, 1998). These genes are known as the 'synthetic multivulva' (synMuv) genes that form two

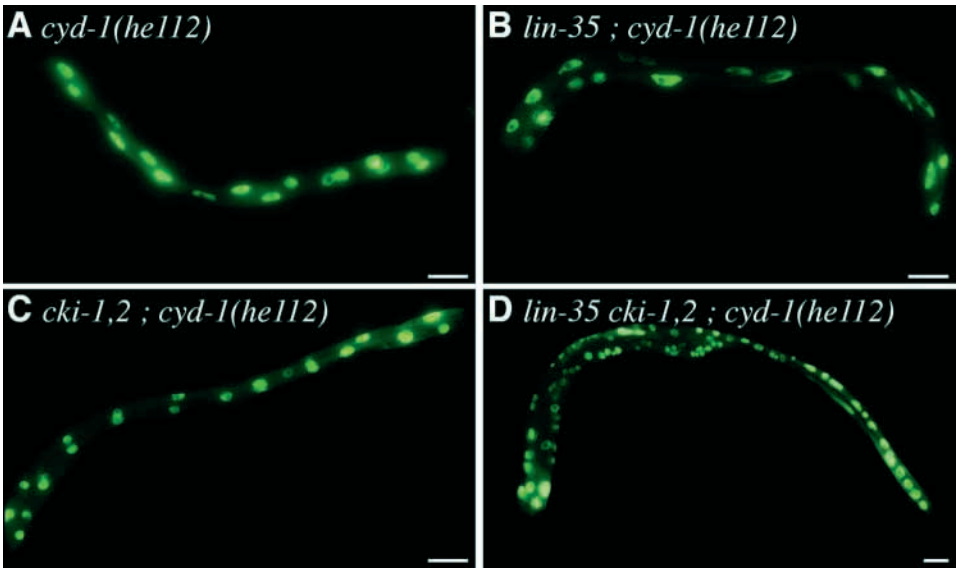

Fig. 7. Cip/Kip and Rb family members cooperate in regulating G1 progression. Epifluorescent images demonstrating the number of intestinal divisions in (A) *cyd-1(he112); elt-2::GFP* mutant larvae and animals of the same genotype after (B) *lin-35* RNAi, (C) *cki-1,2* RNAi and (D) *lin-35; cki-1,2* double RNAi. Intestinal nuclei express the GFP under the control of the *elt-2* promoter region. Scale bars: 25 μm.

functionally redundant classes. Inactivation of both a class A and a class B synMuv gene causes inappropriate induction of vulval fates, resulting in a Multivulva phenotype (Ferguson and Horvitz, 1985; Ferguson and Horvitz, 1989). Genetic epistasis experiments have shown that the synMuv genes act to antagonize a receptor tyrosine kinase/Ras-mediated signaling pathway (Lu and Horvitz, 1998). A role in cell-division has not been described previously for the class B synMuv gene *lin-35* Rb. *lin-35* null mutants are viable and appear to develop normally (Lu and Horvitz, 1998). In addition, the absence of *lin-35* function did not affect the timing of S-phase entry. These findings are surprising as *lin-35* is the only member of the Rb family in *C. elegans*, and loss of Rb is lethal in mice (Jacks et al., 1992) as well as in *Drosophila* (Du and Dyson, 1999). Moreover, mouse embryonic fibroblasts that lack all three Rb-family members show severe cell-cycle defects in tissue culture (Dannenberg et al., 2000; Sage et al., 2000). Interestingly, in chimeric mouse experiments, adult mice with largely normal tissues were found to contain high percentages of $Rb^{-/-}$ cells (Maandag et al., 1994; Williams et al., 1994). Thus, the developmental requirement for a functional *Rb* gene may be limited to specific tissues or cell types.

lin-35 Rb and *cki-1* Cip/Kip cooperate in G1/S control

Our results demonstrate that in the absence of *lin-35* Rb function additional levels of control are sufficient to maintain the correct timing of S phase in *C. elegans*. Based on results in other systems, we reasoned that such controls are likely to involve inhibitors of the Cip/Kip family. Indeed, several results indicate that *cki-1,2* Cip/Kip and *lin-35* Rb cooperate in controlling the G1/S transition in *C. elegans*. The strongest argument for cooperation between *cki-1,2* and *lin-35* is the observed synergistic effect of double inactivation on supernumerary cell divisions. Most strikingly, animals contained on average 84 intestinal nuclei following inactivation of *cki-1,2* as well as *lin-35*, while adult wild-type animals, *lin-35* mutants, and *cki-1,2(RNAi)* animals averaged 34, 34 and 47 nuclei, respectively. Such increased numbers of intestinal divisions have not been reported previously in *C. elegans*. That *cki-1* and *lin-35* act at least in part in parallel pathways is further indicated by their distinct loss-of-function phenotypes. Loss of *lin-35* does not lead to precocious S phase but does allow multiple rounds of DNA replication in *cyd-1* or *cdk-4* mutant animals. By contrast, inactivation of *cki-1* by RNAi results in premature S phase, yet permits only a single round of DNA synthesis in *cyd-1* and *cdk-4* mutants. These results agree well with those obtained for *dacapo*, a Cip/Kip inhibitor in *Drosophila* (de Nooij et al., 1996; Lane et al., 1996). The *dacapo* mutant phenotype has been proposed to result from failure to inactivate residual cyclin E kinase (de Nooij et al., 1996). For further rounds of cell division, cyclin E needs to be transcribed, which requires inactivation of RBF/E2F-mediated transcriptional repression. We expect that similar mechanisms explain the cooperative effects of *cki-1* and *lin-35* Rb in *C. elegans*.

Several observations in mammalian systems also suggest cooperation. In tissue culture, $p21^{-/-}$ $pRb^{-/-}$ mouse cells are more defective in G1 control than cells lacking either single gene (Brugarolas et al., 1998). Moreover, double inactivation of genes in both pathways increases tumor formation in mouse models (Brugarolas et al., 1998; Franklin et al., 1998; Park et al., 1999). As p21 is an important downstream target of the p53 tumor suppressor, cooperation between p21 and pRb in cell-cycle control may contribute to the strong selective pressure for dual inactivation of p53 and pRb in human cancer. Thus, although control by Cip/Kip inhibitors may be more rate limiting in *C. elegans*, cooperation between Cip/Kip and pRb family members in G1/S control is probably shared among metazoans.

Additional functions of Cyclin D-CDK4/6 kinases

The absence of pRb family proteins in *lin-35* putative null mutants was not sufficient to fully overcome the requirement for a cyclin D-dependent kinase in *C. elegans*. This indicates that *cyd-1* and *cdk-4* do not act solely to inactivate *lin-35*. A second activity described for cyclin D-CDK4/6 complexes is sequestration of Cip/Kip inhibitors, which allows activation of cyclin E/CDK2 complexes (Sherr and Roberts, 1999). It is possible that sequestering CKI-1 and CKI-2 is the detected additional function of the CYD-1/CDK-4 kinase. Consistent

with this idea, we found that CYD-1, as well as CYE-1, interacts with CKI-1 in the two hybrid system. Interestingly, the single *Drosophila* Cip/Kip family member Dacapo does not bind to CycD-Cdk4 (Meyer et al., 2000). Possibly, alternative ways to control Cip/Kip activity have been developed in *Drosophila*, which may explain the reduced role of the cyclin D-CDK4/6 kinase in cell-cycle progression.

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