

REPORT

CDC-25.2, a *C. elegans* ortholog of *cdc25*, is essential for the progression of intestinal divisions

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ABSTRACT

Intestinal divisions in *Caenorhabditis elegans* take place in 3 stages: (1) cell divisions during embryogenesis, (2) binucleations at the L1 stage, and (3) endoreduplications at the end of each larval stage. Here, we report that CDC-25.2, a *C. elegans* ortholog of *Cdc25*, is required for these specialized division cycles between the 16E cell stage and the onset of endoreduplication. Results of our genetic analyses suggest that CDC-25.2 regulates intestinal cell divisions and binucleations by counteracting WEE-1.3 and by activating the CDK-1/CYB-1 complex. CDC-25.2 activity is then repressed by LIN-23 E3 ubiquitin ligase before the onset of intestinal endoreduplication, and this repression is maintained by LIN-35, the *C. elegans* ortholog of Retinoblastoma (Rb). These findings indicate that timely regulation of CDC-25.2 activity is essential for the progression of specialized division cycles and development of the *C. elegans* intestine.

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Introduction


Cell cycle regulation is essential during development. Therefore, defects in cell cycle regulation are often teratogenic and may cause various diseases such as cancer. Understanding of the molecular mechanisms regulating the cell cycle may potentially lead to development of treatments for such diseases. *Caenorhabditis elegans* is an ideal model organism for studying cell cycle regulation during organ development because it has an invariant cell lineage and a well-defined anatomy. The intestine is involved in multiple fundamental biological processes, such as food digestion, nutrition distribution, immunity, stress response, and aging.^{1–4} All intestinal cells of *C. elegans* originate from a single embryonic blastomere, E, which undergoes 4 rounds of nearly symmetrical cell divisions to produce 16 intestinal cells, the 16E cells. 16E cells exist as pairs of bilateral cells, and the structure of each cell pair is called an “int ring.” Thus, there are 8 int rings, int1 to int8, at the 16E cell stage. The structural framework of the intestine is established at this 16E cell stage.^{5–7} Thereafter, additional cell divisions occur in the anterior-most int1 cells and the posterior-most int8 cells. As a result, the int1 ring consists of 4 cells through dorsal/ventral division, and the int8 ring divides into 2 new int rings, int8 and int9, through anterior/posterior division; in total, the intestine comprises 20 cells in 9 int rings before hatching.^{6–8} After hatching, intestinal cells further undergo specialized division cycles. At the first larval stage (L1), some intestinal nuclei undergo nuclear divisions without cytokinesis, resulting in binucleated cells, during a process called binucleation. Int1 and int2 cells

never binucleate, while int3–7 cells always binucleate, and int8 and int9 cells occasionally binucleate.⁹ Therefore, worms in the late L1 stage have 30 to 34 intestinal nuclei in 20 cells. Subsequently, all intestinal nuclei undergo endoreduplication before each larval molting, which double DNA content without nuclear division and cytokinesis.¹⁰ Since intestinal nuclei endoreduplicate 4 times in the 4 larval stages, they finally contain 32C DNA content (1C is the amount of DNA content equivalent to a haploid genome set) in the adult stage. Taken together, the intestinal divisions of *C. elegans* involve transition of the cell cycle mode from cell division, binucleation, to endoreduplication. Therefore, studies of intestinal divisions in *C. elegans* may reveal not only the regulatory mechanisms by which respective division modes are controlled, but also the mechanisms by which their timely transitions are regulated.

Cdc25 phosphatase promotes cell cycle progression through dephosphorylation of cyclin-dependent kinases (Cdks) that are phosphorylated, and thus inactivated, by Wee1/Myt1 kinases.^{11,12} *cdc-25.1* gain-of-function (*gf*) mutants displayed intestinal hyperplasia, while *cdc-25.1* loss-of-function (*lf*) mutants exhibited defects in embryonic intestinal divisions and in germline proliferation.^{13–18} Prolonged CDC-25.1 activity in *cdc-25.1* (*gf*) mutants caused extra cell divisions between the 8E and the 16E cell stages of embryonic intestinal development.^{16,19} In contrast, division patterns were not affected before the 8E and after the 16E cell stages. This indicates that timely inactivation of CDC-25.1 is required for normal intestinal development. The *cdc-25.1* (*gf*) mutant phenotype also suggests that, after the

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16E cell stage, another member of the CDC-25 family plays a pivotal role in intestinal division. There are 4 *cdc25* homologs in *C. elegans*: *cdc-25.1*, *cdc-25.2*, *cdc-25.3*, and *cdc-25.4*.²⁰ We previously reported that *cdc-25.2* regulates oocyte maturation.²¹ Also it has been reported that RNAi of *cdc-25.2* resulted in embryonic defects in neuroblasts, ABarp lineage, and C and E lineages.²² *cdc-25.3* and *cdc-25.4* mRNAs are expressed but their functions are yet largely unknown. Cdc25 phosphatases are evolutionarily conserved from yeast to mammals.^{20,23–26} Although yeast has one *cdc25*, fruit flies, mice, and humans have multiple *cdc25* family members that function in distinct developmental stages. *Drosophila* has 2 *cdc25* homologs, *string* and *twine*.^{23,26} *string* regulates mitotic cell cycles during embryogenesis and germline development, while *twine* regulates meiotic cell cycles in the germline.^{27,28} In mammals, there are 3 *cdc25* family members, *CDC25A*, *CDC25B*, and *CDC25C*.^{24,25} *CDC25A* predominantly functions during the G1 to S phase transition,²⁹ while *CDC25B* and *CDC25C* regulate the G2 to M phase transition.^{30,31} Nevertheless, mice lacking both *Cdc25B* and *Cdc25C* develop almost normally without any obvious abnormalities except female sterility caused by lack of *Cdc25B*. This indicates that, although the functions of *Cdc25B* and *Cdc25C* are distinct from that of *Cdc25A* during development, *Cdc25A* can largely compensate for their functions when they are absent.³²

In this study, we report that *C. elegans* CDC-25.2 solely regulates intestinal cell divisions and binucleations after the 16E cell stage by counteracting WEE-1.3 and by activating the CDK-1/CYB-1 complex. After that, CDC-25.2 activity is repressed by LIN-23 before the onset of intestinal endoreduplications, and this negative regulation is maintained by LIN-35. These findings indicate that CDC-25.2 has a unique function during *C. elegans* intestinal development and its timely regulation is essential for the transition between specialized division cycles during the development.

Results

Deletion of *cdc-25.2* causes an arrest of intestinal division at the 16E cell stage

To explore the roles of *cdc-25* family genes during *C. elegans* intestinal development, we scored the number of intestinal nuclei in each deletion mutant (Table 1). To facilitate the analysis, an intestine-specific reporter transgene, *rrIs01[elt-2::GFP]*,^{15,16,33} was introduced into each mutant strain used in this study. When each *cdc-25* gene was knocked out, only the deletion mutant of *cdc-25.2*, *ok597*, showed a decreased number of intestinal nuclei compared to wild type (Table 1).

The intestinal development originating from the single E blastomere increased the number of cells to 20 by the “1.5-fold” embryonic stage in wild type (Fig. 1A). This number remained the same until the early L1 stage (Fig. 1C, E, and I).^{5,6} Post-embryonic nuclear divisions in the L1 stage increased the number of intestinal nuclei to 30–34, although the int2 nuclei remained undivided (Fig. 1G and I).⁹ After the L1 nuclear divisions, all intestinal nuclei doubled their DNA content before each larval molting via

Table 1. The number of intestinal nuclei per worm in respective genotypes.

Genotype	Number of intestinal nuclei	
	L1	Adult
wild type	20.0 ± 0.4 (n = 22)	31.5 ± 1.8 (n = 22)
<i>cdc-25.1(nr2036)</i>	n.d.	33.2 ± 1.0 (n = 12)
<i>cdc-25.2(ok597)</i>	16.0 ± 0 (n = 22)	16.0 ± 0 (n = 23)
<i>cdc-25.3(ok358)</i>	n.d.	31.3 ± 1.8 (n = 15)
<i>cdc-25.4(tm4088)</i>	n.d.	32.0 ± 1.3 (n = 16)
M ⁺ Z ⁺ <i>cdc-25.2(ok597)/nT1[qls51]</i>	n.d.	29.2 ± 3.3 (n = 32)
M ⁺ Z ⁻ <i>cdc-25.2(ok597)/cdc-25.2(ok597)</i>	n.d.	16.0 ± 0 (n = 48)
M ⁻ Z ⁻ <i>cdc-25.2(ok597)/cdc-25.2(ok597)</i>	n.d.	16.0 ± 0.2 (n = 27)

M, maternally loaded product; Z, zygotically expressed product. Most of the worm strains examined contained a transgene, *rrIs01[elt-2::GFP]*, to specifically mark intestinal nuclei with GFP. GFP-positive nuclei were counted as intestinal nuclei in each worm. n.d., not determined.

endoreduplication. In contrast, there were 16 intestinal cells at the “1.5-fold” embryonic stage in *cdc-25.2(ok597)* mutants (Fig. 1B), and this number remained the same during the later developmental stages until the adult stage due to lack of additional cell divisions and binucleations (Fig. 1D, F, H and J). As a result, the int1 ring contained only 2 nuclei (Fig. 1F and H, arrowheads), and the int9 ring was not generated (Fig. 1J). Because binucleations also failed to occur in the int3–7 and the int8, the *cdc-25.2* mutant adults contained only 16 intestinal nuclei that extended along the body axis (Fig. 1H). On the other hand, int2 division patterns were identical between wild type and the *cdc-25.2* mutants because additional cell divisions and binucleations do not occur after the 16E cell stage in int2 nuclei (Fig. 1I and J). To evaluate the contribution of *cdc-25.2* to endoreduplication, we took advantage of the fact that the int2 division pattern was identical between wild type and *cdc-25.2* mutants. “Relative DNA content of int2 nuclei” was determined as the average DNA staining intensity of int2 nuclei in each genotype at the adult stage, which was normalized with that of int2 nuclei in controls. We found that the relative DNA content of int2 nuclei was not different between wild type and *cdc-25.2* mutants (Table 2). This result suggests that the *cdc-25.2* mutants underwent the same endoreduplications as did the wild type. Taken together, these findings indicate that the *cdc-25.2* mutants had defective intestinal divisions beginning in the 16E cell stage, but the mutants’ endoreduplications before each larval molting were normal. Specifically, the 4 cell divisions after the 16E cell stage during embryogenesis and all the binucleations at the L1 stage were omitted in the *cdc-25.2* mutants (Fig. 1J, thick lines). This intestinal defect was also observed in *cdc-25.2(ok597)* mutant males (data not shown), indicating that the function of *cdc-25.2* is not sex-dependent.

cdc-25.2 is transiently expressed and functions in the intestine

Because *cdc-25.2* activity is required for normal intestinal development, the spatiotemporal expression of *cdc-25.2* during larval development was examined by whole-mount *in situ* hybridization.³⁴ As expected from the mutant

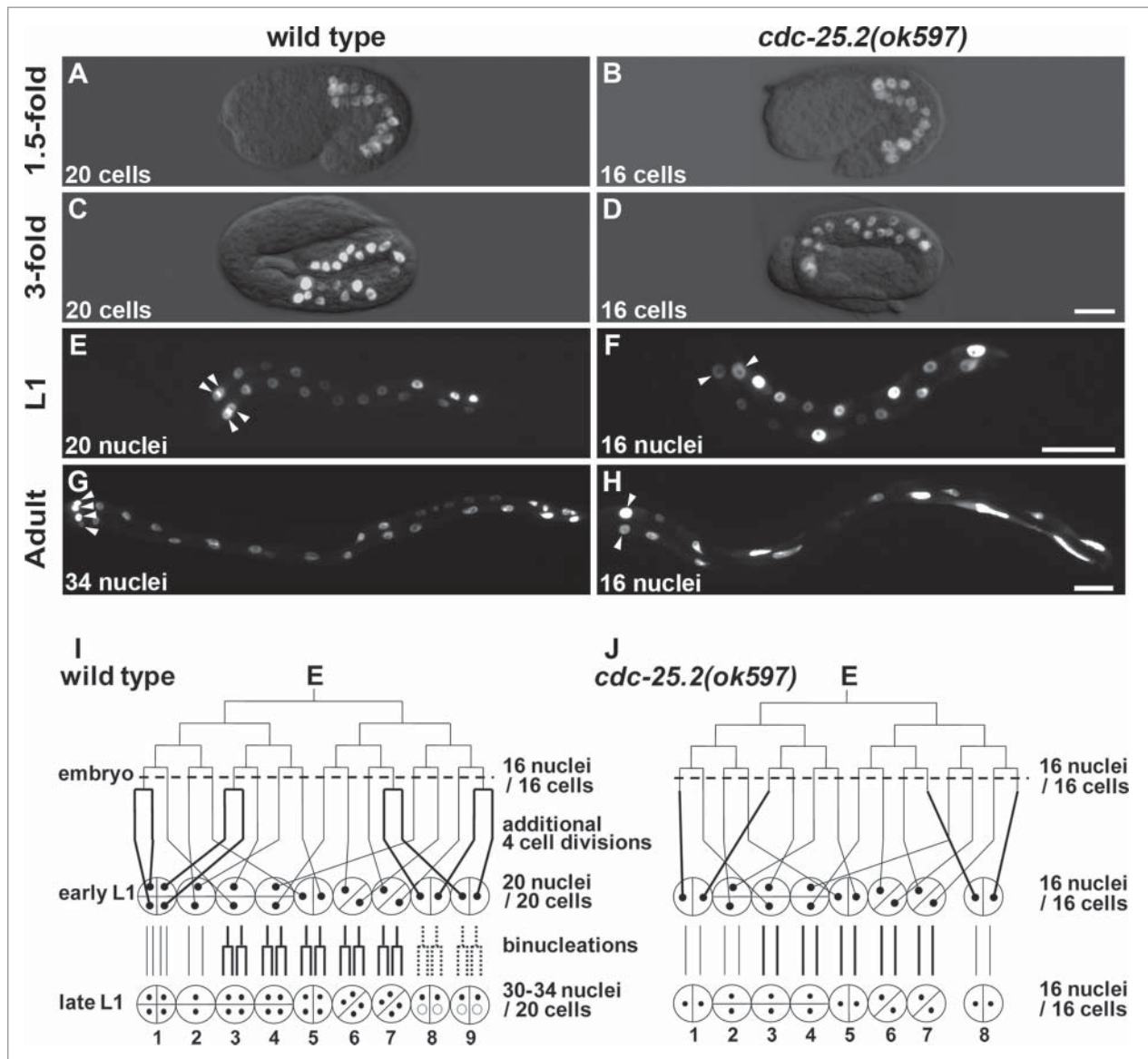


Figure 1. Intestinal development was arrested at the 16E cell stage in *cdc-25.2* mutants. (A-H) GFP-marked intestinal nuclei in wild type (A, C, E and G) and *cdc-25.2(ok597)* mutants (B, D, F and H) at different developmental stages: (A, B) 1.5-fold embryos, (C, D) 3-fold embryos, (E, F) L1-stage larvae, (G, H) adult worms. Left, the anterior side. Arrowheads in (E-H) indicate locations of int1 nuclei. Scale bars, 10 μm in (D), 25 μm in (F) and 50 μm in (H). (I, J) Schematic diagrams of intestinal divisions in wild type (I) and *cdc-25.2(ok597)* mutants (J). Vertical and horizontal lines indicate division patterns of the E lineage. Broken horizontal lines indicate the 16E cell stage. Bold lines indicate different division patterns between wild type and *cdc-25.2(ok597)* mutants. Small black dots indicate intestinal nuclei. Half circles and quarter circles indicate int ring cells. Bottom numbers indicate the numbering of each int ring. In wild type, int8-9 nuclei do not always divide at the L1 stage. Therefore, they are indicated by dotted lines and small dotted circles.

Table 2. Relative DNA content of int2 nuclei in respective genotypes.

Genotype	Relative DNA content of int2 nuclei	<i>p</i> value
wild type	1.0 ± 0.1 (n = 14)	
<i>cdc-25.2(ok597)</i>	1.0 ± 0.1 (n = 14)	= 0.5
mock RNAi	1.0 ± 0.3 (n = 21)	
<i>cdk-1</i> RNAi	1.1 ± 0.3 (n = 19)	= 0.23
<i>cdk-2</i> RNAi	0.6 ± 0.3 (n = 19)	< 0.05
<i>cdk-4</i> RNAi	0.7 ± 0.4 (n = 13)	< 0.05
<i>cyb-1</i> RNAi	1.1 ± 0.3 (n = 22)	= 0.48
<i>cyd-1</i> RNAi	1.0 ± 0.2 (n = 16)	= 0.85
<i>cye-1</i> RNAi	0.4 ± 0.2 (n = 24)	< 0.05

Intestine-specific RNAi was performed using the OLB11 strain. The relative DNA content of int2 nuclei in each genotype was determined as the average DNA staining intensity of int2 nuclei in each genotype normalized with that of int2 nuclei in the control (wild type or mock RNAi) measured at the adult stage. n, number of examined nuclei. *P* values are calculated against the appropriate controls.

phenotype, we found that *cdc-25.2* mRNA was expressed in the intestine at the L1 stage (Fig. 2B, between the broken lines). In contrast, a *cdc-25.2* mRNA signal was not detected in the L2- to L3-stage larvae (Fig. 2D), but reappeared after the L4 stage in the gonad (Fig. 2F). These results indicate that *cdc-25.2* is transiently expressed in the intestine at the L1 stage.

Furthermore, when a *cdc-25.2* transgene was expressed under the control of the intestine-specific *elt-2* promoter, the transgene induced intestinal hyperplasia in the wild-type background (Fig. 2G and I). In addition, when expressed in the *cdc-25.2* mutant background, the transgene partially rescued the intestinal hypoplasia phenotype of the mutants (Fig. 2H and I). These results support the idea that *cdc-25.2* functions in the intestine to regulate intestinal divisions.

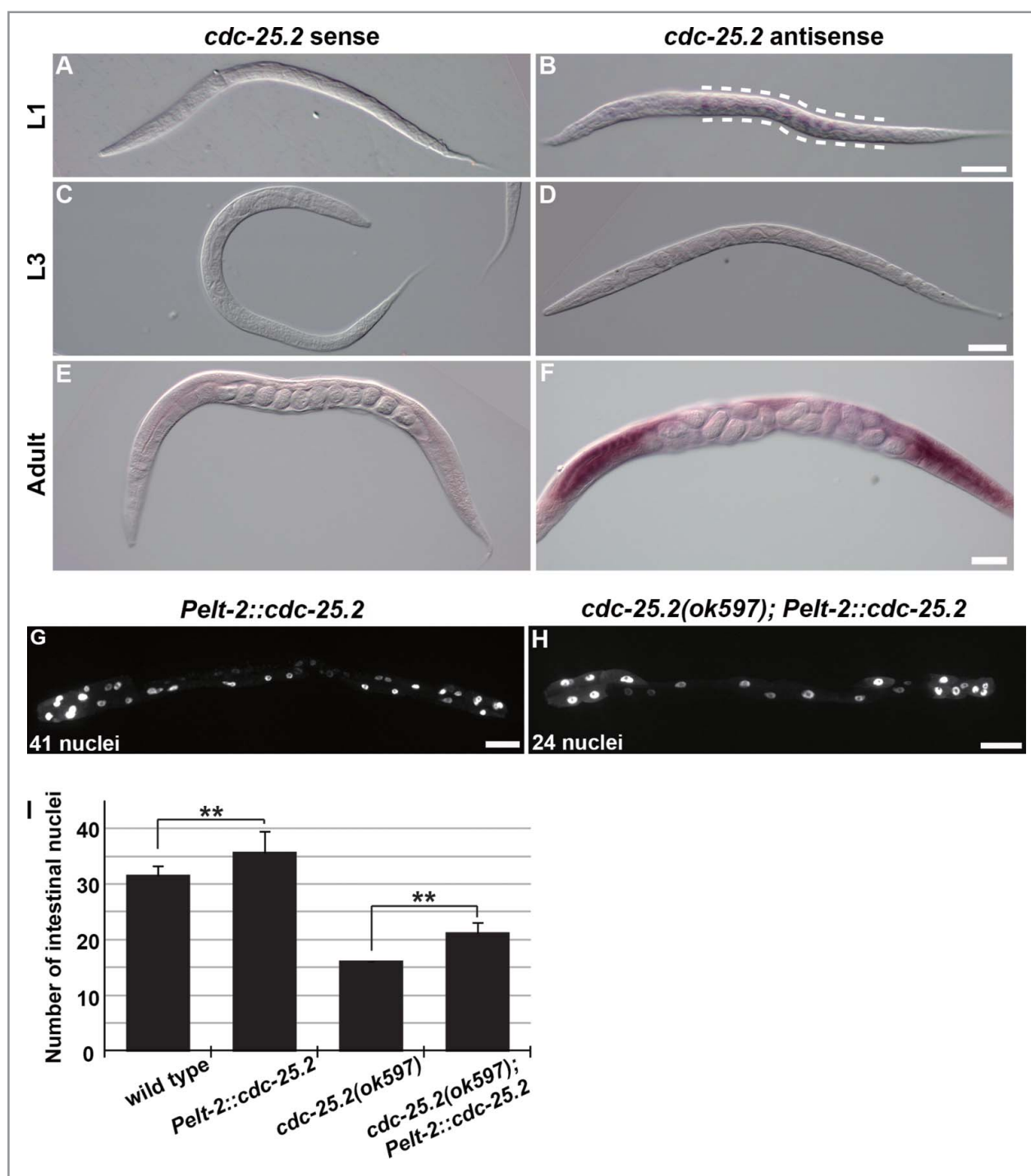


Figure 2. *In situ* expression pattern of *cdc-25.2* mRNA and partial rescue of the *cdc-25.2* mutant phenotype by intestinal *cdc-25.2* transgene expression. (A-F) *In situ* hybridization of *cdc-25.2* during larval development with a *cdc-25.2* sense probe (A, C and E) and a *cdc-25.2* antisense probe (B, D and F): (A, B) L1 stage; broken lines in (B) indicate the region of *cdc-25.2* mRNA expression, (C, D) L3 stage, (E, F) adult stage; *cdc-25.2* mRNA expression was observed in the gonads. Scale bars, 25 μ m in (B), 50 μ m in (D), and 100 μ m in (F). (G) GFP-marked intestinal nuclei in a *Pelt-2::cdc-25.2* transgenic adult worm, in which a *cdc-25.2* transgene under the control of the intestine-specific *elt-2* promoter was expressed in a wild-type background. (H) GFP-marked intestinal nuclei in a *cdc-25.2(ok597); Pelt-2::cdc-25.2* transgenic adult worm, in which the *Pelt-2::cdc-25.2* transgene was expressed in the *cdc-25.2* mutant background. Left, the anterior side. Scale bars, 50 μ m. (I) Average numbers of intestinal nuclei in wild-type adults (n = 22), *Pelt-2::cdc-25.2* adults (n = 19), *cdc-25.2(ok597)* adults (n = 23), and *cdc-25.2(ok597); Pelt-2::cdc-25.2* adults (n = 30). ** $p < 0.001$.

***cdc-25.2* is not required for intestinal divisions up to the 16E cell stage**

Although embryonic cell divisions after the 16E cell stage and postembryonic binucleations were omitted, the E blastomeres divided normally up to the 16E cell stage in the *cdc-25.2*

mutants (Fig. 1). This suggests that E cell divisions up to the 16E cell stage are regulated either by maternally loaded *cdc-25.2* or by the activity of another *cdc-25* family member. Because many aspects of early embryonic development are controlled by maternally loaded gene products (M) instead of zygotic gene products (Z) in *C. elegans*,³⁵ maternal *cdc-25.2*

may be required for early intestinal divisions. Although the *cdc-25.2* mutants are sterile, the penetrance of the sterility is approximately 85% at 20°C.²¹ Thus, a few homozygous *cdc-25.2* mutant hermaphrodites produced from heterozygous mothers ($M^+ Z^-$) can produce homozygous *cdc-25.2* mutant progeny in which neither the maternal load nor the zygotic product of *cdc-25.2* is present ($M^- Z^-$). When the intestinal phenotype of $M^- Z^-$ *cdc-25.2* homozygous mutant progeny was examined, we found that they produced 16 intestinal nuclei, as did their $M^+ Z^-$ *cdc-25.2* homozygous mutant mothers (Table 1). This result indicates that maternally loaded *cdc-25.2* is not required for intestinal divisions up to the 16E cell stage. Rather, it suggests that another *cdc-25* family member is involved in the intestinal divisions before the 16E cell stage.

***cdc-25.1(gf)* mutation suppresses the embryonic but not the postembryonic intestinal defect of *cdc-25.2(ok597)* mutants**

The phenotype of *cdc-25.1(rr31gf)* mutants suggests that *cdc-25.1* functions around the 8E cell stage or earlier during embryonic intestinal development.¹⁶ The observation that RNAi against *cdc-25.1* in adult hermaphrodites caused embryonic lethality of the progeny also implies that maternally loaded *cdc-25.1* is essential for early embryonic cell cycles.^{13,15} To examine whether the prolonged presence of *cdc-25.1* activity can compensate for the lack of *cdc-25.2* activity, we introduced *cdc-25.1(rr31gf)* mutation into *cdc-25.2* mutants. The intestinal divisions of this double mutant were then examined (Fig. 3). The

cdc-25.1(gf) single mutants contained an increased number of intestinal nuclei at the L1 stage (Fig. 3A and E, on average 39.6 ± 1.1); this number further increased to almost 60 nuclei at the adult stage (Fig. 3C and E, on average 59.0 ± 3.4), as previously described.^{16,19} On the other hand, although the *cdc-25.1(gf); cdc-25.2* double mutants contained more than the wild-type number (20) of intestinal nuclei at the L1 stage (Fig. 3B and E, on average 24.1 ± 1.9), the double mutants ended up containing fewer than the wild-type number (30–34) of intestinal nuclei at the adult stage (Fig. 3D and E, on average 27.6 ± 5.3). These results indicate that, although extra cell divisions occurred during embryogenesis, postembryonic nuclear divisions did not occur properly in the double mutant intestine (Fig. 3E). In other words, although the *cdc-25.1(gf)* mutation suppressed the defect of embryonic intestinal cell divisions, it failed to suppress the defect of postembryonic intestinal nuclear divisions of the *cdc-25.2* mutants. This finding supports the view that *cdc-25.1* regulates intestinal divisions before the 16E cell stage, and then *cdc-25.2* takes over this role until binucleations are accomplished at the L1 stage.

***CDC-25.2* counteracts *WEE-1.3* and positively regulates *CDK-1/CYB-1* to promote intestinal divisions**

In *C. elegans*, there are 3 Wee1/Myt1 homolog genes: *wee-1.1*, *wee-1.2*, and *wee-1.3*. Among them, *wee-1.2* is most likely a pseudogene.³⁶ It was previously shown that RNAi depletion of *wee-1.3* partially suppressed the oocyte maturation defect of the *cdc-25.2* mutants.²¹ We found that RNAi against *wee-1.3*, but

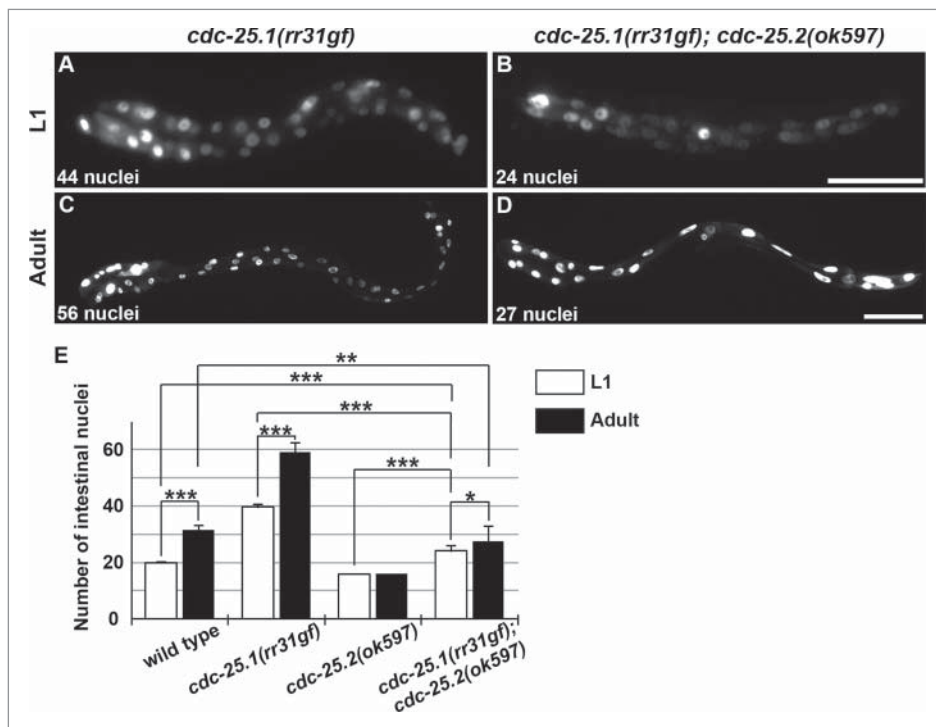


Figure 3. *cdc-25.1(rr31gf)* mutation partially suppressed the *cdc-25.2* mutant intestinal phenotype. GFP-marked intestinal nuclei in (A) a *cdc-25.1(rr31gf)* mutant L1 larva, (B) a *cdc-25.1(rr31gf); cdc-25.2(ok597)* double-mutant L1 larva, (C) a *cdc-25.1(rr31gf)* mutant adult hermaphrodite, and (D) a *cdc-25.1(rr31gf); cdc-25.2(ok597)* double-mutant adult hermaphrodite. Left, the anterior side. Scale bars, 25 μ m in (B), and 50 μ m in (D). (E) Average numbers of intestinal nuclei in wild-type L1 and adult ($n = 22$ and 22, respectively), in *cdc-25.1(rr31gf)* mutant L1 and adult ($n = 22$ and 19, respectively), in *cdc-25.2(ok597)* mutant L1 and adult ($n = 22$ and 23, respectively), and in *cdc-25.1(rr31gf); cdc-25.2(ok597)* double-mutant L1 and adult ($n = 19$ and 59, respectively). * $p = 0.0068$, ** $p = 0.0015$, *** $p < 0.001$.

not *wee-1.1*, partially suppressed the defect in intestinal division in the *cdc-25.2* mutants (Table 3).

To identify a possible target CDK regulated by CDC-25.2 and WEE-1.3, as well as its cyclin partner in intestinal development, we tested RNAi depletion of *C. elegans* *cdk* and *cyclin* homolog genes. CDKs and cyclins are essential in many tissues. Thus, RNAi depletion of most of them in wild-type N2 induced pleiotropic defects leading to developmental arrest or retardation, such as embryonic lethality (Emb), larval lethality (Lvl), growth retardation (Sck, Dpy, Slo), and germline sterility (Ste) (Table 4), as previously reported.³⁷⁻⁴⁰ This hindered our ability to identify which CDKs and cyclins function during intestinal development. To overcome this obstacle, we used the *C. elegans* strain OLB11 and its derivative. Because OLB11 is an *rde-1* loss-of-function mutant but expresses RDE-1 under the control of the intestine-specific *elt-2* promoter, only the intestine in this strain is susceptible to RNAi.⁴¹ An *elt-2::gfp* transgene, *rrIs01*, was also introduced into OLB11 to facilitate observation of the intestinal nuclei (Table S1). Among the *cdk* homolog genes, RNAi depletion of *cdk-1*, *cdk-2*, and *cdk-4* reduced the number of intestinal nuclei in OLB11 (Table 4). *cdk-1* RNAi reduced the number of nuclei to approximately 20. However, the relative DNA content of int2 nuclei measured by fluorescence intensity did not differ from that of mock RNAi controls (Table 2). Although RNAi depletion of *cdk-2* and *cdk-4* also reduced the number of intestinal nuclei, the level of reduction was not nearly as significant (Table 4). Furthermore, the relative DNA content of int2 nuclei was also significantly reduced after RNAi depletion of *cdk-2* and *cdk-4* compared to mock RNAi controls (Table 2). Among the *cyclin* homolog genes, RNAi depletion of *cyb-1*, *cyd-1*, *cye-1*, and *cyl-1* decreased the number of intestinal nuclei in OLB11 (Table 4). Like *cdk-1* RNAi, although *cyb-1* RNAi and *cyd-1* RNAi reduced the number of intestinal nuclei, the relative DNA content of int2 nuclei was not different from that of mock RNAi controls (Table 2). In contrast, RNAi against *cye-1* not only reduced the number of intestinal nuclei but also significantly decreased the relative DNA content of int2 nuclei, similar to RNAi against *cdk-2* and *cdk-4*. On the other hand, *cyb-3* RNAi induced early-stage embryonic lethality in both the N2 and OLB11 strains for unknown reasons (Table 4). It should be noted that the RNAi phenotypes of the *cdk* homolog and the *cyclin* homolog genes observed in the OLB11 background were much weaker than the corresponding mutant phenotypes or the RNAi phenotypes reported in previous studies.^{38,42-44} The incomplete RNAi phenotypes observed in OLB11 were possibly caused by insufficient recovery of the Rde-1(+) phenotype in OLB11 intestines

during early embryogenesis (see Discussion). Although the RNAi phenotypes were incomplete, it can still be assumed that zygotic gene activities expressed during postembryonic intestinal development were effectively abrogated by RNAi in OLB11. Therefore, the results are still sufficient to conclude that CDK-1 and CYB-1 are the most likely candidates for CDK and cyclin that function in intestinal divisions after the 16E cell stage under the control of CDC-25.2 and WEE-1.3.

Extra nuclear divisions in *lin-23* and *lin-35* mutants are suppressed by *cdc-25.2* RNAi

Because extra intestinal nuclear divisions were observed in place of endoreduplication in *lin-23(e1883)* L1 larvae,⁴⁵ it is thought that LIN-23 is required during the L1 stage and has a role in restricting intestinal nuclear divisions. In other words, LIN-23 activity may be required for the shift from binucleation to endoreduplication, probably through ubiquitin-mediated degradation of a cell-cycle regulator essential for intestinal divisions. We hypothesized that CDC-25.2 is negatively regulated by LIN-23 during this shift. To test this possibility, we examined whether the intestinal hyper-nucleation of *lin-23(e1883)* mutants is suppressed by *cdc-25.2* RNAi. If the hyper-nucleation is caused by a failure to repress CDC-25.2 activity, *cdc-25.2* RNAi should suppress the *lin-23* mutant phenotype. Indeed, we observed that the intestinal hyper-nucleation of *lin-23(e1883)* mutants was strongly suppressed by *cdc-25.2* RNAi (Fig. 4A, B and E). In contrast, *cdc-25.1* RNAi had virtually no effect on the *lin-23* mutant phenotype (Fig. 4E).

Intestinal hyper-nucleation in place of endoreduplication was also reported in *lin-35(rr33)* early larvae.⁴⁶ Because LIN-35 is a *C. elegans* homolog of the mammalian transcriptional repressor, Retinoblastoma (Rb), this hyper-nucleation phenotype could be a consequence of a failure to repress transcription of a cell-cycle regulator gene. We assumed that *cdc-25.2* could also be transcriptionally repressed either directly or indirectly by LIN-35 during early larval stages. Therefore, if *cdc-25.2* was not properly repressed in the *lin-35* mutants, extra intestinal nuclear divisions might be induced instead of endoreduplication. To test this possibility, we examined whether the intestinal hyper-nucleation of *lin-35(rr33)* mutants was suppressed by *cdc-25.2* RNAi. Indeed, we found that RNAi against *cdc-25.2*, but not *cdc-25.1*, suppressed the intestinal hyper-nucleation of the *lin-35* mutants (Fig. 4C-E).

To test whether the level of *cdc-25.2* mRNA is upregulated in *lin-35* mutants when additional nuclear divisions occur, we quantified *cdc-25.2* mRNA levels in synchronized wild-type and *lin-35(rr33)* mutant populations during the late L1 to early

Table 3. RNAi depletion of *wee-1* family genes in wild type and *cdc-25.2* mutants.

Genotype	L4440	<i>wee-1.1</i> (RNAi)	<i>wee-1.3</i> (RNAi)	<i>p</i> value
wild type	30.6 ± 1.4 (n = 14)	30.7 ± 1.4 (n = 14)	29.5 ± 1.9 (n = 13)	= 0.1
<i>cdc-25.2(ok597)</i>	15.9 ± 0.6 (n = 15)	15.9 ± 0.5 (n = 17)	22.4 ± 3.0 (n = 9)	< 0.001

After RNAi treatment by the feeding method,⁵³ adult progeny were fixed, stained with Hoechst 33342, and the numbers of their intestinal nuclei were counted. *n*, number of examined worms. *P* values were calculated by comparing L4440 controls and *wee-1.3*(RNAi) worms.

Table 4. RNAi depletion of genes encoding cell-cycle regulators, *cdks*, and *cyclins* in N2 and OLB11.

Gene	Phenotype		Number of intestinal nuclei in OLB11
	N2	OLB11	
mock	wild type	wild type	29.7 ± 1.2 (n = 20)
<i>cdc-25.1</i>	Emb	wild type	31.6 ± 1.7 (n = 28)
<i>cdc-25.2</i>	Emb	number reduced	19.2 ± 5.3 (n = 29)**
<i>cdc-25.3</i>	wild type	wild type	32.1 ± 1.5 (n = 31)
<i>cdc-25.4</i>	wild type	wild type	29.8 ± 0.9 (n = 21)
<i>wee-1.3</i>	P0 sterile	wild type	33.3 ± 1.9 (n = 28)
<i>cdk-1</i>	1-cell Emb	number reduced	19.5 ± 1.6 (n = 24)**
<i>cdk-2</i>	Ste	number/DNA reduced	27.9 ± 1.9 (n = 34)**
<i>cdk-4</i>	Sck/ Dpy/ Lvl/ Emb	number/DNA reduced	26.8 ± 3.3 (n = 29)**
<i>cdk-5</i>	wild type	wild type	31.2 ± 1.6 (n = 29)
<i>cdk-7</i>	Slo/ Ste/ Lvl/ Emb	wild type	31.5 ± 1.2 (n = 32)
<i>cdk-8</i>	Ste	wild type	31.8 ± 1.6 (n = 16)
<i>cdk-9</i>	Emb	wild type	30.5 ± 1.4 (n = 25)
<i>cyb-1</i>	Emb	number reduced	25.1 ± 1.7 (n = 25)**
<i>cyb-2.1</i>	wild type	wild type	31.5 ± 1.7 (n = 25)
<i>cyb-2.2</i>	Lvl/ Emb	wild type	31.3 ± 1.5 (n = 27)
<i>cyb-3</i>	Emb	Emb	n.d.
<i>cyd-1</i>	Lvl	number reduced	25.1 ± 2.3 (n = 18)**
<i>cye-1</i>	Emb	number/DNA reduced	27.7 ± 2.4 (n = 20)*
<i>cyh-1</i>	Ste/ Emb	wild type	31.6 ± 1.5 (n = 25)
<i>cyl-1</i>	Emb	L3 arrest	27.0 ± 3.8 (n = 22)*

The indicated dsRNA solutions were injected into N2 or OLB11: *rde-1(ne219) V; dusIs[Pelt-2::rde-1 + rol-6(+)]* worms, and the resulting phenotype and the number of intestinal nuclei in OLB11 were examined in their progeny. Only the intestine is susceptible to RNAi in OLB11. Buffer injection was used as the mock control. Abbreviations used are as follows: Sck, sick; Dpy, dumpy; Ste, sterile; Lvl, larval lethal; Slo, slow growth; Emb, embryonic lethal; number reduced, the number of intestinal nuclei reduced; DNA reduced, the DNA content of intestinal nuclei reduced; number/DNA reduced, both the number and the DNA content of intestinal nuclei reduced; n.d., not determined.

* $p < 0.05$, ** $p < 0.001$.

L2 stages by qRT-PCR, while simultaneously observing their intestinal nuclear divisions (Fig. 5). To minimize delay or fluctuation of development in *lin-35* mutants as compared to wild type, the populations were synchronized and cultured at 15°C, the lowest permissive temperature, and progression of development was carefully monitored through observation of intestinal

nuclear divisions and larval molting. We found that both wild type and *lin-35(rr33)* mutants started L1 nuclear divisions at 25 hours of cultivation and molted between 25 and 27 hours of cultivation, indicating that there was no significant difference in the developmental rate between wild type and *lin-35* mutants at 15°C, as previously described (Fig. 5A and C).⁴⁶ We then

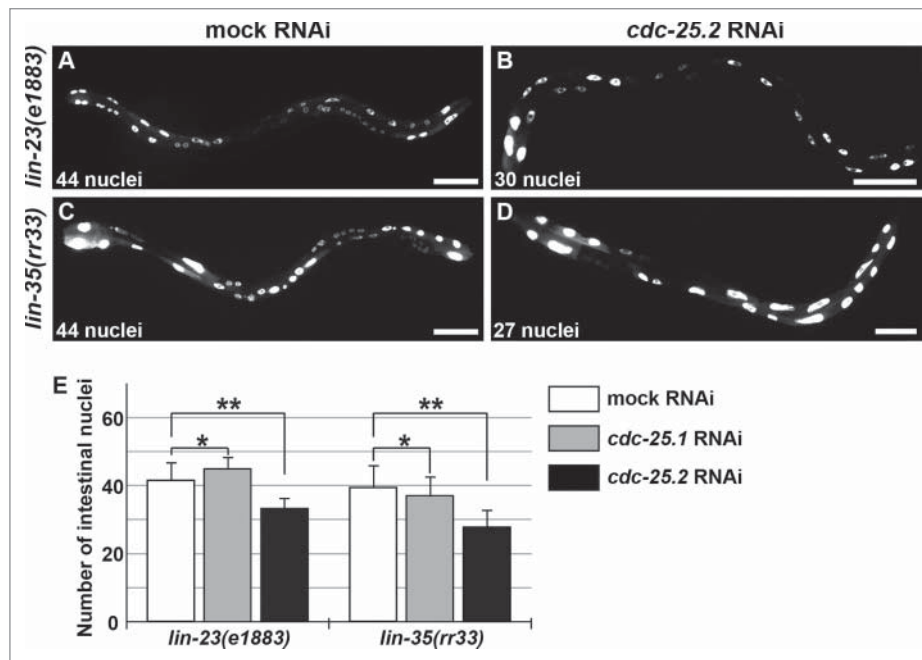


Figure 4. *cdc-25.2* RNAi suppressed the intestinal hyper-nucleation phenotype of the *lin-23* and *lin-35* mutants. (A) Mock RNAi-treated *lin-23(e1883)* mutant adult. (B) *cdc-25.2* RNAi-treated *lin-23(e1883)* mutant adult. (C) Mock RNAi-treated *lin-35(rr33)* mutant adult. (D) *cdc-25.2* RNAi-treated *lin-35(rr33)* mutant adult. Left, the anterior side. Scale bars, 50 μ m. (E) Average numbers of intestinal nuclei after RNAi depletion of mock, *cdc-25.1*, and *cdc-25.2* in *lin-23(e1883)* mutants (n = 33, 15, and 38, respectively), and in *lin-35(rr33)* mutants (n = 33, 54, and 25, respectively). * $p = 0.025$ and 0.7 in *lin-23(e1883)* and *lin-35(rr33)*, respectively. ** $p < 0.001$.

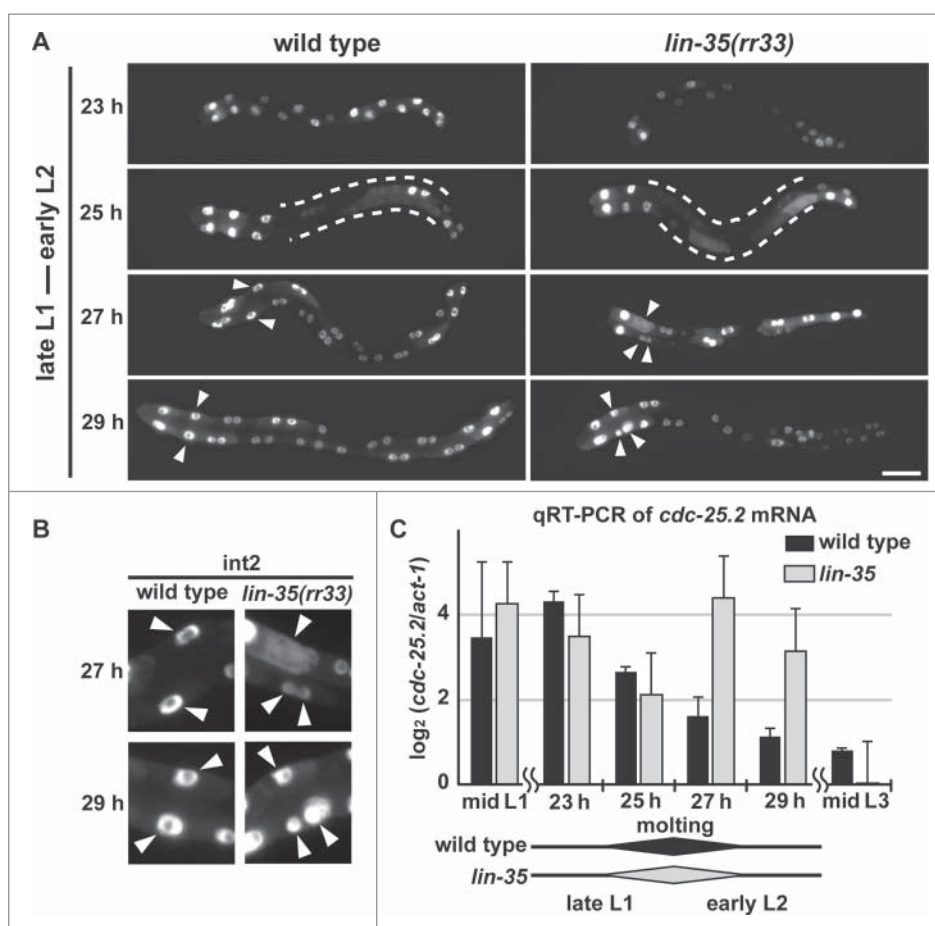


Figure 5. *cdc-25.2* mRNA levels were negatively regulated by LIN-35. (A) GFP-marked intestinal nuclei in wild type and *lin-35(rr33)* mutants during early larval development. Hours (h) indicate the feeding period of synchronized L1 larvae in culture plates at 15°C. Dotted lines indicate regions of intestine in which GFP signals temporarily dispersed, and therefore intestinal nuclei were not clearly identified. Arrowheads indicate nuclei of int2 cells. Left, the anterior side. Scale bar, 25 μ m. (B) Enlarged images of int2 nuclei. Excessive numbers of int2 nuclei were observed in 27 h- and 29 h-old *lin-35(rr33)* mutant early larvae (arrowheads). In contrast, the number of int2 nuclei was consistently 2 in wild-type larvae. (C) A time course of *cdc-25.2* mRNA levels during early larval development in wild type and *lin-35(rr33)* mutants. The *cdc-25.2* mRNA level at each time point in wild type and *lin-35(rr33)* mutants was measured 3 times by qRT-PCR, averaged, normalized to that of *act-1* mRNA, and shown as the \log_2 value. Rhomboid shapes at the bottom indicate the duration of L1-to-L2 molting observed in wild type and *lin-35(rr33)* mutant larval samples.

observed additional nuclear divisions in *lin-35* mutants, but not in wild type, at 27 and 29 hours of cultivation (Fig. 5A). These additional nuclear divisions in *lin-35* mutants were clearly identified by observing int2 nuclei at 27 and 29 hours of cultivation (Fig. 5B). Notably, we found that the level of *cdc-25.2* mRNA was upregulated during the 27-to-29-hour cultivation window in *lin-35* mutants, but not in wild type, as assessed by qRT-PCR quantification (Fig. 5C). These results indicate that *cdc-25.2* mRNA was temporarily upregulated in *lin-35* mutants when additional nuclear divisions occurred after the L1-to-L2 molting. These results also suggest that *cdc-25.2* is negatively regulated by *lin-35* at the transcriptional level during the early L2 stage, possibly to promote transition of cell cycle mode from nuclear division to endoreduplication.

Discussion

The intestinal development of *C. elegans* is an excellent model system to study cell division cycles because it consists of 3 distinct periods that employ different division modes (Figs. 1I and 6). The first is the period of cell divisions during embryogenesis. The second is the period of

binucleations that occur in the int3-7 cells (occasionally also in the int8-9 cells) at the L1 larval stage, which produces 30-34 nuclei in the 20 cells. The third is the period of endoreduplications that occur before each larval molting and increase DNA content. In this study, we demonstrated that CDC-25.2 serves an essential role in intestinal development in the later stage of the first period and through the second period. First, we showed that CDC-25.2 is required for intestinal divisions only after the 16E cell stage. Homozygous progeny produced from *cdc-25.2* heterozygous mothers ($M^+ Z^-$) and rare homozygous progeny produced from *cdc-25.2* homozygous mothers ($M^- Z^-$) generated the same 16 intestinal nuclei (Table 1), indicating that there was no contribution of maternal *cdc-25.2* product to the intestinal divisions before the 16E cell stage. In addition, the *cdc-25.1* (*gf*) mutation that causes intestinal hyperplasia did not completely suppress the intestinal division defects of the *cdc-25.2* mutants (Fig. 3). Furthermore, *cdc-25.1* RNAi was less suppressive than *cdc-25.2* RNAi on intestinal hypernucleation in *lin-23(e1883)* and *lin-35(rr33)* mutants (Fig. 4). These results and studies by others strongly suggest that, although they may overlap, the functional periods of

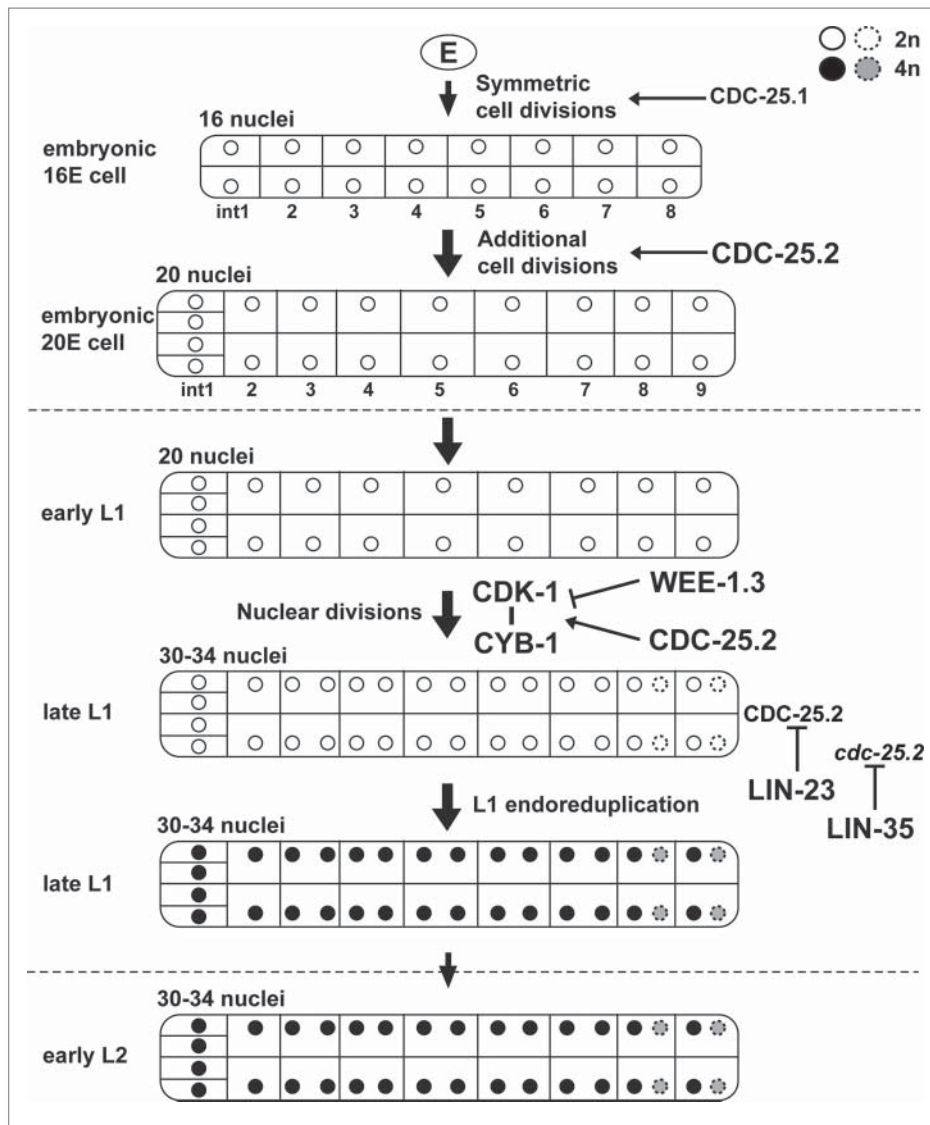


Figure 6. A proposed model for the function and regulation of CDC-25.2 during *C. elegans* intestinal development. Tubes and internal compartments indicate the whole intestine and intestinal cells, respectively. Circles indicate intestinal nuclei and the gray scale indicates different DNA contents of the nuclei after endoreduplications (white, 2n; black and gray, 4n). Broken circles indicate intestinal nuclei that occasionally divided during nuclear divisions. Vertical arrows indicate the progression of intestinal divisions. Broken horizontal lines indicate the borders of developmental stages. Horizontal arrows indicate that respective intestinal divisions are positively regulated by CDC-25.1 or CDC-25.2. The horizontal T-bar indicates the inhibition of the CDK-1/CYB-1 complex by WEE-1.3. The vertical T-bars indicate the inhibition of CDC-25.2 protein and *cdc-25.2* mRNA by LIN-23 and LIN-35, respectively.

CDC-25.1 and CDC-25.2 are different. That is, CDC-25.1 functions earlier than CDC-25.2, probably up to the 16E cell stage, in embryonic intestinal cell divisions (Fig. 6).^{19,47} Because neither *cdc-25.3* nor *cdc-25.4* deletion mutants showed any obvious intestinal division defects (Table 1), CDC-25.2 appears to be the only member of the family that regulates intestinal divisions after the 16E cell stage to accomplish the rest of the embryonic cell divisions as well as the entire postembryonic nuclear divisions (Fig. 6).

In the wild type, after completion of binucleations in the L1 stage, although the number of intestinal nuclei (30–34) does not increase any further, all the intestinal nuclei double their DNA content via endoreduplication before each larval molting.¹⁰ It was previously reported that endoreduplication at the end of the L1 stage was replaced by extra nuclear divisions in *lin-23(e1883)* mutants, which resulted in intestinal hyper-

nucleation.⁴⁵ Since it was also reported that LIN-23 may negatively regulate embryonic intestinal cell divisions through ubiquitin-mediated degradation of CDC-25.1,^{19,47} we hypothesized that LIN-23 might be required during the L1 stage to restrict intestinal nuclear divisions through inactivation of CDC-25.2. Indeed, we found that the intestinal hyper-nucleation of *lin-23(e1883)* mutants was almost completely suppressed by *cdc-25.2* RNAi (Fig. 4). This result suggests that LIN-23 negatively regulates CDC-25.2 at the end of the L1 stage to successfully shift the intestinal division mode from binucleation to endoreduplication (Fig. 6). However, whether LIN-23 inactivates CDC-25.2 directly or indirectly remains to be elucidated. Extra nuclear divisions in place of endoreduplication was also observed in *lin-35(rr33)* mutants at the early L2 stage.⁴⁶ We found that the intestinal hyper-nucleation of *lin-35(rr33)* mutants was also almost completely suppressed by *cdc-25.2* RNAi (Fig. 4), and

that the level of *cdc-25.2* mRNA was transiently upregulated after the L1-to-L2 molting in the *lin-35* mutants (Fig. 5C). These results indicate that the level of *cdc-25.2* mRNA needs to be repressed in a timely manner by LIN-35 in order to accomplish L1 endoreduplication (Fig. 6). However, precisely how this process occurs is not yet clear. Taken together, our results indicate that *cdc-25.2* activity (CDC-25.2 protein and *cdc-25.2* mRNA) needs to be negatively regulated by both LIN-23 and LIN-35 at the end of the L1 stage or at the beginning of the L2 stage to achieve successful transition from the nuclear division to L1-stage endoreduplication (Fig. 6).

We previously reported that an absence of CDC-25.2 during oogenesis interfered with normal oocyte maturation and caused the endomitotic oocyte (Emo) phenotype.²¹ In this study, we found that, although additional 4 cell divisions during embryogenesis and binucleations at the L1 stage were totally abrogated, intestinal endoreduplication was not affected in the *cdc-25.2* mutants; int2 nuclei had the same DNA levels in wild type and *cdc-25.2* mutants (Table 2). In fact, inactivation of CDC-25.2 appeared to be essential for initiating and accomplishing intestinal endoreduplication. These observations suggest that, under certain circumstances, inactivation of CDC-25.2 causes endomitosis or endoreduplication instead of merely arresting the cell cycle. Endoreduplication is a specialized cell cycle in which the M phase is bypassed, but the other 3 phases, G1, S, and G2, continue to occur (reviewed in ref. 48). It would be intriguing to understand the mechanism by which inactivation of CDC-25.2 induces endoreduplication instead of cell cycle arrest.

To better understand the regulatory mechanism of intestinal divisions in *C. elegans*, we tested possible involvement of other conserved cell-cycle regulators by performing systematic RNAi analysis. First, among the genes of *wee-1* family, RNAi depletion of *wee-1.3* partially suppressed the *cdc-25.2* intestinal phenotype. As *wee-1.3* RNAi also suppressed the *cdc-25.2* Emo phenotype during oogenesis,²¹ this suppression in the intestine indicates that the counteractive interaction between CDC-25.2 and WEE-1.3 is conserved between the organs in *C. elegans*. To identify the specific CDK and cyclin that function during *C. elegans* intestinal divisions, we screened the available *C. elegans* orthologs via intestine-specific RNAi analysis using the OLB11 strain. Among the *cdk* genes, RNAi depletion of *cdk-1*, *cdk-2*, and *cdk-4*, and among the *cyclin* genes, RNAi depletion of *cyb-1*, *cyd-1*, *cye-1*, and *cyl-1* reduced the number of intestinal nuclei in OLB11 (Table 4). Furthermore, RNAi depletion of *cdk-1*, *cyb-1*, and *cyd-1* did not affect relative DNA-staining intensity of int2 nuclei at the adult stage, nor did the *cdc-25.2* (*ok597*) mutation (Table 2), suggesting that these 3 genes are most likely not involved in the regulation of endoreduplication. However, in a previous study, it was reported that intestinal development in 2 *cyd-1* mutants, *he112* and *he116*, was arrested at the 16E cell stage with 2C DNA content, indicating that both binucleations and endoreduplications were abrogated in the *cyd-1* mutants.⁴² Therefore, our results on *cyd-1* RNAi might not be accurate. The simplest explanation for this discrepancy is that our *cyd-1* RNAi treatment in OLB11 did not completely inactivate the gene. The OLB11 strain contains an *rde-1* transgene that is driven by an intestine-specific *elt-2* promoter in the *rde-1* mutant background, which restores RNAi activity specifically in the intestine (Table S1).⁴¹ However, because *elt-2* begins

to be expressed at the embryonic 2E cell stage, maternal gene products that are already loaded before the 2E cell stage are not effectively inactivated by RNAi in OLB11.³³ Therefore, the effectiveness of RNAi depletion in OLB11 differs among genes depending on their timing of expression during development. This difference in RNAi effectiveness may at least partially explain why intestine-specific RNAi phenotypes of *cdk* and *cyclin* homolog genes in OLB11 were weaker than those seen in previous studies. Nevertheless, our RNAi results and those of previous studies^{37,42} are consistent with the view that CDK-1 is the most likely candidate CDK that is required for intestinal nuclear divisions but not for endoreduplications. Because *cyb-1* RNAi displayed the phenotype that was most similar to that of *cdk-1* RNAi among the *cyclin* genes in OLB11, and because CYB-1 and CYB-3 were previously shown to associate with CDK-1,⁴⁰ we assume that CYB-1 is one of the most likely candidates for the cyclin that works in concert with CDK-1 during intestinal nuclear divisions. Because *cyb-3* RNAi in OLB11 caused embryonic lethality, we could not determine whether CYB-3 also functions after the 16E cell stage, possibly together with CDK-1. Taken together, based on our intestine-specific RNAi analysis and previous studies, we hypothesize that, after the 16E cell stage, CDC-25.2 positively and WEE-1.3 negatively regulates CDK-1, which forms a complex with CYB-1, to control intestinal nuclear divisions (Fig. 6). On the other hand, RNAi depletion of *cdk-2*, *cdk-4*, and *cye-1* in OLB11 not only reduced the number of intestinal nuclei, but also significantly reduced the relative DNA content of int2 nuclei (Table 2). These results suggest that CDK-2, CDK-4, CYE-1, and also possibly CYD-1 (see above) are required not only for nuclear divisions but also for endoreduplications in the intestine. Previous studies on *cye-1* mutations, which revealed defects in both binucleation and endoreduplication,^{43,44} support this role of CYE-1.

In this study, we demonstrated that CDC-25.2 and its regulation are essential for the progression of diversified intestinal divisions including embryonic cell divisions, postembryonic nuclear divisions, and endoreduplications. Nevertheless, the biological significance of CDC-25.2's role in intestinal development is still elusive, notably because the intestine of *cdc-25.2* mutants, which contains only 16 nuclei instead of 30–34 nuclei, seems to function normally, at least under standard growth conditions. Further analysis is required to better understand the biological importance of this specialized developmental process, in which CDC-25.2 is involved.

Materials and methods

Strains

C. elegans strains used in this study are listed in a separate Table (Table S1). Worms were cultured and handled at 20°C using standard procedures.⁴⁹

Construction and microscopic observation of transgenic animals

To visualize intestinal nuclei, An *elt-2::gfp* transgene, *rrIs01* [*Pelt-2::GFP*], was introduced into some strains by genetic

crossing (Table S1). To express *cdc-25.2* under the control of the intestine-specific *elt-2* promoter, a full-length *cdc-25.2* cDNA was inserted into the pOLB1872 plasmid between the *Bam*HI and *Sma*I restriction sites, which was then microinjected into recipient worms with the pRF4 injection marker plasmid as previously described.⁵⁰ The generated transgenic worms containing the heritable extrachromosomal array, *kkuEx03[Pelt-2::cdc-25.2::mCherry]*, were then crossed with wild-type and *cdc-25.2(ok597)* heterozygous *rrIs01[Pelt-2::GFP]* transgenic worms for overexpression and rescue experiments, respectively (Table S1). For staging, newly hatched larvae were first synchronized at the L1 stage in the absence of food, then cultured with food until they reached the desired developmental stage. To examine the number of intestinal nuclei, the staged worms were transferred to M9 buffer on a poly-L-lysine-coated glass slide, covered with a coverslip, and observed under a fluorescence microscope (Zeiss Axioskop 2). Images were recorded using an Orca-ERG digital camera (Hamamatsu) with Openlab 5 software (Improvision).

Immunohistochemistry and confocal microscopy

Embryonic specimens were prepared as previously described⁵¹ and immunostained with rabbit polyclonal anti-GFP primary antibody (Invitrogen A11122, 1:400) and Alexa Fluor 488 goat anti-rabbit IgG (H⁺L) secondary antibody (Invitrogen A11008, 1:200). Images were recorded using a FluoView FV1000 confocal microscope (Olympus).

In situ hybridization

The *C. elegans* cDNA clone yk472b2 was used to generate RNA probes for *cdc-25.2*. Digoxigenin (DIG) labeling of RNA probes was performed according to the manufacturer's instructions (Roche, DIG application manual for *in situ* hybridization; http://www.roche-applied-science.com/PROD_INF/MANUALS/InSitu/InSi_toc.htm). Larvae were synchronized, fixed, and hybridized with sense or antisense RNA probe as previously described.³⁴ Fixed specimens were hybridized with 1:500 diluted *cdc-25.2* sense or antisense RNA probe at 60°C for 16 hours. *In situ* hybridization images were taken using an AxioCam MRc 5 digital camera and processed with AxioVision software (Zeiss).

DNA quantification

DNA staining of intact worms was carried out as previously described.^{10,52} Fluorescence images were taken with an Orca-ERG digital camera (Hamamatsu) using the same exposure conditions in all cases. Thereafter, the fluorescence intensities of Hoechst 33342-stained intestinal nuclei were measured using Openlab 5 software (Improvision). The area of each intestinal nucleus was set as region of interest (ROI), and the average fluorescence intensity of each ROI (i_{av}) was measured. Average background intensity (b_{av}) was also measured and subtracted from the i_{av} of each intestinal nucleus ($i_{av}-b_{av}$). The DNA content of each intestinal nucleus was calculated as $(i_{av}-b_{av}) \times \text{area of ROI}$. This value was normalized to that of wild-type int2 for quantitative comparison.

RNA interference

RNAi depletion of *wee-1* family genes in the wild-type and *cdc-25.2(ok597)* worms was performed by the feeding RNAi method,⁵³ with the *E. coli* strain HT115(DE3) that harbored L4440-derived vectors in which a *C. elegans* genomic insert, F35H8.7 (*wee-1.1*) or Y53C12A.1 (*wee-1.3*), was inserted. Gravid adult hermaphrodites were transferred to each feeding RNAi plate, and the resulting progeny were examined for their intestinal phenotypes when they became adults. RNAi depletion of *cdc-25.1* and *cdc-25.2* in *lin-23(e1883)* and *lin-35(rr33)* mutant worms was performed by the soaking RNAi method:⁵⁴ dsRNA was transcribed *in vitro* from corresponding yk EST clones (Table S2) and dissolved in soaking buffer. Synchronized L1 larvae were soaked in the dsRNA solution and incubated for 48 hours at 20°C. Then, the soaked worms were placed onto OP50-seeded NGM plates and grown until they reached the L4 or young adult stage, at which time their intestinal phenotypes were observed. For RNAi depletion of cell-cycle regulator genes in N2 and OLB11 worms, dsRNA of each gene prepared by *in vitro* transcription was microinjected into the gonads of young adult hermaphrodites, and their progeny were examined for the resulting intestinal phenotypes when they became adults. cDNA inserts of corresponding yk EST clones (Table S2) were PCR-amplified using the T7 primer and the CMo422 primer pair (Table S3) as DNA templates for *in vitro* transcription. Those DNA templates for which yk clones were not available were PCR-amplified from an N2 cDNA pool using gene-specific primers (Table S3).

Quantitative real-time RT-PCR

To quantify mRNA levels of *cdc-25.2*, total RNA was prepared from synchronized wild-type N2 and *lin-35(rr33)* mutant worms at respective developmental stages. To collect mid-L1 and mid-L3 stage worms, synchronized L1 worms were fed for 10 hours and 30 hours at 20°C, respectively, before harvesting. To collect late-L1 to early-L2 stage worms, synchronized L1 worms were fed for 23, 25, 27, and 29 hours at 15°C. Real-time measurement of *cdc-25.2* mRNA levels was performed as previously described.²¹ The measured values were normalized to that of *act-1* mRNA and presented as \log_2 values for quantitative comparisons.

Statistical analysis

P values were calculated by Student's t-test, and most of the data were obtained from 3 independent experiments with multiple sample numbers (*n*), which are described in the figure legends.

Abbreviations

CDC	cell division cycle
CDK	cyclin-dependent kinase
16E	16 great-great-granddaughter cells of the founder blastomere E

L1 to L4 the first to the fourth larval stage
 M maternally loaded
 Z zygotically expressed
 Emo endomitotic oocyte

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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