Development 126, 2227-2239 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV6396

The *Caenorhabditis elegans* gene *ncc-1* encodes a *cdc2*-related kinase required for M phase in meiotic and mitotic cell divisions, but not for S phase

Mike Boxem, Dayalan G. Srinivasan and Sander van den Heuvel*

Massachusetts General Hospital Cancer Center, Building 149, 13th Street, Charlestown, MA 02129, USA
*Author for correspondence (e-mail: heuvel@helix.mgh.harvard.edu)

Accepted 22 February; published on WWW 19 April 1999

SUMMARY

We have identified six protein kinases that belong to the family of cdc2-related kinases in *Caenorhabditis elegans*. Results from RNA interference experiments indicate that at least one of these kinases is required for cell-cycle progression during meiosis and mitosis. This kinase, encoded by the *ncc-1* gene, is closely related to human *Cdk1/Cdc2, Cdk2* and *Cdk3* and yeast *CDC28/cdc2*$^{+}$. We addressed whether *ncc-1* acts to promote passage through a single transition or multiple transitions in the cell cycle, analogous to *Cdks* in vertebrates or yeasts, respectively. We isolated five recessive *ncc-1* mutations in a genetic screen for mutants that resemble larval arrested *ncc-1(RNAi)* animals. Our results indicate that maternal *ncc-1* product is sufficient for embryogenesis, and that zygotic expression is required for cell divisions during larval development. Cells that form the postembryonic lineages in wild-type animals do not enter mitosis in *ncc-1* mutants, as indicated by lack of chromosome condensation and nuclear envelope breakdown. However, progression through G_1 and S phase appears unaffected, as revealed by expression of ribonucleotide reductase, incorporation of BrdU and DNA quantitation. Our results indicate that *C. elegans* uses multiple Cdks to regulate cell-cycle transitions and that *ncc-1* is the *C. elegans* ortholog of *Cdk1/Cdc2* in other metazoans, required for M phase in meiotic as well as mitotic cell cycles.

Key words: *Caenorhabditis elegans*, *ncc-1*, Cell cycle, Cdc2, Cdk, *cdk5*, PCTAIRE, Cell-division

INTRODUCTION

A fundamental question in biology is how cell division, growth and differentiation are coordinately regulated during development of multicellular organisms. This coordination likely involves connections between developmental signals and the cell-cycle machinery. Cyclin-dependent kinases (Cdks) have been identified as key components of the cell-cycle machinery in all eukaryotes studied (Forsburg and Nurse, 1991; Nigg, 1995; Norbury and Nurse, 1992). In yeasts, progression through the cell cycle is controlled by a single Cdk, encoded by the *CDC28* and *cdc2*$^{+}$ genes in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively. The CDC28/cdc2 catalytic subunit requires association with a cyclin regulatory partner for kinase activity, and different cyclins are involved in progression through G_1, S and M phase. Regulation of cell-cycle progression is more complex in multicellular organisms; a large family of kinases related to CDC28/cdc2 has been identified in metazoans and several of these kinases have been implicated in cell-cycle regulation (reviewed by Nigg, 1995). In addition, various cyclins are expressed during distinct phases of the cell cycle in metazoans. Thus, progression through the cell cycle in higher eukaryotes is thought to require successive activation of different Cdks, regulated in part by transient associations with distinct cyclins. Other levels of regulation include association with kinase inhibitors and activating as well as inactivating phosphorylations.

When vertebrate cells enter a division cycle, the first CDKs to become activated are Cdk4 and/or Cdk6 in association with D-type cyclins (Sherr, 1994, 1996). Late in G_1, Cdk2 is activated and can be found associated first with cyclin E and subsequently cyclin A (Tsai et al., 1993a). Kinase activity of Cdk1/Cdc2 in combination with A- and B-type cyclins peaks at the G_2/M transition (Draetta and Beach, 1988; Tsai et al., 1993a). Several lines of evidence indicate that these Cdks regulate specific cell-cycle transitions. Extracellular signals, such as growth factors, influence expression levels of D-type cyclins and association with Cdk4 and Cdk6 (Matsushime et al., 1994, 1991). Ectopic expression of cyclin D, as well as cyclin E, shortens G_1 phase and accelerates entry into S phase (Ohtsubo and Roberts, 1993; Quelle et al., 1993). Inhibition of either cyclin A activity or Cdk2 activity inhibits DNA replication (Fang and Newport, 1991; Girard et al., 1991; Pagano et al., 1993; Tsai et al., 1993a; van den Heuvel and Harlow, 1993), while inhibition of Cdk1/Cdc2 prevents entry into mitosis (Fang and Newport, 1991; Riabowol et al., 1989; Th'ng et al., 1990; van den Heuvel and Harlow, 1993). The function of several other cdc2-related kinases is currently either unknown or, for Cdk5, appears unrelated to cell-cycle progression (Chae et al., 1997; Gilmore et al., 1998; Nikolic et

al., 1996). Thus far, genetic analysis of *Cdk* loss-of-function mutations has been limited in multicellular organisms. A family of cdc2-related kinases has been identified in *Drosophila* and one of these kinases, Dmcdc2, has been implicated in regulation of mitosis and inhibition of DNA replication (Hayashi, 1996; Sauer et al., 1996; Stern et al., 1993). A second kinase, Dmcdc2c, appears active during S phase (Lehner and O'Farrell, 1990).

The nematode *C. elegans* is uniquely suited as an animal model to study developmental control of cell division. The transparency of these animals allows monitoring of cell divisions in living animals. In addition, the near invariance of the cell lineage has allowed a precise description of the time of division for every somatic cell (Sulston and Horvitz, 1977; Sulston et al., 1983). Combined with effective genetics, these characteristics allow efficient isolation and detailed examination of cell-cycle mutants.

Genetic studies have already revealed several genetic pathways that regulate postembryonic cell divisions. For instance, pathways that involve TGF-β and insulin-related signaling cascades control entry into and exit from a developmentally arrested dauer stage (Massague, 1998; Riddle and Albert, 1997). In addition, a pathway of heterochronic genes controls the timing of cell division in several postembryonic lineages (reviewed by Slack and Ruvkun, 1997). Furthermore, a Notch-like signaling pathway that includes the *lag-2* ligand and *glp-1* receptor controls the switch between mitotic versus meiotic division in the germline (Francis et al., 1995; Kadyk and Kimble, 1998; Kimble and Simpson, 1997). The targets of such developmental signaling pathways likely include components of the general cell-cycle machinery, which may be regulated through intermediates such as *cki-1*, a developmentally regulated Cdk inhibitor of the CIP/KIP family (Hong et al., 1998).

To be able to explore connections between developmental signals and the cell-cycle machinery, we have initiated analysis of the cyclin-dependent kinases present in *C. elegans*. We have identified six putative kinases that share extensive homology with cdc2-related kinases in higher eukaryotes. RNA-interference of only one of these kinases caused complete arrest of cell division. A cDNA clone of this kinase, named *ncc-1* for nematode cell cycle, was previously identified and shown to complement the *CDC28-1N* mutation in *S. cerevisiae* (Mori et al., 1994). Here we show that *ncc-1* is required for progression through M phase in meiotic and mitotic cell cycles, but not for G_1 and S phase progression. Thus, *ncc-1* performs a role similar to *Cdk1/Cdc2* in higher eukaryotes.

MATERIALS AND METHODS

Culture conditions and strains

Worm strains used were derived from the wild-type Bristol strain N2 and Bergerac strain RW7000. Worms were cultured using standard techniques as described by Brenner (1974). We used the following mutations, descriptions of which can be found in Riddle et al. (1997) or cited references:

LGIII: *dpy-17(e164), unc-36(e251), dpy-19(e1259), unc-32(e189), glp-1(q158), ncc-1(he5, he6, he24, he25 and n3064)* (this paper), *unc-47(e307), unc-49(e382), tra-1(e1099) and dpy-18(e364).* Rearrangements: *qDp3(III,f)* (Austin and Kimble, 1987), *ctDp6(III,f)* (Hunter and Wood, 1990), *nDf40* (Graham and Kimble, 1993), *qC1 dpy-19(e1259) glp-1(q339)* (Edgley et al., 1995). Expression of an rnr::GFP reporter was examined in strains VT774 *unc36(e251); maIs103[rnr::GFP unc-36(+)]* (Hong et al., 1998) and SV61 *ncc-1(n3064)/dpy-19(e1259) unc-47(e307); maIs103[rnr::GFP unc-36(+)].*

Cloning of *cdc2*-related genes

To clone *cdc2*-related genes from *C. elegans,* we used RT-PCR amplification with degenerate oligonucleotide primers, low stringency hybridization of a cDNA library and antibody screening of an expression library. For the reverse transcriptase PCR approach, RNA was isolated from mixed stage *C. elegans* cultures grown in bulk (Wood, 1988). First strand cDNA was prepared from total RNA and amplified by PCR, using degenerate oligonucleotides derived from the highly conserved motifs EKIGEGTY and EGVPSTA as 5′ primers and DLKPQNL and WYRSPEV as 3′ primers (primer sequences available upon request). PCR fragments were cloned into pBluescript II SK (Stratagene) and sequenced by the dideoxy method. Partial clones were obtained this way from *ncc-1*, *cdk5* and *pct-1*. To obtain full length cDNAs, a nematode cDNA library cloned in the Uni-ZAP vector (Stratagene) was probed with PCR fragments labeled with [α-^{32}P]dCTP. Although low stringency conditions were used, we obtained only cDNA clones from *ncc-1, cdk5* and *pct-1*. The same library was induced with IPTG and probed with a mouse monoclonal antibody recognizing the PSTAIRE motif (a kind gift from M. Yamashita). This resulted in identification of multiple cDNAs that were all derived from the *ncc-1* gene.

We expect the predicted kinases to be full length based on the isolation of multiple cDNAs of similar size, homology in the N terminus with the human kinases and a nonsense codon preceding the ATG initiation codon in the *pct-1* gene. The genomic localization was identified for each gene by probing YAC contigs, followed by PCR analysis of cosmids from the region. Cosmids T05G5, C07G1 and R11C7 were found to contain the *ncc-1*, *pct-1* and *cdk5* genes, respectively, and were used to determine the gene structures by Southern blotting and DNA sequencing. Subsequently, all sequences have been confirmed by the *C. elegans* sequencing project.

RNA interference

Plasmids containing full length *ncc-1*, *cdk-5* and *pct-1* cDNAs were used for in vitro transcription. For B0285.1, a genomic fragment was amplified by PCR using primers 5′-agaattcacaatgcttgatcaaattggc and 5′-aaggatccagattgtttttcttttccg. For, K03E5.3 a genomic fragment was amplified using primers 5′-aatccgcaattttgatgactccag and 5′-caaccgatatcgctccaggtaagc. These fragments were cloned into pBluescript SK. A plasmid containing cDNA yk492e2, derived from H06A10.1, was kindly provided by M. Park, M. Krause and Y. Kohara. Templates were linearized and transcribed in vitro with T3 or T7 RNA polymerase. RNA was phenol extracted, ethanol precipitated and dissolved in TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) to a final concentration of 1 mg/ml. A 1:1 mixture of the two strands was injected into young adult N2 hermaphrodites as described (Fire et al., 1998).

Screening for *ncc-1* alleles

N2 animals were mutagenized with 50 mM ethylmethanesulfonate (EMS) as described by Brenner (1974). Mutagenized L4 hermaphrodites were picked to 100 mm plates, 30 per plate, and allowed to produce self progeny at 25°C. F_1 animals in the fourth larval stage were picked to 60 mm plates, four per plate, and their progeny were examined for mutants that resemble larval-arrested *ncc-1(RNAi)* animals. Mutants that were sterile, uncoordinated, slightly dumpy and had relatively large head regions were examined by Nomarski microscopy to determine defects in cell division. Candidate mutations were recovered from siblings and were mapped initially by PCR, making use of primers based on polymorphic Sequence-Tagged Sites in the RW7000 Bergerac strain (Williams et al., 1992). *ncc-1* mutations mapped to chromosome III and were placed by standard

three factor crosses (Wood, 1988) between *dpy-19(e1259)* and *unc-47(e307)* or *dpy-17(e164)* and *tra-1(e1099),* about 1.06 map units to the right of *dpy-19* at position 0.89. In addition, all mutations failed to complement *nDf40* and allele *n3064*.

Antibodies

Antibodies were used in the following dilutions: mouse monoclonal antibody DM1A against α-tubulin (Sigma) diluted 1:1000 for western blot analysis and 1:500 for immunostaining, rabbit polyclonal antibody against phosphorylated histone H3 (Upstate Biotechnology) diluted 1:400, mouse monoclonal antibody BU-33 against BrdU (Sigma) diluted 1:60, mouse monoclonal antibody against the PSTAIRE region of Cdc2 (a kind gift from M. Yamashita), used 1:1000 diluted for immunoblotting and 1:250 diluted for immunostaining. A polyclonal antiserum raised against the C terminus of NCC-1 was diluted 1:1000 for immunostaining (a kind gift from J. Schumacher and A. Golden). Donkey-anti-mouse and donkey-anti-rabbit IgG secondary antibodies, rhodamine- or FITC-conjugated, were from Jackson labs and diluted 1:200.

Western blot analysis and Immunostaining

To obtain protein samples, synchronized wild-type animals were washed 4 times in M9 buffer and dissolved by boiling for 5 minutes in 1× Laemmli protein loading buffer (Harlow and Lane, 1988). Protein samples were separated by SDS-PAGE followed by coomassie staining, to determine protein concentration, or immunoblotted using standard procedures (Harlow and Lane, 1988).

For antibody staining of germlines and embryos, gravid wild-type hermaphrodites were dissected in a drop of M9 buffer on a gelatin-coated slide. A coverslip was applied and the slide frozen on dry ice. After 10 minutes the coverslip was cracked off and slides were incubated in methanol at −20°C for 20 minutes and acetone at −20°C for 20 minutes. For immunostaining of larvae, animals were fixed in Bouin's solution and antibody staining was performed according to standard procedure (Harlow and Lane, 1988; Nonet et al., 1997). For staining of DNA only, animals were fixed overnight in Carnoys solution (60% ethanol, 30% glacial acetic acid, 10% chloroform) and slowly rehydrated in PBS + 0.1% Tween-20. In all procedures, DNA was stained with either propidium iodide (PI) 1 mg/ml, following treatment with 200 μg/ml RNAse A for 30 minutes at 37°C, or with 1mg/ml 4,6-diamidino-2-phenylindole (DAPI) (Sigma). Samples were mounted on slides in 10% PBS, 2.3% Dabco, glycerol.

Images were obtained using a Sensys cooled CCD camera (Photometrics, Tucson, AZ) and a Power Macintosh computer. Image analysis and computational deconvolution was performed with Openlabs software version 1.7.8 (Improvision, Boston, MA). Images were pseudocolored and merged using Adobe Photoshop.

BrdU labeling

Synchronized cultures were obtained from embryos isolated by hypochlorite treatment and hatched in the absence of food (Wood, 1988). The resulting early L1 arrested larvae were transferred to plates with bacteria, which triggers highly synchronized development. DNA synthesis was visualized by incorporation of BrdU. A solution of BrdU in S-medium was added to plates with synchronized larvae to a final concentration of 0.5 mg/ml. Animals were fixed in Bouin's fixative and stained with anti-BrdU antibodies. The staining procedure was as described above with one addition; prior to preadsorbtion, DNA was denatured by incubating the fixed animals for 2 hours at room temperature in a 1:1 mixture of 4 M HCl and PBS + 1% BSA + 0.5% Triton X-100.

DNA quantitation

To quantitate DNA content, series of 32 Z-sections were taken of propidium iodide-stained animals with a confocal scanning laser microscope (Leica). Total pixel intensities were calculated for each section on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Pixel intensities of all 32 sections were added and recalculated to DNA content, using body wall muscle nuclei as a 2n DNA standard.

RESULTS

Identification of Cdc2 family members in *C. elegans*

Using several molecular approaches, we obtained cDNA clones from three *C. elegans* cdc2-related kinases (Materials and Methods and Fig. 1A). The kinase most closely related to human CDK1 (64% amino acid identity) was identified previously and named *ncc-1* (Mori et al., 1994). The predicted NCC-1 protein of 332 amino acids diverges from other Cdk1 and Cdk2 kinases at two striking positions: it has an N-terminal extension of 18 amino acids and contains an Ile-to-Val substitution in the highly conserved PSTA<u>I</u>RE region of the protein that is involved in cyclin binding (Jeffrey et al., 1995). A second kinase gene encodes a member of the PCTAIRE subfamily and was therefore named *pct-1*. Within the catalytic core, the predicted PCT-1 protein of 577 amino acids shares ~80% identity with any one of three human PCTAIRE kinases. In addition, PCT-1 has N-terminal and C-terminal extensions that are less conserved but similar in size to the human kinases. A third *C. elegans* kinase is a close homolog of human Cdk5 (74% amino-acid identity) and was named *cdk-5*. For each of these three kinases we sequenced the longest cDNAs and determined the genomic structure (Fig. 1A).

Three additional kinases that are more distantly related to Cdc2 were identified in BLAST homology searches (Table 1). The predicted gene H06A10.1 encodes a kinase that is most related to Cdk4 and Cdk6. K03E5.3 encodes a kinase that is 42%, 43% and 41% identical to human Cdk1, Cdk2 and Cdk3, respectively. Finally, the predicted product of B0285.1 shares 47% amino acid identity with human CHED kinase, and 41% with Cdk2 and Cdk3. As the sequencing of the *C. elegans* genome has been completed (The *C. elegans* Sequencing Consortium, 1998), we can conclude that these are the *C.*

Table 1. *cdc2*-related kinases in *C. elegans*

C. elegans gene	Human homolog	Amino acid identity (%)	RNAi phenotype
ncc-1	*Cdk1* *Cdk2* *Cdk3*	64 59 60	One-cell stage embryonic arrest. In the first 12 hours after injection, up to 10 progeny arrest cell division at the L1 stage.
K03E5.3	*Cdk1* *Cdk2* *Cdk3*	42 43 41	Embryonic lethal, larval arrest without growth, larval arrest with growth, sterile animals with protruding vulva (see text).
H06A10.1	*Cdk4* *Cdk6*	37 33	Variable cell division defects in larval development*.
cdk-5	*Cdk5*	74	No phenotype detected.
B0285.1	*Ched* *Cdk2* *Cdk3*	47 41 41	Late L3 or early L4 arrest.
pct-1	*Pctaire-1* *Pctaire-2* *Pctaire-3*	52 50 60	No phenotype detected.

*M. Park and M. Krause, personal communication.

elegans kinases that are most closely related to members of the Cdk1/2/3, Cdk5, Cdk4/6 and PCTAIRE subfamilies of cdc2-related kinases.

ncc-1 is a candidate cell-cycle regulator

We used RNA interference (RNAi) to determine which of these kinases may be involved in regulation of the *C. elegans* cell cycle. RNAi has been shown to cause specific loss-of-function phenotypes for many different genes in *C. elegans*, although the mechanism of action is not understood (Guo and Kemphues, 1996; Tabara et al., 1998). Double-stranded RNA (dsRNA) is at least one order of magnitude more potent in interfering activity than either single strand (Fire et al., 1998). We transcribed both strands of each of the six kinases in vitro and injected dsRNAs into wild-type hermaphrodites. Injection of *ncc-1* dsRNA caused a completely penetrant embryonic arrest at the one cell stage in all progeny produced after 12 hours post-injection. In addition, some of the earlier progeny arrested during larval development (31 larval mutants from 10 injected animals). These arrested larvae were uncoordinated, sterile, slightly dumpy and had disproportionally large head regions. Examination by Nomarski microscopy revealed that no or very few cells in the postembryonic lineages had divided. Most striking was the absence of any division of the blast cells Z1 to Z4 that normally form the somatic gonad and germ-line. These gonad precursor cells divide multiple times in several *C. elegans* cell-cycle mutants, including *lin-5* and *lin-6* mutants (our unpublished observations; Sulston and Horvitz, 1981). Both the embryonic and L1-arrest phenotypes implicated *ncc-1* as an important cell-cycle regulator.

RNAi for two *Cdc2* related genes, *cdk-5* and *pct-1*, did not result in any apparent phenotypes (data not shown). RNAi for each of the remaining three *cdc2*-related genes caused obvious developmental defects but not a strict arrest of cell division. A clear but highly variable effect was observed following dsRNA injection of K03E5.3: the progeny arrested either as embryos (12%, *n*=69), early larvae (17%, *n*=98), late larvae (9%, *n*=50), sterile adults (39%, *n*=228) or developed to wild-type adults (24%, *n*=132). Progeny from B0285.1 dsRNA-injected animals arrested late in the third larval stage (L3) or early L4 stage. Most cell divisions have been completed at this stage with the exception of germ-line divisions. Finally, H06A10.1 RNAi resulted in slightly abnormal progeny but no apparent cell-cycle defects. Variable defects in cell division have been observed to result from H06A10.1 RNAi by M. Park and M. Krause (personal communication). Redundancy between different kinases might mask individual gene functions. However, injection of a mix of dsRNAs from K03E5.3, F18H3.5, B0285.1 and *cdk-5* did not cause stronger defects: the phenotype observed closely mimicked that of K03E5.3 RNAi. Thus, based on these RNAi results, *ncc-1* is the best candidate for a general cell-cycle kinase in *C. elegans*.

Table 2. Timing of events following ovulation in *ncc-1(RNAi)* embryos

	N2 (*n*=5)*	*ncc-1 RNAi* (*n*=6)
Entry to uterus	5.3±0.5	5.8±0.8
Appearance of paternal pronucleus	31.8±3.9	28.5±7.9
Pseudocleavage	33.4±4.8	does not occur
Appearance of maternal pronucleus	33.5±4.6	29.3±5.5
Pronuclear meeting	39.5±4.7	58.6±2.4
Nuclear envelope breakdown	45.2±4.8	does not occur
Onset of cytokinesis	49.7±5.9	does not occur
Completion of cell division	56.2±6.7	does not occur

Time is in minutes measured from ovulation.

*Similar results have been described by (Kirby et al., 1990; McCarter et al., 1998; Rose et al., 1997).

NCC-1 is closely related to yeast CDC28/cdc2 as well as mammalian Cdk1 and Cdk2. Therefore, *ncc-1* could act analogously to *CDC28*/*cdc2*$^+$ in yeasts and regulate multiple transitions in the cell cycle. Alternatively, *ncc-1* could play a role similar to one of the human kinases, regulating either G_1/S

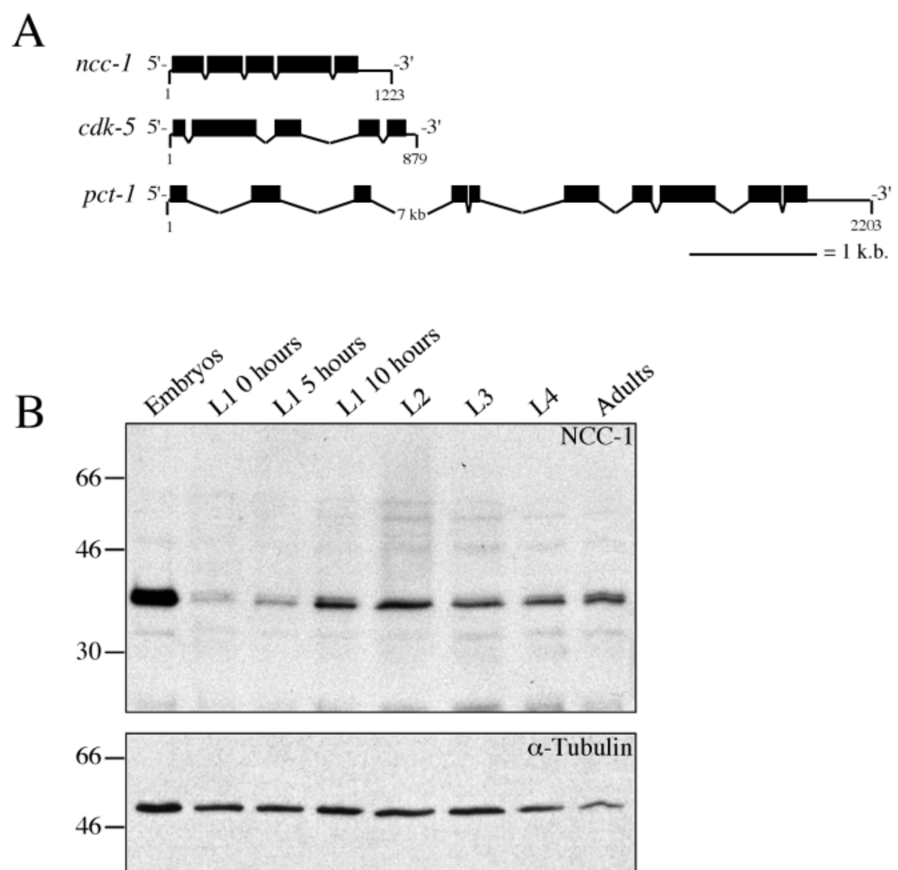

Fig. 1. (A) Gene structure of the *C. elegans cdc2*-related genes *ncc-1, cdk-5* and *pct-1*. Gene structures were determined by Southern blotting and DNA sequencing. Structures have been confirmed by the *C. elegans* sequencing project. (B) NCC-1 protein levels correlate with cell divisions. Protein lysates of synchronized wild-type animal populations were immunoblotted and probed with anti-PSTAIRE antibodies. α-tubulin protein levels were determined as a loading control.

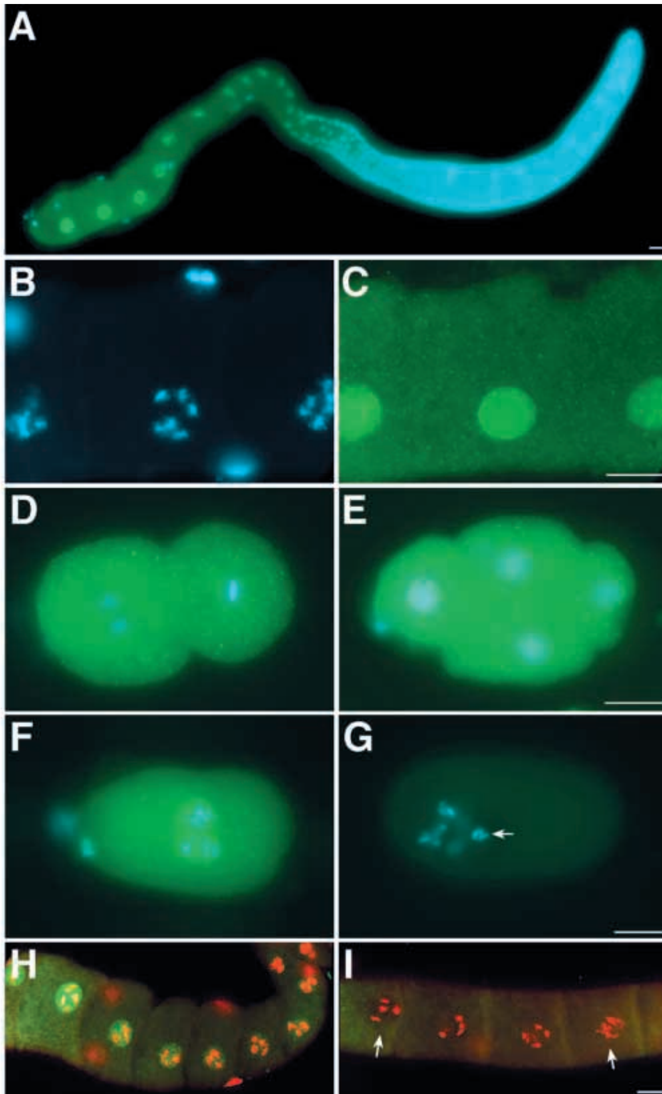

Fig. 2. NCC-1 protein localization in wild-type and *ncc-1(RNAi)* germ cells and embryos. (A-G) Staining of NCC-1 is in green and DNA is in blue (DAPI). (A) Nuclear NCC-1 expression levels increase during oocyte development (from right to left). A close-up of oocytes is shown in which DNA (B) and NCC-1 (C) are stained. NCC-1 localization is cytoplasmic during mitosis; (D) a wild-type 2 cell embryo in which the AB cell is in anaphase (left) and the P1 cell in metaphase (right). (E) Nuclear and cytoplasmic NCC-1 localization in a 4 cell wild-type embryo. (F,G) Comparison of NCC-1 staining in wild-type embryo (F) and *ncc-1(RNAi)* embryo (G). Exposure times and processing were identical for both images. NCC-1 staining is absent in *ncc-1(RNAi)* embryos. An arrow indicates condensed DNA in the paternal pronucleus. (H,I) NCC-1 (green) and DNA (PI, red) staining in wild-type and *ncc-1(RNAi)* oocytes. NCC-1 accumulates in nuclei of developing oocytes (from right to left) in the wild-type (H) but not in dsRNA-injected gonads (I). Note that chromosomes fully condense (compare the earlier (right) and later (left) nuclei indicated) to the diakinesis state in oocytes following RNAi treatment. Scale bars approx. 10 μm.

progression or mitosis. To distinguish between these possibilities, we determined the requirements of *ncc-1* in the *C. elegans* cell cycle.

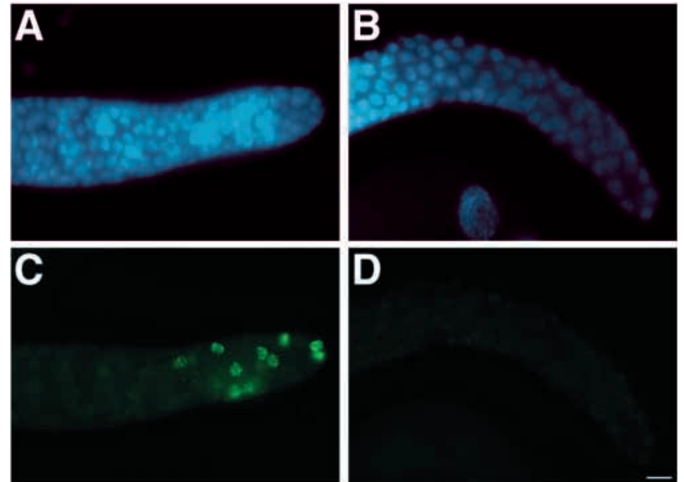

Fig. 3. Arrest of mitotic divisions in the germline by *ncc-1(RNAi)*. Germ cells in the distal, mitotic region of the gonad were stained with the DNA stain DAPI (A,B) and antibodies recognizing phosphorylated histone H3 (C,D). Wild-type germ cells in mitosis stain with the phosphorylated histone H3 antibody (C), whereas staining is not detected following *ncc-1* RNAi (D). Scale bar approx. 10 μm.

NCC-1 protein expression correlates with cell division and is abolished by RNAi

If *ncc-1* is required for cell division in general, NCC-1 protein is expected to be present in dividing cells. NCC-1 protein levels were found to correlate with the number of cell divisions during various stages of development, as was shown by western blot analysis (Fig. 1B). The highest NCC-1 levels were detected in embryos and the lowest levels in L1 larvae that were arrested by food deprivation. Upon release from L1 arrest, and resumption of cell division, NCC-1 protein levels were found to increase.

We used two different antibodies to detect the localization of NCC-1: a monoclonal antibody that recognizes the PSTAIRE peptide and an antiserum raised against a C-terminal NCC-1 peptide. Identical staining patterns were obtained with both antibodies. NCC-1 was found in the nucleus of oocytes and embryonic cells and, in addition, diffusely localized in the cytoplasm (Fig. 2A-F). During growth of oocytes in the proximal gonad, NCC-1 levels gradually increase (Fig. 2A,H). Following nuclear membrane breakdown, staining was diffuse in mitotic cells (Fig. 2D). NCC-1 appeared excluded from the DNA in metaphase and, to a lesser extent, in anaphase cells. NCC-1 was not detectable in oocytes or embryos of RNAi-treated animals (Fig. 2G,I). This result confirms the staining specificity and supports that *ncc-1(RNAi)* results in strongly reduced NCC-1 protein levels.

ncc-1 is required for meiotic maturation

To determine the cause of arrest in *ncc-1(RNAi)* embryos, we analyzed gametogenesis and early embryogenesis in the injected hermaphrodites. In wild-type animals, germ-precursor nuclei are produced by mitotic divisions in the distal ends of the syncytial gonad (Kimble and White, 1981). As these nuclei move further from the distal tip cell, they exit the mitotic cycle, initiate meiosis and progress through pachytene of meiotic

prophase I. Oocyte formation begins when germ nuclei reach the flexure in the U-shaped gonad; they exit from pachytene, cellularize, continue to enlarge and progress to diakinesis of Meiosis I. Shortly before fertilization, meiotic maturation and nuclear envelope degradation are initiated. Mature oocytes ovulate by entering the spermatheca at the proximal end of the gonad. Meiotic maturation is completed after fertilization and two polar bodies are expelled from the fertilized egg. The maternal pronucleus subsequently migrates towards the paternal pronucleus, while the egg undergoes pseudocleavage. The two pronuclei meet in the posterior of the egg, migrate to the middle and initiate a first mitotic division.

We examined the consequences of *ncc-1* dsRNA injection on these maturation and fertilization events in live animals by Nomarski microscopy. Two types of abnormalities were apparent: at the distal ends of the gonad fewer germ cells were produced (see below) and at the proximal ends many of the events that follow ovulation and fertilization of oocytes were aberrant (Table 2). Gonads were examined at various time points following dsRNA injection by Nomarski microscopy of living animals and fluorescence microscopy of fixed worms stained with DAPI for analysis of DNA organization. Formation and growth of oocytes was normal. The morphology and progression from pachytene to late stage oocytes, with six fully condensed bivalents in typical diakinesis arrangement, was also normal (Fig. 2I). Based on the position of the most distal oocyte with fully condensed chromosomes, relative to the flexure in the gonad, progress through pachytene appeared slightly delayed in about half of the injected animals (data not shown).

The first apparent defect was abnormal breakdown of the nuclear membrane in the mature oocyte, which required about 20 minutes to complete instead of 5 minutes in the wild type. Cytological analysis demonstrated that the transition from diakinesis to metaphase of meiosis I failed to occur as chromosomes did not congress to form a metaphase plate. No polar bodies were observed consistent with a complete failure of the meiotic divisions (Fig. 2G). However, fertilization did trigger formation of an eggshell. The maternal pronucleus enlarged and became about twice the wild-type size. Migration of the maternal pronucleus toward the paternal pronucleus was initiated but meeting of the nuclei was delayed and pseudocleavage did not occur (Table 2). The chromosomes in the paternal pronucleus failed to decondense and this nucleus remained small (Fig. 2G). Meeting of the pronuclei was followed by stable arrest: the nuclear membranes did not break down and mitosis did not occur. By immunostaining with tubulin-specific antibodies, we detected a bipolar spindle around the maternal nucleus in about half of the embryos (data not shown). We cannot exclude that formation of this spindle is dependent on residual activity of *ncc-1* in the *ncc-1(RNAi)* embryos (see Discussion).

The earliest defect seen in *ncc-1(RNAi)* animals is lack of meiotic maturation. Thus, these results suggest that *ncc-1* is required to promote meiotic maturation. The subsequent abnormalities may be secondary consequences of the meiotic maturation defect.

ncc-1 is required for mitotic divisions in the germ-line

We noticed that *ncc-1* dsRNA-injected animals produced fewer oocytes than wild-type hermaphrodites. Upon closer examination, the number of germ cells was found to decrease gradually over time in *ncc-1* RNA-injected animals. This reduction was first apparent in the mitotic region of the gonad arms, starting at around 12 hours after injection, at 20°C. The gonad was essentially empty by day three to four. During the same period, control injected animals did not show a noticeable change in the number of germ cells or general appearance of the gonad.

The germ precursor nuclei that were present in the distal region at 20 hours after injection were enlarged (Fig. 3B). Mitotic cells were not observed in gonads fixed and stained with DAPI. As metaphase cells are not always unambiguously detected in wild-type gonads, we also examined the presence of a mitotic phosphorylated epitope. Chromosome condensation in mitosis has been described to coincide with phosphorylation of histone H3 on Ser-10, which forms part of a consensus Cdc2-phosphorylation site (Ajiro et al., 1996; Hendzel et al., 1997). A rabbit polyclonal antiserum raised against a phosphorylated H3 peptide also recognizes a mitotic phospho-epitope in *C. elegans* (Kadyk and Kimble, 1998; Lieb et al., 1998). We immunostained *ncc-1* dsRNA-injected and control injected animals 20 hours after injection to examine the presence of the phosphorylated histone H3 epitope. Staining was absent in *ncc-1* RNA-injected germlines whereas control injected animals stained similarly to wild type (Fig. 3C,D). NCC-1 could directly or indirectly cause phosphorylation of histone H3, or be required at an earlier step in germ cell division. In either case, when taken together, these observations indicate that *ncc-1* is required for mitotic divisions in the distal part of the gonad.

Isolation of *ncc-1* mutants based on the larval *ncc-1 (RNAi)* phenotype

To further characterize the role of *ncc-1* in cell division, we isolated *ncc-1* mutations in a genetic screen. The larval arrested *ncc-1(RNAi)* animals may have received functional *ncc-1* maternal product yet fail zygotic expression. Based on this interpretation, we screened for uncoordinated, sterile mutants with disproportionally large head regions in the F_2 progeny of ethylmethanesulfonate (EMS) treated animals, in order to isolate *ncc-1* mutants.

In a semi-clonal screen of about 22000 haploid genomes, we obtained 21 independent recessive mutations that caused uncoordinated, sterile, large head phenotypes and defects in cell division during the first larval stage. Based on standard complementation assays and genetic mapping, 14 of these mutations likely affect the same gene. We characterized 4 of these mutations in detail. In addition, we also characterized *n3064* which displays a very similar phenotype and was isolated in a previous screen for potential cell-cycle mutants (S. v.d.H. and H. R. Horvitz, unpublished results). All five of these mutations mapped in close proximity to *ncc-1* and failed to complement *nDf40*, a deletion that removes the *ncc-1* locus. In addition, DNA sequencing revealed mutations within the *ncc-1* ORF in all five mutants (Fig. 4). The mutant alleles all contain G-C to A-T transitions, the type of mutation most commonly induced by EMS. The best candidate null allele is *he5*. This allele contains a deletion of 131bp, that removes the entire PSTAIRE domain, as well as a nonsense mutation that should cause a truncated product (Fig. 4). Allele *he26* also contains a nonsense mutation and encodes a product that lacks

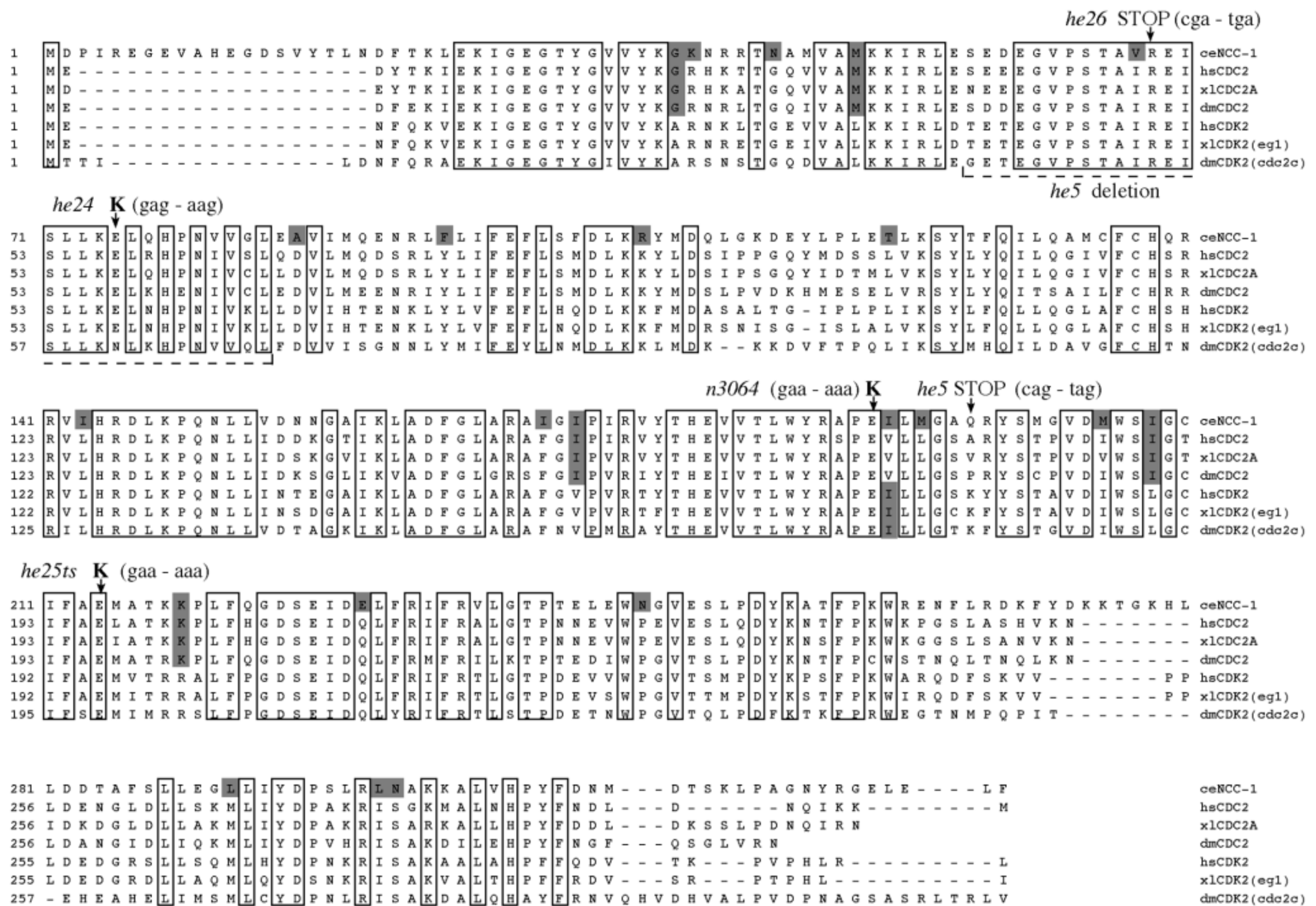

Fig. 4. Alignment of NCC-1 protein with Cdk1/Cdc2 and Cdk2 kinases from human, *Xenopus laevis* and *Drosophila melanogaster*. Boxed regions indicate regions of amino acid identity. Shaded small squares indicate residues that are conserved in Cdc2 and Cdk2 kinases but not in NCC-1. Shaded rectangles indicate residues conserved between NCC-1 and Cdc2 kinases, but distinct in Cdk2 kinases. One shaded box (near *n3064* mutation) indicates a residue shared between NCC-1 and Cdk2 kinases, but not present in Cdc2 kinases.

the C-terminal 265 out of 332 kinase residues. The other three alleles, *he24, n3064* and *he25*, contain missense mutations at positions 75, 191 and 214, respectively, replacing negatively charged glutamates by positively charged lysine residues. Each mutation affects a residue that is highly conserved within the Cdc2 family, in *he24* and *he25,* or in all protein kinases, in *n3064* (Fig. 4).

ncc-1 is required for postembryonic cell divisions

We characterized the nature of the *ncc-1* alleles and cell-cycle phenotypes in more detail. As homozygous mutants are fully penetrant sterile, the *ncc-1* mutations were maintained in heterozygous strains. Derived from heterozygous (*ncc-1/+*) mothers, *ncc-1* mutant progeny completed embryogenesis, presumably as a result of maternal gene function, but displayed no cell divisions after hatching. The percentage mutant progeny was close to 25% for all mutations, confirming the recessive nature of the alleles (Table 3). At 25°C, homozygous mutants and *trans*-heterozygotes with *nDf40* were phenotypically indistinguishable. Thus, all five mutations cause fully penetrant strong loss-of-function phenotypes at 25°C. Similar results were obtained at 15°C for all alleles except *he25*. Homozygous *he25* mutants were of more wild-type appearance at 15°C, although shorter and fully penetrant sterile. Based on our genetic and molecular characterizations, 4 of the 5 alleles are strong loss-of-function or null mutations, whereas *he25* causes incomplete loss of function at 15°C.

Nomarski microscopy observations indicated that all blast cells that give rise to the postembryonic lineages in wild-type animals are present in *ncc-1* mutants. However, these cells did not initiate mitotic divisions, with the possible exception of a rare division of an intestinal nucleus. Examination of fixed and DNA-stained animals confirmed these findings (Table 3). We quantified the cell-cycle defects for each of the mutations in three postembryonic lineages, by examining the first divisions of the ventral cord precursor cells (P), the intestinal cells (I) and the precursor cells of the somatic gonad and germline (Z1, Z2, Z3 and Z4). In wild-type animals, these cells start to divide about halfway through L1 (P and Z1-4) or late in L1 stage (I). All five mutations caused very similar defects in cell division. Not a single division of cells in the gonad primordium or P_{1-10} was observed in 20 animals examined (Table 3). Several observations indicate that *ncc-1* is directly required for cell division and that the lack of division was not caused by

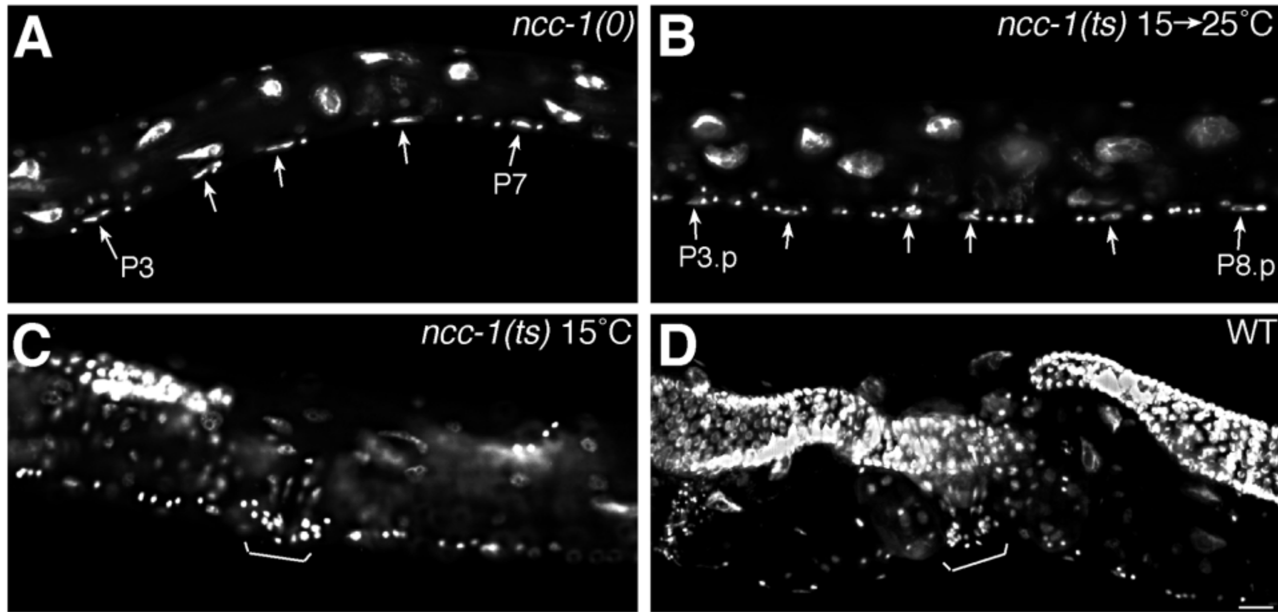

Fig. 5. (A,B) *ncc-1* is required for postembryonic blast cell divisions. DNA is stained with propidium iodide, all panels show animals of similar age (adults). (A) An *ncc-1(he5)* mutant, in which P cell divisions have not occured. P3-P7 are indicated by arrows. (B) A temperature sensitive *ncc-1(he25)* mutant shifted from 15°C to 25°C in the third larval stage, which caused the Pn.p divisions to fail. Undivided P3.p-P8.p cells are indicated by arrows. (C) An *ncc-1(he25)* animal grown at 15°C, which allowed most divisions in the P lineages as well as formation of a vulva (D) Wild-type adult. The bracket indicates the position of the vulva. Scale bar approx. 10 μm.

sickness or lethality. The *ncc-1* mutants were viable and continued to grow to 80% of the wild-type size (the length of young adult *ncc-1* and wild-type animals was 0.73±0.04 mm and 0.91±0.08 mm, respectively). In addition, the precursor cells of the ventral nerve cord migrated at the normal time from a more lateral to a ventral position (data not shown).

Requirement for *ncc-1* is not restricted to the first postembryonic or L1 divisions, as was shown in *he25ts* animals. At 15°C, the P cells undergo extensive divisions during the L1 stage in *he25ts* mutants and the P3.p to P8.p (Pn.p) daughters divide in late L3 to form the vulva (Fig. 5C). All P cells failed to divide in *he25* animals shifted to 25°C during the early L1 stage, thereby resembling *ncc-1(0)* animals (Table 3). In contrast, temperature shift during the L2 or L3 stages allowed the P cells to divide during L1 but prevented division of the Pn.p daughters (Fig. 5B).

These results are consistent with a role for *ncc-1* as a general cell-cycle regulator, which is in agreement with the high degree of identity between NCC-1 and the Cdk1 and Cdk2 kinases in other species (Fig. 4).

ncc-1 is required for mitosis, but not for DNA replication

We observed that the precursor cells of the postembryonic lineages did not initiate chromosome condensation or nuclear envelope breakdown in *ncc-1* mutants. Apparently, the cells arrest prior to entry into mitosis, yet this arrest could occur in G_1, S or G_2 phase. We examined whether cells in *ncc-1* mutants initiate S phase to determine the point of arrest.

As a first assay, we used an S phase reporter that expresses the green fluorescent protein (GFP) under the control of a *C. elegans* ribonucleotide reductase (*rnr*) promoter. Expression of *rnr* tightly correlates with S phase in other eukaryotes (Fernandez-Sarabia and Fantes, 1990). In addition, rnr::GFP expression in transgenic worms that contain the integrated *rnr::gfp* reporter coincides with the previously reported time of S phase (Hong et al., 1998). We examined GFP expression in L1 larvae that were double homozygous for the *ncc-1(n3064)* mutation and integrated *rnr::gfp*. Wild-type animals and *ncc-1* mutants were found to express rnr::GFP at the same time and in the same cell types (Fig. 6A). Thus, blast cells of the postembryonic lineages complete G_1 and appear to become competent for S phase in *ncc-1* mutants.

To directly address whether *ncc-1* mutants synthesize DNA,

Table 3. Characterization of *ncc-1* alleles

Parental genotype	% larval arrest of progeny (*n*)	Postembryonic blast cell divisions (no.) P1-P10 (*n*=20)	I (*n*=20)	Z1-Z4 (*n*=20)
Wild type	0.0 (>2000)	200	263	80
he5/+	23.2 (1393)	0	2	0
he24/+	25.0 (1106)	0	9	0
he25/+ at 15°C	26.4 (1346)	ND	ND	ND
he25/+ at 25°C	25.6 (1047)	0	11	0
he26/+	25.4 (1405)	0	7	0
n3064/+	26.9 (1039)	0	2	0

Total progeny of four *ncc-1/+* heterozygotes was counted to determine percentage larval arrest. Divisions of P-cells and I cells were scored in mutants fixed and stained with the DNA stain PI. Divisions of P_{11} and P_{12} could not be unambiguously determined and are therefore excluded. For P-cell divisions, animals were fixed 24 hours at 15°C after release from an L1 arrest, except *ncc-1(he25)* mutants, which were fixed after 9 hours at 25°C. For I-cell divisions, animals were fixed as adults. Z-cell divisions were examined in vivo by Nomarski microscopy, 28 hours after release from L1 arrest at 20°C, except *ncc-1(he25)* mutants, which were scored 24 hours at 25°C after release from the L1 arrest. *n*=number of animals examined.

we examined incorporation of the thymidine analog BrdU. Progeny from animals heterozygous for *he5*, *he26* or *n3064* were incubated with BrdU from 3 to 9 hours of L1 development at 25°C and subsequently stained with anti-BrdU antibodies. In wild-type animals as well as *ncc-1* mutants, BrdU incorporation was detected in all cells that were previously reported to undergo S phase during this developmental phase (Hedgecock and White, 1985). For example, BrdU staining was detected in the ventral cord precursor cells W and P1-P12 (Fig. 6B), and these cells divided in the wild-type but not in *ncc-1* mutants. Other cells that stained strongly with BrdU in wild-type as well as homozygous *ncc-1* animals include the lateral ectoblasts H1,H2, V1-V6 and T and cells in the gonad primordium (data not shown). These results show that neither the timing of S-phase nor the specific cells that enter S-phase are affected by the *ncc-1* mutations.

The complete genome is replicated in *ncc-1* mutants

The results described above indicate that *ncc-1* is strictly required for M phase and not for G_1 progression or DNA replication. However, we wished to examine two alternative explanations. First, it remained possible that cells in *ncc-1* mutants initiate DNA synthesis but fail during the elongation phase. Second, perdurance of maternal product could formally allow initiation of the first division cycle in the postembryonic lineages. To address these issues, we compared the amounts of DNA in intestinal nuclei of adult wild-type animals and *ncc-1* mutants. Wild-type animals hatch with 20 diploid intestinal cells. In general, the 14 most posterior of these undergo one round of DNA synthesis followed by nuclear division in late L1 stage (Hedgecock and White, 1985; Sulston and Horvitz, 1977 and Table 2). In addition, during each larval stage all intestinal nuclei undergo S-phase without mitosis, leading to a 32n DNA content by the adult stage.

We fixed adult wild-type and *ncc-1* animals and stained them with the DNA stain propidium iodide, and determined the DNA content of the intestinal cells by quantitative confocal analysis. Body wall muscles are diploid throughout postembryonic development in wild-type animals. Using body muscle nuclei as an internal 2n DNA standard, we found that the intestinal nuclei in *ncc-1* mutants and wild-type animals were equally polyploid and contained close to 32n amounts of DNA (Figs 6C, 7). The posterior nuclei did not have higher amounts of DNA than the anterior nuclei, suggesting that the lack of mitosis in *ncc-1* mutants prevents the second S phase that normally follows this division during L1 development. Together, these results show that cells in *ncc-1* mutants can replicate their entire genome even late in development. Thus, *ncc-1* appears to be required specifically for M phase.

DISCUSSION

The family of cdc2-related kinases in *C. elegans*

In this study, we have identified members of the family of cdc2-related kinases in *C. elegans*. In vertebrates, the kinases that are most closely related to cdc2, can be divided into 4 different groups (Meyerson et al., 1992), and a member of each of these subfamilies appears to be present in *C. elegans*.

The most extensively studied subfamily consists of Cdk1/Cdc2, Cdk2 and Cdk3. These kinases are closely related in sequence, and each of the human kinases can complement *CDC28* mutations in *S. cerevisiae*. Cdk2 and Cdk1/Cdc2 are activated sequentially in the cell cycle and studies in a number of systems indicate distinct functions. Immunodepletion of the *Xenopus* homologs showed a specific requirement for Cdk2/Eg1 during S-phase and for Cdc2 during M phase (Fang and Newport, 1991). Antibody-injection studies and expression of dominant-negative mutants implicated Cdk2 and Cdk3 in G_1-S control and Cdk1 in G_2-M regulation in mammalian cells (Pagano et al., 1993; Riabowol et al., 1989; Tsai et al., 1993a; van den Heuvel and Harlow, 1993). Finally, a Cdk1 mutation has been identified as the genetic lesion in a

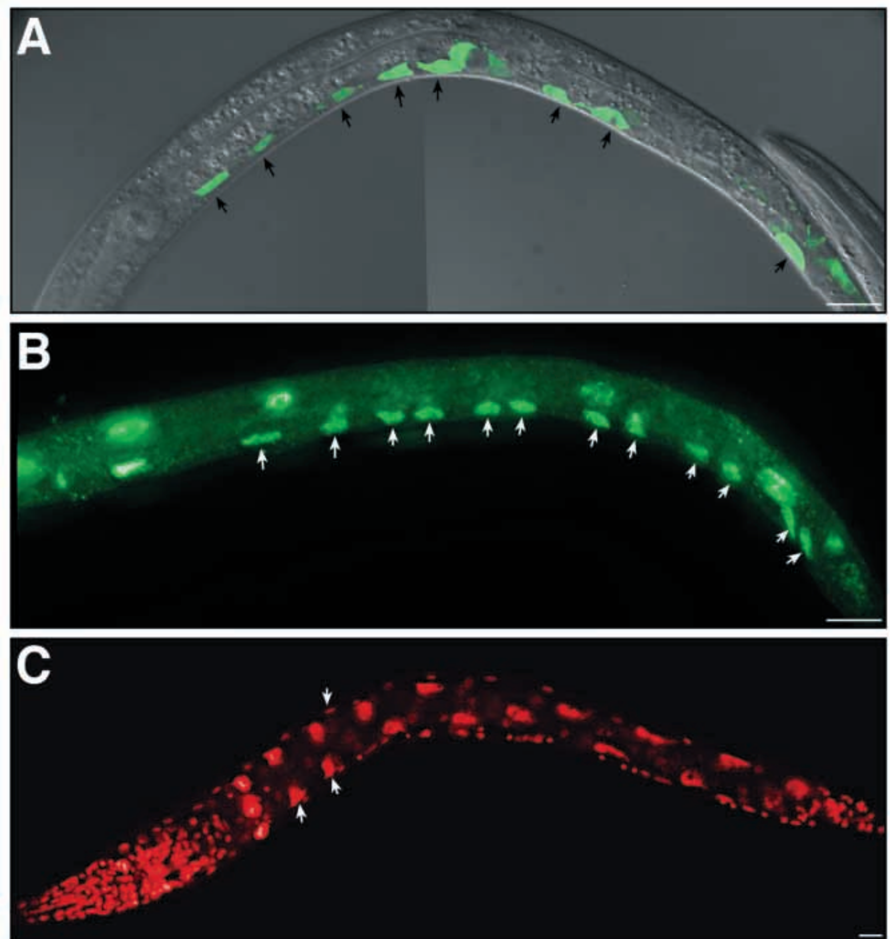

Fig. 6. *ncc-1* mutants complete G_1 and S phase. (A) GFP expressed from the *rnr::GFP* reporter construct in an *ncc-1(n3064)* mutant. Several P-cells expressing *rnr::GFP* are indicated. (B) BrdU incorporation in an *ncc-1(he5)* mutant. Incorporation of BrdU can be detected in all P-cells (arrows). (C) DNA stained with propidium iodide in adult *he24* animal. Arrows indicate body wall muscle cell (2n, top) and polyploid intestinal cells (32n, bottom). Scale bars approx. 10 μm.

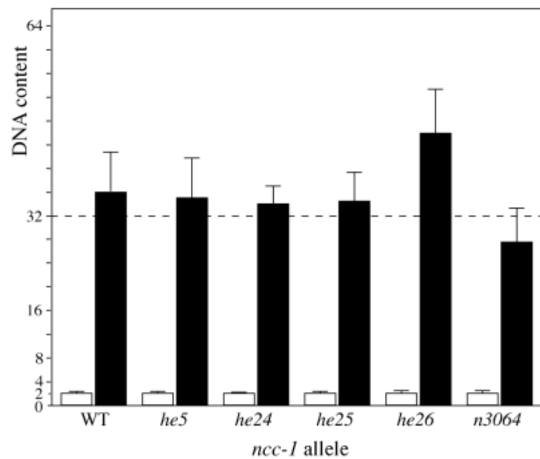

Fig. 7. Intestinal cells in wild-type and *ncc-1* animals are equally polyploid (black bars). Body muscle cells are used as an internal 2n standard (white bars). Each bar indicates the average DNA content ± s.d., of 10 nuclei in 2 independent animals.

mouse cell line with a temperature-sensitive G_2 arrest phenotype (Th'ng et al., 1990). The *C. elegans* kinase *ncc-1* belongs in this subfamily; it shares more than 60% identity with the three human kinases in this group and can complement the *CDC28-1N* mutation in *S. cerevisiae* (Mori et al., 1994). Our studies further define NCC-1 as the ortholog of Cdk1/Cdc2. At present, no homologs of Cdk2 or Cdk3 have been identified, although K03E5.3 and B0285.1 are predicted to encode candidates that are 42% and 41% identical to Cdk2 (see below).

Human Cdk4 and Cdk6 form a subgroup of two kinases that share 71% amino acid identity among each other. These kinases are more distantly related to the Cdk1/2/3 subfamily and they have not been observed to complement *CDC28* mutations in yeast (Meyerson et al., 1992). Based on their association with D-type cyclins and timing of activation, Cdk4 and Cdk6 have been implicated in G_1 control. Moreover, the kinase inhibitor p16 can bind to and inactivate Cdk4 and Cdk6 and overexpression of p16 can cause G_1 arrest in human cells (Sherr, 1996; Sherr and Roberts, 1995). Results from studies in mammals suggest a pathway for G_1 control in which the tumor suppressor p16 acts upstream of the oncogene products Cdk4, Cdk6 and Cyclin D, which negatively regulate the retinoblastoma protein, pRB (Sherr, 1996). At present, neither Cdk4 nor Cdk6 have been shown directly to have essential cell-cycle functions. In fact, deletion of the *Drosophila cdk4/6* gene was found to cause reduced fertility but not a general cell-cycle arrest (C. Lehner, personal communication). The product of H06A10.1 is most related to this subgroup and is predicted to share 37% identity with human Cdk4 (33% with Cdk6). In RNAi experiments with this kinase, we and others have only identified very limited defects in cell division (M. Park and M. Krause, personal communication).

A third subfamily consists of three closely related PCTAIRE kinases in humans. *C. elegans* PCT-1 is ~80% identical to any one of the three human PCTAIRE kinases within a 240 amino-acid fragment of the catalytic core. Cyclin association has not been described for these kinases. In fact, PCTAIRE kinases contain N-terminal and C-terminal extensions that could act as regulators in *cis*. Such N- and C-terminal extensions are also present in the *C. elegans* PCT-1 kinase, and measure 234 and 48 amino acids respectively. PCTAIRE message and protein are abundantly expressed in human and rodent brains (Hirose et al., 1997; Le Bouffant et al., 1998; Meyerson et al., 1992). By developmental northern blotting, we detected *pct-1* message during all developmental stages in the worm (data not shown). The *pct-1* mRNA levels were not found to correlate with mitotic divisions but were strongly elevated in young adults. We did not detect any abnormalities in *pct-1* dsRNA-injected animals or their progeny.

A single kinase, Cdk5, that is highly conserved in metazoans defines a distinct subfamily. Cdk5 is closely related to the Cdk1/2/3 kinases and yet no cell-cycle related functions have been identified. By contrast, Cdk5 kinase activity can be detected specifically in post-mitotic neuronal cells (Tsai et al., 1993b). Cdk5 has been implicated in neurite outgrowth during neuronal differentiation (Nikolic et al., 1996), and mice lacking *Cdk5* or its activator *p35* display severe defects in cortical lamination (Chae et al., 1997; Gilmore et al., 1998). We did not observe obvious defects in neural functions in *cdk-5(RNAi)* animals.

Implications from RNAi studies

For a remarkable number of genes RNA interference has now been shown to cause strong loss-of-function phenotypes (e.g.: Fire et al., 1998; Tabara et al., 1998). However, absence of an RNAi phenotype is little informative, especially because the mechanism of interference is not understood. Two aspects of RNA interference have been particularly useful in this study: RNAi offers the possibility to examine the function of maternally contributed product and RNAi phenotypes can be used to direct the isolation of mutant alleles in genetic screens.

The first aspect relates to a common problem in studying genes that are required in early as well as later development. A single wild-type copy from the mother generally appears sufficient to provide maternal function and, despite early gene requirements, loss-of-function phenotypes may first become detectable during late embryogenesis or early larval development (Ahnn and Fire, 1994; Storfer-Glazer and Wood, 1994). However, RNA interference is particularly efficient in blocking maternal contribution, and RNAi allowed us to examine *ncc-1* gene function during early embryogenesis. Following RNAi, NCC-1 protein levels were reduced below the detectable level even in the germline of injected animals. This way, we were able to detect requirement for *ncc-1* in mitotic and meiotic division in the germline.

Another application of RNAi is first demonstrated in this study: we used RNAi to predict the *ncc-1* loss-of-function phenotype and subsequently identified *ncc-1* mutations in animals with similar phenotypes. In an F_2 screen, homozygous mutants are likely derived from mothers with one functional gene copy and hence display zygotic phenotypes. For three genes that are both maternally and zygotically required, *lin-5*, *lin-6* and *ncc-1*, we have observed that some of the early brood after RNA injection closely mimic zygotic phenotypes. Such RNAi larval phenotypes may provide a powerful reverse-genetics approach for many other genes in *C. elegans*.

ncc-1 acts to promote entry into mitosis

NCC-1 is closely related to yeast CDC28/cdc2 as well as

Cdk1/Cdc2, Cdk2 and Cdk3 kinases in vertebrates. A second kinase with such a high degree of homology was not detected. Does *ncc-1* perform a role similar to *CDC28*/*cdc2*$^{+}$, regulating multiple transitions in the cell cycle, or does it act to regulate progression through a specific phase of the cell cycle? The data described here show that *ncc-1* is required specifically for meiotic and mitotic M phase, indicating that *ncc-1* is the ortholog of Cdk1/Cdc2 in other metazoans.

The strongest support for this conclusion stems from the cell-cycle arrest caused by *ncc-1* mutations. Several lines of evidence indicate that four of the *ncc-1* mutations create strong or complete loss of function. Two of the alleles should produce truncated products that lack most of the conserved kinase domains: *he5* contains a deletion and nonsense mutation and *he26* contains a nonsense mutation at codon 68. In addition, the missense mutation identified in *n3064* causes a Glu-to-Lys substitution of one of nine residues that are nearly invariant in protein kinases. Based on crystal structure studies, this glutamate residue is expected to contact an equally conserved and oppositely charged Arg in the C-terminal end of the catalytic core (De Bondt et al., 1993; Knighton et al., 1991). The missense mutation in *he24* results in a Glu-to-Lys change next to the PSTAIRE motif. The corresponding Glu 57 in human Cdk2 is believed to form a hydrogen bond with Tyr 185 in cyclin A (Jeffrey et al., 1995). Binding of cyclin A to the PSTAIRE domain probably contributes to kinase activation by changing both the orientation of ATP and the position of a kinase domain that blocks the catalytic cleft. The *he25* allele contains a missense mutation that affects a glutamate residue that is conserved in Cdk1/Cdc2 and Cdk2 but not in kinases outside the Cdc2 family. The phenotype caused by the *he25* mutation is partly temperature sensitive, indicating that this mutation does not create a null allele.

The *ncc-1* mutants developed normally through embryogenesis and failed all cell divisions after hatching. The first postembryonic cell cycles in *ncc-1* mutants appear to be normal in timing of G_1 and S phase, based on rnr::GFP expression and BrdU incorporation. However, chromosome condensation and nuclear envelope degradation do not occur. Thus, the cells likely arrest in G_2 and *ncc-1* appears required to promote progression into mitosis. This function is consistent with the role of Cdk1/Cdc2 kinases in other metazoans. One minor difference exists with *Drosophila*, in which inactivation of the mitotic kinase activity has been observed to result in polyploidy (Hayashi, 1996). This difference may reflect the fact that many cells in *Drosophila* undergo endoreduplication after final division.

We expect that *ncc-1* is essential for all embryonic and postembryonic divisions. This is based on the observed *ncc-1* requirement for cell divisions in all postembryonic lineages and the fact that homologous *Cdks* are required for all mitotic divisions in other species. As explained above, maternal *ncc-1* product likely is sufficient for embryogenesis. However, this assumption lacks formal proof, as *ncc-1(RNAi)* embryos arrest prior to the zygotic division.

ncc-1 is required for meiosis I

Following *ncc-1* RNAi, fertilized oocytes fail to complete meiotic maturation. At the time of injection, a large number of germ precursor cells are in pachytene of meiosis I. These nuclei progress through meiotic prophase I but do not initiate metaphase, indicating that *ncc-1* is required to promote the transition from prophase to metaphase in meiosis. A role in meiosis is consistent with the function of Cdk1/Cdc2 in other eukaryotes. In fact, *Xenopus* p34^{cdc2} was discovered by the biochemical characterization of maturation promoting factor (MPF), a cytoplasmic factor that induces meiotic maturation when injected into immature oocytes (Masui and Markert, 1971). Completion of meiosis I and II is a two step process in amphibians. Progesterone, or injection of MPF, triggers oocytes that are arrested in diplotene of prophase I to progress through meiosis I. Mature oocytes will subsequently arrest in metaphase of meiosis II and can be triggered by fertilization to complete meiosis (Masui and Markert, 1971). Meiotic maturation in *C. elegans* is induced by a factor in sperm that is independent from the sperm's function at fertilization (McCarter et al., 1999). In the absence of sperm, *C. elegans* oocytes arrest for prolonged periods in diakinesis (McCarter et al., 1999). Future studies may reveal whether progesterone and the 'sperm factor' activate similar pathways that induce *p34*^{cdc2} and *ncc-1*, respectively, and trigger progression to meiotic metaphase I.

Does progression through meiotic prophase require *ncc-1* function? In the RNA-injected animals, oocyte development appears normal and chromosomes become fully condensed. We cannot exclude that inactivation of *ncc-1* by RNAi was incomplete. However, several observations support effective inactivation. First, upon injection of *ncc-1* dsRNA, NCC-1 protein levels were reduced below the level of detection (Fig. 2G,I). Secondly, *ncc-1* RNAi interfered with cell division in the mitotic part of the germ-line and neutralized phosphorylation of histone H3 in mitotic germ cells as well as oocytes. Thus, a potent effect from RNAi was detected throughout the entire gonad. Finally, injection of dsRNA at a concentration of 0.01 μg/μl was sufficient to completely arrest meiotic maturation, whereas even a 100-fold higher concentration did not affect progression through prophase of meiosis I. If inactivation was complete, entry into meiotic development, meiotic prophase progression, condensation of chromosomes to diakinesis bivalents and oocyte development can all occur independent of *ncc-1*. If these processes do require *ncc-1* activity, this must be less activity than required for germ cell proliferation and for the transition from diakinesis to meiosis I. As yet, formation of diakinesis bivalents of normal morphology in the absence of Cdk1 activity has not been described in any eukaryote. During spermatogenesis in *Drosophila,* DNA condensation can occur in the apparent absence of *Dmcdc2* activity (Sigrist et al., 1995). However, male meiosis in *Drosophila* does not involve meiotic recombination and attachment of bivalents by chiasmata.

Nuclear envelope breakdown occurs slowly in mature *ncc-1(RNAi)* oocytes; by the time it is accomplished meiosis would normally have been completed. The fact that degradation still occurs could indicate incomplete inactivation of *ncc-1*, as Cdk1 is believed to phosphorylate nuclear lamins and to trigger nuclear envelope breakdown in mitosis (Peter et al., 1990). However, other kinases have also been implicated in nuclear lamin phosphorylation, including S6 kinase II (Ward and Kirschner, 1990) and protein kinase C (Hocevar et al., 1993). Such kinases may trigger nuclear enveloppe breakdown in the absence of *ncc-1* activity.

How is progression through G_1/S regulated in *C. elegans*?

Our results provide an interesting paradox: *ncc-1* appears specifically required for the regulation of M phase, yet essential regulators of the G_1-S transition have not been identified. Such regulators are especially important as regulation of cell division by developmental signals in most cells occurs during G_1 phase. Ectopic expression of the Cdk inhibitor *cki-1* causes cell division arrest prior to S phase in *C. elegans* (Hong et al., 1998), which is consistent with the assumption that G_1-S progression is dependent on Cdk activity. In other metazoans, Cdk2 appears to promote G_1/S transition, although genetic null phenotypes have not been described as yet. As the *C. elegans* genome has been completely sequenced, the *C. elegans* kinase that is most similar to Cdk2 is likely encoded by the K03E5.3 gene. RNAi for this gene caused severe but highly variable developmental and cell division defects. More severe defects where seen when the same RNA was injected into heterozygous *ncc-1*/+ animals (data not shown). We are currently exploring whether K03E5.3 and *ncc-1* can act redundantly in G_1/S progression.

We are indebted to Ed Harlow, in whose lab this work was initiated. We express our gratitude to the members of the Harlow-Dyson laboratories for stimulating discussions, and Li Huei Tsai for help with the sequence analysis of cdk-5. Anne Hart, Marc Vidal, Mako Saito and Nick Dyson are acknowledged for critically reading the manuscript, M. Park, M. Krause, Y. Kohara and Andy Golden for kind gifts of plasmids and antibodies, Alan Coulson for cosmids and Michael Hengartner and Bob Horvitz for help and advice. The *Caenorhabditis* Genetics Center, supported by the NIH National Center for Research Resources, provided several strains for this work. S. v.d.H. received grants from the Helen Hay Whitney Foundation and the Jessie B. Cox Charitable Trust and the Medical Foundation. D. G. S. was supported in part by a William A. Starr Fellowship and NIH, National Research Services Award CA09642-09.

REFERENCES

Ahnn, J. and Fire, A. (1994). A screen for genetic loci required for body-wall muscle development during embryogenesis in *Caenorhabditis elegans*. *Genetics* **137**, 483-498.

Ajiro, K., Yasuda, H. and Tsuji, H. (1996). Vanadate triggers the transition from chromosome condensation to decondensation in a mitotic mutant (tsTM13) inactivation of p34cdc2/H1 kinase and dephosphorylation of mitosis-specific histone H3. *Eur. J. Biochem.* **241**, 923-930.

Austin, J. and Kimble, J. (1987). *glp-1* is required in the germ line for regulation of the decision between mitosis and meiosis in *C. elegans*. *Cell* **51**, 589-599.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Chae, T., Kwon, Y. T., Bronson, R., Dikkes, P., Li, E. and Tsai, L. H. (1997). Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. *Neuron* **18**, 29-42.

De Bondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O. and Kim, S. H. (1993). Crystal structure of cyclin-dependent kinase 2. *Nature* **363**, 595-602.

Draetta, G. and Beach, D. (1988). Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement. *Cell* **54**, 17-26.

Edgley, M. L., Baillie, D. L., Riddle, D. L. and Rose, A. M. (1995). Genetic balancers. In *Methods Cell Biol*, vol. 48 (ed. H. F. Epstein and D. C. Shakes), pp. 147-184. San Diego: Academic Press.

Fang, F. and Newport, J. W. (1991). Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes. *Cell* **66**, 731-742.

Fernandez-Sarabia, M. J. and Fantes, P. A. (1990). Ribonucleotide reductase and its regulation during the cell cycle. *Trends Genet.* **6**, 275-276.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811.

Forsburg, S. L. and Nurse, P. (1991). Cell cycle regulation in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *Annu. Rev. Cell Biol.* **7**, 227-256.

Francis, R., Barton, M. K., Kimble, J. and Schedl, T. (1995). *gld-1*, a tumor suppressor gene required for oocyte development in *Caenorhabditis elegans*. *Genetics* **139**, 579-606.

Gilmore, E. C., Ohshima, T., Goffinet, A. M., Kulkarni, A. B. and Herrup, K. (1998). Cyclin-dependent kinase 5-deficient mice demonstrate novel developmental arrest in cerebral cortex. *J. Neurosci.* **18**, 6370-6377.

Girard, F., Strausfeld, U., Fernandez, A. and Lamb, N. J. (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell* **67**, 1169-1179.

Graham, P. L. and Kimble, J. (1993). The *mog-1* gene is required for the switch from spermatogenesis to oogenesis in *Caenorhabditis elegans*. *Genetics* **133**, 919-931.

Guo, S. and Kemphues, K. J. (1996). A non-muscle myosin required for embryonic polarity in *Caenorhabditis elegans*. *Nature* **382**, 455-458.

Harlow, E. and Lane, D. (1988). *Antibodies, A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Hayashi, S. (1996). A Cdc2 dependent checkpoint maintains diploidy in *Drosophila*. *Development* **122**, 1051-1058.

Hedgecock, E. M. and White, J. G. (1985). Polyploid tissues in the nematode *Caenorhabditis elegans*. *Dev. Biol.* **107**, 128-133.

Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P. and Allis, C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348-360.

Hirose, T., Tamaru, T., Okumura, N., Nagai, K. and Okada, M. (1997). PCTAIRE 2, a Cdc2-related serine/threonine kinase, is predominantly expressed in terminally differentiated neurons. *Eur. J. Biochem.* **249**, 481-488.

Hocevar, B. A., Burns, D. J. and Fields, A. P. (1993). Identification of protein kinase C (PKC) phosphorylation sites on human lamin B. Potential role of PKC in nuclear lamina structural dynamics. *J. Biol. Chem.* **268**, 7545-7552.

Hong, Y., Roy, R. and Ambros, V. (1998). Developmental regulation of a cyclin-dependent kinase inhibitor controls postembryonic cell cycle progression in *Caenorhabditis elegans*. *Development* **125**, 3585-3597.

Hunter, C. P. and Wood, W. B. (1990). The *tra-1* gene determines sexual phenotype cell-autonomously in *C. elegans*. *Cell* **63**, 1193-1204.

Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J. and Pavletich, N. P. (1995). Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature* **376**, 313-320.

Kadyk, L. C. and Kimble, J. (1998). Genetic regulation of entry into meiosis in *Caenorhabditis elegans*. *Development* **125**, 1803-1813.

Kimble, J. and Simpson, P. (1997). The LIN-12/Notch signaling pathway and its regulation. *Annu. Rev. Cell Dev. Biol.* **13**, 333-361.

Kimble, J. E. and White, J. G. (1981). On the control of germ cell development in *Caenorhabditis elegans*. *Dev. Biol.* **81**, 208-219.

Kirby, C., Kusch, M. and Kemphues, K. (1990). Mutations in the par genes of Caenorhabditis elegans affect cytoplasmic reorganization during the first cell cycle. *Dev. Biol.* **142**, 203-215.

Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S. and Sowadski, J. M. (1991). Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**, 407-414.

Le Bouffant, F., Capdevielle, J., Guillemot, J. C. and Sladeczek, F. (1998). Characterization of brain PCTAIRE-1 kinase immunoreactivity and its interactions with p11 and 14-3-3 proteins. *Eur. J. Biochem.* **257**, 112-120.

Lehner, C. F. and O'Farrell, P. H. (1990). *Drosophila* cdc2 homologs: a functional homolog is coexpressed with a cognate variant. *EMBO J.* **9**, 3573-3581.

Lieb, J. D., Albrecht, M. R., Chuang, P. T. and Meyer, B. J. (1998). MIX-1: an essential component of the *C. elegans* mitotic machinery executes X chromosome dosage compensation. *Cell* **92**, 265-277.

Massague, J. (1998). TGF-beta signal transduction. *Annu. Rev. Biochem.* **67**, 753-791.

Masui, Y. and Markert, C. L. (1971). Cytoplasmic control of nuclear

behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* **177**, 129-145.

Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J. and Kato, J. Y. (1994). D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.* **14**, 2066-2076.

Matsushime, H., Roussel, M. F., Ashmun, R. A. and Sherr, C. J. (1991). Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* **65**, 701-713.

McCarter, J., Bartlett, B., Dang, T. and Schedl, T. (1999). On the control of oocyte meiotic maturation and ovulation in *Caenorhabditis elegans*. *Dev. Biol.* **205**, 111-128.

Meyerson, M., Enders, G. H., Wu, C. L., Su, L. K., Gorka, C., Nelson, C., Harlow, E. and Tsai, L. H. (1992). A family of human cdc2-related protein kinases. *EMBO J.* **11**, 2909-2917.

Mori, H., Palmer, R. E. and Sternberg, P. W. (1994). The identification of a *Caenorhabditis elegans* homolog of p34cdc2 kinase. *Mol. Gen. Genet.* **245**, 781-786.

Nigg, E. A. (1995). Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *BioEssays* **17**, 471-480.

Nikolic, M., Dudek, H., Kwon, Y. T., Ramos, Y. F. and Tsai, L. H. (1996). The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. *Genes Dev.* **10**, 816-825.

Nonet, M. L., Staunton, J. E., Kilgard, M. P., Fergestad, T., Hartwieg, E., Horvitz, H. R., Jorgensen, E. M. and Meyer, B. J. (1997). *Caenorhabditis elegans rab-3* mutant synapses exhibit impaired function and are partially depleted of vesicles. *J. Neurosci.* **17**, 8061-8073.

Norbury, C. and Nurse, P. (1992). Animal cell cycles and their control. *Annu. Rev. Biochem.* **61**, 441-470.

Ohtsubo, M. and Roberts, J. M. (1993). Cyclin-dependent regulation of G1 in mammalian fibroblasts. *Science* **259**, 1908-1912.

Pagano, M., Pepperkok, R., Lukas, J., Baldin, V., Ansorge, W., Bartek, J. and Draetta, G. (1993). Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts. *J. Cell Biol.* **121**, 101-111.

Peter, M., Nakagawa, J., Doree, M., Labbe, J. C. and Nigg, E. A. (1990). In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell* **61**, 591-602.

Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J. Y., Bar-Sagi, D., Roussel, M. F. and Sherr, C. J. (1993). Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev.* **7**, 1559-1571.

Riabowol, K., Draetta, G., Brizuela, L., Vandre, D. and Beach, D. (1989). The cdc2 kinase is a nuclear protein that is essential for mitosis in mammalian cells. *Cell* **57**, 393-401.

Riddle, D. L. and Albert, P. S. (1997). Genetic and environmental regulation of dauer larva development. In *C. elegans II*, (ed. D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess), pp. 739-768. New York: Cold Spring Harbor Laboratory Press.

Riddle, D. L., Blumenthal, T., Meyer, B. J. and Priess, J. R. (1997). *C. elegans II*. New York: Cold Spring Harbor Laboratory Press.

Rose, K. L., Winfrey, V. P., Hoffman, L. H., Hall, D. H., Furuta, T. and Greenstein, D. (1997). The POU gene ceh-18 promotes gonadal sheath cell differentiation and function required for meiotic maturation and ovulation in Caenorhabditis elegans. *Dev. Biol.* **192**, 59-77.

Sauer, K., Weigmann, K., Sigrist, S. and Lehner, C. F. (1996). Novel members of the cdc2-related kinase family in *Drosophila*: cdk4/6, cdk5, PFTAIRE, and PITSLRE kinase. *Mol. Biol. Cell* **7**, 1759-1769.

Sherr, C. J. (1994). G1 phase progression: cycling on cue. *Cell* **79**, 551-555.

Sherr, C. J. (1996). Cancer cell cycles. *Science* **274**, 1672-1677.

Sherr, C. J. and Roberts, J. M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.* **9**, 1149-1163.

Sigrist, S., Ried, G. and Lehner, C. F. (1995). Dmcdc2 kinase is required for both meiotic divisions during *Drosophila* spermatogenesis and is activated by the Twine/cdc25 phosphatase. *Mech. Dev.* **53**, 247-260.

Slack, F. and Ruvkun, G. (1997). Temporal pattern formation by heterochronic genes. *Annu. Rev. Genet.* **31**, 611-634.

Stern, B., Ried, G., Clegg, N. J., Grigliatti, T. A. and Lehner, C. F. (1993). Genetic analysis of the *Drosophila* cdc2 homolog. *Development* **117**, 219-232.

Storfer-Glazer, F. A. and Wood, W. B. (1994). Effects of chromosomal deficiencies on early cleavage patterning and terminal phenotype in *Caenorhabditis elegans* embryos. *Genetics* **137**, 499-508.

Sulston, J. E. and Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev. Biol.* **56**, 110-156.

Sulston, J. E. and Horvitz, H. R. (1981). Abnormal cell lineages in mutants of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **82**, 41-55.

Sulston, J. E., Schierenberg, E., White, J. G. and Thomson, J. N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100**, 64-119.

Tabara, H., Grishok, A. and Mello, C. C. (1998). RNAi in *C. elegans*: soaking in the genome sequence. *Science* **282**, 430-431.

The *C. elegans* Sequencing Consortium. (1998). Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* **282**, 2012-2018.

Th'ng, J. P., Wright, P. S., Hamaguchi, J., Lee, M. G., Norbury, C. J., Nurse, P. and Bradbury, E. M. (1990). The FT210 cell line is a mouse G2 phase mutant with a temperature-sensitive CDC2 gene product. *Cell* **63**, 313-324.

Tsai, L. H., Lees, E., Faha, B., Harlow, E. and Riabowol, K. (1993a). The cdk2 kinase is required for the G1-to-S transition in mammalian cells. *Oncogene* **8**, 1593-1602.

Tsai, L. H., Takahashi, T., Caviness, V. S., Jr. and Harlow, E. (1993b). Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. *Development* **119**, 1029-1040.

van den Heuvel, S. and Harlow, E. (1993). Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **262**, 2050-2054.

Ward, G. E. and Kirschner, M. W. (1990). Identification of cell cycle-regulated phosphorylation sites on nuclear lamin C. *Cell* **61**, 561-577.

Williams, B. D., Schrank, B., Huynh, C., Shownkeen, R. and Waterston, R. H. (1992). A genetic mapping system in *Caenorhabditis elegans* based on polymorphic sequence-tagged sites. *Genetics* **131**, 609-624.

Wood, W. B. (1988). *The nematode* C. elegans. New York: Cold Spring Harbor Laboratory Press.