A mouse *cdc25* homolog is differentially and developmentally expressed

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The timing and activation of the p34^{cdc2} kinase in mammals is associated with dephosphorylation of phosphotyrosine and phosphothreonine residues on the p34^{cdc2} kinase. For fission yeast, the timing of mitosis is regulated by cyclic accumulation of *cdc25*, which promotes dephosphorylation of p34^{cdc2} and concomitant protein kinase activation. We report the identification and characterization of a structural and functional mouse homolog, Cdc25M2, of the *cdc25* phosphatase. Cdc25M2 shows high sequence identity to the previously reported human homolog *cdc25Hu2*. Cdc25M2 can functionally complement for a *Schizosaccharomyces pombe cdc25^{ts}* mutation, and when expressed in *Escherichia coli* and purified, Cdc25M2 is an active phosphatase. *cdc25M2* mRNA shows variation in expression in different tissues in the mouse embryo and is expressed in a developmental and cell-cycle-dependent fashion. We suggest that the expression and accumulation of the *cdc25* mitotic inducer may play a critical role in the regulation of mouse development.

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Chromosomal replication in S phase and chromosome segregation in M phase are the two major cell cycle events that are conserved among eukaryotic cells. Central to these events is a group of regulatory gene products that control the passage through S phase and mitosis. These regulators act as intrinsic controls during cell cycle progression and respond to extracellular conditions and signals to drive the cell cycle. They include a number of protein kinases and phosphatases that were defined initially by genetic studies in budding and fission yeast (Reed 1980; Nurse and Bissett 1981) and by biochemical studies in *Xenopus* and sea urchin (Masui and Markert 1971; Evans et al. 1983). Of particular interest is a universal control that regulates M-phase onset (for review, see Nurse 1990), and central to this control is the conserved p34^{cdc2} protein kinase. Activation of p34^{cdc2} induces M phase, and high p34^{cdc2} kinase activity maintains the cell in M-phase state. A second component of this cell cycle progression control is a family of proteins called cyclins. Cyclins are regulatory components of the *cdc2* family of protein kinases, and cyclin B is required for p34^{cdc2} kinase activation. There are various types of cyclins (for review, see Pines and Hunter 1990, 1991; Hunter and Pines 1991; Reed 1991) that appear to have roles at different points in the cell cycle.

In fission yeast, the cell cycle timing of M-phase entry is governed by two mitotic inducer genes, *cdc25⁺* and *cdi1⁺/nim1⁺* (Russell and Nurse 1986; Feilott et al.

1991), and two mitotic inhibitor genes, *wee1⁺* and *mik1⁺* (Russell and Nurse 1987; Lundgren et al. 1991). The products of these genes act together to regulate the function of p34^{cdc2} for the initiation of M phase. The *cdi1⁺/nim1⁺*, *wee1⁺*, and *mik1⁺* genes encode protein kinases, whereas the *cdc25⁺* gene product promotes dephosphorylation of p34^{cdc2}. In wild-type *Schizosaccharomyces pombe* cells, the *cdc25⁺* mitotic inducer is required for entry into mitosis, for p34^{cdc2} dephosphorylation and for p34^{cdc2} protein kinase activation. It appears that the level of *cdc25⁺* expression in fission yeast is rate limiting for M-phase entry in *S. pombe*. Incremental increases in *cdc25⁺* gene dosage cause a decrease in cell size at mitosis, and the levels of *cdc25⁺* mRNA and protein increase as cells approach mitosis (Russell and Nurse 1986). For fission yeast, these data suggest that the timing of mitosis is regulated by cyclic accumulation of *cdc25* and that *cdc25* regulates dephosphorylation of p34^{cdc2} and subsequent protein kinase activation.

The mitotic activity of fission yeast *cdc25⁺* is highly conserved among eukaryotes. First, in the budding yeast *Saccharomyces cerevisiae*, a mitotic inducer gene called *MIH1* can complement *S. pombe* temperature-conditional *cdc25⁻* mutants (Russell et al. 1989). Unlike *S. pombe*, the *S. cerevisiae* *MIH1* gene is not essential and can be mutated without causing a significant growth defect. However, when the *S. pombe* *wee1⁺* protein kinase is expressed in an *S. cerevisiae* *mih1* mutant, the trans-

genic yeast strain arrests in G₂-M phase. Second, in *Drosophila*, a mitotic activator called *string* (*stg*) has been described that, like *cdc25*, encodes an essential function (Edgar and O'Farrell 1990).

Human homologs (*CDC25Hs1* and *CDC25Hu2*) of fission yeast *cdc25* have been described previously (Sadhu et al. 1990; Nagata et al. 1991). The *CDC25Hs1* cDNA was identified through a polymerase chain reaction (PCR)-based strategy that amplified a region of the human gene that is conserved with the budding yeast and *Drosophila* *cdc25* proteins, whereas *CDC25Hu2* was isolated by its ability to functionally complement a *S. pombe* *cdc25^{ts}* mutant. Similarly, *CDC25Hs1* also complements a *cdc25^{ts}* mutant. Antisera raised against *CDC25Hs1* and microinjected into HeLa cells result in G₂-phase arrest (Millar et al. 1991). This suggests that *cdc25* may be an important regulator of the onset of mitosis in mammalian cells.

There is increasing evidence from vertebrate systems that, as in fission yeast, the onset of mitosis is controlled by the p34^{cdc2}/cyclin B complex. Once cyclin B associates with *cdc2* in G₂ phase, *cdc2* is phosphorylated on tyrosine-15 (Y-15) and threonine-14 (T-14), which are dephosphorylated as cells enter mitosis. These residues are within the predicted nucleotide-binding region of *cdc2*. Mutation of these sites suggests that phosphorylation of Y-15 and T-14 inhibit p34^{cdc2}/cyclin B kinase activity (Krek and Nigg 1991; Norbury et al. 1991). Purified bacterially expressed *cdc25* has been shown to activate p34^{cdc2}/cyclin B in vitro by dephosphorylating *cdc2* (Gautier et al. 1991; Kumagai and Dunphy 1991; Strausfeld et al. 1991). These observations suggest that the activity of the p34^{cdc2}/cyclin B kinase is negatively regulated by phosphorylation at Y-15 and T-14.

To investigate further the nature of mitotic control in mammalian cells, we report here the identification and isolation of a *cdc25* homolog from mouse. The product of the mouse *cdc25* homolog, which we call *Cdc25M2* because of its similarity to *CDC25Hu2*, rescues a fission yeast *cdc25^{ts}* mutant and can dephosphorylate *p*-nitrophenyl phosphate (*p*-NPP), indicating conservation of structure and function in the *cdc25* family. We also report that this mouse *cdc25* homolog shows variation in expression in different tissues in the mouse embryo and is expressed in a developmental and cell cycle-dependent fashion, indicating that the expression and accumulation of the *cdc25* mitotic inducer may play a critical role in the regulation of development.

Results

Isolation of the mouse *cdc25* cDNA

Protein sequence comparisons of the known *cdc25* homologs revealed a highly conserved carboxy-terminal region of ~150 amino acids. To isolate a mouse *cdc25* cDNA, we used the *Drosophila* *stg* cDNA encoding these amino acids as a probe for low-stringency hybridization. The longest cDNA (clone 6, 2.9 kb, Fig. 1) obtained from a mouse P19 teratocarcinoma cell cDNA

library contained a 1.7-kb open reading frame that potentially encodes a 576-amino-acid protein. Another related clone (clone 4) contained an insert encoding a stop codon upstream of methionine 200 of clone 6. This insert is 76 nucleotides long and occurs after nucleotide 560 in clone 6. PCR was used in an attempt to detect the presence of this insert in P19 mRNA. A single detectable fragment of the size predicted by clone 6 was produced (data not shown). This analysis suggests that clone 6 is the more physiologically relevant form of *cdc25M2* mRNA in P19 cells.

The protein encoded by *cdc25M2* mRNA shares a conserved carboxy-terminal region with the other known *cdc25* proteins. Most of the homology between *Cdc25M2* and all other *cdc25*-like gene products is located in a region in *Cdc25M2* that starts at amino acid position 388 and extends for 155 amino acids to residue 542 (Figs. 1 and 2). Within this region of homology, *Cdc25M2* shows greatest identity to the human *CDC25Hu2* (91%) and least identity to the *S. cerevisiae* *MIH1* protein (30%).

The highest region of homology within the five *cdc25* proteins can be found between residues 433 and 453 and 475 and 495 in the *Cdc25M2* protein. These regions contain the *cdc25* shared motifs of IXDCRYPYEYXG-GHIXXAVNL and KRXILXFHCEFSSERGPXMXR. Because these motifs are so highly conserved between species (81% for *Cdc25M2*), they might represent the active functional region in all of these proteins. For example, the HCEFSSER motif is similar to the invariant HCX-AGXXR motif (Cool et al. 1989; Guan et al. 1990, 1991) surrounding the active site of various protein tyrosine phosphatases (PTPases) and is essential to *cdc25* activity (Dunphy and Kumagai 1991; Gautier et al. 1991).

Unlike any of the other *cdc25* homologs, *Cdc25M2* shows substantial amino acid identity to *CDC25Hu2* throughout the entire protein (Figs. 2 and 3). The overall identity between *Cdc25M2* and *CDC25Hs1* and *Hu2* is 37% and 81%, respectively. Outside the conserved catalytic domain of the *cdc25* family, *Cdc25M2* is most closely related to *CDC25Hu2* (76%) and shows little identity to *CDC25Hs1* (20%) (Fig. 2). It is for this reason that we have named this mouse homolog of *cdc25* *cdc25M2*.

cdc25M2 is a functional homolog of *S. pombe* *cdc25*

To investigate whether the mouse *cdc25M2* gene and *S. pombe* *cdc25⁺* gene have similar roles in mitotic control, we determined whether *cdc25M2* could rescue a fission yeast temperature-sensitive *cdc25⁻* mutation. The *cdc25M2* cDNA was isolated and ligated into a *LEU2*, 2 μ origin-based plasmid containing the SV40 early promoter. The resulting plasmid as well as the parental plasmid were transformed into a *S. pombe* *cdc25-22 leu1-32* strain. Transformants were isolated and examined for their ability to complement for the temperature-sensitive *cdc25-22* lesion. The mouse *cdc25M2* cDNA allowed *cdc25-22* cells to form colonies at 35°C,

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1   TCCCCTACCCCCAGGCTCACTCTCGGAGCTGAGCCAGCTGGGTGCGGCTGTGCTGGCCGCTGTACTGTGGCCCTCTAGCTAGCCTTTGCC
91  CGCCCCGCCACGATGGAGGTACCCCTGCAGAAGTCTGCGCCGGGTTCAGCTCTCAGTCTGCCCGCTGCTGGGTGGCATTACGCGGCCG
    M E V P L Q K S A P G S A L S P A R V L G G I Q R P
181 CGCCACCTCTCGGTCTTTGAGTTTGAAGTCTGATGGCTTCTGGGGTCTCCGAGCCTACAGCTTCTCCTCTCCGGTTACCACTCTTACA
    R H L S V F E F E S D G F L G S P E P T A S S S P V T T L T
271 CAGACCATGCACAACCTCGTGGGCTCGGCAGTGAGCCTCCAAAAGCTCAAGTAGGAAGCCTGTCGTTCCAGAACAGGCTGGCAGACCTA
    Q T M H N L A G L G S E P P K A Q V G S L S F Q N R L A D L
361 TCCCTGTCCAGGCGCACCTCTGAGTGCTCCCTGTCTGAGTCTCAGAATCTTCGGATGCAGGTCTGTGCATGGACTCCCCAGCCCT
    S L S R R T S E C S L S S E S S E S S D A G L C M D S P S P
451 GTGGACCCGAGATGGCAGAGCGCACGTTTGAACAGGCCATTAGGCAGCCAGTCCGGTCAATCAAATGAGCAGTTTACCATAAAACGC
    V D P Q M A E R T F E Q A I Q A A S R V I Q N E Q F T I K R
541 TTCCGATCCTTACCAGTGAGGCTGCTGGAACACAGTCCGGTGTGTCAGAGCATCACCAACTCCCGAGCACTGGACAGTGGAGGAAAAC
    F R S L P V R L L E H S P V L G S I T N S R A L D S W R K T
631 GAAGCAGGCTACCGAGCCGCCCAATAGTCTCTGGGAGGACAAAGAGAATGATGGATATATCTTCAAGATGCCACAGGAGCTCCCTCAT
    E A G Y R A A A N S P G E D K E N D G Y I F K M P Q E L P H
721 TCCAGCTCTGCCAAGCTTTGGCAGAATGGGTCAGCCGACAGAGCCCTTATCCAGAGGCCAGCTCAGCCCTGACTTGATGTGTCTT
    S S S R R T S E C S L S S E S S E S S D A G L C M D S P S P
811 ACCACTGAGTGAAGATGGAAGTAGAGGAGCTGAGCCCGGTGGCACAGTCTTCTTCTTGAAGGCTCTGCGAAAGGCTCTGAAGAAGAT
    T T E W K M E V E E L S P V A Q S S S L T P V E R A S E E D
901 GACGGATTGTGGACATCCTGGAGAGTGATTTAAAGGATGACGAGAAGGTCGCCCGGGCATGGAGAACCTCATTAGTGCCCACTGGTC
    D G F V D L I E S D L K D R D V P A G M E N L I S P L V
991 AAAAAAGCTGGATAAGGAAGAGGAACAGGATCTCATGTTCAGCAAGTGCCAGAGGCTCTTCCGCTCCCCATCCATGCCATGCAGTGTG
    K K L D K E E E Q D L I M F S K C Q R L F R S P S M P C S V
1081 ATCCGACCATCTCAAGAGGCTAGAGCGGCCCCAGGACCGGATGTGCCTGTCCAGAGCAAGCGCAGGAAAAGTGTGACACCCCTGGAA
    I R P I L V K R L E R P V D R D V P A G M E N L I S P L V
1171 GAGCAGCAGCTGAAGAACCTAAGGCCGTGTCTTTCGCTCAAAGTCTGTGTCTGATGAGATTGAGAATCCTGGATAGTGACCCCGT
    E Q Q L E E P K A R V F R S K S L C H E I E N I L D S D H R
1261 GGACTGATCGGAGATTACTTAAGGCTTCTCTGTCAGACCGTGGATGGCAACACCAAGACCTTAAGTACATCTCACCAGAACTATG
    G L I G D V S K A F L L Q D T V D G K H Q D L K Y I S P E T M
1351 GTGGCCCTGTTAAGCAGCAAGTTCAGCAACATCGTGGAGAAATTTGTCATTGTGGACTGCAGATACCCCTATGAGTATGAAGCGGGCAT
    V A L L T G K F S N I V E K F V I V D C R Y P Y E Y E G G H
1441 ATCAAGAATGCTGTGAACCTGCCCTTGAACGGGATGCTGAGACTTTCTGTCTGACGCTCCCATCATGCCTTGTAGCCTGGACAAGAGA
    I K N A V L P L E R D A E T F L L Q R P I M P C S L D K R
1531 ATCATCTCTATTTTCACTGTGAATTCGTCTGAGCGTGGACCAAGATGTGCCGCTTCATCAGGGAACGGGACCGTGCAGCTAACGAC
    I I L I F H C E F S S E R G P R M C R F I R E R D R A A N D
1621 TACCCAGCCTGTACTACCCGAGATGTACATCTCAAAGCGGGCTACAAGGAGTCTTCCACAGCATCCGAACCTTTGTGAGCCCCAG
    Y P C S I Y P F M Y L K G G Y K E F F P Q H P N F T C E P Q
1711 GACTACCGACCATGAACACGAGGCTTTCAAGGATGAGCTGAGGAACCTTTCGCTTAAGACTGCAGCTGGGCTGGGGAACGGAGCAGG
    D Y R P M N H E A F R D E L R N F R L K T R S W A G E R S R
1801 AGGGAACCTTTGTAGCAGGCTGCAAGACCACTGATGATGAGCTGCTGCCATCCTTACCTCGTGAGGCTTGGGAGCCAGTGGGTCCCATGG
    R E L C S R L Q D Q *
1891 GCCTGTGAGGCCACCTACCTTATAGAGGCTCAGGTGCTATAGGGGGTGGGGGCATGGTGTGGTGTACATCTGTCTGCCCTGTCTC
1981 AATTTTCTGTCTCACTCCACTTATTTTCTGTATCTTGGTACTGGTCCCAGCTTAAAGAGCTGAACCTGAGGGTGGATGCCAGCTGAGGA
2071 GAAGTCTGTGTGTTCAAGTGGGAGTCTTTTACAGTCTTTTCTTGTGTTGACTCTTTGTCTTCCCGCTTTTGGGAGAGCCT
2161 CAGCCCCGTTAGGATGGCAGAGTAGCTTCTTGCATGGCCTGGATGCAGGATGCTACTCTGCTACACAGGGCTGTGTCTACTCTCTCTCC
2251 TGGCCACTGGCATAGACTTATGCTCTCCATTGGAAGCATAAGTGGCTTTCTTCTGTGTAGTCTTACCTGTAGCTTTCATTTTGT
2341 CTGTCTGACACACTGACCGCTGGGGAGGCTTCTTGTACAGCTTGGGGCTAGAGATTCAAATATCACTTACTCGTTAGGCTTGGCTTTTG
2431 TTAGCCCAAGAAAGGATCTTGCCTTTAAGACCCCAAGGCTAGAGGGAAGGCTGACTCAGGAGCCTCTGGGAGCCCATCTCTACTAC
2521 TGTGAACCCAGAGCCTCGTGGTGCAGCACTTGTGCTGGGCAATGGACAGTACCGAGCTTTGCTGTTGATCCTTCTCTGCCCTTCC
2611 CTGTTTCTCTTCTATATCTGCCCAGAAGACCTCTTGTATGTGTGTTTCTGTGTGTACTAGTACTGAGTCTAGGCCCTTGTGTT
2701 GCATGGTCATGGATGCACAGTGCCTTATATACATGTATGCACACAACCGGGTCCAAGTATTTGGTAAGCATGATGGCCTATGGCAGGA
2791 GTGTGTGTGCGCGTGTGAACAAAGTACATCTACTGTTTGGAAATGTTAAAGAAGCATTGTTATTATGGGAGGGGGGAGCAACC
2881 TCTGGGTTCAGAACTACATATGCTGGAAGGCCCAATGAGTCTCTGTTGGGGG 2935

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Figure 1. Sequence of the mouse *cdc25M2* cDNA. The mouse *cdc25M2* cDNA was identified in a λ ZAPII cDNA library by low-stringency hybridization with a *Drosophila stg* probe. The cDNA was excised by superinfection with R408 helper phage and double-stranded DNA prepared for sequencing. A 2.9-kb cDNA was sequenced, as discussed in Materials and methods, and the predicted open reading frame is shown. The upstream stop codon is indicated in boldface type, as is methionine 200, the potential alternative start site for translation (see text).

whereas the control transformants were unable to support robust growth on solid medium (Fig. 4a). Similarly, the *cdc25M2* cDNA was capable of rescuing the *cdc25-22* G₂ arrest when cells were shifted from 25°C to 35°C, whereas the control plasmid did not (Fig. 4b). We found that the degree of rescue was variable among individual cells, but most cells divided at 1–1.5 times wild-type size. These data establish that Cdc25M2 is able to function as a mitotic inducer in fission yeast and suggest that

Cdc25M2 is likely to function in mitotic control in mouse cells.

Cdc25M2 has phosphatase activity that can dephosphorylate p-NPP

Because of the structural similarity between Cdc25M2 and PTPases, we determined whether Cdc25M2 had associated phosphatase activity. We used the phosphatase

Figure 2. Comparison of the structure and similarities between *cdc25* proteins from various species. (a) Conceptual alignment of *cdc25* proteins from various species. Open boxes indicate regions with little amino acid similarity, hatched boxes indicate homology outside of the conserved carboxyl terminus, and shaded boxes indicate the ~155-amino-acid region of high identity between all *cdc25* proteins. (b) A comparison of the level of amino acid identity among various *cdc25* proteins through the shared region of homology (shaded box in a). The species shown are mouse (M2), human (Hs1 and Hu2), *Drosophila* (D), *S. pombe* (P), and *S. cerevisiae* (C).

assay described by Guan et al. (1990, 1991), which measures the colorimetric conversion of *p*-NPP to *p*-nitrophenol (*p*-NP). This assay has been used previously to measure the phosphatase activity associated with PTPases like *PTP1* from *S. cerevisiae*, rat brain PTPase, and vaccinia virus PTPase (Guan and Dixon 1991). Residues 278–576 of Cdc25M2 were expressed in *E. coli* as a glutathione *S*-transferase (GST) fusion protein, purified from *E. coli* extracts with glutathione-coupled Sepharose (see Materials and methods), and used to measure *p*-NPP conversion to *p*-NP. Purified, bacterially expressed GST–Cdc25M2 dephosphorylated *p*-NPP in a concentration-dependent manner (Fig. 5). Identically expressed and purified GST did not exhibit any phosphatase activity under the same reaction conditions. This confirms the structural observation that Cdc25M2 is related to protein tyrosine phosphatases and suggests that Cdc25M2 is similar functionally to other members of the *cdc25* family.

cdc25M2 expression is regulated temporally and developmentally

To examine further the possibility that Cdc25M2 functions as a mitotic inducer in mouse cells, we determined whether *cdc25M2* mRNA is expressed periodically in the mouse cell cycle by examining *cdc25M2* mRNA lev-

els after serum addition to quiescent cells. Swiss 3T3 cells were serum starved for 72 hr to enrich for G₀ cells and were then released into fresh medium containing 10% fetal bovine serum (FBS) (Stein and Stein 1989). Cell cycle progression was followed for 42 hr by measuring the incorporation of radioactive DNA precursors (see Materials and methods). We observed a synchronous round of DNA synthesis as seen by a peak of [³H]thymidine incorporation (Fig. 6a). RNA was prepared from these cells, and *cdc25M2* levels were measured by Northern blot analysis (Fig. 6b). We observed that *cdc25M2* mRNA levels were low in G₀ phase of the cell cycle, remained low through G₁ (which occurs 9–15 hr after addition of serum in these experiments), increased during S phase (which occurs 15–30 hr after serum addition), and reached a plateau ~21–24 hr after the start of this experiment. The peak *cdc25M2* mRNA levels reached maximum midway through S phase and remained elevated through the completion of S phase and into G₂–M. Densitometric scanning indicated that the level of *cdc25M2* increased about three- to fivefold from G₁ to G₂ levels. Similar to *CDC25Hs1* in HeLa cells, these data establish that the level of mouse *cdc25M2* mRNA increases as cells progress through the cell cycle from G₁ and approach mitosis. We also found that *cdc25M2* mRNA levels were readily detectable in logarithmic cells (Fig. 6b), and we note that *cdc25M2* mRNA levels do not drop dramatically as cells progress through the cell cycle.

To characterize further the expression of *cdc25M2* mRNA, we examined its levels in various tissues from an adult rat (Fig. 7a) and at different stages during mouse development by Northern analysis (Fig. 7b). We found that *cdc25M2* mRNA was detectable in most adult tissues. The level of *cdc25M2* mRNA was lowest in adrenal, kidney, liver, and muscle, was moderately expressed in brain, heart, and intestine and was expressed highest in lung and spleen. We examined the amount of *cdc25M2* expression during embryonic development by Northern blotting of mRNA samples from day-10.5 to day-18.5 embryos. We found that total *cdc25M2* mRNA levels fluctuate during embryonic development and that *cdc25M2* mRNA peaks between day 12.5 and 13.5 and remains elevated until day 15.5. After this period, *cdc25M2* mRNA levels gradually diminish. We are unable to determine by this analysis whether variation in *cdc25M2* mRNA level is the result of altered transcription rates, mRNA stability, or merely variation among tissue types.

Because of the variation in mRNA levels in adult tissues and during embryonic development, we determined the spatial distribution of *cdc25M2* mRNA in embryonic tissues by in situ hybridization. We observed a high and widespread distribution of *cdc25M2* transcripts in differentiating tissues containing dividing cells (days 10.5–16.5). In particular, the liver shows high expression in a day-13.5 embryo (Fig. 8a,b), but at day 16.5, the signal has decreased to near-background level (Fig. 8c). We also found high levels of *cdc25M2* throughout the central nervous system in the early embryo. As cells differenti-

Figure 3. Sequence alignment of the amino-terminal regions of mammalian *cdc25* proteins. An alignment of the amino acid residues 1–387 of Cdc25M2 with CDC25Hs1 and CDC25Hu2. Amino acid identity is indicated by boxed letters. The alignment was performed with the UWGCG program (Devereaux et al. 1984).

ate, this expression becomes restricted to regions containing rapidly proliferating cells. For example, as cells in the telencephalon (endbrain) become postmitotic, they migrate toward the periphery and eventually differentiate into the basal ganglia and cerebral cortex. We observed that *cdc25M2* expression is concentrated to the ventricular surface of the telencephalon and that there is only weak labeling in the periphery (Fig. 9). There also appears to be a correlation between the order in which the brain vesicles divide and *cdc25M2* expression. Cells in the mesencephalon (midbrain) divide after those in the metencephalon (afterbrain) (Kandel and Schwartz 1985). The metencephalon shows high expression of *cdc25M2* mRNA in a day-10.5 embryo (not shown) but is reduced to near-background level in a day-13.5 embryo, whereas at this point the mesencephalon (midbrain) shows a strong, highly localized signal (Fig. 8).

Extracellular signals, including growth factors and morphogens, are widely used for controlling mammalian cell proliferation and development. Although the specific intracellular targets for these signals are still obscure, they must change the expression patterns of genes involved in growth control and differentiation. To understand the alteration in expression of *cdc25M2* during mouse development, we determined whether *cdc25M2* mRNA levels are responsive to a growth factor (serum) that induced immediately early gene expression (Treis-

man 1985) and to a morphogen (retinoic acid) that induces proliferation during development (Roberts and Sporn 1984). We starved Swiss 3T3 cells of serum for 72 hr, added 10% serum, and examined *cdc25M2* levels immediately after the induction. In contrast to the well-characterized immediate-early response to serum seen with genes like *fos* (Kruijer et al. 1984), we found that *cdc25M2* transcription was not induced rapidly by serum (Fig. 6).

To determine whether *cdc25M2* levels are responsive to retinoic acid, we examined the induction of *cdc25M2* after an 8-hr treatment of embryonal carcinoma P19 cells with varying concentrations of retinoic acid. As shown in Figure 10, we found that *cdc25M2* mRNA was induced 5- to 10-fold by retinoic acid treatment. The time course of retinoic acid induction of *cdc25M2* mRNA is similar to the retinoic acid induction seen for retinoic acid receptor β mRNA (*RAR β*) (Song and Siu 1989). To test whether retinoic acid induction was a primary event, we determined whether induction occurred in the presence of cycloheximide. However, similar to many highly unstable mRNAs (Graves et al. 1987; Müllner and Kühn 1988; Shyu et al. 1989), we found that the addition of cycloheximide resulted in higher steady-state *cdc25M2* mRNA levels after induction with retinoic acid. This precludes determination of whether retinoic acid induction is a primary event.

Figure 4. The mouse *cdc25M2* gene is a functional homolog of *S. pombe cdc25*. (a) *S. pombe* strains were transformed to *Leu*⁺ and grown at room temperature. Both wild-type *cdc25*⁺ and *cdc25*^{ts} strains were transformed. Transformants were single-colony-purified on EMM medium, streaked for single colonies on YES medium, and incubated at the *cdc25*^{ts} nonpermissive temperature of 35°C. Sector 1 shows a vector-control *Leu*⁺ *cdc25*⁺ transformant; sector 2 contains a *cdc25*^{ts} strain transformed to *Leu*⁺ with a *cdc25M2*-containing plasmid; sector 3 shows a *Leu*⁺ vector-alone *cdc25*^{ts} strain. (b) Various *S. pombe* strains were grown overnight in EMM liquid medium to mid-log phase. Cultures were transferred by dilution into YES media, grown for several hours at permissive temperature, and shifted to nonpermissive temperature (35°C). Growth was monitored as described (Russell and Nurse 1987). Arrow indicates the time point when cultures were shifted to nonpermissive temperature (35°C). (×) *cdc25*-22 + *cdc25M2*; (■) *cdc25*-22 + vector; (□) *cdc25*⁺; (◆) *cdc25*-22.

Discussion

To understand how extracellular signal molecules such as growth factors and morphogens act in mammalian cell proliferation and development, we have isolated a mouse homolog of the *Drosophila stg* gene. The *D. melanogaster stg* gene product is a regulator of embryonic cell cycles (Edgar and O'Farrell 1990). The *stg* gene product is functionally identical to the fission yeast rate-limiting mitotic inducer *cdc25* and is one possible candidate for a developmental target for growth control and differentiation.

cdc25M2 is a functional homolog of the *S. pombe*

cdc25 gene. The mouse gene is fully capable of restoring the temperature-conditional growth defect associated with the *cdc25*-22 temperature-sensitive mutation and allows *cdc25M2* transgenic fission yeast to grow at nearly wild-type rates in broth medium, to form normal colonies on agar medium at the nonpermissive temperature, and to divide at nearly normal cell size (Fig. 4). Complementation occurs despite the fact that *cdc25M2* expression is not cell cycle regulated in *S. pombe*, as the protein is overexpressed from the SV40 promoter.

Cdc25M2 shows striking amino acid identity throughout its amino-terminal noncatalytic region to *CDC25Hu2*, whereas neither of these predicted gene

Figure 5. Mouse Cdc25M2 can dephosphorylate *p*-NPP. The indicated amounts of purified recombinant GST-Cdc25M2 protein (□) or GST protein (◆) were incubated with *p*-NPP as described in Materials and methods. The reactions were incubated for 45 min at 30°C, terminated with the addition of 0.2 M NaOH, and *p*-NP production was measured by absorbance at 410 nm. Under these conditions, the dephosphorylation of *p*-NPP is linear with respect to protein concentration and with time (not shown).

products shows any significant identity to the corresponding region of CDC25Hs1 (Fig. 2). This suggests that at least two distinct types of *cdc25* homologs exist in mammals. A second and structurally distinct *cdc25* homolog in P19 cells has been identified, suggesting that multiple *cdc25* homologs exist within a single cell (B.

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The importance of *cdc25* homologs in regulating the onset of mitosis has been established in a number of systems. The *cdc25* homologs identified in *S. pombe* and *D. melanogaster* are essential (Russell and Nurse 1987;

Figure 6. Expression of *cdc25M2* is regulated during the mouse cell cycle. (a) The incorporation of [³H]thymidine into DNA was monitored at various times after addition of 10% serum; the average of two measurements is shown. The y-axis corresponds to cpm measured; the x-axis corresponds to hours after serum addition. Periods in the cell cycle are shown below the diagram. (b) Total RNA was isolated at various times after addition of serum to quiescent Swiss 3T3 cells. Five micrograms of total RNA for each time point was analyzed by Northern blotting. Numbers indicate hours after serum addition. Log indicates cells in log phase grown continuously in the presence of serum. The filter was probed with *cdc25M2* cDNA. (c) Ethidium bromide staining of 18S and 28S rRNA for the RNAs analyzed above. Samples from the 0- and 15-min time points showed weak rRNA staining.

Developmental expression of a mouse *cdc25* homolog

Figure 7. CDC25M2 expression varies during embryonic development and between tissues. (a) Five micrograms of poly(A)⁺ RNA from various adult rat tissues was analyzed by Northern blotting. The probe was CDC25M2 cDNA. (b) Ten micrograms of poly(A)⁺ RNA from various stage mouse embryos was examined by Northern blotting.

Edgar and O'Farrell 1990). *S. pombe cdc25⁺* is required for viability and cell cycle progression, and *cdc25* mutants can only be maintained if they contain temperature-conditional mutations or if they contain additional mutations in the *wee1* protein kinase. In *Drosophila*, *stg* mutants are unable to complete early embryonic development, and cell cycle progression is prevented after cellularization. The observation that antibodies raised to CDC25Hs1 microinjected into HeLa cells cause G₂ arrest suggests that *cdc25* homologs might also play an important role in the regulation of mitosis in mammalian cells (Millar et al. 1991).

The products of the fission yeast *cdc25⁺* gene and its homologs *MIH1* in budding yeast and the *stg* gene product in *Drosophila* are positive regulators of the G₂ M-phase transition. In fission yeast, the mutation *cdc2-F15*, whose product cannot be phosphorylated on amino acid residue tyrosine 15, bypasses the requirement for *cdc25* function (Gould and Nurse 1989), and a human PTPase can rescue a *cdc25^{ts}* mutation (Gould et al. 1990). These experiments support the view that *cdc25* protein is required for promoting the dephosphorylation of Y-15 in *S. pombe* p34^{cdc2}.

Biochemical and structural evidence support this conclusion. A number of reports have shown that *cdc25* is a protein phosphatase that can dephosphorylate Y-15 of *cdc2* in vitro. Addition of bacterially produced *stg* protein into *Xenopus* extracts efficiently induces the dephosphorylation of the *Xenopus cdc2* protein and appears to activate the p34^{cdc2}/cyclin B complex (Gautier et al. 1991; Kumagai and Dunphy 1991). Similarly, purified bacterially expressed CDC25Hs1 protein can de-

phosphorylate and activate starfish p34^{cdc2}/cyclin B complex in vitro (Strausfeld et al. 1991). Structural comparisons of *cdc25⁺* from *S. pombe* to known PTPases identified an "HCXXXXXR" motif that is found in the vaccinia virus VH1 phosphatase and is conserved among all *cdc25* homologs (Guan et al. 1991; Moreno and Nurse 1991). The cysteine in this motif is essential to VH1 phosphatase activity (Guan et al. 1991). When this cysteine is mutated, bacterially expressed *cdc25* loses its phosphatase activity and its ability to activate p34^{cdc2}/cyclin B (Dunphy and Kumagai 1991; Gautier et al. 1991).

We believe that Cdc25M2 also acts as a protein phosphatase. A purified bacterially expressed GST-Cdc25M2 fusion protein dephosphorylates *p*-NPP (Fig. 5). Given the evidence discussed above, a possible in vivo substrate for Cdc25M2 is p34^{cdc2}/cyclin B. Vertebrate p34^{cdc2}/cyclin B activity is inhibited by phosphorylation on T-14 as well as Y-15 (Krek and Nigg 1991; Norbury et al. 1991). This raises the possibility that Cdc25M2 is perhaps a "dual-specificity" phosphatase that dephosphorylates both Y-15 and T-14; alternatively, two different phosphatases may be required—one for each residue.

Whereas expression of *cdc25* is rate limiting for mitosis in *S. pombe* and varies throughout the cell cycle (Russell and Nurse 1987; Moreno et al. 1990), a similar relationship has not been established for mammalian cells. CDC25Hs1 protein levels appear to be relatively constant throughout the cell cycle (Millar et al. 1991). Despite the variation in CDC25Hs1 mRNA (Sadhu et al. 1990), CDC25Hu2 mRNA varies only slightly (Nagata et al. 1991). Upon stimulating Swiss 3T3 cells to

Figure 8. *cdc25M2* mRNA is localized to specific regions of the mouse embryo. (a) Saggital section of an embryonic day-13.5 (E13.5) mouse embryo. (Tl) Telencephalon; (Pz) proliferative zone; (Ms) mesencephalon; (Mt) metencephalon; (Sc) spinal cord; (Li) liver (V) vibrissae. (b) A dark-field autoradiograph showing in situ hybridization of *cdc25M2* antisense mRNA to the bright-field section shown in a. (c) A dark-field autoradiograph showing in situ hybridization of *cdc25M2* antisense mRNA to a saggital section of an E16.5 mouse embryo. (Ob) Olfactory bulb; (Pi) pituitary; (Sc) spinal cord; (Th) thymus; (Li) liver; (Na) nasal cavity.

enter the cell cycle from G_0 , we found that *cdc25M2* mRNA levels are first detected during late G_1 to early S phase and are elevated three- to fivefold above early G_1 levels, but not fall later in the cycle.

In *Drosophila*, increasing levels of regulation act upon mitosis as an embryo develops. The first 13 rapid cycles in the fly syncytial embryo rely on maternal gene products (for review, see Glover 1991). These 13 divisions

Figure 9. *cdc25M2* expression in the central nervous system of a mouse embryo. (a) A coronal section of the head of an E12.5 mouse embryo. (Ne) Neuroepithelium; (TI) telencephalon; (R) retina; (Na) nasal cavity; (Fm) facial mesenchyme; (E) eye. (b) Dark-field autoradiograph showing expression of *cdc25M2* in the embryonic section in a.

lack the *cdc25*-dependent feedback regulation that monitors completion of S phase. Such regulation is introduced in cycle 14 with the beginning of an apparent G_2 phase and a coordinated network of mitotic control that is under the overall control of *stg*. After the onset of cellularization in the fourteenth division cycle, fly somatic cells undergo mitotic divisions that are coordinated with the process of gastrulation and morphogenesis. In wild-type embryos, short-lived zygotic *stg* transcripts precede and induce the spatiotemporal onset of mitosis.

We anticipated that the universal mitotic control defined by developmental studies in *D. melanogaster* may hold true in mouse development. To test the notion that high levels of *cdc25M2* might correlate with proliferating cells, we analyzed the levels of *cdc25M2* mRNA in the developing mouse. The *cdc25M2* gene shows a temporal and spatial pattern of expression during embryonic development. *cdc25M2* mRNA is most abundant during days 13.5–16.5 in development; after day 15.5, this level declines rapidly. In situ hybridizations of *cdc25M2* antisense RNA show that the *cdc25M2* gene is widely expressed in differentiating tissues containing dividing cells. In particular, there appears to be a correlation between *cdc25M2* expression and the presumed region and timing of mitosis in cells in the liver and the central nervous system. *cdc25M2* expression in the liver is found in day-13.5 embryos, during which rapid cell division occurs, and has decreased in the liver in a day-16.5 embryo, at which point proliferation in liver cells has presumably dropped sharply (Fig. 8). A high level of *cdc25M2* transcript was detected in regions in the nervous system containing rapidly dividing cells such as the

neuroepithelium (Fig. 9) and proliferative zone (Fig. 8b), whereas strong signals were not observed in the periphery of the neural tube, which contains postmitotic, differentiated cells (Fujita 1964). Finally, the timing of *cdc25M2* expression in the brain vesicles such as the telencephalon, mesencephalon, and metencephalon is consistent with the order in which the cells in these regions divide. The differential localization of *cdc25M2* in the mouse embryo suggests that elucidation of the developmental role of each type of *cdc25* protein within an organism might be informative.

Mammalian cell proliferation and development are responsive to diverse extracellular signals. We found that *cdc25M2* mRNA levels were clearly inducible by retinoic acid treatment in P19 cells and that *cdc25M2* mRNA could be stabilized by cycloheximide treatment. This suggests that *cdc25M2* mRNA is unstable and might be prone to regulation by cotranslational processing in a fashion similar to the regulation of early response genes like *fos* and *myc* or by cell cycle-controlled instability as seen for histone mRNA or for tubulin. In addition, retinoic acid induction of *cdc25M2* expression suggests that *cdc25M2* gene is one potential intracellular target for growth control and differentiation. The precise mechanism of *cdc25M2* induction will require the isolation of its promoter element. Furthermore, it is unclear whether the degradation of *cdc25M2* mRNA is translation dependent like tubulin mRNA (Pachter et al. 1987; Gay et al. 1989) or cell cycle controlled like histone mRNA (Gallwitz 1975; DeLisle et al. 1983; Heintz et al. 1983). However, these results indicate that a universal mitotic control element can act either as a direct or indirect target for extracellular developmental signals.

Figure 10. CDC25M2 expression is induced by retinoic acid in mouse P19 embryonic teratocarcinoma cells. (a) RNA samples were prepared from P19 cells treated with 10 μ g/ml of cycloheximide without retinoic acid (lanes 1,2) and with 10⁻⁹ M (lanes 3,4) or 5 $\times$ 10⁻⁷ M (lanes 5,6) of retinoic acid for 8 hr. Paired samples of 15 μ g of total RNA (lanes 1,3,5) and 5 μ g of poly(A)⁺ RNA (lanes 2,4,6) from each treatment were analyzed by Northern blotting using CDC25M2 cDNA as a probe. (b) The filter above was rehybridized with a cDNA whose expression level was not affected by the presence of retinoic acid. RNA samples and retinoic acid treatment correspond to the lanes described in a.

Materials and methods

Isolation of cdc25M2 cDNA

Poly(A)⁺ RNA from mouse P19 teratocarcinoma cells was used as a source material for cDNA library construction. Isolation of total RNA and purification of poly(A)⁺ RNA were carried out as described [Chomczynski and Sacchi 1987; Kakizuka et al. 1988]. First-strand synthesis was primed with oligo(dT), and the double-stranded cDNA was prepared as described previously [Kakizuka et al. 1991]. The library was constructed in λ ZAPII (Stratagene). A portion of the *Drosophila stg* gene encoding the conserved catalytic domain of the cdc25 homologs was isolated as a PCR fragment and used as a probe in low-stringency hybridization [Edgar and O'Farrell 1990] to screen for mouse homologs. The hybridization of the membrane filters (Colony/Plaque Screen, Dupont) was carried out in 1 M NaCl, 50 mM Tris-HCl (pH 7.5), 1% SDS, 10% dextran sulfate, 100 μ g/ml of denatured salmon sperm DNA, 200 μ g/ml of yeast RNA, and 5 ng/ml of ³²P-labeled probe ($\sim 5 \times 10^8$ cpm/ μ g) at 60°C. The filters were washed in 2 $\times$ SSC and 0.1% SDS at 55°C. From 25,000 recombinant phages, 10 positives were identified and one of them showed amino acid similarity to that of *stg*. Recombinant phages (300,000) were rescreened using the cDNA as a probe, and 20 positives were identified. The cDNA inserts in these

phages were excised and restriction mapped. Clone 4 was sequenced entirely. Restriction analysis showed that clones 2 and 6 contained additional 5' sequence, and the 5' end of clone 6 was sequenced up to this point on both strands. Three hundred nucleotides of the 3' sequence of clone 2 were also sequenced to verify that it corresponded exactly to that of clone 4. Standard methods were used for phage and *E. coli* propagation, and methods for DNA and RNA manipulations were as described [Sambrook et al. 1989].

PCR analysis of cdc25M2 mRNA

PCR primers were designed to amplify the portion of CDC25M2 RNA corresponding to the region containing an insert in clone 4. Primer A included an *Sau*3AI site and sequence upstream of nucleotide 560 (5'-GATCTGAACAGGCCATTTCAGGCAG-3'), and primer B contained sequence on the opposite strand downstream of nucleotide 560 (5'-GATCCTCGGTAGCCTGCTTCAGTT-3'). Reverse transcription of mouse P19 mRNA and subsequent PCR was performed as described with minor modifications [Sambrook et al. 1989; Middlemas et al. 1991].

Expression of cdc25M2 in *S. pombe*

To express *cdc25M2* in *S. pombe*, we fused the *cdc25M2* cDNA to the SV40 early promoter [Lee and Nurse 1987]. The *cdc25M2* cDNA was cloned as a *Sall*-*Bam*HI fragment into a *LEU2*, 2 μ origin-based plasmid. The SV40 early promoter from plasmid pCH110 (Pharmacia-LKB) was cloned adjacent to the 5' end of *cdc25M2*. This plasmid and a control vector lacking the *cdc25M2* cDNA were transformed into a *leu1 cdc25-22* strain (kindly provided by P. Nurse and K. Gould), and *Leu*⁺ transformants were selected at 24°C on EMM media [Moreno et al. 1991]. *S. pombe* transformation followed standard procedures. Transformants were grown at room temperature to mid-log phase in EMM media, and the cultures were split into YES media [Moreno et al. 1991] and shifted to the *cdc25*^{ts} nonpermissive temperature (35°C). Growth of the culture was followed by microscopic examination of Calcofluor-stained cells [Russell and Nurse 1987] and by cell number determination with a Coulter counter as described [Russell and Nurse 1987; Sadhu et al. 1990].

Expression and purification of a GST-CDC25M2 fusion protein and detection of phosphatase activity

A 1-kb *Dra*I-*Nco*I fragment was isolated from the cloned cDNA of *cdc25M2* (Fig. 1) and ligated into *Sma*I-*Nco*I-digested pGEX-KG [Guan and Dixon 1991] to produce pGEX-K288. *E. coli* strain BL21 containing plasmid pLysS was transformed with pGEX-K288, and cells containing both plasmids were grown in 800 ml of L broth at 37°C to mid-log phase and treated with 0.6 mM IPTG for 2 hr at 25°C. Cells were harvested, washed in TD (150 mM NaCl, 10 mM Tris-HCl at pH 7.5), resuspended in TD containing both 1% Tween 20 and 1% Triton X-100, and the mixture was sonicated for 30 sec. The extract was centrifuged twice at 2500 rpm for 10 min in a Beckman J6B centrifuge, and the clarified supernatant was collected. Five hundred microliters [50% (vol/vol)] of Sepharose (Pharmacia), coupled with glutathione, was added to the extract, and the slurry was incubated for 2–3 hr at 4°C. The beads were collected by centrifugation for 10 sec at 1000 rpm and washed extensively in 500 mM NaCl, 10 mM DTT, and 10 mM HEPES (pH 8.0). Protein was eluted by incubating the beads for 3–4 hr at 4°C in 100 mM Tris-HCl (pH 8.0), 10 mM DTT, and 5 mM glutathione. GST from pGEX-KG was purified in an identical manner. Protein concentration was determined by gel electrophoresis.

To assay the activity of the purified GST-Cdc25M2 fusion protein, we measured dephosphorylation of *p*-NPP using the conditions described by Guan et al. (1990, 1991). Reaction mixtures (200 μ l) contained 50 mM imidazole (pH 7.4), 0.5% β -mercaptoethanol, and 10 mM *p*-NPP, and either purified GST-Cdc25M2 fusion protein or GST, and the reactions were incubated at 30°C for 45 min. Reactions were terminated by adding 800 μ l of 0.2 M NaOH. Absorbance at 410 nm was measured.

Analysis of *cdc25M2* mRNA levels

cdc25M2 mRNA levels were analyzed by Northern blots using a randomly primed *cdc25M2* cDNA insert as a probe. Total and poly(A)⁺ mRNA was isolated as described above. Total or poly(A)⁺ RNA was separated by electrophoresis in a 1% agarose gel containing 0.66 M formaldehyde, transferred to a Nytran filter (Schleicher & Schuell), and hybridized with *cdc25M2* cDNA. Hybridization was carried out at 42°C in a buffer containing 50% formamide, 50 mM sodium phosphate buffer (pH 6.5), 5 $\times$ SSC, 4 $\times$ Denhardt's solution, 0.1% SDS, 100 μ g/ml of denatured salmon sperm DNA, 200 μ g/ml of yeast RNA, and 5 ng/ml of ³²P-labeled probe ($\sim 5 \times 10^8$ cpm/ μ g). The filters were washed with 0.5 $\times$ SSC and 0.1% SDS at 50°C and exposed to film with an intensifying screen. Total RNA from adult male rat tissues and mouse embryos was isolated as described previously (Chomczynski and Sacchi 1987).

To examine *cdc25M2* mRNA levels during cell cycle progression, the methods described by Lee et al. (1988) were used. Briefly, Swiss 3T3 cells were maintained in DMEM with 0.5% FBS for 72 hr. At this time we estimate that >90% of the cells were in G₀. Fresh medium (DMEM) containing 10% FBS was added to the serum-depleted cells, and samples were taken at various times after addition. Progression through the cell cycle was monitored by labeling cells for 30 min with 10 μ Ci of [³H]thymidine (83 Ci/mmol, Amersham) prior to harvesting. Incorporation of [³H]thymidine into DNA was measured as described (Stein and Stein 1989) but briefly involved washing the media from cells, lysing the cells, precipitating DNA with cold trichloroacetic acid (TCA), and measuring incorporation of ³H into TCA-precipitated DNA. Parallel samples of cells were harvested for RNA preparation and Northern analysis.

Retinoic acid induction experiments were performed as follows: Monolayer cultures of P19 cells were maintained in α MEM with 10% FBS and were treated by 10 μ g/ml of cycloheximide (Calbiochem) with or without 10^{-9} M or 5×10^{-7} M retinoic acid (Sigma) for 8 hr. The effect of retinoic acid was monitored by Northern blot using the RAR β cDNA as a probe (Song and Siu 1989).

In situ hybridization of *cdc25M* to developing mouse embryos

In situ hybridization was performed as described by Bettler et al. (1990). Two antisense RNA probes covering nucleotides 738–1274 and 1360–1703 (Fig. 1) were prepared using ³⁵S-labeled UTP (1500 Ci/mmol, Dupont) and T3 RNA polymerase (Promega). Both probes gave identical results. Unrelated probes (Bettler et al. 1990) were used to determine specificity (data not shown). The occurrence of a vaginal plug was defined as EO.

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Note added in proof

Galaktionov and Beach (1991, *Cell* 67: 1181–1194) reported the third member of human *cdc25*.

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