M glycine and 1 mM EGTA (pH 2.7) for 20 min. This elution step was repeated, and the pooled eluates were quickly neutralized with tris base.
31. Identical volumes of cell cultures were harvested at an optical density at 598 nm of ~1.3 and washed in 1.2 M sorbitol. Cells were resuspended in precipitation buffer [50 mM tris (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM Na_3VO_4, 0.07% Triton X-100] containing a protease inhibitor cocktail and 1% bovine serum albumin, and broken with glass beads in a Mini Beadbeater (Biospec). After two sequential centrifugations at 5000 and 10,000*g* (each for 4 min), the supernatant was incubated with antibodies for 1 hour. Antibody-antigen complexes were incubated with protein A– or protein G–Sepharose beads (1:1) (Pharmacia) for polyclonal and monoclonal antibodies, respectively. Beads were sedimented at 1000*g*, washed three times with precipitation buffer, and resuspended in equal volumes of lysis buffer. Equal volumes of the various precipitations were analyzed by immunoblot analysis. Samples were separated by SDS–polyacrylamide gel electrophoresis (8 or 10% gels) [U. K. Laemmli, *Nature* **227**, 680 (1970)] and transferred electrophoretically (Multiphor chamber, Pharmacia) to nitrocellulose filters. Filters were incubated sequentially with primary antibodies (rabbit or mouse) and secondary antibodies (horseradish peroxidase–conjugated goat antibodies to rabbit or mouse immunoglobulin G) (Bio-Rad) in tris-buffered saline containing 1% bovine serum albumin. Immunoblots were developed with the Amersham ECL kit.
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CDC25 Phosphatases as Potential Human Oncogenes

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Cyclin-dependent kinases (CDKs) are activated by CDC25 phosphatases, which remove inhibitory phosphate from tyrosine and threonine residues. In human cells, CDC25 proteins are encoded by a multigene family, consisting of *CDC25A*, *CDC25B*, and *CDC25C*. In rodent cells, human CDC25A or CDC25B but not CDC25C phosphatases cooperate with either Ha-*RAS*^{G12V} or loss of *RB1* in oncogenic focus formation. Such transformants were highly aneuploid, grew in soft agar, and formed high-grade tumors in nude mice. Overexpression of CDC25B was detected in 32 percent of human primary breast cancers tested. The CDC25 phosphatases may contribute to the development of human cancer.

A family of related cyclin-dependent kinases (CDKs) regulates progression through each phase of the cell division cycle (*1*). These proteins are positively regulated by association with cyclins (*2*) and activating phosphorylation by the CDK-activating kinase (CAK) (*3*). Negative regulation of the CDKs is achieved independently by at least two different mechanisms: binding of the inhibitory subunits (p21, p16, p15, p27, and p18) (*4*) and phosphorylation of conserved threonine and tyrosine residues, usually at positions 14 and 15 in CDKs (*5*). In fission yeast, phosphorylation on inhibitory residues is negated by the CDC25 dual specificity phosphatase, whose only known function is the removal of inhibitory phosphate on the CDC2 kinase (*6*). In humans, there are three *CDC25*-related genes that share approximately 40 to 50% amino acid identity (*7*, *8*). Human *CDC25* genes function at the G_1 or S phase of the cell cycle (*9*) and at the G_2 or M phase (*8*, *10*). Several mammalian cell cycle genes participate in neoplastic transformation (*1*). As a rule, these genes function early in the cell cycle in G_1 or at the G_1-S border. Because some of the *CDC25* genes have been implicated in the progression from G_1 to the S phase, we reasoned that *CDC25* might display oncogenic properties in mammalian cells.

We introduced *CDC25A*, *B*, or C on a mammalian constitutive expression vector into normal mouse embryo fibroblasts at early passage (*11*). Cells were transfected with these plasmids either alone or in combination with oncogenic versions of H-*RAS* in which Gly^{12} was changed to Val (G12V) or mutant *p53* [where Glu^{258} was changed to Lys (E258K)]. The cells were then plated in either nonselective or selective (G418) media. After 4 to 5 weeks, the plates were stained and photographed to detect the formation of potentially transformed foci. In these assays, we observed oncogenic cooperation between *CDC25A* or *CDC25B* and H-*RAS*^{G12V} (Fig. 1A). No cooperation between *CDC25C* and *RAS* was detected. A few weak foci were formed upon transfection of *CDC25A* alone, and only slightly more were observed with the combination of *CDC25A* and mutant *p53* (Fig. 1A). No focus formation was observed with H-*RAS*^{G12V} alone (*12*). Clones isolated from the *CDC25A-RAS* and *CDC25B-RAS* foci mildly overexpressed CDC25 proteins (two to three times more protein than that in parental cells) (*13*). G418-selected colonies were counted to assess transformation efficiency (*14*). In all experiments, similar numbers of the G418-selected colonies were obtained.

Microscopical examination of the cells expressing CDC25A and RAS or CDC25B and RAS revealed a transformed cell morphology indicated by multilayer growth, loose attachment to the substrate, and aneuploidy (*14*). Cells from individual foci readily grew in the presence of G418, which demonstrates that they represent cells transfected with the plasmids rather than spontaneously transformed mouse cells. Cells cotransfected with *CDC25A* or *CDC25B* and H-*RAS*^{G12V} together with relevant controls were tested for the ability to form colonies in soft agar (*15*). At 3 weeks we detected formation of tight colonies with cells derived from any of three independent foci of the cells transfected with *RAS* and *CDC25A* or *RAS* and *CDC25B* cotransfected cells (Fig. 2). To verify the tumorigenic potential of these cells, we introduced them into nude mice (*15*). After 20 to 25 days, we detected tumor formation in all experimental animals injected with cells transfected with *CDC25A* and *RAS* or *CDC25B* and *RAS* (in each case eight mice were injected). The average size of tumors 25 days after injection was 5.6 ± 1.7 mm for *CDC25A-RAS* and 7.4 ± 2.5 mm for *CDC25B-RAS* transfection. No tumors were detected in mice injected with G418-selected cells that had been transfected with either *RAS* or *CDC25* alone or with the parental vector plasmid (Table 1).

In human tumors, mutations in *RAS* often coincide with mutations or deletions in the tumor suppressor genes *p53* and *RB1*. We therefore investigated whether defects

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in *p53* and *RB1* could render normal fibroblasts susceptible to the oncogenic effects of *CDC25A* overexpression. We introduced *CDC25A* into $RB^{-/-}$ or $p53^{-/-}$ fibroblasts (*16*) and after 4 weeks scored the formation of oncogenic foci. Focus formation was observed in the $RB^{-/-}$ cells (Fig. 1B). In the case of $p53^{-/-}$ cells, we selected transformants with G418 and hygromycin B because $p53^{-/-}$ cells overgrow upon periodic addition of fresh media and foci cannot be visually detected and scored on the background of untransfected cells. $RB^{-/-}$ or $p53^{-/-}$ cells transfected with *CDC25A* or a parental vector plasmid were further grown in the continuous presence of G418 and hygromycin selection. Experiments assaying growth in soft agar and in nude mice demonstrated the oncogenic properties of *CDC25A*-transfected $RB^{-/-}$ cells but not those of $p53^{-/-}$ cells (Fig. 2 and Table 1). All cells transfected with control plasmids were nononcogenic (Fig. 2 and Table 1).

In light of the in vitro evidence that *CDC25* can act as an oncogene, we analyzed *CDC25* expression in cell lines derived from various human tumors. High levels of *CDC25* expression were detected in several cell lines, including those that originated from human breast cancer (*17*). We therefore investigated the expression of *CDC25* in a characterized series of human primary breast cancers. The study population (*18*) consisted of a retrospective series of tissues from 124 patients with axillary node–negative invasive breast cancer who were treated between 1972 and 1982. The patients were all treated by modified radical mastectomy, without perioperative adjuvant therapy, and had a median follow-up of 11 years. Cancerous tissue from these patients had been characterized in terms of size, histologic and nuclear grade, mitotic rate, peritumoral lymphovascular invasion, microvessel density, and *p53* status (*19*, *20*).

Normal and neoplastic archival tissue was used for in situ hybridization with antisense riboprobes for *CDC25A*, *B*, and *C* (*21*). There was no detectable expression of *CDC25C* in normal or tumor tissue, and no detectable expression of CDC25B by in situ hybridization was seen in normal breast tissue (Fig. 3A). However, *CDC25B* was overexpressed in 32% of the neoplastic tissue samples (Fig. 3A). In many tumors (Fig. 3, A and B), practically all cells displayed large amounts of CDC25B mRNA, further underscoring the full deregulation of its expression. No correlation between the mitotic index of the particular tumor and CDC25B overexpression was found ($P = 0.849$).

To further investigate the significance of the observed tumor-specific CDC25 overexpression, we correlated CDC25B expression with other tumor-specific markers. There was a strong correlation between CDC25B overexpression and microvessel density (*19*, *20*) ($P = 0.038$), a negative prognostic feature indicative of augmented angiogenesis in breast cancer (*20*). CDC25B positivity was more frequently seen in higher histologic grade cancers ($P = 0.02$) in which nuclear

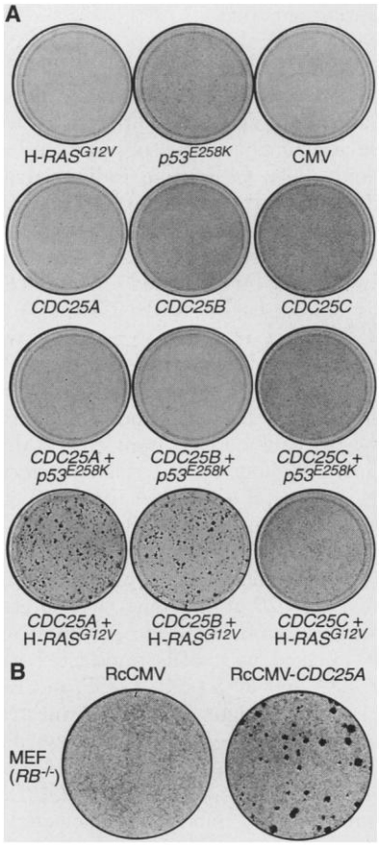

Fig. 1. Oncogenic cooperation between *CDC25* and H-*RAS*^{G12V} and oncogenic foci of the $RB^{-/-}$ cells, transfected with *CDC25A*. (**A**) Foci formation after transfection of normal mouse fibroblasts with various plasmids. (**B**) $RB^{-/-}$ primary fibroblasts, transfected with *CDC25A* or control plasmid (RcCMV). Photomicrographs of the transformed foci were on the lawn of the $RB^{-/-}$ cells.

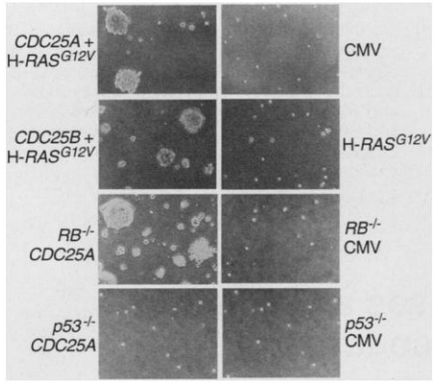

Fig. 2. Photomicrographs of the colonies in semisolid agarose. Mouse embryo fibroblasts (MEFs) were transfected with vector alone (CMV) or with vectors with *CDC25A* or H-*RAS*^{G12V}, *CDC25B*, and H-*RAS*^{V12}, as indicated; $RB^{-/-}$ cells or $p53^{-/-}$ cells were also transfected with *CDC25A* and RcCMV as indicated. All cells were grown in semisolid agarose for 3 weeks.

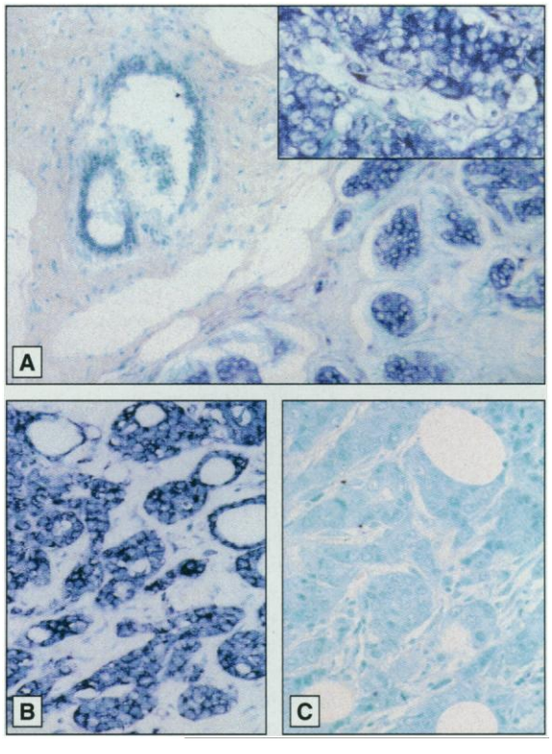

Fig. 3. Tumor-specific overexpression of *CDC25B* in breast cancer patients. CDC25B expression was visualized after hybridization with a digoxigenin-labeled *CDC25B* probe (*21*) with NBT-BCIP (*21*). (**A**) Stained breast cancer cells are on the right, normal breast cells are on the left. (**B** and **C**) CDC25B-positive (B) and CDC25B-negative (C) tumors. Almost all visible cells of the CDC25B-positive tumor are overexpressing CDC25B. All cells were counterstained with methyl green. (**Inset**) Up to 100% of the cells in the tumor are overexpressing CDC25B. Magnification; ×200, (A); ×400, (B) and (C). Inset, ×400.

atypias are more frequent. Patients with high levels of CDC25B expression in tumor cells had a distant recurrence rate of 41.9%, whereas those who expressed little or no CDC25B mRNA had a 29.1% recurrence rate at 10 years. Similarly, 37.2% of overexpressors versus only 19.9% of *CDC25B*-negative patients were dead of their disease after 10 years.

CDC25A and possibly CDC25B phosphatases associate with Raf1, a kinase that is activated by *RAS* (*13*). Furthermore, Raf1 phosphorylates and activates CDC25 in vitro, which suggests a possible mechanism for the observed synergism between *RAS* and *CDC25*. The difference in oncogenic potential between various CDC25 proteins might be attributed either to differences in their cell cycle timing or substrate specificity (*8–10*).

The most dramatic effect of *CDC25A* expression was observed in fibroblasts lacking the tumor suppressor *RB1*, in which introduction of the *CDC25A* gene alone causes oncogenic transformation (Fig. 2). The ability of CDC25 phosphatases to show oncogenic cooperation with either oncogenic *RAS* mutants or *RB1* deletion mutants underscores the potential significance of CDC25 overexpression in the development of human malignancies. In support of this suggestion, CDC25B was found to be highly expressed in 32% of the primary breast cancers. Tumor-specific expression of *CDC25B* in human breast carcinomas correlates with less favorable prognosis and survival. Our results suggest that alterations in the function of *CDC25A* and *CDC25B* by overexpression might promote oncogenic transformation in vivo and further suggest that CDC25 phosphatases (A and B) are novel potential oncogenes.

Table 1. Oncogenic transformation of the wild-type mouse fibroblasts by the ectopic expression of *CDC25* genes in cooperation with H-*RAS*^{G12V} or *RB*$^{-/-}$ fibroblasts by *CDC25A* alone. ++ and +++ indicate foci formation or growth in semisolid agarose; +/− indicates infrequent formation of less transformed foci upon MEF transfection with CDC25A or CDC25B plasmids. NA indicates that none of the mice had tumors (0/8); for those that had tumors (8/8), the time until the tumors reached 5 mm in size is an average of their occurrence in eight of eight mice. ND, not done; − indicates no foci formation or no growth in semisolid agarose.

Plasmids	MEF cells	Foci	Soft agar	Days on average for tumor to reach 5 mm
CDC25A + H-RAS^{V12}	WT	+++	++	25
CDC25A	WT	+/−	−	NA
CDC25B + H-RAS^{V12}	WT	+++	++	20
CDC25B	WT	+/−	−	NA
H-RAS^{V12}	WT	−	−	NA
RcCMV	WT	−	−	NA
CDC25A	RB$^{-/-}$	+++	+++	17
RcCMV	RB$^{-/-}$	−	−	NA
CDC25A	p53$^{-/-}$	ND	−	NA
RcCMV	p53$^{-/-}$	ND	−	NA

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11. Human genes *CDC25A*, *B*, and *C* were subcloned into RcCMV, pcDNA3, or pCEP4 vectors (Invitrogen). A plasmid with mutant *p53*^{E258K} was expressed from the cytomegalovirus (CMV) promoter. Plasmid (pNV) containing oncogenic c-Ha-RAS^{Val12} was under control of the murine leukemia virus long terminal repeat. Mouse (Balb/c) embryonic fibroblast cells were obtained at passage 2. Cells were split 1:4 every 2 to 3 days. Cells were transfected at passages 4 to 6 with calcium phosphate precipitates (24 to 48 hours after the split). Special precautions were taken for plasmid purification and buffer preparations. Detailed transfection protocols are available on request. Precipitates containing 20 to 30 μg of plasmid or plasmids without carrier DNA were incubated with cells on 100-mm plates for 12 to 14 hours. Cells were washed with phosphate-buffered saline (PBS), treated with 12.5% glycerol for 2 min, washed in PBS three times, and allowed to recover in complete medium for 24 to 36 hours before splitting 1:2 or 1:3 on selective (G418 or hygromycin) or nonselective medium. In the latter case, cells were fed once every 4 to 5 days, and after 4 to 5 weeks were stained with 0.4% crystal violet in 20% ethanol. Simultaneous transfection of *CDC25A* and *CDC25B* was toxic to fibroblasts. Less than one-tenth of the overall number of G418-selected colonies was observed in this case compared to those observed after *CDC25A* and *RAS* cotransfection.
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21. Digoxigenin-labeled probes were used for in situ hybridization. One microgram of the recombinant plasmid containing a 611–base pair fragment of human *CDC25B* complementary DNA corresponding to the NH_2-terminus of *CDC25B* was linearized by Sal I and Eco RI to generate sense and antisense transcripts, respectively. Riboprobes were generated with T3 and T7 RNA polymerase for 1 hour at 37°C in ×1 transcription buffer (Promega, Madison, WI), 10 mM dithiothreitol, 40 U of ribonuclease (RNase) inhibitor, adenosine, cytosine, and guanosine triphosphates (1 mM each), and a mixture of cold uridine triphosphate (UTP) and digoxigenin-UTP (6.5 and 3.5 mM, respectively, for a total concentration of 1 mM) (Boehringer Mannheim, Indianapolis, IN). Formalin-fixed paraffin-embedded sections (5 μM) were dewaxed, rehydrated, and then washed in PBS. Sections were digested with proteinase K (50 μg/ml) in 1 M tris-EDTA buffer (pH 8) for 8 min at 37°C, washed in PBS, and dried. Prehybridization was done at 37°C for 15 min in 50% formamide and ×2 saline sodium citrate (SSC). Hybridization was performed at 42°C overnight with the application of 10 pM digoxigenin-labeled riboprobe in 50 μl of hybridization buffer [50% deionized formamide, ×2 SSC, 10% dextran sulfate, 1% SDS, and denatured herring sperm DNA (10 mg/ml)] per section under a liquid cover slip (Ventana Medical Systems, Tucson, AZ) for 60 min. The highest stringency of posthybridization washes was 42°C in ×0.1 SSC for 15 min. Antibody to digoxigenin (1:500) was applied for 30 min at 37°C. Detection was accomplished with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) for 12 min for all sections. Sections were counterstained with methyl green, dehydrated, and mounted. Controls consisted of sense probes and tissue pretreatment with RNase A. An automated in situ hybridization instrument (Ventana Medical Systems, Tucson, AZ) in which the duration and temperature of all the steps were standardized was used for all cases. The extent of mRNA expression was thus directly comparable from case to case. Cases were judged to be CDC25-positive when >50% of tumor cells showed a strong cytoplasmic signal for the corresponding mRNA.

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