slips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated for 24 hours. Cells were then incubated in DMEM containing 0.25% FBS for 48 hours before microinjection. One to 2 hours after microinjection, cells were switched to medium containing 10% FBS and a 1:1000 dilution of BrdU (Amersham, Arlington Heights, IL). After 18 to 24 hours of incubation, cells were fixed and processed (*16*). After permeabilization, cells were denatured with 1 M HCl for 30 min. For detection of human integrin expression, cells were probed with the human integrin β_1–specific mAbs TS2/16 (*6*) or P5D2 (Developmental Hybridoma Bank). After incubation with secondary antibodies, cells were blocked with mouse immunoglobulin IgG before staining with fluorescein isothiocyanate–labeled antibodies to BrdU (Becton Dickinson, San Jose, CA). The cDNAs were injected at a concentration of 0.1 mg/ml. In control experiments, injection of pBJ-1 vector alone at twice the molar concentration of β_{1C} cDNA had no effect on DNA synthesis. Injection of 10 times this concentration of vector alone inhibited DNA synthesis by 46%, substantially lower than the inhibition by β_{1C} (95%). Approximately 100 expressing cells, with equivalent expression levels as determined by microfluorimetry (*11*), were analyzed per variant.

19. C3H 10T1/2 cells were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% FBS (Gemini Bioproducts, Calbasas, CA). Nuclear microinjections were performed as described [L. D. Chong *et al.*, *Cell* **79**, 507 (1994)]. Twenty-four hours before microinjection, cells were plated on etched glass cover slips. In a typical experiment, ~50 cells were injected with either β_1 (0.1 mg/ml) or β_{1C} (0.2 mg/ml) expression vector [cDNA concentrations were adjusted to generate cells that expressed equivalent amounts of protein as determined by microfluorimetry (*11*)]. Approximately 50% of the injected cells expressed protein.
20. The D130A mutation of β_1 was generated as previously described (*14*). An Xba I–Hind III fragment of the 5′ end of β_1 cDNA containing this mutation was then used to construct D130A β_{1C} (*17*).
21. Quiescent 10T1/2 cells that had been maintained in 0.25% serum (*18*) were injected with β_{1C} cDNA (0.2 mg/ml) 0, 4, 8, 12, or 16 hours after the addition of DMEM containing 10% FBS. BrdU (1:1000 dilution) was added 18 hours after the switch to 10% FBS, and after incubation for an additional 6 hours, cells were fixed and stained as previously described (*18*). In control experiments, maximal expression was achieved 1 hour after cDNA injection. Approximately 100 expressing cells were analyzed per time point.
22. Quiescent 10T1/2 cells that had been maintained in 0.25% serum (*18*) were injected with β_{1C} cDNA (0.1 mg/ml) 1 to 4 hours after the addition of DMEM supplemented with 10% FBS. After 18 hours, cells were fixed and stained (*16*). Cells were probed with mAb P5D2 to integrin β_1 and affinity-purified rabbit antibodies to cyclin A or cyclin E [M. Ohtsubo, A. M. Theodoras, J. Schumacher, J. M. Roberts, M. Pagano, *Mol. Cell. Biol.* **15**, 2612 (1995)]. Approximately 50 expressing cells were analyzed for each cyclin.
23. The cytoplasmic truncation of β_1 (β_1^{Δ}) was generated by inserting an Xba I linker into the Hind III site of the β_1 cDNA clone B3 (*14*). The COOH-terminal sequence of β_1^{Δ} is Gly-Leu-Ala-Leu-Leu-Leu-Ile-Trp-Lys-Leu^{733}. An Xba I fragment containing this mutation was then subcloned into the Xba I site of pBJ-1 to generate β_1^{Δ}. The β_{1C} cytoplasmic truncations Δ766, Δ774, and Δ792 were generated, with the use of site-directed mutagenesis [W. P. Deng and J. A. Nickoloff, *Anal. Biochem.* **200**, 81 (1992)], by introducing stop codons at positions 767, 775, and 793 of β_{1C}, respectively. Mutants were verified by DNA sequencing.
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Association of the Yeast Pheromone Response G Protein βγ Subunits with the MAP Kinase Scaffold Ste5p

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The mating response pathway of the yeast *Saccharomyces cerevisiae* includes a heterotrimeric guanine nucleotide–binding protein (G protein) that activates a mitogen-activated protein (MAP) kinase cascade by an unknown mechanism. An amino-terminal fragment of the MAP kinase scaffold protein Ste5p that interfered with pheromone-induced cell cycle arrest was identified. A haploid-specific interaction between the amino terminus of Ste5p and the G protein β subunit Ste4p was also detected in a two-hybrid assay, and the product of a signaling-defective allele of *STE4* was defective in this interaction. In cells with a constitutively activated pheromone response pathway, epitope-tagged Ste4p was coimmunoprecipitated with Ste5p. Thus, association of the G protein and the MAP kinase cassette via the scaffolding protein Ste5p may transmit the G protein signal.

The pheromone response pathway of *S. cerevisiae* is a well-studied system of signal transduction (*1*, *2*). Many genes that are required for haploid yeast cells to mate have been identified in genetic screens. Characterization of the products of these genes has provided an outline of the molecular mechanism of this pathway, which includes a G protein encoded by the genes *GPA1* (Gα subunit) (*3*), *STE4* (Gβ subunit), and *STE18* (Gγ subunit) (*4*). This G protein is coupled to cell type–specific receptor proteins encoded by the *STE2* and *STE3* genes (*5*). The βγ subunits of the activated G protein stimulate the downstream elements of the pathway (*4*), which include the Ste20p protein kinase (*6*, *7*) and a set of kinases that are similar to MEK kinase, MEK (MAP kinase kinase), and MAP kinase of higher cells and are encoded by the *STE11* (*8*), *STE7* (*9*), and *FUS3/KSS1* (*10*) genes, respectively.

The *STE5* gene encodes a protein (Ste5p) of 917 amino acids, different regions of which physically interact with the members of the MAP kinase cascade (*11*–*13*). Thus, Ste5p has been proposed to act as a scaffold to organize the kinases (*2*, *11*). However, it is not clear how the elements of this cascade are activated in response to the βγ subunits of the G protein. Because MAP kinase activation is the hallmark of many signaling pathways in mammalian cells, characterization of this process is of general importance. We now show that, in the yeast pheromone response pathway, the G protein β subunit can associate with the NH_2-terminus of the MAP kinase scaffold Ste5p, suggesting a mechanism for direct activation.

Oligonucleotide-directed random mutagenesis (*14*, *15*) was performed on two regions of *STE5* (*16*, *17*), and the mutant libraries were screened to identify dominant-negative mutants of Ste5p (*18*). For this screen, the *STE5* libraries were transformed into strain YEL106 (W303-1a *sst1::LEU2*) and tested for galactose-dependent interference with the α factor–specific growth arrest signal (*15*). Plasmids from clones with a strong interference phenotype were rescued and retransformed into YEL106 to confirm that the phenotype was plasmid dependent. Four of the mutations resulted in major structural changes to Ste5p; CW1-2 was a deletion of amino acids 376 to 546, and CW4-8, CW3-1, and CW2-1 were COOH-terminal truncations leaving the NH_2-terminal 394, 241, and 214 amino acids, respectively. In addition, CW2-8 was a deletion of amino acids 215 and 216. All these mutants blocked the constitutive cell cycle arrest caused by deletion of *GPA1*. The two internal deletions, CW2-8 and CW1-2, allowed weak mating of a *STE5*-deleted strain, whereas the three COOH-terminal deletions did not.

Although expression of mutant CW2-1 blocked both the constitutive cell cycle arrest caused by deletion of *GPA1* and cell cycle arrest induced by mating pheromone (Fig. 1), it did not block cell cycle arrest caused by overexpression of the hyperactive *ste11-1* allele (Fig. 1A). This observation suggests that the Ste5p dominant-negative mutant may act at a step between the G protein and the MAP kinase cascade, a result consistent with the placement of Ste5p function in the pheromone response pathway by analysis of hyperactive and null

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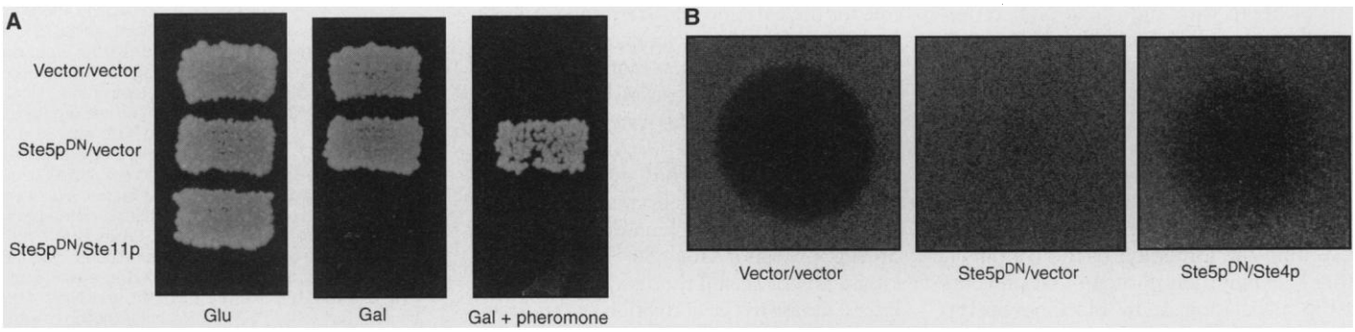

Fig. 1. Effect of dominant-negative Ste5p on cell cycle arrest. (**A**) Block of pheromone-induced cell cycle arrest, but not hyperactive Ste11p–mediated arrest, by dominant-negative Ste5p. Cells of the pheromone-supersensitive strain YEL106 (*6*) transformed with various plasmids were tested for cell cycle arrest. First row, growth of cells containing the two vector plasmids pRS313 (*URA3*$^+$) and pRS316 (*HIS3*$^+$) (*35*). Second row, growth of cells containing plasmid pRS316 and plasmid pCW2-1, which overexpresses the NH_2-terminus of Ste5p. Third row, growth of cells overexpressing both the NH_2-terminus of Ste5p and a hyperactive allele of *STE11* (*36*). DN, dominant negative; Glu, glucose; Gal, galactose; Gal + pheromone, galactose plus 5 μg of α-factor. (**B**) Suppression of dominant-negative Ste5p by overproduction of Ste4p. Cells of strain YEL106 transformed with various plasmids were embedded in galactose medium lacking histidine and uracil, and treated with 20 μl of synthetic α-factor (100 μg/ml) (Sigma) as described (*4*). (**Left panel**) The response of cells containing the two control plasmids, pRS313 and pRS316; (**center panel**) the response of cells containing plasmids pRS316 and pCW2-1; (**right panel**) the response of cells containing plasmid pCW2-1 and plasmid pL19 (*29*), which overexpresses Ste4p.

mutations (*19*). We tested whether the NH_2-terminus of Ste5p functioned in a dominant-negative manner through sequestration of components other than the members of the MAP kinase cascade. Overexpression of Ste4p together with the NH_2-terminal fragment of Ste5p reestablished cell cycle arrest in response to pheromone (Fig. 1B). Therefore, the dominant-negative phenotype of the Ste5p NH_2-terminus results, at least in part, from the creation of a limiting concentration of Ste4p.

To investigate whether the NH_2-terminus of Ste5p physically interacts with Ste4p, we fused the nucleotide sequence encoding the NH_2-terminal 214 amino acids of Ste5p to that encoding the LexA binding domain of plasmid pBTM116 (*20*), to generate plasmid M276p16 (*21*). We then used the two-hybrid assay (*22*) to test for interaction with Ste4p. When transformed into yeast strain L40 (*12*), the combination of plasmid M276p16 and plasmid pKB40.1 (*23*), which encodes a fusion of Ste4p and the Gal4p trans-activation domain, allowed histidine-independent growth and increased β-galactosidase expression several hundredfold (Table 1). Thus, the two-hybrid assay detected interaction between Ste4p and the NH_2-terminus of Ste5p. However, β-galactosidase activity was not increased when these two plasmids were introduced into strain DL40 (*24*), an a/α diploid derivative of L40, showing that the interaction is haploid specific. Introduction of plasmid pKB24.5 (*23*), which encodes Ste18p fused to the Gal4p trans-activation domain, into these diploid cells increased β-galactosidase expression. This increase required all three plasmids, showing that the haploid specificity of the interaction of Ste4p with the NH_2-terminus of Ste5p resulted from the absence of Ste18p in diploid cells. The β and γ subunits of G proteins are tightly associated and function as a single unit (*23*, *25*), and both Ste4p and Ste18p are required for activation of downstream elements of the pheromone response pathway (*4*).

We investigated the biological relevance of the association between Ste4p and Ste5p by examining the association of mutant ver-

Table 1. Two-hybrid analysis of Ste4p and Ste5p association.

Strain	pGAD plasmid	LexA plasmid	β-Gal activity*
L40	Control†	Ste5 NT	1.2 ± 0.05
L40	Ste4 wt	Control‡	0.3 ± 0.1
L40	Ste4 wt	Ste5 NT	830 ± 200
L40	Ste4 Hpl21-1	Ste5 NT	275 ± 75
L40	Ste4 Hpl21-3	Ste5 NT	690 ± 420
L40	Ste4-19	Ste5 NT	2.0 ± 1.3
L40	Ste4 D62N	Ste5 NT	240 ± 20
L40	Ste18 wt	Ste5 NT	4.0 ± 1.6
DL40	Control§	Ste5 NT	0.7 ± 0.1
DL40	Ste18 wt	Ste5 NT	1.2 ± 0.3
DL40	Ste4 wt	Ste5 NT	1.0 ± 0.1

*β-Galactosidase activity was determined by standard techniques (*23*). Values are means of duplicate assays of two independent transformants. †Control plasmid was NST1 expressing a COOH-terminal fragment of the Bem1 protein. ‡Control plasmid was LexA lamin (*20*). §Control plasmid was pGAD2F.N (*23*). wt, wild type; NT, NH_2-terminus.

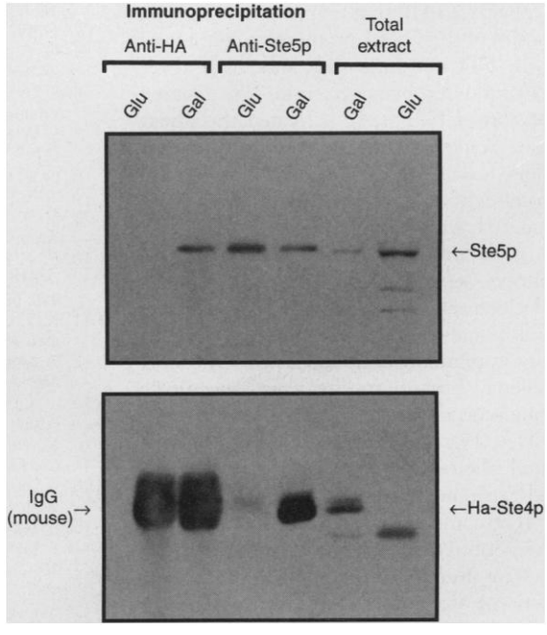

Fig. 2. Immunoprecipitation of proteins from yeast extracts with antibodies to HA-Ste4p and to Ste5p. (**Upper panel**) A specific band for Ste5p was detected after immunoprecipitation with antibodies to HA-Ste4p in an extract of yeast induced by galactose (Gal) but not after growth in medium containing glucose (Glu). The detection of Ste5p after immunoprecipitation with antibodies to Ste5p shows that comparable amounts of Ste5p were present in cells grown in glucose or galactose. (**Lower panel**) A specific band for HA-tagged Ste4p was detected after immunoprecipitation with rabbit antibodies to Ste5p in extracts of yeast induced with galactose. The faint band detected in the extract of noninduced cells results from a weak cross-reaction of the secondary antibodies to mouse immunoglobulin G (IgG) with rabbit IgG. Because of its comigration with immunoglobulins, HA-Ste4p cannot be detected with antibodies to mouse IgG after immunoprecipitation with mouse antibodies. The lower molecular weight band that corresponds to a cross-reaction with an unknown protein is more marked in the total extract lane for the cells grown in glucose because more protein was loaded.

sions of Ste4p with the Ste5p NH_2-terminus. Plasmid pKB41.1 is identical to plasmid pKB40.1 with the exception that it contains allele *4-19* of *STE4*, which encodes a change at amino acid 278 (F to FF) of Ste4p that affects its ability to interact with Ste18p and to signal (*23*). This mutant version of Ste4p did not interact with the NH_2-terminus of Ste5p (Table 1). Thus, preventing the formation of the βγ dimer, either by a mutation in Ste4p that prevents Ste18p association or by blocking Ste18p expression in a diploid, eliminates the association of Ste4p with the NH_2-terminus of Ste5p. A similar correlation between mutants defective in signaling and in association was observed for Ras and Raf in the two-hybrid assay (*20*, *26*).

In contrast, Ste4p encoded by the haploid-lethal *Hpl21-1* and *Hpl21-3* alleles in plasmids pKB86.6 and pKB85.60, respectively (*27*), did interact with the Ste5p NH_2-terminus (Table 1), consistent with previous data showing that these mutant Ste4 proteins do not interact with Gpa1p but still associate with Ste18p and activate downstream elements of the pheromone response pathway. A dominant-negative mutant of Ste4p (D62N) (*15*) also associated with the NH_2-terminus of Ste5p (Table 1), consistent with the notion that such mutants interact with, but cannot activate, an effector protein (*15*).

We used coimmunoprecipitation to establish biochemically that Ste4p and Ste5p associate. Strain YEL121 (W303-1A *ste4::LEU2*) was transformed with plasmid pL55 and the transformants were grown in medium containing galactose; plasmid pL55 is pRS313GAL that expresses, under the control of the *GAL1* promoter, Ste4p tagged at the NH_2-terminus with an epitope (HA) from influenza hemagglutinin (*28*). Overexpression of HA-Ste4p activated the pheromone response pathway, resulting in morphological changes and cell cycle arrest (*29*). Proteins from these cells were immunoprecipitated with either antibodies to Ste5p (*30*) or antibodies (12CA5) to the HA epitope, separated by SDS–polyacrylamide gel electrophoresis, and detected by immunoblot analysis (*31*). HA-Ste4p was specifically coimmunoprecipitated with the antibodies to Ste5p, and Ste5p was specifically coimmunoprecipitated with the antibodies to HA (Fig. 2). Thus, in cells with an activated pheromone response pathway, Ste4p and Ste5p are present in a complex.

It remains to be determined whether this interaction between Ste4p and Ste5p occurs in uninduced cells or is a transient result of activation of the pheromone response pathway. Other researchers have not detected an interaction between Ste4p and Ste5p deleted for the NH_2-terminal 24 amino acids (*11*, *13*). This result may reflect a role for the extreme NH_2-terminus of Ste5p in interactions with Ste4p, or it may be consistent with studies that have shown that protein domains can give stronger signals in the two-hybrid system than complete proteins (*20*).

Genetic evidence has established that the Ste20p kinase acts between the G protein and the MAP kinase cascade (*6*). Ste20p phosphorylates Ste11p, the MEK kinase homolog, and thus may act as a MEK kinase kinase in the activation of the downstream elements of the pheromone response pathway (*32*). Binding of the G protein subunits to Ste5p may facilitate interaction of Ste20p with Ste11p. An alternative model is that Ste20p-mediated phosphorylation of Ste11p may occur in the absence of the G protein association with Ste5p, but subsequent activation of the MAP kinase cassette may require a signal generated by G protein–Ste5p association. Ste20p acts upstream of the Ste11p kinase in the pseudohyphal growth pathway, but this pathway is independent of both Ste5p and the G protein subunits (*33*). Our identification of a physical association of Ste4p with Ste5p suggests that receptor-mediated activation of the G protein can be transmitted to the MAP kinase cascade through this association.

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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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37. We thank D. Dignard for technical assistance, S. Fields for materials, R. Brousseau and A. Mazza for oligonucleotides, and Glaxo Group Research Limited for their support. T.L. was supported by a fellowship from the Deutsche Forschungsgemeinschaft and A.F.-L. by INSERM (France). National Research Council of Canada publication 38543.

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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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