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The eukaryotic cell division cycle is primary controlled by a family of protein serine/threonine kinases, the cyclin-dependent-kinases (CDKs). Activation of CDKs is dependent upon the interaction with an activating cyclin subunit. Presumably, these kinases progress the cell cycle by phosphorylation of critical cellular proteins. The detection of these transient kinase-substrate protein-protein interactions using conventional methods presents a major technical challenge, and is primarily responsible for our poor understanding of CDK substrates. The yeast two-hybrid system is a method for detecting protein-protein interactions. A major limitation of this method is the fact that only binary interactions can be detected. Using a modified version of the two-hybrid system, I am able to detect the interaction of ternary protein complexes. Using the cyclin D1-CDK6 protein complex as bait, I have identified the interaction of this complex with the tumor suppressor protein tuberin. Tuberin interacts specifically with the D-type cyclins, and this interaction has been found to exist in lysates from whole mouse embryos. Using the yeast three-hybrid system, it may be possible to detect the cellular substrates of cyclin-CDK complexes in human breast cells and further our understanding of the pathogenesis of human breast cancer.

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Background

A hallmark of many tumor cells is their ability to continuously cycle under conditions where normal cells would either be quiescent or proliferating at a reduced rate. Therefore, the molecular pathways controlling the cell cycle must inevitably interact with pathways which regulate cell growth and suppress tumorigenesis. Eukaryotic cell cycle progression is composed of multiple transitions between different cell cycle states. One of the most important transitions is during late G1, defined as START in yeast or the restriction (R) point in mammalian cells. Until this point, cells are sensitive to a wide variety of growth regulation, including extracellular growth factors, DNA damage, and inducers of cellular differentiation. Once past the START or R point, cells become largely refractory to these regulations and are committed to complete a new round of DNA replication. Deregulation of the START or R checkpoint may therefore allow cells to bypass normal restrictions on entry into S phase and promote an uncontrolled growth phenotype characteristic of tumor cells.

Biochemically, transitions through different cell cycle stages are primarily regulated by a family of closely related protein kinases, the cyclin-dependent kinases (CDKs), presumably by phosphorylating critical cellular proteins. The activity of CDKs are regulated at three different levels: binding with an activating subunit, a cyclin; subunit phosphorylation, and inhibition by a CDK inhibitor {544, 681, 486}. Both CDK and cyclins constitute a multigene family, different members presumably functioning to regulate different stages of the cell cycle through their combinatorial interactions (Fig. 1). The cyclins that are most closely linked to the regulation of mammalian START are the D-type cyclin-dependent CDK4 and CDK6 (Figure 1). Mammalian cyclin D1 was initially isolated by virtue of its ability to rescue yeast cells deficient for G1 cyclin function [CLN1, CLN2 and CLN3, {50}], its induction in response to colony-stimulating factor 1 {108}, and in a search for the putative oncogene *PRAD1* rearranged in parathyroid tumors {110}. Cyclin D1 is amplified in 15-20% of human breast cancers as part of the 11q13 amplicon and is also genetically linked to the *bcl-1* oncogene, a locus activated by translocation to an immunoglobulin gene enhancer in some B cell lymphomas and leukemias. Examination of human mammary tumors for the expression of the 11q13 locus indicated that the cyclin D1 gene, but not other proto-oncogenes such as *INT2* and *HST1*, is located at the position where a relevant oncogene is expected, based on detailed Southern analysis, and was found to be consistently amplified in breast cancer cells {16, 83, 127}. Transgenic expression of cyclin D1 in mammary tissue lead to the development of mammary hyperplasia and carcinoma {714}. Mice lacking cyclin D1 fail to undergo the massive proliferation of the mammary epithelial compartment associated with pregnancy {576}. The molecular mechanism by which altered cyclin D1 expression contributes to breast cancer development is not clear. One hypothesis is that the amplification of cyclin D1 leads to the elevated kinase activity of cyclin D1-CDK4/CDK6, resulting in inappropriate phosphorylation of cyclin D1 targeted substrate protein(s) whose function is involved in controlling mammary cell growth and whose activity is critically regulated by the cyclin D1-dependent kinases in G1. Thus far, the only substrate of cyclin D-CDK4/6 kinases that has been identified is the retinoblastoma susceptibility gene product, pRb whose growth suppressing activity is negatively regulated by the CDKs {97, 181}. Using a novel three-hybrid system that I have recently developed, I propose to address this critical issue by identifying cellular protein(s) that specifically interact with the cyclin D1-CDK6 complex in normal mammary epithelial cells (HMECs) and breast cancer cells.

The major negative regulation of CDKs in mammalian cells is accomplished by CDK inhibitors {486}. Two distinct families of CDK inhibitors, represented by two prototype CDK inhibitors, p21 and p16, have been identified in mammalian cells. p21 was first discovered in normal human fibroblast cells as a component of cyclin D-CDK quaternary complexes that also contain proliferating cell nuclear antigen [PCNA, {58}]. *p21* (also known as *WAF1, CIP1, SDI1, PIC1*, CAP20) encodes a potent inhibitor of multiple cyclin-CDK enzymes. The expression of p21 is transcriptionally activated by the tumor suppresser p53 {69}. Mice lacking p21 are defective in G1 checkpoint control when exposed to gamma-irradiation {578, 574, 661}. The second member of this family, p27, has recently been shown to be a direct target of the viral oncoprotein E1A {715}, suggesting a potential mechanism by which a viral oncoprotein deregulates normal cell cycle control. Mice lacking p27 display multi-organ hyperplasia, suggesting that this protein is necessary for normal growth control {708, 709, 710}. p16/INK4a (also known as MTS1, CDK4I) was first observed as a CDK4-associated protein in human cells {60} and was subsequently cloned and characterized as a specific inhibitor of the CDK4/6-cyclin D kinases {291}. Three additional members of the INK4 family have since been isolated: p15^{INK4b} {321, 387, 417}, p18^{INK4c} {417, 488} and p19^{INK4d} {487, 488, 469}. p16 and its neighboring p15, have been found to be mutated or deleted in many human tumor derived cell lines as well as human primary tumors {486}. p16 is now believed to represent the first melanoma susceptibility gene, located on chromosome 9p21. p16 null mice develop multiple tumors at an early age, further suggesting a role for p16 as a tumor suppresser {679}, but this interpretation has been complicated due to the recent disovery of a second gene, p19^{ARF} (Alternative Reading Frame), occupying the same locus. p19ARF was discovered as a gene that shares the second exon of p16, albeit in a different

reading frame. Splicing of the first exon of ARF to the second exon of p16, results in a protein with no homology to any known CDK inhibitor. (Kamijo, Cell 91(5), 1997) It was subsequently found that ARF is capable of degrading the cellular protein mdm2, whose role is the degradation of p53. Thus, ARF is able to stabilize p53, inducing a cell cycle arrest. [Zhang, 92(6) 1998]. Interaction of members of the p21 inhibitor family with CDKs is stimulated by or dependent on the cyclin subunit, suggesting that the p21 family of inhibitors preferentially interact with cyclin-CDK complexes {469, 540}. As an example, human p27*Kip1* was isolated using CDK4 as bait in combination with cyclin D1 {327}. Co-expression of cyclin D1 and CDK6 using the yeast three-hybrid vector may allow me to identify potential novel members of the p21 CDK inhibitor family. In addition, expression of some CDK inhibitor genes, in particular members of the p16 family, exhibit remarkable tissue specificity {417, 469}. A screen specific for CDK4 and CDK6 interacting proteins has not previously been conducted in normal HMECs or breast cancer cells.

Hypothesis and Purpose

The yeast two-hybrid system is a method for detecting protein-protein interactions {340}. The assay takes advantage of the modular nature of the GAL4 transcription factor which contains separable DNA binding (GAL4-BD)and transcriptional activation domains (GAL4-AD). In this system, two plasmids are introduced into yeast-one plasmid that encodes the GAL4-BD fused to the protein of interest, the "bait", and the other encoding the GAL4-AD fused to another protein, "the prey", often introduced as a cDNA library. If the bait and the prey proteins interact, the GAL4-BD and the GAL4-AD are brought into close proximity and activate expression of a nutritional reporter gene under the control of a GAL4-responsive promoter, allowing yeast cells to grow in the absence of the nutrient. Numerous protein-protein interactions have since been discovered using this system, including the interaction of the CDK inhibitor, p18*INK4c*, with human CDK6 in this laboratory {417}.

A major limitation of the two-hybrid system is that only one gene can be expressed from the bait plasmid. As a result, only proteins that form a binary complex with the bait protein can be identified. It can not detect interactions that require the bait protein complexed with a second protein. This becomes critical in identifying substrates of CDK enzymes since formation of the cyclin-CDK complex is necessary to possess enzymatic activity toward its substrate. In addition, members of the p21 CDK inhibitor family are known to interact preferentially, if not exclusively, with the cyclin-CDK complexes. Here the P.I. describes the development of a yeast three-hybrid system (Fig. 2) that allows the co-expression of the GAL4 BD-bait (CDK6) fusion and a second gene (cyclin D1), which then form a complex capable of interacting with the GAL4 AD-prey fusion. This approach will be used to screen both a normal HMEC library as well as a human breast cancer library. Identification of cellular protein(s) interacting with the cyclin D1-CDK6 complex and any changes between normal and transformed cells may lend insight into the process of cellular transformation associated with breast cancer.

Methods

To conduct a yeast three-hybrid screen, two vectors, pGBT6 and pGBT7 (Fig. 3), were constructed (see figure legend for details).

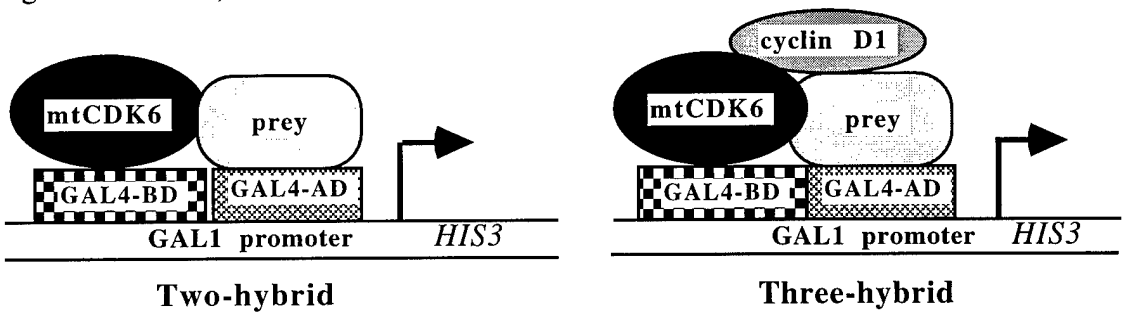

Fig. 2. Schematic comparison of yeast two-hybrid and three-hybrid systems.

Catalytically inactive CDK4(K35M), but not wild type, in association with its partner cyclin D1 forms a stable ternary complex with its substrate pRb {104}. In order to stabilize the substrate interaction with cyclin D1-CDK6, I have constructed a similar catalytically inactive CDK6 mutant, mtCDK6, by mutating the lysine residue at codon 43 to methionine (K43M) in the ATP binding domain through PCR mediated site-directed mutagenesis. This mutant CDK6 cDNA was fused in-frame with the GAL4-BD in both pGBT6 and pGBT7. The human cyclin D1 was inserted with (pGBT7-mtK6/D1) or without (pGBT6-mtK6/D1) fusion with the NLS. Both constructs were introduced into the yeast strain HF7c (*his3-200, leu2-3, trp1-901*). To test the three-hybrid system, prey plasmids encoding two types of Cyclin D1-CDK6 interacting proteins were transformed into Hf7c in combiantion with either pGBT6-mCDK6/D1, pGBT7-mCDK6/D1, or pGBT7-mCDK6.

In the first assay, the C-terminus of pRb that is commonly used as the substrate of *in vitro* kinase assays for cyclin D-CDK4 and cyclin D-CDK6 enzymes was fused in-frame with the GAL4-AD in a yeast expression plasmid, pGAD, that carries the leu marker. Yeast cells transformed with pGAD-Rb with pGBT6-mtCDK6/D1, pGBT7-mtCDK6/D1, but not with pGBT7-mtCDK6 that does not express cyclin D1, grew on media lacking leucine, tryptophan and histidine (figure 4), indicating that mtCDK6 and D1 were interacting with pRb protein and that this interaction is cyclin D1 dependent.

In the second assay, the CDK inhibitor p21 was fused in-frame with the GAL4-AD of pGAD, as described above. Yeast cells transformed with pGBT7-mCDK6/D1 and pGAD-p21 grew on media in the absence of leucine, tryptophan and histidine, while yeast transformed with pGBT7-mCDK6 and pGAD-p21 did not (figure 4). In this experiment, pGBT6-mCDK6/D1 was not assayed.

Because of the low level of leakage of the GAL1 promoter and the presence of dying yeast cells whose histidine may be secreted into the media to support the growth of false positive yeast cells, a second independent assay for the bait and prey protein interaction was carried out. Interestingly, while both pGBT7 (cyclin D1 fused with NLS) and pGBT6 (cyclin D1 synthesized without the NLS) are capable of activating the expression of the *HIS3* gene, only pGBT7 is able to activate the expression of the *lacZ* gene (data not shown), suggesting that the nuclear localization signal enhances the interaction of cyclin D1 with CDK6(K43M) and the pRb.

Yeast Three-Hybrid Screen

The major effort of this proposal is to use the three-hybrid system to identify cellular proteins, from normal HMECs and breast cancer cells, that interact with the cyclin D1-CDK6 complex. Prior to generating the needed HMEC cDNA libraries, however, the three-hybrid system was tested for its ability to detect cyclin D1-CDK6 interacting proteins in an actual library sceen. There was some concern that in actual use the three hybrid system may either primarily detect interactions with the GAL4-BD fusion protein, or that there may be an unworkably high background, due to the expression of three proteins in a single yeast cell. In order to address these concerns, an available human keratinocyte cDNA library was used to assess the ability of the three hybrid system to detect cyclin D1-CDK6 interacting proteins.

One hundred micrograms of plasmid DNA from a human keratinocyte (HaCat) library subcloned into pACT2 was transformed separately into yeast HF7c cells pGBT7-mtCDK6/D1, pGBT7-mtCDK6/D3 or pGBT7-mtCDK6 using the lithium acetate method. After incubating on selective media at 30°C for between three and four days, positive colonies that grew on histidine, leucine and tryptophan deficient media were patched onto the same selective media to confirm their growth phenotype and then assayed for the expression of β-gal activity by a qualitative filter assay. The library plasmid insert was amplified from positive yeast colonies by PCR using a pair of oligo primers specific to the pACT2 prey plasmid. PCR products from all positive colonies were digested by a frequently cutting restriction enzyme (either Alu I or HaeIII). cDNA clones with identical digestion patterns were grouped and a representative clone from each group was sequenced. The results from these screens is summarized in Table 1.

Importantly, both p21 and the pRb family member p130 were isolated using pGBT7-mtCDK6/D1 but not pGBT7-mtCDK6. p21 is known to preferentailly bind to cyclin-CDK complexes and p130 is a known substrate of the cyclin D/CDK6 complex. These results are a strong indication that the three-hybrid system is working as expected. In order to examine both the validity, specificity, and the type of interactions being detected using the three-hybrid screen, experiments were conducted to confirm the interaction in yeast, assay the interaction in a system outside of yeast, and to determine whether the isolates were indeed capable of being phosphorylated by the Cyclin D1/CDK6 complex.

Confirmation and characterization of positive clones interacting with mtCDK6/cyclin D

For most of the positive clones listed in Table 1, the library plasmid was rescued, confirmed by restriction mapping and re-transformed into Hf7c yeast. The only exceptions are those clones that were either known or suspected false positives. In addition, the positive clones were tested by two-hybrid assay for their ability to interact with cyclin D1 alone, cyclin D3 alone, mtCDK6 alone, and by three-hybrid assay for their ability to interact with the mtCDK6/cyclin D1 complex and the mtCDK6/cyclin D3 complex. Each positive clone was able to interact with cyclin D alone, but none were capable of interacting with mtCDK6 alone, indicating a cyclin D dependent interaction between isolated proteins and CDK6 and formation of a ternary CDK6-cyclin D-prey protein complex in yeast cells (data not shown).

Of the proteins isolated from the two three-hybrid screens (cyclin D1/mtCDK6 and cyclin D3/mtCDK6), fibronectin receptor shows a preference for cyclin D3 over cyclin D1, while tuberin, thymidine kinase and peroxisomal enoyl- CoA-hydratase (ECH) show a preference for cyclin D1. In addition, both tuberin and ECH interact with mtCDK6/cyclin D1 in the presence of the *HIS3* inhibitor, 30 mM 3-amino-triazole (3-AT), indicative of a strong interaction. In contrast, tuberin and ECH interact weakly with the mtCDK6/cyclin D3 complex, as judged by poor growth on media lacking histidine and a lack of interaction detected during the library screening. These results suggest that despite a high degree of sequence similarity between cyclin D1 and D3, they may target the same catalytic CDK6 subunit to distinct substrate proteins.

As an initial independent confirmation of clones believed to be interacting with the mtCDK6/cyclin D complex, GST fusion proteins were purified for some positive clones. These GST fusion expression plasmids were transformed into the protease deficient bacterial strain BL21(DE3) and cultured in LB media with 100μg/mL ampicillin. At an OD_{600} between 0.5 and 1.0, the cultures were induced with IPTG. Briefly, cultures were lysed in NP-40 lysis buffer, treated with lysozyme and sonicated. Supernatants were then incubated with GST-sepharose beads for one hour. The beads were washed three times with lysis buffer and then used for either a GST pulldown assay or a CDK6/cyclin D1 kinase assay. (Isolates from the mtCDK6/cyclin D3 screen have not been tested). For the GST pulldown assay, cyclin D1was *in vitro* translated and incubated with the GST fusion protein on beads. Samples were run on a 12.5% SDS-PAGE and exposed to film. Tuberin, thymidine kinase and H4, a novel protein, were found to bind to cyclin D1 in this assay (data not shown). These results indicate that the cyclin D1-mtCDK6 interacting proteins isolated form the three-hybrid screen are able to interact in a context other that yeast, suggesting that the isolates represent true positives. In order to examine whether any of the isolates may represent subtrates of the cyclin D1-CDK6 complex, an *in vitro* kinase assay was utilized.

For the kinase assay, GST fusion proteins were incubated on beads with baculovirus extract expressing either CDK6 alone, cyclin D1 alone, or CDK6 and cyclin D1 together. Positive clones were found to be phosphorylated when incubated with both CDK6 and cyclin D1 both not with either component alone (data not shown). In this assay, consistent with the GST pulldown assay, tuberin, thymidine kinase and clone H4, a novel protein, were found to be phosphorylated. ECH and TRAP1 do not appear to be phosphorylated, although it should be noted that these are not the full length proteins. Based on these experiments and the aims of this project, I chose to further characterize the interaction of the cyclin D/CDK6 complex with tuberin.

To confirm the interaction of tuberin with the $CDK6^{K43M}$ /cyclin D1 complex, we investigated the interaction in both mammalian and insect cell systems. A cDNA encoding the hemagglutinin-tagged (HA) tub-C region was placed under the control of the cytomegalovirus promoter and transfected into human Saos-2 osteosarcoma cells either alone or in combination with one of the three D-type cyclins or cyclin E. Immunoprecipitation coupled with western blotting revealed that tub-C is capable of interacting with all of the D-type cyclins, but not cyclin E (Fig. 5). This observation was confirmed in insect Sf9 cells infected with baculovirus including cyclins D, A, B, C, and E (data not shown). These results indicate that tuberin specifically binds the D-type cyclins and can bind to cyclin D in a binary fashion. This data also suggests that the CDK and tuberin binding site are distinct, allowing the formation of a ternary complex as observed in yeast.

To confirm that the observed interactions of cyclin D with tuberin occur at endogenous protein levels *in vivo*, we examined the interaction in whole mouse embryo cell extracts from day 9.5 of gestation. Immunoprecipitation with antibody to tuberin followed by western blotting using a monoclonal antibody to cyclin D3 revealed that tuberin was able to co-immunoprecipitate cyclin D3 (Fig.6). The majority of cellular cyclin D3 is not complexed with tuberin, but cyclin D3 associated with the total cellular tuberin at a proportionally high level. Although tuberin is expressed at a low level its role in development is essential, as rats deficient for tuberin die during embryogenesis (Rennebeck et al., 1998). This observation suggests that the interaction of cyclin D with tuberin may be limited either to specific tissues or within specific temporal regions of the developing embryo. The absence or low levels of cyclin D1-tuberin complexes may be attributed to the relatively low level of cyclin D1 expression in the embryos.

These results indicate that using the yeast-three hybrid system a previously uncharacterized interaction between the cyclin D/CDK6 complex and a tumor suppressor protein has been identified. It is unclear as to whether this interaction has a role in breast cancer, and further studies will be needed to determine whether tuberin is expressed in breast tissue. Alternatively, this system may be used to screen a human breast cancer cDNA library.

Table 1. Summary of HaCaT cDNA library screens

	Bait		
	pGBT7-mtCDK6	pGBT7-CDK6/cyc D1	pGBT7-CDK6/cyc D3
Total number screened	$1X10^6$	$1X10^6$	$2.53X10^6$
Efficiency	$1X10^4/\mu g$	$1X10^4/\mu g$	$2.5X10^4/\mu g$
Number of clones analyzed	57	135	43
Gene			
CyclinD1	65%	23%	28%
Cyclin D2	5%	3%	5%
Cyclin D3	7%	0	0
p15INK4B	12%	11%	9%
p16 INK4A	5%	1	1
p21	0	7%	16%
p130	0	2%	1
Tuberin	0	15%	5%
CoxIII	0	15%	0
Peroxisomal enoyl-CoA-hydratase	0	11%	0
Thymidine Kinase	0	5%	0
Fibronectin receptor	0	0	5%
Dopachrome tautomerase	0	0	5%
Ubiquinone	0	0	5%
Novel	4%	8%	12%
Other	2%	3%	0

III-B.4.b Illustrations/Diagrams/Chemical Syntheses

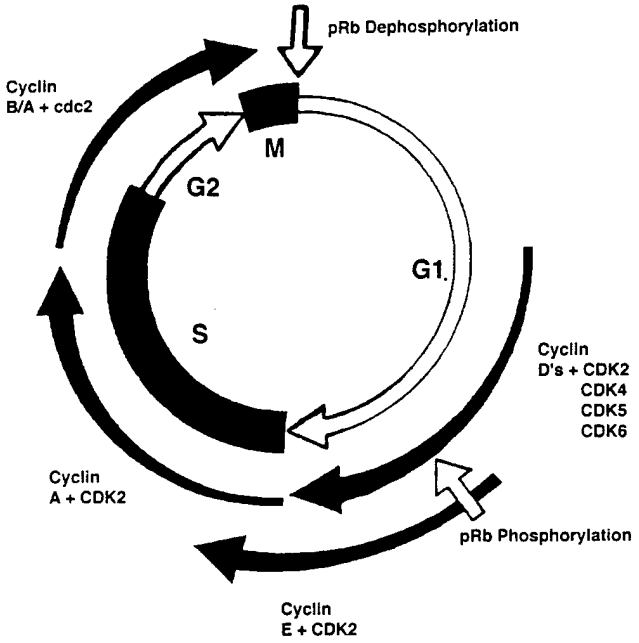

Figure 1. **General schematic of the mammalian cell cycle.**
Shown are the interactions of different cyclins and CDKs as well as the functional status of pRb. Adapted from Sherr, 1993.

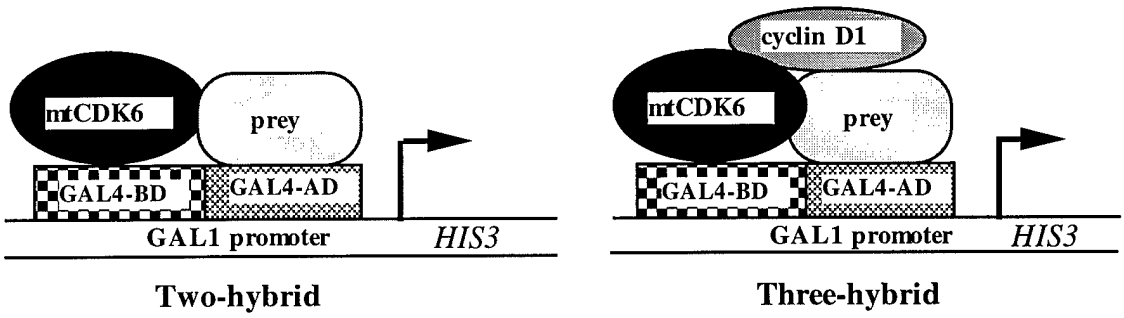

Figure 2. **Schematic comparison of the yeast two-hybrid and three-hybrid systems.**
While the two-hybrid system is suitable for detecting binary interactions, the three-hybrid is capable of detecting proteins in a ternary complex.

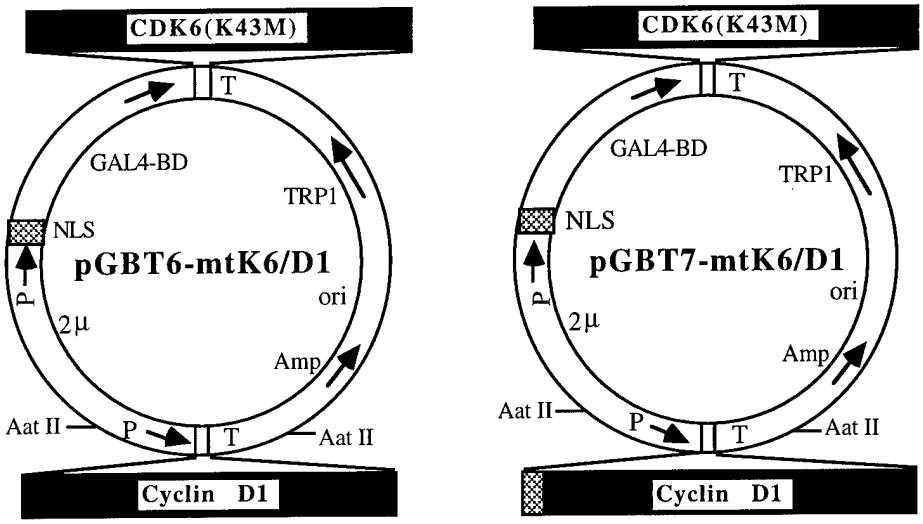

Fig. 3. **Yeast three-hybrid vectors.** Key: P: yeast ADH promoter, T: yeast ADH terminator, Ori: E.coli origin of replication, NLS: nuclear localization signal, GAL4-BD: GAL4 DNA binding domain, Amp: β-lactamase, TRP1: tryptophan

Both plasmids were derived from pGBT8, a modified form of pGBT9 that has been widely used for yeast two-hybrid screening. In addition to the yeast 2m origin of replication, the E.coli origin of replication, and the ampicillin resistance gene, pGBT8 carries the tryptophan marker (TRP1) and a strong yeast alcohol dehydrogenase (ADH) promoter directing the expression of the yeast GAL4-BD followed by a multiple cloning sequence (MCS) for the insertion of the bait cDNA. A 915 base pair Aat II restriction fragment was generated from pGBT8 by PCR that contains an ADH promoter followed by a sequence encoding a nuclear localization signal (NLS, the first 73 amino acids of the GAL4), a unique MCS and the ADH termination sequence. This cassette was inserted into the Aat II site of pGBT8, resulting in pGBT7. A similar experimental methodology was used to generate pGBT6 that does not retain the nuclear localization signal. Both pGBT7 and pGTB6 were confirmed by restriction mapping and partial sequencing.

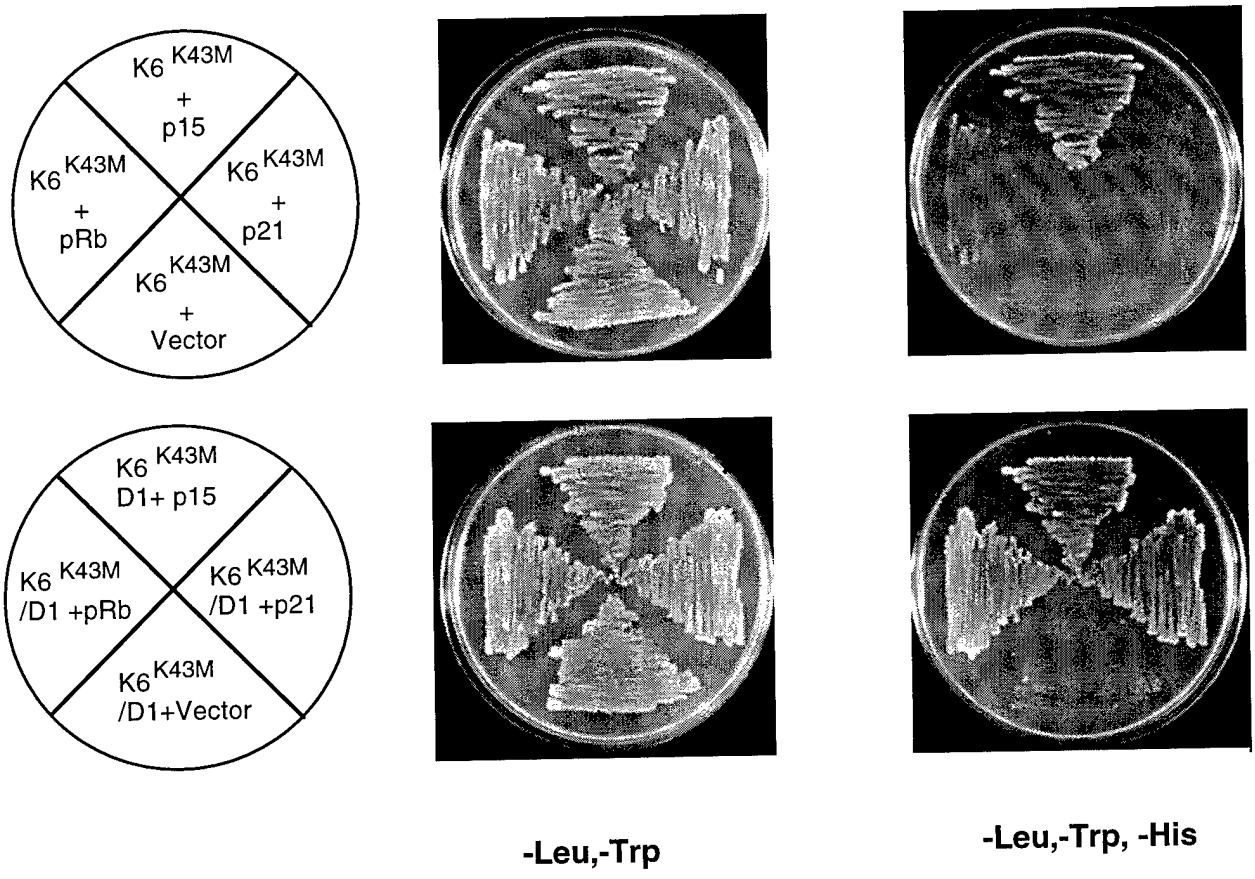

Figure 4. **pRb and p21 interact with mtCDK6 in a cyclin D dependent fashion.** Yeast cells were transformed with either pBGT7-mtCDK6 or pGBT7-mtCDK6/D1 as bait and various prey plasmids expressing the indicated gene subcloned into pGADGH. Yeast were plated onto -leucine, -tryptophan to show that both plasmids were present and that the yeast were viable. To select for yeast expressing interacting proteins, yeast were plated onto media lacking leucine, tryptophan and histidine. Plates were incubated at 30 celsius for three days and re-streaked onto fresh plates. These plates were again incubated at 30 celsius for three days.

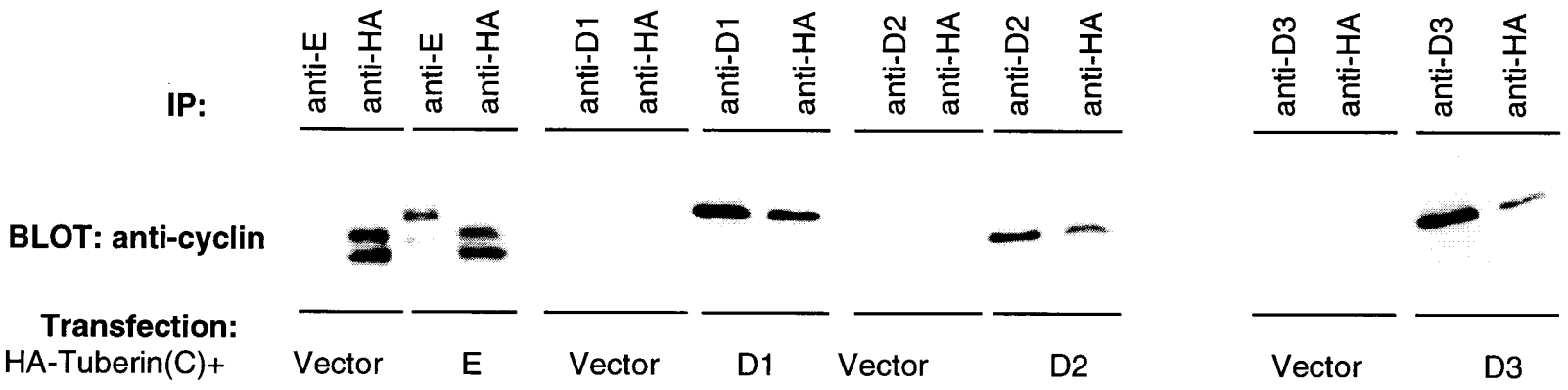

Fig.5 Tuberin interacts specifically with the D-type cyclins

Saos-2 cells were transiently transfected using lipofectamine reagent with plasmids encoding HA-Tub-c and the indicated cyclin or empty control vector under the control of the cytomegalovirus promoter.

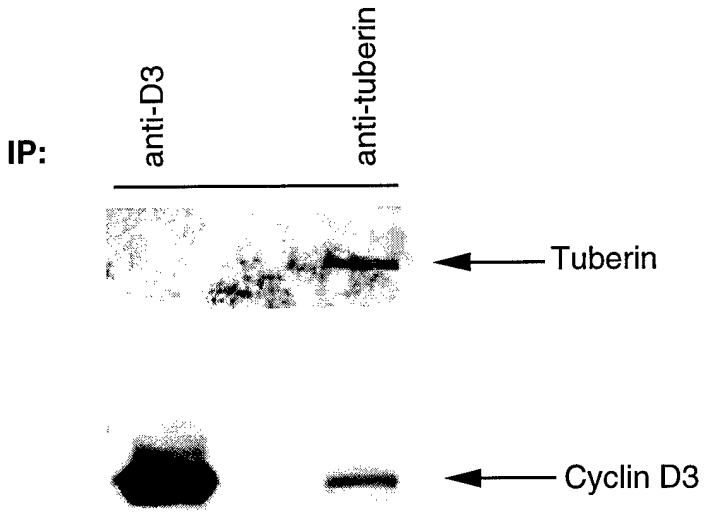

Fig. 6 In vivo association of tuberin with cyclin D3

Whole mouse embryos at day 9.5 of gestation were homogenized and lysed using NP-40 lysis buffer. Lysates were cleared and 300mg immunoprecipitated using either a monoclonal antibody to cyclin D3 (Neomarkers) or polyclonal antibody to the carboxyl-terminus of tuberin, a kind gift of Dr. Raymond Yeung. Samples were separated on a 12.5% polyacrylamide gel and transferred to nitrocellulose. Membranes were blotted with the same antibody used for immunoprecipitation for both tuberin (upper panel) and cyclin D3 (lower panel).