

ORIGINAL ARTICLE

Identification of mutations that disrupt phosphorylation-dependent nuclear export of cyclin D1

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Although cyclin D1 is overexpressed in a significant number of human cancers, overexpression alone is insufficient to promote tumorigenesis. *In vitro* studies have revealed that inhibition of cyclin D1 nuclear export unmasks its neoplastic potential. Cyclin D1 nuclear export depends upon phosphorylation of a C-terminal residue, threonine 286, (Thr-286) which in turn promotes association with the nuclear exportin, CRM1. Mutation of Thr-286 to a non-phosphorylatable residue results in a constitutively nuclear cyclin D1 protein with significantly increased oncogenic potential. To determine whether cyclin D1 is subject to mutations that inhibit its nuclear export in human cancer, we have sequenced exon 5 of cyclin D1 in primary esophageal carcinoma samples and in cell lines derived from esophageal cancer. Our work reveals that cyclin D1 is subject to mutations in primary human cancer. The mutations identified specifically disrupt phosphorylation of cyclin D1 at Thr-286, thereby enforcing nuclear accumulation of cyclin D1. Through characterization of these mutants, we also define an acidic residue within the C-terminus of cyclin D1 that is necessary for recognition and phosphorylation of cyclin D1 by glycogen synthase kinase-3 beta. Finally, through construction of compound mutants, we demonstrate that cell transformation by the cancer-derived cyclin D1 alleles correlates with their ability to associate with and activate CDK4. Our data reveal that cyclin D1 is subject to mutations in primary human cancer that specifically disrupt phosphorylation-dependent nuclear export of cyclin D1 and suggest that such mutations contribute to the genesis and progression of neoplastic growth.

Oncogene (2006) 25, 6291–6303. doi:10.1038/sj.onc.1209644; published online 29 May 2006

Keywords: cyclin D1; GSK-3 β ; CDK4; nuclear export; cancer

Introduction

The cell cycle is a tightly regulated cellular clock composed of four distinct phases: two gap phases, G1 and G2, separating the DNA synthetic (S) phase from mitosis (M). Progression through each phase of the cell cycle is driven by complexes minimally composed of a regulatory, cyclin, and a catalytic, CDK, subunit. Progression through G1 phase is initiated by mitogenic stimulation, which in turn initiates the expression and assembly of the D-type cyclins (D1, D2, D3) with their cognate catalytic partners, CDK4/6.

The cyclin D/CDK4 holoenzyme has two functions necessary for cell cycle progression. The first is the initiation of phosphorylation-dependent inactivation of the retinoblastoma family of proteins, Rb, p107 and p130 (Hatakeyama *et al.*, 1994; Harbour *et al.*, 1999; Calbo *et al.*, 2002; Farkas *et al.*, 2002; Leng *et al.*, 2002), which is subsequently completed by the cyclin E/CDK2 complex (Harbour *et al.*, 1999). The second involves the stoichiometric association of cyclin D/CDK4 with Cip/Kip family proteins. This association facilitates both cyclin D1/CDK4 activity through increased nuclear retention and subunit assembly, while simultaneously preventing Cip/Kip access to cyclin E/CDK2 complexes (Cheng *et al.*, 1998, 1999; Sherr and Roberts, 1999; Muraoka *et al.*, 2001, 2002; Alt *et al.*, 2002).

The D-type cyclins are unique in responding directly to mitogenic signaling pathways rather than signaling intrinsic to cell cycle progression. Expression of cyclin D1 requires Ras-dependent activation of the Raf–Mek–Erk kinase module (Albanese *et al.*, 1995; Lavoie *et al.*, 1996; Winston *et al.*, 1996; Aktas *et al.*, 1997; Kerkhoff and Rapp, 1997; Cheng *et al.*, 1998). In addition, maximal accumulation of cyclin D1 depends on phosphatidylinositol 3'-kinase-dependent activation of Akt and the concomitant inactivation of glycogen synthase kinase-3 beta (GSK-3 β) (Rodriguez-Viciana *et al.*, 1994; Kauffmann-Zeh *et al.*, 1997). Glycogen synthase kinase-3 beta-dependent phosphorylation of cyclin D1 on threonine 286 (Thr-286) triggers the nuclear export and cytoplasmic proteolysis of cyclin D1 via the 26S proteasome (Diehl *et al.*, 1998).

Perturbation of the cell cycle machinery is one of the hallmarks of cancer (Collecchi *et al.*, 2000). Cyclin D1,

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Received 7 February 2006; revised 27 March 2006; accepted 27 March 2006; published online 29 May 2006

the Bcl1 oncogene, is involved in the t(11;14)(q13;32) chromosomal translocation associated with mantle cell lymphomas (Lukas *et al.*, 1994; Lovec *et al.*, 1994a, b) and is amplified in a subset of breast, bladder, esophageal, lung and squamous cell carcinomas (Hall and Peters, 1996; Diehl, 2002). Yet, despite its overexpression in a number of malignancies, overexpression of wild-type cyclin D1 is not by itself sufficient to induce a transformed cellular phenotype in cell-based systems (Quelle *et al.*, 1993; Alt *et al.*, 2000). In contrast, as we have previously shown, expression of an artificially engineered cyclin D1-T286A or a naturally occurring alternative splice variant of cyclin D1 lacking the fifth exon, cyclin D1b – neither D1-T286A nor cyclin D1b is phosphorylated by GSK-3 β and thus, is refractory to phosphorylation-dependent nuclear export – has the capacity to drive transformation of murine fibroblasts in the absence of a collaborating oncogene (Alt *et al.*, 2000; Lu *et al.*, 2003). Cumulatively, our results support a model wherein dysregulation of cyclin D1 nuclear export increases its oncogenic potential, emphasizing the biological relevance of identifying cyclin D1 mutations in human cancer that target residues essential for these regulatory functions.

Little evidence exists for the identification of cyclin D1 mutations in human cancer that specifically target its phosphorylation at Thr-286 and/or nuclear export. Herein, we report the identification of such cyclin D1 mutations in esophageal cancer. The mutations specifically disrupt cyclin D1 phosphorylation on Thr-286, resulting in inhibition of S-phase specific nuclear export. In addition, through structure–function analysis of cancer-derived deletion mutants, we have identified structural requirements in cyclin D1 necessary for its recognition by GSK-3 β that serve to coordinate GSK-3 β -mediated phosphorylation of cyclin D1 on Thr-286 (Benzeno and Diehl, 2004). Indeed, our data demonstrate the occurrence of deregulated cyclin D1 nucleocytoplasmic shuttling in human cancer.

Results

Identification of cyclin D1 mutations in human cancer

Overexpression of nuclear export-defective cyclin D1 mutants in murine fibroblasts or in transgenic mice confers a neoplastic phenotype on expressing cells demonstrating the distinctive oncogenic potential of such cyclin D1 mutants (Alt *et al.*, 2000; Lu *et al.*, 2003; Gladden *et al.*, 2005). These data suggest that cyclin D1 may be subject to mutations in human cancer that specifically disrupt its nuclear export. To address this issue, we screened a panel of 90 primary human esophageal tumor-derived patient DNA samples for cyclin D1 mutations. We chose esophageal cancer owing to the high prevalence of cyclin D1 overexpression (40–60%) and its nuclear localization in esophageal adenocarcinoma and squamous cell carcinomas, a striking percentage of which are not associated with gene amplification (Adelaide *et al.*, 1995; Arber *et al.*, 1996; Bani-Hani *et al.*, 2000; Lin and Beerm, 2004). Sequencing exon 5 of

the cyclin D1 gene in this set of tumor samples revealed a threonine to arginine substitution at codon 286 (T286R) and a deletion encompassing codons 266–295 of cyclin D1 (Δ 266–295). The deletion is reminiscent of cyclin D1b, which is expressed as a consequence of cancer-specific alternative splicing (Lu *et al.*, 2003; Solomon *et al.*, 2003). Importantly, no such mutations were identified in either matched normal or in 100 unmatched normal esophageal tissue samples. Together, our results provide strong evidence that these cyclin D1 alterations are not a result of inherent polymorphisms in the cyclin D1 gene. Supporting evidence stems from the identification of a second mutation at Thr-286 (T/H) through a search of the available expressed sequence tag (EST) database. Unfortunately, we were unable to obtain the cell line from which this cyclin D1 allele was sequenced in order to confirm the presence of this mutation.

To characterize the properties of the cancer-derived mutants, we engineered NIH-3T3 cell lines that express either D1-T286R isolated from esophageal cancer or cyclin D1- Δ 289–292, a deletion mutant recently identified in endometrial cancer (Moreno-Bueno *et al.*, 2003). Neither D1-T286R nor D1- Δ 289–292 was phosphorylated at Thr-286 *in vivo* (data not shown). Consistent with inhibition of Thr-286 phosphorylation, both D1-T286R and D1- Δ 289–292 exhibited a significantly increased half-life relative to wild-type cyclin D1 (Figure 1a). In addition, the patient-derived cyclin D1 mutants featured constitutive nuclear localization as assessed by indirect immunofluorescence (Figure 1b and c) and retained their ability to support CDK4 catalytic activity as assessed by *in vitro* Rb kinase assays (Figure 1d).

To expand our analysis, we screened a panel of 20 independently derived human esophageal carcinoma cell lines for mutations in exon 5 of cyclin D1. Three of these, TE3, TE7 and TE12, harbored a mutation in cyclin D1 resulting in a proline to alanine substitution at codon 287. The proline-287 (P287)A mutation was confirmed in TE3 and TE7 cell lines obtained from an independent source. The expression of the alternatively spliced cyclin D1b was not detected in either TE3 or TE7, but could be detected in TE12 (Lu *et al.*, 2003). The capacity of GSK-3 β to function as a proline-directed kinase (Doble and Woodgett, 2003) implies that P287 is likely to be required for Thr-286 phosphorylation (Diehl *et al.*, 1997). To test this notion, NIH-3T3 cell lines were engineered to express the cyclin D1-P287A mutant. Immunoblot with a phosphospecific antibody revealed the absence of Thr-286 phosphorylation in the D1-P287A mutant (Figure 2a). Consistent with inhibition of Thr-286 phosphorylation, D1-P287A accumulated predominantly in the nucleus of expressing cells (Figure 2b and c). We also examined the subcellular localization of D1-P287A in TE3 (squamous cell carcinoma) and TE7 (adenocarcinoma), which harbor the endogenous cyclin D1-P287A mutant. D1-P287A was almost entirely nuclear in both TE3 and TE7, whereas wild-type cyclin D1, which is expressed in KYSE520 cells, exhibited both nuclear and cytoplasmic

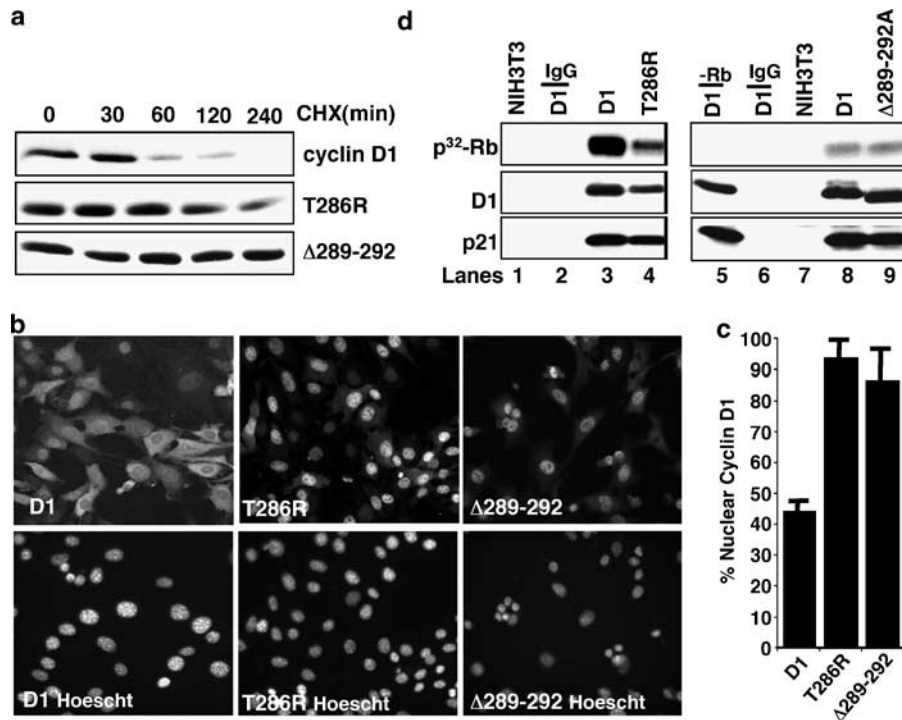


Figure 1 Cancer-derived cyclin D1 mutants are constitutively nuclear and retain kinase activity. (a) Cyclin D1 cancer-derived mutants are stabilized. NIH-3T3 cells stably overexpressing the indicated cyclin D1 mutants were treated with cycloheximide for the indicated intervals. Lysates prepared from the respective cell lines were processed for Western analysis using the cyclin D1 antibody. (b) NIH-3T3 cells stably overexpressing wild-type cyclin D1, D1-T286R and D1-Δ289-292 were fixed and the localization of either cyclin D1 protein was determined by immunofluorescence. Corresponding Hoechst staining is shown. (c) Quantification of immunofluorescence shown in (b). (d) D1, D1-T286R, and D1-Δ289-292 were precipitated from NIH-3T3 cells stably overexpressing the respective Flag-tagged protein and subjected to *in vitro* kinase assays using recombinant glutathione-*S*-transferase-Rb. Phosphorylated Rb protein was visualized by autoradiography following transfer to nitrocellulose membrane. The same membrane was immunoblotted for cyclin D1 and co-precipitated p21^{Cip1} protein. IP with normal rabbit immunoglobulin G was used as a control.

staining (Figure 3a and b). As the P287A mutation only occurs in one cyclin D1 allele in TE3/T37 cell lines, the absence of cytoplasmic staining suggests that accumulation of cyclin D1-P287A greatly exceeds that of cyclin D1 in these cells.

Phosphorylation of Thr-286 not only directs cyclin D1 nuclear export but also promotes rapid proteasome-dependent destruction of cyclin D1 (Diehl *et al.*, 1997). We therefore predicted that cyclin D1-P287A would exhibit an extended half-life relative to wild-type cyclin D1. We examined turnover of cyclin D1 versus D1-P287A in both the esophageal carcinoma-derived cell lines and NIH-3T3 cell engineered to express D1-P287A ectopically. Consistent with loss of Thr-286 phosphorylation and increased nuclear retention, cyclin D1-P287A exhibited decreased kinetics of turnover in both NIH-3T3 cells and the esophageal cancer cell lines, TE3 and TE7, harboring the constitutively nuclear D1-P287A mutant (Figure 3c). However, endogenous wild-type cyclin D1, in NIH-3T3 and KYSE520 cells, exhibited the expected short half-life of approximately 30 min (Figure 3c). Importantly, cyclin D1-P287A retained the ability to support CDK4 activation as demonstrated by phosphorylation of recombinant Rb using *in vitro* kinase assays (Figure 3d).

C-terminal acidic residues within cyclin D1 coordinate glycogen synthase kinase-3 beta-dependent phosphorylation

Although our data demonstrate that the deletion of amino acids 289–292 disrupts Thr-286 phosphorylation, the underlying mechanism is not inherently obvious. Through careful examination of the C-terminus of all three D-type cyclins, we noted the presence of aspartic acid residues positioned at ($n+3$) and ($n+6$) relative to Thr-286 (n) that are spatially conserved in other D-type cyclins (Figure 4a). Importantly, the deletion of amino acids 289–292 of cyclin D1 in endometrial cancer encompasses both aspartic acids D289 and D292. The crystal structure of GSK-3 β revealed a unique priming mechanism that regulates GSK-3 β -mediated phosphorylation of its substrates (Bax *et al.*, 2001; Dajani *et al.*, 2001; Frame *et al.*, 2001). Priming phosphorylation occurs at position ($n+4$) relative to the *bona fide* residue of GSK-3 β phosphorylation (n). Because cyclin D1 is not a recipient of a priming phosphorylation, we considered the possibility that a proximal negatively charged acidic residue (aspartic acid) within cyclin D1 might facilitate substrate binding within the dense positively charged substrate-binding groove of GSK-3 β . We generated cell lines expressing either double

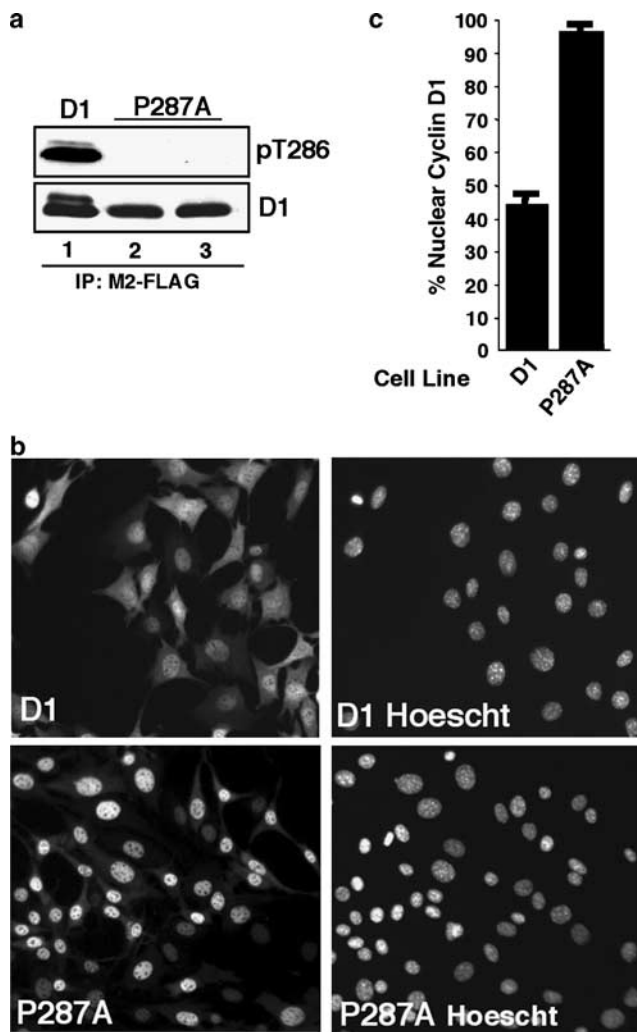


Figure 2 The tumor-derived cyclin D1-P287A mutant is constitutively nuclear. (a) Abrogated D1-P287A phosphorylation at threonine 286. Sf9 cells were infected with D1 or D1-P287A. Flag-tagged protein was precipitated with M2 antibody and assayed for phosphorylation at threonine 286 by Western blot using phosphospecific threonine 286 antibody. Total immunoprecipitated cyclin D1 was confirmed using cyclin D1 antibody. (b) D1-3T3 or D1-P287A-3T3 cells were fixed and the localization of cyclin D1 protein was determined by immunofluorescence. Corresponding Hoechst staining is shown. (c) Quantification of immunofluorescence shown in (b).

D1-D289/D292A, or the single D1-D289A and D1-D292A, alanine substitution mutants to assess their role in the regulation of Thr-286 phosphorylation. In contrast to D1-D292A, neither D1-D289/D292A nor D1-D289A was detectably phosphorylated on Thr-286 (Figure 4b, lanes 3–5), demonstrating that D289 positioned ($n+3$) from Thr-286 contributes to GSK-3 β -dependent phosphorylation of cyclin D1. In addition, to ensure these results were not owing to epitope masking, we tested the ability of GSK-3 β to phosphorylate wild-type cyclin D1 and D1-D289A *in vitro*. Cyclin D1/CDK4 and D1-D289A/CDK4 complexes purified from Sf9 lysates were used as substrates. Because a

majority of wild-type cyclin D1 molecules are phosphorylated at Thr-286 in Sf9 cells, we first subjected purified cyclin D1 and D1-D289A to phosphatase treatment. Following extensive washing to remove phosphatase, equivalent concentrations of the respective cyclin substrates were incubated with increasing concentrations of recombinant GSK-3 β . Wild-type cyclin D1 was efficiently phosphorylated by GSK-3 β , whereas phosphorylation of D1-D289A was significantly attenuated (Figure 4c and d). Similar results were obtained using the phospho-286 antibody to assess phosphorylation (data not shown). Importantly, all mutants retained the ability to associate with GSK-3 β (Figure 4e). Thus, abrogated cyclin D1 phosphorylation at Thr-286 is not the result of a disrupted cyclin D1–GSK-3 β complex. Our results identify residue D289, positioned ($n+3$) relative to Thr-286, as critical for efficient GSK-3 β -mediated phosphorylation of cyclin D1.

These data demonstrate that Asp-289 is required for Thr-286 phosphorylation and suggest that the negative character of the aspartic acid residue might mimic that associated with a phosphate group and direct GSK-3 β phosphorylation of cyclin D1. To test whether cyclin D1 is recognized by GSK-3 β as a primed substrate, we assessed the ability of the GID polypeptide (Gsk-3 β interacting domain of axin) to inhibit GSK-3 β phosphorylation. GSK-3 β -interacting domain of axin preferentially inhibits GSK-3 β activity toward unprimed substrates (Yost *et al.*, 1998; Hedgepeth *et al.*, 1999; Fraser *et al.*, 2002; Zhang *et al.*, 2003). For these experiments, the C-terminal 41 residues of either cyclin D1 or D1-T286A were fused to glutathione-S-transferase (GST), expressed in bacteria and used as substrates (Diehl *et al.*, 1998), whereas protein phosphatase-1 inhibitor 2 (I-2) protein was used as an unprimed substrate control (Park *et al.*, 1994; Zhang *et al.*, 2003). As expected, GSK-3 β phosphorylation of I-2 was inhibited by GID in a concentration-dependent manner (Figure 4f (lanes 7–9) and g). In contrast, GID did not alter GSK-3 β -mediated phosphorylation of cyclin D1 (Figure 4f (lanes 2–4) and g), demonstrating that phosphorylation of cyclin D1 by GSK-3 β differs from that of a true unprimed substrate.

The absence of Thr-286 phosphorylation in the D1-D289/292A and D1-D289A mutants suggested that both might accumulate in the nuclear compartment and be refractory to proteasomal degradation. Indeed, both mutants exhibited exclusively nuclear localization patterns (Figure 5a and b) as determined by immunofluorescent staining. Consistent with the absence of Thr-286 phosphorylation and their nuclear accumulation, both D1-D289A and D1-D289/292A exhibit a half-life in excess of 3 h, which is comparable to cyclin D1-T286A (Figure 5c). Although phosphorylation at Thr-286 was absent in both D1-D289A and D1-D289/292A mutants, both retained the ability to bind to Cip/Kip proteins and to support Rb kinase activity (Figure 5d), emphasizing the retained structural and functional integrity of these mutants. These data support a model wherein D289 functions to coordinate GSK-3 β -mediated phosphorylation of cyclin D1.

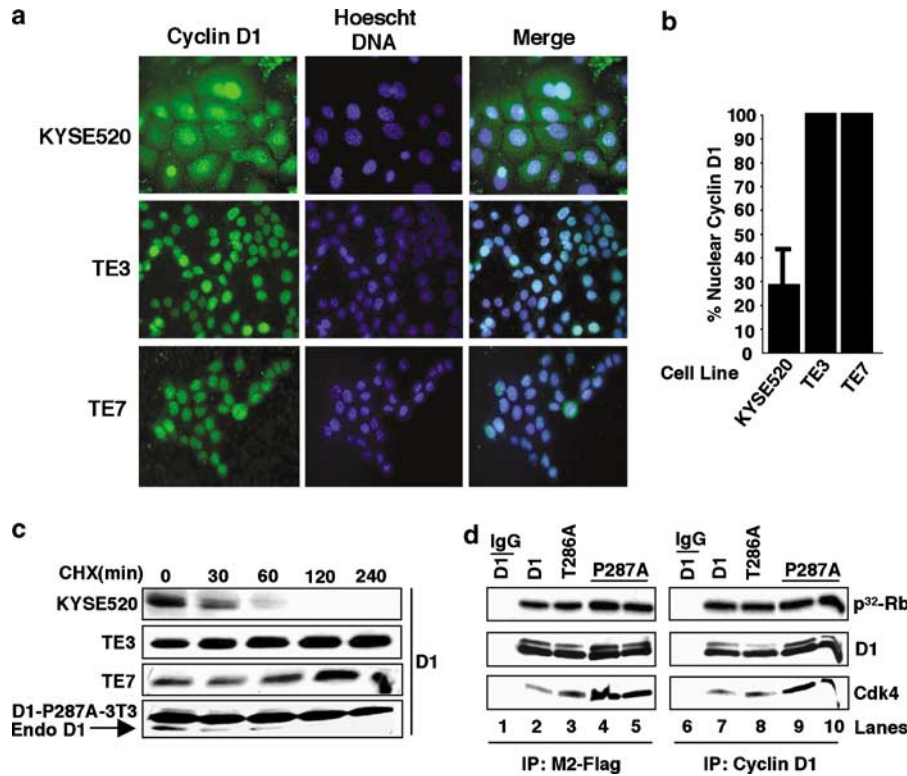


Figure 3 Identification of esophageal carcinoma-derived cell lines harboring cyclin D1-P287A. (a) The subcellular localization of endogenous cyclin D1 protein in KYSE520 and D1-P287A in TE3 and TE7 cell lines was determined by immunofluorescence using a cyclin D1 antibody (Ab3, Calbiochem, San Diego, CA, USA) followed by fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (green). Corresponding Hoechst DNA staining is shown (blue) and a merged view (Merge) of both channels (green/blue). (b) Quantification of immunofluorescence is shown in (a). (c) NIH-3T3 cells stably overexpressing D1-P287A and patient-derived KYSE520, TE3 and TE7 cell lines were treated with cycloheximide for the indicated intervals. Lysates from the indicated cell lines were assayed for cyclin D1 expression by Western analysis using the cyclin D1 antibody. (d) Following co-infection of D1, D1-T286A and D1-P287A with CDK4 in Sf9 cells, cyclin D1 protein was immunoprecipitated using either the M2 antibody or cyclin D1 antibody and assayed for its ability to support Rb kinase activity, visualized by autoradiography following transfer onto nitrocellulose membrane. The same membrane was processed for Western blot analysis of phospho-serine 780 Rb, cyclin D1 and co-precipitating CDK4.

Cancer-derived cyclin D1 mutants exhibit oncogenic properties relative to wild-type cyclin D1

Cells engineered to overexpress cyclin D1 display a contracted G1 interval (Quelle *et al.*, 1993) but do not exhibit a transformed phenotype. In contrast, a cyclin D1 mutant that cannot be phosphorylated at Thr-286 (D1-T286A) and is constitutively nuclear promotes cellular transformation (Alt *et al.*, 2000; Gladden *et al.*, 2005), demonstrating that disruption of cyclin D1 nuclear export is an oncogenic event. Thus, we hypothesized that cancer-derived cyclin D1 mutants that cannot be phosphorylated at Thr-286 and are thus constitutively nuclear should exhibit an increased transforming potential relative to wild-type cyclin D1. We tested this hypothesis using NIH-3T3 or NIH-3T3 derivatives that overexpress Flag-tagged mutant cyclin D1 isoforms and analysed these cells for characteristics of transformation. As published previously (Alt *et al.*, 2000), early passage cell lines displayed normal growth control (data not shown). In contrast, over the course of five independent experiments utilizing cell lines of passage p10 or later, cyclin D1 mutants, D1-T286R and D1-Δ289–292, reproducibly formed foci (Figure 6a). Consistent with these results, identical

results were obtained in experiments analysing anchorage-independent growth in soft agar, a more stringent criterion for oncogenic potential (Figure 6b and c). These data demonstrate that tumor-derived cyclin D1 mutations targeting Thr-286 phosphorylation and nuclear export confer an oncogenic advantage to cells expressing mutant forms of cyclin D1 that exhibit an increased capacity to drive neoplastic growth in the absence of a collaborating oncogene.

Transformation by constitutively nuclear cyclin D1 correlates with CDK4 activation

Cyclin D1 was initially characterized as the allosteric regulatory subunit for CDK4/6 kinases. However, accumulating evidence suggests that cyclin D1 can also function as a transcriptional regulator independent of its capacity to regulate CDK activity (Fu *et al.*, 2004). These observations prompted us to determine whether transformation by cyclin D1 is kinase dependent. To do so, we generated a cyclin D1-K114E (and compound T286A/R point mutants) that was previously characterized *in vitro* as ‘kinase-dead’ (Hinds *et al.*, 1994; Coqueret, 2002). This single amino-acid change,

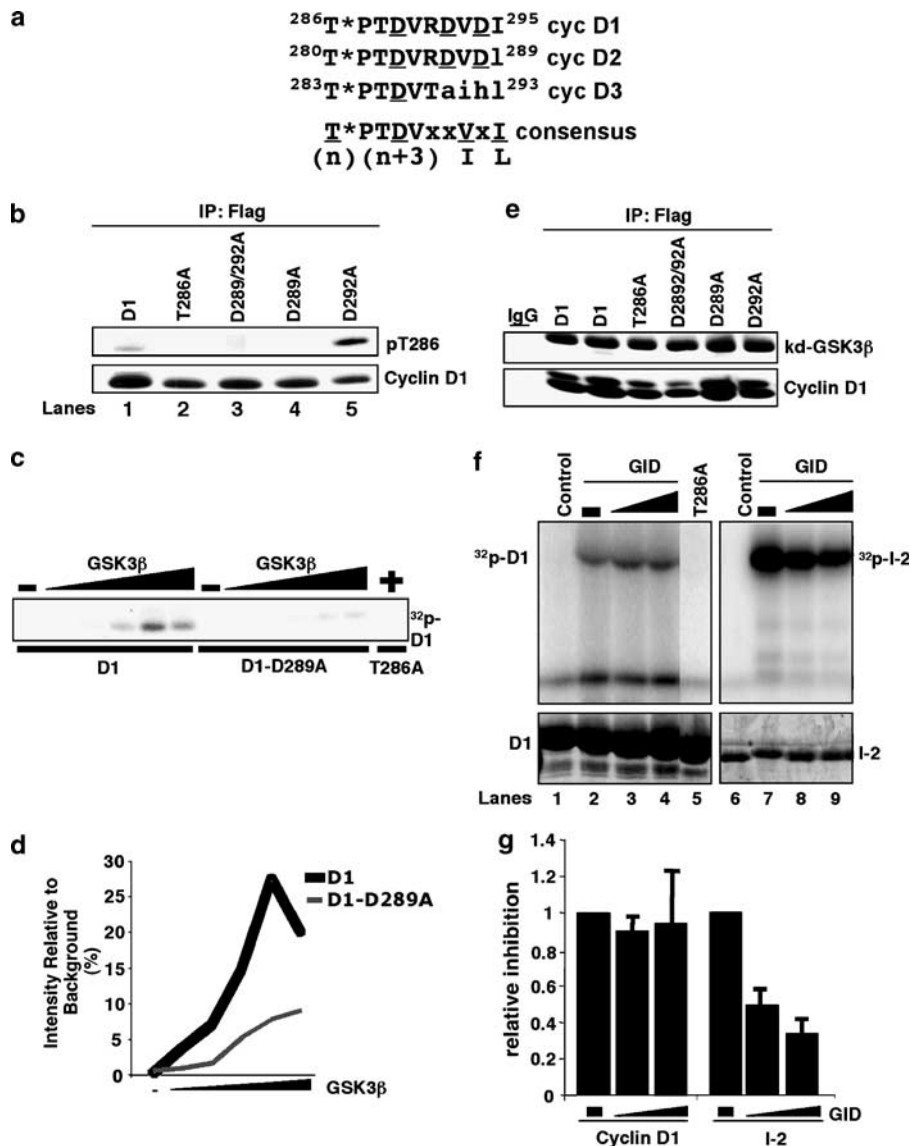


Figure 4 GSK-3 β phosphorylation of cyclin D1 depends on the integrity of aspartic acid D289. **(a)** Schematic of spatially conserved acidic aspartic acid residues within the C-terminus of D-type cyclins. (x) = Non-conserved amino acids, (n) = GSK-3 β phosphorylation site and (n + 3) = conserved proximal aspartic acid 289. **(b)** threonine 286 phosphorylation status of cyclin D1 mutants. Cyclin D1 was precipitated using M2 antibody, and threonine 286 phosphorylation was assayed by immunoblot using the phospho-threonine 286 antibody. Total cyclin D1 was confirmed using an antibody that recognizes both phosphorylated and unphosphorylated species. **(c)** Purified cyclin D1 or D1-D289A were incubated with increasing concentrations of recombinant GSK-3 β and [γ - 32 P]ATP. D1-T286A, as a negative control, was incubated with the highest concentration of GSK-3 β (+). Cyclin D1 phosphorylation was visualized by autoradiography. **(d)** Quantification of γ - 32 P-incorporation is shown in (c). **(e)** GSK-3 β co-precipitates with wild-type and mutant cyclin D1 isoforms. Sf9 lysates co-expressing kinase-defective GSK-3 β and the indicated Flag-D1 isoforms were precipitated with the M2 antibody. Cyclin D1 and co-precipitating GSK-3 β levels were confirmed by immunoblot. IP with immunoglobulin G was used as a control. **(f)** GSK-3 β -interacting-domain of axin (GID) fails to inhibit GSK-3 β phosphorylation of cyclin D1. Purified C-terminal glutathione-S-transferase-cyclin D1 or recombinant inhibitor 2 (I-2) was added to *in vitro* kinase reactions in the presence of GSK-3 β and [γ - 32 P]ATP. Phosphorylated proteins were separated on a denaturing polyacrylamide gel and visualized by autoradiography. Synthesized GID₃₈₀₋₄₀₄ peptide (GID) was added at increasing concentrations (black triangle). Cyclin D1 and I-2 protein levels were visualized by Coomassie staining. (-) = No GSK-3 β and control = reactions in the absence of GID. D1-T286A (T286A) is a negative control for cyclin D1 phosphorylation. **(g)** Quantification of cyclin D1 and I-2 phosphorylation. Y-axis = normalized phosphorylation relative to control reaction. Results are representative of at least four independent experiments.

Lys114→Glu (K114E), is thought to disrupt cyclin D1-CDK4 binding. We generated NIH-3T3 cells that stably express cyclin D1-T286R-KE, D1-KE and T286A-KE. All three KE mutants exhibited localization and degradation kinetics identical to their counterparts:

D1, D1-T286A, D1-T286R (Figure 7a and b). To confirm whether the K114E mutation disrupts cyclin-CDK interactions, lysates prepared from NIH-3T3 cell lines overexpressing the respective K114E cyclin D1 mutant were precipitated with the M2 monoclonal

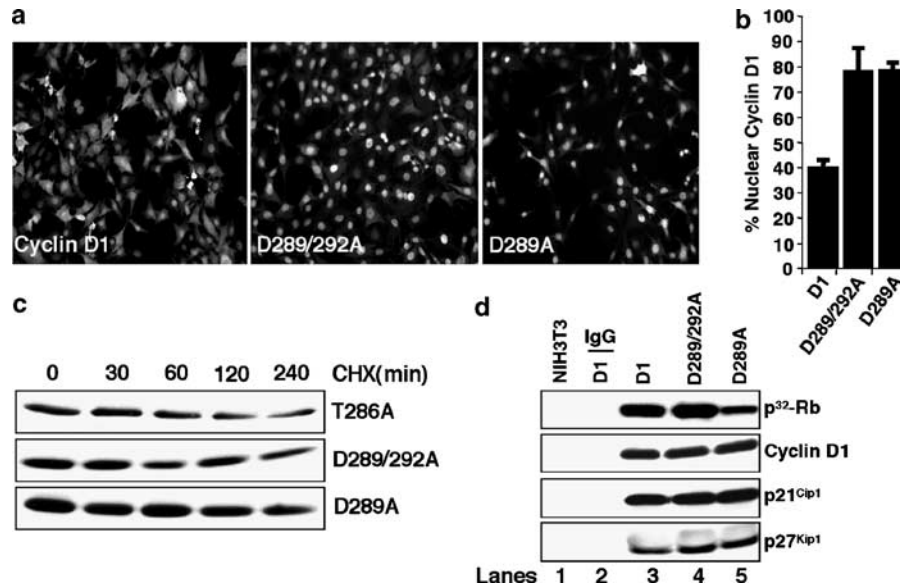


Figure 5 Mutation of D289 results in a constitutively nuclear and stable cyclin D1. (a) D1-, D1-D289/292A- and D1-D289A-3T3 cells were fixed and cyclin D1 localization determined by indirect immunofluorescence. (b) Quantification of cyclin D1 immunofluorescence shown in (a). (c) Stabilization of cyclin D1 mutants. NIH-3T3 cells stably overexpressing the indicated cyclin D1 proteins were treated with cycloheximide for the indicated intervals. Lysates were subjected to Western analysis using the cyclin D1 antibody. (d) Cyclin D1 mutants are kinase competent. Proteins from NIH-3T3 cells stably overexpressing the indicated Flag-tagged cyclin D1 were precipitated using M2 antibody and assayed for the ability to support Rb kinase activity. Phosphorylated proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE), transferred onto a nitrocellulose membrane and visualized by autoradiography. The same nitrocellulose membrane was processed for Western blot using antibodies for cyclin D1 and co-precipitating p21^{Cip1} and p27^{Kip1}.

antibody followed by immunoblot for CDK4. Surprisingly, endogenous CDK4 efficiently co-precipitated with all K114E derivative proteins, demonstrating that cyclin D1-CDK4 binding is not abolished *in vivo* (Figure 7c). Although mutant cyclins appear to associate effectively with CDK4 in cultured cells, immune complex kinase assays using the respective cyclin–CDK complexes revealed that all K114E mutant holoenzymes exhibited reduced kinase activity relative to wild-type counterparts, as assessed by total γ^{32} -P incorporation and immunoblotting for Rb phosphorylation at serine 780 (Ser780) (Kitagawa *et al.*, 1996) (Figure 7c). Densitometric scanning revealed KE mutants retain approximately 20% of wild-type cyclin D1 activity towards Rb.

Although the K114E mutants retain 20% of wild-type kinase activity, we reasoned that if kinase activation were critical for transformation of mouse fibroblasts, K114E mutant activity would be compromised. We assessed the ability of K114E mutant cyclin D1 derivatives to induce growth of NIH-3T3 cells in semisolid medium. Neither wild-type cyclin D1 nor the D1-KE triggered significant growth (Figure 6d). Although cell lines of passage 10 or greater expressing constitutively nuclear TA-KE and TR-KE were capable of growth in soft agar, growth, as assessed by colony number, was reduced by greater than 50% relative to both D1-T286A and D1-T286R, respectively (Figure 6d and e). As the KE-T286A mutant retained the ability to accelerate G1 progression (Figure 7d), their reduced transforming capacity cannot be attributed to defects in G1 progression. These data demonstrate that cyclin D1

mutants with compromised kinase activity are also compromised with respect to transforming potential.

To further investigate the mechanism underlying the retained kinase activity and assembly of the K114E cyclin D1 mutants with CDK4, we considered the possibility that association of D1-KE mutants with CDK4 *in vivo* was facilitated by a cellular cofactor. Indeed, p21^{Cip1} and p27^{Kip1} have been shown to promote stable assembly of the cyclin D1/CDK4 kinase (LaBaer *et al.*, 1997; Cheng *et al.*, 1999). We used insect Sf9 cells, which express no detectable endogenous p21^{Cip1}, to determine whether it facilitates the association of mutant cyclin D1 with CDK4. Sf9 were co-infected with baculovirus encoding wild-type cyclin D1 or D1-KE along with CDK4 and increasing concentrations of p21^{Cip1}. Western analysis of the cyclin D1 immune complexes revealed markedly reduced levels of CDK4 in D1-KE relative to wild-type cyclin D1 precipitates in the absence of p21^{Cip1} (Figure 8a, compare lanes 4 and 10). Serial titration of the p21^{Cip1} baculovirus increased co-precipitation of CDK4 with either immunoprecipitated D1 or D1-KE.

We subsequently investigated whether cyclin D1 KE-CDK4 complexes assembled by p21^{Cip1} in Sf9 cells were functionally active (Figure 8b and c). In the absence of p21^{Cip1}, D1-KE retained severely reduced kinase activity (Figure 8b, lanes 4, 5 and 9, 10) relative to wild-type D1 (Figure 8b, lanes 2 and 7) but above that of controls (Figure 8b, lanes 1 and 6). Immunoblot for phospho-Ser780 Rb confirmed reduced Rb phosphorylation by mutant D1-KE. Co-expression of p21^{Cip1} increased CDK4 association with D1-KE (Figure 8a)

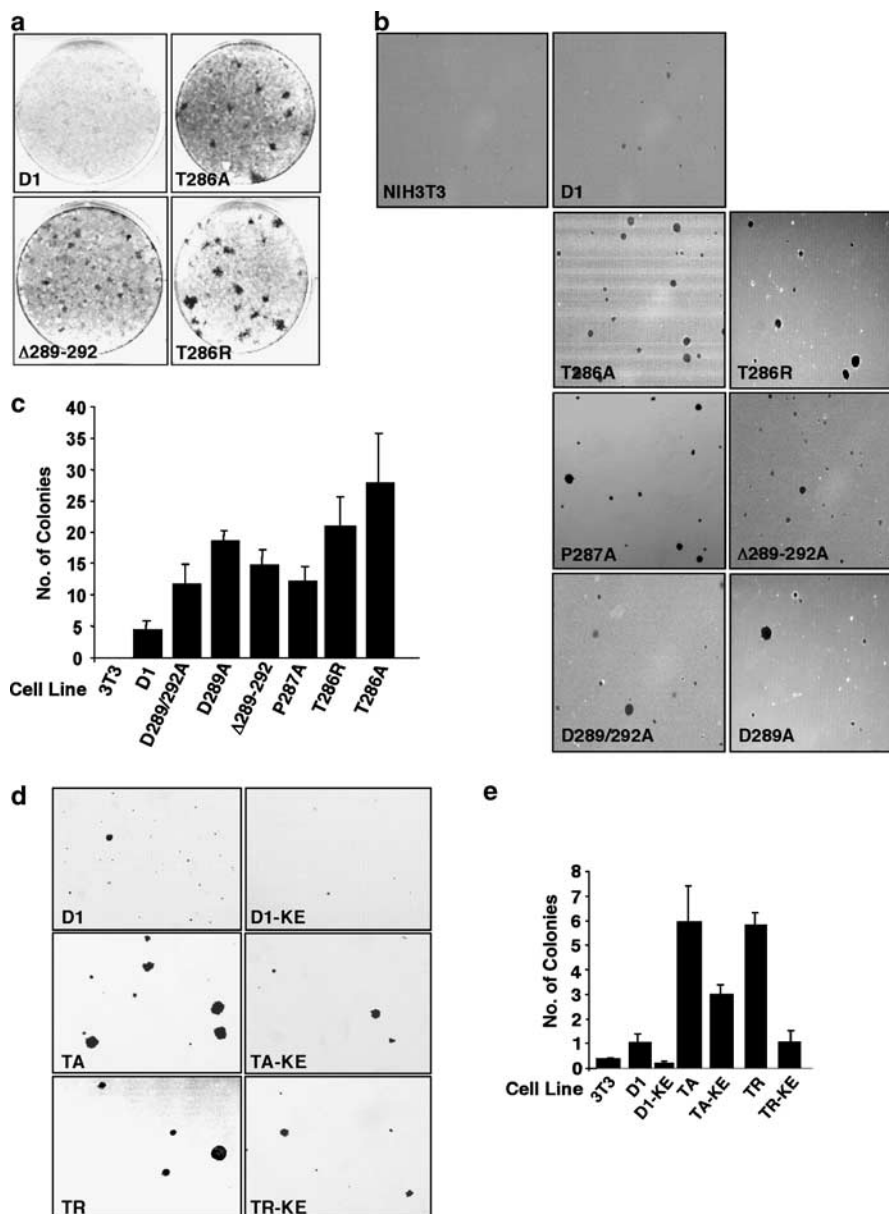


Figure 6 Tumor-derived cyclin D1 mutants transform murine fibroblasts. (a) Cells were plated in six-well dishes in medium supplemented with 5% fetal calf serum and stained with Giemsa to visualize foci following 21 days of growth. (b) NIH-3T3 cells stably overexpressing the indicated cyclin D1 proteins were plated in semisolid medium and allowed to proliferate for 21 days. Colonies were visualized by 0.01% neutral red stain. (c) Quantification of colonies scored in (b). (d) NIH-3T3 cells stably expressing the indicated cyclin D1 proteins were plated in semisolid medium and allowed to proliferate for 21 days. Colonies were visualized by 0.01% neutral red stain. (e) Quantification of colonies scored is shown in (d).

and increased the kinase activity of the D1-KE holoenzyme (Figure 8c). Strikingly, whereas high concentrations of p21^{Cip1} rapidly inhibit wild-cyclin D1 activity, inhibition of D1-KE kinase activity by p21^{Cip1} is achieved only at the highest p21^{Cip1} concentrations (Figure 8a and c). These results indicate that in the absence of a physiologic assembly factor *in vitro*, a K114E mutant fails to associate with CDK4. However, expression of p21^{Cip1} *in vivo* or its supplementation *in vitro* is sufficient to maintain assembly of a KE cyclin D1-CDK4 complex, providing for attenuated yet

sustained catalytic potential. As the K114E mutation does not result in differential association of cyclin D1 with the general transcriptional apparatus, our results suggest that reduced cellular transformation in the presence of a K114E mutation results from reduced nuclear CDK4 activation.

Discussion

Previous work has suggested that phosphorylation-dependent nuclear export of cyclin D1 is critical for

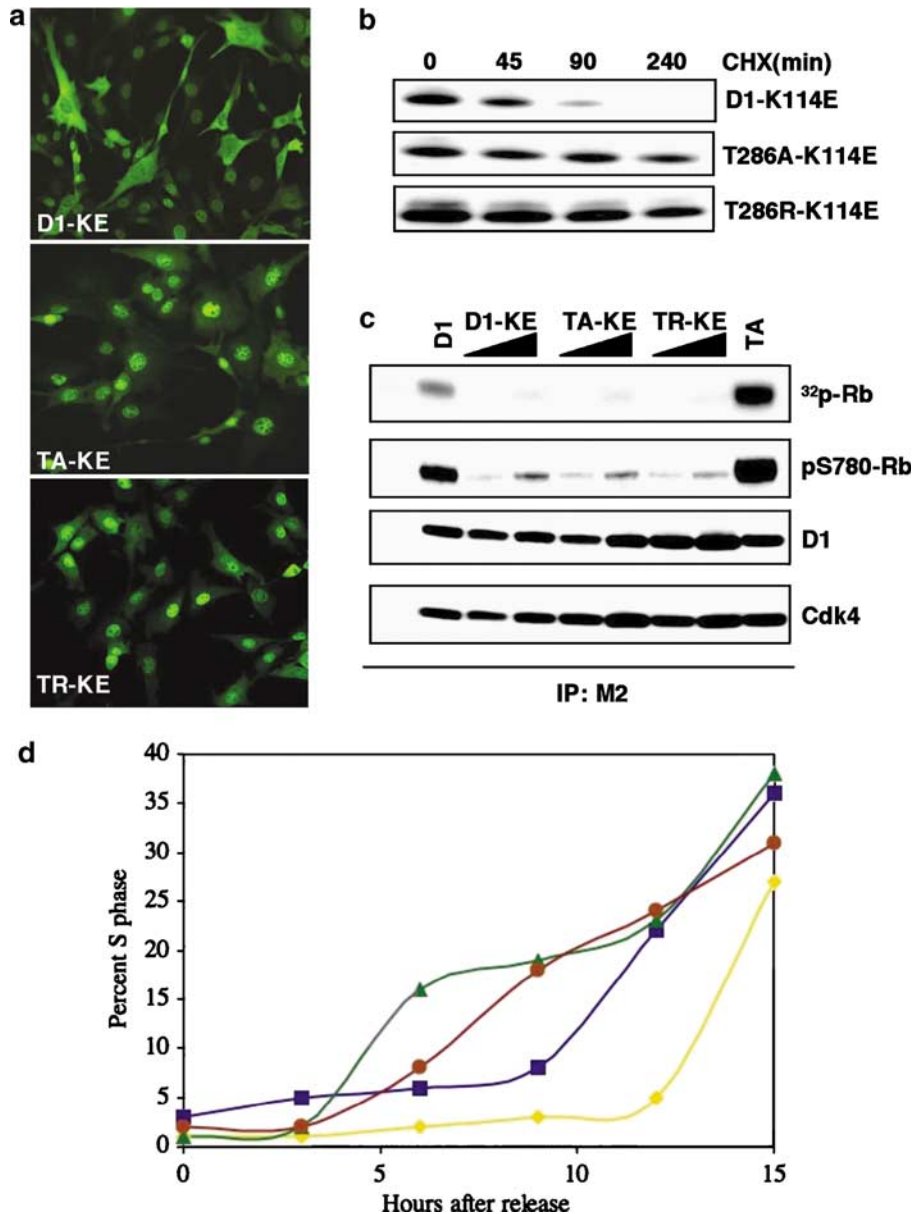


Figure 7 K114E mutants confer a hypomorphic phenotype. (a) NIH-3T3 cells stably expressing D1-KE, TA-KE and TR-KE were fixed, and cyclin D1 localization determined by indirect immunofluorescence. (b) NIH-3T3 cells stably expressing the indicated proteins were treated with cycloheximide. Lysates from the respective cell lines were processed for Western blot analysis using cyclin D1 antibody. (c) Lysates from NIH-3T3 cell lines stably expressing the indicated Flag-tagged proteins were processed for precipitation with M2 antibody and the resulting precipitates were assayed for the ability to support Rb kinase activity. Phosphorylated Rb was visualized by autoradiography, following SDS-polyacrylamide gel electrophoresis (PAGE) and transfer to nitrocellulose membrane. The same membrane was probed with antibodies specific for phospho-serine 780 Rb. (d) NIH-3T3 (yellow), D1-3T3 (blue), D1-T286A (green) and TA-KE-3T3 (maroon) cell lines synchronized by culturing in media containing 0.1% serum for 24 h were stimulated to re-enter the cell cycle by the addition of serum-derived growth factors and 5-bromo-2'-deoxyuridine. S-phase entry was monitored by flow cytometry.

the prevention of aberrant cell growth *in vitro* and in mouse models (Alt *et al.*, 2000; Gladden *et al.*, 2005). However, there is little data demonstrating the existence of cyclin D1 mutations that disrupt this regulatory process in human cancer. With the identification of critical regulatory residues within the C-terminus of cyclin D1 that maps to exon 5 of the cyclin D1 gene, we have identified and characterized mutations in cyclin D1 isolated from primary human cancer. We have identified

an arginine for threonine substitution at position 286 in primary esophageal cancers, a histidine for threonine substitution at position 286 through an EST tag search, a deletion of amino acids 266–295 in primary esophageal cancer and a proline to alanine substitution at position 287, D1-P287A, identified in three independently derived esophageal cancer cell lines, TE3, TE7 and TE12. These mutations target residues responsible for Thr-286 phosphorylation and therefore, directly impact

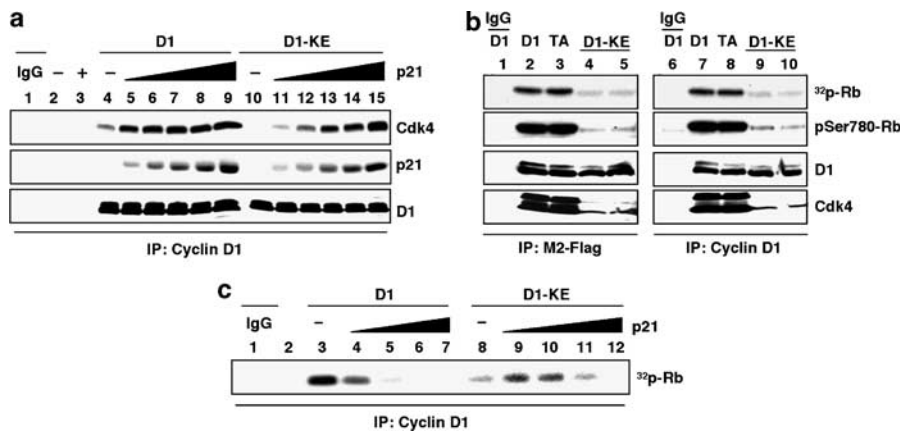


Figure 8 D1-K114E binds CDK4 and retains kinase activity in the presence of p21. (a) Cyclin D1 complexes were immunoprecipitated from Sf9 lysates prepared following co-infection with either wild-type cyclin D1 or D1-KE along with CDK4 and increases concentrations of baculovirus encoding p21^{Cip1} using cyclin D1 antibody. The resulting immunoprecipitates were assayed for co-precipitated CDK4 and p21^{Cip1}. Total immunoprecipitated cyclin D1 is confirmed by immunoblot using cyclin D1 antibody. (b) Following co-infection of CDK4 either with D1, D1-T286A or D1-KE in Sf9 cells, cyclin D1 was immunoprecipitated using either M2 or cyclin D1 antibody and assayed for the ability to support Rb kinase activity. Phosphorylated Rb was visualized by autoradiography, following transfer onto a nitrocellulose membrane. The same membrane was probed for Rb phospho-serine 780, cyclin D1 and co-precipitating CDK4. (c) Moderate concentrations of p21^{Cip1} promote D1-K114E kinase activity. D1 and D1-K114E *in vitro* kinase assays using recombinant glutathione-S-transferase-Rb and [γ -³²P]ATP in the absence and presence of increasing concentrations of p21^{Cip1}. Phosphorylated Rb was resolved by electrophoresis, transferred onto a nitrocellulose membrane and visualized by autoradiography.

cyclin D1 nuclear export and ubiquitin-dependent proteolysis. Characterization of the identified cyclin D1 mutants revealed wild-type properties with regard to association and activation of CDK4, suggesting that any oncogenic properties do not reflect an increased propensity to induce CDK activity. Rather, the impact of the mutations lies exclusively upon the constitutive nuclear accumulation of active cyclin D1/CDK complexes.

Several conclusions can be drawn regarding cyclin D1 alterations in cancer. First, mutations in the cyclin D1 coding region likely occur at a relatively low frequency, ~4% (four mutations identified in 110 samples including cell lines and primary tissue) in the current study. Although this figure is less than the estimated frequency of cyclin D1 overexpression in esophageal cancer, it is important to consider that B-Raf mutations have been estimated to occur only occur at an estimated frequency of 8% in human cancer (Davies *et al.*, 2002; Garnett and Marais, 2004). Second, the mutations specifically disrupt CRM1 nuclear export resulting in the constitutive nuclear accumulation of an active cyclin D1/CDK4 kinase that is refractory to rapid degradation via the 26S proteasome. Finally, whereas this might argue against a generalized model wherein inactivation of cyclin D1 nuclear export contributes to cancer initiation, it is likely that this mutation frequency dramatically under-represents the actual frequency with which cyclin D1 nuclear export is targeted in human cancer. In point of fact, we have previously demonstrated that cancer-specific alternative splicing of cyclin D1 contributes to the expression of a constitutively nuclear cyclin D1 isoform; our work suggests that this isoform is expressed in approximately 40% of primary esophageal carcinomas (Lu *et al.*, 2003).

Similar to our results, C-terminal cyclin D1 mutants were also identified in endometrial cancer (Moreno-Bueno *et al.*, 2003) that map to residues adjacent to Thr-286. The in-frame deletion of residues 289–292 in D1- Δ 289–292 deletes both a portion of the cyclin D1 nuclear export signal (Benzeno and Diehl, 2004) and a critical aspartic acid, D289, that is essential for subsequent phosphorylation of Thr-286 by GSK-3 β . As predicted from loss of both Thr-286 phosphorylation and deletion of the NES, D1- Δ 289–292 accumulates in the nucleus and drives cellular transformation. Thus, cyclin D1 mutations isolated from both esophageal and endometrial cancer target the same regulatory sites important for nuclear export. Given that deregulation of cyclin D1 is proposed to contribute to the genesis and progression of additional cancers, it will be critical to expand this analysis to ascertain the potential contribution of cyclin D1 mutations to additional malignancies.

The GSK-3 β kinase targets two distinct sets of substrates, primed and unprimed. Canonically primed GSK-3 β substrates are characterized by a preceding phosphorylation event that is ($n+4$) residues from the target GSK-3 β phosphorylation site (n). This substrate priming event potentiates the alignment of β - and α -helical domains within the positively charged binding groove of GSK-3 β and provides optimal coordination of the target phosphorylation residue within the catalytic cleft of this kinase (Dajani *et al.*, 2001; ter Haar *et al.*, 2001). Examples of primed GSK-3 β substrates include glycogen synthase, cyclin E, c-myc and NFAT (Fiol *et al.*, 1988, 1990; Beals *et al.*, 1997; Sears *et al.*, 2000; Welcker *et al.*, 2003, 2004). Unprimed GSK-3 β substrates include the I-2 inhibitor of PP1 and β -catenin (Hagen *et al.*, 2002; Zhang *et al.*, 2003).

As a canonical priming phosphorylation event that is characterized by the transfer of a phosphate group at position ($n+4$) is not required to initiate GSK-3 β -mediated phosphorylation of cyclin D1 at Thr-286, the classification of cyclin D1 as an 'unprimed' GSK-3 β substrate is appropriate at face value (Diehl *et al.*, 1998). Our data, on the other hand, suggest that recognition of cyclin D1 by GSK-3 β requires an acidic residue at the ($n+3$) position (Asp-289); the negative charge provided by Asp-289 may in fact substitute for a canonical priming phosphorylation event at ($n+4$). Indeed, aspartic acid 289 is conserved in both mouse and human cyclin D1 and is spatially conserved in all three D-type cyclins, highlighting its significance. Mutation Asp-289 to a non-charged residue or its deletion inhibits Thr-286 phosphorylation. Previous work has revealed that a specific peptide inhibitor, GID, preferentially inhibits GSK-3 β phosphorylation of unprimed substrates such as I-2 (Hedgepeth *et al.*, 1999; Zhang *et al.*, 2003). Consistent with GSK-3 β recognizing cyclin D1 as a primed substrate (Hedgepeth *et al.*, 1999; Zhang *et al.*, 2003), phosphorylation of cyclin D1 by GSK-3 β is insensitive to the GID peptide inhibitor. These data strongly support the idea that D289 facilitates recognition and phosphorylation of cyclin D1 at Thr-286 and may do so via providing the negative charge generally provided by phosphorylation at the ($n+4$) position.

Nuclear accumulation of cyclin D1 during S phase correlates with its oncogenic capacity and ascribes a gain-of-function activity to cancer-derived, nuclear export-defective cyclin D1 mutants. The precise nature of cyclin D1's nuclear S-phase function remains unclear but may be related to the formation of active complexes with CDK4. Alternatively, there is evidence for the CDK-independent functions of cyclin D1 as well (Hirai and Sherr, 1996; Neuman *et al.*, 1997; Zwijsen *et al.*, 1997; Lamb *et al.*, 2000; Petre *et al.*, 2002; Benzeno *et al.*, 2004). With the advent of anticancer therapeutics that target the active site of cyclin-dependent kinases, it is critical to discern between CDK-dependent versus CDK-independent functions (Fry *et al.*, 2001, 2004). To begin to address the contribution of cyclin D1 kinase activity in cell transformation, we investigated the potential of a K114E mutation in the context of constitutively nuclear TA-KE and TR-KE, to affect cellular transformation *in vitro*. The K114E mutation had previously been reported as 'kinase-dead' owing to its failure to bind CDK4 *in vitro* (Hinds *et al.*, 1994). In contrast, we found that in cells this mutant cyclin D1 associates with both CDK4 and p21^{Cip1} but is characterized by an attenuated and thus, hypomorphic capacity to activate CDK4 catalytic activity. Although not 'kinase-dead', we used these compound mutants as a tool to evaluate the effect of attenuated cyclin D1 activity on transforming potential. Our results reveal that the attenuated activity of constitutively nuclear T286A-KE and T286R-KE correlates with a significant reduction in transformation relative to wild-type counterparts, T286A and T286R, as assessed by anchorage-independent growth. Our data provide strong evidence

that transformation by cyclin D1 is driven by its constitutive nuclear retention. Our results along with recently published work from the Sicinski and Hinds laboratories (Landis *et al.*, 2006; Yu *et al.*, 2006) also suggest that neoplastic growth is the direct result of unrestricted catalytic activity.

Although cyclin D1 is frequently overexpressed in human cancer, it is only weakly oncogenic *in vitro* or in mouse model systems. In contrast, we have demonstrated that cyclin D1 mutants that are refractory to nuclear export exhibit an enhanced capacity to trigger a cell transformation *in vitro*. We have recently demonstrated that transgenic expression of the constitutively nuclear mutant cyclin D1-T286A in murine lymphocytes triggers an aggressive B-cell lymphoma (Gladden *et al.*, 2005). This is again in contrast to wild-type cyclin D1 expression of which is not sufficient to trigger B-cell malignancies in mice (Bodrug *et al.*, 1994; Lovec *et al.*, 1994a). Interpreted in sum, we suggest that overexpression of wild-type cyclin D1, although providing a growth advantage and contributing to the proliferative potential of tumor cells, is not in fact a transforming event. In contrast, in cases where cyclin D1 contributes to cancer initiation, accumulating data suggests that cells will exhibit defects in cyclin D1 nuclear export. These defects could result from mutations that impact the GSK-3 β phosphoacceptor site (data herein), induce alternative splicing of cyclin D1 (Lu *et al.*, 2003) or via upstream mutations that impact the signal-transduction pathway that regulates Thr-286 phosphorylation (Rimerman *et al.*, 2000).

In conclusion, our results emphasize the need for further investigation into the downstream mechanism(s) by which oncogenic mutants of cyclin D1 drive neoplastic growth. The results provided by these experiments will increase our understanding of the contribution of cyclin D1/CDK activity to cyclin D1 transformation in human neoplasia and expand our knowledge of downstream regulators including novel substrates that potentiate the oncogenicity of a constitutively active cyclin D1 mutant protein.

Materials and methods

Cell culture conditions and transfections

All mammalian cell lines were maintained in Dulbecco's modified Eagle's medium containing glutamine supplemented with antibiotics (Cellgro, Mediatech Inc., Herndon, VA, USA) and 10% fetal calf serum (FCS) (BioWhittaker Europe, Belgium). Insect Sf9 cells were grown in Grace's medium supplemented with 10% heat-inactivated FCS. Procedures for manipulation of baculoviruses were described previously (Summers, 1987). All cyclin D1 point mutants corresponding to human somatic cyclin D1 mutations were engineered using pFlex-murine cyclin D1 vector as a template for oligonucleotide-directed mutagenesis with the Quick ChangeTM *in vitro* mutagenesis system (Stratagene, La Jolla, CA, USA). All flag-tagged cyclin D1 mutants were confirmed by sequencing. Derivation of NIH-3T3 cells engineered to overexpress Flag-tagged cyclin D1 and Flag-tagged cyclin D1 mutants were as described previously (Diehl *et al.*, 1998).

Immunoblotting and kinase assays

For Western analysis, cells were lysed in Tween-20 buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid at pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM ethylenediamine tetraacetate, 0.1% Tween-20, 1 mM phenylmethylsulfonyl fluoride, 20 U/ml aprotinin, 5 µg/ml leupeptin, 0.4 mM NaVO₄, 0.4 mM NaF). Proteins were resolved on denaturing polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes (MSI, Westborough, MA, USA), and blotted with the indicated antibodies. For the detection of cyclin D1-dependent kinase activity, cells were harvested in Tween 20 buffer. Following precipitation with the M2 or cyclin D1 antibody, protein kinase assays using 1 µg of recombinant GST-Rb were performed as described previously (Diehl and Sherr, 1997; Rimerman *et al.*, 2000). Detection of GSK-3β was as described previously (Diehl *et al.*, 1998); GSK-3β kinase assays and GID inhibition assays were performed as described previously (Zhang *et al.*, 2003).

Immunofluorescence

NIH-3T3 cells seeded on glass coverslips were fixed using methanol–acetone (1:1). Coverslips were stained with a mouse-specific cyclin D1 monoclonal antibody (D1-17-13G) in phosphate-buffered saline (PBS)/10% FCS. Secondary fluorescein isothiocyanate-conjugated anti-mouse (Amersham Pharmacia Biotech, Uppsala, Sweden) antibody staining was performed for 30 min. DNA was visualized using Hoechst 33258 dye at a 1:500 dilution. Coverslips were mounted on glass slides with Vectashield medium (Vector Laboratories Inc., Burlingame, CA, USA).

Protein turnover analysis

NIH-3T3 cells overexpressing either Flag-tagged wild-type or mutant cyclin D1 were seeded in 10 cm dishes. The following day, cells were treated with the protein synthesis inhibitor cycloheximide (100 µg/ml; Sigma, St Louis, MO, USA) for the

indicated intervals, and harvested in sodium dodecylsulfate (SDS)-sample buffer.

Tumor samples and DNA sequence analysis

Cyclin D1 exon 5 polymerase chain reaction products from normal and tumor-derived genomic DNA samples were generated using human cyclin D1 forward: 5'-CTCAGGTCCAGAG GAGGCAG-3' and human cyclin D1 reverse: 5'-GAGATG GAAGGGGGAAAGAG-3' primers that generated overlapping amplicons. All mutations were confirmed via bidirectional sequencing in two independent polymerase chain reaction reactions. Tumor-derived cell lines utilized were available in our laboratory. Mutations in TE3/7 were confirmed upon acquisition of independent samples of these from the Rustgi laboratory.

Cell transformation assays

NIH-3T3 cells and derivatives overexpressing the indicated cyclin D1 isoforms were plated at 1.5×10^5 cells/well of a six-well dish. Cells were cultured in media containing 5% FCS. Foci were visualized after 21–28 days with Wright–Giemsa stain (Sigma). Anchorage-independent growth was determined by analysing cellular growth in semisolid medium. Cells (5×10^3) were seeded in Iscove's media containing 0.65% noble agar/10% FCS. Cells were grown for 3 weeks in 8% CO₂. For visualization, colonies were incubated with 1 ml/well of 0.01% neutral red (Sigma N7005) in PBS for 1 h at 37°C. Quantification of results represents the average number of colonies scored with standard error calculated from three independent experiments.

Acknowledgements

We thank J Woodgett for providing GSK-3β cDNAs. This work was supported by a grant from the National Institutes of Health (CA93237, CA111360) and the WW Smith Charitable Trust (JAD).

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