Journal of Cellular Biochemistry 96:906–913 (2005)

Location, Location, Location: The Role of Cyclin D1 Nuclear Localization in Cancer

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Abstract The control of cell proliferation is crucial in maintaining cellular homeostasis and loss of this mechanism is a principle hallmark of cancer cells. A primary target of growth factor signaling is the cyclin D1-dependent kinase (D1-CDK4/6) whose activity promotes G_1 phase progression by phosphorylating the retinoblastoma protein (Rb) along with related pocket proteins 107 and p130, relieving inhibition of E2F family transcription factors. Cyclin D1 accumulation is regulated at multiple levels including transcription, post-translational activation and cellular localization throughout the cell cycle. While overexpression of cyclin D1 has been observed in a number of human cancers, mouse cancer models overexpressing D1 have fallen short of establishing a role for cyclin D1 in the initiation of malignant phenotypes suggesting an additional regulatory mechanism exists that prevents cyclin D1-driven cancer. This article will present an overview of current data investigating the regulation of cyclin D1 nuclear localization and the prevalence of these aberrations in cancer. Finally, future avenues of research involving cyclin D1 cellular localization and its regulation in cancer will be addressed. J. Cell. Biochem. 96: 906–913, 2005. © 2005 Wiley-Liss, Inc.

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

Cell division requires high fidelity duplication of the genome (S-phase) and proper distribution of a complete copy of the genome to each of two daughter cells (mitosis). These two distinct phases of the cell cycle are separated by gap phases that serve as intervals where cellular machinery insures the fidelity of these processes. The first gap phase, G_1, is unique among cell cycle phases in that it is the point at which cells are responsive to extracellular cues (e.g., growth factor signaling). As cells enter and transit G_1 phase, cells acquire mass and establish the replication machinery necessary for DNA replication. Thus, G_1 phase serves as the point wherein growth factor signaling integrates with the cell cycle and cell division. A critical cell cycle regulatory target of growth factor signaling pathways are the D-type cyclins. There are three D-type cyclins that are expressed in a tissue specific manner and bind to either of two homologous catalytic subunits, CDK4 or CDK6. Growth factor signaling through Ras-dependent pathways increases cyclin D transcription, translation and ultimately promotes binding to either CDK4 or CDK6 [Sherr, 1993].

Of the three D-type cyclins, cyclin D1 remains the most extensively studied, largely because of its frequent overexpression in human malignancy. In addition to transcriptional and translational regulation, cyclin D1 is subject to growth factor dependent post-translational regulation. Activation of the assembled cyclin D1/CDK4 complex requires phosphorylation by the CDK-activating kinase (CAK) in turn priming the D1/CDK4 kinase to execute its primary role in G_1 regulation, phosphorylation of the retinoblastoma protein [Kato et al., 1994; Diehl and Sherr, 1997]. Following CAK activation, the active D1/CDK4 holoenzyme translocates to the nucleus and performs the initial

Grant sponsor: National Institutes of Health; Grant numbers: CA93237, CA111360.

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Received 22 July 2005; Accepted 25 July 2005

DOI 10.1002/jcb.20613

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phosphorylation of Rb and in concert with activated cyclin E/CDK2 phosphorylation of Rb, relieves the Rb mediated inhibitory action on the E2F transcription factor [Kato et al., 1993; Lundberg and Weinberg, 1998].

Cyclin D1/CDK4 activity is kept in check by several distinct regulatory events. Like most cyclins, cyclin D1 is a highly labile protein with a half-life of 20–30 min [Matsushime et al., 1991]. Proteolysis of cyclin D1 requires poly-ubiquitination, which targets cyclin D1 to the 26S proteasome [Diehl et al., 1997]. Poly-ubiquitination of cyclin D1 requires phosphorylation of a conserved C-terminal threonine, Thr-286, by the glycogen synthase kinase 3 beta, GSK-3β. GSK-3β is excluded from the nucleus during G_1 phase, but enters the nucleus upon S-phase entry thereby gaining access to the nuclear cyclin D1-CDK4 complex [Diehl et al., 1998]. Phosphorylation of cyclin D1 on Thr-286 then triggers a coupled event. Phosphorylated Thr-286 is first bound by the CRM1 nuclear exportin, which then shuttles the cyclin D1 complex to the cytoplasm. Once in the cytoplasm, phospho-cyclin D1 is then targeted by an as yet unidentified E3 ubiquitin ligase, ubiquitinated and thereby marked for destruction (Fig. 1). In addition to regulation via nuclear export coupled proteolysis, cyclin D-CDK kinase activity is opposed by direct binding with small polypeptide inhibitors of which $p16^{INK4a}$ is the prototypical member [Sherr and Roberts, 1999].

The cyclin D1/CDK4 complex drives G_1 progression via both kinase-dependent and kinase-independent mechanisms. The inactivation of Rb, and related pocket proteins, requires direct CDK-dependent phosphorylation. In addition to direct phosphorylation of pocket proteins, the cyclin D-CDK complex serves as a stoichiometric "soak" for the CDK inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ [Cheng et al., 1999]. The binding of $p21^{CIP1}$ and $p27^{KIP1}$ with cyclin D1 and CDK4 serves a dual function. The first is that $p21^{CIP1}$ and $p27^{KIP1}$ facilitate stable association of the cyclin D-CDK4 complex [LaBaer et al., 1997]. Indeed cells lacking both $p21^{CIP1}$ and $p27^{KIP1}$ are unable to form active D1/CDK4 complexes [Soos et al., 1996; LaBaer et al., 1997; Cheng et al., 1999] (Fig. 1). Second, the stable association of $p21^{CIP1}$ and $p27^{KIP1}$ with cyclin D1-CDK4 relieves CDK2 from the CIP/KIP

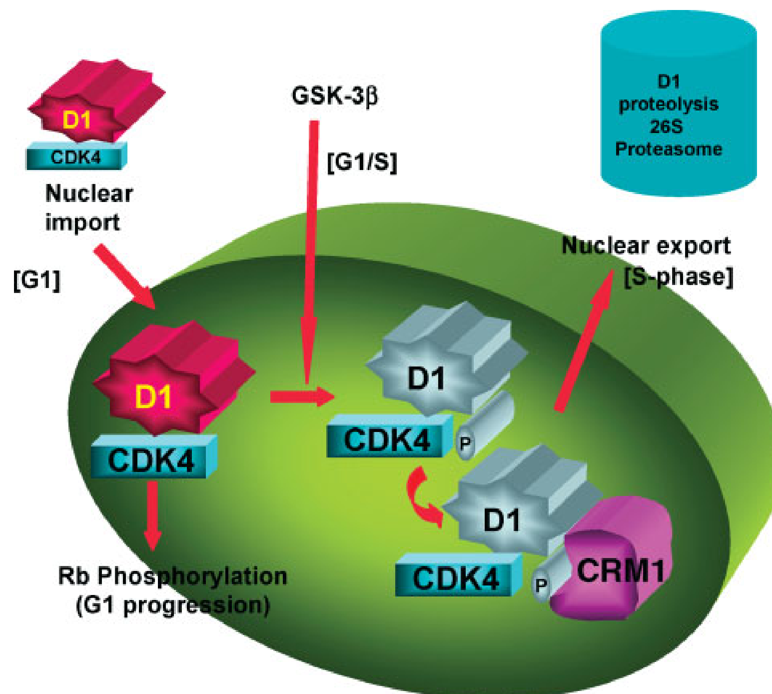

Fig. 1. Regulation of cyclin D1 sub-cellular localization. During G_1 phase cyclin D1 assembles with CDK4 and a CIP/KIP protein where upon the complex enters the nucleus and phosphorylates Rb promoting G_1 to S phase progression. The GSK-3β kinase enters the nucleus during the G_1/S transition and phosphorylates cyclin D1 at Thr-286 triggering D1 nuclear export, ubiquitination and degradation by the 26S proteasome.

inhibitory activities thereby indirectly promoting CDK2 activity. Therefore, induction of cyclin D1 during G_1 drives Rb phosphorylation and promotes CDK2 activity by titrating the CDK2 inhibitory proteins $p21^{CIP1}/p27^{KIP1}$.

More recently, a new G_1 substrate for cyclin D1-CDK4 has been identified and in turn a new mechanism through which D1/CDK4 drives proliferation envisioned. Smad3, a member of the Smad transcription complex, can be directly phosphorylated by the cyclin D-CDK4 kinase [Matsuura et al., 2004]. Smad proteins are regulated by TGF-β signaling and oppose cell proliferation via transcriptional activation of cell cycle inhibitors such as $p27^{KIP}$ and $p15^{INK4b}$ [Shi and Massague, 2003]. Cyclin D-CDK4 phosphorylation appears to inhibit Smad3 transcriptional activity and therefore its antiproliferative function. This data provides additional evidence demonstrating how the induction of D type cyclins by growth factors drives G_1 to S phase progression and restriction point passage.

Increased levels of cyclin D1 occur in a large segment of human cancers. Amplification of the cyclin D1 locus accounts for a low percentage of the total number of cancers that overexpress cyclin D1; the mechanisms contributing to cyclin D1 overexpression in the remainder has not firmly been established. Strikingly, a large body of work strongly suggests that enforced overexpression of cyclin D1 is not likely to be the essential transforming property of cyclin D1 [Quelle et al., 1993; Resnitzky et al., 1994]. More recently, work has revealed that expression of a cyclin D1 mutant that is refractory to nuclear export and proteolytic degradation at the G_1/S boundary is a highly transforming mutant and functions independent of additional oncogenes in vitro [Alt et al., 2000]. This finding reveals a previously unappreciated role for regulated nuclear export in harnessing cyclin D1-CDK4 activity and suggests that retention of this kinase in the nucleus during S-phase is a cancer promoting or predisposing event.

PHOSPHORYLATION-DEPENDENT CYCLIN D1 NUCLEAR EXPORT IN CANCER

Initial work identified the GSK-3β kinase as the protein kinase that phosphorylates cyclin D1 at Thr-286 thereby targeting cyclin D1 for nuclear export and proteolysis [Diehl et al., 1998]. Further observations revealed that this phosphorylation event correlated with the G_1 to S-phase transition in the cell cycle suggesting temporal regulation. Additional studies revealed that GSK-3β access to cyclin D1 complexes was limited to S-phase via regulation of GSK-3β nuclear accumulation. While the mechanisms that determine GSK-3β nuclear entry remain to be firmly established, it has recently been demonstrated that GSK-3β localization is also subject to regulated nuclear export. GSK-3β nuclear entry appears to be restricted through interactions with FRAT/GBP. The latter contains a bona fide nuclear export signal and current data suggest that GSK-3β likely “piggy backs” out of the nucleus with FRAT/GBP [Franca-Koh et al., 2002]. The temporal access of GSK-3β to the nucleus during S-phase suggests that association of FRAT/GBP with GSK-3β will be subject to temporal regulation, but this remains to be established.

The accumulation of GSK-3β in the nucleus following G_1 to S-phase transition and subsequent stoichiometric cyclin D1-CDK4 nuclear export is consistent with the notion that in normal cells nuclear activities of cyclin D1 are important for G_1 progression but likely are counterproductive in S-phase. Consistent with this, early work revealed that overexpression of D type cyclins that are still subject to nuclear export in murine fibroblasts shortens the G_1 interval without a concomitant increase in S-phase transit; however, overexpression of the cyclin alone was not sufficient to induce cellular transformation [Quelle et al., 1993; Resnitzky et al., 1994]. Recent data demonstrates that expression of either the constitutively nuclear mutant, which is refractory to GSK-3β-dependent phosphorylation, cyclin D1-T286A, promotes cellular transformation independent of cooperating oncogenes [Alt et al., 2000]. This suggests that in addition to the well-described G_1 functions of cyclin D1 in growth factor signaling and G_1 to S-phase progression, D1 can have additional mechanisms throughout the cell cycle that support cellular transformation.

If cyclin D1 nuclear export is a key feature of homeostatic cell growth, one might anticipate that it would be targeted during cancer genesis. While there have been few studies that have addressed this question, recent work suggests that cyclin D1 localization may be deregulated and thereby contribute to neoplastic transformation. Two laboratories have recently identified

a novel cyclin D1 isoform, cyclin D1b, whose expression results from alternative splicing [Lu et al., 2003; Solomon et al., 2003]. Unlike the canonical cyclin D1a transcript, D1b lacks the fifth exon containing both the GSK-3β phosphorylation site and the Crm1 binding site; consequently cyclin D1b is constitutively nuclear [Lu et al., 2003]. Cyclin D1b still binds and activates CDK4 and is able to disassociate an Rb/MCM7 complex demonstrating the protein product of the alternative transcript is capable of executing the known functions of the D1/CDK4 holoenzyme. The D1b protein was expressed in several tumor cell lines and was also detected in primary esophageal carcinoma tissue. Expression of cyclin D1b was not detectable in non-malignant tissue demonstrating that the D1b protein expression is cancer specific [Lu et al., 2003]. In addition, immunohistochemical staining for D1b revealed its expression and predominately nuclear localization in primary tumor tissue providing further evidence that alterations promoting cyclin D1 nuclear localization are present in human cancers.

In addition to alternative splicing, sequencing of cyclin D1 in endometrial cancer revealed mutations in the C-terminus [Moreno-Bueno et al., 2003]. While these mutants were not biochemically characterized, the location of these mutations suggests they will perturb cyclin D1 nuclear export. The first mutation was a single base substitution resulting in a proline to serine or threone substitution at residue 287. Given previous reports of GSK-3β functioning as a proline directed kinase, this mutation is predicted to block Thr-286 phosphorylation, resulting in a constitutively nuclear mutant. The second mutation identified by Moreno-Bueno et al., was a deletion of amino acids 289–292, which encompasses residues recently described as the Crm1 binding site [Benzeno and Diehl, 2004]. The identification of these mutants in primary cancer specimens is provocative. However, characterization of these mutants will be necessary to reveal their true biochemical properties. Given the identification of these mutations, analysis of cyclin D1 primary sequence will be necessary to more accurately assess the frequency of such mutations and the spectrum of cancers wherein they occur.

In addition to direct mutations and alternative splicing, signal transduction pathways that regulate cyclin D1 stability and localization are subject to cancer specific alterations in human malignancy. Cyclin D1 localization in normal cells is regulated via Ras-PI3K-Akt-GSK-3β signaling. Flux through this pathway, and specifically activation of Akt, is opposed by the phosphatase activity of PTEN. Essentially all components of this pathway are targeted in human cancer. The PTEN tumor suppressor is itself frequently inactivated in cancer [Di Cristofano and Pandolfi, 2000]. While inactivation of PTEN will indirectly increase Akt activity, and thus down-regulate GSK-3β, Akt is also overexpressed during cancer genesis [Bjornsti and Houghton, 2004]. Biochemical examination of these pathways has confirmed that Akt overexpression [Diehl et al., 1998] and PTEN inactivation [Radu et al., 2003] directly impacts cyclin D1, demonstrating the relevance of such mutations in human cancer.

Finally the machinery the cell utilizes to ubiquitinate phosphorylated cyclin D1 may also be inactivated in tumor cells. Given that cyclin D1 ubiquitination is coupled with cyclin nuclear export, it is conceivable that inactivation of cyclin D1 ubiquitination might also impact on its nuclear accumulation. Currently the E3 ubiquitin ligase that specifically recognizes cyclin D1 is not identified. However, by analogy with the cyclin E ubiquitin ligase Fbw7, which is directly targeted in human cancer [Moberg et al., 2001; Strohmaier et al., 2001], it is reasonable to expect that the cyclin D1 ubiquitin ligase may also be targeted during cancer genesis.

REGULATION OF THE CYCLIN D1b ALTERNATIVE TRANSCRIPT

An alternatively spliced isoform of cyclin D1, cyclin D1b, was recently identified in human cancer-derived cell lines. Characterization of cyclin D1b revealed that it is constitutively nuclear and that its expression in cultured cells results in neoplastic transformation [Lu et al., 2003; Solomon et al., 2003]. Consistent with the notion that cyclin D1b expression may participate in cancer genesis in vivo, cyclin D1b protein was detected in primary esophageal cancer tissue, but not in adjacent normal tissue [Lu et al., 2003]. Further investigation will be required to determine whether cyclin D1b expression is an early or late event; if it indeed plays a role in initiation, one would predict

expression in the early stages of tumorigenesis. Although it is clear that cyclin D1b accumulates at a certain frequency in human malignancy, we are only beginning to assess the frequency of cyclin D1b expression.

The mechanisms that determine expression and accumulation of canonical cyclin D1 have been under intense investigation for almost 15 years. Consequently, the underlying mechanisms are reasonably well understood. In contrast, the mechanisms that would lead to cancer specific alternative splicing of cyclin D1 are poorly understood. Splicing of pre-mRNA is a fundamental process in expression of metazoan genes and is the process whereby various proteins can be expressed from a single gene according to different regulatory programs. The ability of higher organisms to create such a diverse variety of proteins from their genes provides these organisms with the ability to adapt and survive in the presence of negative environmental events. Additionally, this capacity to adapt can also be detrimental to the organism as is observed in cancer where a number of alternative transcripts have been shown to be involved in the evolution of malignancies. The majority of aberrant alternative splicing events are the result of single nucleotide mutations affecting *cis*-elements regulating splicing such as the 3′ or 5′ splice site at the intron/exon boundary or the intronic or exonic enhancer or silencer sites. Early studies on the cyclin D1b transcript identified a single nucleotide polymorphism (G870A) at the 3′ splice donor site in exon 4 and suggested this might contribute to cyclin D1b expression [Betticher et al., 1995; Zheng et al., 2001]. However, additional work has revealed that the presence of the G870A polymorphism is not a prerequisite for expression of cyclin D1b, suggesting that in addition to *cis*-elements, *trans*-elements may be involved in regulating the expression of cyclin D1b [Howe and Lynas, 2001]. Mutations targeting *trans*-elements regulating pre-mRNA splicing are not as common as mutations targeting *cis*-elements and this is likely due to the vital role of the basal splicing machinery in normal mRNA splicing. Nevertheless alterations of the splicing signals and their role in the progression of cancer are starting to emerge as more studies proceed.

Two *trans* acting factors of particular interest with regard to tumorigenesis are the SR protein family members and the splicing regulator PTB. The SR family of proteins binds to exonic splicing enhancers, thereby promoting exon recognition and correct exon splicing. Increased expression of SR proteins has been shown to correlate with a progression from a pre-neoplastic to metastatic cancer in a mouse model of mammary cancer [Stickeler et al., 1999]. Increased SR protein levels also correlated with an increased complexity of CD44 isoforms, a protein commonly involved in metastasis, suggesting that increased SR protein levels may promote expression of alternative transcripts eventually contributing to tumor progression [Gunthert et al., 1991]. In addition to the SR family of proteins, the PTB protein binds intronic splicing regulatory elements promoting exonic recognition and accurate splicing. Like the SR proteins, the PTB regulator has been shown to be increased in glioblastomas where it is linked with the expression of an alternative splice variant of fibroblast growth factor receptor 1 providing a growth advantage for the glioblastoma cells [Yamaguchi et al., 1994].

Although overexpression of the SR proteins and PTB correlates with an increased spectrum of protein transcripts and with tumor progression, the mechanisms through which these splicing factors are regulated in cancer is unknown. There is no evidence that oncogenes such as Ras or Myc play a role in regulating splicing factors; however it is tempting to speculate that expression of potent oncogenes will target splicing machinery in an attempt to diversify the protein pool and thereby assist in tumor survival and progression. Revisiting how expression of cyclin D1b is regulated, two possible scenarios now present themselves. In a model wherein cyclin D1b expression is an early event, polymorphisms present in the cyclin D1 gene contribute to cyclin D1b expression providing the tumor cell with a decreased dependence upon growth factor signaling. In a second scenario, an initiating oncogenic event increases the level of *trans*-elements regulating splicing in turn increasing the incidence of alternative splicing events. Cells expressing cyclin D1b would be selected for because of the growth advantage over the other tumor cells allowing for tumor progression and the expression of D1b in the tumor tissue. Further studies investigating the prevalence of the G870A and D1b expression in addition to splicing experiments involving a cyclin D1 mini-gene would

help in determining how expression of D1b is regulated and whether cyclin D1b expression could be blocked with inhibitors of upstream regulatory elements.

FUNCTIONAL REDUNDANCY OF G_1 CYCLIN DEPENDENT KINASES IN VIVO

Given its prominent role as an integrator of growth factor signaling with the cell cycle, it was reasonable to anticipate an obligatory role for D-type cyclins during embryonic development. Surprisingly this has not been the case. Deletion of any one individual D-type cyclin, D1, D2, or D3 results in viable embryos with only minor defects in specific tissue compartments suggesting all three D type cyclins are forcibly interchangeable during development [Fantl et al., 1995; Sicinski et al., 1995, 1996, 2003]. Targeted deletion of either of the D-type cyclin catalytic subunits, CDK4 or CDK6, does not perturb embryonic development either, although fibroblasts from $CDK4^{-/-}$ animals do display delays in growth factor triggered cell cycle reentry [Rane et al., 1999; Tsutsui et al., 1999]. In contrast, disruption of all three D type cyclins or both CDK4 and CDK6 resulted in embryonic death between days E14 to E16. Lethality in both cases is the result of complete hematopoietic failure indicating that cyclin D/CDK activity is essential for hematopoietic development [Kozar et al., 2004; Malumbres et al., 2004]. Unlike the mitotic cyclins, the absence of early embryonic lethality in mice lacking either the cyclin D-dependent kinase or the CDK2-dependent kinase [Berthet et al., 2003; Ortega et al., 2003] reveals the plasticity and similarity of function of the G_1 and S-phase cyclin/CDK complexes. One function that is commonly observed in the knockout models of the G_1 and S-phase cyclins is that these cyclin/CDK components and their regulatory mechanisms are extremely important for cellular transformation. Consequently these proteins are commonly targeted in human malignancies. The most striking result being that ablation of cyclin D1 prevents Ras- and Neu-dependent mammary cancer development [Yu et al., 2001]. These data support the notion that organism development may not require unique cyclin functions while in contrast cancer genesis, may conversely depend upon as yet unidentified and unique biochemical activities associated with the various CDK/cyclin complexes.

While mouse models of cyclin D ablation have demonstrated a highly overlapping system with other G_1 cyclins, mouse models of cyclin D1 driven malignancies have not provided significant insight into the role of cyclin D1 in cancer initiation and progression. A majority of the work examining the role of the D1/CDK4 complex in tumor promotion have been undertaken in vitro utilizing overexpression models in mouse fibroblasts. These studies indicate that enforced overexpression of cyclin D1 in murine fibroblasts is not sufficient to transform cells with out the actions of a cooperating oncogene [Quelle et al., 1993; Resnitzky et al., 1994]. In contrast, expression of cyclin D1 mutants that lack the Thr-286 phospho-acceptor site, transforms fibroblasts independent of additional oncogenes [Alt et al., 2000; Lu et al., 2003] (Fig. 1). To directly assess the oncogenicity of cyclin D1 in vivo, mice have been engineered to overexpress wild type cyclin D1 in either the mammary gland or in lymphoid cells. Mice overexpressing cyclin D1 in mammary epithelium exhibit predisposition to mammary cancer following a long latency peroiod [Wang et al., 1994]. In contrast, mice expressing a lympoid specific cyclin D1 transgene exhibit essentially no phenotype [Bodrug et al., 1994; Lovec et al., 1994]. This was a surprising result given that mantle cell lymphoma is thought to result from mis-expression of cyclin D1 in B-cells as a result of an 11;14 chromosomal translocation. By analogy with experiments that assess cyclin D1 oncogenicity in cultured cells, it is likely that the weak phenotypes observed in mouse models reflects the capacity of cells to promote nuclear export of overexpressed wild type cyclin D1. If so, one would anticipate that mice engineered to overexpress constitutively nuclear cyclin D1 isoforms would exhibit a significant increase in tumor susceptibility. In addition to models recapitulating mutations of the cyclin D1 phospho-acceptor site, additional mouse models expressing the cyclin D1 alternative transcript, cyclin D1b are needed to provide not only pre-clinical models of cyclin D1-dependent malignancy, but also to fully understand the role of cyclin D1 localization with respect to cancer development.

CONCLUSIONS

Inactivation of the Rb pathway is a frequent occurrence in human malignancy. There are variable mechanisms involved in ablation of the

Rb pathway including inactivation or deletion of the p16^{INK4a} protein, overexpression of cyclin D1, and direct mutation of Rb. While the early work documenting cyclin D1 overexpression pointed to cyclin D1 as a potential cancer promoting protein, current work has revealed that the cell has developed mechanisms to limit access of overexpressed cyclin D1 to nuclear substrates and thereby prevent cancerous outgrowth. Cumulatively, the data suggest that tumor suppression breaks down when the cell is no longer capable of shuttling cyclin D1 from the nucleus during S-phase. Loss of cyclin D1 nuclear export can result from direct mutations within cyclin D1, cancer specific alternative splicing or mutations that target the upstream signaling pathway that regulate phosphorylation-dependent nuclear export of cyclin D1 complexes. Questions now arise regarding the novel mechanism(s) of nuclear cyclin D1/CDK4 complexes during S-phase and the role they have in promoting cancer. The establishment of mouse models to critically evaluate the biological activity of these mutants will facilitate our understanding of their role in cancer development and provide potential pre-clinical models for the evaluation of novel therapeutics. These observations now provide a significant base of initial studies to drive further investigations into the prevalence of nuclear cyclin D1 in human tumors and further biochemical studies into alternative mechanisms that regulate the nuclear localization of cyclin D1.

ACKNOWLEDGMENTS

We would like to thank laboratory members for insightful comments regarding this manuscript and apologize to those whose work could not be cited due to length limitations.

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