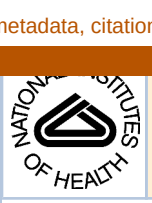



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# Enhanced skin carcinogenesis and lack of thymus hyperplasia in transgenic mice expressing human cyclin D1b (*CCND1b*)

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## Abstract

Cyclin D1b is an alternative transcript of the cyclin D1 gene (*CCND1*) expressed in human tumors. Its abundance is regulated by a single base pair polymorphism at the exon 4/intron 4 boundary (nucleotide 870). Epidemiological studies have shown a correlation between the presence of the G870A allele (that favors the splicing for cyclin D1b) with increased risk and less favorable outcome in several forms of cancer. More recently, it has been shown that, unlike cyclin D1a, the alternative transcript D1b by itself has the capacity to transform fibroblasts in vitro. In order to study the oncogenic potential of cyclin D1b, we developed transgenic mice expressing human cyclin D1b under the control of the bovine K5 promoter (K5D1b mice). Seven founders were obtained and none of them presented any significant phenotype or developed spontaneous tumors. Interestingly, K5D1b mice do not develop the fatal thymic hyperplasia, which is characteristic of the cyclin D1a transgenic mice (K5D1a). Susceptibility to skin carcinogenesis was tested in K5D1b mice using two-stage carcinogenesis protocols. In two independent experiments, K5D1b mice developed higher papilloma multiplicity as compared with wild-type littermates. However, when K5D1b mice were crossed with cyclin D1KO mice, the expression of cyclin D1b was unable to rescue the carcinogenesis-resistant phenotype of the cyclin D1 KO mice. To further explore the role of cyclin D1b in mouse models of carcinogenesis we carried out in silico analysis and in vitro experiments to evaluate the existence of a mouse homologous of the human cyclin D1b transcript. We were unable to find any evidence of an alternatively spliced transcript in mouse *Ccnd1*. These results show that human cyclin D1b has different biological functions than cyclin D1a and confirm its oncogenic properties.

### Keywords

cyclin D1; skin carcinogenesis; DMBA; TPA; transgenic mouse model



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## INTRODUCTION

Cyclin D1, a protein involved in cell cycle progression, is thought to be the point of convergence of mitogenic signal transduction cascades [1][2]. Strictly regulated at the level of transcription, translation and protein stability, cyclin D1 binds and activates CDK4 (or CDK6) forming an active kinase holoenzyme that phosphorylates Rb and other proteins [3]. In addition to this cell cycle related function, it was recently shown that cyclin D1 acts as a transcriptional regulator in a CDK independent manner [4][5].

A large body of literature has shown that genetic alterations of the cyclin D1 gene (*CCND1*) are common in human and experimental cancer. Overexpression, amplification, gene rearrangements, and mutations of this gene have been found in tumors of different origins [6]. In spite of this overwhelming correlative evidence, functional studies in vitro have not shown transforming activity and in vivo transgenic models overexpressing this cyclin have shown either weak or no oncogenic activity [7-10]. Yet, experiments in cyclin D1-null mice (D1KO) or cyclin D1 deficient cells have shown that cyclin D1 is necessary for tumor development [11-14]. This paradox may at least be partly explained by the presence of an alternative splicing product of *CCND1*, whose abundance is modulated in humans by the G870A polymorphism [15][16].

This single base pair G/A polymorphism at the exon 4/intron 4 boundary (nucleotide 870) of *CCND1* does not result in an amino acid substitution but instead modulates splicing of exons 4 and 5 [17]. It is postulated that the G allele favors the normal splicing of *CCND1* exons 4 and 5, to produce the canonical cyclin D1a mRNA, while the A allele enhances alternative gene splicing, resulting in a shorter transcript, cyclin D1b. This alternative product incorporates 99 bp of intron 4 but lacks the complete exon 5 sequence [17].

Several regulatory sequences are encoded by exon 5, including a PEST domain, that has been postulated to destabilize cyclin D1, the threonine 286 phosphorylation site for glycogen synthase kinase 3b (GSK3b), shown to promote nuclear export and protein turnover [18], and a novel regulatory region of cyclin D1 that is required for binding and repression of the androgen receptor (AR) [19]. Thus, based on the sequence analysis it is expected that cyclin D1b will result in a more stable protein with constitutive nuclear localization and altered co-transcriptional activities. Studies by three independent laboratories, including ours, have confirmed that cyclin D1b remains constitutively nuclear; however, changes in the stability of the protein were not consistently shown [20][21]. Unlike cyclin D1a, the alternative cyclin D1b was able to transform cells in vitro and the tumorigenicity of cyclin D1b-transformed cells was demonstrated in immunocompromised SCID mice [21]. Since previous studies by the Diehl laboratory showed that a mutated cyclin D1 (T286A) that is constitutively nuclear becomes tumorigenic [21], it has been postulated that the cyclin D1b oncogenic properties may also be related to its constitutive nuclear location. However, the actual molecular mechanisms involved in cyclin D1b transformation have not been elucidated.

In addition to the in vitro evidence, the role of cyclin D1b in human cancer is also supported by several epidemiological studies showing that the G870A *CCND1* polymorphism is associated with increased susceptibility and poor prognosis in a variety of human cancers, including basal cell carcinoma of the skin and tumors of the prostate, head and neck, urinary bladder, and colon [22-26]. Since G870A enhances cyclin D1b expression, these studies suggest a possible link between the alternative transcript and oncogenesis.

Using transgenic mice expressing a constitutively nuclear mutant cyclin D1 in lymphocytes, Gladden et al. [27] showed the first evidence that nuclear retention of cyclin D1 is oncogenic in vivo. Still, the biology of cyclin D1b is poorly understood. Little is known about expression of cyclin D1b in different stages of neoplasia or premalignant conditions. Also, the occurrence

of the cyclin D1b transcript and the existence of polymorphisms at the exon 4/intron 4 boundary in other species besides humans has not been reported.

In this study, we developed a transgenic mouse model expressing cyclin D1b to analyze its functional significance in cancer development. The human *CCND1b* cDNA was placed under the control of a bovine keratin 5 (K5) promoter that constitutively expressed cyclin D1b in the basal cells of the interfollicular epidermis, the pilosebaceous unit, and to a lesser degree in other organs like thymus, pancreas, prostate, and gall bladder [8][28][29]. Interestingly, K5-cyclin D1b mice (K5D1b) have a different phenotype than K5-cyclin D1a mice (K5D1a), which express the canonical human *CCND1a* under the control of the same K5 promoter [8]. Whereas K5D1a mice display strong thymic hyperplasia and moderate skin hyperplasia, K5D1b mice do not have an obvious thymic or skin phenotype. Although K5D1b transgenic mice do not show histological alterations or develop spontaneous tumors in tissues expressing K5, they exhibited enhanced susceptibility to skin papilloma development when challenged with a chemical carcinogenesis protocol.

## MATERIALS AND METHODS

### Development and Phenotyping of K5D1b Transgenic Mice

K5D1b transgenic mice were developed in our laboratory with the same strategy (pronuclear microinjection of FVB/N fertilized eggs) previously used for K5D1a, K5-cyclin D2, and K5-cyclin D3 lines [30][31]. Seven founders were original identified using genomic DNA and specific PCR reactions for the human cyclin D1b isoform (forward 5′-CTCCGTCTTGAGCATGGCTC-3′ and reverse 5′-TAGCAGAGAGCTACAGACTTCG-3′), and a non-coding region of the transgenic construct (i.e., a human β-globin sequence) (Figure 1). Expression analysis of the transgene was performed by RT-PCR with human cyclin D1b-specific primers (forward 5′-GTGCTGTCT CATCGATTTGGCAA-3′ and reverse 5′-CATATGTCCTTCGAAGTGAGAGA-3′) that amplified a fragment of 1420 bp and by Western blot using antibodies against the transcribed sequence of intron 4 (Sen-2 and D1bAb) [20] and the amino-terminus region (monoclonal antibody DCS-6 from Neomarkers (Fremont, CA); and anti-cyclin D1 Ab-3 from Oncogene, Inc., Cambridge, MA) (Figure 1). Based on these expression analyses, we selected founder # 8 to establish the transgenic line used for phenotyping and carcinogenesis studies. The standard nomenclature for this K5D1b transgenic line is FVB/N-Tg(Krt5-*CCND1b*)8Sprd1.

Twenty transgenic and 20 normal siblings mice ranging from 3-wk to 12-mo old were used to collect different tissues (skin, thymus, esophagus, stomach, bladder and prostate). Mice were injected with 60 mg/g body weight of BrdU 30 min before euthanized. Histopathological analysis was carried out on paraffin-embedded sections stained with hematoxylin and eosin (H&E). Cell proliferation was determined by immunohistochemical (IHC) analysis using anti-BrdU antibody (Becton Dickinson Immunocytometry System, San Jose, CA) and counting the number of nucleated cells in the epidermal basal cell layer in sections of dorsal skin. Apoptosis was evaluated using an anti caspase-3 polyclonal antibody AF835 (R&D Systems, Minneapolis, MN). The seven founder mice were analyzed at 10 mo of age (retired breeders) following the same protocol.

### Development of Congenic Lines

Since our K5D1a transgenic line was maintained on a congenic C57BL/6J background, and given the necessity to establish comparisons between K5D1a and K5D1b transgenic lines, we developed C57BL/6J-K5D1b and FVB/N-K5D1a congenic lines. To develop these lines we used marker assisted “speed congenic” breeding strategies [32]. Fourteen N2, N3, and N4 males (positive for the transgene as tested by PCR) were successively genotyped with polymorphic

simple sequence-length polymorphism (SSLP) markers distributed throughout the genome in order to select those males with the least amount of donor genome. Microsatellite analysis of N5 mice from both congenic lines showed that more than 98% of the alleles were from the recipient strain genome (data not shown).

### Generation of K5D1a/b Bigenic Lines and Transgenic/KO Compound Mice

We generated transgenic/KO compound lines by crossing partially congenic FVB/N; SSIN-*Ccnd1*-/- (D1KO) mice [33] (gift of Dr. Robert A. Weinberg and Dr. Piotr Sicinsky) with the FVB/N-K5D1b congenic line. K5D1a/b bigenic mutant mice were generated by intercrossing FVB/N-K5D1a and FVB/N-K5D1b lines. Identification of the different genotypes was carried out using cyclin D1b specific primers (see above) and two sets of primers identifying the KO and wild-type allele of the *Ccnd1* gene as previously described [11].

### X-Ray Images

X-ray radiographs were taken with a MX-20 Digital X-ray machine (Faxitron X-ray Corporation, Wheeling, IL) at 24 kV/15 s. Mice were immobilized with ketamine (50 mg per kilogram of body weight, intraperitoneally).

### Short-Term UV-Irradiation

Both wild-type and K5D1b transgenic mice were exposed one time to 200 mJ of UV irradiation. Then these mice were terminated at different times after irradiation during a 24 h period (0, 4, 8, 12, 16, and 24 h). The mice were intraperitoneally injected with BrdU 30 min before euthanasia and their skins were collected. Sections of dorsal skin were used for IHC analysis to evaluate p53 expression (#NCL-p53-CM5p; Novocastra Laboratories, New Castle, UK), apoptosis/caspase-3 (R&D Systems), and BrdU (Becton Dickinson Immunocytometry System) incorporation as previously described [31].

### Skin Carcinogenesis and Tissue Collection

Fifteen K5D1b mice plus 15 littermate wild-type controls were used to determine the susceptibility to chemically induced tumorigenesis. The two-stage carcinogenesis protocol was performed by initiating newborn mice (24-72 h age) as previously described [34] with 200 nmol of 7,12-dimethyl-benz[a]anthracene (DMBA) (Sigma Chemical Co., St Louis, MO) in 30 μL of acetone on the dorsal skin. Promotion was started 2 wk after initiation with twice weekly applications of 4 μg of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma Chemical Co.) in 200 μL of acetone. Promotion was stopped after 30 wk and the animals were kept alive for a total of 40 wk after initiation. Tumors were counted weekly and histological analysis of the tumors was performed at the end of the experiment or upon the death of the animal.

### Identification of Putative Murine Cyclin D1b Isoforms

For RNA preparations, skin, thymus and papillomas induced by DMBA/TPA were ground and then homogenized in TriReagent (Sigma Chemical Co.) according to the instructions of the manufacturer. Total RNA preparations were treated with DNAse (Life Technologies, Inc., Grand Island, NY) and then used for reverse transcription (RT) and polymerase chain reaction (PCR) assays. RTs were performed from total mRNA using GeneAmp® RNA PCR kit (Applied Biosystems, Foster City, CA) and the products were separated by 2% agarose gel electrophoresis. The PCR product was purified from the agarose gel, cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and sequenced by BigDye Terminator sequence chemistry. The sequences were compared by BLAST against the NR database at NCBI.

### Statistical Analysis

Statistical significance was determined by calculation of *P* values. Statistical differences for squamous cell carcinoma (SCC) multiplicity were determined with Fisher's exact test. The differences between papilloma per animal (multiplicity) were analyzed by comparison of frequencies using Poisson's regression.

## RESULTS

### K5D1b Mice Demonstrate no Obvious Phenotype

Following pronuclear microinjection of the cyclin D1b transgenic construct into fertilized FVB/N eggs, seven founders were detected by PCR as described in Materials and Methods Section. The expression of the human cyclin D1b transcript was confirmed by RT-PCR in epidermis and thymus from transgenic mice (Figure 1B). In addition, protein expression was confirmed by Western blots in epidermal tissues using four different antibodies (Figure 1C). Two antibodies against the carboxy-terminal sequence derived from intron 4 recognize exclusively the cyclin D1b isoform (Sen-2, D1bAb), whereas the commercial antibodies Ab3 and DCS-6 recognize both, cyclin D1a and cyclin D1b isoforms.

Phenotypic changes were studied extensively in the seven K5D1b founders and first generation offspring, and also in the established K5D1b line up to 1 yr of age. Neither gross observation nor histopathological studies showed abnormal phenotype in the analyzed tissues (skin, thymus, esophagus, stomach, bladder and prostate). This is in sharp contrast to K5D1a mice, which showed epidermal hyperproliferation and severe thymic hyperplasia [8]. Figure 2 shows representative thymus size of FVB/N wild-type, K5D1b and K5D1a mice as observed during necropsy (panel A) or X-ray imaging (panel B). The lack of thymic phenotype was observed not only in seven independent founders with different levels of expression and random integration in the original FVB/N background but also in the congenic line established on C57BL/6 (data not shown). It is worth mentioning that K5D1a mice exhibit the hyperplastic thymic phenotype in the original B6D2 mixed background, as well as in SENCAR and C57BL/6 backgrounds [8][35].

### Epidermal Proliferation in K5D1b Mice

Cell proliferation in the basal layer of untreated dorsal skin from K5D1b mice and control littermates was evaluated by BrdU incorporation. Unlike K5D1a mice that presented a moderate increase in keratinocyte proliferation, K5D1b mice showed no difference in epidermal proliferation when compared with wild-type mice (data not shown). In order to determine whether forced expression of cyclin D1b results in an exacerbated response to TPA-induced keratinocyte proliferation, K5D1b mice and wild-type littermates were treated with a topical application of TPA to the shaved dorsal skin, and BrdU incorporation was measured at 24 h. However, the level of proliferation was similar in both K5D1b and wild-type mice (data not shown).

### Short-Term UV Irradiation Experiments

To test the possibility of alterations in the $G_1$checkpoints in tissues expressing human cyclin D1b, we exposed K5D1b mice and littermate controls to a single dose of 200 mJ/cm$^2$UV and collected skin samples at 4, 8, 12, 16, and 24 h after exposure. As shown in Figure 3A, we observed a higher proliferative response (measured by BrdU incorporation), in K5D1b transgenic keratinocytes, compared to wild-type littermates. In addition, we observed reduced numbers of apoptotic cells in epidermis from transgenic mice compared to wild-type (Figure 3B). Also, IHC analysis showed a decrease in levels of p53 in UV-irradiated K5D1b mice

(Figure 3C). These results suggest that overexpression of cyclin D1b exacerbates UV-induced proliferation.

## K5D1b Mice Show Increased Sensitivity to Two-Stage Skin Carcinogenesis

To determine whether cyclin D1b expression may have an effect on tumor development, we conducted a two-stage skin carcinogenesis protocol using FVB/N-K5D1b mice and wild-type littermates. Initiation was carried out by topical application of DMBA in newborn mice as previously described [34] and promotion was carried out twice a week by topical application of TPA for 30 wk. The tumor multiplicity (papillomas per mouse) was scored in each group for 40 wk. The first papilloma appeared in both K5D1b mice and wild-type siblings between 6 and 7 wk of promotion. The number of papillomas increased rapidly in the transgenic mice reaching a plateau of approximately 8 papillomas per mice at 18 wk, whereas wild-type mice reach a plateau of 5.8 papilloma per mice at 21 wk (data not shown). This is in contrast with K5D1a mice, in which skin tumor development after two-stage carcinogenesis was not affected [36]. This experiment was repeated showing a more pronounced difference between K5D1b and wild-type littermates (see below and Figure 4A). SCC incidence and multiplicity at 30 wk showed no statistically significant differences (Fisher's exact test) between K5D1b mice and wild-type littermates (incidence was: K5D1b = 27.3% and WT = 25%; average number of SCC per mouse was: K5D1b = 0.41 and WT = 0.31).

To study the functional consequences of human cyclin D1b overexpression in skin tumorigenesis in the absence of the endogenous cyclin D1 protein, we established a series of compound lines intercrossing D1KO and K5D1b mice. We carried out a two-stage carcinogenesis experiment using six different genotypes (12-19 mice per genotype). In agreement with our previous experiment, D1+/+; K5D1b mice, expressing normal levels of the endogenous *Ccnd1* gene but expressing human *CCND1b*, showed an increase susceptibility to skin carcinogenesis, since the number of papillomas per mouse was significantly higher ($P < 0.001$) when compared with wild-type mice (D1+/+; WT) (Figure 4A). In agreement with these results, expression of cyclin D1b in a *Ccnd1* haplo-insufficient background (D1+/-; K5D1b compound mutant mice) showed an intermediate susceptibility to DMBA/TPA compared to D1+/+; K5D1b and D1-/-; K5D1b compound mutant mice (Figure 4B). We previously demonstrated that lack of cyclin D1 results in severe reduction in the number of papillomas [11]. Interestingly, the compound mutant line lacking the endogenous *Ccnd1* gene but expressing the human *CCND1b* isoform (D1-/-; K5D1b) also develop a very small number of tumors, showing that cyclin D1b overexpression in keratinocytes can not rescue the resistance of cyclin D1 null mice (D1-/-; WT) to skin tumor development after DMBA/TPA treatment (Figure 4C).

## Lack of Evidence of Cyclin D1b Expression in Mouse Tissues

In order to investigate the existence of cyclin D1b in the mouse we used first an "in silico" approach. The GenBank accession # NT_039437 (*Ccnd1* containing contig) was used to predict a 325 bp transcript unique to mouse cyclin D1b, including the 99 bp sequence derived from intron 4 and the 226 bp sequence from exon 4. This sequence was used to query the NCBI dbEST (www.ncbi.nlm.nih.gov/dbEST) and the RIKEN mouse full-length cDNA collection (http://genome.gsc.riken.jp/home.html) databases. No sequence matched our predicted D1b transcript. Based on the same reference sequence, we designed several primers to amplify a region of the putative cyclin D1b isoform. The target region spanned from exon 3 to the first 30 nucleotides of the mouse *Ccnd1* intron 4. We performed RT-PCR from different tissues (i.e., skin, thymus, and papillomas obtained after DMBA/TPA carcinogenesis) and various inbred mouse strains (i.e., SSIN/Sprd, FVB/N, and 129/Sv), and no PCR products matching predicted sequences for cyclin D1b were obtained.

## DISCUSSION

We describe here the phenotype of transgenic mice (K5D1b) that express human cyclin D1b, an alternative transcript of *CCND1* that has been shown to have oncogenic properties in tissue culture experiments [20][21]. The purpose of this study was to investigate the in vivo effect of cyclin D1b overexpression and to compare the phenotype of the K5D1b mice with our previously described K5D1a mice overexpressing the canonical form of cyclin D1 [8][9].

K5D1b mice were essentially devoid of any macroscopic or microscopic phenotype and did not develop spontaneous tumors. In this regard the K5D1b is not very different from the previously described K5D1a mice [8][9] with only one remarkable difference: the lack of the severe and fatal thymic hyperplasia observed in mice overexpressing the cyclin D1a transcript [8]. The mechanisms by which overexpression of cyclin D1 lead to thymic hyperplasia in K5D1a mice are not well understood but our previous research suggests that it is caused by the expansion of a K5 positive progenitor compartment located in the medulo-cortical junction of the thymus [35]. The absence of the thymus phenotype in K5D1b mice is unlikely to be the result of differences in expression because both transgenic mice, K5D1a and K5D1b, were developed with the same strategy and the absence of the thymus phenotype was observed in several founders with different levels of expression. The more likely explanation for the lack of thymic phenotype in K5D1b mice is a loss of function associated with the absence of exon 5 in the cyclin D1b isoform [20][21].

Experimental evidence from three independent studies, including ours, confirmed the predominant nuclear localization of cyclin D1b but failed to show changes in stability [20] [21]. Functional studies using a cyclin D1 mutant (T286A) confirmed that this residue, a target of GSK-3b, is critical for cyclin D1 nuclear export and also showed that this mutation confers cyclin D1 oncogenic properties [18][27]. The oncogenic potential of constitutively nuclear cyclin D1, was further demonstrated in transgenic mice overexpressing the mutated form of cyclin D1 (T286A) controlled by either the lymphoid-specific Eμ enhancer or the mammary gland MMTV-LTR promoter. Mice that expressed the T286A mutant cyclin D1 in lymphatic tissues developed mature B-cell lymphomas, whereas mice overexpressing the canonical cyclin D1 under the control of the same promoter did not develop neoplastic lesions [27]. Similarly, mice expressing the T286A mutant cyclin D1 in the mammary gland developed tumors at a faster rate than those animals overexpressing wild-type cyclin D1a [37].

Recent findings showing the presence of *CCND1* mutations in endometrial carcinomas and esophageal cancer also support the hypothesis that constitutive nuclear location confers cyclin D1 oncogenic properties [38][39]. Point mutations and deletions in *CCND1*, creating truncated mRNAs, have also been recently reported in mantle cell lymphomas [40]. Based on this large body of information, it can be postulated that the oncogenic properties of cyclin D1b are related to its constitutive nuclear localization. However, the actual molecular mechanisms by which cyclin D1 nuclear retention may affect tumor development are not well understood. A recent study from Diehl's laboratory suggests that nuclear accumulation of cyclin D1 inhibits CDT1 proteolysis resulting in aneuploidy and genome instability [41].

In our transgenic model, the forced expression of cyclin D1b did not result in spontaneous neoplasias but increased the susceptibility to chemically induced skin carcinogenesis. In contrast, our previously published K5D1a transgenic did not produce an increase in susceptibility to the two stage carcinogenesis protocol [36]. Similarly, other transgenic mice overexpressing the canonical cyclin D1a have not shown an oncogenic phenotype or in some case a weak oncogenic phenotype [42-45]. The only exception is the study of Yamamoto et al. [10] reporting enhanced skin tumorigenesis using a complete DMBA-induced carcinogenesis protocol in the relatively resistant C57BL/6 background.

Whether the lack of proliferative activity of cyclin D1b is related to the nuclear retention, changes in substrate recognition or decreased CDK4 activity remains to be determined. Paradoxically, our study showed that when proliferation is stimulated by UV exposure, K5D1b mice show a higher epidermal proliferative response than the wild-type littermates. In addition, K5D1b mice irradiated with a single exposure to UV light had a reduced number of apoptotic cells and a lower percentage of p53 positive cells than the wild-type mice. Collectively, these results suggest that overexpression of cyclin D1b interferes with the proliferation checkpoints facilitating the transit to S phase under conditions of stress. Cyclin D1 activation of CDK4 and CDK6 leading to phosphorylation of RB is still considered an important function of cyclin D1 but it is presently recognized that the cyclin D1/CDK4 complex phosphorylates several other proteins, including SMAD3, CDT1 and the RB related proteins p107 and p130 [15]. Furthermore, it has become clear that cyclin D1 has also cell cycle and CDK-independent functions. Several studies have shown that cyclin D1 acts independently of CDKs as a modifier of gene transcription [4][46]. Transcription factors regulated by cyclin D1 include several members of the nuclear receptor superfamily like estrogen and progesterone receptors, thyroid hormone, and PPARg. Other transcription factors regulated by cyclin D1 are C/EBPb, STAT3, DMP1, and Beta2/NeuroD [26][47][48]. One mechanism involved in transcriptional modification by cyclin D1 is the enhancement of transcription by means of recruitment of coactivators [15]. A specific LxxLL motif important for coactivator recruitment has been identified in exon 5 [49][50]. The lack of the LxxLL motif may play a role in the oncogenicity of cyclin D1b as shown recently by the Knudsen laboratory [26]. They showed that while cyclin D1a association with the AR results in growth inhibition of AR-dependent prostate cells, association of the D1b with AR stimulated proliferation in this cell type [26]. The cotranscriptional activities of cyclin D1 may play important role not only in transformation but also in the development of the thymic hyperplasia.

The discovery of cyclin D1b and its oncogenic properties generate an attractive hypothesis to explain some of the paradoxical results regarding the role of cyclin D1 in cancer development. In spite of the large body of literature providing circumstantial evidence for the oncogenic role of cyclin D1, no tissue culture or transgenic experiments have been able to establish cyclin D1 as a dominant oncogene [15][16]. Yet, cyclin D1 null mice are resistant to chemical skin carcinogenesis [11] as well as Ras and Neu transformation in breast cancer models [14].

In summary, our results show that cyclin D1b has very distinct biological properties compared to canonical cyclin D1a and also confirm cyclin D1b oncogenicity in vivo. However, our data does not provide support for the existence of a murine homologue of the cyclin D1b isoform and shows that human cyclin D1b transgene could not rescue cyclin D1KO mice resistance to epidermal carcinogenesis. In addition, the lack of a thymus phenotype in K5D1b mice opens new possibilities in understanding the role of cyclin D1 in thymus homeostasis.

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## Abbreviations

IHC, immunohistochemical; DMBA, 7,12-dimethyl-benz[a]anthracene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RT, reverse transcription; PCR, polymerase chain reaction; SCC, squamous cell carcinoma; AR, androgen receptor.

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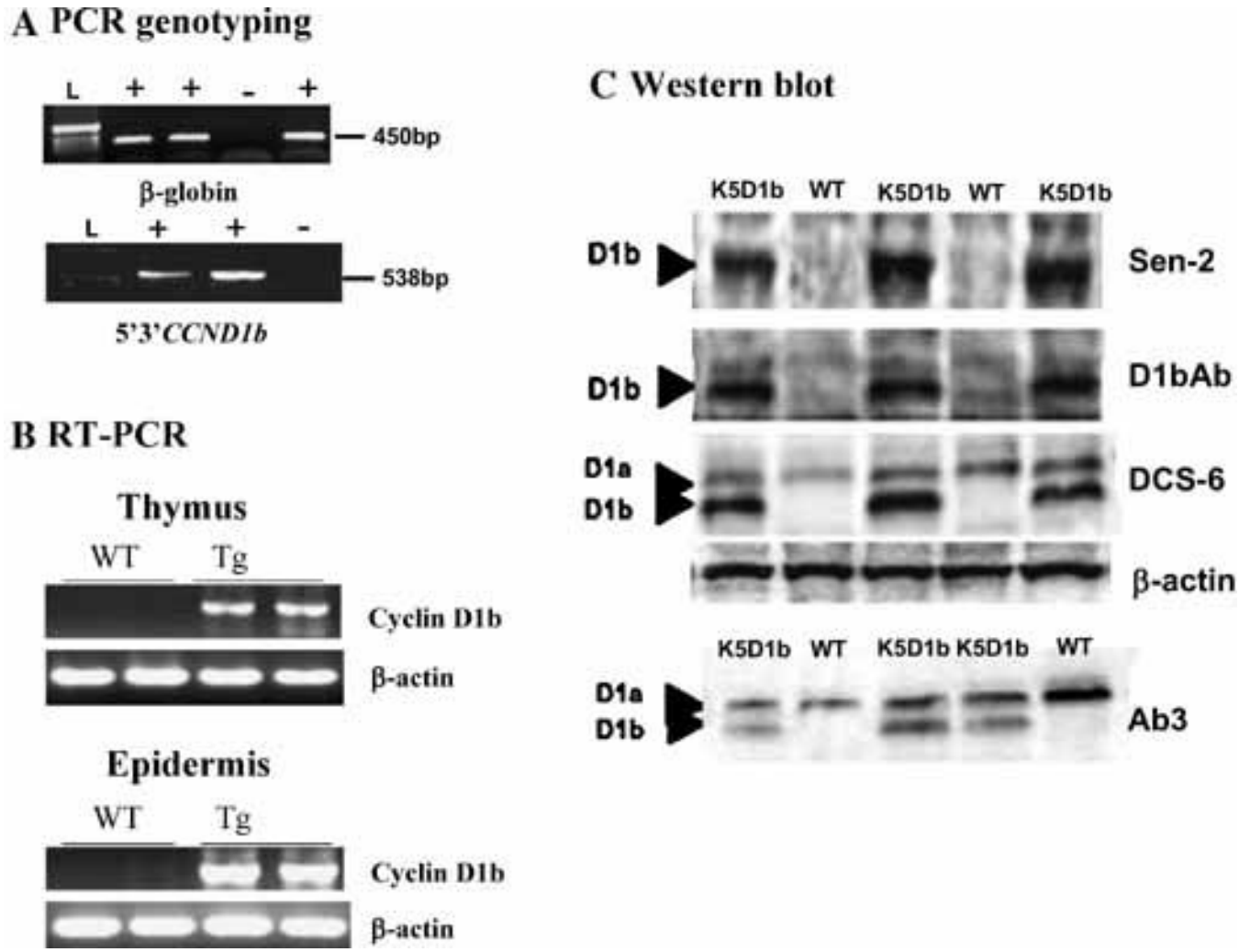


**Figure 1.**

(A) K5-*CCND1B* transgene screening techniques. PCR amplification of DNA extracted from mouse tail clips. Above: β-globin sequence was amplified resulting in a 450 bp product. Below: a cyclin D1b specific sequence was amplified resulting in a 538 bp product (L: molecular weight marker). (B) RT-PCRs were performed from epidermis and thymus total mRNA. Tg, K5D1b transgenic; WT, wild-type littermates. (C) Western blot expression of the K5D1b transgene. Western blots were carried out in epidermal extracts using 4 different antibodies. Two antibodies (Sen-2 and D1bAb) are polyclonal antibodies against the sequences derived from intron 4 and are cyclin D1b specific. The two other antibodies (DCS-6 and Ab3) are commercially available antibodies against the C-terminal region and recognize both cyclin D1 transcripts.

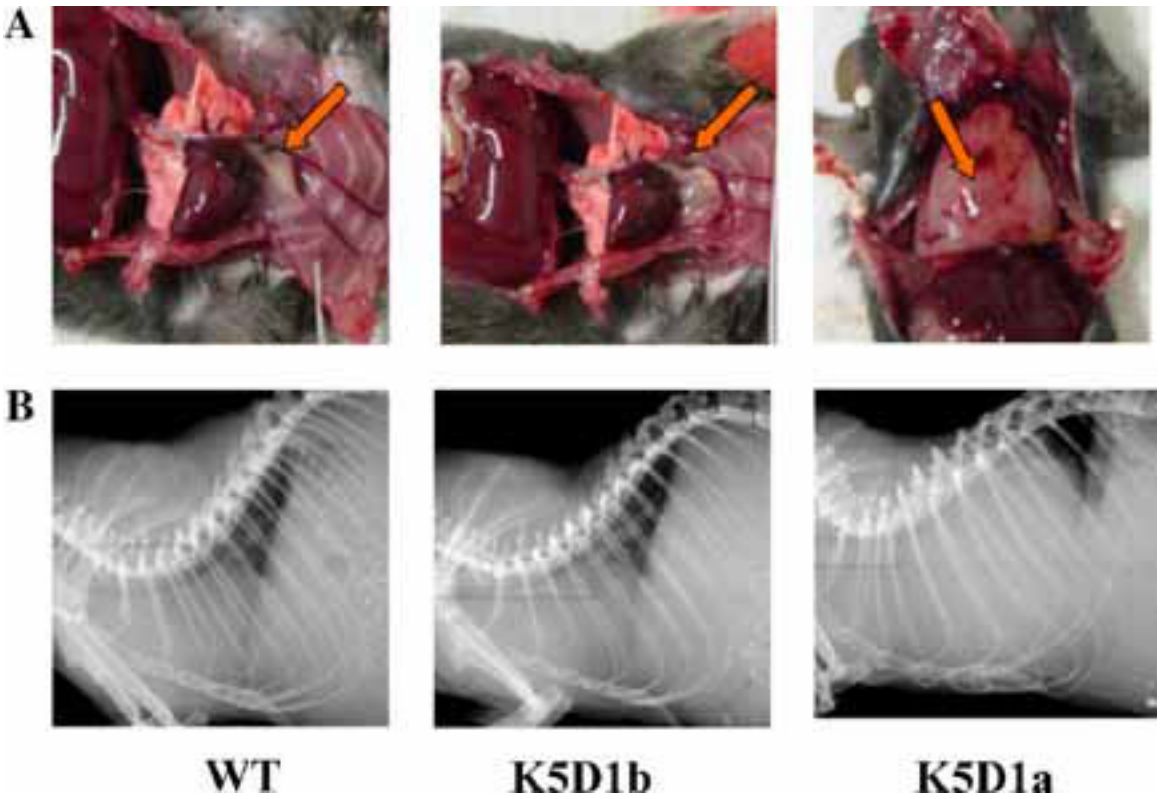


**Figure 2.**
Thymus development in 3-mo-old wild-type, K5D1b and K5D1a mice. (A) Gross morphology of the thoracic cavity of WT, K5D1b and K5D1a FVB/N mice (arrows point at the thymus). (B) X-ray images of the thorax from the same animals.

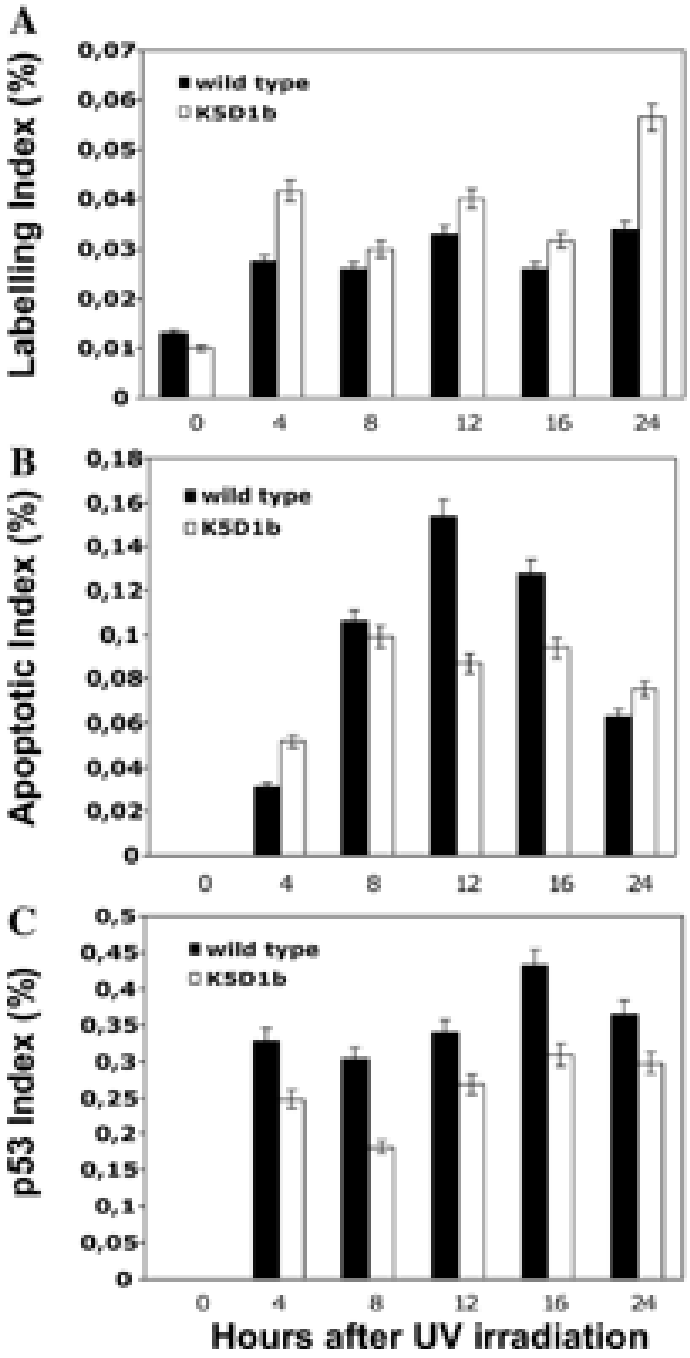


**Figure 3.**

Effect of short term UV exposure on dorsal skin of K5D1b mice. K5D1b mice and littermate controls were irradiated with a single dose of 200 mJ/cm$^2$UV. (A) BrdU incorporation in the basal cells of the epidermis. (B) Apoptosis index determined by caspase-3 immunostaining. (C) p53 nuclear accumulation determined by IHC.

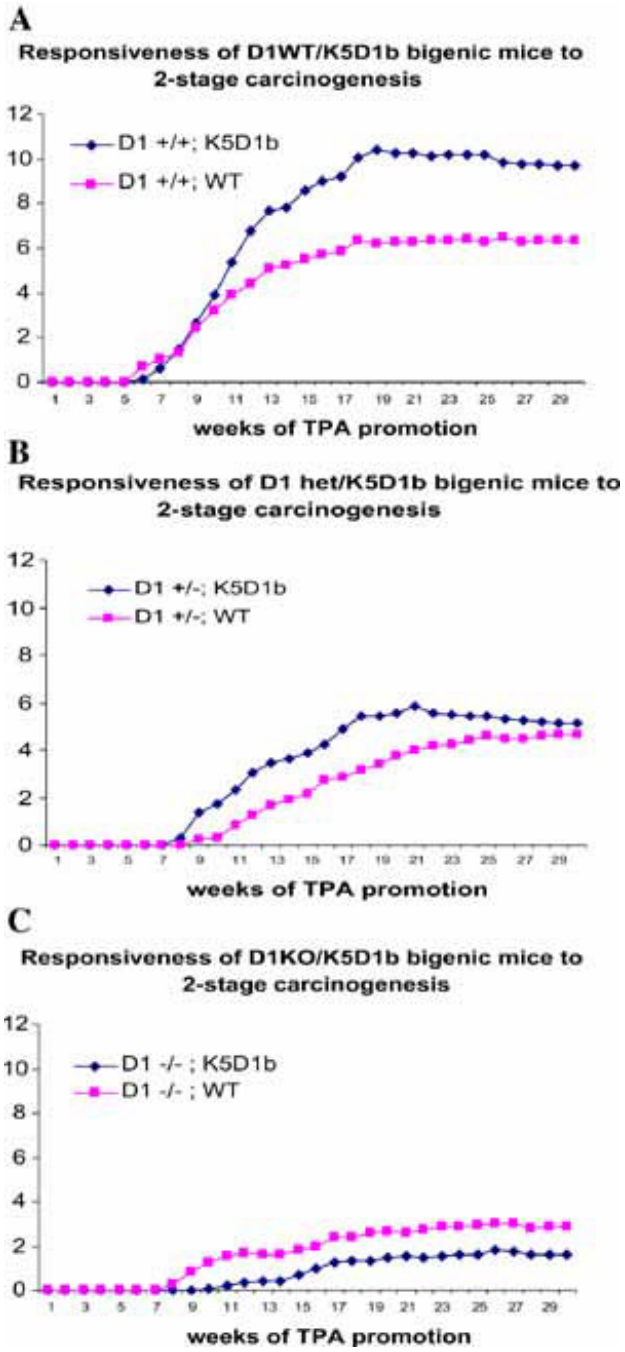


**Figure 4.**

Multiplicity of skin tumors after two-stage carcinogenesis in K5D1b crosses with *Ccnd1* KO mice. (A) D1+/+; K5D1b compound mutant mice, expressing normal levels of *Ccnd1* gene but expressing human *CCND1b*, show a significant increase in susceptibility to carcinogenesis, compared with wild-type mice (D1+/+; WT) ($P < 0.001$). (B) D1+/-; K5D1b compound mutant mice, haplo-insufficient for the endogenous *Ccnd1* gene but overexpressing human *CCND1b*, shows an intermediate susceptibility to DMBA/TPA compared to D1+/+; K5D1b and D1-/-; K5D1b compound mutant mice. (C) D1-/-; K5D1b compound mutant mice, lacking the endogenous *Ccnd1* gene but expressing the human *CCND1b* isoform, does not rescue the resistance of cyclin D1 null mice (D1-/-; WT) to skin tumor development after DMBA/TPA

carcinogenesis. Analysis of differences between groups was performed with Poisson's regression for multiplicity.