# Requirement for cyclin D3 in lymphocyte development and T cell leukemias

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## Summary

**The D-type cyclins (cyclins D1, D2, and D3) are components of the core cell cycle machinery in mammalian cells. Cyclin D3 gene is rearranged and the protein is overexpressed in several human lymphoid malignancies. In order to determine the function of cyclin D3 in development and oncogenesis, we generated and analyzed cyclin D3-deficient mice. We found that cyclin D3$^{-/-}$ animals fail to undergo normal expansion of immature T lymphocytes and show greatly reduced susceptibility to T cell malignancies triggered by specific oncogenic pathways. The requirement for cyclin D3 also operates in human malignancies, as knock-down of cyclin D3 inhibited proliferation of acute lymphoblastic leukemias deriving from immature T lymphocytes. These studies point to cyclin D3 as a potential target for therapeutic intervention in specific human malignancies.**

## Introduction

D-type cyclins are the ultimate recipients of mitogenic and oncogenic signals. Three D cyclins (cyclins D1, D2, and D3) have been described in mammalian cells. The three proteins are encoded by separate genes, but they show substantial amino acid similarity and are expressed in a highly overlapping fashion in all proliferating cells (Sherr and Roberts, 1999).

Once induced, D cyclins bind and activate their associated cyclin-dependent kinases CDK4 and CDK6 (Bates et al., 1994; Kato et al., 1994; Meyerson and Harlow, 1994). Cyclin D-CDK complexes phosphorylate the retinoblastoma tumor suppressor gene product, pRB, and pRB-related proteins p130 and p107 (Bates et al., 1994; Matsushime et al., 1992, 1994; Meyerson and Harlow, 1994). This phosphorylation cancels growth-inhibitory functions of pRB, leads to release or derepression of the E2F transcription factors and allows induction of E2F-target genes that are required for the S phase entry (Dyson, 1998). Hence, D cyclins lie at the interface between upstream oncogenic pathways and the retinoblastoma tumor suppressor protein.

Consistent with their growth-promoting functions, abnormal expression of D cyclins is believed to be a driving force in several human cancers. Indeed, chromosomal abnormalities involving cyclin D loci and overexpression of cyclin D protein were observed in many malignancies. Cyclin D1 gene is rearranged or amplified and the protein is overexpressed in several human cancers such as breast carcinomas; squamous cell carcinomas of head and neck, esophagus, tongue, and larynx; carcinomas of uterine cervix; astrocytomas; non-small cell lung cancers; soft tissue sarcomas; mantle cell lymphomas; and others (Cheung et al., 2001; Fujii et al., 2001; Lammie et al., 1991; Withers et al.,

### SIGNIFICANCE

**D cyclins are the ultimate recipients of oncogenic signals. Amplification of the cyclin D genes and overexpression of cyclin D proteins is seen in several human cancers. Importantly, a large number of human malignancies contain lesions in pathways impacting on D cyclins. In this study, we found that mice lacking cyclin D3 show greatly reduced sensitivity to malignancies triggered by specific oncogenic pathways in immature T lymphocytes. This requirement for cyclin D3 also operates in human malignancies, as knock-down of cyclin D3 inhibited proliferation of T cell Acute Lymphoblastic Leukemia (T-ALL) cells. These studies illustrate the utility of manipulating the mouse genome to understand the molecular pathways operating in human cancer cells, and they point to cyclin D3 as a potential therapeutic target in human malignancies deriving from immature T lymphocytes.**

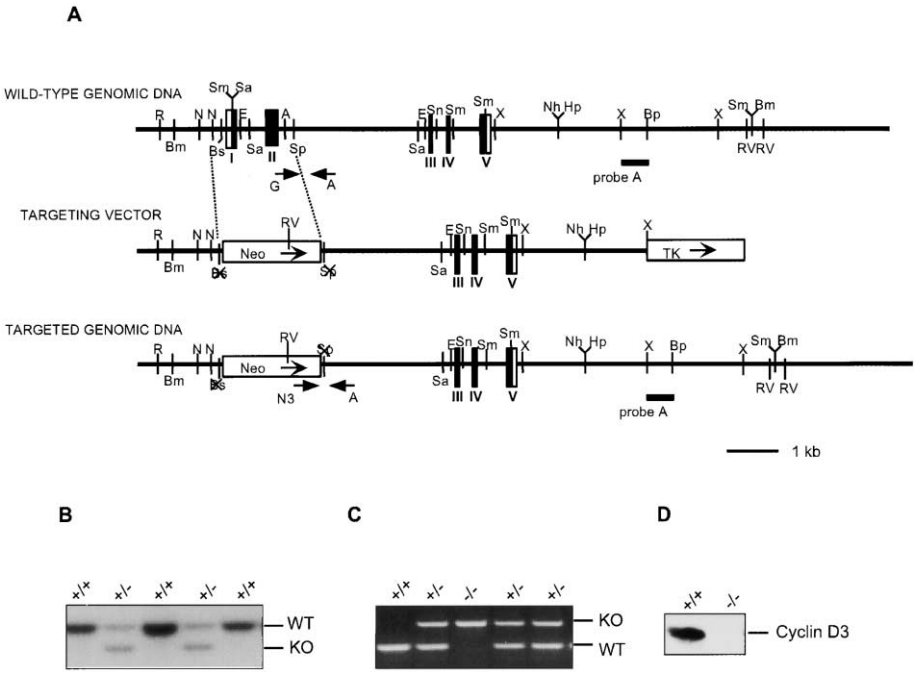


**Figure 1.** Targeted inactivation of the cyclin D3 gene

**A:** Cyclin D3 gene targeting strategy. The coding exons are shown as filled boxes and are numbered; open boxes denote the noncoding portions. Probe A used for Southern blot analysis, as well as PCR primers G, A, and N3 are indicated. Neo, the neomycin phosphotransferase gene; TK, thymidine kinase gene. Arrows show transcriptional orientation of these genes. Restriction enzyme abbreviations: R, EcoRI; Bm, BamHI; N, NruI; Bs, BssHII; Sm, SmaI; Sa, Sac II; E, EagI; A, Asp718; Sp, SpeI; Sn, SnaBI; X, XbaI; Nh, NheI; Hp, HpaI; Bp, BspEI; RV, EcoRV.

**B:** Southern blot analysis of genomic DNA extracted from ES cell clones, digested with EcoRI and EcoRV and hybridized with probe A. The position of wild-type (wt) and disrupted (KO) alleles are indicated.

**C:** PCR analysis of genomic DNA isolated from mouse tails. The position of wild-type (wt) and mutant (KO) amplification products are indicated.

**D:** Immunoblot analysis of mouse embryo lysates probed with anti-cyclin D3 antibody. In **B**, **C**, and **D**, the genotypes are presented above the lanes.

1991; Bartkova et al., 1994; Dickson et al., 1995; Weinstat-Saslow et al., 1995). Cyclin D2 gene is amplified in human testicular tumors (Houldsworth et al., 1997; Sicinski et al., 1996), while the protein is overexpressed in a wide range of B cell malignancies, such as B cell lymphocytic leukemias, lymphoplasmacytic lymphomas (Delmer et al., 1995), and chronic lymphocytic leukemias (Motokura and Arnold, 1993).

Cyclin D3 is the least studied of the D cyclins. It is expressed in nearly all proliferating cells, and it shows the most broad expression pattern of all three D-type cyclins (Bartkova et al., 1998). Like other D cyclins, cyclin D3 is overexpressed in human cancers (Buschges et al., 1999; Hedberg et al., 2002; Ito et al., 2001). A potential role for cyclin D3 in the malignancies of the lymphoid system is suggested by the observations that cyclin D3 gene is rearranged in several neoplastic diseases deriving from this compartment, such as diffuse large B cell lymphomas or multiple myelomas (Filipits et al., 2002; Shaughnessy et al., 2001).

In the present study, we tested the role for cyclin D3 in development and in oncogenesis by generating cyclin D3-deficient mice. We found that cyclin D3 is critically required for the proliferative burst during development of immature T lymphocytes. Importantly, we found that cyclin D3 is essential for specific oncogenic pathways operating in this compartment. Lastly, we demonstrate that this requirement for cyclin D3 also extends to human malignancies deriving from immature T lymphocytes. These studies raise a possibility of novel therapeutic approaches in human T cell leukemias, centered on cyclin D3 inhibition.

## Results

### Thymocyte defects in cyclin D3-deficient mice

Cyclin D3$^{-/-}$ mice were generated by gene targeting in embryonal stem cells, using standard procedures (Figure 1). Cyclin D3-deficient animals were born with expected frequency; they were fertile and appeared normal during the 1.5 year observation period (data not shown). However, we noticed that cyclin D3$^{-/-}$ mice displayed severely hypoplastic thymuses (Figure 2A), which contained 7-fold fewer thymocytes than wild-type littermates (Figure 2B).

To further investigate the defect seen in cyclin D3-deficient thymuses, we analyzed T cell development in mutant animals. The great majority of T cells normally follow the developmental sequence CD4$^-$CD8$^-$ (double-negative)→CD4$^+$CD8$^+$ (double-positive)→CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ (single-positive). We found that the percentage of double-negative cells was increased while the fraction of double-positive cells was decreased in the thymuses of cyclin D3$^{-/-}$ animals, as compared with wild-type littermates (Figure 2C). When the absolute numbers of thymocytes in each fraction were calculated, we found that the total number of double-negative thymocytes was unchanged in mutant animals, as compared with wild-type littermates, while the total number of double-positive cells was reduced over 12-fold (Figure 2D).

### Impaired expansion of immature T lymphocytes

The expansion of immature T lymphocytes within the thymus normally occurs at the double-negative stage (Muljo and Schlissel, 2000). The double-negative population can be further subdivided into four developmental stages, termed DN-1 to DN-4. Up to the DN-3, the proliferation of thymocytes is driven by the interleukins and c-kit (Di Santo et al., 2000). A dramatic shift takes place at the DN-3 stage. At this point, immature lymphocytes rearrange β chains of their T cell receptor (TCR) and assemble the pre-TCR. The proliferation of thymocytes becomes cytokine independent, but it is driven instead by the pre-TCR (Fehling et al., 1995; Hoffman et al., 1996). Signals emanating from the pre-TCR drive expansion of the DN-4 and of "immature single-positive" (ISP) cells, which finally differentiate into double-positive thymocytes and arrest their proliferation.

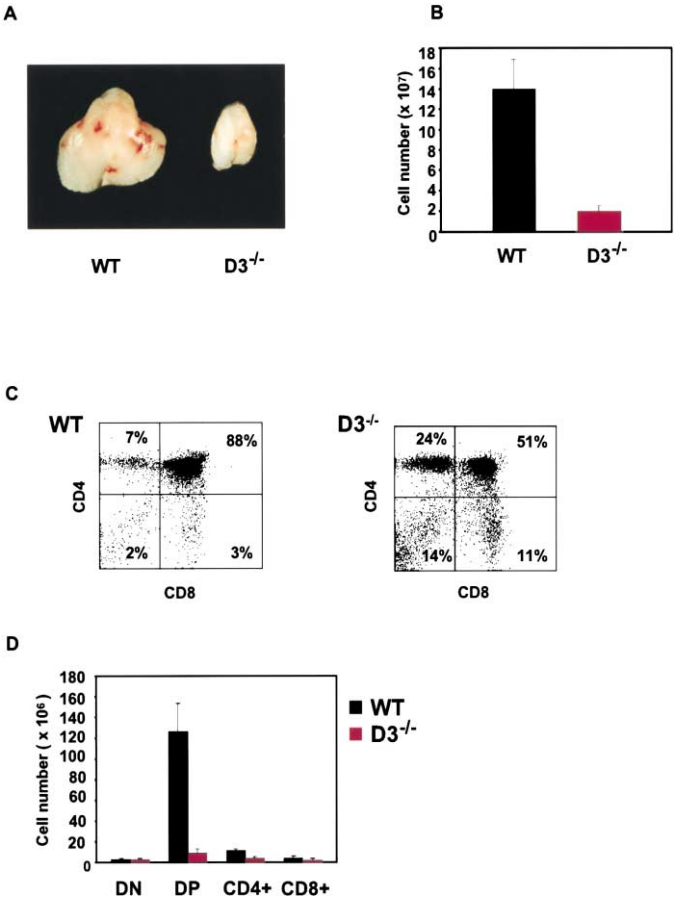


**Figure 2.** Defective thymocyte development in cyclin D3-deficient mice

**A:** Appearance of thymuses dissected from 5-week-old wild-type (wt) and cyclin D3-deficient mice. Magnification ~6×.
**B:** Total number of thymocytes in 4- to 6-week-old wild-type (n = 11) and cyclin D3$^{-/-}$ (n = 11) mice. Error bars denote SD.
**C:** Examples of flow cytometric profiles of thymocytes from 5-week-old mice stained with anti-CD4 and anti-CD8 antibodies. The percentage of cells in each quadrant is shown.
**D:** Total number of CD4$^{-}$CD8$^{-}$ double-negative (DN), CD4$^{+}$CD8$^{+}$ double-positive (DP), CD4$^{+}$, and CD8$^{+}$ thymocytes in 4- to 6-week-old wild-type (n = 8) and cyclin D3$^{-/-}$ mice (n = 9). Error bars denote SD.

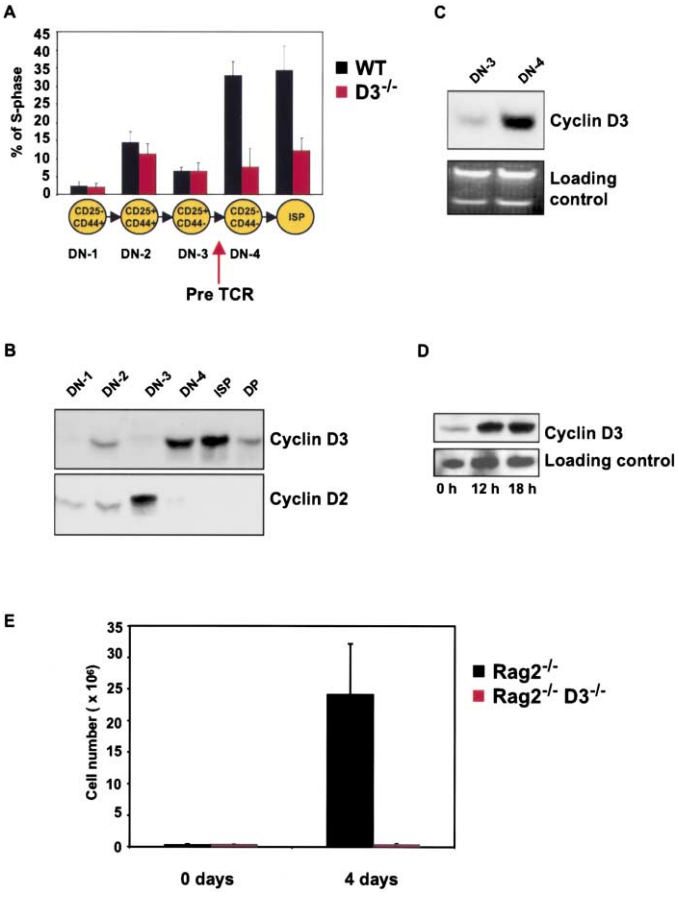


**Figure 3.** Impaired expansion of immature T lymphocytes in cyclin D3-deficient mice

**A:** Percentage of cells in the S phase of the cell cycle among subsets of double-negative (DN) and immature single-positive (ISP) thymocytes. The assembly of the pre-TCR is indicated. Shown are means of four independent experiments, each using pooled thymocytes from 5 mice, 4–6 weeks old. Error bars indicate SD.
**B:** Western blot analysis of thymocyte subsets from wild-type mice. Upper and lower panels represent the same blot sequentially probed with antibodies against cyclin D3 and D2. DP denotes double-positive cells.
**C:** Northern blot analysis of RNA prepared from indicated thymocyte subsets from wild-type mice probed with a cyclin D3-specific probe. Ethidium-bromide-stained gel is shown below (loading control).
**D:** Rag2$^{-/-}$ mice were injected intraperitoneally with anti-CD3ε antibody. Thymocytes were harvested at 0, 12, and 18 hr after stimulation, and cyclin D3 levels were determined by the Western blotting. A cross-reacting band is shown below (loading control). To ensure equal loading, Western blots were reprobed with an anti-actin antibody.
**E:** Rag2$^{-/-}$ or Rag2$^{-/-}$/cyclin D3$^{-/-}$ mice were injected intraperitoneally with anti-CD3ε antibody. Four days after stimulation, thymocyte numbers were determined and compared with thymocyte counts in untreated animals. The values were: untreated Rag2$^{-/-}$ = 0.35 × 10$^{6}$; untreated Rag2$^{-/-}$/cyclin D3$^{-/-}$ = 0.37 × 10$^{6}$; treated Rag2$^{-/-}$ = 24.0 × 10$^{6}$; treated Rag2$^{-/-}$/cyclin D3$^{-/-}$ = 0.42 × 10$^{6}$. Four to five mice were analyzed for each time point. Error bars indicate SE.

To probe the proliferation rate of mutant thymocytes, we flow-sorted double-negative cells into DN-1–DN-4 and ISP populations, and we determined the percentage of proliferating cells in each population. We found that the fraction of cycling cells at the DN-1, DN-2, and DN-3 did not differ significantly between wild-type and cyclin D3-deficient mice (Figure 3A), revealing that cyclin D3 is dispensable for cytokine-driven expansion of immature T lymphocytes. In contrast, the pre-TCR-driven expansion of the DN-4 and ISP thymocytes was very reduced in cyclin D3-deficient mice (Figure 3A). Importantly, we determined that cyclin D3-deficient thymocytes expressed normal levels of TCRβ and of other pre-TCR components (data not shown). Collectively, these observations suggest that cyclin D3 might operate downstream of the pre-TCR in driving proliferation of immature T lymphocytes, and that in the absence of cyclin D3, the normally assembled pre-TCR fails to drive expansion of immature thymocytes.

To address this issue further, we quantified the expression of cyclin D3 protein in sorted double-negative populations of wild-type mice, using Western blotting. We found that cyclin D3 was strongly induced at the DN-4 and the ISP stages, i.e., following pre-TCR assembly (Figure 3B, upper panel). In contrast, cyclin D2 expression was high at cytokine-dependent

stages (DN-1 to DN-3) but was essentially absent once TCRβ rearrangements took place (Figure 3B, lower panel). No expression of cyclin D1 was detected in the thymocytes (data not shown).

Northern blot analyses revealed a strong induction of cyclin D3 mRNA at the DN-4 stage, consistent with increased protein levels (Figure 3C). These observations reinforced the notion that cyclin D3 might be a critical downstream target of the signaling pathways emanating from the pre-TCR.

We next took advantage of mice deficient in recombinase-activating gene (Rag). The thymocytes of these animals are arrested at the DN-3 stage (Mombaerts et al., 1992; Shinkai et al., 1992). This arrest can be overcome by administration of anti-CD3ϵ antibodies, which mimics the signaling from the pre-TCR (Levelt et al., 1993; Shinkai and Alt, 1994). We injected Rag-2-deficient mice with anti-CD3ϵ and determined the expression of cyclin D3 following the stimulation. Administration of anti-CD3ϵ antibodies led to rapid induction of cyclin D3 (Figure 3D). This finding further supports our hypothesis that cyclin D3 operates downstream of the pre-TCR.

We next crossed Rag-2-deficient mice with cyclin D3$^{-/-}$ animals and we generated double-knockout Rag2$^{-/-}$/cyclin D3$^{-/-}$ animals. These mice, along with Rag2$^{-/-}$ mice, were injected with anti-CD3ϵ antibody, and we monitored the response. As expected, Rag2$^{-/-}$ mice responded with rapid cyclin D3 induction followed by a proliferative burst that led to approximately 60-fold increase in thymocyte numbers. In contrast, thymocyte expansion was virtually absent in Rag2$^{-/-}$ mice lacking cyclin D3 (Figure 3E). This result further confirms that cyclin D3 is critically required for pre-TCR-driven expansion of immature thymocytes.

### Cyclin D3 operates downstream of proto-oncogene p56$^{LCK}$ kinase

The signaling pathways operating downstream of the pre-TCR are not fully elucidated. However, it is well established that proto-oncogene tyrosine kinase p56$^{LCK}$ signals downstream of the pre-TCR and plays a key role in transducing mitogenic signals (Fehling et al., 1997; Levelt et al., 1995; Molina et al., 1992; Mombaerts et al., 1994). We noted that the developmental abnormalities in thymuses of cyclin D3-deficient mice bear strong resemblance to the phenotype of mice lacking p56$^{LCK}$ (Levelt et al., 1995; Molina et al., 1992). Hence, we hypothesized that cyclin D3 might operate downstream of p56$^{LCK}$. To address this issue experimentally, we first sorted DN-3 and DN-4 thymocytes from p56$^{LCK}$-deficient and from control, wild-type mice, and we determined the levels of cyclin D3 using Western blotting. As expected from our previous analyses, in wild-type mice we detected a strong induction of cyclin D3 at the DN-4 stage. In contrast, this induction was essentially absent in mice lacking p56$^{LCK}$ (Figure 4A). Hence, genetic ablation of p56$^{LCK}$ abolished the induction of cyclin D3 after the pre-TCR formation, suggesting that the induction of cyclin D3 is controlled via p56$^{LCK}$-dependent pathway.

To further explore this notion, we compared cyclin D3 mRNA and protein levels in the p56$^{LCK}$-deficient Jurkat T cell leukemia cell line, J.CaM, and in J.CaM cells that we engineered to express activated p56$^{LCK}$. We found that ectopic expression of activated p56$^{LCK}$ led to strong upregulation of cyclin D3 (Figure 4B). These findings were further strengthened by our observation that cotransfection of the constitutively active form of p56$^{LCK}$ together with a cyclin D3-promoter-reporter construct resulted in over 3-fold induction of the cyclin D3 promoter (data not shown).

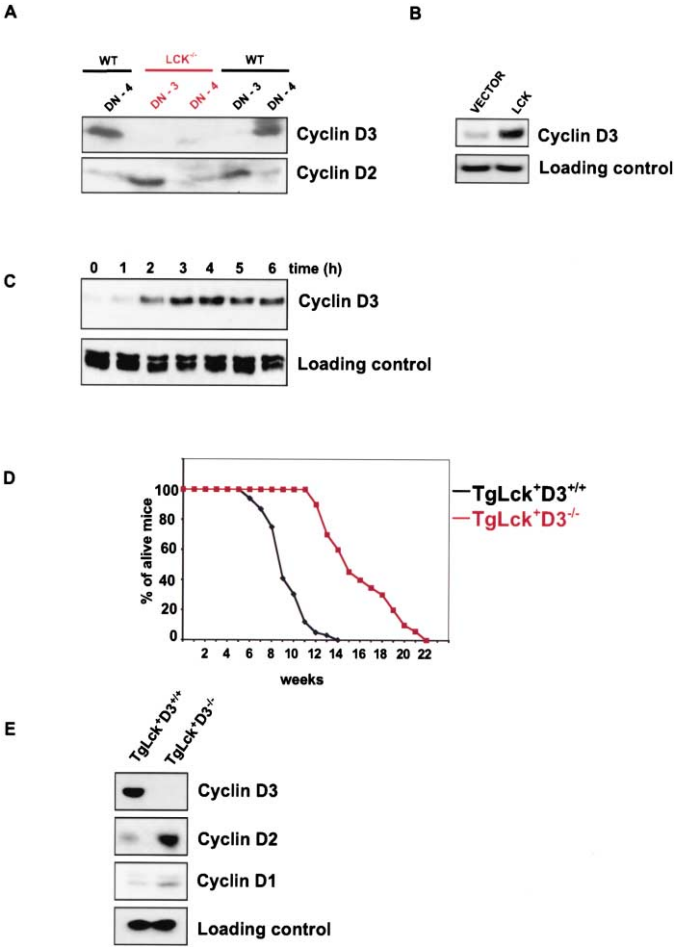


**Figure 4.** Cyclin D3 operates downstream of p56$^{LCK}$ kinase

**A:** Western blot analyses of sorted DN-3 and DN-4 thymocytes derived from wild-type (wt) or p56$^{LCK-/-}$ mice. Upper and lower panel represent the same blot sequentially probed with antibodies against cyclin D3 and D2.

**B:** Northern blot analysis of p56$^{LCK}$-deficient Jurkat derivative infected with an empty vector (VECTOR) or with a retrovirus expressing activated p56$^{LCK}$ (LCK). Blots were probed with a cyclin D3-specific probe and were reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA (loading control).

**C:** Cyclin D3 levels in p56$^{LCK}$-overexpressing thymic lymphoma cell line LGF2046. Cells were treated overnight with PP1, and inhibitor of p56$^{LCK}$. The inhibitor was then washed off and the levels of cyclin D3 were followed by Western blotting. Blots were reprobed with anti-CDK4 antibody (loading control).

**D:** Comparison of tumor susceptibility between p56$^{LCK}$/cyclin D3$^{+/+}$ (n = 10) and p56$^{LCK}$/cyclin D3$^{-/-}$ (n = 12) mice.

**E:** Expression of D cyclins in p56$^{LCK}$-triggered tumors arising in cyclin D3$^{+/+}$ and cyclin D3$^{-/-}$ animals. Western blots were probed with antibodies against cyclins D3, D2, and D1 and with anti-actin antibody (loading control).

Lastly, we tested the connection between p56$^{LCK}$ and cyclin D3 using two p56$^{LCK}$-transformed thymoblast cell lines, LGF2046 and LGF10442. These lines were derived from a thymic lymphoma arising in transgenic mice overexpressing activated p56$^{LCK}$ in immature T cells (Abraham et al., 1991; Lin and Abraham, 1997). The proliferation of these cells is critically dependent

on p56$^{LCK}$ (S.D.L., unpublished observations). To determine the time frame of cyclin D3 induction in response to p56$^{LCK}$, we treated thymoblast cells overnight with PP1, an inhibitor of p56$^{LCK}$ (and of other Src family of kinases), thereby arresting cell proliferation. We next washed off the inhibitor, thereby triggering p56$^{LCK}$ activation, and followed cyclin D3 levels by Western blotting. We found that cyclin D3 was strongly induced as early as 2 hr after triggering the kinase activity of p56$^{LCK}$ (Figure 4C), well before any changes in proliferative status of cells could be observed (data not shown), again pointing to a direct link between p56$^{LCK}$ signaling and cyclin D3. Collectively, our results suggest that cyclin D3 represents a critical target of the pre-TCR→p56$^{LCK}$ pathway operating within immature T cells.

**Requirement for cyclin D3 in mouse T cell malignancies**

The observed role for cyclin D3 in T cell development prompted us to investigate the requirement for cyclin D3 in oncogenesis of this cellular compartment. To address this issue, we utilized three mouse models of T cell malignances: two involving the pre-TCR→p56$^{LCK}$-dependent pathway and one pre-TCR-independent pathway. First, we crossed cyclin D3-deficient mice with mice overexpressing p56$^{LCK}$. These transgenic mice are highly prone to T cell tumors due to targeted overexpression of p56$^{LCK}$ in immature thymocytes (Abraham et al., 1991).

As expected (Abraham et al., 1991), cyclin D3$^{+/+}$/p56$^{LCK}$ transgenic mice rapidly developed T cell malignancies and died starting at 7 weeks of age. In contrast, the incidence of tumors was delayed by 6–8 weeks in cyclin D3$^{-/-}$/p56$^{LCK}$ mice and the survival time was nearly doubled (Figure 4D).

In order to understand a bypass mechanism that eventually allowed delayed tumor appearance in cyclin D3-deficient mice, we analyzed the expression pattern of D cyclins in tumors arising in wild-type and cyclin D3$^{-/-}$ mice. Our analyses revealed that tumors found in wild-type mice expressed high levels of cyclin D3 but only low levels of cyclins D2 and D1. In contrast, tumors arising in cyclin D3$^{-/-}$ animals lacked cyclin D3 (as expected), but instead expressed greatly elevated levels of cyclin D2 (Figure 4E). Importantly, both cyclin D3$^{+/+}$ and cyclin D3$^{-/-}$ tumors were of the same immunophenotype (CD4$^{\pm}$CD8$^{+}$CD25$^{+}$CD44$^{-}$, data not shown). We interpret these findings as an indication that in the absence of cyclin D3, p56$^{LCK}$ can drive tumor cell proliferation via cyclin D2.

We extended these analyses using another model of T cell malignancy, namely leukemias induced by the activated, intracellular (IC) form of the Notch. This model is believed to be reminiscent of human childhood T cell acute lymphoblastic leukemias (T-ALL) (Bellavia et al., 2000). Importantly, it is well established that Notch-driven leukemogenesis critically requires pre-TCR signaling, as mice lacking pre-TCR components are resistant to Notch-induced leukemias (Allman et al., 2001; Bellavia et al., 2002). In our experimental approach, we collected bone marrows from wild-type and cyclin D3-deficient animals, sorted their hematopoietic progenitor cells (HPC), and infected the HPC with retroviruses encoding Notch1 together with GFP (Aster et al., 2000; Pui et al., 1999). These infected cells were injected into sublethally irradiated recipients. As expected (Aster et al., 2000; Pui et al., 1999), infection of wild-type bone marrow with activated Notch led to appearance of T-ALL leukemias (presence of CD4$^{+}$CD8$^{+}$ double-positive T cells in the peripheral blood) in 100% of recipient mice 2 weeks after bone marrow transduction (Figure 5A). Peripheral blood leukemias persisted until 11–13 weeks, when all mice died due to presence of multiple T cell tumors. In striking contrast, mice reconstituted with Notch-expressing cyclin D3$^{-/-}$ bone marrow remained leukemia-free throughout the entire observation period (Figure 5A).

Importantly, we verified that the transduced cyclin D3$^{-/-}$ bone marrow cells contributed to the T cell compartment in the recipient animals. Analyses of peripheral blood revealed that GFP-positive (i.e., transduced by Notch-IC) cyclin D3$^{-/-}$ cells give rise to mature peripheral CD4$^{+}$CD8$^{-}$ and CD4$^{-}$CD8$^{+}$ T

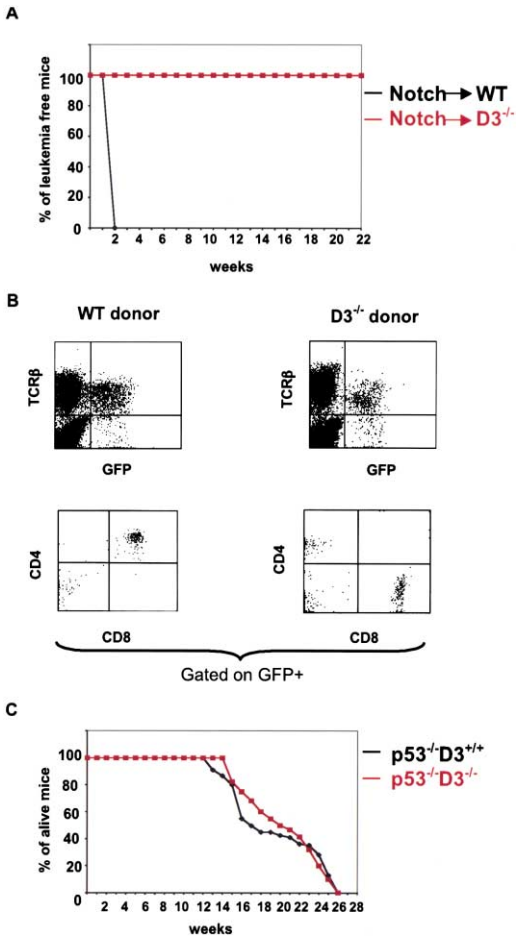


**Figure 5.** Occurrence of T cell malignancies in cyclin D3$^{-/-}$ mice

**A:** Cyclin D3$^{+/+}$ or cyclin D3$^{-/-}$ lineage-negative bone marrow cells were transduced with retroviruses encoding constitutively active intracellular domain of Notch1-IRES-EGFP or IRES-EGFP, and the cells were injected into irradiated Rag2$^{-/-}\gamma c^{-/-}$ recipients. Bone marrow cells collected from 12 cyclin D3$^{+/+}$ donors were used to reconstitute 6 recipients, while bone marrow cells from 14 cyclin D3$^{-/-}$ mice were used to reconstitute 8 recipients. Incidence of leukemia (presence of CD4$^{+}$CD8$^{+}$ leukemic cells in the peripheral blood) is indicated; all cyclin D3$^{+/+}$ mice died at 11–13 weeks and presented multiple tumors. None of cyclin D3$^{-/-}$ mice had any tumors at the end of observation period (22 weeks).

**B:** Upper panels: total, GFP-positive, and GFP-negative peripheral blood lymphocytes were stained with antibodies against TCRβ and analyzed by flow cytometry. Lower panels: peripheral blood lymphocytes were stained with antibodies against CD4 and CD8, gated on GFP-positive cells, and analyzed by flow cytometry.

**C:** Comparison of tumor susceptibility between p53$^{-/-}$/cyclin D3$^{+/+}$ (n = 8) and p53$^{-/-}$/cyclin D3$^{-/-}$ (n = 10) mice.

lymphocytes. These peripheral T cells expressed surface TCRβ, an indication that normal pre-TCR selection took place (Figure 5B). In contrast, wild-type cells transduced with Notch-IC gave rise to double-positive leukemic cells (Figure 5B).

Collectively, these results indicate that cyclin D3 is required for Notch oncogenic pathway that signals through pre-TCR, as well as for p56$^{LCK}$ pathway that signals downstream of the pre-TCR.

We next asked whether a similar requirement for cyclin D3 operated in pre-TCR-independent T cell malignancies. We took advantage of p53-deficient mice that develop T cell tumors in a pre-TCR-independent fashion (Liao et al., 1998; Nacht and Jacks, 1998). Importantly, these tumors involve malignant transformation of the very same T cell subsets (double-positive) as pre-TCR-dependent Notch-driven tumors (Liao et al., 1998; Nacht and Jacks, 1998; Pui et al., 1999). We crossed cyclin D3$^{-/-}$ mice with p53-deficient animals and generated cyclin D3$^{-/-}$p53$^{-/-}$ animals. These cyclin D3$^{-/-}$p53$^{-/-}$ animals displayed normal tumor susceptibility and developed T cell malignancies with similar kinetics as p53$^{-/-}$ mice (Figure 5C). Hence, cyclin D3 is dispensable for this pre-TCR-independent T cell oncogenic pathway.

It should be noted, however, that while our analyses point to a requirement for cyclin D3 in pre-TCR-dependent mouse T cell leukemias, the continued expression of cyclin D3 in these tumors may eventually become pre-TCR independent. Thus, pre-TCR signaling was shown to interfere with the activity of E2A transcription factor, via induction of an inhibitory protein, Id3 (Engel and Murre, 2001), and this may contribute to cyclin D3 induction. On the other hand, activated Notch can also inhibit E2A activity (Ordentlich et al., 1998), thereby mimicking some aspects of pre-TCR signaling, and this action may result in pre-TCR-independent expression of cyclin D3 in Notch-driven tumors.

### Expression of cyclin D3 in human T-ALL

The specific requirement for cyclin D3 function in murine leukemias prompted us to investigate the requirement for cyclin D3 in human T cell malignancies. We decided to focus on T cell acute lymphoblastic leukemias (T-ALL), since this malignancy is believed to arise predominantly from cells corresponding to immature thymocytes. We started our analyses by performing "data mining" on the published global pattern of gene expression in 39 cases of T-ALL (Ferrando et al., 2002). We first conducted a "nearest neighbor" analysis, to determine which genes show expression most closely correlating with the expression of cyclin D3. Strikingly, we found that among the 6817 genes analyzed, p56$^{LCK}$ scored among the top ten genes most closely associated with cyclin D3; the association was very strong (coefficient of the log expression values = 0.77). Hierarchical cluster analysis, shown in Figure 6A, illustrates the highly similar expression profiles of cyclin D3 and p56$^{LCK}$ in all 39 tumors. Significantly, among other genes within the cyclin D3 cluster, there are two direct targets for p56$^{LCK}$ in the pre-TCR pathway, namely CD3ε and ZAP-70 (Figure 6A) (Muljo and Schlissel, 2000).

Analysis of T-ALL tumors arranged according to developmental stage (Ferrando et al., 2002) revealed that cyclin D3, p56$^{LCK}$, and TCRβ were highly expressed in leukemias deriving from later (after pre-TCR formation) stages of T cell development (Figure 6B). In contrast, T-ALL cases deriving from the very early

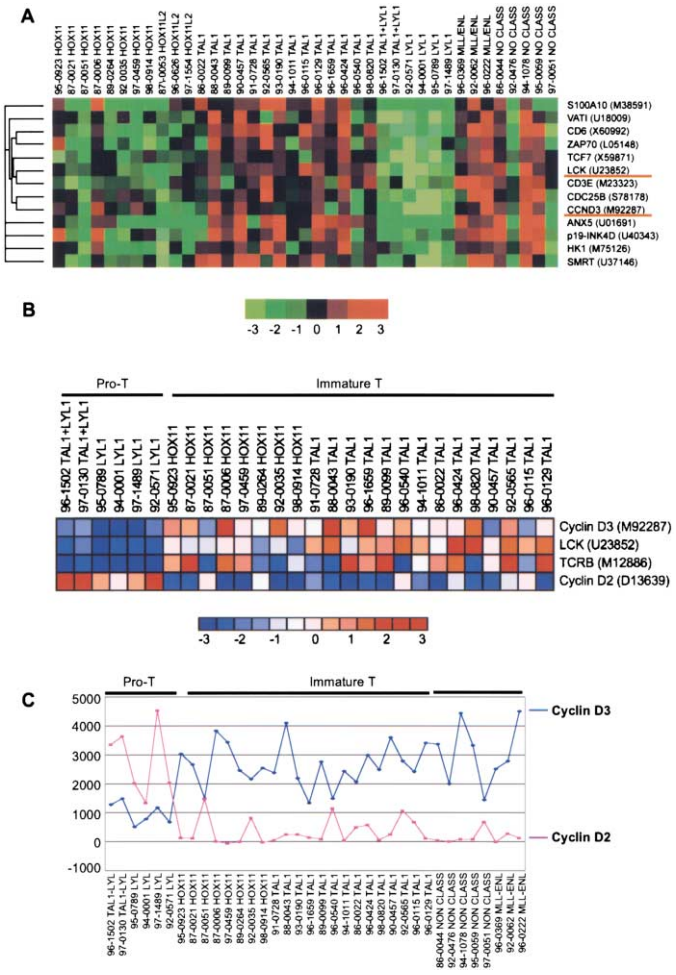


**Figure 6.** Expression of cyclin D3 in human T cell acute lymphoblastic leukemias

**A:** A detailed view of the hierarchical tree of genes (rows) clustered across 39 T cell leukemia samples (columns) focused on the cluster of genes containing cyclin D3. The normalized expression value for each gene is indicated, red representing higher expression and green representing lower expression. Cyclin D3 and p56$^{LCK}$ are underlined.

**B:** Differential expression of cyclin D3, p56$^{LCK}$, TCRβ, and cyclin D2 (rows) across T-ALL leukemia samples (columns) arranged according to known developmental stages of T cell maturation. Samples expressing LYL1 oncogene are associated with an early arrest at the double-negative stage of T cell differentiation prior to pre-TCR expression, while HOX11- and TAL1-positive samples are arrested at the early cortical and late cortical stages of thymocyte differentiation, respectively (Ferrando et al., 2002). The normalized expression value for each gene is indicated, with red representing higher expression and blue representing lower expression.

**C:** Absolute fluorescence values of cyclin D2 and cyclin D3 across all T-ALL samples.

pro-T cells displayed low expression levels of the three genes (Figure 6B).

Comparison of absolute cyclin D3 levels with those of cyclin D2 revealed that leukemias corresponding to pro-T cells expressed high levels of cyclin D2 together with low levels of cyclin D3. In contrast, more mature leukemias corresponding to pre-T cells and double-positive cells often expressed cyclin D3 as a sole D-type cyclin (Figure 6C). This pattern very closely mirrors

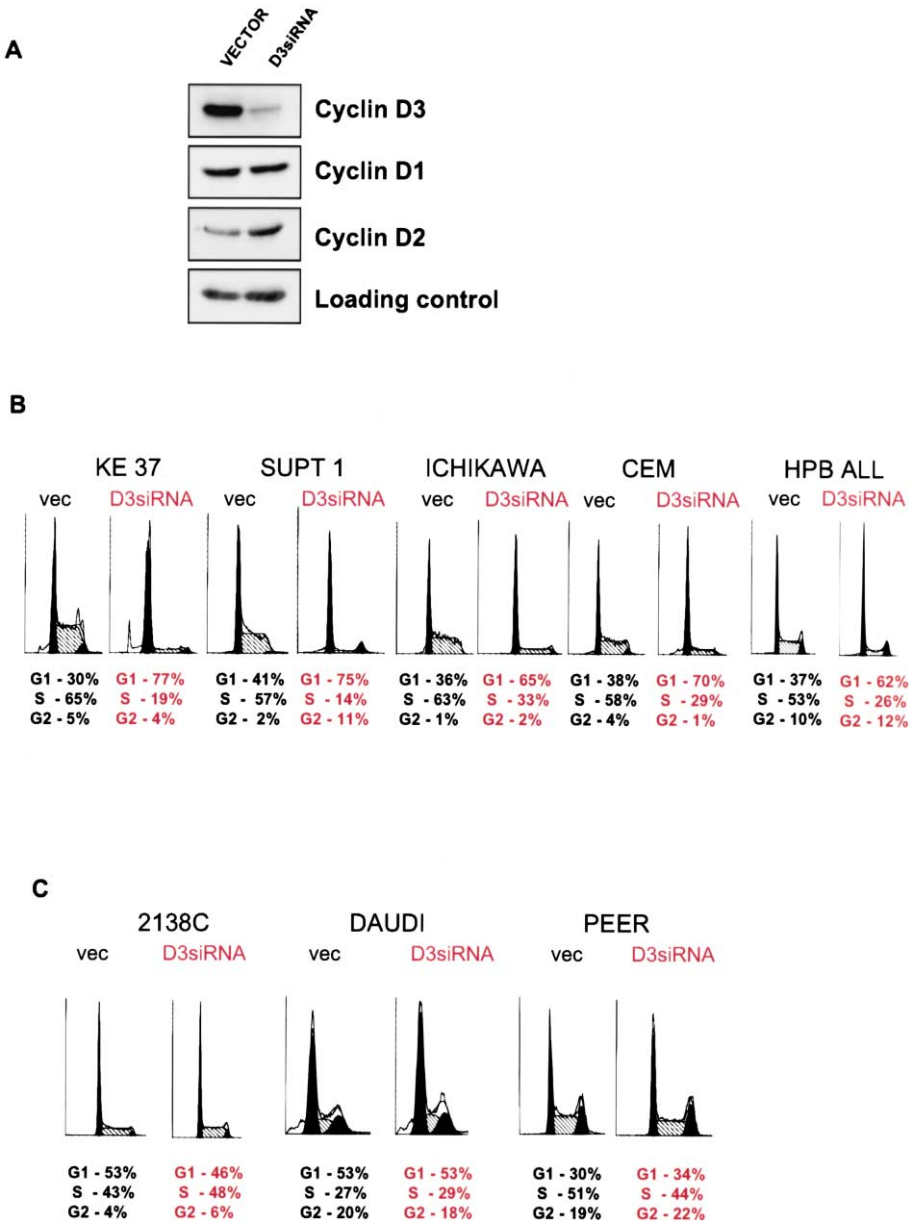


**Figure 7.** Requirement for cyclin D3 in human T cell acute lymphoblastic leukemias

**A:** Western blot analysis of the levels of D cyclins in Hela cells infected with an empty vector or with vector encoding cyclin D3 siRNA. Blots were also probed with anti-actin antibody (loading control).

**B:** Twelve T-ALL cell lines deriving from immature T cells with rearranged TCRβ chains were infected with retroviruses encoding siRNA against cyclin D3, and the impact on cell proliferation was scored by propidium iodide staining followed by FACS. Shown are results from five T-ALL cell lines. Similar results were seen in remaining seven T-ALL lines (see Experimental Procedures).

**C:** For control, we used two T-ALL deriving from γδ lineage of T cells and four B cell leukemias. Shown is one γδ T-ALL line (PEER) and two B cell leukemias (DAUDI and 2138C). Similar results were seen in the remaining lines (see Experimental Procedures).

the expression seen by us in normal thymocyte development (Figure 3B). Hence, the specific expression pattern of D cyclins, $p56^{LCK}$, and TCRβ, seen during lymphocyte development, is largely preserved in malignancies deriving from distinct developmental stages.

**Requirement for cyclin D3 in human T-ALL**

Lastly, we asked whether the requirement for cyclin D3 in oncogenic proliferation, demonstrated by us in mouse T cell malignancies, also operated in human T-ALL cells. To this end, we collected 12 T-ALL cell lines (see Experimental Procedures) corresponding to immature thymocytes with rearranged TCRβ chains, and we cultured them in vitro. We next infected the cells with retroviruses expressing siRNA against cyclin D3. Western blot analyses confirmed a significant reduction of cyclin D3 levels following siRNA expression (Figure 7A). We also verified that this siRNA was cyclin D3 specific, i.e., it did not affect levels of cyclins D1 or D2 (Figure 7A). We next gauged the impact of cyclin D3 knock-down on cell proliferation by comparing cell cycle distribution of cells infected with vectors encoding cyclin D3 siRNA versus cells infected with empty vectors. We found that knock-down of cyclin D3 significantly inhibited proliferation of all 12 human immature T-ALL lines (Figure 7B, and data not shown). For control, we used two T-ALL cell lines derived from T lymphocytes of the γδ lineage and four lines derived from B lymphocytes. Our analyses revealed that the proliferation of these six control cell lines was not significantly affected by the knock-down of cyclin D3 (Figure 7C, and data not shown). Collectively, these results indicate that cyclin D3 is critically required for proliferation of human T-ALL deriving from immature T cells, as an acute ablation of cyclin D3 blocks their proliferation.

## Discussion

While amplification of the cyclin D genes and overexpression of cyclin D proteins were reported in several human malignancies, a large number of human cancers contain lesions in pathways impacting on D cyclins (Ortega et al., 2002). For this reason, elucidation of the normal functions of D cyclins is critically important to understand the role of these proteins in human tumorigenesis.

In the present work, we analyzed the functions of cyclin D3 by generating and studying cyclin D3-deficient mice. The very specific developmental defect that we observed in cyclin D3 null animals led us to analyze the exact molecular pathway that impinges on cyclin D3 in immature T lymphocytes. Through combination of genetic crosses, in vivo studies of mice lacking particular signaling molecules ($p56^{LCK}$, TCRβ), and in vitro cell and molecular analyses, we determined that cyclin D3 is the major downstream target of the pre-TCR→$p56^{LCK}$ pathway.

The specific requirement for cyclin D3 is restricted to this particular signaling pathway, as cytokine-driven expansion of immature T cells proceeds normally in the absence of cyclin D3 (Figure 3A). Moreover, peripheral T lymphocytes isolated from cyclin D3 null animals proliferate normally in response to TCR stimulation (data not shown), again highlighting a very specific requirement for cyclin D3 downstream of "pre-receptor" pre-TCR signaling, but not in signaling downstream of mature TCR.

The critical role for cyclin D3 in transmitting pre-TCR-dependent mitogenic signals prompted us to analyze the requirement for cyclin D3 in specific oncogenic pathways operating in immature T cells. Again, we found that cyclin D3 is essential for oncogenic pathways operating downstream of the pre-TCR, while it is dispensable for a pre-TCR-independent pathway. Specifically, we observed that mice lacking cyclin D3 are resistant to Notch-driven leukemias and show reduced susceptibility to T cell malignancies triggered by $p56^{LCK}$ but remain fully susceptible to a pre-TCR-independent oncogenic pathway. These studies extend our earlier, published observations that mice lacking another component of the cell cycle machinery, cyclin D1, are resistant to Ras- and Neu-driven breast cancers, while being fully susceptible to Wnt-1- and Myc-driven breast tumorigenesis (Yu et al., 2001). Collectively, this work establishes that different D cyclins are critically required for tumorigenesis in specific tissues (cyclin D1, breast; cyclin D3, immature T cells), and they represent key downstream components of defined oncogenic pathways (Neu→Ras in case of cyclin D1; pre-TCR→$p56^{LCK}$ in case of cyclin D3).

In the current study, we extended our analyses to human malignancies. We found that human T-ALL with rearranged TCRβ chains critically require cyclin D3, as knock-down of this protein inhibited the proliferation of tumor cells. Importantly, knock-down of cyclin D3 had no impact on proliferation of two T-ALL cell lines deriving from γδ lineage of T cells, nor of four B cell leukemias (Figure 7C and data not shown). Collectively, these results reveal a requirement for cyclin D3 function in a specific subset of human lymphoid malignancies.

### Are D cyclins good targets in human cancer therapy?

All proliferating cells, including cancer cells, express at least one type of D cyclin (Bartkova et al., 1995, 1998; Lukas et al., 1994, 1995; Tam et al., 1994; Tetsu and McCormick, 2003). Our current understanding of the cell cycle progression is that at least one D cyclin must be present to allow normal cell division. An exception to the rule is provided by the observation that tumor cells that lost the retinoblastoma protein no longer require D cyclins, an indication that pRB is the major target for D cyclins in cell cycle progression (Sherr and Roberts, 1999). However, the dependence of cancer cells on D cyclins has not been systematically analyzed. A recent demonstration that CDK2 activity is dispensable in cancer cells suggests that these tumor cells can proliferate in the absence of certain cell cycle activators (Tetsu and McCormick, 2003). Importantly, cyclin D-CDK4 kinase activity was shown to be critically required for the proliferation of the very same cancer cell lines (Tetsu and McCormick, 2003). It should be noted that the majority of tumor cells express two or even all three D cyclins (Bartkova et al., 1998). Since the three D cyclins are believed to perform overlapping, redundant functions in cell cycle progression (Sherr and Roberts, 1999), one would have to target all D cyclins expressed by the tumor to arrest cancer cell proliferation.

The demonstration that a subset of T-ALL expresses cyclin D3 as a sole D cyclin and critically depends on this cyclin for proliferation offers a unique window of opportunity to use anti-cyclin D3 therapy in selected human lymphoid malignancies. We propose that targeting of a single D cyclin might not be of use in many human tumors that depend on multiple D cyclins (or do not require D cyclins at all). On the other hand, malignancies do exist where the driving oncogenic pathway signals to the cell cycle machinery selectively via a specific D cyclin. These malignancies need to be identified, as they represent prime candidates for anti-cyclin D therapies. Given a very narrow phenotype of cyclin D3-deficient mice, demonstrated here, and equally circumscribed impact of the loss of cyclins D1 (Fantl et al., 1995; Sicinski et al., 1995) or D2 (Sicinski et al., 1996), we hypothesize that agents, which cripple function of particular D cyclins, might be highly selective in shutting off the proliferation of selected human malignancies, while sparing other tissues. We propose that human T-ALL deriving from immature T lymphocytes are good candidates for a therapeutic strategy centered around cyclin D3 inhibition.

### Experimental procedures

#### Generation of cyclin D3-deficient mice

Cyclin D3 gene-targeting construct was assembled in pPNT vector by removing a 1.4 kb BssH II-Spe I fragment of the murine cyclin D3 gene (containing coding exons I and II) and replacing it with the neomycin phosphotransferase gene. The construct was electroporated into embryonal stem (ES) cells and recombinant clones were selected. 18 out of 146 ES cell clones analyzed underwent homologous recombination at the cyclin D3 locus. Two of these clones contributed to the germline tissue when mutant mice were generated using standard procedures (Sicinski et al., 1995). For the genotyping, genomic DNA was digested with Eco RI and Eco RV and analyzed by Southern blotting using a 650 bp Xba I-BspE I fragment of the cyclin D3 gene (probe A in Figure 1A). Alternatively, genomic DNA was PCR amplified using cyclin D3 specific primers: A (5′-GAACGTTGTGACGTAGGAGC-3′), G (5′-TCCAT CCTGCGATGGCTCAC-3′) and a neo gene-specific primer N3 (5′-TGC TGTCCATCTGCACGAGA-3′) by denaturing the DNA at 94°C for 3 min, followed by 36 cycles of amplification: 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 7 min.

#### Other mouse strains and cell lines

$Rag2^{-/-}$ mice were purchased from the Taconic Farms. $Rag2^{-/-}$ or $Rag2^{-/-}$/cyclin $D3^{-/-}$ mice were injected intraperitoneally with 100 μg of anti-CD3 antibody (145-2C11, PharMingen, San Diego, CA). $p56^{LCK}$-deficient (Molina et al., 1992) and p53-deficient (Nacht and Jacks, 1998) mice were purchased

from the Jackson Laboratories. Transgenic line LGY4220, expressing wild-type $p56^{LCK}$ kinase driven by the proximal lck promoter, was kindly provided by Dr. R. Perlmutter.

J.CaM1.6 cells ($p56^{LCK}$-deficient derivative of Jurkat E6-1) were purchased from the ATCC. The Y505F constitutively activated mutant form of the $p56^{LCK}$ kinase, cloned in the retroviral expression vector pGFP-RV, was kindly provided by Dr. Kenneth M. Murphy (Washington University School of Medicine, St. Louis, MO). Three micrograms of the pGFP-LCKY505F or the corresponding empty vector were transfected into the phoenix retroviral packaging cell line using the Fugene 6 reagent (Roche) according to the manufacturer instructions. 72 hr after transfection, culture supernatants were used to infect the J.Cam1.6 cells at the final concentration of 1 million cells/ml for 4 hr in the presence of Polybrene (8 μg/ml). RNA was isolated and processed for Northern blotting.

The $p56^{LCK}$-transformed cell lines LGF2046 and LGF10442 were derived from thymic lymphomas explanted from transgenic mice that overexpressed constitutively activated $p56^{LCK}$ transgene ($p56^{LCKF505}$) driven by the $p56^{LCK}$ proximal promoter, as described (Lin and Abraham, 1997). Cells were cultured for 18 hr in the presence of the Src-family kinase inhibitor PP1 (Hanke et al., 1996). The inhibitor was then washed off and cells were harvested at the indicated time points, lysed, subjected to SDS-PAGE, and immunoblotted for cyclin D3 protein.

**Flow cytometry and FACS**

The following monoclonal antibodies were used: anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD25 (7D4), anti-CD44 (IM7), anti-TCRγδ (GL3), anti-TCRβ (H57-597), and anti-Pan-NK cells (DX5), all from PharMingen (San Diego, CA). These antibodies were directly coupled to phycoerythtin (PE), fluorescein isothiocyanate (FITC), Cy-Chrome, or allophycocyanin (APC).

To sort thymocytes into different populations, cells were first stained with biotinylated anti-CD4 and anti-CD8, followed by depletion with streptavidin-conjugated magnetic beads (Dynal, Oslo, Norway). Depleted cells were then stained with anti-CD4-PE, anti-CD8-PE, anti-DX5-PE, anti-TCRγδ-PE, anti-CD44-Cy-Chrome, and anti-CD25-FITC, and PE-negative cells were gated and sorted. Immature single-positive cells were defined, as $CD4^{-}CD8^{+}TCR\beta^{-}$. Sorted cells were processed immediately for Western or Northern blot analyses. To measure the fraction of proliferating cells, sorted thymocytes were fixed with 0.5% paraformaldehyde for 15 min on ice. After fixation, cells were resuspended in a staining medium that consisted of 50 μg/ml of propidium iodide and 250 μg/ml of RNase A, then incubated at 37°C for 30 min.

Flow cytometric analyses were done on a FACScalibur cytometer (Beckton Dickinson, San Jose, CA) and modeled with the MoDFit program. Cells were sorted by a MoFlo cell sorter (Cytomation, Fort Collins, CO).

**Western and Northern blotting**

Protein extracts were prepared from sorted thymocytes, separated using 10% SDS-PAGE, transferred to Immobilion-P membrane (Millipore), and probed with an anti-cyclin D3 (C-16), anti-cyclin D2 (M-20) anti-cyclin D1, anti-CDK4 (Santa Cruz Biotechnology), and anti-actin (Chemicon) antibodies. As a secondary antibody, peroxidase-conjugated IgG (BioRad) was used, followed by enhanced chemiluminescence (ECL) detection (Sigma). Northern blot analyses were performed as described (Yu et al., 2001).

**Transduction of bone marrow with Notch**

A cassette encoding the constitutively active form of Notch-1 (kindly provided by I. Screpanti) was cloned upstream of an EGFP construct containing an internal ribosomal entry site (IRES, Clontech, Palo Alto, CA). The entire cassette (or only the IRES-EGFP portion as a control) was inserted into a modified MMLV-based retroviral vector (kindly provided by R. Mulligan). Retroviral supernatants were produced by transient transfection of the retroviral vector and packaging plasmids (Ory et al., 1996) into the human embryonic kidney epithelial cell line 293T. Supernatants were concentrated by ultracentrifugation and stored at −80°C. Bone marrow transduction was done as previously described (Aifantis et al., 2002). Briefly, lineage negative (CD3ε, TCRβ, Gr-1, Ter-119, Mac-1, DX5, and CD19-negative) bone marrow cells were sorted from cyclin $D3^{+/+}$ and cyclin $D3^{-/-}$ mice and cultured in the presence of IL-7, SCF, Flt3L, and IL-6 (all from R&D Systems, Minneapolis, MN). On days 2 and 4 after sorting, cells were subject to retroviral transduction by centrifugation at 2200 rpm for 1.5 hr at room temperature with 6 μg/ml polybrene. On day 5, $7 \times 10^4$ $EGFP^{+}$ $Lin^{-}$ cells were injected intravenously into irradiated (500 rad) RAG-$2^{-/-}\gamma c^{-/-}$ hosts (Colucci et al., 2000) (Taconic Farms). Two weeks after injection, and at biweekly intervals thereafter, peripheral blood was stained for CD4 and CD8 expression and analyzed by flow cytometry.

**T cell leukemia samples and microarray hybridization**

Detailed information about T cell leukemia patients, RNA purification, microarray hybridization, and data acquisition have been described before (Ferrando et al., 2002). Briefly, cryopreserved lymphoblast samples of 39 T-ALL cases were obtained and global gene expression analysis was performed using Affymetrix HU6800 arrays (Ferrando et al., 2002).

**Microarray data analysis**

Data reduction was performed by filtering the expression values from Affymetrix HU6800 arrays using Genecluster software 2.0 (floor 100, ceiling 16,000, fold difference >5, max-min difference >500). Data of the 452 genes that passed these filters was analyzed using Cluster software (Eisen et al., 1998). Expression values were adjusted by performing a log transformation followed by normalization and mean centering of the genes. Hierarchical clustering of genes on these adjusted expression values across the 39 samples analyzed was performed using correlation (uncentered) as similarity metric, and the dendrogram resulting from this analysis was visualized using Treeview. Nearest neighbor analysis using the signal-to-noise statistics was performed to identify genes associated with cyclin D3 (Golub et al., 1999). Gene-Cluster analysis software and Cluster and Treeview programs are available together with detailed instructions for microarray data analysis at http://www-genome.wi.mit.edu/cancer/software/software.html and http://rana.lbl.gov/EisenSoftware.htm, respectively.

**siRNA against cyclin D3**

To target cyclin D3 expression, siRNA sequences were designed against the 5′-AAGGAUCUUUGUGGCCAAGGA-3′ sequence of human cyclin D3 transcript. Oligonucleotides were cloned into pMKO.1 retroviral vector, yielding pMKO.1-αD3. T-ALL lines (CEM, Molt 15, Molt 4, P12-Ichikawa, RPMI8402, PF382, Jurkat, HPB ALL, KE-37, CML-T1, SUP-T1, and MKB-1), control γδ T-ALL lines (Loucy and PEER), and control B cell lines (2138C, JVM2, Granta, and Daudi) were grown in 10% calf serum in RPMI, infected with pMKO.1 or with pMKO.1-αD3 retroviruses, and selected in medium containing 1–3 μg/ml puromycin for 2 to 5 days, and the fraction of cells in different phases of the cell cycle was determined by propidium iodide staining followed by FACS. Gating was done on live cells.

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