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Therapeutic Targeting of the Cyclin D3:CDK4/6 Complex in T Cell Leukemia

Catherine M. Sawai,[1,6] Jacquelyn Freund,[1] Philmo Oh,[1] Delphine Ndiaye-Lobry,[1] Jamieson C. Bretz,[2] Alexandros Strikoudis,[1] Lali Genesca,[3] Thomas Trimarchi,[1] Michelle A. Kelliher,[4] Marcus Clark,[5] Jean Soulier,[3] Selina Chen-Kiang,[2] and Iannis Aifantis[1,*]

[1]Department of Pathology and Howard Hughes Medical Institute, New York University School of Medicine, New York, NY 10016, USA
[2]Department of Pathology and Graduate Program in Immunology and Microbial Pathogenesis, Weill Medical College of Cornell University, New York, NY 10065, USA
[3]INSERM U944 and University Paris Diderot, Saint-Louis Hospital, Paris, 75010 France
[4]Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA
[5]Department of Medicine, Section of Rheumatology and Gwen Knapp Center for Lupus Research, University of Chicago, Chicago, IL 60637, USA
[6]Present address: Department of Microbiology and Immunology, Columbia University, New York, NY 10032, USA
*Correspondence: iannis.aifantis@nyumc.org
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SUMMARY

D-type cyclins form complexes with cyclin-dependent kinases (CDK4/6) and promote cell cycle progression. Although cyclin D functions appear largely tissue specific, we demonstrate that cyclin D3 has unique functions in lymphocyte development and cannot be replaced by cyclin D2, which is also expressed during blood differentiation. We show that only combined deletion of $p27^{Kip1}$ and retinoblastoma tumor suppressor (Rb) is sufficient to rescue the development of *Ccnd3*$^{-/-}$ thymocytes. Furthermore, we show that a small molecule targeting the kinase function of cyclin D3:CDK4/6 inhibits both cell cycle entry in human T cell acute lymphoblastic leukemia (T-ALL) and disease progression in animal models of T-ALL. These studies identify unique functions for cyclin D3:CDK4/6 complexes and suggest potential therapeutic protocols for this devastating blood tumor.

INTRODUCTION

D-type cyclins (D1, D2, and D3) bind cyclin-dependent kinases 4 and 6 (CDK4/6), and the activity of cyclin D:CDK4/6 complexes promotes entry into the cell cycle (Sherr, 1995; Sherr and Roberts, 2004). Cyclin D:CDK4/6 complexes are believed to promote cell cycle progression through at least two functions: by interacting with cell cycle inhibitors, such as $p21^{Cip1}$ and $p27^{Kip1}$, and by the phosphorylation of the retinoblastoma tumor suppressor (Rb). Cyclin D:CDK4/6 are thought to form ternary complexes that bind cyclin-dependent kinase inhibitors (CDKIs) of the $p21^{Cip}$/$p27^{Kip1}$ family (Sherr and Roberts, 2004). This facilitates downstream cyclin E:CDK2 complex activity that, along with cyclin D:CDK4/6, inactivates Rb and allows activation of E2F transcription factors and progression through the cell cycle.

The functions of D-type cyclins have been studied using germline gene deletion. Each knockout mouse was viable, but displayed distinct tissue-specific defects (Ciemerych et al., 2002; Kozar et al., 2004; Sicinski et al., 1995, 1996; Sicinska et al., 2003, 2006). When these deficiencies were combined, complete hematopoietic failure was observed, demonstrating the absolute requirement for D-type cyclins within the hematopoietic system (Kozar et al., 2004). Cyclin D2-deficient (*Ccnd2*$^{-/-}$) mice display reduced proliferation of mature splenic B cells and lack $CD5^{+}$ peritoneal B cells (Solvason et al., 2000). Cyclin D3 knockout (*Ccnd3*$^{-/-}$) animals show defects in early B and T cell differentiation, as well as impaired proliferation of

Significance

While dispensable for proliferation of many tissues, D-type cyclins are absolutely required in the hematopoietic system, specifically for early lymphocyte development. Aberrant expression of D-type cyclins is associated with hematopoietic neoplasms; thus deciphering the functions of cyclin D is critical for the development of strategies to prevent an oncogenic cell cycle. We present genetic evidence that combined deletion of $p27^{Kip1}$ and Rb, which regulate progression through the G_1 phase of the cell cycle, rescue the defect in early T cell development from deletion of cyclin D3. Furthermore, we show that inhibition of cyclin D:CDK4/6 activity abrogates proliferation in T-ALL cell lines and primary human cells as well as progression of disease in animal models of T-ALL.

granulocytes (Cooper et al., 2006; Peled et al., 2010; Sicinska et al., 2003, 2006). Cyclin D1 was recently suggested to play a key role in hematopoietic stem cell quiescence and self-renewal (Zou et al., 2011); however, *Ccnd1*$^{-/-}$ mice do not display striking hematopoietic effects, most likely due to redundancy with D2 and D3 (Sicinski et al., 1995).

Previous work has suggested that defects associated with individual cyclin D deficiency stem from their tissue-specific expression and that D-type cyclins are largely functionally redundant. For example, high expression of cyclin D1 protein, but not D2 or D3, is observed in both the retina and mammary tissue, and *Ccnd1*$^{-/-}$animals correspondingly have reduced proliferation of both the cells that contribute to the retina and breast epithelium compartment (Sicinski et al., 1995). Genetic studies in which endogenous *Ccnd1* was substituted with *Ccnd2* complementary DNA (cDNA) have demonstrated that cyclin D2 can largely replace cyclin D1 function in mammary and retina tissue development (Carthon et al., 2005). However, these tissues typically express a single D-type cyclin, so whether D-type cyclins can functionally replace one another in cells that express more than one cyclin, such as developing lymphocytes, remains unclear.

Aberrant cell cycle regulation is a common thread to all forms of cancer (Hunter and Pines, 1994). Deregulated expression of all D-type cyclins is frequently observed in hematopoietic malignancies (Bergsagel et al., 2005; Motokura and Arnold, 1993). We have previously shown that induction of T cell acute lymphoblastic leukemia (T-ALL), a disease caused by transformation of lymphocyte progenitors, requires cyclin D3, as expression of the oncogenic intracellular domain of Notch1 (ICN1) in *Ccnd3*$^{-/-}$ bone marrow progenitors fails to initiate disease. Consistent with these animal studies, cyclin D overexpression is commonly seen in human T-ALL, with specific cyclin D expression associated with distinct T-ALL subsets (Li et al., 2008; Sicinska et al., 2003). Early thymocyte progenitor-ALL is characterized by cyclin D2 overexpression (Coustan-Smith et al., 2009), whereas more mature forms of T-ALL are associated with D3 overexpression (Joshi et al., 2009; Li et al., 2008). Finally, previous data have suggested that Notch signaling directly regulates cyclin D3 expression, and blocking cyclin D3 expression by γ-secretase inhibition of Notch signaling prevents cell cycle progression in human T-ALL cell lines in vitro (Joshi et al., 2009). These data suggested that D-type cyclins and/or their downstream interacting partners could be attractive therapeutic targets in this type of disease.

RESULTS

Unique Roles for Cyclin D3 in Lymphocyte Development

We have previously shown that cyclins D2 and D3 are both expressed during early stages of lymphocytic differentiation; however, only loss of cyclin D3 leads to significant effects on cell differentiation (Cooper et al., 2006; Sicinska et al., 2003). To genetically test the ability of cyclin D2 to replace cyclin D3 function, we generated mice in which *Ccnd2* cDNA was targeted to the *Ccnd3* locus, such that *Ccnd2* was regulated by the *Ccnd3* 5′ and 3′ untranslated region (Figure S1 available online). The unique *Ccnd3/2* transcript generated from the knock-in allele was not detected in wild-type, *Ccnd2*$^{-/-}$, or *Ccnd3*$^{-/-}$ cells using quantitative PCR (qPCR) analysis (Figure 1A). This unique transcript was specifically produced in *Ccnd3*$^{+/Ccnd2\text{-}Neo}$ lymphocytes at low levels, but deletion of the neomycin resistance cassette resulted in a significant increase in messenger RNA (mRNA) expression in *Ccnd3*$^{+/Ccnd2}$ cells. Analysis of total *Ccnd2* mRNA showed comparable expression in *Ccnd3*$^{+/Ccnd2}$ cells to that of wild-type cells. As expected, *Ccnd2* transcripts were decreased in *Ccnd2*$^{+/-}$ thymocytes and diminished in *Ccnd2*$^{-/-}$ cells (Figure 1B). To further confirm knock-in allele expression, we generated *Ccnd2*$^{-/-}$*Ccnd3*$^{+/Ccnd2}$ animals and analyzed cyclin D2 expression in lymphocytes. Although the protein was not detected in *Ccnd2*$^{-/-}$*Ccnd3*$^{+/Ccnd2\text{-}Neo}$ cells that retained the neomycin resistance cassette, cyclin D2 was readily detected in cells from knock-in animals (*Ccnd2*$^{-/-}$ *Ccnd3*$^{+/Ccnd2}$) that had deleted the selection cassette (Figure 1C). Furthermore, cyclin D2 expression was increased in *Ccnd3*$^{Ccnd2/Ccnd2}$ (hereafter, *Ccnd3*$^{D2/D2}$) cells compared to both wild-type and *Ccnd3*$^{-/-}$ cells, demonstrating that cyclin D2 protein was specifically generated from the knock-in allele.

Given the role of cyclin D3 in early thymopoiesis (Sicinska et al., 2003), we investigated T cell development in *Ccnd3*$^{D2/D2}$ animals (Figure 1D). We found that thymus size and total thymus cellularity of *Ccnd3*$^{D2/D2}$ animals was reduced similarly to that of *Ccnd3*$^{-/-}$ mice, and both were significantly decreased from those of controls. Loss of cyclin D3 is associated with an increase in the percentage of CD4^{-}CD8^{-} double negative (DN) thymocytes and a corresponding decrease in CD4^{+}CD8^{+} double positive (DP) cells (Sicinska et al., 2003); although the overall number of DN cells was not altered, the number of *Ccnd3*$^{-/-}$ DP was significantly reduced. We found that the total number of *Ccnd3*$^{D2/D2}$ DN and DP cells was similar to that observed in *Ccnd3*$^{-/-}$ animals (Figure 1E). Although loss of cyclin D3 did not affect the absolute number of DN cells generated, the percentage of DN cells was increased, while the percentage of DP cells was decreased compared to controls (*Ccnd3*$^{-/-}$ DN 10.2% and DP 62.4%; control DN 2.3% and DP 83.8%). As observed in *Ccnd3*$^{-/-}$ thymocytes, the percentage of *Ccnd3*$^{D2/D2}$ DN cells was also increased and the percentage of DP cells was reduced compared to controls (Figure 1F). Finally, we quantified proliferation of *Ccnd3*$^{D2/D2}$ pro- and pre-T cells, which was comparable to that observed in *Ccnd3*$^{-/-}$ pro- and pre-T cells (Figure 1G); however, both of these populations showed a decrease in rates of proliferation in comparison to wild-type cells. Even with high cyclin D2 expression, cyclin D:CDK4/6 specific phosphorylation of Rb (S807/811) was reduced in *Ccnd3*$^{D2/D2}$ thymocytes compared to wild-type and was similar to that observed in *Ccnd3*$^{-/-}$ cells (Figure 1H). These data demonstrated that, in early *Ccnd3*$^{D2/D2}$ thymocytes, despite the abundance of cyclin D2, Rb remained hypophosphorylated and the cells could not efficiently pass into S-phase.

In addition to its role in T cell development, cyclin D3 is also required for early B lymphopoiesis (Cooper et al., 2006). We found that the overall bone marrow cellularity in *Ccnd3*$^{D2/D2}$ animals was reduced to that found in *Ccnd3*$^{-/-}$ mice (Figure S1), while control bone marrow contained a significantly higher number of cells. Furthermore, knock-in animals had a decreased number of pre-B cells compared to controls, and knock-in pre-B cells had an impaired ability to proliferate compared to

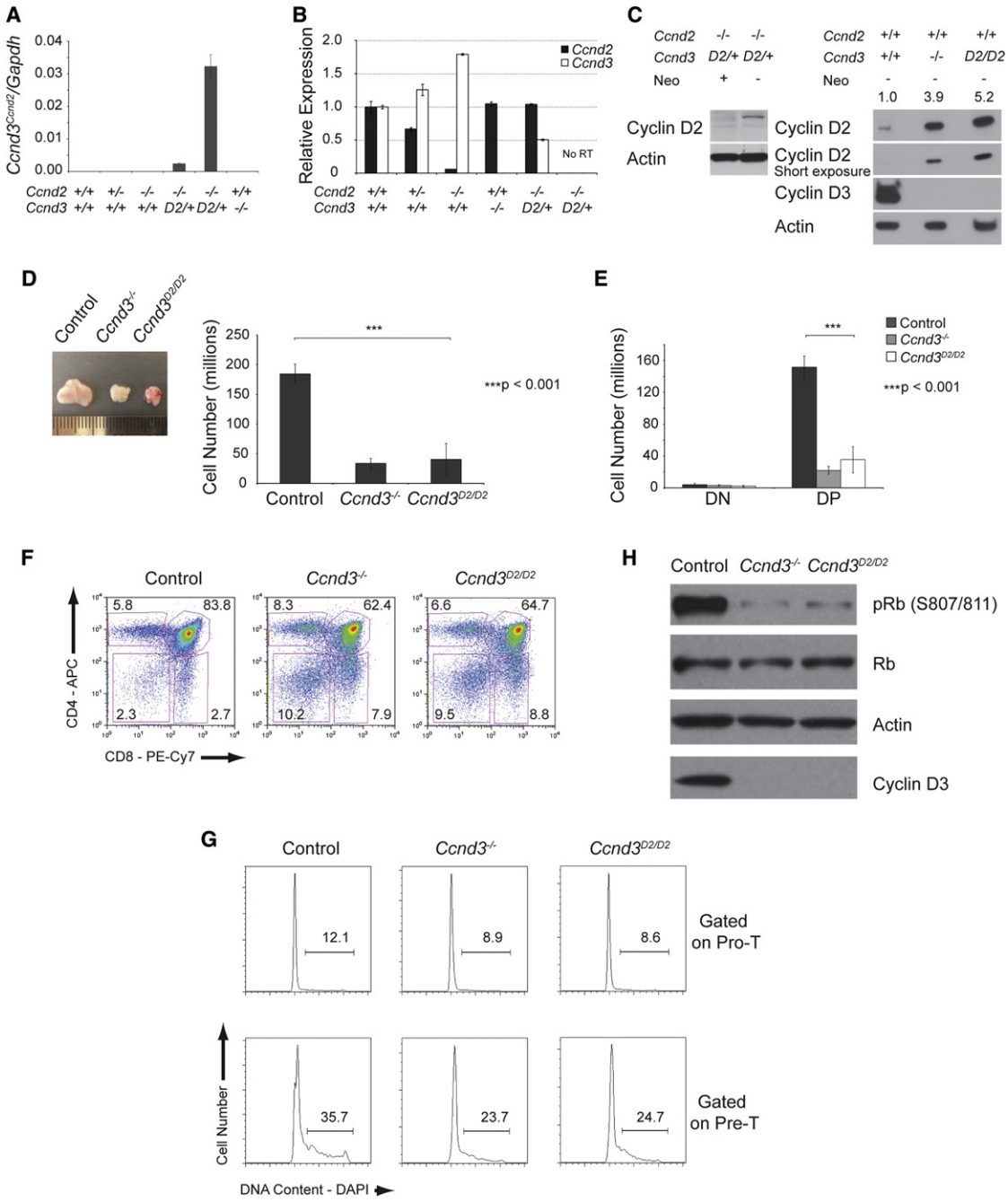

Figure 1. Cyclin D3 Has Unique Functions in Normal Lymphopoiesis

(A–C) Total thymocytes were isolated from wild-type control, *Ccnd2*$^{+/-}$, *Ccnd2*$^{-/-}$, *Ccnd2*$^{-/-}$*Ccnd3*$^{+/Ccnd2\text{-}Neo}$, *Ccnd2*$^{-/-}$*Ccnd3*$^{+/Ccnd2}$, or *Ccnd3*$^{Ccnd2/Ccnd2}$ animals and analyzed for expression of (A) the unique transcript generated from the *Ccnd3*^{Ccnd2} knock-in allele and (B) total *Ccnd2* transcript normalized to *Gapdh* by qPCR or (C) for cyclin D2, D3, and actin (loading control) expression by Western blot. Bands for cyclin D2 and actin were quantified and the relative amount of protein indicated with the wild-type control set to 1.

(D–F) Images of thymuses, left panel (D). Thymus cellularity of control, *Ccnd3*$^{-/-}$, and *Ccnd3*$^{D2/D2}$ mice was measured, and (E) the fraction of DN ($CD4^-CD8^-$) and DP ($CD4^+CD8^+$) cells were calculated using percentages from (F) flow cytometry of CD4 and CD8 expression.

(G) Cell cycle status of pro- ($CD4^-CD8^-CD44^-CD25^+$) and pre-T cells ($CD4^-CD8^-CD44^-CD25^{lo}$) was measured by incorporation of DAPI using FACS analysis.

(H) Wild-type control, *Ccnd3*$^{-/-}$ and *Ccnd3*$^{D2/D2}$ thymocytes were analyzed for Rb (S807/811), total Rb, cyclin D3, and actin by Western blot. Data represent mean ± SD (***p < 0.001) and are representative of at least three independent experiments.

See also Figure S1.

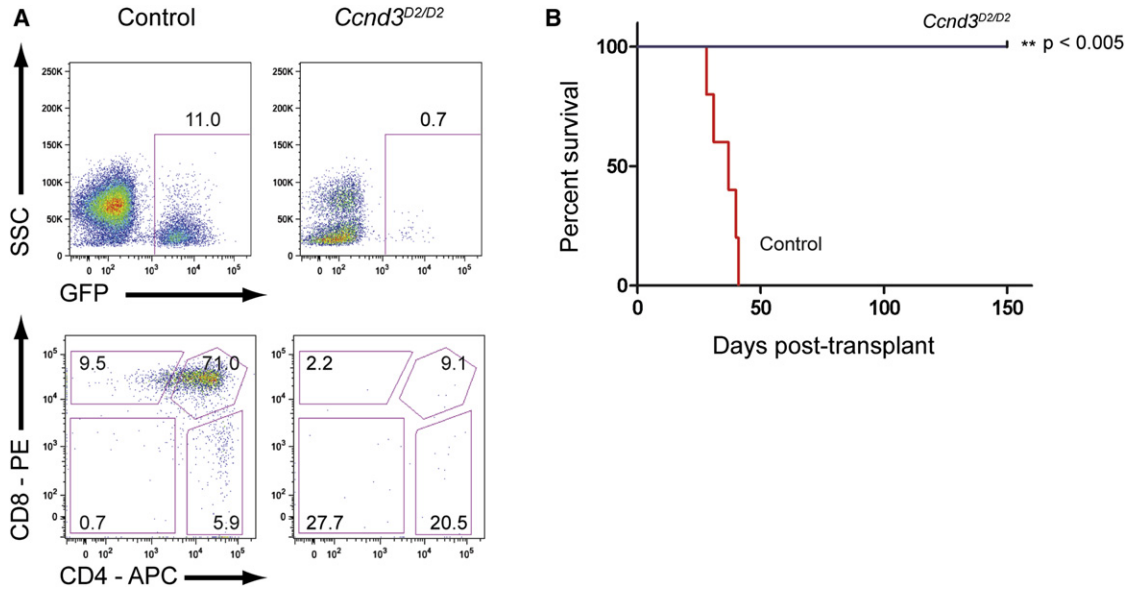

Figure 2. Cyclin D3 Has Nonredundant Functions in the Induction of T-ALL

(A) Lethally irradiated B6.SJL animals were transplanted with 3 × 10^5 pMIGR1 ICN1-IRES-EGFP wild-type or *Ccnd3*$^{D2/D2}$ bone marrow progenitors, and peripheral blood was collected and analyzed for DP cells by FACS. Peripheral blood analysis shown is 31 days upon transplant of transduced cells.

(B) Kaplan-Meier curve shows the survival of transplanted mice for the period of observation (**p < 0.005). Data are representative of two independent experiments with five mice per condition.

controls. Collectively, these results indicated that cyclin D2 expression could not rescue the defects in lymphocyte development caused by the lack of cyclin D3.

Unique Roles for Cyclin D3 in Acute Lymphocytic Leukemia

Although *Ccnd2* expression from the *Ccnd3* locus did not rescue the defect during the normal development of *Ccnd3*$^{-/-}$ early lymphocytes, we wanted to test its ability to replace the requirement for cyclin D3 in oncogenic transformation, specifically in T-ALL (Sicinska et al., 2003). We selected a Notch-driven model of T-ALL, as more than 90% of human T-ALL shows signs of constitutive Notch pathway activation and the majority of human T-ALL lines are addicted to Notch1 function (Palomero et al., 2007; Weng et al., 2004). We transduced lineage$^-$c-Kit$^+$ bone marrow progenitors from wild-type and *Ccnd3*$^{D2/D2}$ mice with retrovirus encoding the constitutively active intracellular domain of Notch1-IRES-enhanced green fluorescent protein (EGFP) (ICN1-EGFP), as previously described (Espinosa et al., 2010; Vilimas et al., 2007; Walkley et al., 2005). These cells were transplanted into lethally irradiated congenic wild-type mice, and animals were monitored for the presence of CD4$^+$CD8$^+$ DP leukemic cells in the peripheral blood. At 2 weeks after transplant, ICN1-EGFP$^+$ leukemic cells were detected in the blood of control animals. Furthermore, 1 month posttransplant, the peripheral blood of control animals contained a significant number of ICN1-EGFP$^+$ cells, of which 71% were DP cells, while only a minute number of ICN1-EGFP$^+$ cells were observed in recipients of *Ccnd3*$^{D2/D2}$ cells (Figure 2A). The development of disease in hosts that received wild-type cells transduced with ICN1 retrovirus was rapid, with all control animals succumbing to the disease 4–6 weeks after transplant (Figure 2B). In contrast, animals that received transduced *Ccnd3*$^{D2/D2}$ cells displayed significant protection from disease, with all animals remaining disease-free for the entire 6-month period of observation. These results demonstrated a specific requirement for cyclin D3, but not cyclin D2, in the induction of T-ALL.

Endogenous Ccnd2 Protein Induction Fails to Rescue *Ccnd3*$^{-/-}$ Phenotypes

Considering that *Ccnd2* knock-in into the *Ccnd3* locus did not rescue the specific requirements for cyclin D3, we further investigated the regulation of expression of these molecules in early lymphocytes. We readily detected cyclin D2 protein in purified wild-type pro-T (CD4$^-$8$^-$25$^+$44$^-$); however, this expression decreased in wild-type pre-T (CD4$^-$8$^-$25$^{low/neg}$44$^-$) cells (Figure S1). However, in *Ccnd3*$^{-/-}$ cells, cyclin D2 protein was significantly increased in pro- and pre-T cells as well as total thymocytes. This finding suggested that "physiological" cyclin D2 overexpression was not able to rescue the developmental block caused by cyclin D3 deficiency, in agreement with our observations from the knock-in described in Figure 1. Cyclin D2 overexpression appears to be posttranscriptional, as no differences in *Ccnd2* mRNA levels were detected from wild-type and *Ccnd3*$^{-/-}$ total thymocytes (Figure S1). Furthermore, immunofluorescence analysis of cyclin D2 and D3 protein from purified DN wild-type thymocytes confirmed previous findings, as it showed preferential induction of cyclin D2 protein in *Ccnd3*$^{-/-}$ thymocyte progenitors (Figure S1). Cyclin D3 expression remained unchanged in response to *Ccnd2* deletion (data not shown; Figure S1). To further investigate regulation of cyclin D2 protein, we treated thymocytes with cycloheximide, which inhibits de novo protein synthesis, and analyzed expression over time. Cyclin D2 half-life was

significantly increased in *Ccnd3*$^{-/-}$ cells (2.5-–4-fold) when compared to wild-type thymocytes (Figure S3D). We also observed that cyclin D2 protein was stabilized upon treatment with the proteasome inhibitor MG-132, suggesting regulation by the ubiquitin-proteasome system (Figure S1). These studies further support our previous hypothesis, as they demonstrate that "physiological" upregulation of cyclin D2 protein is unable to "rescue" normal lymphocytic differentiation and induction of leukemia.

Loss of p27^{Kip1} Partially Restores T Cell Development in *Ccnd3*$^{-/-}$ Mice

We next sought to further define the functions of cyclin D3 in regulating cell cycle during early lymphocyte development. Of the downstream cell cycle regulators, we focused on the cell cycle inhibitor p27^{Kip1}, as mice deficient for p27^{Kip1} show increased thymic cellularity (Fero et al., 1996; Nakayama et al., 1996), and p27^{Kip1} is the only CDKI dynamically regulated at early stages of T cell development (I.A., unpublished data). To genetically test the interaction between cyclin D3 and p27^{Kip1}, we crossed *Ccnd3*$^{-/-}$ and *Cdkn1b*$^{-/-}$ mice. Analysis of cyclin D3 and p27 protein from total thymocytes of *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ mice confirmed the genotypes of the mice (Figure 3A). We measured total thymic cellularity and found that the average number of *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ cells was significantly increased compared to that of *Ccnd3*$^{-/-}$ thymocytes, but remained significantly reduced compared to wild-type controls (Figure 3B). Differences in total thymocyte numbers were associated with differences in percentages and average number of DP cells (Figures 3C and 3D). While *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ animals displayed significantly higher percentages and numbers of DP thymocytes than *Ccnd3*$^{-/-}$ animals, they had significantly fewer DP cells than control mice. Taken together, these observations suggested that ablation of p27^{Kip1} only partially restores development of *Ccnd3*$^{-/-}$ thymocytes.

We have previously shown that the *Ccnd3*$^{-/-}$ T cell defect stems from a reduction in S-phase entry and cell cycle progression (Sicinska et al., 2003); thus, we next assessed the cell cycle status of *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ early lymphocytes. We evaluated cell cycle using DAPI and observed an increase from 11.6% to 17.5% of pre-T cells in S-G_2-M phases of the cell cycle from *Ccnd3*$^{-/-}$ and *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ animals, respectively (Figure 3E). In contrast, the percentage of *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ proliferating pre-T cells was still reduced compared to controls. To further investigate cell cycle, we measured CDK2-associated activity using an in vitro kinase assay. CDK2-containing complexes immunoprecipitated from *Ccnd3*$^{-/-}$ total thymocytes showed little kinase activity toward exogenous Rb (Figure 3F). In contrast, *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ thymocytes showed an increase in CDK2-associated activity, but this activity did not reach the levels of control CDK2 activity. These combined studies indicated that loss of p27^{Kip1} only partially restored the cell cycle and developmental defects of early *Ccnd3*$^{-/-}$ T cells.

Notch1-Induced Transformation of *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ Cells

We also tested the ability of *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ cells to be transformed by Notch1 activation. Peripheral blood analysis 2 weeks after transplant revealed a small population of ICN1-EGFP^{+} DP cells in hosts that received *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ transduced cells (Figure S2A). Similar to control mice that received wild-type ICN1-transduced cells, all recipients of *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ transduced cells developed disease, albeit with slightly delayed kinetics, and succumbed to disease by 10 weeks posttransplant (Figure S2B). These data show that additional loss of p27^{Kip1} was sufficient to rescue transformation and progression to disease of *Ccnd3*$^{-/-}$ cells.

Loss of Rb Partially Restores *Ccnd3*$^{-/-}$ T Cell Development

As loss of p27 did not completely rescue *Ccnd3*$^{-/-}$ lymphopoiesis at the steady state, we hypothesized that remaining activities of additional key cell cycle regulators could prevent cells from properly entering the cell cycle in the absence of cyclin D3. We observed that Rb was expressed and phosphorylated in wild-type pre-T cells and that its expression, specifically the cyclin D:CDK4/6-phosphorylated pRb (S807/811) species, was reduced in *Ccnd3*$^{-/-}$ cells (Figure 4A). To genetically test the importance of Rb regulation, we generated cyclin D3/Rb doubly deficient animals by crossing *Ccnd3*$^{-/-}$ mice to *Rb1*$^{F/F}$ mice carrying the *Mx1*-Cre transgene. Administration of the double-strand RNA mimic poly(I:C) to these animals induced Cre recombinase expression, mediating deletion of *Rb1*, and these animals hereafter are referred to as *Ccnd3*$^{-/-}$*Rb1*$^{-/-}$. Rb was not detectable in total thymocytes from *Ccnd3*$^{-/-}$*Rb1*$^{-/-}$ mice, demonstrating efficient deletion of the floxed *Rb1* alleles (Figure 4B).

As with *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ mice, the thymic cellularity of *Ccnd3*$^{-/-}$*Rb1*$^{-/-}$ animals was significantly increased compared to *Ccnd3*$^{-/-}$ animals, yet significantly decreased compared to controls (Figure 4C). *Ccnd3*$^{-/-}$*Rb1*$^{-/-}$ animals displayed trends in the percentages and average number of DP cells that were similar to those seen in *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ mice (Figures 4D and 4E), indicating that deletion of Rb1 only partially restored *Ccnd3*$^{-/-}$ early T cell development. We next analyzed the cell cycle of *Ccnd3*$^{-/-}$*Rb1*$^{-/-}$ pre-T cells and detected approximately 21% of cells in S-G_2-M phases, whereas only 11.5% of *Ccnd3*$^{-/-}$ pre-T cells had transitioned beyond G_1 (Figure 4F). However, the percentage of *Ccnd3*$^{-/-}$*Rb1*$^{-/-}$ proliferating pre-T cells was significantly lower than that of controls (approximately 30%). We also tested CDK2-associated kinase activity in *Ccnd3*$^{-/-}$*Rb1*$^{-/-}$ total thymocytes by in vitro kinase assay. CDK2-containing complexes isolated from *Ccnd3*$^{-/-}$*Rb1*$^{-/-}$ T cells showed partially restored ability to phosphorylate exogenous Rb compared to *Ccnd3*$^{-/-}$ thymocytes (Figure 4G). However, the CDK2 kinase activity in control thymocytes was significantly higher, providing an explanation for the only partial restoration of progenitor cell proliferation.

Combined Loss of p27^{Kip1} and Rb Restores *Ccnd3*$^{-/-}$ T Cell Development

Our genetic analyses showed that neither p27^{Kip1} nor Rb loss was sufficient to completely rescue *Ccnd3*$^{-/-}$ developmental defects. Although there could be several alternative explanations for this incomplete rescue (including redundancy with other CDKI or Rb pocket proteins), we hypothesized that concomitant inactivation of p27^{Kip1} and Rb could provide efficient rescue, suggesting that cyclin D3 acts by simultaneously altering pRb

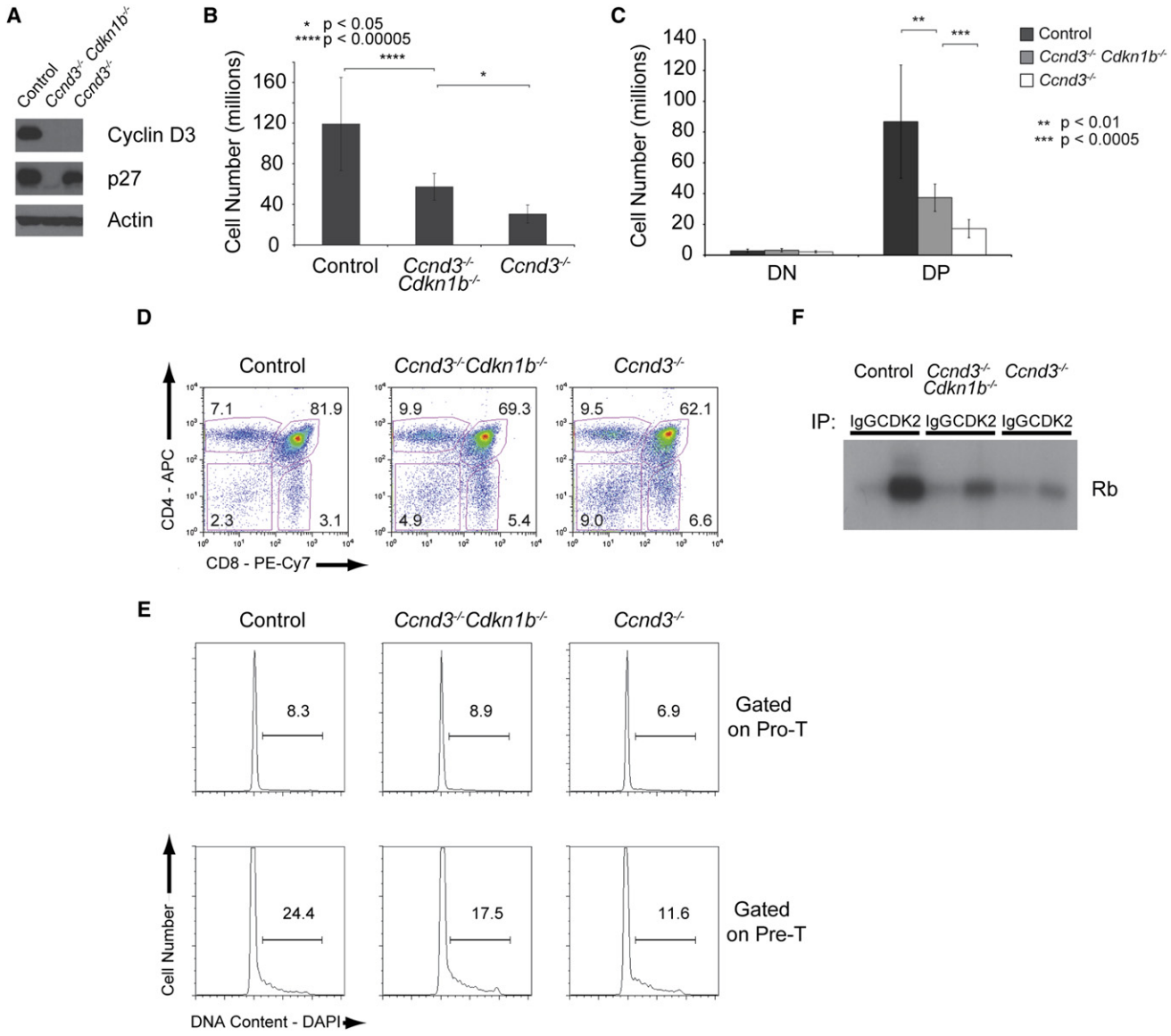

Figure 3. Loss of p27 Partially Rescues the Development of *Ccnd3*$^{-/-}$ T Cell Progenitors

(A) Total thymocytes analyzed for cyclin D3, p27, and actin expression by immunoblot.

(B–D) Total thymus cellularity of littermate control, *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$, and *Ccnd3*$^{-/-}$ mice was quantified (B), and the relative number of DN and DP cells was calculated using total cell numbers and percentages from flow cytometry (C and D).

(E) The proliferation of pro- and pre-T cells was analyzed by measuring DAPI incorporation by flow cytometric analysis.

(F) Protein lysates were subjected to immunoprecipitation with antibody to CDK2 or normal rabbit immunoglobulin G (IgG) as control, and the ability of precipitated complexes to phosphorylate recombinant Rb was measured by in vitro kinase assay. Results represent mean ± SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.0005$, ****$p < 0.00005$) and are representative of three independent experiments of three mice of each genotype.

See also Figure S2.

activity and binding p27^{Kip1}. We thus generated *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$*Rb1*$^{-/-}$ compound mutant animals and analyzed early T cell development. We found that ablation of both p27^{Kip1} and Rb in *Ccnd3*$^{-/-}$ animals resulted in a significant increase in total thymocyte numbers, comparable to that of controls (Figure 5A). The number of DN cells in compound mutant animals was also similar to wild-type thymuses (Figure 5B). Moreover, *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$*Rb1*$^{-/-}$ and control thymuses contained an almost identical number of DP cells (Figures 5B and 5C). Finally, we measured pre-T cell proliferation. Only 13.0% of *Ccnd3*$^{-/-}$ cells were in S-G_2-M phases (Figure 5D). In contrast, approximately 29% of *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$*Rb1*$^{-/-}$ pre-T cells were in S-G_2-M phases, a percentage similar to that found in control littermate animals. These results indicated that only simultaneous genetic deletion of p27^{Kip1} and Rb was sufficient to completely rescue cell cycle progression and the development of *Ccnd3*$^{-/-}$ early T cells.

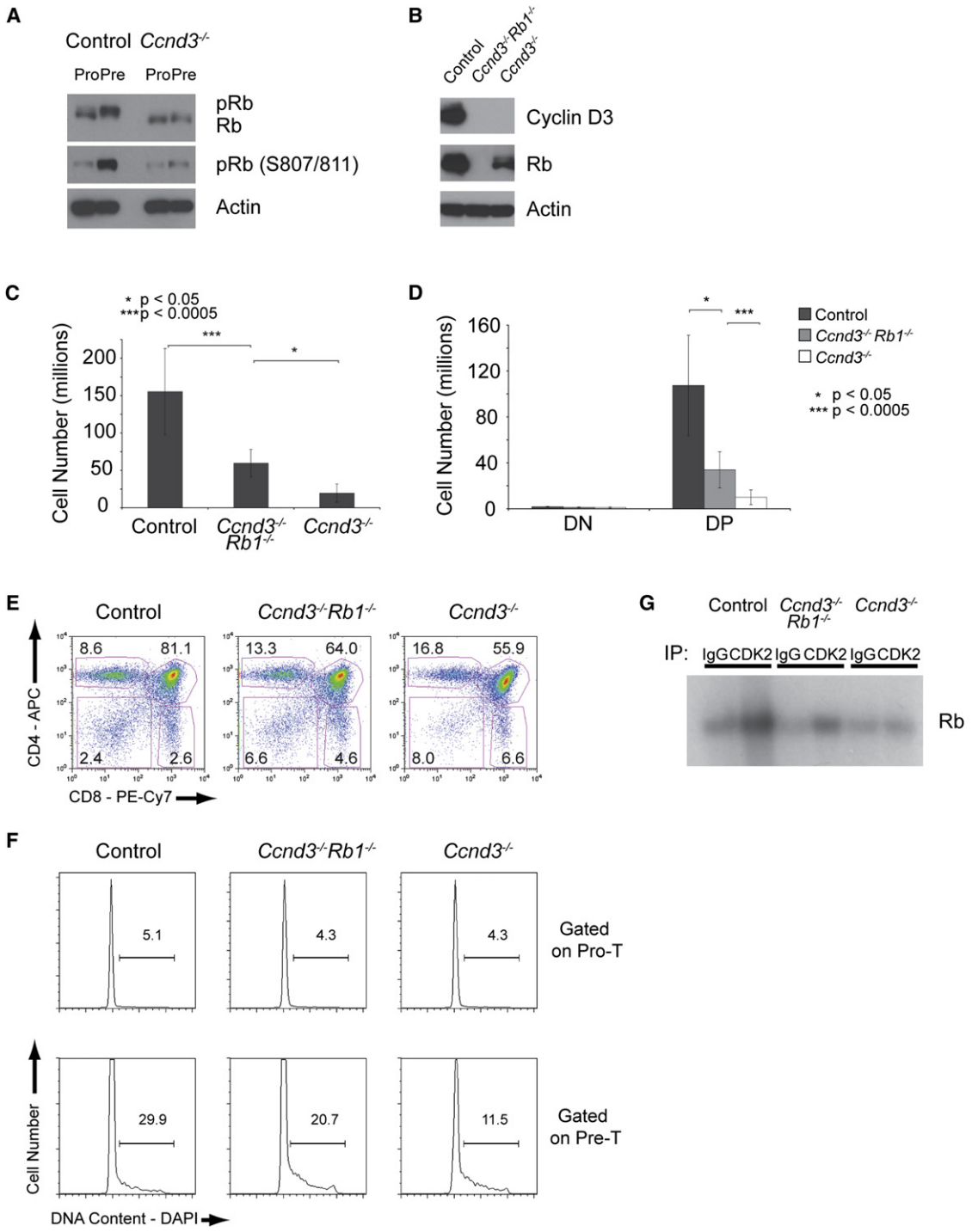

Figure 4. Deletion of Rb Partially Rescues the Development of *Ccnd3*$^{-/-}$ T Cell Progenitors

(A and B) Analysis of total Rb, pRb (S807/811), cyclin D3, and actin by immunoblot from pro- or pre-T purified from wild-type, *Ccnd3*$^{-/-}$*Rb1*$^{-/-}$, and *Ccnd3*$^{-/-}$ total thymocytes.

(C–E) Total thymus numbers of littermate control, *Ccnd3*$^{-/-}$*Rb1*$^{-/-}$, and *Ccnd3*$^{-/-}$ mice were quantified (C), and the relative number of DN and DP cells was calculated using total cell numbers and percentages from flow cytometry (D and E).

(F) Cell cycle analysis of pro- and pre-T cells by DAPI incorporation measured by FACS.

(G) Immune complexes were pulled-down with anti-Cdk2 antibody or normal rabbit IgG, and phosphorylation of exogenous Rb was measured by kinase assay.

Results represent mean ± SD (*p < 0.05, ***p < 0.0005) and are representative of three independent experiments of three mice of each genotype.

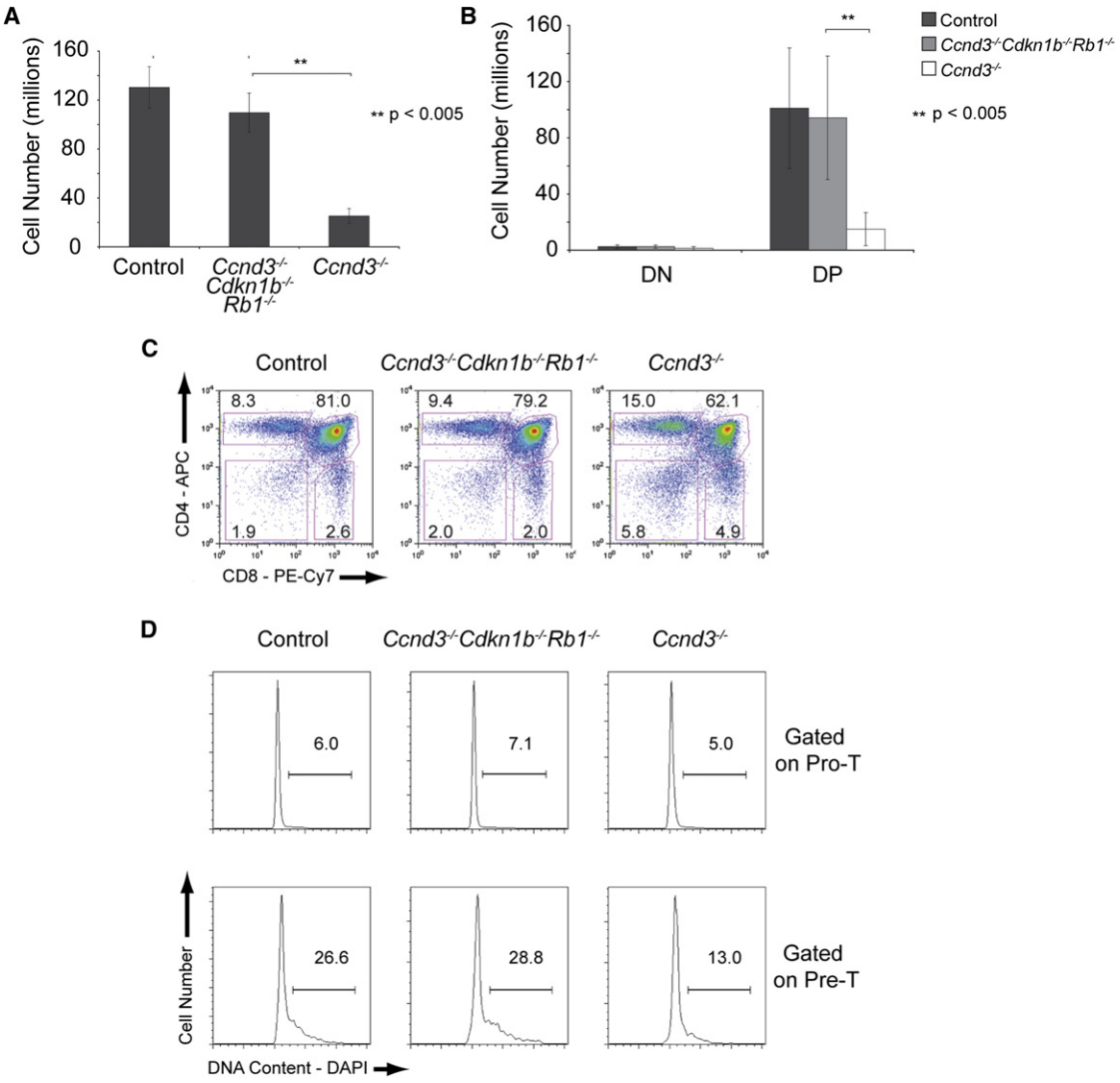

Figure 5. Combined Loss of p27 and Rb Restores *Ccnd3*$^{-/-}$ T Lymphopoiesis

(A–C) Total thymus cellularity of littermate control, *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$*Rb1*$^{-/-}$, and *Ccnd3*$^{-/-}$ mice was quantified (A), and the relative number of DN and DP cells was calculated using total cell numbers and percentages from flow cytometry (B and C).

(D) Cell cycle status of pro- and pre-T cells was measured by DAPI incorporation by flow cytometric analysis. Results represent mean ± SD (**p < 0.005) and are representative of two independent experiments of four mice of each genotype.

Inhibition of Cyclin D3:CDK4/6 Complex Activity Suppresses Human T-ALL Cell Growth

Our studies suggested that cyclin D3 has unique functions in lymphocyte development and transformation, likely in conjunction with CDK4/6, to titrate CDKIs and inactivate Rb. We have previously shown that cyclin D3 expression is essential for the induction of Notch-driven T-ALL (Sicinska et al., 2003). However, these studies did not address the potential of cyclin D3:CDK4/6 targeting during disease progression, a question of significant clinical relevance. To address this question, we used PD-0332991, a CDK4/6 specific small molecule inhibitor currently in clinical trials for multiple myeloma treatment (Baughn et al., 2006; Marzec et al., 2006; Menu et al., 2008). Initially, to confirm interaction between cyclin D3 and CDK4/6 in T cells, we performed immunoprecipitation of endogenous CDK6 in wild-type thymocytes and observed specific interaction with cyclin D3 (Figure 6A). Similar cyclin D3:CDK4/6 interaction was also observed in human T-ALL cell lines. Having confirmed this interaction, we initiated in vitro treatments of mouse and human T-ALL cell lines with PD-0332991. All human T-ALL lines utilized carried *NOTCH1* mutations, and the majority was absolutely dependent on Notch activity. Although the mouse T-ALL lines were driven by overexpression of TAL1, they also contained Notch1-truncating PEST mutations (O'Neil et al., 2006). We tested PD-0332991 at both 0.5 and 1 μM and found similar effects in vitro. PD-0332991 treatment efficiently inhibited S-phase entry of all cell lines within 15 hr, leading to accumulation of cells in G_0/G_1 phases (Figure 6B). The effects of drug treatment were reversible, as removal of PD-0332991 led to efficient re-entry in the cell cycle (Figure S3). To further expand

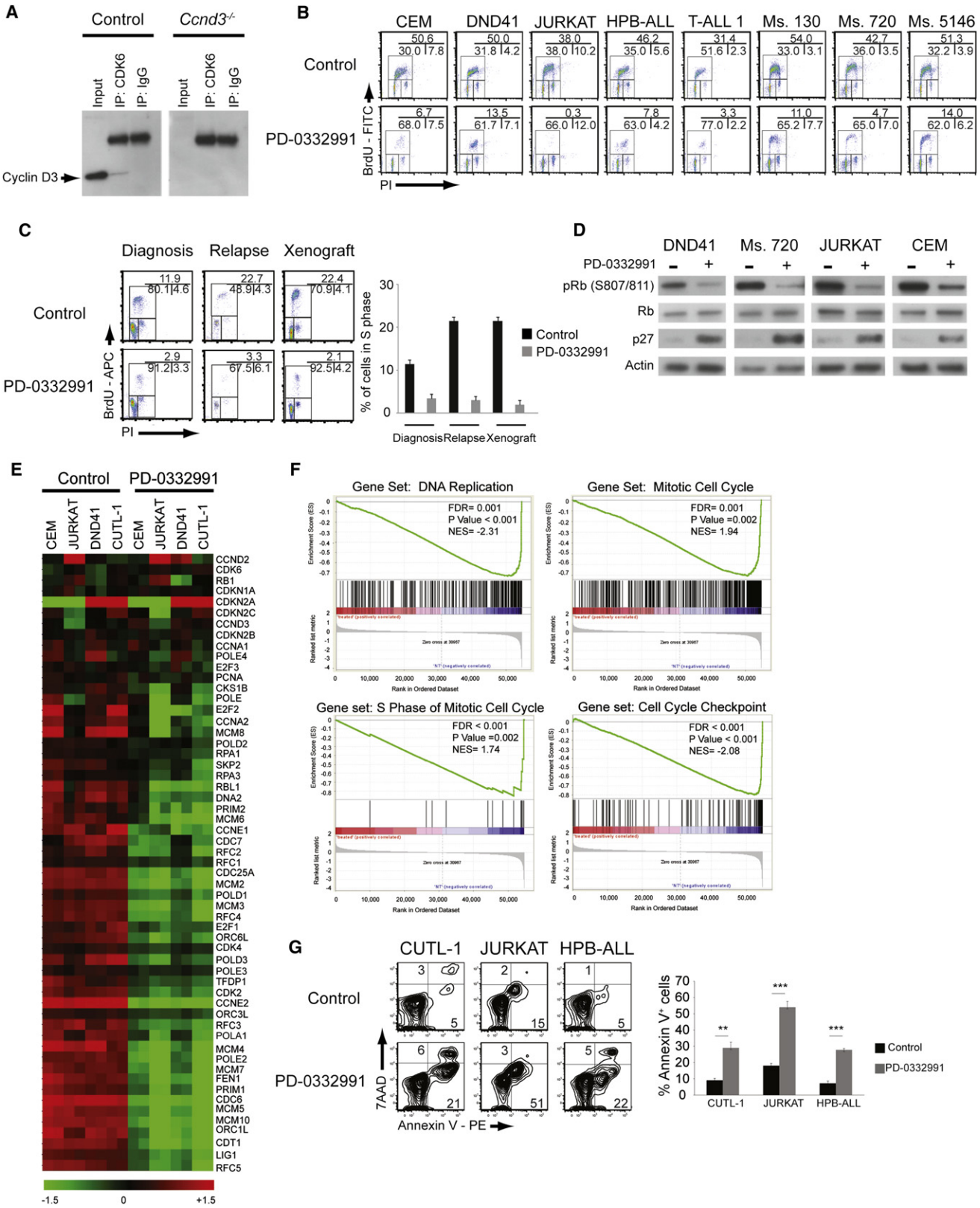

Figure 6. Inhibition of Cyclin D3:CDK4/6 Activity Inhibits T-ALL Cell Growth In Vitro

(A) Protein lysates from wild-type and *Ccnd3*$^{-/-}$ thymocytes were prepared and subjected to pull-down with antibody to CDK6 or normal rabbit IgG and then analyzed by immunoblot cyclin D3 antibody.

these studies using primary leukemia samples, we in vitro treated cells isolated from two T-ALL patients. PD-0332991 treatment led to inhibition of cell cycle progression and accumulation in the G_0/G_1 cell cycle phase in the two T-ALL primary samples (Figure 6C). We also compared primary T-ALL leukemia cells at diagnosis to cells at relapse or after engraftment in immune-deficient mice. We found a strong inhibition in all conditions, despite higher cycling rates in the relapse and xenograft cells, suggesting that PD-0332991 could be efficient in the treatment of relapsed T-ALL (Figure 6C).

To gain a better molecular and biochemical understanding of PD-0332991 function on human T-ALL cells, we have used immunoblotting to define expression and activation of known cell cycle regulators. PD-0332991 treatment efficiently suppressed pRb (S807/811) phosphorylation and increased the expression of the $p27^{Kip1}$ CDKI, both hallmarks of a G_0/G_1 arrest (Figure 6D). Whole-transcriptome analysis using four human T-ALL lines led to similar results (Figures 6E and 6F). PD-0332991 treatment suppressed the expression of key mitosis regulators, including *E2f2*, *Ccna2*, *Skp2*, *Cdc25a*, *Ccne2*, and *Cdt1*. PD-0332991 treatment did not affect expression of *Ccnd3* or *Cdk4*/*Cdk6* mRNA. Gene set enrichment analysis (GSEA) showed that PD-0332991 treatment led to significant gene expression correlation with gene sets related to cell cycle progression, including DNA replication, S-phase entry, and entry into mitosis (Figure 6F). Furthermore, after 4 days exposure to PD-0332991, we observed a significant increase in annexin V expression compared to controls, indicating progression to cell death after treatment (Figure 6G). These combined studies suggested that, by inducing cell cycle arrest and apoptosis of leukemic cells, PD-0332991-mediated inhibition of cyclin D3:CDK4/6 activity could be an attractive therapy for T-ALL.

Inhibition of Cyclin D3:CDK4/6 Complex Activity Inhibits T-ALL Progression In Vivo

To test the ability of PD-0332991 to suppress disease progression in preclinical models of T-ALL, we treated animals that received cells expressing potent oncogenic forms of NOTCH1. Upon establishment of disease at week 3, we initiated treatment of leukemic mice by oral administration of PD-0332991 for ten consecutive days. The effects of the treatment were rapid and significant, as all untreated control mice died by week 12, while the majority of PD-0332991-treated mice survived during the period of observation (Figure 7A). Peripheral white blood counts significantly decreased, and ICN1-EGFP$^+$ DP leukemic cells disappeared from the peripheral blood of the vast majority of the treated animals (Figures 7B and 7C). In contrast, control animals displayed splenomegaly, and analysis of control splenocytes showed that the majority of cells were ICN1-EGFP$^+$ DP leukemic cells (Figures 7D and 7E). Histologic examination demonstrated a significant reduction of leukemic cell infiltrations in all tissues studied from PD-0332991-treated animals (Figure 7F). Furthermore, ICN1-EGFP$^+$ cells showed increased annexin V expression in PD-0332991-treated animals compared to controls (Figure 7G). T-ALL cells were addicted to CDK4/6 function, as interruption of drug administration led to relapse of the disease (data not shown), suggesting that the disease can be reinitiated by a small number of noncycling cells with leukemia-initiating abilities.

To extend these studies using a xenograft model, we transplanted lethally irradiated *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice with the human T-ALL CEM cell line and administered PD-0332991 3 weeks posttransplant for 14 consecutive days. Peripheral blood analysis at 4 weeks posttransplant showed 55% huCD45$^+$ cells from untreated control mice compared to 6% huCD45$^+$ cells from PD-0332991-treated mice. All untreated control mice died within 30 days of transplant, while the majority of treated mice survived during the observation period (Figure S4). Collectively, these studies demonstrated that cyclin D3:CDK4/6 inhibition was able to efficiently suppress T-ALL progression leading to disease regression in vivo.

DISCUSSION

We demonstrate here that the cyclin D3:CDK4/6 complex has unique functions in the expansion of normally developing T cell progenitors and induction of T cell leukemia. We show that cyclin D2, a D-type cyclin also expressed in developing T cell progenitors, cannot replace cyclin D3. Although we detected cyclin D2 expression specifically generated by the knock-in allele, *Ccnd2* is unable to rescue the developmental defects caused by *Ccnd3* deficiency. Two additional findings support the notion that cyclin D2 is unable to sustain expansion of thymocyte progenitors. Initially, we found high expression of cyclin D2 protein in pro-T cells; however, the cells are largely quiescent, suggesting that elevated levels of D-type cyclins do not always correlate with high rates of proliferation. Moreover, we were able to demonstrate that, in the absence of cyclin D3, D2 expression was significantly elevated. This finding suggested that differentiating progenitors respond to the loss of cyclin D3 by upregulating cyclin D2; however, as with *Ccnd3*$^{D2/D2}$ genetic replacement, this endogenous upregulation of cyclin D2 fails to

(B) Human and mouse (Ms.) T-ALL cell lines were treated with 1 μM PD-0332991 for 15 hr, followed by pulse with 30 μM BrdU. The percentage of cells in the S-phase was measured by FACS analysis of BrdU incorporation and propidium iodide (PI).
(C) Primary human T-ALL cells (purified from the same patient) at diagnosis, relapse, and as a xenograft were grown on MS5-DL1 stromal cells for 2 days, then treated with 0.5 μM PD-0332991 for 15 hr and pulsed with 30 μM BrdU. The percentages of cells in the S-phase were calculated using percentages from FACS analysis of BrdU incorporation and PI.
(D) Alternatively, cell lines were treated for 12 hr with 0.5 μM PD-0332991. Protein extracts were analyzed by SDS-PAGE followed by immunoblot with antibodies against p27, Rb, and pRb (Ser807/811), or actin as a loading control.
(E) Heat map showing downregulation of genes associated with the S-phase of cell cycle and DNA replication upon treatment with 1 μM PD-0332991.
(F) GSEA showing significant downregulation of genes associated with DNA replication, mitotic cell cycle, the S-phase of the cell cycle, and the cell cycle checkpoints.
(G) Human T-ALL cell lines were treated with 1 μM PD-0332991 for 4 days and analyzed for apoptosis by annexin V and 7-amino-actinomycin D. Data represent mean ± SD and are representative of at least three independent experiments.
See also Figure S3.

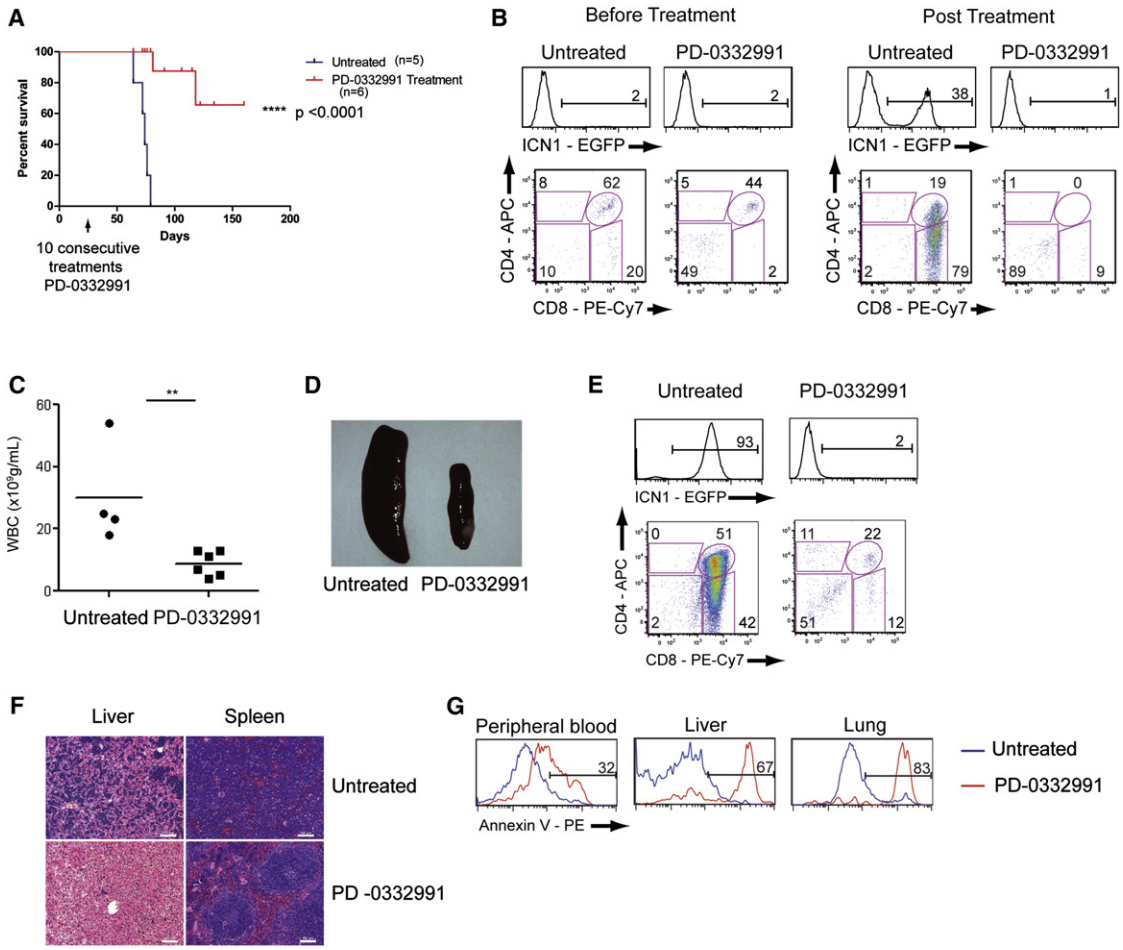

Figure 7. PD-0332991 Inhibits Cyclin D3:CDK4/6 Activity and T-ALL Progression In Vivo

(A and B) Kaplan Meier survival curve of lethally irradiated CD45.1$^+$ B6.SJL mice transplanted with ICN1-EGFP-transduced bone marrow. Mice were treated with 150 mg/kg PD-0332991 or vehicle for ten consecutive days beginning on day 21 (***$p < 0.0001$). Peripheral blood was analyzed for expression of GFP, CD4, and CD8 at both 21 and 28 days posttransplant.

(C) White blood cell counts control and PD-0332991-treated animals were measured at the endpoint analysis, 7 weeks posttransplantation. PD-0332991-treated animals showed significantly reduced blood counts compared to controls (**$p < 0.01$).

(D and E) Spleens were harvested and control mice showed increased splenic mass compared to treated animals. Splenocytes were analyzed for GFP, CD4, and CD8 expression by FACS analysis.

(F) Liver and spleen were paraffin embedded and tissue sections were stained by hematoxylin and eosin staining (H&E). Scale bars correspond to 100 μm at 10X magnification.

(G) Apoptosis in peripheral blood, liver, and lung was measured by expression of annexin V. Data are representative of two independent experiments.

See also Figure S4.

rescue the *Ccnd3*$^{-/-}$ phenotypes. These experiments suggest that the two cyclins have different functions during early hematopoiesis, in agreement with previous reports using distinct tissue systems. Indeed it was demonstrated that D-type cyclins could have distinct cellular localizations and different abilities to bind CDK inhibitors, such as p27 and p21 (Tamamori-Adachi et al., 2008). Moreover, biochemical studies showed differential substrate specificity between cyclin D1:CDK4 and cyclin D3:CDK4 complexes (Sarcevic et al., 1997). Finally, other studies suggested differential utilization of the LxCxE Rb-binding motif between cyclin D1 and D3 (Baker et al., 2005). Although we were able to demonstrate that cyclin D2 is unable to efficiently phosphorylate Rb in the absence of cyclin D3, further studies are required to elucidate the mechanistic differences among the three kinases in hematopoiesis.

In addition to its requirement in normal T cell development, cyclin D3 is required for the induction of T-ALL, a disease with rapid kinetics and characterized by increased rates of cell division. The inhibition of T-ALL induction is not due to a complete inhibition of T cell progenitor differentiation, as *Ccnd3*$^{D2/D2}$ thymuses generate a significant number of mature T cell receptor $\alpha\beta^+$ cells. Finally, the inability of cyclin D2 to rescue the *Ccnd3*$^{-/-}$ phenotypes is unlike previous models of cyclin "rescue" experiments (Carthon et al., 2005; Geng et al., 1999). This phenotypic disparity may reflect the requirement for precise regulation of cell cycle in lymphocytes, a cell type expressing

more than one D-type cyclin, compared to cell types where a single D-type cyclin is expressed.

Since the *Ccnd3*$^{-/-}$ defects stemmed from the failure of lymphocyte progenitors to proliferate optimally, we focused on putative functions of cyclin D3 that directly regulate the cell cycle. Using genetic animal crosses, we demonstrated that the inhibitor p27^{Kip1} is only one of the critical regulators of cell cycle during T cell development. The p27^{Kip1} inhibitor appears to be a key regulator in early T cell development, as its protein expression is immediately decreased upon expression of the pre-T cell receptor, a prerequisite for both progression of T cell differentiation and induction of T-ALL (Aifantis et al., 2008). It would be interesting to determine the specific functions of p27^{Kip1} in developing T cells using specific mutants that disrupt the interaction between p27^{Kip1} and cyclin D:CDK4/6. While elimination of p27^{Kip1} is sufficient to allow normal proliferation of *Ccnd1*$^{-/-}$ mammary and retina cells (Geng et al., 2001), loss of p27^{Kip1} does not restore optimal proliferation of *Ccnd3*$^{-/-}$ T cells. Indeed, phosphorylation and inactivation of Rb function by cyclin D3:CDK4/6 complexes are another critical function, as demonstrated using the *Ccnd3*$^{-/-}$*Rb1*$^{-/-}$ animals. p27^{Kip1} and Rb are thought to have both cell-autonomous and cell-nonautonomous effects (Chien et al., 2006; Walkley et al., 2007). Although we cannot exclude cell-nonautonomous effects from contributing to the rescue of *Ccnd3*$^{-/-}$ thymocyte development, we believe there is a cell-intrinsic component as well, since ICN1-EGFP-transduced *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ cells were able to be transformed and produce disease after transplant into wild-type hosts (Figure S4). Our data provide genetic evidence suggesting that only simultaneous elimination of both layers of regulation sufficiently lowers the threshold for developing progenitors to enter the cell cycle.

These data suggested that targeting cyclin D3:CDK4/6 complex function can directly target induction and progression of T cell leukemia and, at the same time, cause minimal hematologic side effects, based on the phenotypes of animals lacking expression of cyclin D3 or CDK4/6 (Cooper et al., 2006; Malumbres et al., 2004; Rane et al., 1999; Sicinska et al., 2003, 2006; Tsutsui et al., 1999). To pharmacologically target this kinase complex, we have used PD-0332991, a small molecule selectively inhibiting CDK4/6 function (Fry et al., 2004). PD-0332991 is a pyridopyrimidine that exhibits an IC_{50} value less than 0.01 μmol/L against cyclin D3:CDK4/6 complexes. PD-0332991 is currently in clinical trials for the treatment of multiple myeloma (Baughn et al., 2006; Menu et al., 2008), advanced adult solid tumors, and refractory non-Hodgkin's lymphoma (Schwartz et al., 2011). The drug has a relatively long half-life and is generally well tolerated with minimal side effects. Our results and studies now demonstrate that PD-0332991 could be an efficient treatment for pediatric and adult T cell leukemia. Indeed, we were able to show rapid induction of cell cycle arrest in both mouse and human T-ALL cell lines. Furthermore, we show that the treatment results in cell cycle arrest of primary human leukemia cells both at diagnosis and relapse, as well as loss of leukemic cells by apoptosis, and inhibition of disease progression. Previous data have shown that PD-0332991 effectively inhibits the cell cycle in a breast cancer cell line, and this is dependent on Rb, as chronic loss of Rb eventually leads to resistance to PD-0332991 (Dean et al., 2010). Further studies are required to determine whether Rb is required for PD-0332991-induced cell cycle arrest in T-ALL. PD-0332991 efficiency against relapsed disease is particularly exciting, as such leukemia cells are hyperproliferating, particularly aggressive, and usually nonresponsive to current therapeutic protocols. Although we have observed that PD-0332991 administration can efficiently suppress disease progression, it most likely does not target putative leukemia-initiating cells, as the disease slowly relapses upon discontinuation of treatment. To further potentiate its activity and achieve sustained remission, PD-0332991 could be used in combinatorial treatment protocols together with either conventional chemotherapy or next generation T-ALL-targeted therapeutic compounds, including γ-secretase inhibitors (Real et al., 2009) or Velcade (Menu et al., 2008; Vilimas et al., 2007).

EXPERIMENTAL PROCEDURES

Mice

For generation of *Ccnd3*$^{D2/D2}$ mice, see Supplemental Experimental Procedures. *Ccnd2*$^{-/-}$, *Ccnd3*$^{-/-}$, and *Rb1*$^{F/F}$ mice were kindly provided by Piotr Sicinski and Kay Macleod, respectively, and genotyped following published procedures (Ciemerych et al., 2002). *Rb1*$^{F/F}$ mice were subsequently crossed to *Mx1*-Cre^{+} mice (Jackson Laboratory). Deletion of floxed alleles was induced by intraperitoneal injection of 20 mg/kg polyI:C. Mice were injected five times, once every other day, and analyzed 2 weeks after the last injection. C57BL/6 and *Cdkn1b*$^{-/-}$ mice were purchased from the Jackson Laboratory. All animals were used between 4–10 weeks of age and housed in the sterile Smilow Animal Facility at NYU Medical Center (New York, NY). All experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of NYU Medical Center.

Notch1 Transduction and T-ALL Induction

Retroviral supernatant was generated by transfection of HEK293T cells with pMIGR1-Notch1-IC-IRES-EGFP retroviral construct by the calcium phosphate method. c-Kit^{+} bone marrow progenitors from wild-type control, *Ccnd3*$^{Ccnd2/Ccnd2}$, *Ccnd3*$^{-/-}$, or *Ccnd3*$^{-/-}$*Cdkn1b*$^{-/-}$ animals were purified by magnetic selection and cultured in complete OPTI-MEM supplemented with stem cell factor and Flt3 at 50 ng/ml and interleukin (IL)-6 and IL-7 at 10 ng/ml. Cells were incubated with retroviral supernatant plus 8 μg/ml polybrene and subjected to three rounds of spinoculation prior to transplant. Prior to transplantation, green fluorescent protein (GFP) expression was analyzed by flow cytometry, and 3 × 10^{5} ICN1-EGFP^{+} cells were injected retro-orbitally into lethally irradiated C57B6.SJL (CD45.1^{+}) hosts. Mice were monitored for symptoms of disease by peripheral blood analysis for the presence of CD4^{+}CD8^{+} DP cells.

RNA and Protein Analyses

RNA and protein expression analyses were performed using standard molecular biology procedures. Please refer to the Supplemental Experimental Procedures for further details.

Flow Cytometry

Flow cytometry was performed essentially, as previously described (Cooper et al., 2006). Additional details are found in Supplemental Experimental Procedures.

PD-0332991 Treatment and Microarray Analysis

The CDK4/6-specific inhibitor PD-0332991 was generously provided by Pfizer Global Research and Development. Human T-ALL lines (CEM, DND41, HPB-ALL, Jurkat, and TAL1) and mouse (#130, #720, and #5146) were treated with 0.5, 1, or 5 μM of PD-0332991, with concentrations of 0.5 and 1 μM yielding similar results in vitro. Total RNA was extracted from PD-0332991-treated cells using the RNEasy plus mini kit (QIAGEN). RNA was amplified with the Ovation RNA Amplification System V2 (Nugen) for

complementary RNA (cRNA) amplification and labeling. Labeled cRNA was then hybridized to Human Genome U133 Plus 2.0 GeneChips (Affymetrix) for microarray analysis. Affymetrix gene expression profiling data were normalized with the robust multiarray average algorithm using GeneSpring GX software (Agilent). The gene expression intensity presentation was generated with Multi Experiment Viewer software (http://www.tm4/org/mev/). Gene-set enrichment analysis was performed using GSEA software (http://www.broadinstitute.org/gsea/) using phenotype as permutation type, 1,000 permutations, and signal-to-noise ratio as metric for ranking genes (Mootha et al., 2003; Subramanian et al., 2005). Gene sets used in the analysis were taken from the MSig database of the Broad Institute (http://www.broadinstitute.org/gsea/msigdb/cards/).

For in vivo studies of PD-0332991 treatment, 3 weeks after transplant of Notch1-expressing cells, transplanted mice received either 150 mg/kg PD-0332991 in 50 mM sodium lactate or vehicle control by gavage daily for ten consecutive days. Four weeks after transplant, peripheral blood was analyzed for GFP, CD4, and CD8 expression. Animals were sacrificed 7 to 8 weeks posttransplant as control mice became moribund. Upon autopsy, tissues were harvested and prepared for fluorescence-activated cell sorting (FACS) and histological analysis. Tissues were fixed for 24 hr in 10% buffered formalin, dehydrated, and then embedded in paraffin. Paraffin blocks were sectioned at 5 μm and stained with hematoxylin and eosin. For xenograft studies, sublethally irradiated *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice were transplanted with 5×10^5 human T-ALL CEM cells. Three weeks posttransplant, mice were given either vehicle control or 150 mg/kg PD-0332991 by gavage for 14 days. Peripheral blood was analyzed for huCD45$^+$ cells 4 weeks posttransplant, and survival of mice was monitored.

Primary Human T-ALL Proliferation Assays

The study was approved by the Institut Universitaire d'Hematologie Institutional Review Board (Saint-Louis Hospital, Paris, France), and informed consent was obtained from the patients. Human and xenografted T-ALL cell samples were thawed in T-ALL medium and seeded onto MS5-DLH1 stromal cells, as previously described (Clappier et al., 2011). After 48 hr, blasts were reseeded onto new MS5-DLH1 (6 mm multiwell at 10^6 cells/ml) and treated overnight with 0.5 μM of PD-0332991 inhibitor or solvent (DMSO), followed by 10 μM bromodeoxyuridine (BrdU) pulse for 30 min. Bulk cultures were harvested, and stromal cells were removed from the suspension by two rounds of filtration through a 70 μm strainer and panning for 30 min. The cell suspension was then incubated with an anti-CD45 antibody, fixed and labeled with BrdU antibody.

Statistical Analysis

All data for cell numbers, expression analysis by qPCR, and FACS analysis of cell cycle and apoptosis represent mean ± SD. Statistical significance was calculated using Student's t test, with $p < 0.05$ considered significant. Significance of survival differences was determined by log rank test.

ACCESSION NUMBERS

The gene expression data from treated T-ALL cell lines can be found at the GEO database (http://www.ncbi.nlm.nih.gov/geo/) using the accession number GSE40635.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2012.09.016.

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