

A Requirement for Cyclin-Dependent Kinase 6 in Thymocyte Development and Tumorigenesis

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Abstract

Cyclin-dependent kinase 6 (CDK6) promotes cell cycle progression and is overexpressed in human lymphoid malignancies. To determine the role of CDK6 in development and tumorigenesis, we generated and analyzed knockout mice. *Cdk6*-deficient mice show pronounced thymic atrophy due to reduced proliferative fractions and concomitant transitional blocks in the double-negative stages. Using the OP9-DL1 system to deliver temporally controlled Notch receptor-dependent signaling, we show that CDK6 is required for Notch-dependent survival, proliferation, and differentiation. Furthermore, CDK6-deficient mice were resistant to lymphomagenesis induced by active Akt, a downstream target of Notch signaling. These results show a critical requirement for CDK6 in Notch/Akt-dependent T-cell development and tumorigenesis and strongly support CDK6 as a specific therapeutic target in human lymphoid malignancies. [Cancer Res 2009; 69(3):810–8]

Introduction

Notch signaling is essential in early thymic T-cell development (1). Multipotent T-cell precursors from the bone marrow (BM) commit to the T-cell lineage following stimulation of Notch on entry to the thymus (2, 3). In vertebrates, Notch ligands consist of Jagged and Delta-like family members. Only Delta class ligands can efficiently support T-cell development (4). Interaction of Notch receptor with ligands results in proteolytic cleavage of Notch, releasing the intracellular domain, which then translocates to the nucleus and activates transcription of target genes for T-cell development (2).

Notch signaling is also continuously required for specification, proliferation, differentiation, and survival during the double-negative (DN) stages but must be attenuated in DP thymocytes to allow their further maturation and to prevent their oncogenic transformation (5). However, the mechanism underlying this requirement for T-cell development is unknown. It has been shown that Notch-promoted survival and trophic effects in pre-T cells are mediated by the phosphatidylinositol 3-kinase (PI3K)-Akt

pathway (6). However, very little information exists connecting Notch function to cell cycle control. Therefore, the identification of the cell cycle proteins involved in these responses is likely to provide important insights into development and tumorigenesis.

Cyclin-dependent kinase 6 (CDK6), a cyclin D-responsive regulator of the retinoblastoma protein (pRB) pathway, seems to be of central importance in T-cell development. CDK6 is a multifunctional protein that negatively regulates both pRB and p27^{KIP1} (p27) activity (7) and modulates differentiation of certain cells (8). The activity of CDK6 is very tightly controlled by association with D-cyclins (7) and two families of CDK inhibitors, including the CIP/KIP family and the inhibitors of CDK4 (INK4) family proteins (9).

Of the three D-type cyclins, cyclins D2 and D3 are predominant in thymocytes (10). Cyclin D3 is strongly induced in the DN4 and immature single-positive (ISP) compartments following pre-T-cell receptor (TCR) assembly. In contrast, cyclin D2 expression is high in the DN1-DN3 stages before pre-TCR assembly and barely detectable after pre-TCR assembly (10), suggesting that CDK6 may use different cyclin partners in the thymocyte developmental stages. Consistent with a key role for CDK6 as a D-cyclin partner in thymocytes, knockout (KO) of CDK4 or CDK2 was shown to have no significant effect on T-cell development (11, 12), but significantly decreased thymic cellularity was observed in a CDK6 KO animal (13). Moreover, its predominant expression in hematopoietic cell types (14, 15) and overexpression in human T-cell lymphoblastic lymphomaleukemia (T-LBL/ALL; refs. 15–18) suggest a role for CDK6 in T-cell malignancies. However, the specific role of CDK6 in thymocyte development is unclear and the effect of CDK6 loss on T-cell tumorigenesis has not been ascertained.

In the present study, we tested the role of CDK6 in murine thymocyte development and tumorigenesis by generating *Cdk6*-deficient thymocytes using a knock-in approach. We report that CDK6 acts as a downstream target of Notch and Akt and is essential for T-cell development and tumorigenesis.

Materials and Methods

Construction of *Cdk6* KO and knock-in mice. To disrupt the mouse *Cdk6* locus, we used a Lox-STOP-Lox (LSL) cassette as described in detail in Supplementary Fig. S1 and Supplementary Materials and Methods. Chimeric mice were generated by microinjecting two independent, karyotypically normal ES cell clones. Mating to nestin-cre mice allowed excision of the LSL cassette and reexpression of CDK6 (WT-Δ strains). Subsequent crosses to C57BL/6 wild-type (WT) mice ensured the removal of the cre transgene. Resulting heterozygotes were mated to homozygosity. *Cdk6*-LSL animals that were not mated to nestin-cre were defined as KO animals.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Western blotting, immunoprecipitation, and *in vitro* kinase assays.

Thymi were dissected and lysed. Western blotting, immunoprecipitation-Westerns, and *in vitro* kinase assays were performed as described (19). Antibodies used in this study included cyclin D1 (72-13G, Santa Cruz Biotechnology), cyclin D2 (M-20, Santa Cruz Biotechnology), cyclin D3 (C-16, Santa Cruz Biotechnology), p18 (M-20, Santa Cruz Biotechnology), p27 (BD Transduction Laboratories), CDK2 (BD Transduction Laboratories), CDK6 (C-21, Santa Cruz Biotechnology), CDK4 (C-22, Santa Cruz Biotechnology), actin (Sigma), HA (mono HA.11; Covance), and Akt (phospho-Akt pathway sample kit; Cell Signaling), respectively.

Flow cytometry and fluorescence-activated cell sorting. For cell surface staining and 7-amino-actinomycin D (7AAD) and Annexin V staining, four-color flow cytometry was done as described (20, 21). Thymocytes were sorted by a MoFlo Cell Sorter. Mature cells were stained with antibodies against cell surface markers CD4, CD8, and TCR- β . For DN1-DN4 populations, cells were stained with the biotinylated "cocktail" of lineage-specific antibodies described (20, 21) followed by depletion with streptavidin-conjugated magnetic beads (Dyna). Lineage-depleted cell suspensions were then surface stained with anti-CD44, anti-CD25, and streptavidin. For ISP-CD8⁺ population, cells were stained with the biotinylated cocktail of lineage-specific antibodies. Enriched cell suspensions were then surface stained with anti-CD4-FITC, anti-CD8-APC, and anti-TCR- β -PE. The ISP-CD8⁺ population was defined as with CD8⁺/CD4⁻/TCR- β ⁻. Sorted cells were processed immediately for Western blot analysis.

Purification of BM cells and OP9/OP9-DL1 cocultures. BM cells obtained from WT and KO mice were depleted of CD3⁺, Gr-1⁺, Mac-1⁺, Ter119⁺, and B220⁺ cells through labeling with the corresponding biotinylated antibodies followed by incubation with streptavidin-coated magnetic beads. Lineage-depleted BM cells were stained with c-Kit-APC and streptavidin-FITC, and c-Kit⁺Lin⁻ cells were sorted.

OP9 and OP9-DL1 stromal cells were cultured in MEM- α (Invitrogen), supplemented with 20% fetal bovine serum (Invitrogen), 100 units/mL penicillin, and 100 μ g/mL streptomycin, and plated 1 or 2 d before use. Cocultures were initiated with $\sim 2 \times 10^5$ c-Kit⁺Lin⁻ BM cells in the presence of 5 ng/mL Flt3-L and 5 ng/mL interleukin-7 (IL-7; PeproTech). Cocultures were harvested by forceful pipetting at the indicated time points. Cells were counted and fluorescence-activated cell sorting (FACS) analysis was performed as described above.

Statistics. Data from different pairs of mice are compared by a two-tailed Student's *t* test.

Results

Generation of *Cdk6* KO mice. Recent analysis of mice lacking CDK6 suggested a role for this enzyme in thymocyte proliferation but did not address the specific roles of CDK6 in thymocyte developmental stages. Further, these studies did not address the role of the kinase in leukemia/lymphoma (13). To study the role of CDK6 in T-cell development and tumorigenesis, we generated KO mice using a LSL cassette (Supplementary Fig. S1A). Animals with an intact LSL cassette in the *Cdk6* locus are hereafter referred to as KO mice. To exclude the possibility that any defects observed in the KO strains resulted from nonspecific effects, we also removed the LSL cassette through germ-line expression of cre. The cre-reactivated WT allele (referred to as WT- Δ hereafter) expresses the WT CDK6 from the endogenous locus with regulatory controls intact. Southern blot and PCR analysis confirmed the expected allele structure (Supplementary Fig. S1D and E), and sequence analysis of the entire coding region of exon 1 confirmed the presence of the expected LSL cassette and no other changes (data not shown).

Altered thymocyte numbers in *Cdk6* KO mice. Consistent with a previous report (13), our *CDK6* KO mice were found on dissection to have defects in thymus development. KO mice display significant thymic hypoplasia (Fig. 1A, left) at birth and this

deficiency continues with increasing age. These thymuses contained ~ 4 -fold fewer thymocytes than those from WT littermates (Fig. 1A, middle), although body weights were comparable (Fig. 1A, right). Therefore, our initial analysis of KO mice recapitulates a reported defect in thymocyte formation in a different strain, underscoring our success at achieving KO of CDK6 with the LSL strategy.

Biochemical properties of cell cycle regulators in KO and WT- Δ thymocytes. Extracts of thymocytes derived from KO mice showed a complete loss of detectable CDK6 protein (Fig. 1B, left). Further, excision of the LSL cassette in WT- Δ animals restored expression of CDK6 to normal levels. Consistent with this, WT- Δ animals have normal-appearing thymuses and thymocyte numbers. Next, we assessed the expression level of other cell cycle regulators in thymus extracts. Levels of CDK2 and CDK4 were comparable in all strains of mice. The expression levels of cyclins D3, D2, A, and E, p18, and p27 were very similar in WT, KO, and WT- Δ animals. No expression of cyclin D1, p15, p16, p21, or p57 was observed in the unstressed thymus (data not shown), consistent with other groups' observations (14, 22).

In the absence of CDK6, profound differences in other CDK activities may be observed because D-type cyclins, p27, and INK4 family proteins no longer bind to CDK6 in KO mice and may bind in excess to CDK4 or CDK2 (13) instead. To examine potential cyclin/CDK/INK reassembly, we analyzed the composition of CDK6/D3-containing complexes in thymus lysates (Fig. 1B, middle and right). We found that the ability of CDK6 expressed in WT- Δ to bind cyclins D3, p18, and p27 is similar to that of WT. As expected, no cyclin D3, p18, or p27 coimmunoprecipitated with anti-CDK6 antibodies using extracts from KO thymuses. In the absence of CDK6 (KO lane), anti-cyclin D3 immunoprecipitation revealed that the level of cyclin D3-bound CDK4 increased, whereas the level of cyclin D3-bound CDK2 and p27 closely resembled that in extracts derived from WT littermate thymus (Fig. 1B, right). Further, the level of p27-bound CDK4 or CDK2 (Supplementary Fig. S2A) and phosphorylation of pRB was very similar in WT, WT- Δ , and KO thymocytes (Supplementary Fig. S2B), whereas the binding of p27 to cyclin E/CDK2 was reported to be increased and accompanied by impaired RB phosphorylation in *CDK4*^{-/-} fibroblasts (23). This difference suggests that p27 may coordinate CDK4 and CDK2 activation, but not CDK6 and CDK2 activation. Thus, in the absence of CDK6, changes in p27 association with different CDK complexes are not a major determinant of the proliferative or differentiative effects observed.

To assess CDK activity in thymocyte lysates, we performed *in vitro* kinase assays (Fig. 1C). CDK6 activity from WT- Δ mice was similar to that in WT animals. In contrast, KO extracts contain very little CDK6 kinase activity (Fig. 1C, left). To determine whether CDK4 or CDK2 might compensate for the absence of CDK6 in thymocyte development, we performed *in vitro* kinase assays for these enzymes. Whole thymus lysates from KO mice have CDK4 and CDK2 kinase activity similar to that from WT or WT- Δ (Fig. 1C, middle and right). Therefore, there is no obvious compensatory change in CDK4 or CDK2 kinase activity in KO mice in thymocyte populations analyzed together, nor did loss of CDK6 lead to inhibition of CDK2 activity.

In summary, in unstressed thymocytes, CDK6 kinase activity is lost in *CDK6* KO animals. Further, there is no apparent alteration in CDK4 or CDK2 kinase activity in the absence of CDK6. The data shown above clearly show that our KO and WT- Δ animals behave as expected at the biochemical level.

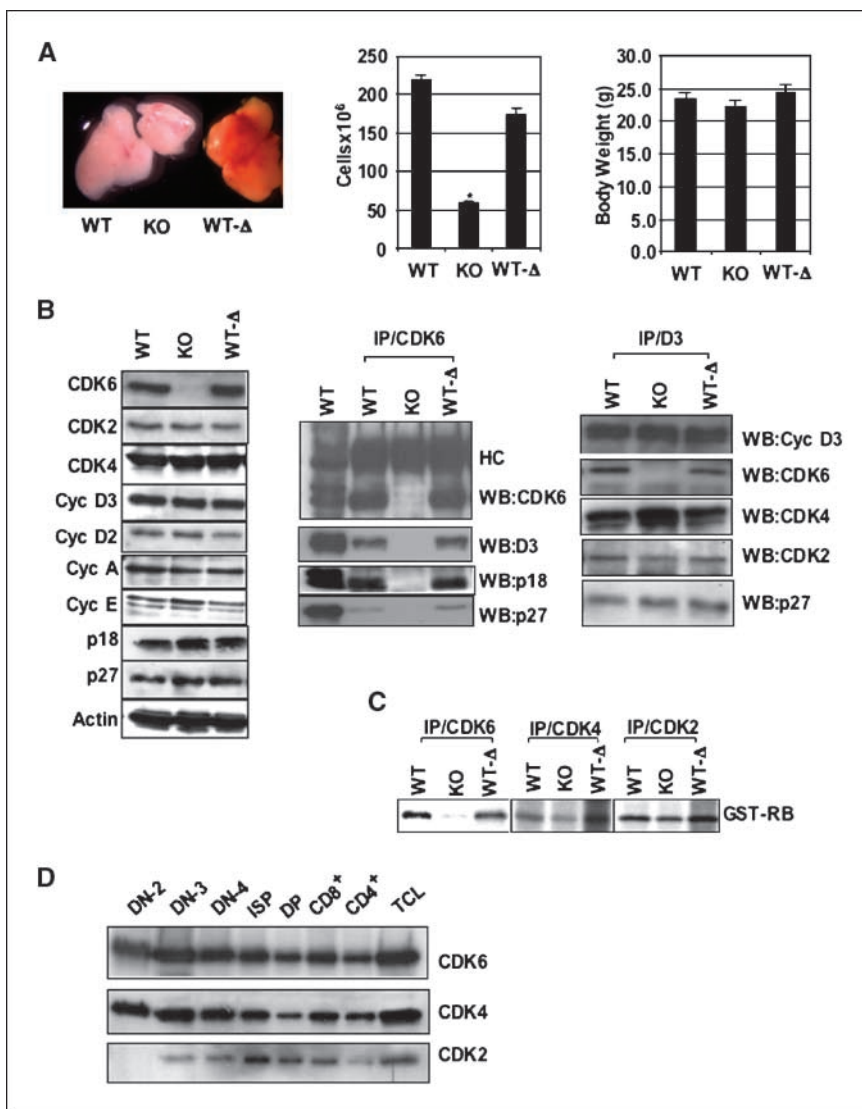


Figure 1. Defective thymocyte development in KO mice and analyses of cell cycle regulatory protein expression and function of kinases. *A, left*, appearance of thymuses dissected from 2-mo-old mice; *middle*, total number of thymocytes from 1- to 3-mo-old mice. *Columns*, mean cell numbers ($n = 15$ for WT and KO, $n = 17$ for WT-Δ); *bars*, SE. *, $P = 1.12508E-09$ for WT and KO. Student's *t* test was used to confirm significant differences between pairs of animals. *Right*, body weights of 1- to 3-mo-old mice. *Columns*, mean (g) body weight; *bars*, SE. *B, left*, immunoblots were probed with different antibodies as indicated. Actin was used as internal control to ensure equal loading; *middle and right*, immunoprecipitation-Westerns. CDK6 or cyclin D3 was immunoprecipitated from thymocyte extracts and the immunoblots were probed with the indicated antibodies. *HC*, IgG heavy chain. *C*, *in vitro* kinase assay. CDK6, CDK4, or CDK2 was immunoprecipitated from thymocyte extracts and an *in vitro* kinase assay was performed using the recombinant pRB (*GST-RB*) as a substrate. *D*, immunoblot analysis of sorted thymocyte subsets (3 million cells of each subset) from WT mice. *Top, middle, and bottom*, the same blot sequentially probed with antibodies against CDK6, CDK4, and CDK2.

Altered thymocyte development in *Cdk6* mutant mice. In an effort to understand the observed deficiency in thymic development caused by CDK6 loss, we first analyzed expression of CDK6 during thymocyte development (Fig. 1D). We found that expression of CDK6 is relatively constant in sorted subpopulations compared with that reported for cyclin D3, which is very dynamic (10). The level of CDK6 was high within DN2, DN3, DN4, ISP, and CD8⁺ subsets. It remained relatively moderate on transition to the DP and CD4⁺ single-positive stage. The expression profile of CDK4 is very similar to that of CDK6 during thymocyte development. In contrast, the expression profile of CDK2 differed from those of CDK4 and CDK6. There was no detectable CDK2 within the DN2 stage. CDK2 was detectable in other subsets and peaked at the ISP stage. Thus, CDK4 and CDK6 protein levels in thymocytes are highest in pro-T cells, are lower after the DN to DP transition, and remain low in CD4⁺ SP and CD8⁺ SP cells.

To determine if *Cdk6* loss affected thymocyte development, we established the precise distribution of thymocytes in KO mice using flow cytometry (Fig. 2A). The fraction of DP cells was decreased in KO mice, and SP populations increased. The DP compartment was

reduced to $\sim 86 \pm 1.5\%$ of that of WT (Fig. 2A), whereas the CD4⁺ SP population increased by $210 \pm 16\%$ and CD8⁺ SP population increased by $184 \pm 13\%$. In contrast, changes in the DN population did not reach statistical significance. The absolute number of thymocytes from each compartment was reduced to different degrees. DP thymocytes were reduced ~ 4 -fold in KO mice, CD4⁺ SP 1.5-fold, CD8⁺ SP 2-fold, and DN 3-fold (Fig. 2B), compared with WT littermates. Hence, CDK6 loss causes reduction in thymocyte numbers in all the compartments, and immature compartments are more profoundly altered than the mature SP compartments. Importantly, CDK6 expression in WT-Δ restored the percentage of thymocyte subsets to a normal level (Fig. 2A) and restored the normal fractions of DN subsets (Fig. 2C), as discussed below. Coupled with the normal size and cellularity of the thymus in WT-Δ animals (Fig. 1A), these data show that the thymic defects observed in our KO animals are the result of changes in CDK6 protein level resulting from the presence of the LSL cassette.

To determine the effect of CDK6 loss on early thymocyte development more precisely, Lin⁻ thymocytes were analyzed for the expression of CD44 and CD25, which define distinct immature

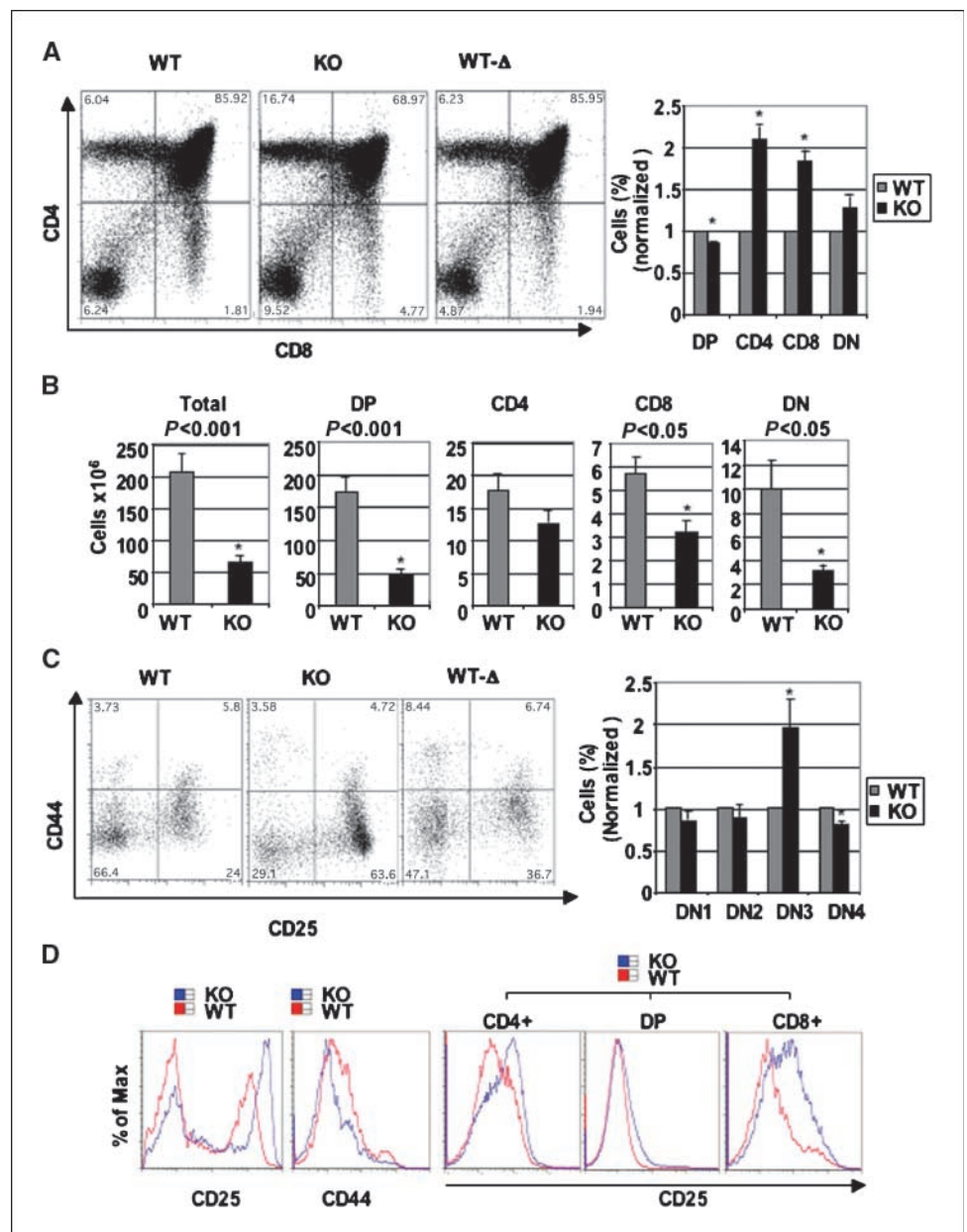
T-cell populations (24). Although the fraction of thymocytes was very similar in DN1 and DN2 compartments of WT and KO mice (Fig. 2C), KO mice showed a $\sim 2 \pm 0.33$ -fold increase in DN3 cells, indicating a potential developmental block at this stage. In contrast, the fraction of DN4 cells was reduced 1.25 ± 0.04 -fold. The expression level of CD25 was elevated and that of CD44 reduced in DN cells from KO thymocytes (Fig. 2D). CD25 was also elevated in CD8 SP cells. There was no difference in the expression level of CD25 and CD44 between WT and WT- Δ (data not shown). KO thymocytes expressed normal levels of TCR- β and of other pre-TCR components (data not shown). We found that DN3 stage cells from WT and KO animals were of similar cell size (data not shown).

Taken together, these data suggest that loss of CDK6 causes reduced thymocyte cell numbers in most developmental subsets. Further, loss of CDK6 resulted in increased CD25 expression and reduced CD44 expression. However, this alteration of CD25 and

CD44 expression and partial block in DN3 is not sufficient to explain the profound reduction in thymic cellularity. Thus, additional effects of CDK6 loss on cellular proliferation or survival may also influence T-cell development, particularly in the DN3 compartment.

Proliferation and apoptosis in thymocyte subsets. Reduced thymic cellularity in KO may reflect reduced proliferation and/or increased apoptosis. We assessed the proliferative and apoptotic fractions in thymocytes at different developmental stages using the radiometric DNA dye 7AAD or Annexin V, respectively, followed by flow cytometry. We found a significantly decreased fraction of cycling cells in KO thymocytes at the DN2, DN3, ISP-CD8⁺, and CD8⁺ stages that averaged $29 \pm 2.9\%$, $43 \pm 7.1\%$, $59 \pm 5.6\%$, and $64 \pm 6.5\%$ of WT, respectively (Supplementary Fig. S3A). Thus, the reduced proliferative fraction in KO mice may contribute to reduced thymic cellularity. However, Annexin V staining revealed a

Figure 2. Alteration of thymocyte subset fractions in thymuses of KO and WT- Δ mice. *A, left*, examples of flow cytometric profiles of thymocytes from 2-mo-old mice stained with anti-CD4, anti-CD8, and TCR- β antibodies. The percentage of cells in each quadrant is shown. *Right*, the bar graph summarizes percentage of each subset from separate experiments ($n = 8$). *Columns*, mean; *bars*, SE. *, $P < 0.05$, significantly different from the control levels, which were arbitrarily defined as 1 unit (100%). *B*, absolute cell numbers ($\times 10^6$) for the subsets were calculated and are shown as bar diagrams. *Columns*, mean cell numbers; *bars*, SE. The statistical significance was calculated using the Student's *t* test. *P* values are indicated above the bars. $n = 8$ for WT and KO. *, $P < 0.001$ or $P < 0.05$ as indicated, significantly different from the control levels, which were arbitrarily defined as 1 unit (100%). *C*, alteration in DN subsets. *Left*, thymocytes from 2-mo-old CDK6 KO or controls were stained with the cocktail of lineage-specific antibodies and anti-CD44 and anti-CD25. Lineage-positive cells were electronically "gated out" and CD44-versus-CD25 profiles of the lineage-negative compartments are presented. Numbers in quadrants indicate the percentage of cells in each subset. *Right*, the bar graph summarizes percentage of each subset from separate ($n = 8$) experiments. *Columns*, mean; *bars*, SE. *, $P < 0.05$, significantly different from the control levels, which were arbitrarily defined as 1 unit. *D*, examples of flow cytometric analysis of CD25 (left) or CD44 (middle) expression in DN thymocytes from WT and KO. *Right*, examples of flow cytometric analysis of CD25 expression in CD4⁺, DP, and CD8⁺ subsets from WT and KO thymocytes.



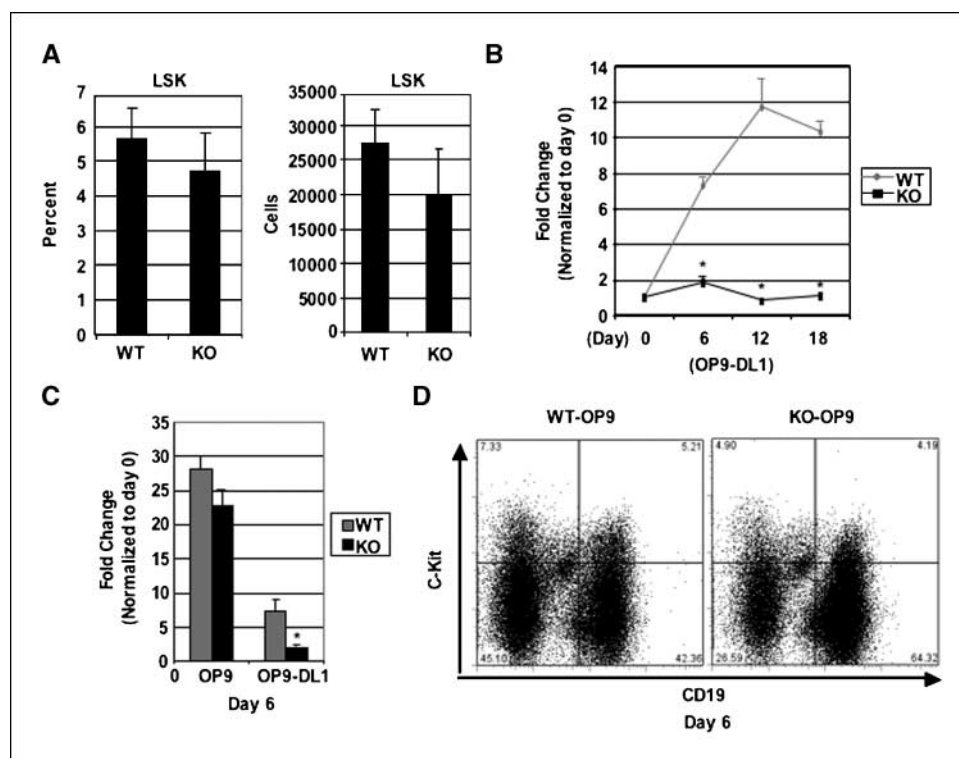


Figure 3. CDK6 is required for Notch-dependent proliferation. **A**, BM cells obtained from WT and KO mice (from humerus and femur) were counted and stained with PE-conjugated antibodies specific for lineage markers (CD3, CD4, CD8, B220, Mac-1, Gr-1, and Ter119) and Sca-1-FITC and c-Kit-APC. Labeled cells were subsequently analyzed by FACS. *Left, columns*, mean percentage of LSK population ($n = 4$ for WT and KO); *bars*, SE. The absolute cell numbers ($\times 10^6$) for the LSK (*right*) were calculated based on the total number of cells and the percentage of LSK in the total cell population and are shown as bar diagrams. *Columns*, mean cell numbers ($n = 4$ for WT and KO); *bars*, SE. **B**, c-Kit⁺Lin⁻ BM cells were cocultured on OP9-DL1 cells and counted at the times indicated. The expansion fold change was calculated based on the ratio between the total number counted at the time indicated versus the total c-Kit⁺Lin⁻ cell number plated at day 0, which was arbitrarily defined as 1 unit. *Points*, mean ($n = 5$); *bars*, SE. *, $P < 0.05$, significantly different from the control levels. **C**, c-Kit⁺Lin⁻ BM cells were cocultured on OP9 or OP9-DL1 cells and counted at day 6 as indicated. The bar graph summarizes the fold changes over day 0 (which were arbitrarily defined as 1 unit) from separate ($n = 6$) experiments. *Columns*, mean; *bars*, SE. *, $P < 0.05$, significantly different from the control levels, which were arbitrarily defined as 1 unit. **D**, representative example of FACS analysis for surface expression of CD19 and c-Kit of pre-B cells sorted from BM and cultured for 6 d on OP9 cells.

substantial reduction of apoptosis in DN3, DN4, CD4, and CD8 compartments in KO mice, averaging $55 \pm 6.7\%$, $76 \pm 4.5\%$, $59 \pm 5.6\%$, and $56 \pm 6.0\%$ of WT, respectively (Supplementary Fig. S3B). Therefore, in most compartments, the reduced proliferation rate is accompanied by a reduced apoptosis rate (Supplementary Fig. S3C), apparently limiting the effect of proliferative changes on thymocyte cell numbers. The exception to this is observed in the DN2 compartment in KO, where the severely reduced proliferative fraction occurs in the absence of changes in apoptosis (Supplementary Fig. S3C). A key implication of these data is that thymocyte cell number changes may not be fully explained by the changes observed in thymocyte development. Thus, alteration of thymic progenitors in the BM, or their ability to respond properly to Notch or other signaling pathways on populating the thymus, may be key to alterations in thymocyte cell numbers.

CDK6 is required for Notch-dependent proliferation, survival, and differentiation. In the BM, progenitors with efficient T lineage potential are found within the Lin⁻Sca-1⁺c-Kit⁺ (LSK) fraction (25). We sought to investigate if CDK6 loss disrupts the proliferative capacity of these BM T-cell precursors. The LSK populations from BM were similar between WT and KO mice (Fig. 3A, left). When the absolute numbers of LSK in each strain were calculated, we found that the absolute number was also similar between WT and KO mice (Fig. 3A, right), consistent with other groups' observations (13). These data indicate that CDK6 loss

does not have a profound effect before seeding of the thymus by progenitor cells, leaving open the possibility that CDK6 might affect the ability of the progenitor cells to respond properly to Notch or other signaling pathways on entering the thymus.

To investigate if CDK6 affects the ability of progenitor cells to respond properly to Notch signaling, we took advantage of a cell culture system (OP9-DL1) that has the capacity to induce the differentiation of hematopoietic progenitors into T cells through expression of Delta-like 1 on BM stromal cells, thus mimicking the thymic stroma (4). c-Kit⁺Lin⁻ BM cells were cocultured on OP9-DL1 cells in the presence of the cytokines Flt3-L and IL-7 and analyzed by cell counting. There was ~ 8 -fold increase in cellularity by day 6 in WT cells, with the cell yields continuing to increase by ~ 12 -fold by day 12. By day 18, the cell number reached a plateau (Fig. 3B). In contrast, c-Kit⁺Lin⁻ progenitors from KO BM failed to expand throughout the coculture period. These findings suggested that CDK6 is required for either cytokine or Notch signaling in cell proliferation.

To further understand the origin of these defects, c-Kit⁺Lin⁻ progenitors from WT and KO BM were cocultured on OP9 or OP9-DL1 stromal cells in parallel and analyzed by cell counting at day 6. Notably, both systems use the cytokines Flt3-L and IL-7 in the growth medium; the difference between these two culture systems is the presence of Delta-like 1 on OP9-DL1 (4). In the absence of this Notch ligand, OP9 cells support proliferation and

differentiation of B-cell precursors. Consistent with published results, WT progenitors showed the expected expansion on OP9-control cells, resulting in a 28-fold population expansion by day 6 of culture (Fig. 3C) and differentiation of ~40% of the cells to mature B cells as evidenced by surface expression of CD19⁺c-Kit⁻ (Fig. 3D). We observed a similar increase in the number of KO cells during the first 6 days of culture on OP9 cells, with the development of over 60% mature B cells (Fig. 3D). Therefore, there was no significant difference in cell expansion between WT and KO progenitor cells when plated on OP9 cells, suggesting that the profound reduction in response to plating on OP9-DL1 cells reflected a lack of response to Delta-like 1-mediated Notch signaling.

We next examined the levels of proliferation and apoptosis of cells cultured on OP9-DL1 cells. As shown in Supplementary Fig. S4A, we observed an ~3-fold reduction in the proliferative fractions in KO cells compared with WT cells at 12 days after plating. Moreover, the apoptotic fraction of thymocyte subsets at day 12 was increased by 1.4- to 6.5-fold in each developmental stage of KO thymocyte cultures (Supplementary Fig. S4B and C). Thus, the reduced proliferation and increased apoptosis of KO progenitors cultured on OP9-DL1 cells supports a role for CDK6 in mediating proliferation and survival in response to Notch signals and may explain the significant reduction in thymic cellularity *in vivo*.

Because OP9-DL1 cocultures can recapitulate most stages of thymocyte development, we next compared differentiation of KO and WT progenitors plated on OP9-DL1. Notably, the cells from KO mice showed a moderate decrease in the percentage of mature single-positive CD8⁺ cells and a dramatic decrease in DP cells (24-, 5-, and 7-fold reduction at days 6, 12, and 18, respectively; Fig. 4A). Reexpression of CDK6 in WT-Δ mice restored the differentiation into mature thymocytes to a normal level comparable with WT (Supplementary Fig. S5).

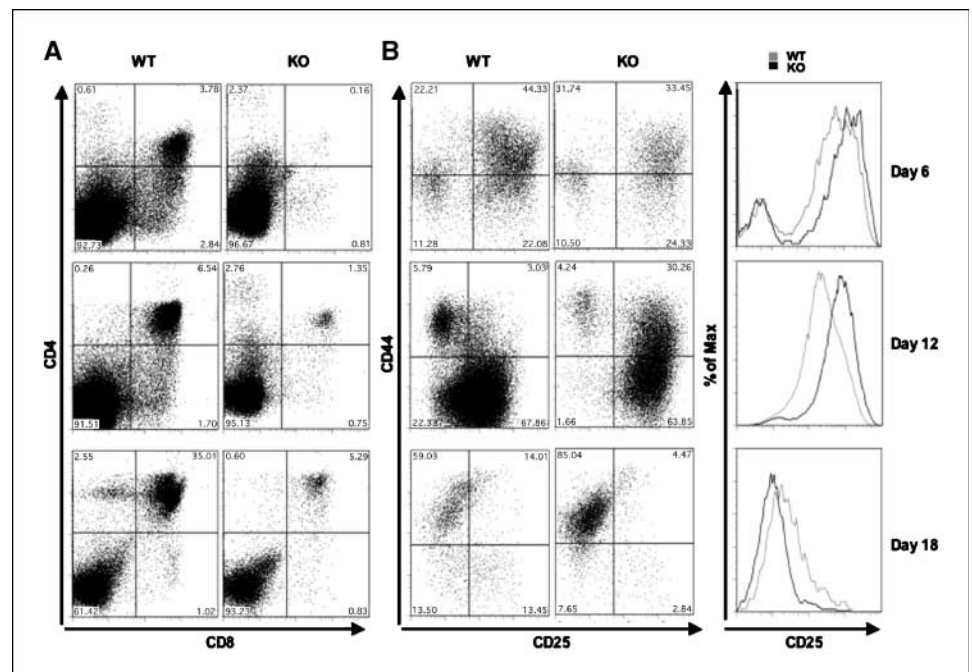
Upon analysis of CD25 and CD44 expression, we found no apparent difference in DN subsets between WT and KO (Fig. 4B, top) littermates at day 6, except CD25 was up-regulated in KO cells

(Fig. 4C, top). At day 12, ~94% of KO cells had developed to the DN2 and DN3 stages and CD44⁻CD25⁻ (DN4) cells were virtually undetectable (Fig. 4B, middle). At day 18, surprisingly, the majority of KO cells in the DN compartment were found in the DN1 stage, suggesting that a fraction of the BM cells may respond poorly to OP9-DL1 culture. Thus, these data largely reproduce the defect seen in KO thymocytes *in vivo*. Surviving cells are delayed or blocked at the DN3 stage, whereas a fraction of thymocytes that progressed to the DP stage efficiently developed into CD4⁺ or CD8⁺ single positives.

In summary, both *in vivo* and cell culture results strongly suggest that CDK6 loss interferes with the proliferation and survival signals instigated by Notch signaling. Further, those thymocytes that survive CDK6 loss display defects in maturation consistent with a role for CDK6 in potentiating early stages and inhibiting late stages of Notch-regulated differentiation.

CDK6-deficient mice are resistant to thymic lymphoma formation induced by active Akt. Notch-promoted survival and trophic effects in pre-T cells are mediated by the PI3K-Akt pathway (6), and activation of Notch is critical to proliferation in both normal and tumor cells. We thus sought to examine if CDK6 loss impairs Akt1 activation in T-cell progenitors or if Akt1 might depend on CDK6, for example, as a partner for D-cyclins stabilized by activated Akt (26, 27). To explore this notion, we used a transgenic mouse strain expressing constitutively active, myristylated Akt1 (MyrAkt1) from the proximal *Lck* gene promoter. The transgenic mice develop thymic lymphomas at ~5 months of age (28). Because recent evidence indicates that Notch and protein kinase B/Akt are frequently hyperactive in many types of human and murine T-cell tumors (28–30), this model may well reflect the critical role of Akt1 in T-cell tumors. The *Cdk6*^{-/-} allele was crossed onto the *Lck-MyrAkt1* background and the resulting *Akt1;Cdk6*^{-/-}, *Akt1;Cdk6*^{+/-}, and *Akt1;Cdk6*^{+/+} compound mutant mice were observed for tumor occurrence. As expected, *Akt1;Cdk6*^{+/+} mice began to develop thymic lymphomas and became moribund beginning at ~5 weeks of age (Fig. 5A), and

Figure 4. CDK6 is required for Notch-dependent differentiation. **A**, flow cytometry of WT and KO cells stained with CD4 and CD8. c-Kit⁺Lin⁻ BM cells were cocultured on OP9-DL1 stromal cells and analyzed by FACS at various time points as indicated. **B**, flow cytometry of WT and KO cells stained with the cocktail of lineage-specific antibodies and anti-CD44 and anti-CD25. Lineage-positive cells were electronically gated out and CD44-versus-CD25 profiles of the lineage-negative compartments are presented. Numbers in quadrants indicate the percentage of cells in each subset stained with CD44 and CD25. **C**, comparisons of flow cytometric analysis of CD25 expression in DN thymocytes from WT and KO cells (from **B**) collected from OP9-DL1 cocultures.



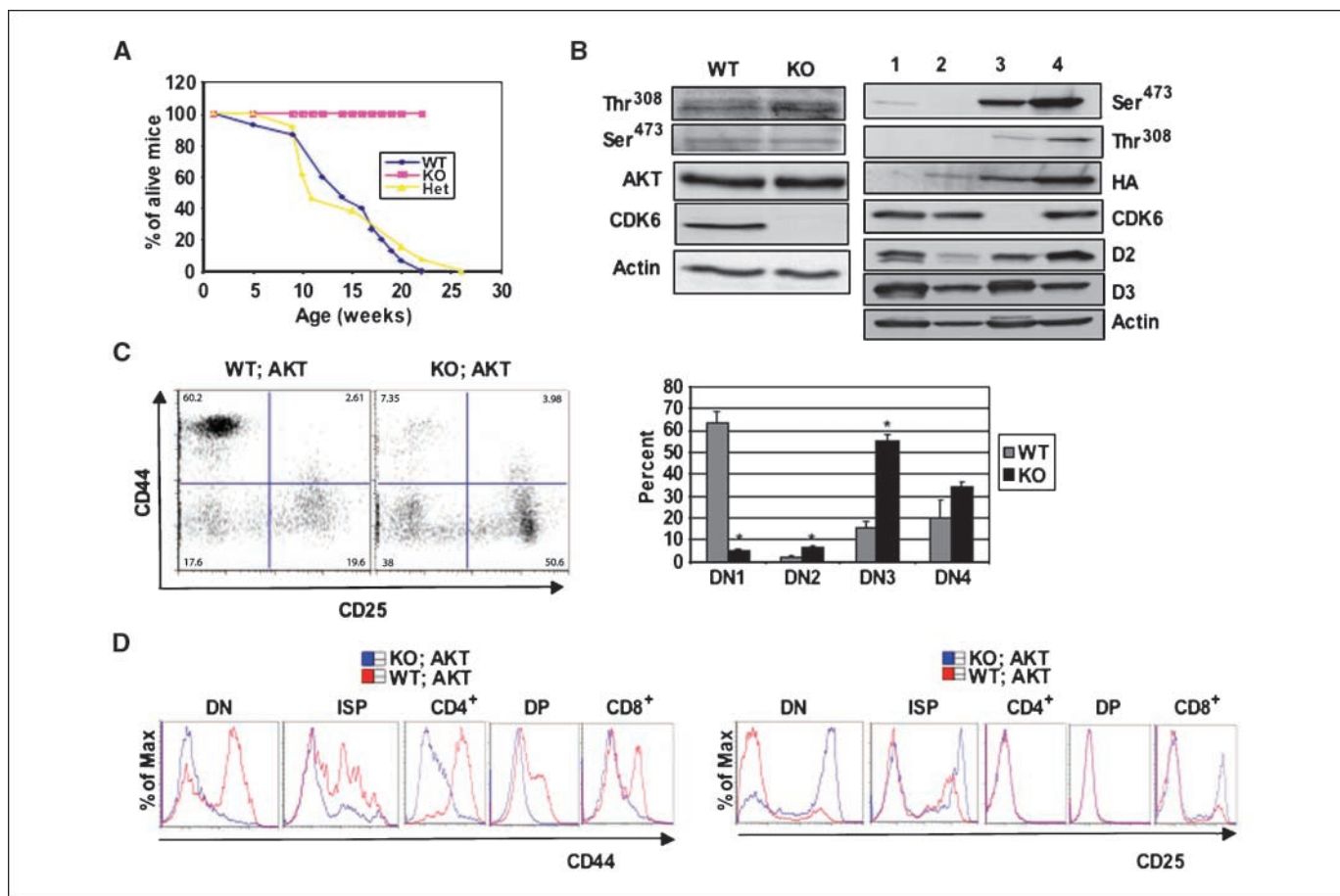


Figure 5. CDK6 operates downstream of Akt kinase. *A*, comparison of tumor susceptibility between WT;Akt ($n = 21$), Het;Akt ($n = 17$), and KO;Akt ($n = 15$) mice. *B*, Western blot analysis of thymocyte lysates derived from 4-wk-old WT or KO mice (left) or WT (lane 1) or WT;Akt (pretumor, lane 2) or 4-mo-old KO;Akt (lane 3) mice or WT;Akt (tumor cells, lane 4, right). Immunoblots were probed with different antibodies as indicated. Actin was used as an internal control to ensure equal loading. Anti-HA was used to detect transgene MyrAkt and distinguish it from endogenous Akt. *C*, left, the MyrAkt transgene failed to promote β -selection in the absence of CDK6. DN thymocytes from 4-wk-old WT;Akt and KO;Akt mice stained with the cocktail of lineage-specific antibodies and anti-CD44 and anti-CD25. Lineage-positive cells were electronically gated out and CD44-versus-CD25 profiles of the lineage-negative compartments are presented. Numbers in quadrants indicate the percentage of cells in each subset stained with CD44 and CD25. The bar graph (right) summarizes percentage of each subset from separate ($n = 3$ for WT;Akt, $n = 4$ for KO;Akt) experiments. Columns, mean; bars, SE. *, $P < 0.05$, significantly different from the control levels. *D*, comparisons of flow cytometric analysis of CD44 (left) or CD25 (right) expression in some compartments from WT;Akt and KO;Akt.

virtually all had developed tumors by 26 weeks of age, consistent with previous reports (28). Similarly, most *Akt1;Cdk6*^{+/-} mice had developed tumors by 22 weeks of age. In contrast, none of the *Akt1;Cdk6*^{-/-} mice developed tumors by the age of 26 weeks (Fig. 5A). Indeed, we continued to follow these mice out to 52 weeks of age and never observed thymic lymphomas. Based on these results, we conclude that mice lacking CDK6 are resistant to T-cell malignancies triggered by constitutively active Akt1.

To determine how CDK6 loss impaired tumor formation, we examined the phosphorylation of Akt on Ser⁴⁷³ and Thr³⁰⁸ because activation of Akt depends on these modifications (31). No difference between WT and KO animals was observed (Fig. 5B, left). This is consistent with a role for CDK6 downstream of Akt1 activation. Next, we analyzed the expression of the *MyrAkt1* transgene and the level of phosphorylation on Ser⁴⁷³ and Thr³⁰⁸ in transgene-positive animals. To achieve this, thymocytes were prepared from young healthy (Fig. 5B, right, lane 2) or tumor-bearing (lane 4) WT;Akt1 mice. Thymocytes were also prepared from young WT (lane 1) and KO;Akt1 (lane 3) thymi. Thymocyte extracts were blotted with anti-HA antibody, which specifically recognizes the HA-tagged, transgene-encoded MyrAkt1. This

revealed that HA-MyrAkt1 was readily detected in WT;Akt1 thymocyte extracts but not in those lacking the transgene (lane 1). Surprisingly, KO;Akt1 (lane 3) thymocyte extracts contained much higher levels of transgene-encoded Akt1 despite the complete lack of tumor formation noted above. This may indicate that CDK6 can negatively affect the expression from the *Lck* promoter, thus increasing transgene expression in KO cells. More likely, however, is the possibility that *CDK6* KO cells accumulate in a stage that allows high levels of *Lck-MyrAkt1* expression, but are blocked from further progression to tumorigenesis.

To examine this possibility, we next analyzed the expression of cyclins D2 and D3. As had been previously observed, cyclin D3 was readily detectable in WT;Akt1 thymocyte extracts but at a slightly lower level than is found in total thymocytes lacking transgene *Akt1* (Fig. 5B, right). Consistent with a defect in progression to tumorigenesis, we observed that the cyclin D2 level was much higher in WT;Akt1 tumors than is found in KO;Akt1 thymocytes at the same age.

We next determined the effect of CDK6 loss on early lymphomagenesis by analyzing Lin⁻ thymocytes because constitutively active *Lck-MyrAkt1* transgenes promote the transition of DN3 cells to

the DN4 stage (32). Consistent with previous observations, staining DN thymocytes from *WT;Akt1* with anti-CD44 and anti-CD25 revealed an unexpected dramatic increase of DN1 cells, with a parallel decrease of both DN3 and DN4 cells (Fig. 5C). The increased DN1 population results from the Akt1-mediated up-regulation of CD44 in DN4 thymocytes, making them appear as DN1 cells (32). In contrast, the majority of DN thymocytes from *KO;Akt* mice were found in the DN3 stage, similar to the observation in KO mice (Fig. 2C). As expected, the expression level of CD44 was increased in all the compartments examined in *WT;Akt1* thymocytes but not in those from *KO;Akt1* mice (Fig. 5D, left), whereas the expression level of CD25 in *KO;Akt1* mice was increased in DN, ISP, and CD8⁺ stages compared with that of *WT;Akt1* (Fig. 5D, right). There was no change in the expression level of intracellular TCR- β in *WT;Akt1* and *KO;Akt1* mice (data not shown). Therefore, we conclude that the *MyrAkt1* transgene cannot promote the transition of DN3 cells to the DN4 stage in the absence of CDK6, nor can the transgene drive high levels of CD44 expression in *KO;Akt1* mice. Thus, constitutive Akt1 signaling requires CDK6 to promote tumorigenesis in the thymus.

Discussion

The G₁ cell cycle control protein CDK6 has been linked to thymocyte proliferation (13), and CDK6 gene amplification or overexpression is commonly observed in T-LBL/ALL (15, 17). Using CDK6 KO and reconstituted control mice, we have found that immature thymocytes from CDK6 KO mice fail to undergo normal expansion and show complete resistance to lymphomagenesis triggered by constitutively active MyrAkt1. Using the OP9-DL1 thymic stroma mimetic system (4) to deliver temporally controlled, Notch receptor-dependent signaling, we show that CDK6 is

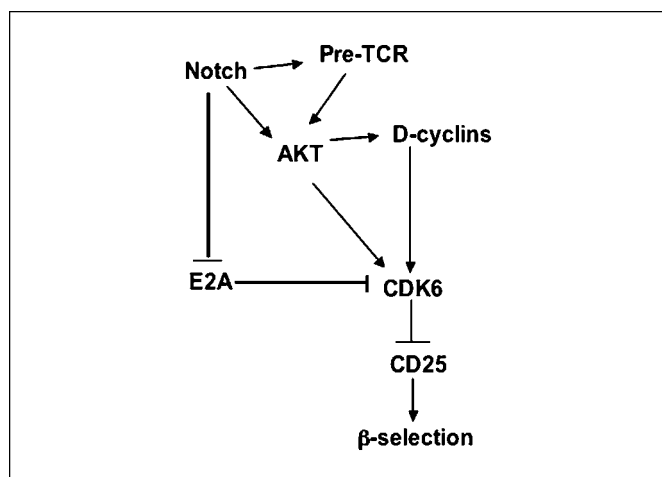


Figure 6. Working model for CDK6 regulation of T-cell development and tumorigenesis. Activation of Notch leads to (a) activation of Akt, (b) inhibition of E2A, and (c) induction of pre-T α transcript (one component of pre-TCR). Akt has also been shown to work downstream of pre-TCR at the β -selection checkpoint. CDK6 as a downstream mediator of Akt can be activated by (a) activated Akt, (b) elevated D-cyclins, and (c) increased gene expression because of reduced E2A proteins. The activated CDK6 in turn suppresses the expression of CD25, ensuring DN3 stage cells transit to DN4. Work presented here shows a need for CDK6 in Notch-dependent development of thymocytes and in Akt-driven lymphomagenesis; the model postulates that CDK6 also has a role in Notch-dependent tumor formation and in mediating Akt-dependent development. Future verification of this model would provide a clear indication of links between important developmental and cell cycle pathways in thymocytes and their exploitation by tumorigenic processes.

required for Notch-dependent T-lineage survival, proliferation, and differentiation. The failed expansion of BM progenitors in the absence of CDK6 stems in part from reduced proliferative fractions, increased apoptosis, and concomitant transitional blocks in the DN stages. Furthermore, loss of CDK6 resulted in increased CD25 expression and reduced CD44 expression, indicating that CDK6 acts in part to regulate these key T-cell markers. Taken together, these data show a critical requirement for CDK6 in Notch-dependent thymocyte development and Akt1-dependent T-cell tumorigenesis and strongly support CDK6 as a specific therapeutic target in human lymphoid malignancies.

Because CDK6 is an obligate catalytic partner for cyclin D3, the developmental defects in thymocytes from *CDK6*^{-/-} mice would be predicted to resemble the phenotype of *cyclin D3*^{-/-} mice. However, a distinct difference in phenotype is also observed, perhaps due to differences in the expression pattern of CDK6 and D-cyclins. For instance, the proliferative fractions of *CDK6* KO thymocytes were dramatically reduced in DN2 (~30% of WT) and DN3 (~50% of WT) stages (Supplementary Fig. S3A) before pre-TCR assembly, whereas the proliferative fractions were dramatically reduced in cyclin *D3*^{-/-} mice only in the DN4 and ISP stages (10) after pre-TCR assembly. Therefore, cyclin D3 acts primarily downstream of the pre-TCR but CDK6 can act before pre-TCR formation. Nevertheless, cyclin D3 is required for Notch-driven lymphomagenesis. It is intriguing that CDK6 loss has such profound consequences, given the readily detectable expression of CDK4 in all T-cell developmental stages. This in turn implies that CDK6 plays specific roles in T-cell development (and cancer) that cannot be fulfilled by CDK4.

This notion is further supported by the observation that *CDK6* KO progenitors failed to undergo normal proliferation and differentiation *in vitro* by using the OP9-DL1 system (Figs. 3 and 4; Supplementary Fig. S4). These data strongly support the hypothesis that CDK6 is required for Notch-dependent initiation of T-cell commitment by BM progenitors, likely acting downstream of the serine-threonine kinase Akt. This key component of the signal transduction cascade regulating pre-T-cell growth and survival downstream of Notch (6) can potentially phosphorylate over 9,000 proteins in mammalian cells (33). However, it is not known which downstream effectors of Akt are most critical for the genesis of cancer. Our data clearly indicate that CDK6 is a crucial downstream target of Akt, but the nature of the relationship between Akt and CDK6 remains to be determined. The activation of CDK6 may be due to up-regulation of cyclin D2 (26, 27). Alternatively, or in addition, *CDK6* gene expression may be regulated by Notch activation because Notch signaling collaborates with the pre-TCR to suppress the activity of E2A by inducing E2A ubiquitination and degradation (34–36), which in turn relieves suppression of *CDK6* gene expression (18). The aberrant activation of CDK6 in E47-deficient T-lineage cells contributes to the development of lymphoid malignancy (18).

Based on our data and published evidence, we propose a working model of the role of CDK6 in transmitting signals from Notch/Akt to regulate T-cell development and tumorigenesis (Fig. 6). Activation of Notch leads to or is accompanied by (a) activation of Akt, (b) inhibition of E2A, and (c) induction of *pre-T α* transcript (one component of the pre-TCR; ref. 37). Akt has also been shown to work downstream of the pre-TCR at the β -selection checkpoint (32). CDK6 can then be activated by (a) Akt, (b) elevated D-cyclins, and (c) increased gene expression because of reduced E2A proteins. The activated CDK6 in turn suppresses the expression of CD25,

ensuring DN3 stage cells transit to DN4. This model leads to the hypothesis that CDK6 loss would also block Notch-driven lymphomagenesis; this speculative connection between Notch, Akt, and CDK6 in tumor formation and the molecular basis for these unique functions of CDK6 are currently under investigation.

It is interesting to note that the apparently absolute requirement for CDK6 in Akt-driven thymic lymphoma joins an increasing list of tumors highly dependent on G₁ cyclin/CDK functions. This contrasts with the development of thymocytes *in vivo*, which shows a significant but more limited regulation of thymocyte reduction in the *CDK6* KO animals. Finally, we note that CDK6 is expressed in ES cells and is a transcriptional target of nanog, a pluripotency factor, yet development of most tissues in mice is not profoundly altered by CDK6 loss (38). Together, these observations support the notion that normal stem and progenitor cells have considerable plasticity in the event of loss of individual cell cycle control genes, but tumor-initiating cells may be much more

sensitive to such disruption. This in turn suggests that enzymes such as CDK6 need serious consideration as therapeutic targets in cancer and other developmental diseases where the initiating cell may differ significantly from its normal counterpart.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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