Notch signaling mediates G_1/S cell-cycle progression in T cells via cyclin D3 and its dependent kinases

Ila Joshi, Lisa M. Minter, Janice Telfer, Renée M. Demarest, Anthony J. Capobianco, Jon C. Aster, Piotr Sicinski, Abdul Fauq, Todd E. Golde, and Barbara A. Osborne

1Program in Molecular and Cell Biology and 2Department of Veterinary & Animal Sciences, University of Massachusetts, Amherst; 3Molecular and Cellular Oncogenesis, The Wistar Institute, Philadelphia, PA; 4Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA; 5Department of Cancer Biology, Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, MA; and 6Department of Neuroscience, Mayo Clinic Jacksonville, FL

Notch signaling plays a role in normal lymphocyte development and function. Activating Notch1-mutations, leading to aberrant downstream signaling, have been identified in human T-cell acute lymphoblastic leukemia (T-ALL). While this highlights the contribution of Notch signaling to T-ALL pathogenesis, the mechanisms by which Notch regulates proliferation and survival in normal and leukemic T cells are not fully understood. Our findings identify a role for Notch signaling in G_1/S progression of cell cycle in T cells. Here we show that expression of the G_1 proteins, cyclin D3, CDK4, and CDK6, is Notch-dependent both in vitro and in vivo, and we outline a possible mechanism for the regulated expression of cyclin D3 in activated T cells via CSL (CBF-1, mammals; suppressor of hairless, Drosophila melanogaster; Lag-1, Caenorhabditis elegans), as well as a noncanonical Notch signaling pathway. While cyclin D3 expression contributes to cell-cycle progression in Notch-dependent human T-ALL cell lines, ectopic expression of CDK4 or CDK6 together with cyclin D3 shows partial rescue from γ-secretase inhibitor (GSI)-induced G_1 arrest in these cell lines. Importantly, cyclin D3 and CDK4 are highly overexpressed in Notch-dependent T-cell lymphomas, justifying the combined use of cell-cycle inhibitors and GSI in treating human T-cell malignancies. (Blood. 2009; 113:1689-1698)

Introduction

Notch proteins are a family of ligand-activated large (300 kDa) single-pass transmembrane heterodimeric receptors. Notch controls multiple cell fate decisions and differentiation processes during lymphocyte development and function and is required at various stages of T-cell development. Deregulated Notch signaling during T-cell development leads to malignant transformation, including the cancer most closely associated with aberrant Notch expression in humans, acute T-cell acute lymphoblastic leukemia (T-ALL), which constitutes approximately 15% to 20% of ALLs seen in adults and children. The oncogenic potential of Notch was first identified in (t7;9) chromosomal rearrangement in approximately 2% of human T-ALL, whereby intracellular Notch1 is translocated to the T-cell receptor (TCR) β gene. More than 50% of human T-ALLs bear mutations in Notch1, indicating a prominent role for Notch in this T-cell malignancy. Inhibitors of Notch signaling abrogate the growth of human and murine T-ALL cell lines bearing Notch1 gain-of-function mutations, indicating Notch is required in established tumors.

In vertebrates, 4 notch receptors (Notch 1-4) are activated by 5 different Notch ligands expressed on various cell types: Jagged1, Jagged2, and Delta-like (DL)1, DL3, and DL4. After ligand-binding, proteolytic cleavage by γ-secretase releases the signaling-competent intracellular domain of Notch (NIC). NIC is composed of a RAM domain, ankyrin repeats (ANK) that mediate protein-protein interactions, nuclear localization sequences, a trans-activation domain (TAD), and a C-terminal PEST domain regulating protein turnover. Human T-ALL cases frequently bear activating mutations in the extracellular heterodimerization domain and/or the C-terminal PEST domain of Notch1, resulting in ligand-independent activation. During canonical Notch signaling, NIC translocates to the nucleus, engages its nuclear binding protein CSL (CBF-1, mammals; suppressor of hairless, Drosophila melanogaster; Lag-1, Caenorhabditis elegans) and transcribes downstream target genes, including the hes family of transcriptional repressors. In the absence of NIC, CSL recruits repressor complexes to the regulatory regions of Notch/CSL target genes, inhibiting transcription. NIC interaction with CSL acts as a switch that promotes the assembly of CSL coactivator complexes. γ-Secretase inhibitors (GSIs) block proteolytic cleavage of Notch receptors, thereby preventing activation of Notch. Use of GSI in activated T cells results in down-regulation of nuclear factor (NF)-κB activity, cytokine (interleukin-2 [IL-2] and interferon-γ [IFN-γ]) production, and cell proliferation.

In T-cell lymphomas, context-specific putative target genes have been identified through which Notch1 may promote transformation by altering cell-growth kinetics. The D-type cyclins (cyclins D1, D2, and D3) are the first cyclins to be induced as cells enter the G_1 phase of the cell cycle, and, thus, if regulated by Notch signaling, are highly relevant to the mechanism of Notch-induced lymphomagenesis. D-type cyclins associate with and activate cyclin-dependent kinases (CDK), CDK4 and CDK6. Activation of CDKs at specific time points during the cell cycle

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regulates phosphorylation and inactivation of the retinoblastoma protein (Rb) and derepression of E2F transcription factors, driving the cell into S phase. Notch activation up-regulates cyclin D1 and CDK2 activity in rat kidney epithelial cells increasing cell proliferation. Notch induces transcription of S phase kinase-associated protein 2 (SKP2), which targets CDK-inhibitors p27 and p16 for proteosomal degradation, resulting in premature entry into S phase. In pancreatic cancer cells, down-regulating Notch1 induces a G0/G1 cell-cycle arrest associated with reduced levels of cyclin D1 expression and increased p27kip1 expression. Notch signaling is also a potent regulator of cell cycle in human T-ALL cell lines, because treating them with GSI induces G0/G1 cell-cycle arrest. In addition, cyclin D3 is frequently overexpressed in cancers of the lymphoid system.

Based on these data, we sought to determine how Notch regulates cell-cycle progression in T cells and whether cyclin D3 is a direct target of Notch. Here we demonstrate that Notch regulates cyclin D3 expression in mature T cells and transformed T lymphoblasts, and propose a possible mechanism for regulated cyclin D3 expression in activated T cells. D-type cyclins function together with their catalytic partners CDK4 and CDK6 to facilitate cell-cycle progression, and we also show CDK4 and CDK6 are Notch target genes in peripheral T cells and in lymphomas arising in transgenic animals overexpressing Notch1. Our study reveals the oncogenic potential of aberrant Notch signaling in T cells through its deregulation of the cell cycle. It further suggests that cell-cycle regulation by Notch may represent a target for therapeutic intervention using small molecule inhibitors/modifiers in Notch-driven T-cell leukemias.

**Methods**

**Mice, CD4+ T-cell preparation, and GSI treatment**

C57BL/6 mice were housed in the University of Massachusetts animal care facility in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. CD4+ T cells were isolated from 2- to 3-month-old mice using the IMag magnetic bead system (BD Pharmingen, BD Biosciences, San Jose, CA), and 2.25×10^9/mL mouse CD3, 1 mg/mL anti-CD28 for indicated time periods (0, 3, 6, and 12 hours) in the presence of GSI (IL-CHO, 50 μM) or DMSO (0.1%).

**Lysate preparation, immunoblotting, and antibodies**

Whole cell lysates were prepared with radio immunoprecipitation assay (RIPA) buffer. Preparation of nuclear extracts was described previously. Immunoblotting was performed using anti-cyclin D3, anti-CDK4, anti-CDK6 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-GAPDH (Chemicon International, Billerica, MA); anti–cleaved Notch1 (eBiosciences, San Diego, CA); anti–phospho-Ser780-pRb, and anti–phospho-Ser795-pRb (Cell Signaling Technologies, Danvers, MA).

**Dual luciferase assay and reverse transcription–polymerase chain reaction**

293T cells (10^6) were transfected with indicated Notch expression vectors or empty vector using Fugene6 (Roche, Indianapolis, IN). We used 0.4 μg of D3-Luc reporter plasmid in each experiment and 0.1 μg PRL-CMV (pRL-CMV Vector contains the CMV enhancer and early promoter elements to provide expression of Renilla luciferase in cotransfected mammalian cells). Luciferase assays were performed at 48 hours, following the manufacturer’s instruction (Dual Luciferase Assay System; Promega, Madison, WI). For reverse transcription–polymerase chain reaction (RT-PCR), total RNA was isolated using RNA-BEE (Tel-test, Friendswood, TX), following the manufacturer’s protocol. Reverse transcription was performed with oligo-dT primers (Invitrogen, Carlsbad, CA); cDNA amplified by PCR using cyclin D3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and conditions as described.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) analysis was performed on 10^6 CD4+ T cells using ChIP assay kit (Cell Signaling Solutions; Upstate, Millipore, Billerica, MA) following the manufacturer’s protocol. Eluted material was reverse cross-linked and DNA-purified with PCR purification kit (QIAGEN, Valencia, CA) for PCR. Primer Set1 (227 bp, annealing temperature: 52°C) forward: 5’-GAGAATTCGATCTACTCTTTTCAATC-3’, reverse: 5’-CCCGCCAGTCCCTTGTTTATGAGT-3’; Primer Set2 (297 bp, annealing temperature: 54°C) forward: 5’-AGAAGACTTCTTCTTCAATTTCAGG-3’, reverse: 5’-CTCTGTCCAGCTCCGAGGAG-3’. Primer Set3 (125 bp, annealing temperature: 54°C) forward: 5’-CAGAAGACTTCTTCTTCAATTTCAGG-3’, reverse: 5’-CTCTGTCCAGCTCCGAGGAG-3’.

**Figure 1. Cyclin D3 expression is dependent on Notch signaling in CD4+ T-cells.** Whole cell lysates of CD4+ T cells were analyzed by immunoblotting for cyclin D3 or Notch1IC expression. CD4+ T cells were stimulated with 1 μg/mL anti-CD3e and 1 μg/mL anti-CD28 for indicated time periods (0, 3, 6, and 12 hours) in the presence of GSI (IL-CHO, 50 μM) or DMSO (0.1%). (B) Later time points (24, 48, and 72 hours) after 1 μg/mL anti-CD3e and 1 μg/mL anti-CD28 stimulation were analyzed for differences in cyclin D3 and Notch1IC expression with or without GSI treatment. (C) Whole cell lysates from stimulated CD4+ T cells (for 24, 48, and 72 hours) that were isolated from C57BL/6 mice treated in vivo with GSI for 14 days were analyzed for cyclin D3 and Notch1IC. GAPDH was used as a loading control. LY indicates GSI in rodent chow; and C, control rodent chow.
Human T-ALL cell lines, GSI treatment

Human T-ALL cell lines DND-41, HPB-ALL, and T-ALL1 were maintained in media as described. For GSI treatment, 3 μM zL-CHO were used for 7 days with 0.1% DMSO used as vehicle control.

Cell-cycle analysis

DND-41, HPB-ALL, and T-ALL1 were analyzed for cell-cycle progression using DRAQ5 (AXXORA, San Diego, CA). At time of analysis, cells were resuspended in 3 × 10^6 cells/mL phosphate-buffered saline (PBS), and 5 μL DRAQ5 were added directly to the tube and gently mixed. Cells were incubated at room temperature for 20 minutes and acquired on LSRII using FacsDiva software (both from Becton Dickinson, BD, Franklin Lakes, NJ). Data were analyzed using FlowJo (TreeStar, Ashland, OR).

Plasmids and retroviral infection

pMSCV-IREs-YFP-cyclin D3 expression plasmid was a kind gift from Dr Suzanne J. Baker (St Jude Children’s Research Hospital, Memphis, TN). pMSCV-IREs-DSRed-CDK4 or -CDK6 retroviral construct was generated by subcloning CDK4, CDK6 (pCMV-CDK4 or CDK6 expression plasmid, kind gift of Dr Phil W. Hinds (Tufts-New England Medical Center, Boston, MA)) into pMSCV-IREs-DSRed vector (gift from Dr Dario A.A. Vignali, St Jude Children’s Research Hospital). Expressed proteins of expected size were confirmed by transient expression studies with 293T cells. For retroviral supernatants, 1.5 × 10^7 293T cells were plated onto 60-mm dishes, 1 day before transfection. The next day, 4 μg of the expression plasmid and 2 μg of the retroviral packaging plasmid pCL-Ampho (Imgenex) were transfected using 18 μL of Fugene6. Forty-eight hours after transfection, viral supernatant was harvested and cleared by spinning at 290g (1500 rpm) for 5 minutes. Two milliliters supernatant were mixed with 16 μL Fugene6 and added to cell suspension (human T-ALL cell line, 10^6 cells/mL) in 6-well culture dishes. Cells were spin-infected at 804 (2500 rpm) for 1 hour at 30°C, then placed into 100-mm culture dishes with fresh supernatant. Cells were cultured at 37°C with 5% CO2 for 48 hours, then placed in fresh media. Twenty-four hours after replacement, infection efficiency was analyzed on an LSRII. After 7 days’ culture, cells were sorted for enrichment by gating for fluorescent protein expression (yellow fluorescent protein [YFP], cyclin D3; YFP and DsRed, cyclin D3 and CDK4 or CDK6) using a FACSVantage (Becton Dickinson).

Calculating relative G1 rescue index

The relative G1 rescue index (RI) was calculated to represent the rescue from GSI-induced G1 arrest observed in transduced human T-ALL cell lines relative to G1 arrest observed in wild-type human T-ALL cell lines (Tables S1,2, available on the Blood website; see the Supplemental Materials link at the top of the online article). Difference in percentage of cells in G1 (ΔG1) was calculated by subtracting percentage of cells in G1 with DMSO treatment from percentage of cells in G1 after 7 days’ GSI treatment: ΔG1 = percentage of cells in G1 with DMSO - percentage of cells in G1 at 7 days’ GSI treatment. The relative G1 RI was calculated by subtracting ΔG1 from 100 for human T-ALL cell lines and represented graphically as: RI_{G1} = 100 - ΔG1. Complete G1 rescue is defined as RI = 100.

Analysis of TOP-Notch, MIG-N6 leukemic mice

To generate TOP-Notch/MIT-RX leukemic mice, bone marrow from TOP-Notch animals was transduced with the tetracycline transactivator expression construct, MIT-RX as described previously. To generate MIG-N6 leukemic mice, wild-type FVB bone marrow was infected with MIG-N6.transIT vector as described above. Spleens from diseased animals or wild-type FVB controls were used. Leukemia transplant experiments for tumor regression studies were performed using TOP-Notch/E4.1TA mice as described in Document S1.

Results

Notch signaling regulates cyclin D3 expression in CD4^+ T cells in vitro and in vivo

Previous experiments in our laboratory demonstrated Notch expression is induced by TCR signaling. To examine whether Notch signaling is required for expression of cyclin D3 in primary T cells, CD4^+ T cells isolated from spleens of C57BL/6 mice were stimulated with anti-CD3e and anti-CD28 for different length of times. There was no significant increase in cyclin D3 expression up to 12 hours after stimulation (Figure 1A). We also stimulated CD4^+ T cells in the presence of the GSI, 50 μM IL-CHO, after determining that this concentration reduced Notch-1 levels (Figure S1), and
found there was little or no effect on cyclin D3 expression compared with vehicle control, DMSO, during the first 12 hours (Figure 1A). However, by 24 hours after stimulation, there was an increase in cyclin D3 protein that continued to rise up to 72 hours after stimulation (Figure 1B). Abrogating Notch signaling with GSI-treatment decreased cyclin D3 expression at 24, 48, and 72 hours after stimulation (Figure 1B). We confirmed our observations using another GSI, DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester), and found similar effects, confirming decreased cyclin D3 expression was a result of blocking Notch activity. Next, C57BL/6 mice were treated with GSI (LY-411,575) formulated in rodent chow to deliver 5 mg/kg per day for 2 weeks (Figure S2) to determine how attenuating Notch/CSL signaling in vivo affects cyclin D3 expression. CD4+ T cells from GSI-treated and control chow-fed mice were isolated and stimulated with anti-CD3 and anti-CD28 up to 72 hours. Consistent with in vitro effects of GSI treatment on cyclin D3 expression, treating mice in vivo with GSI also reduced cyclin D3 expression, compared with control animals, and this correlated with reduced Notch1 expression (Figure 1C). Together, these data suggest, in stimulated CD4+ T cells, cyclin D3 expression is a downstream target of Notch signaling.

**Notch1IC directly regulates cyclin D3 promoter activity**

Next, we asked whether Notch signaling influences cyclin D3 expression at the transcript level. CD4+ T cells isolated from spleens of C57BL/6 mice were pretreated with GSI or DMSO then stimulated with anti-CD3ε and anti-CD28. After 24 hours, we assessed cyclin D3 transcript levels by RT-PCR. After stimulation, transcription of cyclin D3 was decreased in cells treated with GSI, compared with controls (Figure 2A). In vivo abrogation of Notch signaling also resulted in reduced transcript levels (Figure 2A). We used the dual luciferase assay to determine whether Notch1 regulates expression from the cyclin D3 promoter. Ectopic expression of Notch1IC increased cyclin D3 reporter activity greater than 10-fold, compared with the control plasmid (Figure 2B). To characterize the domain of Notch1IC that is required for regulating the cyclin D3 promoter, we performed dual luciferase reporter gene assays. Cotransfection of various Notch1IC constructs with the cyclin D3 reporter revealed that the C-terminal region was required for cyclin D3 promoter activity (Figure 2B). To determine whether Notch binds directly to the cyclin D3 promoter, we performed ChIP using CD4+ T cells isolated from spleens of C57BL/6 mice, and stimulated with anti-CD3ε and anti-CD28 for 24 hours. We immunoprecipitated protein-bound DNA using anti-Notch1, followed by PCR using different primer sets specific for the cyclin D3 promoter region. We were able to amplify a 227-bp region (−1764 to −1537) of the cyclin D3 promoter, indicating direct binding of Notch1 to the cyclin D3 promoter (Figure 2C). Inhibiting Notch activation with GSI treatment abrogated the recruitment of Notch1 to the cyclin D3 promoter (Figure 2C). To further investigate whether CSL is involved in Notch-mediated regulation of the cyclin D3 promoter, we performed ChIP with an antibody that immunoprecipitates CSL. We were able to amplify the same 227-bp region (−1764 to −1534) using anti-CSL, as when using anti-Notch1 and, again, GSI treatment diminished the recruitment of CSL to the promoter region (Figure 2C). We also examined other regions within the cyclin D3 promoter for putative Notch/CSL binding. We observed that a 297-bp region (−1322 to −1025) was not amplified by PCR after immunoprecipitation with either antibody (Figure 2C), suggesting that Notch/CSL interaction on the cyclin D3 promoter is specific, and both binding partners are recruited to a unique location within the promoter region. Together, these data indicate that cyclin D3 is a direct target of the Notch/CSL signaling pathway.

**NF-κB binds to the cyclin D3 promoter in primary T cells augmenting Notch-dependent cyclin D3 promoter activity**

NF-κB has recently emerged as a nuclear binding partner for Notch, and both signaling pathways have been shown to be interconnected.34,38,39 Studies by our laboratory and others have documented that signaling through the TCR activates NF-κB, and various NF-κB family members have been shown to modulate cell cycle by regulating the cyclin D1 promoter.34,40-42 Our laboratory has also shown that Notch directly regulates the IFN-γ promoter, through complexes with p50. Therefore, we asked whether NF-κB regulates cyclin D3 in T cells, in conjunction with Notch signaling. To address this, we cotransfected 293T cells with the NF-κB subunit p50, along with Notch1IC and a cyclin D3 luciferase reporter construct. Our results show that coexpression of p50 induces a greater than 2-fold increase in Notch1IC-dependent transactivation of the cyclin D3 reporter (Figure 3A). Although initial cotransfection of p50 with equal amounts of Notch1IC correlates with increases in reporter activity, Notch1IC was the limiting factor in p50-mediated regulation. Furthermore, these data show that D3 reporter activity is Notch-dependent and p50 cooperates with Notch for this effect.

We next sought to determine which functional domains of Notch1IC are critical for its interaction with p50 in regulating the cyclin D3 promoter. Mutant Notch1IC constructs lacking the C-terminal region showed approximately 50% reduction in reporter activity compared with full-length Notch1IC. Consistent with our previous results, we observed that TAD was required for facilitating p50-mediated cyclin D3 promoter activity (Figure 3B). We then analyzed the cyclin D3 promoter for consensus NF-κB binding sites and found 2 putative binding sites within the 227-bp region (−1764 to −1537) that we previously immunoprecipitated for ChIP using anti-Notch and anti-CSL (Figure 2C). Together, with our previous ChIP data (Figure 2C), these results indicate p50, CSL, and Notch1IC interact at distinct sites of the cyclin D3 promoter to augment cyclin D3 promoter activity. Consistent with our previous work,34 NF-κB binding sites are critical for its interaction with p50 in regulating the cyclin D3 promoter. Further, our previous work34 has also shown that Notch directly regulates the IFN-γ promoter, through complexes with p50. Therefore, we asked whether NF-κB regulates cyclin D3 expression in human T-ALL cell lines. We successfully amplified this region in DMSO-treated samples, indicating the p50 subunit of NF-κB binds directly to the cyclin D3 promoter and GSI treatment abrogates this interaction (Figure 3C). As a negative control, we used an additional primer set to amplify −1322 to −1025 of the cyclin D3 promoter that did not immunoprecipitate using antibodies against Notch1 or CSL. We were unable to amplify this region after retrieving de-cross-linked DNA fragments, confirming the specificity with which p50 binds to the cyclin D3 promoter (Figure 3C). Together, with our previous ChIP data (Figure 2C), these results indicate p50, CSL, and Notch1IC interact at distinct sites of the cyclin D3 promoter to augment cyclin D3 promoter activity.
overexpression of cyclin D3 could rescue these cell lines from the effects of GSI. As reported previously, DND-41, HPB-ALL, and T-ALL1 treated with GSI show G1 arrest, reiterating that transit through the cell cycle is Notch-dependent in these cell lines (Figure 4A). Next, we examined whether G1 arrest in DND-41, HPB-ALL, and T-ALL1 cell lines correlated with changes in cyclin D3 expression. Analysis of protein extracts from cell lines treated with GSI revealed reduced cyclin D3 expression when Notch signaling is abrogated compared with vehicle control, DMSO (Figure 4B). Decreased Notch1 expression in all cell lines confirmed the inhibitory effects of GSI treatment on Notch processing (Figure 4B). We observed similar results with DAPT, confirming cyclin D3 is a target of Notch signaling in human T-ALL cell lines (Figure S3). These results indicate cyclin D3 expression is temporally associated with cell-cycle progression in Notch-dependent human lymphomas. We then asked whether overexpressing cyclin D3 could rescue cell lines from the effects of GSI treatment (Figure S4). DND-41, HPB-ALL, and T-ALL1 cell lines were retrovirally transduced with MSCV-cyclin D3-IRES-YFP or MSCV-IRES-YFP empty vector and sorted to enrich for the YFP-expressing population. Cyclin D3-YFP–expressing, as well as the YFP vector control, cells were divided and treated with either GSI or DMSO. We then compared differences in percentages of cells in G1 under the 2 treatment conditions (GSI or DMSO) in the transduced cell lines (cyclin D3-YFP or YFP-vector) with those of nontransduced cell lines to calculate G1 rescue indices (Table S1). Compared with vector alone, in the DND-41 and T-ALL1 cell lines, overexpressing cyclin D3 resulted in partial rescue from G1 arrest due to abrogated Notch signaling (Figures 4C, S5). However, cyclin D3 overexpression was not sufficient to rescue HPB-ALL from GSI treatment (Figures 4C, S5). Together, these data suggest, in human T-cell lymphomas, that cyclin D3 is an important target of the cell-cycle machinery and is regulated by Notch signaling.

**Notch regulates CDK4 and CDK6 expression and pRb phosphorylation in primary T cells**

The fact that we could only partially restore cell-cycle progression in some human T-ALL cell lines after ectopic expression of cyclin D3 prompted us to investigate other components of the cell cycle that might be influenced by Notch signaling. Cyclins function through their catalytic partners, CDK4 and CDK6, to facilitate cell-cycle progression. Therefore, we considered CDK4 and CDK6 as 2 putative targets of Notch regulation in primary T cells. As described above, we isolated CD4+ T cells from spleens of C57BL/6 mice, then stimulated the cells with anti-CD3e and anti-CD28 in the presence or absence of GSI. At 6 hours after stimulation, we found increased expression both of CDK4 and CDK6 in DMSO-treated cells, but GSI treatment abrogated this up-regulation, signifying that even early induction of CDK4 and CDK6 is Notch-dependent (Figure 5A). Expression of both proteins continued to increase up to 72 hours, with maximal expression observed 48 hours after stimulation (Figure 5B). Interestingly, CDK6 expression was more robust than CDK4 expression at these later time points. Consistent with effects at early time points, inhibiting Notch signaling reduced expression of CDK4 and CDK6 at 24, 48, and 72 hours after stimulation (Figure 5B). These observations led us to ask whether differences in CDK4 or CDK6 expression altered phosphorylation of their target protein, pRb. D-cyclins, along with CDK4 and CDK6, are known to phosphorylate pRb at Ser780 and Ser795. We examined phosphorylation of pRb at these residues in CD4+ T cells stimulated for 24, 48, and 72 hours with anti-CD3e and anti-CD28, with or without GSI treatment. We found Ser780 was phosphorylated by 24 hours and remained phosphorylated up to 72 hours after stimulation (Figure 5C). Phosphorylation of Ser780 was also observed at 24 hours, although to a lesser extent than that of Ser780, and also increased up to 72 hours (Figure
5C). In contrast, GSI treatment abrogated phosphorylation of both pRb residues (Figure 5C), indicating Notch signaling regulates temporal expression of CDK4 and CDK6 and subsequent pRb phosphorylation, thus facilitating cell-cycle progression in primary T cells.

Cotransducing CDK4 or CDK6 provides variable rescue of human T-ALL cell lines from GSI-induced G1 arrest

Having shown CDK4 and CDK6 expression, as well as pRb phosphorylation, are targets of Notch signaling in primary T cells, we wanted to determine whether this was also true for human T-ALL cell lines. We treated the 3 T-ALL cell lines with GSI to abrogate Notch signaling and assessed CDK4 and CDK6 expression by immunoblotting. Our results show that GSI treatment reduces CDK4 and CDK6 expression to variable degrees in these cell lines (Figure 6A), with 25% to 30% reduction in CDK4 and CDK6 in GSI-treated DND-41 and HPB-ALL cells. The T-ALL1 cell line showed maximal reduction (almost 50%) in CDK4 and CDK6 expression (Figure 6A). We next asked whether decreased expression of CDK4 and CDK6 correlated with reduced pRb phosphorylation, as it did in primary T cells. Consistent with those data, we observed phosphorylation of pRb was altered by abrogating Notch signaling using GSI. As expected, reduced phosphorylation was variable and cell line-dependent; however, both Ser780 and Ser795 pRb residues showed reduced phosphorylation when the cell lines were treated with GSI (Figure 6B). These results indicate, in addition to cyclin D3, its catalytic partners CDK4 and CDK6, may also be targets of Notch signaling that cooperate to contribute to unchecked cell-cycle progression in T-cell lymphomas. These data prompted us to ask whether we could rescue human T-ALL lines from GSI-induced G1 arrest by cotransducing CDK4 or CDK6 together with cyclin D3. We used vector-YFP control cell lines and 3 cell lines stably expressing cyclin D3-YFP (Figure 5C and Figure S6) to coexpress DsRed-CDK4 or DsRed-CDK6. Cells expressing both YFP and DsRed were sorted to enrich for cells ectopically expressing cyclin D3 with CDK4 or CDK6. Sorted cells were treated with GSI or DMSO, and cell-cycle analysis was performed. We compared the differences in percentages of cells in G1 under the 2 treatment conditions (GSI or DMSO) to calculate G1 RIs, as before (Table S2). In all 3 cell lines, overexpressing cyclin D3 either with CDK4 or CDK6 could rescue cells from G1 arrest, as evidenced by the increase in RI (Figures 6C, S6). Interestingly, in all 3 cell lines, the G1 rescue effects of coexpressing cyclin D3 with CDK4 or CDK6 were additive, compared with those of expressing cyclin D3 alone (Figures 4C, S1). This was particularly true in HPB-ALL cells, in which we did not observe any rescue with ectopic expression of cyclin D3 alone (Figure 6C, Table S1). Taken together, our data indicate cyclin D3, CDK4, and CDK6 are Notch-regulated targets that promote transit through the cell cycle in T-cell lymphomas.

Cyclin D3 and CDK4 are highly expressed in Notch-dependent T-cell lymphomas in vivo

Finally, we utilized the Top-Notch mouse model of Notch-induced lymphoma to address whether Notch1 regulates expression of cell-cycle proteins in T-cell leukemia in vivo.37 We killed Top-Notch leukemic mice showing overt signs of lymphoma and prepared protein extracts from spleens. We confirmed high levels of N\(^{\text{HC}}\) expression in diseased animals, as well as high levels of cyclin D3 and CDK4 and CDK6 expression, as well as pRb phosphorylation, are targets of Notch signaling in primary T cells, we wanted to determine whether this was also true for human T-ALL cell lines. We treated the 3 T-ALL cell lines with GSI to abrogate Notch signaling and assessed CDK4 and CDK6 expression by immunoblotting. Our results show that GSI treatment reduces CDK4 and CDK6 expression to variable degrees in these cell lines (Figure 6A), with 25% to 30% reduction in CDK4 and CDK6 in GSI-treated DND-41 and HPB-ALL cells. The T-ALL1 cell line showed maximal reduction (almost 50%) in CDK4 and CDK6 expression (Figure 6A). We next asked whether decreased expression of CDK4 and CDK6 correlated with reduced pRb phosphorylation, as it did in primary T cells. Consistent with those data, we observed phosphorylation of pRb was altered by abrogating Notch signaling using GSI. As expected, reduced phosphorylation was variable and cell line-dependent; however, both Ser780 and Ser795 pRb residues showed reduced phosphorylation when the cell lines were treated with GSI (Figure 6B). These results indicate, in addition to cyclin D3, its catalytic partners CDK4 and CDK6, may also be targets of Notch signaling that cooperate to contribute to unchecked cell-cycle progression in T-cell lymphomas. These data prompted us to ask whether we could rescue human T-ALL lines from GSI-induced G1 arrest by cotransducing CDK4 or CDK6 together with cyclin D3. We used vector-YFP control cell lines and 3 cell lines stably expressing cyclin D3-YFP (Figure 5C and Figure S6) to coexpress DsRed-CDK4 or DsRed-CDK6. Cells expressing both YFP and DsRed were sorted to enrich for cells ectopically expressing cyclin D3 with CDK4 or CDK6. Sorted cells were treated with GSI or DMSO, and cell-cycle analysis was performed. We compared the differences in percentages of cells in G1 under the 2 treatment conditions (GSI or DMSO) to calculate G1 RIs, as before (Table S2). In all 3 cell lines, overexpressing cyclin D3 either with CDK4 or CDK6 could rescue cells from G1 arrest, as evidenced by the increase in RI (Figures 6C, S6). Interestingly, in all 3 cell lines, the G1 rescue effects of coexpressing cyclin D3 together with CDK4 or CDK6 were additive, compared with those of expressing cyclin D3 alone (Figures 4C, S1). This was particularly true in HPB-ALL cells, in which we did not observe any rescue with ectopic expression of cyclin D3 alone (Figure 6C, Table S1). Taken together, our data indicate cyclin D3, CDK4, and CDK6 are Notch-regulated targets that promote transit through the cell cycle in T-cell lymphomas.
D3 and CDK4 compared with controls (Figure 7A). Interestingly, we did not find detectable CDK6 expression in these animals (data not shown), suggesting functional redundancy of CDK4 and CDK6. We obtained similar results from MIG-Nic leukemic mice (Figure 7B). Next, we performed leukemia transplantation experiments to show that, in the leukemic cells derived from tTA-Nic mice, cyclin D3 and CDK4 expression is dependent on the continuous expression of the Top-NotchIC transgene. tTA-Nic mice that were moribund due to lymphoma were euthanized, and single-cell suspensions prepared from lymphoid organs were transplanted IV into the tail vein of nontransgenic mice. After these mice developed tumors, they were treated with doxycycline to shut off Notch expression. We performed RT-PCR analysis on these regression samples to determine the time point at which Notch was turned off (Figure 7C). We then prepared protein lysates from this time point and observed that abrogation of Notch results in reduced cyclin D3 as well as CDK4 expression. These results further strengthen the link between Notch signaling and cell-cycle regulation. Together, these findings indicate that, in Notch-dependent lymphomas, cyclin D3, and CDK expression is regulated by Notch1 and may contribute to Notch-driven T-cell leukemogenesis.

**Discussion**

We have identified cyclin D3 as a direct target of Notch1 that promotes cell-cycle progression and proliferation both in peripheral and leukemic T cells. We show cyclin D3 expression in T cells is dependent on Notch1 signaling both in vitro and in vivo. Our data demonstrate that Notch1, along with its canonical binding partner CSL, associates with the cyclin D3 promoter. In addition, we show the NF-κB subunit p50 binds the cyclin D3 promoter and enhances Notch1-dependent promoter activity. Taken together, our data using Notch-dependent human T-ALL lines identify cyclin D3 as a target of constitutively active Notch signaling, since ectopic expression of cyclin D3 can partially rescue these cell lines from GSI-induced G1 arrest. Finally, we show that cyclin-dependent kinases CDK4 and CDK6 are also targets of Notch signaling and, along with cyclin D3, contribute to deregulated cell-cycle progression in leukemic T cells.

We demonstrated that 24 hours after activation, primary T cells show a robust increase in cyclin D3 expression that is abrogated with GSI treatment. We have shown that Notch1 associates with the cyclin D3 promoter and the TAD of Notch1IC is required for regulating cyclin D3 promoter, because its removal almost completely abrogates promoter activity. We also found the ANK region of Notch1IC is critical for expression from the cyclin D3 promoter. These results confirm the importance of the ANK and TAD domains of Notch1 in regulating cyclin D3 and are known to be required for Notch-driven T-cell leukemogenesis.

Interestingly, in addition to the canonical Notch binding partner, CSL, we also found the NF-κB subunit p50 binds to the cyclin D3 promoter. It was recently demonstrated that the Notch transcriptional complex (NTC), consisting of CSL/ICN1/MAML, dimerizes on properly spaced pairs of CSL binding sites and that certain target genes require this architecture for Notch responsiveness. However, Notch-mediated effects are exquisitely context-dependent, a property that likely stems from the existence of response elements containing combinations of binding sites for CSL and other transcription factors. In the case of cyclin D3 promoter, we were able to identify 2 putative NF-κB binding sites in a 277-bp region amplified after ChIP. Consensus NF-κB binding sites may incorporate a nested CSL binding site. This raises the question of whether there is competition between NF-κB and CSL for overlapping binding sites on a promoter or for DNA binding factors, such...
as p50, that cooperate with NTC to transactivate Notch-regulated genes. While we show NF-κB and CSL both are recruited to the cyclin D3 promoter, directly regulating cyclin D3 expression, more work is required to elucidate the mechanism involved in this regulation. Our observations underscore an important role for NF-κB in peripheral T-cell function and, very recently, NF-κB has been implicated as a major downstream target of Notch1 in human T-ALL.50 Our results in primary T cells reveal CSL recruitment to the promoter region is diminished by abrogating Notch activity. The Drosophila homolog of CSL, Su(H), is dynamic in its association with DNA and shows significant increase in target-gene occupancy after Notch activation, highlighting the importance of cooperative binding of higher-order NTCs and the influence of other DNA binding cofactors to stabilize the Su(H) activation-complex on DNA.51 It remains to be determined whether regulation of the cyclin D3 promoter involves a similar mechanism.

Several targets of Notch signaling in human T-ALLs have been identified. Transcriptional regulators c-Myc, Lef-1, and the m-TOR pathway have been shown to be associated with Notch-induced transformations.19-21 Notch-dependent human T-ALL cell lines have previously been used to identify targets of deregulated Notch signaling.19,21 Our results have identified cyclin D3 as a target of Notch signaling in the human T-ALL cell lines DND-41, HPB-ALL, and T-ALL1. However, ectopic expression of cyclin D3 alone could not completely rescue these cell lines from GSI-induced G1 arrest. We have demonstrated that while cyclin D3 is an important direct target of Notch signaling in Notch-dependent cell lines, there are other cell-cycle regulators that may be influenced by GSI treatment.

Cyclin-dependent kinases are important for cell-cycle progression in normal T cells and during leukemic transformation.52 We show that CDK4 and CDK6 both are important for Notch-dependent cell-cycle progression. In activated T cells, there is

Figure 6. CDK4 and CDK6 expression and pRB phosphorylation are targets of Notch signaling in human T-ALL cell lines. (A) Whole cell extracts were prepared from human T-ALL cell lines after 7 days of in vitro GSI or DMSO treatment to analyze differences in CDK4 (top) and CDK6 expression (middle). GAPDH (bottom) was used as a loading control. Band intensities of protein expression were normalized to GAPDH and represented graphically using ImageJ software, version 1.31, supported by Wayne Rasband (NIH). (B) Immunoblot of whole-cell lysates prepared from human T-ALL cell lines that were treated with GSI or DMSO for 7 days to analyze differences in pRB phosphorylation. pRB Ser780 (top panels), pRB Ser795 (middle panels), and GAPDH (bottom panels; loading control). (C) Graphical representation of relative G1 rescue indices. Human T-ALL cell lines DND-41, HPB-ALL, and T-ALL1 were retrovirally infected either with MSCV-IRES-YFP and MSCV-IRES-ΔDT (vector, black bar), MSCV-cyclin D3-IRES-YFP and MSCV-cyclin D3-IRES-ΔDT (CDK4 + D3, gray bar), or MSCV-cyclin D3-IRES-YFP and MSCV-cyclin D3-IRES-ΔDT (CDK6 + D3, white bar). The infected cells were sorted for enrichment and treated with DMSO or GSI for 7 days. Cell-cycle analysis was performed to determine the percentage of cells arrested in G1 with GSI treatment compared with DMSO. Relative G1 rescue indices were calculated for each cell line after normalizing against the GSI-induced G1 arrest in the uninfected cell line.

Figure 7. Regulation of cyclin D3 and CDK4 expression by Notch1 in T-cell lymphomas. (A) Protein extract from spleens of 2 wild-type FVB mice (WT) and 3 Top-Notch mice were used to analyze the expression of cyclin D3, CDK4, and Notch-1. GAPDH was used as a loading control. (B) Protein extracts from spleens of 2 wild-type FVB mice (WT) and 2 MIG-Nic leukemic mice were used to determine cyclin D3, CDK4, and Notch-1 expression, as indicated. GAPDH was used as a loading control. (C) Eμ-TATOP-Notch mice were generated as previously described.36,37 Samples prepared for regression analysis were prepared as described in Document S1. Left panel shows the RT-PCR performed on primary tumor samples and on transplanted samples from mice undergoing doxycycline regression for different lengths of time (0, 3, and 24 hours). Notch transcripts were analyzed, and β-actin was used as a positive control. Right panel shows Western blot analysis performed using total cell lysate for detection of cyclin D3 and CDK4 from primary tumor samples and transplanted samples from mice undergoing doxycycline regression for various lengths of time (0 and 24 hours).
increased expression of CDK4 and CDK6 that is abrogated by GSI treatment. Unlike cyclin D3, their expression is induced at early time points, with robust increase 24 hours after stimulation. We observed an additive rescue from G1 arrest with ectopically expressed CDK4 or CDK6, together with cyclin D3, in all 3 Notch-dependent cell lines, suggesting cyclin D3 and its catalytic CDKs may be co-opted as part of the pro-oncogenic Notch pathway. CDK4 levels have been shown to be reduced in T-ALL lines after treatment with the GSI, compound E.21 Failure to effect a complete rescue from GSI-induced G1 arrest could be attributed to inhibition by endogenous cyclin-dependent kinase inhibitors (CKIs) that are negatively regulated by Notch activation.26 CKI p27 was up-regulated after GSI treatment in Notch-dependent human T-ALL cell lines.21 The exact mechanism by which Notch regulates D cyclins and their kinases remains to be explored.

Data presented here reveal a possible mechanism, downstream of deregulated Notch signaling, that may facilitate leukemic transformation. These observations identify cell-cycle components as adjunct therapeutic targets for designing combination treatment strategies using γ-secretase inhibitors for T-ALL treatment.

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Authorship

Contribution: I.J. designed and performed experiments and wrote the manuscript; L.M.M. and I.T. contributed to the design of experiments and interpretation of data; R.D. provided experimental samples for the analysis of TOP-Notch and MIG-NFκB mice; A.J.C., J.C.A., and P.S. provided essential reagents for experiments and were also involved in review of the manuscript; A.F. and T.E.G. provided GSI for in vitro and in vivo work; and B.A.O. provided support for the work, analyzed and interpreted the data, and reviewed the manuscript.

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Correspondence: Barbara A. Osborne, Department of Veterinary & Animal Sciences, 311 Paige Lab, University of Massachusetts, Amherst, MA 01003; e-mail: osborne@vasci.umass.edu.

References

Notch signaling mediates G1/S cell-cycle progression in T cells via cyclin D3 and its dependent kinases

Ila Joshi, Lisa M. Minter, Janice Telfer, Renée M. Demarest, Anthony J. Capobianco, Jon C. Aster, Piotr Sicinski, Abdul Fauq, Todd E. Golde and Barbara A. Osborne