Activating Mutations of *NOTCH1* in Human T Cell Acute Lymphoblastic Leukemia

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Very rare cases of human T cell acute lymphoblastic leukemia (T-ALL) harbor chromosomal translocations that involve *NOTCH1*, a gene encoding a trans-membrane receptor that regulates normal T cell development. Here, we report that more than 50% of human T-ALLs, including tumors from all major molecular oncogenic subtypes, have activating mutations that involve the extracellular heterodimerization domain and/or the C-terminal PEST domain of NOTCH1. These findings greatly expand the role of activated NOTCH1 in the molecular pathogenesis of human T-ALL and provide a strong rationale for targeted therapies that interfere with NOTCH signaling.

T-ALL is an aggressive cancer that preferentially affects children and adolescents. It is commonly associated with acquired chromososomal translocations and other genetic or epigenetic abnormalities, which lead to aberrant expression of a select group of transcription factors (*1*). *NOTCH1* was discovered as a partner gene in a (7;9) chromosomal translocation found in <1% of T-ALLs (*2*). It encodes a transmembrane receptor that is required for the commitment of pluripotent progenitors to T cell fate (*3*) and the sub-

sequent assembly of pre–T cell receptor complexes in immature thymocytes (*4*).

Cleavage of pro-NOTCH1 by a furinlike protease during transit to the cell surface (*5*) produces a NOTCH1 heterodimer comprised of noncovalently associated extracellular (NEC) and transmembrane (NTM) subunits (*6*). The heterodimerization domain (HD) responsible for stable subunit association consists of a 103 amino acid region of NEC (HD-N) and a 65 amino acid region in NTM (HD-C) (*7*). Physiologic activation of NOTCH receptors occurs when ligands of the Delta-Serrate-Lag2 (DSL) family bind to the NEC subunit and initiate a cascade of proteolytic cleavages in the NTM subunit. The final cleavage, catalyzed by γ-secretase (*8, 9*), generates intracellular NOTCH (ICN), which translocates to the nucleus and forms a large transcriptional activation complex that includes proteins of the Mastermind family (*10–12*).

Prior work has shown that enforced NOTCH1 signaling is a potent inducer of T-

ALL in the mouse (*13–15*) and is required to sustain the growth of a human t(7;9)-positive T-ALL cell line (*16*). To investigate the possibility of a more general role for NOTCH signaling in human T-ALL, we tested T-ALL cell lines lacking the t(7;9) for NOTCH dependency by treating these cells with a γ-secretase inhibitor (*17*). Of 30 human T-ALL cell lines tested, 5 showed a G_0/G_1 cell-cycle arrest that equaled or exceeded that of T6E, a reference NOTCH1-dependent murine T-ALL cell line (Fig. 1A). This drug-induced growth suppression was abrogated by retroviral expression of ICN1 (Fig. 1B) and reproduced (fig. S1) by retroviral expression of dominant negative Mastermindlike-1 (*16*). These results indicated that the growth of these five cell lines depends on NOTCH-transduced signals.

Because physical dissociation of the NOTCH extracellular domain has been linked to receptor activation (*6, 18*), we reasoned that the HD domain of NOTCH1 (*7*) could be the site of gain-of-function mutations. A second logical candidate region for oncogenic mutations is the negative regulatory PEST sequence lying at the C terminus of the NOTCH1 NTM (*19*), as retroviral insertions that cause deletion of this region have been reported in murine T-ALL (*14, 15*). Remarkably, sequencing revealed mutations that involve both the HD-N domain and the PEST domain in four of the five NOTCH-dependent cell lines (summarized in Fig. 2). Missense mutations affecting HD-N caused nonconservative changes at amino acid positions that are invariant in vertebrate NOTCH1 receptors (fig. S2). One cell line, DND-41, had two different HD-N mutations within the same *NOTCH1* allele. The PEST mutations were short insertions or deletions causing shifts in reading frame that are predicted to result in partial or complete deletion of the PEST domain (fig. S4). Sequencing of cDNAs revealed that the

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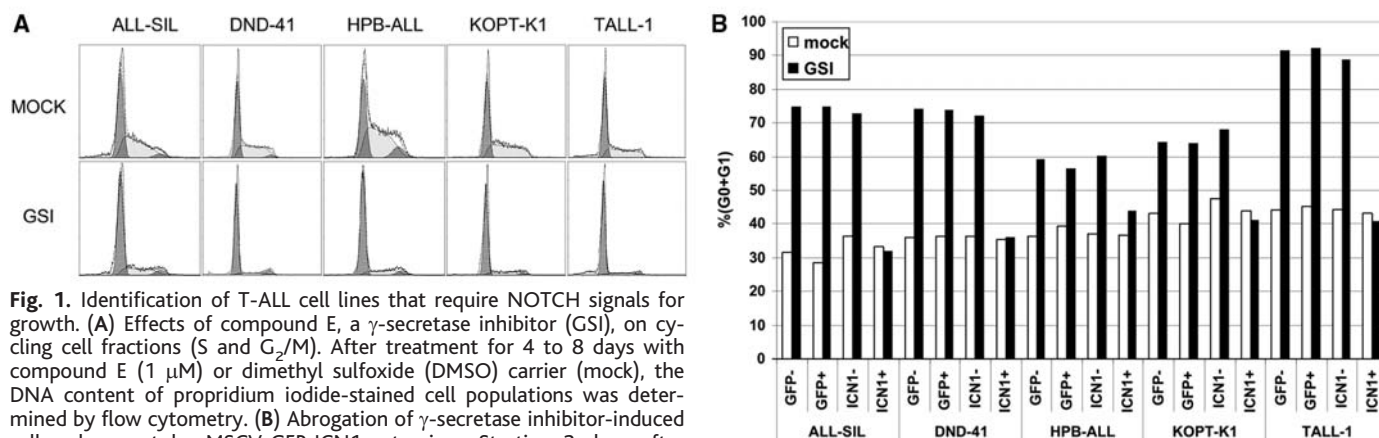
Fig. 1. Identification of T-ALL cell lines that require NOTCH signals for growth. (**A**) Effects of compound E, a γ-secretase inhibitor (GSI), on cycling cell fractions (S and G_2/M). After treatment for 4 to 8 days with compound E (1 μM) or dimethyl sulfoxide (DMSO) carrier (mock), the DNA content of propidium iodide-stained cell populations was determined by flow cytometry. (**B**) Abrogation of γ-secretase inhibitor-induced cell-cycle arrest by MSCV-GFP-ICN1 retrovirus. Starting 2 days after transduction with empty MSCV-GFP or MSCV-GFP-ICN1, cells were treated with either compound E (GSI, 1 μM) or DMSO carrier (mock) for 7 to 10 days. After staining with DRAQ5, the proportion of growth-arrested cells (G_0 + G_1 fractions) in the GFP⁻ and GFP⁺ subpopulations was determined by flow cytometry.
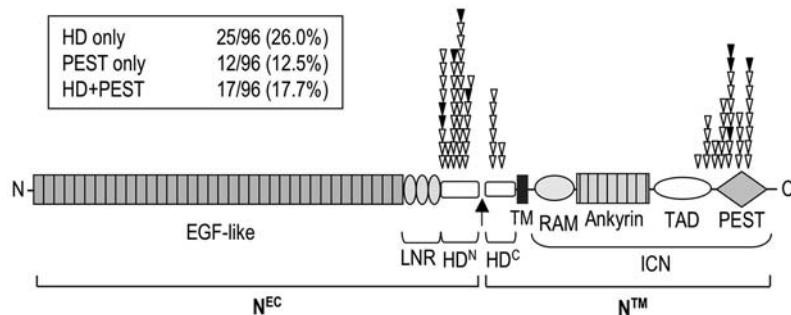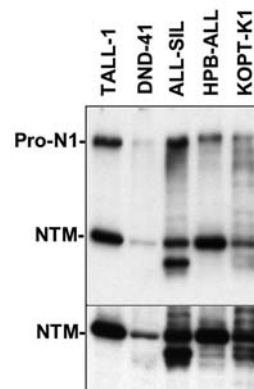
Fig. 2. NOTCH1 HD and PEST domain mutations in human T-ALL. A schematic representation of human NOTCH1 shows the distribution and frequency of HD-N, HD-C, and PEST domain mutations in NOTCH-dependent T-ALL cell lines (black arrowheads) and primary T-ALL samples (white arrowheads). N^{EC}, NOTCH1 extracellular domain; LNR, Lin/NOTCH repeats; HD-N and HD-C, N-terminal and C-terminal halves, respectively, of the heterodimerization domain; N^{TM}, NOTCH transmembrane subunit; TM, transmembrane domain; RAM, RAM domain; ANK, ankyrin repeat domain; TAD, transcriptional activation domain. The arrow denotes the site of furin cleavage.
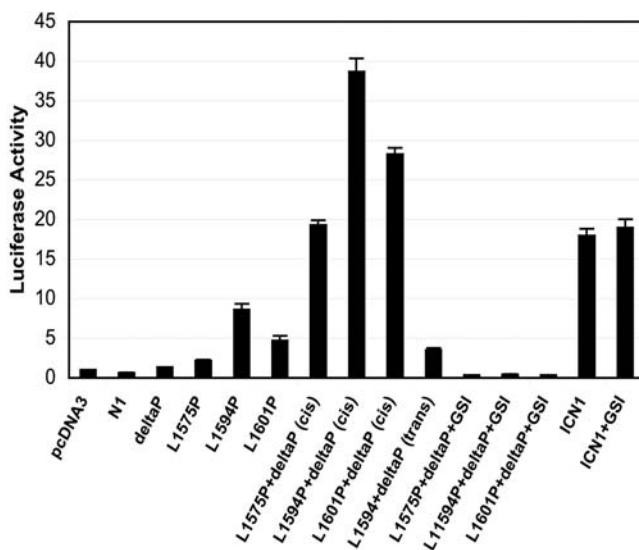

Fig. 3. Western blot analysis of lysates from NOTCH-dependent T-ALL cell lines. Whole-cell extracts were analyzed with a rabbit polyclonal antibody raised against the transcriptional activation domain of NOTCH1 (*13*). The lower panel is a longer exposure of the same blot that accentuates the presence of additional NOTCH1 polypeptides of smaller size than the normal NTM. Each lane contains 25 μg total protein.

HD-N and PEST domain mutations lie in cis in the same *NOTCH1* allele in each of the four cell lines tested (fig. S5). Normal *NOTCH1* cDNA clones were also identified in each cell line, indicating that both alleles are expressed. This is consistent with Western blot analysis (Fig. 3), which revealed that cell lines with HD-N and PEST domain mutations contained a polypeptide of the expected size of NTM and additional smaller polypeptides. We also sequenced a subset of T-ALL cell lines that were insensitive to the γ-secretase inhibitor. This revealed NOTCH1 mutations in 9 out of 19 nonresponsive cell lines, including three lines with dual HD and PEST domain mutations (table S1). The failure of all cell lines with mutations to respond to γ-secretase inhibitors may result from these cell lines having been maintained in tissue culture for many years.

We also identified frequent *NOTCH1* HD and PEST domain mutations in primary T-ALL samples obtained from the bone marrow of 96 children and adolescents at the time of diagnosis (summarized in Fig. 2). At least one mutation was identified in 54 tumors (56.2%). Mutations were seen in tumors associated with misexpression of *HOX11* (2 of 3 cases), *HOX11L2* (10 of 13, or 77%), *TAL1* (12 of 31, or 39%), *LYL1* (9 of 14, or 64%), *MLL-ENL* (1 of 3), or *CALM-AF10* (1 of 2) (table S2), which together define the major molecular subtypes of T-ALL (*1*). The HD domain mutations in primary tumors were clustered in a "hot spot" spanning residues 1574 to 1622 of HD-N and included each of the three L to P missense mutations originally identified in the *NOTCH*-dependent T-ALL cell lines, as well as deletions of 1 to 2 residues and short "in-frame" insertions (fig. S2). In addition, a smaller number of missense mutations were observed in HD-C, which again involved highly conserved residues (fig. S3). PEST domain mutations included insertions or deletions that induced a shift in reading frame

and point mutations that created premature stop codons (fig. S4). In contrast with T-ALLs, B cell ALLs (B-ALLs) (*n* = 89) showed no mutations in these regions of *NOTCH1* (*20*). Mutations were also absent from four remission bone marrow samples obtained from patients whose T-ALLs harbored *NOTCH1* mutations (*20*), indicating that these mutations are acquired within the malignant clones.

To prove that HD domain mutations found in T-ALL patients have effects on function, NOTCH-sensitive reporter-gene assays were conducted in human U2OS cells (Fig. 4). Single L to P mutations within the HD-N domain at residues 1575, 1594, or 1601 caused a 3- to 9-fold increase in luciferase activity, whereas a T-ALL–associated PEST deletion at position 2471 resulted in ~1.5- to 2-fold increase. More strikingly, each HD mutation and the same PEST domain truncation in cis resulted in 20- to 40-fold increases; in contrast, the same mutations in trans produced lesser effects close to the average of each mutation acting alone. The synergistic interaction of HD and PEST domain mutations in cis is consistent with a model (fig. S6) in which (i) HD domain mutations enhance γ-secretase cleavage and increase the rate of production of ICN1 and (ii) truncations that remove the PEST domain increase ICN1 half-life (*19*). The intermediate levels of activation produced by these mutations in trans presumably reflect competition between relatively weak and strong gain-of-function NOTCH1 polypeptides for factors required for processing and signaling. The stimulatory effects of mutated transmembrane NOTCH1 polypeptides were completely abrogated by a γ-secretase inhibitor (Fig. 4), which indicates a requirement for proteolysis at the juxtamembrane site of NTM for signal transduction. In contrast, the stimulation produced by ICN1, which is constitutively nuclear, was unaffected by γ-secretase inhibition (Fig. 4).

Several factors may explain the high frequency of *NOTCH1* mutations in T-ALL. The requirement for NOTCH1 signals during several stages of normal early T cell development provides a functional basis for its frequent involvement. Unlike the t(7;9), which is created during attempted V-D-Jβ rearrangement in committed T cell progenitors, the common point mutations and insertions described here could occur in multipotent hematopoietic progenitors, which normally express *NOTCH1* (*21*). This would be predicted to induce daughter cells to adopt a T cell fate (*22*) and thereby increase the pool of cells at risk for additional leukemogenic events, such as synergistic mutations affecting *NOTCH1* and misexpression of other critical transcription factors. The *NOTCH1* mutations we describe here are currently specific to human T-ALL among vertebrate cancers, but a mutation involving the putative HD domain of the NOTCH homolog GLP-1 causes massive germ-cell proliferation in *Caenorhabditis elegans* (*23*), which suggests that such mutations have a highly conserved capacity to cause abnormal growth in specific cellular contexts.

Of potential clinical relevance, our findings identify the NOTCH pathway as a rational molecular therapeutic target in T-ALL. Although up to 75% of T-ALL patients are currently cured with very intensive cytotoxic chemotherapy regimens (*24*), new therapies are needed for patients with refractory disease, and less toxic, more efficacious drug combinations would be generally beneficial. Potent, specific inhibitors have already been developed (*25*) as a result of the involvement of γ-secretase in the production of amyloidogenic peptides in patients with Alzheimer's disease. On the basis of the results reported

Fig. 4. HD and PEST domain mutations activate NOTCH1 signaling synergistically. Human U2OS cells were transiently co-transfected in 24-well format with the indicated pcDNA3 plasmids, a NOTCH-responsive luciferase reporter gene, and an internal *Renilla* luciferase internal control plasmid, as described previously (*13*). Twenty-five ng of pcDNA3 plasmid was used per well, except for experiments with pcDNA3-ICN1, in which 5 ng of plasmid per well was used. "DeltaP" denotes the presence of a deletion removing NOTCH1 residues 2473 to 2556. Normalized luciferase activities in whole-cell lysates were determined in triplicate and expressed relative to the activity in lysates prepared from vector-transfected cells. Error bars represent standard deviations.

1. A. A. Ferrando *et al.*, *Cancer Cell* **1**, 75 (2002).
2. L. W. Ellisen *et al.*, *Cell* **66**, 649 (1991).
3. F. Radtke *et al.*, *Immunity* **10**, 547 (1999).
4. A. Wolfer, A. Wilson, M. Nemir, H. R. MacDonald, F. Radtke, *Immunity* **16**, 869 (2002).
5. F. Logeat *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8108 (1998).
6. M. D. Rand *et al.*, *Mol. Cell. Biol.* **20**, 1825 (2000).
7. C. Sanchez-Irizarry *et al.*, *Mol. Cell. Biol.*, in press.
8. R. Francis *et al.*, *Dev. Cell* **3**, 85 (2002).
9. W. T. Kimberly *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 6382 (2003).
10. A. E. Wallberg, K. Pedersen, U. Lendahl, R. G. Roeder, *Mol. Cell. Biol.* **22**, 7812 (2002).
11. C. J. Fryer, E. Lamar, I. Turbachova, C. Kintner, K. A. Jones, *Genes Dev.* **16**, 1397 (2002).
12. Y. Nam, A. P. Weng, J. C. Aster, S. C. Blacklow, *J. Biol. Chem.* **278**, 21232 (2003).
13. J. C. Aster *et al.*, *Mol. Cell. Biol.* **20**, 7505 (2000).
14. C. D. Hoemann, N. Beaulieu, L. Girard, N. Rebai, P. Jolicoeur, *Mol. Cell. Biol.* **20**, 3831 (2000).
15. B. J. Feldman, T. Hampton, M. L. Cleary, *Blood* **96**, 1906 (2000).
16. A. P. Weng *et al.*, *Mol. Cell. Biol.* **23**, 655 (2003).
17. Materials and methods are available as supporting material on *Science* Online.
18. H. Kramer, *Sci. STKE* **2000**, pe1 (2000).
19. N. Gupta-Rossi *et al.*, *J. Biol. Chem.* **276**, 34371 (2001).
20. J. C. Aster, A. A. Ferrando, A. P. Weng, unpublished data.
21. L. M. Calvi *et al.*, *Nature* **425**, 841 (2003).
22. D. Allman *et al.*, *J. Exp. Med.* **194**, 99 (2001).
23. L. W. Berry, B. Westlund, T. Schedl, *Development* **124**, 925 (1997).
24. C. H. Pui, M. V. Relling, J. R. Downing, *N. Engl. J. Med.* **350**, 1535 (2004).
25. M. S. Wolfe, *Nature Rev. Drug Discov.* **1**, 859 (2002).
26. G. T. Wong *et al.*, *J. Biol. Chem.* **279**, 12876 (2004).
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here, clinical trials are warranted to test the efficacy and potential side effects (*26*) of this class of NOTCH-pathway inhibitor in patients with T-ALL.

Jun Turnover Is Controlled Through JNK-Dependent Phosphorylation of the E3 Ligase Itch

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The turnover of Jun proteins, like that of other transcription factors, is regulated through ubiquitin-dependent proteolysis. Usually, such processes are regulated by extracellular stimuli through phosphorylation of the target protein, which allows recognition by F box–containing E3 ubiquitin ligases. In the case of c-Jun and JunB, we found that extracellular stimuli also modulate protein turnover by regulating the activity of an E3 ligase by means of its phosphorylation. Activation of the Jun amino-terminal kinase (JNK) mitogen-activated protein kinase cascade after T cell stimulation accelerated degradation of c-Jun and JunB through phosphorylation-dependent activation of the E3 ligase Itch. This pathway modulates cytokine production by effector T cells.

Ubiquitin-dependent proteolysis controls turnover and abundance of transcription factors and other regulatory proteins (*1*). Protein ubiquitination requires the concerted action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) (*2*, *3*). Extracellular stimuli can regulate protein turnover through inducible substrate phosphorylation which confers recognition by F box–containing E3 ligases (*4*). Such E3 ligases, which are devoid of catalytic activity, recognize only the phosphorylated forms of their substrates (*5*). Transcription factors regulated through ubiquitin-dependent turnover include the Jun proteins, components of the AP-1 transcription factor. The activity of c-Jun and JunB is enhanced by phosphorylation of their transcriptional activation domain by JNKs (*6*, *7*). JNK-dependent phosphorylation can also stabilize c-Jun (*8*, *9*). Recently, however, JNK-mediated phosphorylation was shown to accelerate c-Jun degradation by allowing its recognition by the E3 ligase Fbw7-containing Skp/Cullin/F-box protein complex (SCF^Fbw7) (*10*). Here, we provide physiological and biochemical evidence for another pathway through which extracellular stimuli control c-Jun and JunB abundance. This process is based on inducible phosphorylation of an E3 ligase of the homology to the E6-associated protein C terminus (HECT) family, which increases its catalytic activity.

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