Plenary paper

Deletion-based mechanisms of Notch1 activation in T-ALL: key roles for RAG recombines and a conserved internal translational start site in Notch1

Todd D. Ashworth,1 Warren S. Pear,2 Mark Y. Chiang,3,4 Stephen C. Blacklow,1 Jérôme Mastio,5 Lanwei Xu,2 Michelle Kelliher,6 Philippe Kastner,5 Susan Chan,5 and Jon C. Aster1

1Department of Pathology, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA; 2Department of Pathology and 3Division of Hematology/Oncology, Department of Medicine, Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, PA; 4Division of Hematology and Oncology, University of Michigan Cancer Center, Ann Arbor, MI; 5Institut de Génétique et de Biologie Moléculaire et Cellulaire, Department of Cancer Biology, Université de Strasbourg, Strasbourg, France; and 6Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA

Point mutations that trigger ligand-independent proteolysis of the Notch1 ectodomain occur frequently in human T-cell acute lymphoblastic leukemia (T-ALL) but are rare in murine T-ALL, suggesting that other mechanisms account for Notch1 activation in murine tumors. Here we show that most murine T-ALLs harbor Notch1 deletions that fall into 2 types, both leading to ligand-independent Notch1 activation. Type 1 deletions remove exon 1 and the proximal promoter, appear to be RAG-mediated, and are associated with mRNA transcripts that initiate from 3′ regions of Notch1. In line with the RAG dependency of these rearrangements, RAG2 binds to the 5′ end of Notch1 in normal thymocytes near the deletion breakpoints. Type 2 deletions remove sequences between exon 1 and exons 26 to 28 of Notch1, appear to be RAG-independent, and are associated with transcripts in which exon 1 is spliced out of frame to 3′ Notch1 exons. Translation of both types of transcripts initiates at a conserved methionine residue, M1727, which lies within the Notch1 transmembrane domain. Polypeptides initiating at M1727 insert into membranes and are subject to constitutive cleavage by γ-secretase. Thus, like human T-ALL, murine T-ALL is often associated with acquired mutations that cause ligand-independent Notch1 activation. (Blood. 2010;116(25):5455-5464)

Introduction

Notch receptors participate in a signaling pathway of broad importance in development, immunity, and disease, including cancer. The clearest association of Notch and cancer is in T cell acute lymphoblastic leukemia/lymphoma (T-ALL).1 Somatic gain-of-function mutations in Notch1 occur in the majority of human and murine T-ALLs, but the most common mutations reported to date differ in kind between the 2 species. In human T-ALL, the most frequent Notch1 mutations are point substitutions or small in-frame insertions or deletions in the Notch1 negative regulatory region (NRR),2 an extracellular domain composed of 3 Lin12/Notch repeats and a heterodimerization domain that holds Notch receptors in the “off-state” in the absence of ligand.3 NRR mutations abrogate NRR function4,5 and lead to successive ligand-independent activations. The first cleavage is carried out by metallo-proteases of the ADAM family6 at a site just external to the transmembrane domain, which primes the protein for cleavage within its transmembrane domain by γ-secretase.7 γ-Secretase cleavage releases intracellular Notch1 (ICN1), allowing it to translocate to the nucleus and form a transcription activation complex with the DNA-binding factor CSL and coactivators of the Mastermind-like family.

In contrast, the most common Notch1 mutations in murine T-ALL described to date are stop codon or frameshift mutations that result in deletion of a C-terminal PEST degron domain. PEST deletions occur at frequencies of 30% to 80% in murine T-ALL, depending on the genetic background.8-12 PEST deletions also occur in 10% to 20% of human T-ALLs, often in cis with NRR mutations in the same allele.2 When combined with NRR mutations, PEST deletions cause synergistic increases in Notch1 signal dose by stabilizing ICN1, but PEST deletions alone have little or no intrinsic signaling activity and are not oncogenic.12 Of note, most cell lines derived from murine T-ALLs have detectable ICN1 and are sensitive to γ-secretase inhibitors (GSIs),8-12 indicating a dependency on Notch signaling for growth and survival. Given the absence of NRR mutations, the basis for Notch1 activation in murine T-ALL has been unclear. A clue comes from studies of murine T-ALLs arising after irradiation or in the context of RAG or ATM deficiency.13,15 Such tumors often have deletions involving 5′ portions of Notch1, but the relevance of these events to Notch1 activation in other genetic contexts has not been explored.

In this report, we describe 2 types of somatic deletions in the 5′ end of Notch1 in murine T-ALLs that cause ligand-independent Notch1 activation. Both types of deletions create Notch1 alleles that express truncated mRNAs encoding Notch1 polypeptides lacking the NRR. These findings highlight 2 common mechanisms of Notch1 activation in murine T-ALL and support the existence of strong selection for ligand-independent activation of Notch1 in both human and murine disease.

Methods

Cell culture

Mouse T-ALL cell lines were cultured in Opti-MEM medium supplemented with 8% fetal bovine serum, 1% penicillin/streptomycin, 1 mM glutamine, the relevant data supplement and payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734.

© 2010 by The American Society of Hematology
and 0.1% β-mercaptoethanol. Human CUTLL1 T-ALL cells were grown in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. U2OS cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were maintained at 37°C less than 5% CO₂.

Cell growth assays
Approximately 1 × 10⁶ cells/well in 96-well plates were cultured in the presence of human IgG (10 µg/mL), anti-Notch1 inhibitory antibodies (10 µg/mL), or 1 µM compound E (Tocris). Cell growth was assessed 24, 48, and 72 hours after treatment using the Cell Titer Glo viability assay (Promega). Treatments were performed in triplicate.

IC1N reconstitution assays
MigIR retroviruses were used to transduce T-ALL cells as described. Transduced cells were treated with vehicle or the GSI compound E (1 µM; Tocris) for 72 hours. Cells were stained with propidium iodide, and sub-G₀/G₁ fractions were determined by flow cytometry as described.

Northern blot analysis
Total RNA (50 µg) was isolated with Trizol (Invitrogen) and subjected to polyA+ selection on magnetic oligo-dT beads (Invitrogen). PolyA-RNA was denatured, electrophoresed in formaldehyde–0.8% agarose gels, and transferred onto nylon membranes (GE Healthcare, Hybond-XL) in 5× saline sodium citrate–10mM NaOH. Membranes were UV cross-linked (Stratagene, QuikHyb) at 68°C for 10 minutes. DNA probes (10 µg) were labeled with [α³²P]-dCTP using Ready-To-Go beads (GE Healthcare). Hybridization was performed at 68°C for 1 hour followed by 2 washes with 2 times saline sodium citrate for 15 minutes at 68°C and 1 wash with 1 time saline sodium citrate for 30 minutes at 68°C.

Southern blot analysis
Genomic DNA (10 µg) was digested with EcoRI (8 U/µg DNA) at 37°C overnight. After electrophoresis in 0.8% agarose gels, the DNA was depurinated for 20 minutes in 0.25M HCl and denatured in 0.4M NaOH for 1 hour at 25°C. DNA was transferred onto nylon membranes overnight in 0.4M NaOH. Hybridization conditions, DNA probe labeling, and subsequent washing were carried out as described for Northern blot analysis.

Western blot analysis
Whole-cell detergent lysates or T-ALL cells or transiently transfected U2OS cells were analyzed on Western blots stained with antibodies against activated ICN1-specific antibody (V-1744, Cell Signaling Technologies), β-actin (Cell Signaling Technologies), or the N-terminus of Ikaros (H-100, Santa Cruz Biotechnology).

Notch1 and Ikaros mutation analysis
Exons 26, 27, and 34 of murine Notch1 were amplified from genomic DNA according to Lin et al and sequenced. The Notch1 mutational status of the Tal1 transgenic cell lines has been described. The region of Ikaros encoding the zinc finger domains (nucleotides 373-1001) was polymerase chain reaction (PCR) amplified from T-ALL cell line cDNAs and sequenced.

5′-RACE
5′-Rapid amplification of cDNA ends (RACE) with total RNA (5 µg) was done using the FirstChoice RLM-RACE kit (Ambion). Products were amplified using primer specific for the 5′-adapter and Notch1 exon 27 with the Expand Long Template kit (Roche Diagnostics) and the following conditions: 94°C for 2 minutes; 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 5 minutes, 32 cycles.

Results
GSI-sensitive murine T-ALLs are resistant to Notch1-selective inhibitory antibodies
Given the absence of known abnormalities involving the Notch1 ectodomain in murine T-ALL, we anticipated that Notch1-selective inhibitory antibodies specific for the ligand-binding domain (EGF repeats 11-13) or the NRR of Notch1 would suppress the growth of GSI-sensitive murine T-ALL lines. However, all 3 GSI-sensitive T-ALL cell lines tested were completely resistant to both types of inhibitory Notch1 antibodies (supplemental Figure 1, available on the Blood Web site; see the Supplemental Material link at the top of the online article). To confirm that GSI was acting through Notch1 inhibition, we tested whether IC1N, which is downstream of γ-secretase, could rescue the lines from γ-secretase inhibition. Supplemental Figure 1 shows a representative result, in which transduction of ICN1 into the cell line 330 abrogated GSI-induced cell death.
Table 1. Murine T-ALL cell lines

<table>
<thead>
<tr>
<th>Line</th>
<th>Genetic background</th>
<th>GSI-sensitive</th>
<th>PEST mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>140</td>
<td>Kras-G12D</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>144</td>
<td>Kras-G12D</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>171</td>
<td>Kras-G12D</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>330</td>
<td>Kras-G12D</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>385</td>
<td>Kras-G12D</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>430</td>
<td>Kras-G12D</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>435</td>
<td>Kras-G12D</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>SCID-adh</td>
<td>SCID-adh</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>G4A2</td>
<td>Bcr-Ab1 retrovirus</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>130</td>
<td>Tal1 transgenic/Heb</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>135.1</td>
<td>Tal1 transgenic/Heb</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>135.2</td>
<td>Tal1 transgenic/Heb</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>720</td>
<td>Tal1 transgenic/Heb</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5406</td>
<td>Tal1 transgenic/p16</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Murine T-ALLs express truncated Notch1 transcripts

The insensitivity of murine T-ALLs to inhibitory antibodies suggested that these lines expressed constitutively active Notch1 polypeptides without intact ectodomains. To investigate this possibility, we collected 14 murine T-ALL cell lines derived from tumors arising in diverse genetic backgrounds (Table 1), and initially selected 10 of these cell lines for detailed study. These lines all have detectable ICN1 (supplemental Figure 2).

Northern blot analysis performed with a probe from exon 34, the most 3' Notch1 exon, revealed that in addition to full-length Notch1 transcripts of approximately 10 kb in size, all 10 cell lines expressed major Notch1 transcripts of approximately 5.5 kb and approximately 4.5 kb in size (Figure 1A and data not shown). Additional Northern blots revealed that the short Notch1 mRNAs were of 2 types. In 7 cell lines, the short transcripts hybridized to an exon 26-specific probe; whereas in 3 cell lines (SCID-adh and the lines 135.1 and 135.2, subclones derived from a single primary tumor), no hybridization was observed (Figure 1B). By contrast, an exon 1-specific probe hybridized to the short transcripts in SCID-adh, 135.1, and 135.2, subclones derived from a single primary tumor), no hybridization was observed (Figure 1B). By contrast, an exon 1-specific probe hybridized to the short transcripts in SCID-adh, 135.1, and 135.2, cells and not to the short transcripts in the other 7 cell lines (Figure 1C; and data not shown). We designated the short transcripts that contain exon 26 and lack exon 1 “type 1” transcripts and the short transcripts that contain exon 1 and lack exon 26 “type 2” transcripts. Tsuji et al used RT-PCR to detect Notch1 transcripts in murine T-ALLs that initiated in alternative 5’ exons they designated 1a and 1b.13 However, probes specific for exons 1a and 1b failed to detect Notch1 transcripts on Northern blots in any of our lines (data not shown), suggesting that such transcripts are of low abundance and unlikely to be of functional significance.

Two polyadenylation sites have been identified in the 3'-untranslated region of Notch1.13 Only the longest type 1 and type 2 transcripts hybridized to a probe lying 3' of the first polyadenylation site (supplemental Figure 3; and data not shown). The same probe detected the approximately 10-kb Notch1 transcripts in normal thymus and the T-ALL cell lines (data not shown) but did not hybridize to the approximately 9-kb Notch1 transcript in G4A2 cells (supplemental Figure 3). These results are consistent with 2 alternative 3' polyadenylation sites in the type 1 and type 2 Notch1 transcripts, as well as in the long Notch1 transcripts expressed in G4A2 cells.

Structure of truncated Notch1 transcripts

The absence of exon 1 sequences from type 1 transcripts suggested an origin from an internal transcriptional start site. 5'-RACE using cDNA prepared from 144 cells produced several products, all with 5’ ends within exon 25 at nucleotides 35184, 35219, and 35243, respectively (Figure 2A); here and elsewhere, the nucleotide numbering used for the Notch1 is relative to the A residue in the ATG start codon in exon 1, which is designated position 1. The longest RACE product began at an A residue within the sequence TCACCTT, a sequence conserved between mouse and humans that matches the consensus sequence for an initiator (Inr) element (YYANWYY). Additional Northern blots showed that the major type 1 transcripts hybridized to a 3’ exon 25 probe and failed to hybridize to a 5’ exon 25 probe (Figure 2B). Thus, type 1 transcripts initiate mainly within or immediately 3’ of exon 25, which encodes the second Lin12/Notch repeats of Notch1.

Guided by the Northern blot data, we were able to determine the structure of type 2 transcripts by performing RT-PCR with primers specific for exon 1 and exon 28. All lines expressing type 2 transcripts yielded RT-PCR products (Figure 2C), whereas cell lines expressing type 1 transcripts did not. Sequencing of the...
RT-PCR products from 135.1 and 135.2 cells revealed a transcript in which exon 1 was spliced out of frame to the exon 27 via the normal splice donor and acceptor sites. In contrast, SCID-adh cells yielded a transcript consisting of exon 1 and 81 bp of noncontiguous intron 1 sequence joined to exon 28 at a site 12 bp 3’ of the exon 28 splice acceptor site. The point of joining of exon1 to intron 1 lay immediately 3’ of a consensus splice acceptor sequence (CAG) in intron 1 germline sequences, consistent with splicing of exon1 to a cryptic intron 1 acceptor site.

Association of aberrant transcripts with 5’ Notch1 deletions
To determine the origin of type 1 and type 2 transcripts, we assessed the integrity of Notch1 by Southern blot analysis (Figure 3). Probe A, specific for a region lying approximately 12 kb 5’ of exon 1, detected genomic rearrangements in all 7 cell lines expressing type 1 transcripts, whereas cell lines expressing type 2 transcripts gave germline bands (Figure 3A). The rearranged fragment detected with probe A in the cell line 130 was slightly...
smaller than the rearranged fragments in other “type 1” cell lines, whereas the rearranged fragment in the G4A2 cell line was amplified at least several-fold relative to the germline fragment.

Southern blots hybridized to probes specific for sequences lying approximately 4.8 kb 5’ of exon 1 (B) or within exon 1 (C) detected genomic rearrangements in cell lines expressing type 2 transcripts, but not in cell lines expressing type 1 transcripts (Figure 3B-C). The cell line 135.2 showed a rearranged band and no germline band with probe C, which is consistent with the absence of normal Notch1 transcripts in this line (Figure 1). Additional Southern blots (data not shown) hybridized to probes specific for the region from intron 2 to intron 8 revealed that 135.1 and SCID-adh DNA yielded germline-sized restriction fragments of reduced signal intensity and that 135.2 cells produced no hybridization signals, suggesting that these lines all have intragenic Notch1 deletions involving regions 3’ of exon 1.

In the course of our studies, we became aware of the work of Jeannet et al showing that the expression of truncated Notch1 transcripts could be induced by Cre-mediated deletion of exon 1 and proximal Notch1 promoter sequences. Based on this insight, the Southern blot data, and prior work by Tsuji et al, we designed PCR s that allowed us to deduce a common deleted region of exon 1 and proximal Notch1 promoter sequences. Based on this insight, the Southern blot data, and prior work by Tsuji et al, we designed PCRs that allowed us to deduce a common deleted region of exon 1 and proximal Notch1 promoter sequences.

We subsequently screened 4 additional cell lines (140, 171, 385, and 5406, described in Table 1) for the presence of type 1 deletions by PCR, all of which were all positive (Figure 4A). In addition, using this PCR method, Jeannet et al detected deletions involving the same RSS sites in approximately 75% of T-ALLs arising in an Ikaro hypomorphic background.

To further explore the possible role of RAG in type 1 rearrangements, we mined the ChIP-Seq data of Ji et al to determine whether RAG2 associates with the 5’ end of Notch1 in normal thymocytes. Binding of RAG2 near the transcriptional start site in exon 1 of Notch1 was readily detectable in wild-type thymocytes and in thymocytes expressing RAG1 with a D708A mutation that is permissive for binding to chromatin but abolishes RAG catalytic function (Figure 4B). The major RAG2 binding peak overlapped with the ChIP-Seq peak for histone H3K4 trimethylation (H3K4-me3), an activation mark that may be recognized by the plant homology domain of RAG2. Thus, RAG2 associates with Notch1 in thymocytes near the ectopic RSSs that give rise to type 1 rearrangements.

Sequencing of RT-PCR products and Southern blot results made it probable that the deletions associated with the type 2 transcripts extended close to exon 27 in 135.1 cells and 135.2 cells and exon 29 in SCID-adh cells. A PCR strategy based on this reasoning amplified the DNA sequences flanking the deleted regions in these cell lines (Figure 4A). We subsequently screened 5 additional cell lines (140, 171, 385, and 5406, described in Table 1) for the presence of type 1 deletions by PCR, all of which were all positive (Figure 4A). In addition, using this PCR method, Jeannet et al detected deletions involving the same RSS sites in approximately 75% of T-ALLs arising in an Ikaro hypomorphic background.

To further explore the possible role of RAG in type 1 rearrangements, we mined the ChIP-Seq data of Ji et al to determine whether RAG2 associates with the 5’ end of Notch1 in normal thymocytes. Binding of RAG2 near the transcriptional start site in exon 1 of Notch1 was readily detectable in wild-type thymocytes and in thymocytes expressing RAG1 with a D708A mutation that is permissive for binding to chromatin but abolishes RAG catalytic function (Figure 4B). The major RAG2 binding peak overlapped with the ChIP-Seq peak for histone H3K4 trimethylation (H3K4-me3), an activation mark that may be recognized by the plant homology domain of RAG2. Thus, RAG2 associates with Notch1 in thymocytes near the ectopic RSSs that give rise to type 1 rearrangements.

Sequencing of RT-PCR products and Southern blot results made it probable that the deletions associated with the type 2 transcripts extended close to exon 27 in 135.1 cells and 135.2 cells and exon 29 in SCID-adh cells. A PCR strategy based on this reasoning amplified the DNA sequences flanking the deleted regions in these cell lines (Figure 4A). We subsequently screened 5 additional cell lines (140, 171, 385, and 5406, described in Table 1) for the presence of type 1 deletions by PCR, all of which were all positive (Figure 4A). In addition, using this PCR method, Jeannet et al detected deletions involving the same RSS sites in approximately 75% of T-ALLs arising in an Ikaro hypomorphic background. We subsequently screened 4 additional cell lines (140, 171, 385, and 5406, described in Table 1) for the presence of type 1 deletions by PCR, all of which were all positive (Figure 4A). In addition, using this PCR method, Jeannet et al detected deletions involving the same RSS sites in approximately 75% of T-ALLs arising in an Ikaro hypomorphic background.
135.1 and 135.2 cells harbored the same complex genomic rearrangement (Figure 4A). In both lines, breakpoints lying within intron 1 and exon 26 were joined via a 184-bp insertion derived from a region approximately 21 kb 5′ of exon 1. The 3′ end of the inserted region contains a 7-bp sequence homologous to DNA at the site of breakage in exon 26, suggesting the involvement of microhomology-mediated end-joining, a mechanism implicated in many chromosomal aberrations found in lymphoid malignancies.23 None of the genomic breakpoints in lines expressing type 2 transcripts has recognizable flanking RSS-like sequences.

**Ikaroś mutations are common, but not universal, in cell lines expressing aberrant Notch1 transcripts**

Jeannot et al note that aberrant Notch1 transcripts are expressed by tumors arising in Ik L/L mice bearing a germline hypomorphic Ikaros mutation and a conditional deletion of exon 1 of tumors arising in Ik L/L mice bearing a germline hypomorphic transcription from the 3′ end of Ikaros. Ikaros function might be a key factor in the activation of loss-of-function phenotypes in prior work, suggesting that loss of Ikaros function might be a key factor in the activation of transcription from the 3′ end of Notch1. Ikaros function can be lost because of mutations that create splice variants encoding smaller, dominant negative protein isoforms, or by point mutations involving the zinc-finger region that abrogate binding to DNA. Western blots (supplemental Figure 4) revealed Ikaroś isoforms consistent with dominant negative polyepitides in 2 of the type 2 lines (135.1 and 135.2) and one of the type 1 lines (130). In addition, SCID.adh cells (a type 2 line) contained decreased amounts of an Ikaroś isoform of approximately normal size. Five of the remaining cell lines were analyzed by sequencing of Ikaroś cDNAs. A heterozygous T to C mutation that results in a L188P substitution in the third zinc finger was found in the 435 line, and a heterozygous G to A heterozygous T to C mutation that results in a L188P substitution in the third zinc finger was found in the 720 line (data not shown). Both mutations are close to the position of other Ikaroś mutations described in murine T-ALL in previous studies by other workers in the field24 and probably have deleterious effects. The remaining lines, 130, 330, and 430, had wild-type Ikaroś sequences.

These data are consistent with past work showing that Ikaroś loss-of-function mutations are found in a substantial fraction of murine T-ALL24 and are strongly selected in the context of Notch1 activation.25,26 However, because 3 of the 6 lines with type 1 deletions we analyzed expressed apparently normal forms of Ikaroś, loss of Ikaroś function does not appear to be essential for activation of transcription from the 3′ end of Notch1.

**An intramembranous methionine residue serves as the translational start site in aberrant Notch1 transcripts**

Murine T-ALLs are generally Notch1-dependent and sensitive to inhibition by γ-secretase (supplemental Figure 1 and data not shown).5,8,10,12,16 which cleaves intramembranously.7 Virtual translation of type 2 transcripts revealed that the start codon in exon 1 was either spliced out of frame (135.1 and 135.2 cells) or was followed by an intronic stop codon (SCID.adh cells; Figures 2C, 5A). As a result, the first in-frame ATG in both kinds of type 2 transcripts encodes M1727, a residue lying within the N-terminal portion of the Notch1 transmembrane domain. Virtual translation of the longest type 1 transcript showed the presence of 3 in-frame ATG codons, M1616, M1659, and M1727 (Figure 5A). M1616 and M1659 are located in the C-terminal portion of the HD domain, making it uncertain whether the resulting polypeptides would insert into membranes. In contrast, a polypeptide initiating with M1727 would have a hydrophobic N-terminus that could promote membrane insertion.

The same floxed Ikaros mutation and a conditional deletion of exon 1 of tumors arising in Ik L/L mice bearing a germline hypomorphic transcription from the 3′ end of Notch1 might be a key factor in the activation of loss-of-function phenotypes in prior work, suggesting that loss of the position of other zinc finger was found in the 435 line, and a heterozygous G to A mutation that results in a L188P substitution in the third zinc finger was found in the 720 line (data not shown). Both mutations are close to the position of other Ikaroś mutations described in murine T-ALL in previous studies by other workers in the field and probably have deleterious effects. The remaining lines, 130, 330, and 430, had wild-type Ikaroś sequences.

These data are consistent with past work showing that Ikaroś loss-of-function mutations are found in a substantial fraction of murine T-ALL, and are strongly selected in the context of Notch1 activation. However, because 3 of the 6 lines with type 1 deletions we analyzed expressed apparently normal forms of Ikaroś, loss of Ikaroś function does not appear to be essential for activation of transcription from the 3′ end of Notch1.

**Notch1 deletions and truncated transcripts appear before extrathyMIC spread of T-ALL**

IntrathyMIC T-cell development depends on activation of Notch1 by ligands, such as DLL4,9 expressed on thymic stromal cells. If ligand is limiting within the thymic niche, cells acquiring Notch1 mutations that lead to ligand-independent activation would gain a selective advantage during intrathyMIC stages of T-ALL development. To explore this issue, we analyzed primary thymic T-ALLs arising in Tal1/Lmo2 transgenic mice. PCR amplification of genomic DNAs from 2 of 3 primary thymic T-ALLs yielded products that were proven by sequencing to contain “type 1” Notch1 deletions (Figure 6A). A ratiometric quantitative RT-PCR assay that compares the levels of Notch1 5′ and 3′ transcripts revealed a significant excess of 3′ transcripts in the 2 tumors with type 1 deletions as well as in positive control T-ALL cell lines expressing type 1 (144) or type 2 (135.2) transcripts (Figure 6B). By contrast, normal thymocytes and a primary tumor lacking Notch1 deletions expressed equal numbers of 5′ and 3′ transcripts. These results indicate that deletions that produce ligand-independent Notch1 activation are selected during intrathyMIC stages of T-ALL development.
Discussion

We have described 2 deletion-based mechanisms of Notch1 activation in detail (summarized in Figure 7). “Type 1” deletions appear to be RAG-mediated, remove the 5' proximal promoter and exon 1 of Notch1, and activate transcription from internal sites in or adjacent to exon 25. RAG activity appears early in T-cell development in CD4+CD8-“double-negative” cells and is maintained through the early CD4+CD8+ double-positive stage of T-cell development, defining a developmental window within which these deletions occur. Of note, “ChIP-Seq” analyses show RAG2 binding to the 5' end of Notch1 in normal thymocytes in a distribution that is largely coincident with H3K4-me3 marks, which are enriched in the proximal promoters of transcribed genes. Presumably, once RAG2 binds the Notch1 promoter, it can recruit RAG1, forming a functional recombinase that may catalyze deletions involving nearby ectopic RSSs. “Type 2” deletions remove DNA between exon 2 to exons 26 to 28, leading to expression of aberrant Notch1 mRNA splice variants from the 5' proximal promoter. These deletions are similar to those seen in radiation-induced murine T-ALLs and presumably arise through random DNA breaks and either nonhomologous end joining reactions or microhomology-based DNA repair.

Prior work has pointed to the importance of truncated Notch1 transcripts similar to those described here. In the original report, chromosomal translocations involving NOTCH1 in human T-ALL, the DNA breaks in NOTCH1 clustered in intron 24, which was joined to TCRβ enhancer/promoter sequences. The NOTCH1 transcriptional start sites in these T-ALLs mapped to exon 25, close to the conserved initiator (Inr) site identified in murine tumors with type 1 deletions. Other studies have identified the 5' portion of
Notch1 spanning the region from exon 25 through intron 27 as a common site of retrovirus or transposon insertion in murine T-ALL.32-34 Finally, Tsuji et al described T-ALLs arising in irradiated ATM−/− and SCID mice that were associated with 5′ deletions in Notch1 and abnormally short Notch1 transcripts.13-15 Our work extends these studies to show that 5′ deletions in Notch1 are common in tumors arising independent of irradiation in a variety of T-ALL-prone genetic backgrounds.

In tumors with type 2 deletions, the expression of Notch1 transcripts is presumably governed by the 5′ promoter elements and associated factors that regulate normal Notch1 expression. Of greater interest is the transcriptional regulation of Notch1 in T-ALLs with type 1 deletions. The deleted region contains several binding sites for positive regulators of T-ALLs with type 1 deletions. The deleted region contains several asterisk. N nucleotides and P nucleotides (underlined) are shown. GL indicates by a 12- or 23-bp spacer and the sequence ACAAAAAAC) are denoted with an breakage and joining, as deduced from sequencing of PCR products, are shown. Residues matching the consensus RAG recognition sequence (CACAGTG followed by a 12- or 23-bp spacer and the sequence ACAAAAAAC) are denoted with an asterisk. N nucleotides and P nucleotides (underlined) are also shown. GL indicates germline DNA flanking the breakpoints. Boxes represent sequences resembling RAG signal sequences. (B) Ratiometric Notch1 quantitative RT-PCR analysis. The relative amounts of transcripts containing 5′ (exons 23 and 24) and 3′ (exons 30 and 31) Notch1 sequences were determined for the tumors in panel A and normal murine thymus, a cell line with a homozygous type 2 deletion (135.2), and a cell line with a heterozygous type 1 deletion (144). Each determination was made in triplicate. The results shown are representative of 2 independent experiments.

The authors thank Yiping He for technical support, Grace Teng and David Schatz (Yale University) for providing RAG2 and H3K4-methylated cDNA,37 and proviral insertions into 5′ portions of Notch1 in murine T-ALL drive the expression of transcripts in which the first in-frame ATG encodes M1727.32,34,38 The structure of the TCRB-NOTCH1 fusion transcript in the human T-ALL cell line CUTLL1 suggests that the same methionine serves as a translational start site in some human T-ALLs as well. Interestingly, a leukemogenic form of Notch2 transduced by feline leukemia virus also initiates translation from a methionine located within the transmembrane domain of Notch2.39 Thus, a latent ability to express truncated polypeptides that generate ligand-independent signals is a feature of several vertebrate Notch receptors and warrants further study.

One model for Notch activation proposes that nicastrin binds the extracellular “stub” produced by metalloprotease cleavage of Notch and thereby delivers Notch to the active site of γ-secretase.40 The activity and γ-secretase dependence of the Notch1 polypeptide created by translational initiation at M1727 argues against this model and is consistent with other recent genetic studies indicating that nicastrin stabilizes γ-secretase, but is nonessential for γ-secretase recognition and cleavage of Notch.41,42

Although murine T-ALL is commonly associated with 5′ deletions in Notch1, analysis of close to 200 human T-ALLs (on Affymetrix arrays, with either 615 000 features (250K + 50K arrays) or SNP 6 arrays (1.8 million features) has not revealed focal copy number variants in NOTCH1 (Dr Charles Mullighan, St Jude Children’s Research Hospital, written communication, August 4, 2010). It thus appears that the relatively large deletions that are observed frequently in murine Notch1 are rare, if they occur at all, in NOTCH1 in human T-ALL, with the important caveat that additional studies are needed to exclude the presence of smaller deletions or point mutations involving the 5′ end of the gene. The paucity of deletions involving NOTCH1 in human T-ALL may be related to divergence in the ectopic RSSs, particularly the heptamer sequences of the 2 RSSs that are most commonly involved in murine Notch1 deletions (supplemental Figure 6).

A proposed strategy for selective targeting of Notch receptors in cancer and other diseases is the use of inhibitory antibodies that recognize either the ligand-binding domain or NRR.18,43 The expression of truncated polypeptides lacking these regions, which is common in murine T-ALL and also occurs in human T-ALLs bearing the t(7;9), reveals limitations in this strategy and suggests a possible mechanism for resistance to therapeutics directed against the ectodomain of Notch1. Based on these insights, a multipronged approach to Notch targeting, including protease inhibitors and novel agents that target Notch in the nucleus,44 seems prudent.

Acknowledgments

The authors thank Yiping He for technical support, Grace Teng and David Schatz (Yale University) for providing RAG2 and H3K4me3 ChIP-Seq data across the Notch1 locus, and Dr Hans Huber and Dr Jose Aste-Amezaga (Merck Inc) for providing Notch1 inhibitory antibodies.

This work was supported by the National Institutes of Health (J.C.A., W.S.P., and S.C.B.).
Figure 7. Mechanisms of ligand-independent ICN1 production in T-ALLs bearing Notch1 deletions. (A-B) Structure and functional consequences of type 1 and type 2 Notch1 deletions. (C) Conservation of M1727 in vertebrate Notch1 receptors. "Identical residues. ":: indicates conserved residues. TM indicates transmembrane domain; S3, site of intramembranous γ-secretase cleavage; and ICN, intracellular Notch.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

The current affiliation for M.Y.C. is Division of Hematology and Oncology, University of Michigan Cancer Center, Ann Arbor, MI.

Correspondence: Jon C. Aster, Department of Pathology, Brigham and Women's Hospital, Room 630E, New Research Bldg, 77 Ave Louis Pasteur, Boston, MA 02115; e-mail: jaster@rics.bwh.harvard.edu.

References

14. Tsuji H, Ishii-Ohba H, Katsube T, et al. Involvement of illegitimate V(D)J recombination or micro-homology-mediated nonhomologous end-joining in the formation of intragenic deletions of the


24. Kakinuma S, Nishimura M, Sasanauma S, et al. Spectrum of Znfn1a1 (ikaros) inactivation and its association with loss of heterozygosity in radio-
Deletion-based mechanisms of Notch1 activation in T-ALL: key roles for RAG recombinase and a conserved internal translational start site in Notch1

Todd D. Ashworth, Warren S. Pear, Mark Y. Chiang, Stephen C. Blacklow, Jérôme Mastio, Lanwei Xu, Michelle Kelliher, Philippe Kastner, Susan Chan and Jon C. Aster