making the article by Guech-Ongey and colleagues in this issue the first to demonstrate the link between CD4+ count and BL to this convincing degree of detail.

The multimodal nature of AIDS-related BL is in distinction to the progressive increase of other lymphomas with age. Similar to non-AIDS populations, Guech-Ongey and colleagues found pediatric, young adult, and geriatric peaks in BL incidence; they postulate that age is a reflection of cumulative exposures to viruses and other pressures ultimately leading to lymphoma formation, and that AIDS-related immunosuppression alone cannot explain BL in this population.

In summary, we now have compelling data in a large cohort showing that BL rarely occurs at the lowest CD4 counts in persons with AIDS. Furthermore, BL retains a multimodal incidence in HIV similar to immunocompetent patients, and is not influenced by HIV transmission category or antiretroviral therapy as reflected by the era of treatment. In contrast, other lymphomas continue to increase with lower CD4 counts and with increasing age. Why should BL be the exception to this process? The hypothesis that BL may require functional CD4 cells is provocative, and the findings therefore raise more questions than answers regarding the genesis of BL.

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● ● ● LYMPHOID NEOPLASIA

Comment on Jeannet et al, page 5443, and on Ashworth et al, page 5455

"Cryptic" Notch1 messages induce T-ALL

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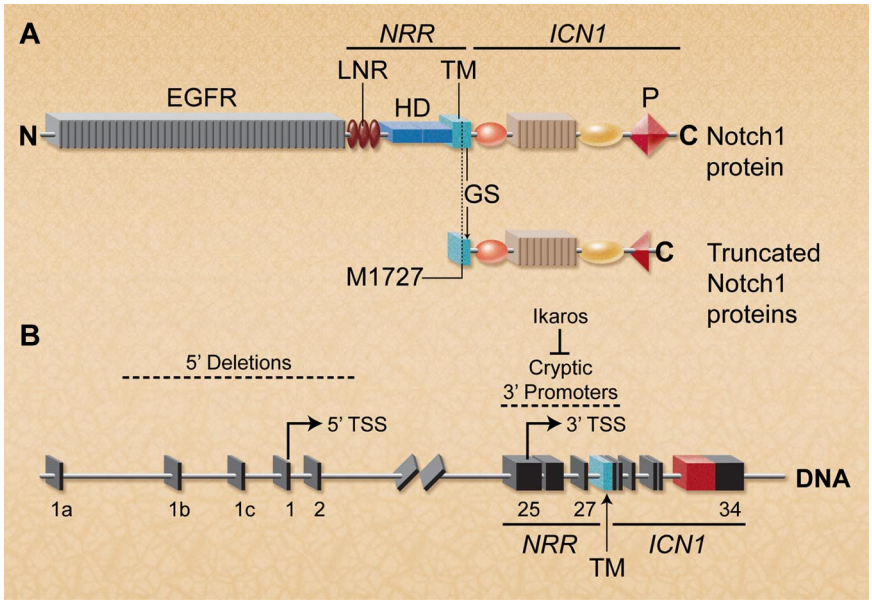
In this issue of *Blood*, 2 articles define a new class of somatic mutations that allow transcription of oncogenic Notch1 from cryptic internal promoters.

Notch1 signaling is essential for T-lineage commitment as well as for survival and proliferation of committed T-cell progenitors in the thymus. Not surprisingly, Notch1-activating mutations are found in more than 50% of human T-cell acute lymphoblastic leukemias (T-ALLs).[1] Rodent T-ALLs induced by a variety of genetic manipulations also exhibit frequent Notch1 mutations that render them Notch1-dependent for survival and proliferation in vitro.[1] Thus, aberrant Notch1 activation represents a key event in the multistep pathway of T-cell leukemogenesis in rodents and humans. Two articles in this issue report the unexpected finding that spontaneous or Cre-LoxP–targeted deletion of the 5′ *Notch1* promoter allows generation of leukemogenic *Notch1* from "cryptic" internal promoters.[2,3] Interestingly, loss-of-function mutations in Ikaros, a transcriptional repressor



Deletion of the 5′ *Notch1* promoter activates cryptic 3′ internal promoters to allow expression of truncated Notch1 proteins lacking the ectodomain. (A) Schematic depiction of full-length wild-type and mutant Notch1 proteins. The ectodomain includes the EGF repeats and the NRR, which contains 3 Lin/Notch repeats (LNR) and the heterodimerization domain (HD). The Notch1 intracellular region (ICN1) contains a C-terminal PEST domain (P). *Notch1* transcripts lacking most of the ectodomain are translated using a conserved internal initiator methionine (M1727) that is just upstream of the GS cleavage site in the TM region. In T-ALLs characterized by both groups, these truncated Notch1 proteins also frequently exhibited PEST domain truncations within ICN1. (B) Spontaneous or targeted *Notch1* deletions that include the 5′ promoter and transcriptional start site (TSS) in exon 1 allow transcription of truncated *Notch1* mRNAs that initiate in exons 25-27 (indicated by arrow). Ikaros loss facilitates epigenetic remodeling to permit transcription from 3′ cryptic promoters in this region. (Professional illustration by A. Y. Chen.)

and T-cell tumor suppressor, potently enhance transcription from these cryptic internal *Notch1* promoters.[3]

Notch receptors have large extracellular regions consisting of many epidermal growth factor repeats (EGFR) and a negative regulatory region (NRR) that autoinhibits Notch activation in the absence of Notch ligands (see figure panel A). Notch binding to its ligands triggers a conformational change in the NRR, allowing gamma secretase (GS)–mediated cleavage within the transmembrane (TM) region, releasing the Notch1 intracellular (ICN1) domain to travel to the nucleus and regulate transcription of Notch target genes.[1] ICN1 does not bind DNA directly, but interacts with DNA-bound RPBJk to induce expression of Notch target genes. Many T-ALL cases have NRR mutations that allow ligand-independent Notch1 activation.[1] A subset has truncating mutations within the ICN1 C-terminal PEST domain, increasing ICN1 stability. However, PEST mutations only weakly activate Notch1 signaling in reporter assays and are poorly leukemogenic in mice,[4] most likely because Notch1 activation remains ligand-dependent.

The strong selection for ligand-independent Notch1 mutant alleles during human T-cell leukemogenesis makes sense because intrathymic Notch ligands required for Notch1 activation are functionally limiting for normal T-cell development.[5] Surprisingly, however, PEST truncations are common but NRR mutations are rare in murine T-ALLs.[1] Therefore, Ashworth and colleagues examined a panel of murine T-ALL cell lines for other abnormalities that could render Notch1 activation ligand-independent. They identified 2 types of truncated *Notch1* transcripts that lacked most of the extracellular domain but retained the TM and ICN1 regions. These transcripts arose from mutant *Notch1* alleles that had large 5′ genomic deletions (see figure, panel B). The most common deletion removed the 5′ *Notch1* promoter as well as the initiator ATG codon in exon 1. Interestingly, deletion breakpoints bore features suggesting that they arose by illegitimate V(D)J recombination, similar to those characterized by Tsuji and colleagues in radiation-induced and $Atm^{-/-}$ thymic lymphomas.[6] A few cell lines had other types of large intragenic *Notch1* deletions that lacked hallmarks of illegitimate V(D)J recombination. In transient assays, transcripts derived from both types of 5′-deleted *Notch1* alleles strongly activated transcription of a Notch reporter construct, results comparable to those induced by a strong human NRR mutant allele. Thus, this study describes a novel mechanism in which 5′ Notch1 genomic deletions generate highly active Notch1 proteins lacking most of the Notch1 ectodomain, explaining the paucity of NRR *Notch1* mutations in murine T-ALLs.

The study by Jeannet et al supports these conclusions but also demonstrates that loss of Ikaros strongly potentiates transcription of *Notch1* alleles harboring 5′ *Notch1* deletions. Loss-of-function Ikaros mutations significantly cooperate with activating Notch1 mutations to promote murine T-ALL.[7,8] Ikaros can bind RBPJk sites and repress certain Notch-regulated genes, suggesting that Ikaros loss may promote T-cell leukemogenesis by enhancing expression of Notch target genes. In support of this notion, the Jeannet study demonstrated that conditional CD4-Cre–mediated deletion of *RBPJk* markedly delayed T-ALL onset in $Ik^{L/L}$ mice expressing hypomorphic Ikaros. Thus, T-cell leukemogenesis induced by Ikaros loss requires canonical Notch signaling. Surprisingly, however, T-ALL onset in $Ik^{L/L}$ mice was greatly accelerated, rather than delayed, by CD4-Cre–mediated deletion of the 5′ end of a floxed *Notch1* allele. This targeted deletion is smaller than the spontaneous ones found by Ashworth et al, but still removes the 5′ *Notch1* promoter and leader peptide encoded by exon 1. Nonetheless, T-ALLs from $Ik^{L/L}$ $Notch1^{f/f}$ $CD4\text{-}Cre^{+}$ mice expressed truncated *Notch1* transcripts lacking most of the Notch1 ectodomain and high levels of active and frequently PEST-mutated ICN1 protein. Furthermore, T-ALLs arising in $Ik^{L/L}$ $Notch1^{+/+}$ mice had spontaneously deleted the 5′ end of *Notch1*, similar to the T-ALL cell lines described by Ashworth.

How is *Notch1* transcribed from alleles bearing targeted or spontaneous deletions of the *Notch1* promoter? The truncated transcripts described in both articles typically initiated in exons 25-27, suggesting the existence of cryptic promoters in this region (figure panel B). Using ChIP-Seq analysis, Jeannet et al identified high levels of acetylated histone 3, an epigenetic mark associated with active chromatin near promoters, centered over the entire 3′ end of *Notch1* beginning abruptly around exon 25. This region lacked acetylated histone 3 in $Ik^{L/L}$ T-ALLs with intact 5′ *Notch1*. Thus, deletion of the 5′ *Notch1* promoter facilitates epigenetic remodeling at the 3′ end of the locus, allowing transcription from cryptic internal promoters. Although this remodeling was also seen in nontransformed *Notch1*-deleted thymocytes expressing wild-type Ikaros, it was greatly increased in the absence of Ikaros, suggesting that Ikaros represses transcription from 3′ cryptic promoters (figure panel B). However, some murine T-ALLs in the Ashworth study expressed wild-type Ikaros, indicating that other factors likely regulate transcription from the cryptic promoters.

Ashworth et al demonstrated that mutant *Notch1* transcripts lacking exon 1 could be translated using an internal initiator methionine (M1727). This residue lies within the Notch TM domain just upstream of the GS cleavage site (figure panel A), and thus generates a Notch1 protein lacking the entire ectodomain but which should still require cleavage by GS. Indeed in both studies, activation of 5′ truncated Notch1 proteins in T-ALLs was blocked by GS inhibitors. Interestingly, this internal methionine is conserved in the Notch TM across many vertebrate species.[2] It will thus be important to determine whether generation of ligand-independent Notch1 proteins has a role in normal T-cell development.

The absence of the Notch1 ectodomain likely allows ligand-independent GS cleavage of these mutant Notch proteins. Nonetheless, PEST mutations were also strongly selected during leukemogenesis in both studies. Some human T-ALLs exhibit NRR and PEST Notch1 mutations in *cis*, and these synergistically increased Notch-dependent transcription as well as leukemogenesis in mice.[4] Therefore, even when Notch1 activation is rendered ligand-independent by deletion of the ectodomain, increasing ICN1 stability by PEST domain truncation further increases Notch transcriptional activity and leukemogenic potential in mice and humans.

Despite the high frequency of 5′ *Notch1* deletions in murine T-ALL, such lesions were not detected in a large panel of human T-ALLs,[2] although small deletions could have been missed. This observation most likely reflects poor conservation of the "cryptic" recombination signal sequences that mediate illegitimate V(D)J recombination at the 5′ end of murine *Notch1*. Nonetheless, in rare cases of human T-ALL with (7,9) chromosomal translocations, *Notch1* transcription appears to initiate from internal promoters around exon 25,

and the conserved TM methionine can be used to generate human Notch1 protein lacking its ectodomain.[2] It is therefore important to identify other mechanisms by which 5′-truncated Notch proteins can be generated in human T-ALL, as this would allow cells to evade therapies currently in development that target Notch ectodomains.[9,10]

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● ● ● PLATELETS & THROMBOPOIESIS

Comment on Poirault-Chassac et al, page 5670

Notch: of mice and men?

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A recent study has demonstrated that Notch is a positive regulator of murine megakaryopoiesis.[1] In this issue of *Blood*, Poirault-Chassac et al report the opposite: Notch inhibits the terminal differentiation of human megakaryocytes.[2] Does this discrepancy arise from species-specific activities of Notch or a difference in experimental design?

Notch signaling plays critical roles in cell fate determination, proliferation, and survival. In the hematopoietic system, Notch is perhaps best known as a positive regulator of normal T-cell development and, when mutated, as a driver of T-cell leukemia.[3] A recent study by Mercher and colleagues revealed that Notch is a positive regulator of megakaryocyte development.[1] In their study, murine LSK hematopoietic progenitor cells plated on OP9 stroma expressing the Delta-like1 (DL1) ligand of Notch gave rise to far more $CD41^+$ megakaryocytes than progenitors cultured on OP9-GFP cells. Furthermore, megakaryocytes derived from OP9-DL1 cultures stained intensely with acetylcholinesterase and showed increased expression of CD42, elevated ploidy and an increased capacity to form megakaryocyte colonies (CFU-MK) relative to cells derived from OP9-GFP stroma. These effects were diminished when canonical Notch signaling was inhibited with either a gamma secretase inhibitor or by expression of dominant-negative MAML1, whereas activation of Notch signaling by expression of ICN4 led to increased CFU-MK colonies and increased absolute numbers of megakaryocytes.

In contrast to these observations in mice, a previous study reported that Notch1 activation inhibits megakaryocytic differentiation of human K562 cells and also $Lin^-Sca\text{-}1^+$ murine progenitors cultured on 3T3 cells expressing the Notch ligand Jagged1.[4] Poirault-Chassac and colleagues confirm the human versus mouse discrepancy by providing new insights into Notch-regulated human megakaryopoiesis. They discovered that culturing human $CD34^+$ cells with either an immobilized chimeric form of the Notch ligand Delta-like4 (Dll4Fc) or on OP9-DL4 and OP9-DL1 stroma led to significantly reduced production of mature $CD41^+CD42^+$ megakaryocytes and diminished proplatelet formation.[2] These effects were reversed by culture with a gamma secretase inhibitor or by expression of dominant-negative MAML1. Of note, the negative effect of Notch required exposure to DL4 in the first 5 days of culture. This is surprising because activation of Notch signaling interfered with terminal differentiation, but did not alter production of CFU-MK or megakaryocyte erythroid precursors (MEPs) from $CD34^+$ cells. Importantly, Poirault-Chassac and colleagues ruled out several obvious experimental differences and also verified that exposure of murine LSK cells to DL1 or DL4 enhanced production of $CD41^+$ cells in culture.

What explains this species-selective effect of Notch on megakaryocytes? One explanation may lie in the starting material for the 2 studies: although they are both considered to be populations enriched for stem cells, human $CD34^+$ and murine LSK cells are not equivalent. A more precise study might compare the effect of Notch activation on megakaryocyte differentiation from highly purified mouse versus human MEPs. Alternatively, the source of the progenitor cells might also be crucial determinant: Poirault-Chassac et al assayed the effect of Notch modulation on human megakaryocytes derived from cord blood or mobilized adult $CD34^+$ cells, whereas Mercher et al examined differentiation of murine bone marrow progenitors.

It is possible that progenitors from different compartments retain a memory of their origin and respond to Notch differently. It is also formally possible that Notch activates overlapping, but distinct, downstream effectors in human and mouse myeloid progenitors. Additional experiments should address these issues. Finally, although there is a difference in the response of human and mouse progenitors to Notch, its dysregulation appears to have the same effect in both species: increased Notch activation has been observed in both humans with and a mouse model of t(1;22) acute megakaryoblastic leukemia.[5] Thus, even though the precise function of Notch in normal megakaryopoiesis is uncertain, it is clear that Notch participates in regulation of hematopoietic cell differentiation beyond the lymphoid compartment.