Convergence of the ZMIZ1 and NOTCH1 Pathways at C-MYC in Acute T Lymphoblastic Leukemias

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Abstract
Activating NOTCH1 mutations are found in 50% to 60% of human T-cell acute lymphoblastic leukemia (T-ALL) samples. In mouse models, these mutations generally fail to induce leukemia. This observation suggests that NOTCH1 activation must collaborate with other genetic events. Mutagenesis screens previously implicated ZMIZ1 as a possible NOTCH1 collaborator in leukemia. ZMIZ1 is a transcriptional coactivator of the protein inhibitor of activated STAT (PIAS)-like family. Its role in oncogenesis is unknown. Here, we show that activated NOTCH1 and ZMIZ1 collaborate to induce T-ALL in mice. ZMIZ1 and activated NOTCH1 are coexpressed in a subset of human T-ALL patients and cell lines. ZMIZ1 inhibition slowed growth and sensitized leukemic cells to corticosteroids and NOTCH inhibitors. Gene expression profiling identified C-MYC, but not other NOTCH-regulated genes, as an essential downstream target of ZMIZ1. ZMIZ1 functionally interacts with NOTCH1 to promote C-MYC transcription and activity. The mechanism does not involve the NOTCH pathway and appears to be indirect and mediated independently of canonical PIAS functions through a novel N-terminal domain. Our study shows the importance of identifying genetic collaborations between parallel leukemic pathways that may be therapeutically targeted. They also raise new inquiries into potential NOTCH–ZMIZ1 collaboration in a variety of C–MYC-driven cancers. Cancer Res; 73(2); 930–41. ©2012 AACR.

Introduction
Supraphysiologic levels of NOTCH signaling have been implicated in a wide variety of cancers including breast cancer, chronic lymphocytic leukemia, T-cell acute lymphoblastic leukemia (T-ALL), and many others (reviewed in ref. 1). Normally, NOTCH receptors reside at the cell membrane in an inactive state (reviewed in ref. 2). NOTCH signaling is restrained by the negative regulatory region (NRR), which consists of the Lin-12/Notch repeats (LNRR) domain and the heterodimerization domain (HD). NOTCH becomes activated after it engages the ligand. γ-Secretase cleaves NOTCH, which releases the intracellular domain of NOTCH (ICN). γ-Secretase inhibitors (GSI) are compounds that inhibit this cleavage step. ICN translocates to the nucleus where it engages the CSL/RBP-Jκ DNA-binding factor. ICN is quickly targeted for proteosomal degradation by multiple "degron" signals in its C-terminal PEST domain (3–6).

In T-ALL, NOTCH1 is constitutively activated through mutations in the HD and PEST domains in approximately 50% to 60% of patient samples (7). HD mutations destabilize the NRR and trigger ligand-independent activation (8, 9). PEST domain mutations remove C-terminal degron sequences, which enhances ICN stability. In mouse models, most leukemia-associated NOTCH1 mutations cannot initiate T-ALL (10). However, diverse human T-ALL–associated oncogenes such as TAL1/SCL and LMO2 collaborate with Notch1 mutations to induce leukemia in mouse models (reviewed in ref. 11). In addition, murine retroviral insertional mutagenesis screens identified dominant-negative Ikaros isoforms as NOTCH1 collaborators (12–14). Alterations in IKAROS have been reported in human T-ALL (13–15). In recent mutagenesis screens, leukemia samples with insertions that activated Notch1 frequently had insertions in the 5′-region upstream of the Zmiz1 gene that led to overexpression of Zmiz1 without disrupting the coding sequence (16–18).

ZMIZ1 (also known as ZIMP10 or RAII7) is a transcriptional coactivator that is related to members of the protein inhibitor of activated STAT (PIAS) family. PIAS proteins bind and regulate transcription factors such as NF-κB, STAT, and SMAD (reviewed in ref. 19). Like PIAS members, ZMIZ1 has a highly conserved MIZ (Mx-responsive zinc finger) domain that is important for protein–protein interactions and sumoylation (20, 21). ZMIZ1 has a strong transcriptional activation domain and regulates the activity of diverse group transcription factors including the androgen receptor, SMAD3, and p53 (20, 22, 23). Mice deficient in Zmiz1 die at embryonic day 10.5, apparently from impaired vasculogenesis (24). Inhibition of ZMIZ1...
impaired the growth of a human prostate cancer cell line (25). The N-terminal portion of ZMIZ1 was discovered as a fusion partner of ABL in a single case of Ph-negative ALL (26).

In the present study, we investigated the role of ZMIZ1 in T-ALL induction and maintenance. We show that ectopic NOTCH1 and ZMIZ1 expression cooperated to induce T-ALL in mice. ZMIZ1 was expressed in a subset of human primary T-ALL samples and cell lines. Inhibition of ZMIZ1 impaired cell growth and sensitized cells to NOTCH inhibitors and glucocorticoids. Mechanistic studies suggested that ZMIZ1 contributes to oncogenesis by indirectly inducing C-MYC through a unique MIZ-independent mechanism.

Materials and Methods

Mice
We obtained 4- to 8-week-old C57BL/6 mice and NOD-SCID γ-chain-deficient mice from Taconic. All mice were housed in specific pathogen-free facilities at the University of Pennsylvania, Philadelphia, PA, and the University of Michigan, Ann Arbor, MI. Experiments were conducted according to guidelines from the National Institutes of Health with approved protocols from the Institutional Animal Care and Use Committees at the University of Pennsylvania (Permit #466100) and the University of Michigan (Permit #10298).

Cell lines
HBX-ALL, PF382, and JURKAT were obtained from Jon Aster (Brigham and Women’s Hospital; September, 2004). ALL-SIL, DND41, LOUCY, SKW-3, MOLT-3, TALL1, and RPMI8402 were obtained from Andrew Weng (Terry Fox Laboratory). The CEM cell line was obtained from Katherine Collins (University of Michigan; November, 2010). The 8946 cell line was obtained from Warren Pear (University of Pennsylvania; September, 2007) and maintained as previously described (27). These cells were tested for C-MYC dependence in the presence of doxycycline monthly. All cell lines were cultured for less than 6 months after resuscitation, maintained as previously described (27), authenticated using the variable number of tandem repeats (VNTR) PCR assay, and tested for contaminants using MycoAlert (Lonza) every 3 months.

Antibodies
Flow cytometry antibodies used were as follows: CD4 (RM4-5), CD8 (53-6.7); LINGFR (ME20.4-LH4), CD1 (HI149), CD5 (L17F12), CD7 (4H9), CD8 (RPA-T8), CD11b (ICRF44), CD13 (WM15), CD33 (HIM-34), CD34 (4H-11), CD45 (HI-30), CD117 (10D2), and HLA-DR (L243). Antibodies used for Western blotting were as follows: cleaved Notch1 (V1744; Cell Signaling Technology); ZMIZ1 (RB1963, Abgent); and C-MYC (N-262; Santa Cruz Biotechnology).

Human T-ALL samples
This study was approved by the University of Michigan Institutional Review Board (Permit #HUM00049758). Frozen samples of T-ALL cells were obtained from the University of Pennsylvania, Mignon Loh, and the Children’s Oncology Group, Linda Holmfeldt, and Charles Mulligan.

Gene expression profiling
We used Affymetrix human HG_U133_plus_2 arrays to assay quadruplicate samples of the CEM cell line transduced with ZMIZ1 short hairpin RNA (shRNA) as well as control shRNA for 72 hours in the absence of puromycin. The array data have been deposited in NCBI’s Gene Expression Omnibus (GEO) as series GSE13252. We collapsed our 3,980 differentially expressed probe sets (with \( P < 0.01 \)) and fold-change at least 1.3-fold) to 1,930 distinct genes increased with ZMIZ1 knockdown, and 935 decreased genes. Our up and down gene lists were separately tested for over-representation in MYC-related curated gene sets from version 3 of MSigDB, using 1-sided Fisher Exact tests.

Quantitative real-time PCR
Mouse Deltax1 (Mm00492297_m1), CD25 (Mm00434261_m1), Hes1 (Mm00468601_m1), and c-Myc (Mm00487803_m1) were obtained from Applied Biosystems. Sybr green primer sequences are shown in Supplementary Table S1. All target gene expression values were normalized to 18S RNA.

Statistical analysis
Linear regression analysis, ANOVA, \( t \) test, and survival curves were carried out using Prism. Unless otherwise indicated, \( P \) values were derived from 2 sample \( t \) tests and values are shown as means ± SD.

Results
ZMIZ1 cooperates with NOTCH1 to induce T-ALL
Murine insertional mutagenesis studies previously suggested that activated Notch1 and Zmiz1 cooperate to induce T-ALL (16–18). To test this possibility, we cloned ZMIZ1 into the retroviral NGFR vector, which expresses ZMIZ1 and a truncated nerve growth factor receptor (NGFR). L1601P is a common leukemia-associated mutation in the HD domain of NOTCH1. \( \Delta P \) is a PEST domain mutation found in the ALL-SIL T-ALL cell line that deletes amino acids 2,473–2,555. L1601PΔP signifies the mutations L1601P and \( \Delta P \) in cis. L1601PΔP rarely induces leukemia in mice (10). L1601PΔP was cloned into the retroviral GFP vector, which expresses L1601PΔP and GFP. NGFR, ZMIZ1, and L1601PΔP retroviruses, normalized to equal titers among all conditions, were transduced into adult murine BM progenitors (Supplementary Fig. S1A and B). These progenitors were then transplanted into lethally irradiated mice. Mice expressing L1601PΔP alone transiently generated circulating CD4+CD8+ double-positive (DP) T-cells as previously described (ref. 10 and Fig. 1A). In contrast, mice expressing both ZMIZ1 and L1601PΔP developed a sustained rise in aberrant DP T-cells (Fig. 1B). None of the control, ZMIZ1, or L1601PΔP mice developed T-ALL (Fig. 1C). In contrast, mice expressing both ZMIZ1 and L1601PΔP developed T-ALL with a penetrance of approximately 60% at 126 days after transplant. The leukemia resembled the previously described T-ALL induced by strong NOTCH1 alleles with infiltration of the bone marrow, spleen, lymph node, and thymus with leukemic DP T-cells (Fig. 1D; ref. 10). Aberrant DP T-cells were not found in the GFP “NGFR” compartment (Fig. 1D, bottom).
ZMIZ1 is expressed in a subset of primary human T-ALL samples

To begin determining the relevance of ZMIZ1 to human T-ALL, we screened 14 T-ALL cell lines for ZMIZ1 expression. CEM and T6E cells expressed the ZMIZ1 protein (Fig. 2A). Protein in several lines was not detectable; RNA was detected, but at levels 5-fold less or lower compared with cell lines expressing ZMIZ1 protein (data not shown). ETP-ALL is a novel high-risk pediatric T-ALL identified by Coustan-Smith and colleagues at the St. Jude Children’s Hospital (28). Homminga and colleagues found that many ETP-ALL samples overexpressed MEF2C (29). The mean RNA expression of ZMIZ1 was approximately 2.1-fold higher \( (P < 10^{-5}) \) in ETP-ALL than typical T-ALL samples in the Coustan-Smith data set (Supplementary Fig. S2A) and approximately 2.4-fold higher \( (P < 10^{-5}) \) in the Homminga data set (Supplementary Fig. S2B). We obtained 12 pediatric ETP-ALL and 12 pediatric non-ETP (i.e., typical) T-ALL RNA samples from the St. Jude Children’s Hospital and the Children’s Oncology Group. We obtained 15 adult T-ALL samples from the University of
Pennsylvania. Mean ZMIZ1 RNA expression was approximately 22% higher in ETP-ALL than typical T-ALL samples, although not statistically significant (Fig. 2B). ZMIZ1 was significantly more highly expressed in the MEF2C<sup>lo</sup> subset than the MEF2C<sup>hi</sup> subset of ETP-ALLs (Fig. 2C). Like activated NOTCH, ZMIZ1 is expressed in diverse “oncogenetic” clusters besides ETP-ALLs (Supplementary Fig. S2C). The ZMIZ1 protein was detected in 1 ETP-ALL sample (#7), 2 typical T-ALL samples (#5 and #11), and 3 adult T-ALL samples (#498, #711, and #790; Fig. 2D). Activated NOTCH1 was detected in 5 of the 6 samples that expressed ZMIZ1.

**ZMIZ1 is differentially expressed during thymopoiesis**

Human T-cell development begins at the ETP/DN1 cell stage and progresses in orderly fashion through immature stages – DN2, DN3, Immature Single Positive (ISP), CD3<sup>+</sup> DP, CD3<sup>+</sup> DP – before developing into the mature CD4 and CD8 cells. We analyzed expression of ZMIZ1 in the data set of Dik and colleagues (30). ZMIZ1 was highest in CD34<sup>+</sup> cord blood and ETP/DN1 cell stages and decreased with maturation (Supplementary Fig. S2D). We then sorted the analogous subpopulations from murine thymus and LSK cells (Lineage<sup>-</sup>/Sca-1<sup>+</sup>/Kit<sup>+</sup>) from the bone marrow (Fig. 2E). The LSK subset contains hematopoietic stem cells and multipotent progenitor cells. Similar to humans, the expression of mouse Zmiz1 was highest in the most primitive thymocyte fractions and significantly decreased with maturation (Fig. 2E). ZMIZ1 expression spanned approximately 5-fold (Fig. 2E). In contrast, ZMIZ1 expression in human T-ALL samples spanned approximately 1,000-fold (Fig. 2B).

**Inhibition of ZMIZ1 function impacts T-ALL proliferation, survival, and metabolism**

We transduced CEM and T6E cells with multiple ZMIZ1 shRNA and verified knockdown of RNA and protein (Supple-
mentary Fig. S3A–D). CEM cells transduced with ZMIZ1-silencing shRNA were approximately 92% fewer than controls after 9 days of growth (Fig. 3A). T6E cells transduced with ZMIZ1-silencing shRNA were approximately 75% fewer than controls (Fig. 3B). For our next experiments, we used the CEM cell line because it is derived from a human T-ALL and because the effect of ZMIZ1-silencing was relatively strong. ZMIZ1 knockdown delayed tumor growth by 7 to 18 days compared with controls after xenotransplantation (Fig. 3C and D). ZMIZ1 silencing significantly increased apoptosis by 7-AAD or Annexin V staining (Fig. 3E and F; Supplementary Fig. S4A and B). ZMIZ1 knockdown slowed cell-cycle progression at the G1–S checkpoint (Fig. 3G and H). ZMIZ1 knockdown produced supernatant with a higher pH (Supplementary Fig. S4C). The ZMIZ1-silenced cells were smaller (Supplementary Fig. S4D).

After normalizing for effects on cell number, ZMIZ1 inhibition significantly reduced NH4+ production (Fig. 3I), lactic acid production (Fig. 3J), glutamine consumption (Supplementary Fig. S4E), and glucose consumption (Supplementary Fig. S4F). ZMIZ1 inhibition had no effect on differentiation state (Supplementary Fig. S4G). These studies suggest that ZMIZ1 stimulates cell growth through multiple mechanisms.

**ZMIZ1 regulates the C-MYC pathway**

We next sought to determine the ZMIZ1-regulated gene set. We transduced CEM cells with nonsilencing shRNA control or ZMIZ1-silencing shRNA and carried out gene expression profiling. We compared expression values of selected probe sets giving P values less than 0.01 and average fold-changes of at least 1.5-fold, which selected 3,980 probe sets as differentially expressed.
expressed (2,611 up and 1,369 down). An identical analysis of the 34 different data sets that can be obtained by permuting the sample labels without giving back the original data gave an average of 35.9 qualifying probe sets, so that we expect less than 1% of our 3,980 selected probe sets to be false positives. A subset of these probe sets that satisfied more stringent selection criteria is shown in Fig. 4A. ZMIZ1 knockdown repressed C-MYC expression by roughly 70% to 80% (Fig. 4B and C) as early as 48 hours after ZMIZ1 knockdown (Supplementary Fig. S5A and B). Primary and mature (spliced) C-MYC transcripts were similarly reduced (Supplementary Fig. S5C and D). We carried out enrichment analysis of the effect of ZMIZ1 knockdown on the 16 curated gene sets involving C-MYC in the Molecular Signatures Database (Supplementary Table S2). The genes that decreased on ZMIZ1 knockdown were significantly enriched in several lists of genes that increased with MYC overexpression. Conversely, the genes that increased with ZMIZ1 knockdown were significantly enriched in lists of genes...
that decreased with MYC overexpression. For example, ZMIZ1 knockdown significantly reduced the expression of C-MYC target genes CCND2 (Cyclin D2), SLC1A4 (ASCT1), SLC16A1 (MCT1), DKC1, and ODC1 (Fig. 4B and C). Silencing ZMIZ1 with multiple shRNAs reduced C-MYC expression by Western blot (Fig. 5A) and quantitative real-time PCR (qPCR; Fig. 5B). These data suggest that ZMIZ1 regulates the expression and activity of C-MYC.

**ZMIZ1 cooperates with NOTCH1 to induce the C-MYC pathway**

C-MYC is a direct target of NOTCH1 (31–33). The 8946 cell line is derived from a murine T-ALL driven by an inducible human C-MYC transgene. It does not express Zmiz1 (data not shown). The 8946 cells die on addition of doxycycline, which represses C-MYC expression, but can be rescued by transduction of strong NOTCH1 alleles that upregulate the expression of endogenous murine c-Myc (31). N1D is the NOTCH1 allele with the D mutation alone. In terms of NOTCH signal strength, N1D is very weak, L1610P is weak, and L1601P D is moderately strong (10). We transduced N1D, L1601P, or L1601P D into 8946 cells in combination with either the NGFR vector or ZMIZ1. ZMIZ1 alone failed to rescue 8946 cells after withdrawal of C-MYC (Fig. 5C, I). N1D, L1601P, or L1601P D alone weakly rescued 8946 cells if at all (Fig. 5C, J–L). In contrast, ZMIZ1 strongly enhanced the ability of N1D, L1601P, or L1601P D to rescue 8946 cells.
L1601P, or L1601PAP to rescue 8946 cells by 18- to 36-fold (Fig. 5C, J-L). Similarly, ZMIZ1 strongly enhanced the ability of N1AP, L1601P, or L1601PAP to induce expression of c-Myc (Fig. 5D) and its target gene Cad (Fig. 5E). These data suggest that ZMIZ1 cooperates with NOTCH1 to induce C-MYC transcription and activity.

**ZMIZ1 appears to regulate C-MYC indirectly through a novel, MIZ-independent transcriptional mechanism and not through the NOTCH signaling pathway**

To further investigate the mechanism of ZMIZ1, we subcloned two 8946 cells that expressed the NGFR control vector and 2 that expressed ZMIZ1. We turned off C-MYC transcription by adding doxycycline. We did not observe any differences between the rate of loss of C-MYC protein between the ZMIZ1-expressing cells and control cells (Supplementary Fig. S6A). Together with Supplementary Fig. S5C and D, these data suggest that ZMIZ1 does not regulate C-MYC through post-transcriptional mechanisms. ZMIZ1 did not enhance the ability of N1AP, L1601P, or L1601PAP to induce Notch1 target genes (other than c-Myc) such as Dtx1 (Fig. 5F), Il2ra (Fig. 5G), and Hes1 (Fig. 5H). We could not detect association of ZMIZ1 with NOTCH1 using immunoprecipitation assays in ZMIZ1-expressing cell lines (Supplementary Fig. S6B). Microarray analysis described in Fig. 4 showed that ZMIZ1 knockdown did not significantly affect well-established NOTCH1 targets in T-cells such as DTX1, IPI-204, CCR7, LEF1, NOTCH1, NOTCH3, Il2ra, Hes1, Nrar1, CCNd3, and Tcf7 (data not shown). These data suggest that ZMIZ1 does not regulate the NOTCH signaling pathway. Chromatin immunoprecipitates did not show association of ZMIZ1 with the approximately 6-Kb region upstream of the MYC transcriptional start site (data not shown). Furthermore, a chimera consisting of ZMIZ1 fused to the ligand-binding domain of the estrogen receptor (ZMIZ1-ER) induced C-MYC RNA at 48 hours (Supplementary Fig. S7A), but not at 6 hours (Supplementary Fig. S7B) after treatment with 4-OHT. These data suggest that the mechanism of ZMIZ1 is likely indirect. Finally, structure-function analysis (Fig. 6A) showed that the MIZ domain, but not the N-terminal domain, was dispensable for rescue of 8946 cells after C-MYC withdrawal (Fig. 6B) and for induction of c-Myc (Fig. 6C). The N-terminal domain has no known function, but appears highly ordered (Fig. 6D). These data suggest that ZMIZ1 regulates C-MYC through a novel, MIZ-independent mechanism.

**C-MYC is an essential but insufficient effector of ZMIZ1 functions**

To determine whether C-MYC can substitute for ZMIZ1, we transduced C-MYC into CEM cells to maintain C-MYC levels irrespective of ZMIZ1 levels (Supplementary Fig. S8A and B). Ectopic expression of C-MYC did not rescue growth of CEM cells in which ZMIZ1 was silenced. qPCR and Western blot confirmed that C-MYC was ectopically expressed (data not shown; Supplementary Fig. S8C). Enforced C-MYC increased apoptosis (Supplementary Fig. S9A–D) in association with a small increase in the G1–S blockade (Supplementary Fig. S9E and F). These data show that C-MYC is not the sole mediator of ZMIZ1 functions. However, it was still possible that C-MYC is required for ZMIZ1 oncogenic functions. To test whether C-MYC is required for CEM growth, we transduced CEM cells with MAD or A-MAX (Supplementary Fig. S8D and E). MAD and A-MAX are dominant-negative inhibitors of C-MYC (31, 34). MAD and A-MAX significantly inhibited growth of CEM cells. Thus, repression of C-MYC, due to knockdown of ZMIZ1, would be expected to impair cell growth. These data suggest that C-MYC is a required, but insufficient, effector of ZMIZ1 functions. Knockdown of ZMIZ1 may impair cell growth in part due to reduction of C-MYC levels.

**ZMIZ1 silencing sensitizes leukemic cells to NOTCH inhibitors and glucocorticoids**

We next determined whether the collaboration between ZMIZ1 and NOTCH1 could be therapeutically targeted. GSIs are being tested for the treatment of T-ALL (35, 36). THP-6 is a human T-ALL cell line that coexpresses ZMIZ1 and activated NOTCH1 (Supplementary Fig. S10A). CEM and THP-6 cells transduced with control shRNA were fully or partly resistant to GSI (Fig. 7A, S10B). However, GSI compared with dimethyl sulfoxide (DMSO) growth inhibition was significantly greater in ZMIZ1 shRNA-treated cells than control shRNA-treated cells (P < 0.0001). In contrast to CEM and THP-6 cells, T6E cells are sensitive to GSI (31). However, under low-dose GSI conditions, there was significantly greater GSI compared with DMSO growth inhibition in ZMIZ1 shRNA-treated cells than control shRNA-treated cells (Supplementary Fig. S10C, P, 0.0152). To test whether ZMIZ1 inhibition could be added to glucocorticoid therapy given the similarities between the glucocorticoid and androgen receptors, we treated CEM cells with ZMIZ1 shRNA and dexamethasone (Fig. 7B). CEM cells transduced with ZMIZ1 shRNA were significantly more sensitive to dexamethasone than controls (Fig. 7C). Mifepristone, a glucocorticoid inhibitor, reversed the sensitivity of the CEM cells to glucocorticoids (Fig. 7D) and for induction of c-Myc (Fig. 7E). These data suggest that ZMIZ1 inhibition may enhance targeting of a subset of T-ALL cells with GSI or glucocorticoids.

**Discussion**

Activating NOTCH1 mutations can occur before the acquisition of other oncogenic events and well in advance of the clinical appearance of T-ALL (37). Some NOTCH1 alleles such as ICN are sufficiently strong to initiate leukemogenesis in mice. In contrast, the vast majority of leukemia-associated NOTCH1 alleles are insufficient (10). This observation suggests that additional genes collaborate with activated NOTCH1 to induce leukemia. Insertional mutagenesis studies in mice identified ZMIZ1 as a possible NOTCH1 collaborator in leukemia development (16–18). Our studies verified that ZMIZ1 collaborates with leukemia-associated NOTCH1 alleles to induce T-ALL in mice. ZMIZ1 is overexpressed in a subset of T-ALL samples. However, like NOTCH1 activation, ZMIZ1 overexpression does not appear to be limited to a specific oncogenic subset. Indeed, samples that expressed ZMIZ1 frequently expressed activated NOTCH1. Our studies also show
that ZMIZ1 may be required for leukemia growth. Together, these findings suggest that ZMIZ1 is an oncogene. It may be a clinically relevant therapeutic target in a subset of T-ALL patients.

In our previous report (10), the penetrance of T-ALL by L1601PΔP in the retroviral transduction/bone marrow transplantation mouse model was approximately 25%. In the current report, none of the L1601PΔP mice developed T-ALL. The difference between the 2 reports was viral titer. Our current protocol used 4-fold less L1601PΔP virus in order to add the ZMIZ1 virus. This reduction in titer likely explains the difference in penetrance. The dose of NOTCH signaling is critically important for leukemic induction (10). The penetrance of T-ALL of our reports (10) is less than another report by Medyouf and colleagues (38). Our protocol used the C57BL/6 strain in contrast to the C57BL/6 X FvB strain in the Medyouf study. Similar numbers of cells were transferred. Titers and radiation dose may have differed from our protocol, which is detailed in Supplementary Material and Methods.

In addition to ZMIZ1, retroviral insertional mutagenesis screens identified Ikaros dominant-negative isoforms and Lunatic Fringe as collaborators of Notch1 (16). These genes enhance Notch signaling through a global effect on Notch target genes. In contrast, the collaboration between ZMIZ1 and NOTCH1 appears restricted to just 1 NOTCH target—C-MYC. In T-ALL, C-MYC promotes proliferation and glucose/glutamine metabolism (31–33) and can often rescue cell death after withdrawal of NOTCH signaling (31, 39). However, the
importance of C-MYC in T-ALL remains to be determined (40). Murine insertional mutagenesis studies showed that insertions in the Notch1 locus preceded insertions in the Zmiz1 locus (16). Thus, NOTCH1 activation may predispose cells to recruit ZMIZ1 overexpression in order to amplify the C-MYC signal. The dependence on ZMIZ1 may be an example of synthetic lethality of NOTCH1 activation.

Additional studies will be needed to better characterize the mechanism of ZMIZ1. C-MYC appears to be a required, but insufficient, effector of ZMIZ1 oncogenic functions. ZMIZ1, like PIAS proteins, may regulate transcription broadly through multiple target genes to achieve its biological functions. Our data suggests that that C-MYC, combined with additional ZMIZ1 effectors, will drive cell growth. Given the pro-apoptotic functions of C-MYC (refs. 41, 42, Supplementary Fig. S9), we are interrogating our microarray data for effectors that counter apoptosis. We plan to test the ability of these effectors in combination with C-MYC to substitute for ZMIZ1. Additional studies will also be needed to determine how ZMIZ1 collaborates with NOTCH1. ZMIZ1 does not appear to target the NOTCH1 pathway or C-MYC posttranscriptionally. Instead, ZMIZ1 may induce c-MYC transcription through indirect mechanisms. ZMIZ1 has no DNA-binding domain. It functions by binding transcription factors to enhance their transcriptional activity. PIAS and PIAS-like proteins interact with a broad range of transcription factors through the MIZ domain (19). Therefore, identifying a single direct mechanism by which ZMIZ1 regulates C-MYC may not be straightforward. Furthermore, the MIZ domain, but not the N-terminal domain, appears to be dispensable. The N-terminal domain of ZMIZ1 is not shared with PIAS family members and has no known functions. Therefore, the mechanism of ZMIZ1 appears to be novel and independent of canonical PIAS functions. Solving the mechanism will require proteomic approaches to identify direct binding partners of the N-terminal domain.

NOTCH signaling has been implicated in many cancers. The literature, publicly available gene expression data sets, and our own observations in the NCI-60 cell lines suggest that ZMIZ1 is frequently coexpressed with NOTCH in diverse cancers (ref. 25 and data not shown). Thus, it is possible that the collaboration between ZMIZ1 and NOTCH1 may extend to other cancers besides T-ALL. Targeting the ZMIZ1-NOTCH collaboration may prove to be a general alternative method of targeting C-MYC and other pathways.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: M.Y. Chiang
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