Supplemental Materials and Methods

Viral constructs and production. pcDNA3-flag KIAA-1224 (a gift from Zijie Sun) contains the human ZMIZ1 cDNA(1). The ZMIZ1 cDNA was subcloned into the NGFR control vector to create RAI17-NGFR. The nonsilencing shRNA control vector was a gift from Thomas Lanigan. Lentiviral constructs expressing shRNA against human ZMIZ1 or murine Zmiz1 were obtained from Open Biosystems. Catalog numbers are as follows: shRNA-13 - RHS3979-9585102; shRNA-14 - RHS3979-9585102; shRNA-15 - RHS3979-9585104; shRNA-16 - RHS3979-9585105; shRNA-m13 - RMM3981-97074310; shRNA-m14 - RMM3981-97074311; shRNA-m15 - RMM3981-97074312; shRNAm16 - RMM3981-97074313; shRNA-m17 - RMM3981-97074314. Lentiviral supernatants were produced by the University of Michigan Vector Core (Director, Thomas Lanigan). The control GFP-expressing MSCV-IRES-GFP (MigR1) construct and the control NGFR-expressing MSCV-IRES-NGFR construct were previously described(2, 3). Construction and cloning of the Notch1 mutants found in human T-ALL were previously described(4). Mad and MXD1 subcloned into MigR1 were obtained as previously described(5). ZMIZ1-ER was created by fusing the 3' end of the human ZMIZ1 cDNA from RAI17-NGFR to the ligand binding domain of the murine estrogen receptor (a gift from Eric Fearon), which was then subcloned into the NGFR vector. High titer retroviral supernatant was produced using transient transfection of 293T-cells and assessed for GFP or NGFR titer by transducing 8946 cells and measuring %GFP and %NGFR two days later.

Transduction and bone marrow transplantation. Retroviral transduction of BM cells and transfer into lethally irradiated recipients was performed as described(3, 6, 7). The protocol used was identical except that the titer of L1601PΔP virus was four-fold less than used previously(3). Briefly, BM cells were collected from 4-8 week old C57BL/6 mice 4 days after intravenous administration of 5-fluorouracil (5FU, 250 mg/kg). The cells were cultured overnight in the

presence of IL-3 (6 ng/ml), IL-6 (5-10 ng/ml), and SCF (100 ng/ml). The cells were then washed, resuspended in retroviral supernatant containing GFP-expressing retrovirus and NGFR-expressing retrovirus (which had been normalized based on GFP/NGFR-titers), placed in the same cytokine cocktail with the addition of polybrene (4 μg/ml), and centrifuged at 1290 x g for 90 minutes. A second round of "spinoculation" was performed the following morning. After washing with PBS, 4-5 x 10^5 cells were injected intravenously into lethally irradiated (900 rads) syngeneic recipients. Mice were maintained on antibiotics in drinking water for two weeks after BMT. Mice were bled every 3 weeks to monitor blood counts and evaluate the presence of circulating immature T-cell progenitors by flow cytometry

Flow cytometry. Cells were stained on ice in PBS containing 2% fetal bovine serum, 10 mM Hepes and 0.02% NaN_3 after blocking with rat and mouse IgG (Sigma) and 24G2 cell supernatant. Acquisition was performed on a FACS-Calibur (Becton-Dickinson) or C6 (Accuri). Annexin V staining was performed by staining with APC Annexin V antibody according to manufacturer's protocol (Becton-Dickinson). Flow sorting was performed on a FACS-Diva or FACS-Aria. Dead cells and doublets were excluded based on FSC/SSC characteristics and DAPI or propidium iodide (PI) staining. Data were analyzed with FlowJo (Tree Star).

Cell culture. T-ALL cell lines were grown in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, 2-mercaptoethanol (0.0005% (v/v), Sigma), and antibiotics. 293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) with the same supplements except 2-mercaptoethanol. Cells were grown at 37°C under 5% CO_2. Retroviral transduction of 8946 cells, sorting, and analysis in the presence of doxycycline (20 ng/ml) was performed as described(3). DBZ (GSI) was obtained from EMD chemicals. Dexamethasone, mifepristone, 4-OHT, and cycloheximide were obtained from

Sigma. For knockdown experiments, puromycin (Sigma) added to transduced cell cultures for 2 days and then removed by centrifugation.

Western blotting and immunoprecipitation. Cleaved NOTCH1 (i.e. activated NOTCH1) was detected using the rabbit polyclonal antibody (Val1744) recognizing the V1744 epitope (Cell Signaling Technology). ZMIZ1 was detected using a rabbit polyclonal antibody (RB1963) recognizing the C-terminus (Abgent). MEF2C was detected using a rabbit polyclonal antibody (D80C1) recognizing the region surrounding Met182 (Cell Signaling Technology). LYL1 was detected using a mouse polyclonal antibody directed against the full-length human protein. β-ACTIN was detected using the AC-74 antibody (Sigma). C-MYC was detected using the N-262 antibody (Santa Cruz Biotechnology). Immunoprecipitation of 1 mg of protein in whole cell lysates of CEM, 8946, and T6E cells with ZMIZ1 antibody was preformed with Protein G Sepharose Fast Flow beads (GE Healthcare Life Sciences) according to manufacturer's instructions. Control rabbit IgG antibody was obtained from Cell Signaling Technology. Western blotting of T-ALL cell lines was performed 6-8 days after transduction with shRNA.

Metabolic assays. CEM cells were transduced overnight with shRNA lentiviruses. After two days of transduction, the cells were treated with puromycin for 2 days. After 1-3 days of further culturing, the cells were cultured plated in phenol red-free, serum-free media for 2 days. The cells were counted and supernatant was obtained by centrifugation. The concentration of glutamine was measured using the GLN-1 kit (Sigma). The concentration of glucose was measured using the GAG0 kit (Sigma). The concentration of lactic acid was measured using the K607-100 kit (Biovision). The concentration of ammonium was measured using the AA0100 kit (Sigma). From these values, the consumption of each metabolite was calculated and normalized to cell number.

Xenotransplantation. CEM cells were transduced with shRNA lentiviruses overnight. Puromycin was added for 2 days. Puromycin was removed and the cells were cultured for an additional 1-2 days. 3 million transduced CEM cells were subcutaneously injected into the left flank of NOD-SCID γ-chain deficient mice. After 2 weeks, tumor diameters were measured by averaging the cranial-caudal and the transverse dimensions obtained using a caliper. The tumor volume was then estimated by the equation: tumor volume = $4/3 \times r^3 \times \pi$. Mice were euthanized once diameter exceeded 20 mm.

Dexamethasone treatment of T-ALL cell lines. CEM cells were transduced overnight with nonsilencing shRNA or ZMIZ1-targeted shRNA. Puromycin was added on Day 2 and removed on Day 4. After 1-3 days, the cells were then split into 96 well plates in 200 μl volumes at 80,000 cells/ml. Increasing concentrations of dexamethasone from 10^{-9} to 10^{-5} M was added. In some experiments, mifepristone (or an equal volume of ethanol) was added to a final concentration of 1 μM. 72 hours later, cell number was determined with PI-exclusion using flow cytometry.

GSI treatment of T-ALL cell lines. CEM cells, T6E cells, and THP6 cells were transduced with lentiviruses containing nonsilencing shRNA and ZMIZ1-targeted shRNA. 2 days later, the cells were treated with puromycin. 2 days later the puromycin was removed. After 1-3 days, the cells were treated with DMSO or GSI (1 μM for CEM and THP6; 10 nM for T6E) for 9 days in 200 μl volumes in 96 well plates. Every three days the cells were split and live cells were counted by PI-exclusion using flow cytometry.

Supplemental References

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