Metabolic reprogramming induces resistance to anti-NOTCH1 therapies in T cell acute lymphoblastic leukemia

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Activating mutations in NOTCH1 are common in T cell acute lymphoblastic leukemia (T-ALL). Here we identify glutaminolysis as a critical pathway for leukemia cell growth downstream of NOTCH1 and a key determinant of the response to anti-NOTCH1 therapies in vivo. Mechanistically, inhibition of NOTCH1 signaling in T-ALL induces a metabolic shutdown, with prominent inhibition of glutaminolysis and triggers autophagy as a salvage pathway supporting leukemia cell metabolism. Consequently, inhibition of glutaminolysis and inhibition of autophagy strongly and synergistically enhance the antileukemic effects of anti-NOTCH1 therapy in mice harboring T-ALL. Moreover, we demonstrate that Pten loss upregulates glycolysis and consequently rescues leukemic cell metabolism, thereby abrogating the antileukemic effects of NOTCH1 inhibition. Overall, these results identify glutaminolysis as a major node in cancer metabolism controlled by NOTCH1 and as a therapeutic target for the treatment of T-ALL.

NOTCH1 signaling is a conserved signal transduction pathway with a prominent role in cell differentiation and tissue patterning during development1. In the hematopoietic system, NOTCH1 has been implicated in stem cell homeostasis and, most prominently, as a major driver of T cell lineage specification in lymphoid progenitors and a master regulator of thymocyte development2–5. In addition, aberrant NOTCH1 signaling has a major role in the pathogenesis of T-ALL, as more than 60% of T-ALL cases harbor activating mutations in the NOTCH1 gene5. Most notably, NOTCH1 has been proposed as a therapeutic target in NOTCH1-mutant leukemias. Small-molecule γ-secretase inhibitors (GSIs), which effectively block NOTCH1 activation via inhibition of a critical intramembrane proteolytic cleavage required for NOTCH1 signaling6, are now in clinical development for the treatment of relapsed and refractory T-ALL. However, the clinical development of anti-NOTCH1 therapies in T-ALL has been hampered by limited and delayed therapeutic response to these drugs, such that the identification of highly effective and synergistic drug combinations capable of generating strong antileukemic responses is a top priority in the field. In addition, and most troubling, most T-ALL cell lines harboring activating mutations in NOTCH1 fail to respond to GSI therapy, owing to mutational loss of the phosphatase and tensin homolog (PTEN) tumor suppressor, a negative regulator of the PI3K-AKT signaling pathway7. It is thus essential to establish the specific mechanisms by which PTEN inactivation drives resistance to anti-NOTCH1 therapy.

RESULTS
Pten loss confers resistance to NOTCH1 inhibition in T-ALL

To analyze the effects of Pten inactivation on the response of primary NOTCH1-induced leukemia cells to GSI therapy in vivo, we generated a mouse model of NOTCH1-induced T-ALL with conditional and inducible loss of Pten. We infected bone marrow hematopoietic progenitors from tamoxifen-inducible conditional Pten-knockout mice (Rosa26CreERT2/Ptenfl/fl) with retrovirus expressing a constitutively active oncogenic mutant form of the NOTCH1 receptor (L1601P Δ-PEST)8 and transplanted the cells into isogenic recipients, which consequently developed NOTCH1-induced T-ALL. We then injected tumor cells from these mice into secondary recipients and treated them with vehicle or tamoxifen to generate Pten-positive (nondeleted) and Pten-deleted isogenic tumors, respectively. Treatment of Pten-positive leukemia-bearing mice with DBZ, a highly active GSI9, induced a marked therapeutic response, as assessed by in vivo bioimaging (Fig. 1a), and a significant improvement in survival over vehicle-treated controls ($P<0.005$) (Fig. 1b and Supplementary Fig. 1). In contrast, mice harboring isogenic Pten-deleted tumors showed resistance to NOTCH1-inhibition therapy and exhibited disease progression under DBZ treatment (Fig. 1a,b and Supplementary Fig. 1). Analysis of cell proliferation in T-ALL lymphoblasts isolated from Pten-positive and Pten-deleted leukemia-bearing mice treated with DBZ showed that NOTCH1 inhibition decreased cell proliferation in Pten-positive tumors, but this effect was lost in Pten-deleted leukemic...
cells (Fig. 1c). Notably, we observed complete clearance of activated NOTCH1 protein (ICN1) in both Pten-positive and Pten-deleted tumors treated with DBZ (Fig. 1d), indicating that Pten loss does not impair the uptake or intrinsic activity of this GSI. Moreover, Myc, a critical downstream effector of the oncogenic effects of NOTCH1 (ref. 10), was effectively downregulated in both Pten-positive and Pten-deleted leukemia treated with DBZ (Fig. 1d and Supplementary Fig. 1), ruling out increased Myc expression secondary to Pten loss as a potential mechanism of escape from the antileukemic effects of NOTCH1 inhibition. Next, to assess the effects of isogenic Pten loss in human cells xenografted in mice, we infected a human primary xenograft (PD-TALL#19) with lentivirus carrying a small hairpin RNA (shRNA) targeting Pten (shPten) or an shRNA control (shLUC), and confirmed the knockdown of Pten in cells expressing shPten (Supplementary Fig. 2). Expression of shLUC did not alter the response to GSI treatment (Supplementary Fig. 2). In contrast, Pten knockdown restored leukemia cell growth in the context of GSI treatment in vivo (Supplementary Fig. 2).

Overall, these results show that Pten inactivation and consequent constitutive activation of the PI3K-AKT signaling pathway can confer resistance to anti-NOTCH1 GSI therapy in vivo.

To investigate the underlying mechanisms mediating resistance to NOTCH1 inhibition in Pten-deleted T-ALL tumor cells, we performed gene expression profiling of isogenic Pten-positive and Pten-deleted leukemia lymphoblasts after acute treatment with DBZ in vivo. This analysis revealed that, whereas direct NOTCH1 target genes (such as Hes1, Dtx1, Ptcr, Heyl and notch3) were effectively downregulated in both Pten-positive and Pten-deleted tumors (Fig. 1e–g and Supplementary Fig. 1), genetic ablation of Pten elicited a global reversal of much of the transcriptional effects of NOTCH1 inhibition (Fig. 1f,h and Supplementary Fig. 1). Functional annotation of genes downregulated by NOTCH1 inhibition whose expression was restored by Pten loss revealed a marked enrichment in pathways associated with cell anabolism, such as ribosomal RNA processing and amino
NOTCH1 inhibition in T-ALL leads to impaired metabolism

Following up on these results, we explored the metabolic effects of NOTCH1 inhibition and Pten loss using a broad-based metabolomic analysis. We performed gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–tandem MS (LC-MS/MS) of isogenic Pten-positive and Pten-deleted NOTCH1-induced leukemia cells treated with DBZ in vivo. This analysis showed that inhibition of NOTCH1 signaling by DBZ in NOTCH1-induced Pten-positive tumors resulted in the accumulation of glucose and its proximal (glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-biphosphate) and distal (3-phosphoglycerate and 2,3-diphosphoglycerate) glycolytic intermediates (Fig. 2a), coupled with higher levels of ribose-5-phosphate, ribulose-5-phosphate and xylulose-5-phosphate in the pentose phosphate pathway. Moreover, inhibition of NOTCH1 signaling was associated with elevated levels of free amino acids (Supplementary Fig. 3), potentially linked with increased autophagy and higher concentrations of glutamine but not glutamate (Fig. 2a). Notably, loss of Pten resulted in increased lactate concentrations (Fig. 2a) and reversed the accumulation of glycolytic intermediates induced by NOTCH1 inhibition (Fig. 2a).

To directly assess the role of impaired carbon metabolism in mediating the antileukemic effects of GSI treatment, we evaluated the capacity of methyl pyruvate, a membrane-soluble metabolite that bypasses glycolysis and can be incorporated directly into the tricarboxylic acid (TCA) cycle, to rescue the effects of NOTCH1 inhibition in DND41 cells, a NOTCH1-mutated and Pten-positive T-ALL cell line. Consistent with previous reports, inhibition of NOTCH1 signaling with DBZ resulted in decreased cell size and proliferation, with cell cycle arrest in G1 (Fig. 2b–d). Methyl pyruvate treatment induced a slight increase in cell size and abrogated the antileukemic effects of NOTCH1 inhibition on cell growth (10.5% and 2.6% reduction in cell size by DBZ in cells treated with vehicle only and methyl pyruvate, respectively; >0.001) and proliferation (Fig. 2b–d).
**Figure 3** Autophagy supports leukemic cell growth in response to NOTCH1 inhibition. (a) Left, representative electron microscopy micrographs of Pten-positive and Pten-deleted lymphoblasts from three independent mice acutely treated with vehicle or DBZ. Right, percentage of cells with autophagosomes (n = 3 mice per group). White arrows indicate autophagosomes. Scale bars, 500 nm. (b) Ratio of LC3a-II to LC3a-I levels after acute treatment of leukemic mice with vehicle only or DBZ. P values were calculated using two-tailed Student’s t-test. Error bars, mean ± s.d. (n = 3 mice per group). (c) Western blot analysis of Atg7 expression in leukemia cells isolated from mice harboring tamoxifen-inducible conditional Atg7-knockout leukemia treated with vehicle (control) or tamoxifen (TMX). (d) Kaplan-Meier survival curves of mice treated with four cycles of vehicle (control) or DBZ (5 mg per kg) on a 4-d-on (red blocks) and 3-d-off schedule (n = 5 per group). NS, not significant.

Similarly, bypass of glutaminolysis by treatment of the cells with membrane-soluble dimethyl α-ketoglutarate effectively antagonized the inhibitory effects of NOTCH1 inhibition on cell size (7.7% and 2.6% reduction in cell size by DBZ in cells treated with vehicle only and dimethyl α-ketoglutarate, respectively; P < 0.001) and proliferation (Fig. 2c–g). Taken together, these results support the concept that inhibition of carbon metabolism has a major role in the antileukemic effects of NOTCH1 inhibition in T-ALL. We obtained similar results for a second NOTCH1-mutated PTEN-positive cell line (HPB-ALL) (Supplementary Fig. 4).

**Figure 4** Glucose and glutamine metabolic flux analysis of T-ALL cells upon NOTCH1 inhibition and Pten loss. (a) Percentage of 13C incorporation into lactate and glutamate after incubation of primary NOTCH1-induced T-ALL cells with [13C]glucose and [13C]glutamine, respectively. (b) Percentage of 13C incorporation into TCA cycle intermediates after incubation of primary NOTCH1-induced T-ALL cells with [13C]glucose and [13C]glutamine, respectively. (c) Extracellular concentration of lactate produced by Pten-positive and Pten-deleted leukemia cells after 6 h of cell culture in vitro using dialyzed medium. (d) Kinetic analysis of glucose incorporation in primary lymphoblasts incubated with [13C]glucose. (e) Kinetic analysis of glutamine incorporation in primary lymphoblasts incubated with [13C]glutamine. M + 2, M + 3, M + 4 and M + 5 indicate compounds that contain 2, 3, 4 or 5 13C atoms, respectively. Error bars, mean ± s.d. (a–e). **P < 0.005; ***P < 0.001; *P < 0.05; Student’s t-test; n = 3 mice per group.
**Figure 5** The antileukemic effects of GSI in *Pten*-positive leukemias can be rescued by myristoylated AKT (MyrAKT), GLS and PKM2 overexpression. (a) Western blot analysis of intracellular activated NOTCH1, AKT phosphorylated at T308 (p(T308)AKT), total AKT, *Pten* and Gls (kidney-type glutaminase isoform, KGA) expression in *Pten*-positive leukemias progressing after 4 cycles of treatment with vehicle or DBZ (5 mg/kg) on a 4-d-on (red blocks) and 3-d-off schedule. (b-g) Peripheral blood leukemia infiltration in mice harboring NOTCH1-induced *Pten*-positive T-ALL cells expressing mCherry and MyrAKT (b), GLS (c), PKM2 (d), PHGDH (e), IL-7R (f) or pre-T cell antigen receptor-α (Ptcra) (g) upon continuous daily treatment with DBZ. Changes in leukemia cell counts of noninfected (mCherry-negative) cells are shown as an internal control. Error bars, median ± s.d. (b-g); *P* values were calculated using two-tailed Student’s *t*-test (*n* = 5 mice per group); NS, not significant.

**Pten**-positive T-ALLs rely on autophagy upon NOTCH1 inhibition

The transcriptional and metabolic changes associated with NOTCH1 inhibition in *Pten*-positive T-ALL tumor cells are indicative of decreased anabolism and a concomitant increase in catabolic processes. Consistent with this, electron microscopy analysis of *Pten*-positive NOTCH1-induced leukemia in vivo showed increased autophagy upon DBZ treatment; this increase did not occur in DBZ-deleted leukemia (Fig. 3a). In addition, western blot analyses showed that DBZ treatment resulted in increased levels of the autophagic marker LC3-II in *Pten*-positive leukemia, whereas *Pten* loss blocked this effect (Fig. 3b and Supplementary Fig. 5).

On the basis of these observations, we hypothesized that induction of autophagy can help sustain cell survival during NOTCH1 inhibition by recycling essential metabolites required for leukemic cell metabolism. To examine the effect of NOTCH1 inhibition in autophagy-defective T-ALL, we generated mice bearing NOTCH1-induced T-ALL using tamoxifen-inducible conditional Atg7-/- (Fig. 4). The transcription and metabolic changes associated with NOTCH1 inhibition in *Pten*-positive leukemias reversed the anabolic (downregulated) and catabolic (upregulated) transcriptional changes associated with NOTCH1 inhibition (Supplementary Fig. 7), reversed blockage of glycolysis and glutaminolysis and the increase in free amino acids, as assessed by the lactate pool (~80% labeling) and contributed to 40–60% of the TCA cycle intermediates citrate, fumarate and malate (Fig. 4a,b). *Pten* deletion resulted in increased lactate production (Fig. 4c), which suggests increased glycolysis. Moreover, kinetic profiling analysis of glucose- and glutamine-derived 13C incorporation into the TCA cycle showed that NOTCH1 inhibition with DBZ decreased the fractional contribution of glucose to lactate, but not to TCA cycle intermediates (Fig. 4d). In contrast, DBZ treatment decreased glutamine labeling of glutamate and TCA cycle intermediates (Fig. 4e). Notably, the DBZ-induced block in glycolysis observed in NOTCH1-induced *Pten*-positive leukemic lymphoblasts was attenuated by isogenic deletion of *Pten* (Fig. 4d). In addition, *Pten* loss attenuated the DBZ-induced block in glutamine-to-glutamate conversion but had no clear effects on the incorporation of glutamine-derived carbon to the TCA cycle (Fig. 4e).

Despite an improvement in survival after four cycles of DBZ treatment, mice harboring *Pten*-positive NOTCH1-induced leukemia eventually showed disease progression (Fig. 1b and Supplementary Fig. 1). At the time of disease progression, *Pten*-positive NOTCH1-induced leukemic cells showed lower expression of *Pten* and higher expression of phosphorylated AKT and glutaminase (Gls) than did leukemic cells from vehicle-treated mice (Fig. 5a). These results suggest that T-ALL lymphoblasts treated with GSI in vivo overcome the therapeutic effects of NOTCH1 inhibition by downregulating *Pten* and upregulating Gls expression.

To further explore the mechanisms downstream of NOTCH1 and PTEN in the control of leukemic cell growth, we tested the effects of overexpressing metabolic regulators and NOTCH1-controlled signaling factors on the response to GSI treatment in vivo. Overexpression of interleukin 7 receptor (IL-7R) or pre-T cell antigen receptor-α (Ptcra), both of which are downregulated by GSI treatment in both *Pten*-positive and *Pten*-deleted T-ALL cells, had no effect on the response of *Pten*-positive cells to vehicle or GSI therapy in vivo (Fig. 5b–g and Supplementary Fig. 6). In contrast, overexpression of constitutively active AKT (myristoylated-AKT) in *Pten*-positive T-ALL tumor cells reversed the anabolic (downregulated) and catabolic (upregulated) transcriptional changes associated with NOTCH1 inhibition (Supplementary Fig. 7), reversed blockage of glycolysis and glutaminolysis and the increase in free amino acids, as assessed by
Supplementary Fig. 9 (c, d) Representative flow cytometry plots from triplicate samples of annexin V (apoptotic cells) and 7-AAD (dead cells) staining (c) and quantification of apoptosis (d) of HPB-ALL cells treated with vehicle (DMSO), BPTES, DBZ or BPTES and DBZ (n = 4 for HPB-ALL DBZ + BPTES; n = 5 for all other groups). (e, f) Representative images from four or five treated mice (left) and quantification (right) of changes in tumor burden as assessed by bioimaging in mice xenografted with human primary T-ALL cells PDTALL#19 (e) or PDTALL#10 (f) and treated with vehicle, BPTES, DBZ or BPTES and DBZ (n = 4 for PDTALL#19 DBZ + BPTES; n = 5 for all other groups). (g, h) Representative images (left) and quantification (right) of changes in tumor burden as assessed by bioimaging in mice allografted with NOTCH1-induced Pten-positive (g) or Pten-deleted (h) mouse leukemia lymphoblasts and treated as indicated (Pten+/− vehicle, n = 4; Pten−/− BPTES, n = 3; all other groups, n = 5). Horizontal lines in e–h indicate the mean values. (i) Western blot analysis of Gls expression in leukemia cells isolated from mice harboring tamoxifen-inducible conditional Gls-knockout leukemia treated with vehicle (control) or tamoxifen (TMX). (j) Kaplan-Meier survival curves of mice harboring Gls-positive and Gls-deleted isogenic leukemias treated with 4 cycles of vehicle or DBZ (5 mg/kg) on a 4-d-on (red blocks) and 3-d-off schedule (log-rank test; ***P < 0.005; n = 10 per group). P values (a, d–h) were calculated using two-tailed Student’s t-test. Error bars, mean ± s.d. of triplicate samples (a, d). Scale bars, 1 cm (e–h).

In contrast, overexpression of 3-phosphoglycerate dehydrogenase (PHGDH), which catalyzes the first and rate-limiting step in the phosphorylated pathway of serine biosynthesis and is associated with tumor cell growth, had limited or no effect on the response of tumor cells to NOTCH1 inhibition (Fig. 5e). Cells overexpressing Gls showed an increase in glutamine utilization (Supplementary Fig. 9); nevertheless, kinetic labeling analysis using glutamine-derived 13C showed that DBZ treatment downregulated glutaminolysis in these cells (Supplementary Fig. 9), suggesting that Gls overexpression may confer resistance to DBZ treatment. Overall, these results demonstrate that metabolic manipulation can modify the in vivo response of NOTCH1-induced leukemia to GSI treatment.

Therapeutic targeting of NOTCH1 and Gls in T-ALL

The identification of glutaminolysis as a critical effector of the antileukemic response to NOTCH1 inhibition in T-ALL suggests that pharmacologic inhibition of Gls may impair leukemic cell growth and sensitize T-ALL cells to NOTCH1 inhibition therapy. To test this hypothesis, we analyzed the effects of Gls inhibition on leukemia cell growth and survival. Treatment of HPB-ALL and DND41 T-ALL cells with BPTES, a potent and specific Gls inhibitor, impaired leukemia cell growth (Fig. 6a and Supplementary Fig. 10) and showed strong and significantly synergistic antileukemic effects in combination with NOTCH1 inhibition using DBZ (combination index at effective dose 50 (ED50) = 0.012 in HPB-ALL and 0.666 in DND-41 cells) (Fig. 6b).
and Supplementary Fig. 10). Notably, this synergistic effect was driven primarily by increased cytotoxicity in cells treated with a combination of BPTES and DBZ (Fig. 6c,d and Supplementary Fig. 10). In contrast, treatment with 2-deoxyglucose, a nonmetabolizable glucose analog that inhibits glycolysis17, showed only additive antileukemic effects in combination with DBZ (Supplementary Fig. 11), supporting the concept that glutaminolysis has a dominant role over glycolysis downstream of NOTCH1 signaling in T-ALL. Notably, CRISPR-Cas9–mediated PTEN inactivation in HPB-ALL cells conferred resistance to treatment with DBZ alone or combined with BPTES (Supplementary Fig. 12), consistent with the idea that PTEN loss is a driver of resistance to GSI therapy in humans.

To further explore the clinical relevance of GLS inhibition therapies in combination with GSIs, we tested the efficacy of combining NOTCH1 and GLS inhibition in human leukemia xenografts. Mice bearing xenografts from either of two independent NOTCH1-mutant and PTEN wild-type human–derived primary T-ALL xenografts (PDTALL#10 (NOTCH1 p.Leu1601Pro; Arg1609His) and PDTALL#19 (NOTCH1 p.Pro1606delinsLeuVal; Val2412fs)) were treated with DBZ, BPTES or both (Fig. 6e,f). Mice treated with BPTES showed progressive tumor growth similar to that observed in controls treated with vehicle (DMSO) only, whereas DBZ induced significant antitumor responses, and these were significantly increased by combined BPTES treatment (Fig. 6e,f).

To better assess the interaction between NOTCH1 signaling and Pten loss in the response to anti-NOTCH1 and GLS inhibition therapies, we analyzed the effects of treatment with BPTES, DBZ or both in mice transplanted with NOTCH1–induced Pten-positive or NOTCH1–induced Pten-deleted mouse isogenic tumor cells. In mice bearing NOTCH1–induced Pten-positive T-ALL, BPTES treatment significantly increased the antileukemic effects of DBZ (Fig. 6g and Supplementary Fig. 13), which translated to increased survival (Supplementary Fig. 14). In contrast, Pten-deleted T-ALL cells showed an impaired response to GSI therapy and to treatment with DBZ combined with BPTES (Fig. 6h). These results are consistent with our metabolic studies showing that Pten loss induces a hyperglycolytic phenotype, which would render T-ALL cells not only resistant to NOTCH1 inhibition but also less sensitive to the inhibition of glutaminolysis as a result of increased glucose-derived carbon input to the TCA cycle.

Finally, we tested the interaction between NOTCH1 signaling and GLS in vivo and rule out potential off-target effects of BPTES, we generated NOTCH1–induced T-ALL using tamoxifen–inducible conditional Glu-knockout (Rosa26Cre-ERT2/+/GluER) mouse hematopoietic progenitors. Leukemic cells were transplanted into secondary recipients, which were treated with vehicle only or tamoxifen to generate Glu-positive and Glu-deleted isogenic tumors, respectively (Fig. 6i). Treatment of NOTCH1–induced Glu-positive T-ALL–bearing mice with DBZ induced a marked in vivo antileukemic response and significantly improved survival (P < 0.005) (Fig. 6j). In addition, tamoxifen–induced deletion of Glus resulted in overt antileukemic effects, which were markedly and significantly enhanced (P < 0.005; 40% complete remission) by GSI treatment (Fig. 6j). This finding fully validates our pharmacological results and highlights glutaminolysis as a key player and therapeutic target in NOTCH1–induced T-ALL.

**DISCUSSION**

Co-occurring activating mutations in NOTCH1 and mutations and deletions in PTEN are characteristic of GSI-resistant T-ALL cell lines2 and can also be found in primary patient samples5,18. However, PTEN-mutant leukemias may still be at least partially responsive to NOTCH1 inhibition19, and epigenetic reprogramming in NOTCH1-driven T-ALL cell lines has been implicated in tumor escape from anti-NOTCH1 therapies after prolonged in vitro treatment with GSIs20, suggesting that additional genetic and epigenetic factors may help to drive resistance to GSI-based anti-NOTCH1 therapies. To formally address the specific role of Pten inactivation as a driver of resistance to GSI therapy, we engineered a mouse model of NOTCH1–induced leukemia with inducible loss of Pten, which allowed for the direct comparison of Pten-positive and Pten-deleted isogenic tumors in immune-competent isogenic secondary recipients in vivo. In this model, mice bearing NOTCH1–induced leukemia showed marked but variable antileukemic responses to GSI therapy. Although Pten deletion did not render leukemic cells completely insensitive to NOTCH1 inhibition using GSI treatment, mutational loss of Pten substantially impaired the response to GSI treatment in each of the three independently generated Pten conditional-knockout tumors tested. All mice harboring Pten-deleted leukemias showed overt progression under therapy, a hallmark of therapy resistance in the clinic, and all mice died by the end of the fourth cycle of treatment. Similarly, PTEN knockdown in a primary human T-ALL xenografted in mice impaired the therapeutic efficacy of GSI treatment. These results demonstrate conclusively that mutational loss of Pten can induce resistance to GSI therapy in primary tumor cells in vivo.

Mechanistically, gene expression–profiling analyses revealed that inhibition of the γ-secretase complex effectively suppressed NOTCH1 activation and that key genes controlled by NOTCH1 were effectively downregulated in Pten-positive and Pten-deleted tumors. In addition, pathway analysis of gene expression programs controlled by NOTCH1 in Pten-positive and Pten-deleted T-ALL tumor cells revealed a dominant effect of NOTCH1 inhibition on the suppression of anabolic genes with concomitant upregulation of catabolic genes. The broad transcriptional effects of NOTCH1 inhibition across different metabolic pathways are reminiscent of those induced by KrasG12D inactivation in pancreatic ductal adenocarcinoma tumor cells21 and support the idea that the antileukemic effects of NOTCH1 inhibition in T-ALL may be mediated, at least in part, by inhibition of cell metabolism. Notably, this model is consistent with the decreased cell growth and cell size phenotypes typically induced by NOTCH1 inhibition in human T-ALL cell lines that carry wild-type PTEN and are sensitive to GSI5,7. Unexpectedly, whereas Pten deletion did not alter the inhibitory effects of GSI treatment on direct gene targets of NOTCH1, Pten deletion had profound effects on the transcriptional response of NOTCH1–induced leukemia to anti-NOTCH1 therapy, reversing GSI-induced anabolic gene downregulation and catabolic gene upregulation. These results suggest that the PI3K-AKT signaling pathway in T-ALL has a substantial transcriptional role in the control of cell metabolism, and that these effects on cell metabolism are antagonistic to those resulting from suppression of oncogenic NOTCH1. Moreover, we observed increased autophagy in Pten-positive, but not Pten-deleted, leukemias upon NOTCH1 inhibition. Activation of autophagy upon GSI treatment and increased antileukemic effects of GSI in autophagy-deficient Aytg-7-null tumors support the concept that NOTCH1 inhibition in Pten-positive T-ALL forces tumor cells to use this catabolic mechanism to obtain critical metabolic intermediates for sustained cell growth22.

Global metabolic profiling and metabolic isotope tracing analyses revealed a negative effect of NOTCH1 inhibition on glycolysis and glutaminolysis in Pten-positive T-ALL. Notably, genetic loss of Pten or activated AKT expression resulted in increased intracellular
Acute T-cell leukemias remain dependent on Notch signaling. Notably, the therapeutic response to combined NOTCH and GLS inhibition was abrogated by loss of Pten. The resistance of Pten-deleted leukemia to combined treatment can be explained by the observation that loss of Pten switches T-ALL cells to a hyperglycolytic phenotype, increasing glucose-derived carbon input to the TCA cycle and making them less dependent on glutaminolysis.

Overall, our results highlight the fundamental importance of NOTCH1 signaling in the control of leukemic cell metabolism, extend our understanding of the prominent role of PTEN and the PI3K pathway in the control of oncogenic cell growth and provide the basis for the design of new therapeutic strategies targeting cell metabolism for the treatment of T-ALL.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Gene Expression Omnibus: microarray gene expression data are available under accession codes GSE71087 and GSE71089.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

D.H. carried out most of the experiments. A.A.-I. analyzed gene expression profile signatures. J.S. performed metabolic studies. M.S.-M. performed some in vivo and in vitro drug response analyses. L.B. analyzed PTEN levels by intracellular FACS staining. V.T. generated some of the NOTCH1-induced primary leukemias. L.X. performed some animal studies with D.H. A.A.W. performed some experiments with human primary T-ALL samples. M.C. conducted histological examination of tumor development and response to therapy. J.E.H. performed some in vivo experiments. J.M. and J.M.M. contributed reagents. S.R. generated the G6 conditional knockout mice. A.L.K. conceived and supervised bioimaging studies. C.C.-C. supervised histological analyses. R.J.D. supervised metabolomic isotope tracing analyses. A.A.F. designed the study, supervised research and wrote the manuscript with D.H.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mouse studies. We maintained all animals in specific pathogen-free facilities at the Irving Cancer Research Center at the Columbia University Medical Campus. The Columbia University Institutional Animal Care and Use Committee (IACUC) approved all animal procedures. Rosa26CreERT2/mice expressing a tamoxifen-inducible form of Cre recombinase from the ubiquitously expressed Rosa26 locus23. Pten conditional knockout (Ptenfl/fl) and Atg7 conditional knockout (Atg7fl/fl) mice have been previously described. To generate NOTCH1-induced T-ALL tumors in mice, we isolated bone marrow lineage negative cells using magnetic beads (Miltenyi kit #130-090-858, following the manufacturer’s guidelines) and performed retroviral transduction by spinoculation with retrovirus encoding activated forms of the NOTCH1 oncogene. Specifically, retrovirus encoding NOTCH1 L1601P ΔPEST was transduced into Pten cels and retrovirus encoding ΔE-NOTCH1 (ref. 14) was transduced into Atg7 or Glb cells. Cells were then transplanted via intravenous injection into lethally irradiated recipients as previously described8.

For all subsequent studies, we used secondary recipient 6–8-week-old C57BL/6 female mice (Taconic Farms). Animals were randomly assigned to the different treatment groups, and investigators were not blinded to group allocation. For survival studies, we transplanted leukemia cells from primary recipients into sublethally irradiated (4 Gy) secondary recipients. Two days after the transplant, we treated the mice with tamoxifen (5 mg per mouse by intraperitoneal injection) to induce deletion of the Pten, Atg7 or Glb, or with vehicle only (corn oil) for the control group; 4 d later, we subdivided the mice into groups treated either with vehicle only (2.3% DMSO in 0.005% methycellulose, 0.1% Tween-80) or with DBZ (5 mg per kg) on a 4-d-on and 3-d-off schedule.

For the DBZ acute treatment analyses in Pten-positive and Pten-deleted isogenic tumors, we transplanted leukemia cells from primary recipients into secondary recipients, which were treated with vehicle or tamoxifen (to induce Pten deletion), as described above. We monitored the peripheral blood of the mice until more than 70% of cells were GFP positive; then, mice were treated twice, 16 h apart, with vehicle only or DBZ (5 mg per kg). 4 h after the second treatment, mice were sacrificed and spleen samples were collected and analyzed by 13C labeling or were snap-frozen for further analyses (microarray, reverse transcription PCR, western bloting, metabolomics).

For the experiment to test the effects of overexpressing metabolic regulators or NOTCH1-controlled signaling factors on the response to DBZ in vivo, we infected Pten-positive leukemic cells with retroviruses driving the expression of the cherry fluorescent protein alone (pMSCV-IRES-mCherry FP) or retroviruses driving bicistronic expression of the cherry fluorescent protein together with either IL-7R (pMSCV-IL-7R-IRES-mCherry FP), pre-TCRα (pMSCV-Ptcrα-IRES-mCherry FP), myristoylated-AKT (pMSCV-Myr-AKT-IRES-mCherry FP), glutaminase (pMSCV-GLS-IRES-mCherry FP), PKM2 (MSCV-PKM2-IRES-mCherry FP) or PHGDH (pMSCV-PHGHDH-IRES-mCherry FP). DNA sequences for myristoylated-AKT and complementary DNAs for genes encoding IL-7R, pre-TCRα, PKM2 and PHGDH were amplified by PCR using primers containing unique restriction enzyme sites, cloned into the multiple cloning sites of the pMSCV-mCherry FP vector (Addgene plasmid #52114), and sequence verified. The infected cells were then injected into sublethally irradiated C57BL/6 mice (4 Gy).

For drug synergism studies in vivo, we infected Pten conditional knockout leukemia cells from primary recipients with retroviruses expressing a fusion protein consisting of the cherry fluorescent protein fused to luciferase (MigR1-mCherry-Luc). A DNA sequence encoding the mCherry-Luc fusion protein was generated by PCR amplification, cloned into the MigR1 vector (Addgene plasmid #27490), and sequence verified. Infected cells were then injected into sub-lethally irradiated C57BL/6 male mice (4 Gy). For the experiment using human primary leukemia xenografts, we infected two primary xenografts generated by injection of primary T-ALL clinical samples into immune-deficient mice (PDTALL19 and PDTALL10) with lentiviruses expressing the cherry fluorescent protein and luciferase (FUW-mCherry-Puro-Luc)20; the cells were then injected into male or female 6–8-week old NRG mice (the Jackson Laboratory). We analyzed the efficacy of glutaminase inhibition with BPTES in combination with DBZ in secondary NRG recipient mice transplanted with these mCherry-luciferase–expressing cells. In these experiments, we treated the mice with daily intraperitoneal doses of vehicle (DMSO), BPTES (25 mg/kg), DBZ (5 mg/kg) or BPTES (25 mg/kg) plus DBZ (5 mg/kg) for 6 d. We evaluated disease progression and therapy response by in vivo bioimaging with the In vivo Imaging System (IVIS, Xenogen).

Microarray gene expression profiling. We isolated RNA from leukemic cells from spleen samples collected from mice acutely treated with vehicle or DBZ using RNAeasy Plus Mini kit (Qiagen) according to the manufacturer’s protocol. RNA (1 µg) from Pten-positive and Pten-deleted leukemic cells, from mice treated with vehicle or DBZ, was amplified and labeled using the 3′ IVT Express Kit (Affymetrix) and hybridized on GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix) according to the manufacturer’s protocol. RNA (1 µg) from Pten-positive mCherry and Pten-positive MyrAKT leukemic cells, from mice treated with vehicle or DBZ, was amplified and labeled with the Illumina TotalprepRNA kit and hybridized on Illumina MouseWG-6 v2.0 BeadChip arrays according to the manufacturer’s protocol. For the Affymetrix arrays, we performed inter-array normalization with the GC-RMA algorithm using open-source Bioconductor software30. For the Illumina arrays, expression values were log-transformed and quantile normalized. We evaluated group differences using t-test and fold change.

For the pathway enrichment analyses, gene sets of interest, including genes whose change in expression in response to GSI was reversed by Pten deletion, were tested for enrichment in functional annotations using the web-based DAVID bioinformatics tools (http://david.abcc.ncifcrf.gov).

To compare the enrichment of genes differentially expressed by GSI and vehicle treatment in Pten-positive and Pten-deleted tumors with that of genes differentially expressed by GSI and vehicle treatment in Pten-positive mCherry expressing tumors and Pten-positive mCherry-MyrAKT expressing tumors, we used gene set enrichment analysis31 and the t-test metric, with 10,000 permutations of the gene list.

Metabolic analyses. We analyzed leukemic spleen samples from mice acutely treated with vehicle or DBZ by GC/MS and LC/MS/MS. Briefly, we collected spleen samples 4 h after the second round of in vivo treatment with vehicle or DBZ and snap froze them. We then extracted the samples and prepared them for analysis using standard solvent extraction methods. The extracted samples were split into equal parts for analysis on the GC/MS and LC/MS/MS platforms of Metabolon, Inc. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards. Following log transformation of GC/MS and LC/MS/MS signal and imputation with minimum observed values for each compound, we used ANOVA contrasts for pairwise comparisons to identify biochemicals whose amounts differed significantly between experimental groups. Analysis by two-way ANOVA identified biochemicals exhibiting significant interaction and main effects for the experimental parameters of Pten status and DBZ treatment. We calculated an estimate of the false discovery rate (q value) to take into account the multiple comparisons that normally occur in metabolomic-based studies. For example, when analyzing 200 compounds, we would expect to see about 10 compounds meeting the P ≤0.05 cutoff by random chance.

13C labeling studies. We cultured leukemia cells collected from the spleens of acutely treated mice (as described above) in RPMI supplemented with 10% dialyzed FBS and 10 mM [13C]glucose or 4 mM [13C]glutamine (Cambridge Isotope Laboratories). Cells were cultured for 6 h for the steady-state glucose and glutamine experiments and for 30 min, 1 h, 2 h and 6 h for the glucose and glutamine kinetics experiment. After labeling, we rinsed the cells in ice-cold normal saline and lysed them with three freeze-thaw cycles in cold 50% methanol. The lysates were centrifuged to remove precipitated protein, 50 nmol of sodium-2-exobutylate was added as an internal standard and the samples were evaporated and derivatized by trimethylsilylation (Tri-Sil HTP reagent, Thermo Scientific). We then injected three microliters of the derivatized material onto an Agilent 6970 Gas Chromatograph equipped with a fused silica capilary GC column (30 m length, 0.25 mm diameter) and networked to an Agilent 5973 Mass Selective Detector. Retention times of all metabolites of interest were validated using pure standards. The measured distribution of mass isotopomers was corrected for the natural abundance of 13C.

Extracellular lactate was measured in 100 µl of RPMI supernatant from cells that had been cultured for 6 h in media made with dialyzed serum. Removal of lipids and proteins from the media was accomplished with a 1:1:

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chloroform-methanol-water extraction. Prior to evaporation, a uniformly labeled $^{13}$C Lactate standard (Cambridge) was added to the aqueous phase. After being dried, the sample was derivatized with 100 µl of Tri-Sil (Thermo Scientific) for 10 min at 75 °C, followed by 10 min at room temperature. 5 µl of the derivatized sample was injected onto an Agilent 6890 Gas Chromatograph networked to an Agilent 5975 Mass Selective Detector. Comparison of the labeled lactate standard to the unlabeled lactate pool derived from cellular metabolism allowed for the quantitation of lactate abundance in the sample. We measured ammonia production with a spectrophotometric assay (Megazyme).

**Quantitative real-time PCR.** We generated cDNA using the ThermoScript RT-PCR system (Invitrogen) and performed quantitative real-time PCR (FastStart Universal SYBR Green Master Mix (Roche) using a 7300 Real-Time PCR System (Applied Biosystems). Relative expression levels were based on Gapdh as a reference control.

**Western blotting.** We performed western blots using standard procedures. Antibodies against Cleaved Notch1 (#4147), GAPDH (#5174), LC3a (#4599), p(S473)AKT (#9271), p(T308)AKT (#9275), AKT total (#9272) and PTEN (#9188) were from Cell Signaling Technologies (1:1,000 dilution). Glutaminase antibody was from Proteintech (1:1,000, #20170-1-AP) or a purified rabbit polyclonal antibody against GLS (1:1,000 dilution). ATG7 antibody was from Sigma-Aldrich (1:1,000, #A2856). Myc antibody (1:200, N-262) was obtained from Santa Cruz. GFP antibody (1:1,000, #11814460001) was obtained from Roche.

**Inhibitors and drugs.** DBZ (S)-2-(3,5-Difluorophenyl)acetamido)-N-(S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)propanamide) was obtained from Syncom (SIC-020042). BPTES (Bis-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide; #SML0601), 2-deoxylucose (#D6134), tamoxifen (#T5648), dimethyl ketoglutamate (#349631) and methyl pyruvate (#9188) were from Cell Signaling Technologies (1:1,000 dilution). Antibodies against Cleaved Notch1 (#4147), GAPDH (#5174), LC3a (#4599), Cat #560002, clone A2B1, dilution 1:5); we used PE-labeled mouse IgG1 isotype (Cat #17-0459-42, clone HI30, dilution 1:200). Next, we performed fixation and permeabilization using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience Cat #00-5523), following the manufacturer’s instructions. Finally, we performed intracellular staining with anti-PTEN-PE (BD Phosflow, Cat #560002, clone A2B1, dilution 1:5); we used PE-labeled mouse IgG1 isotype as a control (BD Pharamingen, Cat #551436, dilution 1:5).

**Cell viability, cell size and flow cytometric analyses.** We analyzed cell viability upon treatment with BPTES (10 µM) and DBZ (250 nM) alone and in combination using the Cell Proliferation Kit I (Roche). We analyzed apoptosis by flow cytometry with the APC AnnexinV Apoptosis Kit I (BD Biosciences). We used propidium iodide (Sigma) DNA staining to analyze cell cycle distribution. For the metabolic rescue experiments, cells were maintained in RPMI buffered with 40 mM HEPES, and treated with methyl pyruvate (10 mM) or dimethyl ketoglutarate (8 mM) and/or DBZ (250 nM). We added new medium every 2 d.

For measurement of PTEN intracellular levels in leukemic cells from mice bearing xenografts of peripheral primary human PTDALL#19 cells, we removed red cells from peripheral blood samples by incubation with red blood cell lysis buffer (155 mM NH$_4$Cl, 12 mM KHCO$_3$, and 0.1 mM EDTA) for 5 min at room temperature. We then stained the samples with anti-human-CD45 (eBioscience, Cat #17-0459-42, clone HI30, dilution 1:200). Next, we performed fixation and permeabilization using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience Cat #00-5523), following the manufacturer’s instructions. Finally, we performed intracellular staining with anti-PTEN-PE (BD Phosflow, Cat #560002, clone A2B1, dilution 1:5); we used PE-labeled mouse IgG1 isotype as a control (BD Pharamingen, Cat #551436, dilution 1:5).

**Statistical analyses.** We performed statistical analysis by Student’s t-test. We considered results with $P < 0.05$ as statistically significant. We analyzed drug synergism using the Chou-Talalay method and used CalcuSyn software (Biosoft) to calculate the combination index (CI) and perform isobologram analysis of drug interactions. Survival in mouse experiments was represented with Kaplan-Meier curves and significance was estimated with the log-rank test (Prism GraphPad).
Corrigendum: Metabolic reprogramming induces resistance to anti-NOTCH1 therapies in T cell acute lymphoblastic leukemia

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Mouse images are duplicated in Fig. 6e (day 0 Vehicle and day 0 BPTES) and in Fig. 6f (day 0 DBZ + BPTES and day 6 DBZ + BPTES). The authors made these errors in assembling the figure panels. The authors have now supplied corrected versions of these panels, in which the correct micrographs for Fig. 6e (day 0 BPTES) and Fig. 6f (day 6 DBZ + BPTES) are included. These errors do not affect the data shown in the graphs in Fig. 6e-f. The errors have been corrected in the HTML and PDF versions of the article.