Dietary thiamine influences L-asparaginase sensitivity in a subset of leukemia cells

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Tumor environment influences anticancer therapy response but which extracellular nutrients affect drug sensitivity is largely unknown. Using functional genomics, we determine modifiers of L-asparaginase (ASNase) response and identify thiamine pyrophosphate kinase 1 as a metabolic dependency under ASNase treatment. While thiamine is generally not limiting for cell proliferation, a DNA-barcode competition assay identifies leukemia cell lines that grow suboptimally under low thiamine and are characterized by low expression of solute carrier family 19 member 2 (SLC19A2), a thiamine transporter. SLC19A2 is necessary for optimal growth and ASNase resistance, when standard medium thiamine is lowered ~100-fold to human plasma concentrations. In addition, humanizing blood thiamine content of mice through diet sensitizes SLC19A2-low leukemia cells to ASNase in vivo. Together, our work reveals that thiamine utilization is a determinant of ASNase response for some cancer cells and that oversupplying vitamins may affect therapeutic response in leukemia.

INTRODUCTION

Nutrient availability in the tumor environment influences the metabolism of cancer cells and may result in dependencies that can be exploited for therapy (1, 2). Deprivation of highly consumed extracellular nutrients in tumors may impose cancer cells to use specific metabolic pathways for proliferation and survival. For instance, low tumor glucose concentrations up-regulate oxidative phosphorylation, an essential adaptation that can be targeted by the biguanide class of drugs (3). Similarly, environmental pyruvate and glutamine levels may determine the anaplerotic substrate on which cancer cells rely (4). Exogenous modulation of tumor nutrient supply can also alter metabolic programs and, consequently, therapeutic response (5). Through effects on one-carbon metabolism, histidine supplementation or dietary methionine restriction sensitizes cancer cells to commonly used chemotherapeutic agents (6, 7). The use of physiological cell culture media has further demonstrated that nutrient environment induces metabolic changes that affect drug sensitivity (8). In these recent studies, simply bringing medium concentrations of uric acid and cystine to physiological levels altered cancer cell responses to 5-fluorouracil and CB-839, respectively (9, 10). Together, these examples illustrate the need for further investigation into which nutrients affect therapeutically relevant metabolic pathways. In particular, it is poorly understood whether vitamins, which act as cofactors for many metabolic reactions, can modulate responses to anticancer drugs.

L-asparaginase (ASNase) is a first-line chemotherapeutic for acute lymphoblastic leukemia (ALL) that depletes the amino acid asparagine from blood, thereby targeting leukemia cells that are unable to synthesize sufficient asparagine (11, 12). Major determinants of ASNase sensitivity include basal expression of asparagine synthetase (ASNS), a key enzyme that converts aspartate to asparagine, as well as the capacity to up-regulate ASNS through ZBTB1 (Zinc Finger and BTB domain-containing protein 1) and activating transcription factor 4 (ATF4) (13, 14). Recent work has identified alternative ways of increasing asparagine availability, such as through proteasome degradation (15) or aspartate uptake by solute carrier family 1 member 3 (SLC1A3) (16). In addition, other cell types can secrete asparagine or its precursors, which ALLs can use, attenuating ASNase cytotoxicity (17, 18). It remains unclear, however, whether extracellular nutrients that are not asparagine precursors can influence sensitivity to ASNase.

Using a CRISPR-Cas9–based genetic screen, here, we interrogated the metabolic determinants of ASNase sensitivity in leukemia. Our analysis pinpointed thiamine pyrophosphate (TPP) kinase 1 (TPK1) as essential for proliferation under ASNase treatment. TPK1 converts TPP to TPP, a cofactor necessary for the activity of the α-ketoglutarate dehydrogenase (AKGDH) complex. In glutamine-anaplerotic leukemia cells, TPP availability enables asparagine synthesis from extracellular glutamine, when asparagine is depleted. We further identified that physiological levels of thiamine do not limit cell proliferation or ASNase response in most cancer cell lines, except for those that have low expression of SLC19A2, a thiamine transporter. These cancer cell lines become sensitive to ASNase when thiamine concentration is lowered to human plasma levels, which is ~100-fold lower than standard culture conditions. Consistent with this observation, a diet that humanizes mouse plasma thiamine levels sensitized leukemia xenografts with endogenously low SLC19A2 to ASNase. Together, our results suggest that SLC19A2 expression may be a determinant of ASNase response and that supraphysiological thiamine supplementation could potentially ablate such an association. Furthermore, our work provides a proof of concept that using human physiological vitamin levels can alter cancer cell sensitivity to therapies.

RESULTS

Functional genomics identifies metabolic determinants of proliferation under ASNase treatment

To begin to understand how tumor environment affects ASNase response of diverse cancer types, we used a pool of 62 DNA-barcoded cell lines, consisting mainly of hematopoietic and lymphoid cancers,
and generated subcutaneous xenografts in mice treated with either ASNase or vehicle (Fig. 1A and fig. S1, A and B). While most cancer cell lines did not respond to ASNase treatment, this competition assay revealed a set of cell lines as strongly sensitive to it. Consistent with previous work (19), many of these lines belong to ALLs that express low levels of ASNS mRNA (Fig. 1A). Despite the evidence that ASNS expression is a reasonable predictor for ASNase response, we found cancer cell lines with high expression of ASNS that appeared to have some sensitivity to ASNase treatment in vivo, whereas some with low expression were resistant. Overall, these data suggest that there may be other factors that affect ASNase response.

To further investigate this possibility, we performed metabolism-focused CRISPR-Cas9–based screens in Jurkat, a T-ALL cell line resistant in our in vivo competition assay (Fig. 1B). Consistent with the essential role of asparagine synthesis under asparagine depletions, our screens yielded ASNS as the top-scoring gene, with six of eight ASNS guides differentially depleted under ASNase treatment (Fig. 1, C and D). In addition to ASNS, we noted scoring of tricarboxylic acid (TCA) cycle genes fumarate hydratase and malate dehydrogenase 2 (MDH2), as well as glutamic-oxaloacetic transaminase 2 (GOT2), highlighting a route to aspartate, the substrate for ASNS and precursor of asparagine (Fig. 1, C to E, and fig. S1C). Furthermore, single-guide RNAs (sgRNAs) targeting SLC1A5, the major glutamine transporter, and SLC25A12, a mitochondrial glutamate/aspartate exchanger, were also substantially depleted under ASNase treatment (fig. S1C). Since many cancers use glutamine as a major anaplerotic source (20), our results confirm that the pathway from glutamine uptake to asparagine synthesis through the oxidative TCA cycle is necessary for maintaining cell proliferation under ASNase treatment (Fig. 1E). The second top-scoring gene in our screens was TPK1, for which all eight guides were differentially depleted when we treated cells with ASNase (Fig. 1, C and D). TPK1 is a ubiquitously expressed cytosolic kinase that uses imported thiamine and adenosine triphosphate to produce TPP, the thiamine derivative that serves as a cofactor for many enzymes, including AKGDH in the TCA cycle (21). To our surprise, TPK1, though predicted to be a cell-essential gene, did not score under standard culture conditions, suggesting the presence of sufficient TPP for proliferation. Given the lack of any previous connection between thiamine metabolism and ASNase response, we next focused on TPK1.

**TPP enables de novo asparagine synthesis and proliferation under ASNase treatment**

To understand how TPK1 loss sensitizes leukemia cells to ASNase, we generated CRISPR-Cas9–mediated clonal knockouts (KO) of TPK1, in which TPK1 protein levels were undetectable (Fig. 2A).

Unlike in our genetic screening conditions, TPK1-null Jurkat cells die in culture unless supplemented with TPP. This suggests that TPP is essential for proliferation and that TPP from other cells with functional TPK1 may have fueled the growth of TPK1-null cells during the course of the genetic screens. To determine whether TPP limitation is sufficient to sensitize Jurkat cells to ASNase, we first determined a TPP dose that enabled suboptimal proliferation of TPK1-null cells under standard culture conditions (2.5 nM). At this dose, TPK1-null cells were substantially more sensitive to ASNase treatment compared to parental controls (Fig. 2B), consistent with our screen results. Overexpressing sgRNA-resistant TPK1 complementary DNA (cDNA) rescued both TPP dependency and the TPP-dependent ASNase sensitivity of TPK1-null cells (fig. S2, A and B). Together, these results show that TPP availability enables proliferation of leukemia cells upon ASNase treatment.

TPP is used as a cofactor by various enzyme complexes, including the AKGDH complex, which catalyzes the oxidative decarboxylation of α-ketoglutarate in the TCA cycle along the pathway from glutamine to asparagine. To directly determine whether de novo asparagine synthesis from glutamine requires TPP, we measured the generation of TCA cycle intermediates, aspartate, and asparagine from uniformly heavy carbon-labeled glutamine ([U-13C]-l-glutamine) (Fig. 2C), in the presence or absence of TPP and asparagine. We performed this in parental and TPK1-null Jurkat cells expressing either a vector or TPK1 cDNA. In asparagine-replete conditions, we did not detect any asparagine labeling, indicating the lack of asparagine synthesis (Fig. 2D). In contrast, when asparagine was absent, ~50% of the asparagine in parental cells, and TPK1-null cells supplemented with TPP or expressing TPK1 cDNA, was derived from oxidative metabolism of [U-13C]-l-glutamine (Fig. 2D), in line with ATF4-mediated up-regulation of ASNS. In the absence of both asparagine and TPP, we observed a substantial decrease in total as well as oxidatively labeled (M + 4) asparagine, aspartate, and malate in TPK1-null cells (Fig. 2, D to F). Addition of TPP or expression of TPK1 cDNA rescued all decreases in these metabolite levels (Fig. 2, D to F). Notably, TPK1 loss also led to the massive accumulation of total alpha-ketoglutarate (Fig. 2G), despite total levels of the upstream metabolite glutamate being comparable across all conditions (Fig. 2H). Together with the depletion of total metabolites downstream from alpha-keto glutarate in the absence of TPP, this is consistent with the cofactor role of TPP for the AKGDH complex. Furthermore, in fractional enrichment profiles, TPP limitation decreased oxidatively labeled (M + 4) asparagine (under asparagine depletion), aspartate, and malate (fig. S2, C to E), compared to TPP-replete conditions and to the oxidative labeling (M + 5) seen for alpha-ketoglutarate (fig. S2F). In contrast, we did not observe any change between the fractional enrichment profiles of alpha-keto glutarate and glutamate (fig. S2, F and G), suggesting that TPP limitation affects glutamine anaplerosis at a point downstream from alpha-keto glutarate in the TCA cycle. Together, these metabolite profiles were in line with our genetic screen data by highlighting that utilization of glutamine for the oxidative TCA cycle is essential for asparagine synthesis under exogenous asparagine limitation in Jurkat cells. Furthermore, these results suggest that by enabling AKGDH activity in the TCA cycle, TPP allows asparagine synthesis from glutamine under extracellular asparagine depletion.

**SLC19A2 expression is a determinant of growth at physiologically relevant thiamine concentrations**

Given that thiamine-derived TPP availability enables growth under ASNase treatment, we considered that conventional RPMI 1640 (RPMI) medium provides supraphysiological (3 μM) thiamine levels. This is approximately 100-fold the content of normal human plasma, which ranges from 6.6 to 43 nM (22). Building upon the observation that TPK1-null cells display increased ASNase sensitivity under limiting TPP concentrations, we explored two questions: first, that physiological thiamine levels may be a growth limitation for a subset of cancer cell lines; and second, that culturing such a subset in limiting thiamine environments may affect ASNase responses. To address the first question, we performed cell line competition assays in RPMI, with and without added thiamine [supplemented with 10% dialyzed fetal bovine serum (FBS)] (Fig. 3A). Notably, the trace thiamine in dialyzed FBS led to ~1 nM total thiamine in culture,
Fig. 1. Functional genomics identifies metabolic determinants of proliferation under ASNase treatment. (A) Right: Schematic outlining cell line competition assay. Left: Log2 fold change in abundance from initial pool, of barcodes (n = 3) representing indicated cell lines in the competition assay, for ASNase-treated tumors (n = 10) relative to mean of vehicle-treated tumors (n = 10). Boxes represent the median and first and third quartiles, and whiskers represent the minimum and maximum of all data points. Statistics: false discovery rate (FDR)–adjusted P, by two-tailed unpaired t test for unequal variances, of ASNase tumor group versus vehicle tumor group. Individual CCLE RNA-seq ASNS expression levels of cell lines are also shown (x indicates no data available). (B) Schematic depicting pooled CRISPR screen under ASNase treatment (0.25 U/ml) using a metabolism-focused single-guide RNA (sgRNA) library. (C) Left: The top 25 genes differentially required under ASNase treatment are shown. Right: Gene scores for Jurkat cells grown in untreated versus ASNase-treated vessels. Most genes, as well as nontargeting control sgRNAs, gave similar scores in untreated and treated vessels. AA, amino acid. (D) Log2 fold change in the abundance of individual sgRNAs in untreated (black) or ASNase-treated (gray) for top-scoring genes, ASNS, TPK1, GOT2, and MDH2. (E) Schematic demonstrating that top-scoring genes in the CRISPR screen highlight a specific route from glutamine to asparagine as essential under ASNase treatment.
Glutamate

TPK1

TPK1

TPK1


Fig. 2. TPP enables de novo asparagine synthesis and proliferation under ASNase treatment. (A) Immunoblot analysis of vector control and two clonal TPK1 KOs made from Jurkat cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (B) Fold change in cell number (log2) of vector control, TPK1_KO1, and TPK1_KO2 Jurkat cells, after untreated or 0.0005 U/ml ASNase conditions for 5 days (mean ± SD, n = 3). Statistics: *P < 0.05 by two-tailed unpaired t test for equal variances, for all 15 untreated-treated pairs. (C) Schematic depicting a metabolic route of asparagine synthesis from glutamine. Filled circles represent 13C atoms derived from [U-13C]-glutamine. (D to H) Total abundance (a.u., arbitrary units) of indicated metabolites derived from labeled glutamine in vector control, TPK1_KO1, and cDNA-rescued TPK1_KO1 Jurkat cells. Cells were incubated for 24 hours in medium containing [U-13C]-glutamine (2 mM) in the presence or absence of asparagine (378 μM) and TPP (3 μM). Colors indicate mass isotopologs (mean ± SD, n = 3).

which approximated the content of human plasma. While many cancer cell lines grew similarly in both conditions, we observed that a small subset was depleted in the competition assay specifically under low thiamine, suggesting that low thiamine content limited their proliferation. We next determined whether the expression of any particular thiamine metabolism gene is predictive of growth responses to thiamine limitation. Correlating growth in low thiamine with mRNA expression data of metabolic genes from CCLE (Cancer Cell Line Encyclopedia) revealed the plasma membrane thiamine transporter SLC19A2 as a top-scoring gene (Fig. 3B). Expression of SLC19A3, the other canonical thiamine transporter, was not a predictor of growth under low thiamine, and low mRNA levels of SLC19A3 compared to SLC19A2 in CCLE (median TPM (transcripts per million) of 0.1 versus 6.6) suggested that SLC19A2 may be the primary transporter expressed in cancer lines. To further support the competition assay results, we simultaneously performed a metabolism-focused CRISPR-Cas9–based genetic screen using Jurkat cells under the same low- and high-thiamine conditions. Similar to the correlation results from the cell competition assays, SLC19A2 was the top-scoring gene, with five of eight guides being selectively depleted in low-thiamine medium (Fig. 3, C and D). Notably, additional genes with no known connections to thiamine uptake scored low in our screen, suggesting that other metabolic dependencies exist when thiamine is low. Together, our unbiased competition assay and genetic screen approaches both pinpoint that SLC19A2 expression may be a determinant of growth at physiologically relevant thiamine concentrations. Given the connection between thiamine utilization and ASNase response, we next asked whether cell lines with low SLC19A2 are more sensitive to ASNase under limiting physiological thiamine. To address this, we identified three B-ALL cell lines, KOPN8, REH, and NALM6, in which SLC19A2 mRNA was undetectable, consistent with CCLE data (Fig. 3E).
Physiological thiamine is limiting for ASNsase response of SLC19A2-low cells

We had previously noted that REH and NALM6 were ASNsase resistant in barcoded competitions in mice, but we considered that the standard mouse chow that we used may have supplied supraphysiological thiamine. Consistent with the in vivo competition results, REH cells proliferated similarly in the presence and absence of ASNsase under standard culture conditions (3 μM thiamine). However, under low-thiamine (~60 nM) conditions, untreated cells grew slightly less than at 3 μM (two versus four doublings in 6 days) but now, notably, ASNsase treatment severely impaired cell growth (no doublings in 6 days). Furthermore, overexpression of SLC19A2 was sufficient to rescue both the decreased growth of untreated cells and the increased ASNsase response at ~60 nM thiamine (Fig. 4A). This suggests that SLC19A2 expression is a determinant of both growth and ASNsase sensitivity in low thiamine. Consistent with this, as we further lowered thiamine toward 10 to 40 nM to be within the human plasma range, we found that for SLC19A2-low NALM6 and REH cells, thiamine levels constrained growth and ASNsase had a greater effect, compared to cells cultured in supraphysiological thiamine (Fig. 4B and fig. S3A). In contrast, SLC19A2-high Jurkat, ST486, and RCH-ACV cells were unaffected by physiological thiamine concentrations, and their ASNsase response was similar at high and low thiamine (Fig. 4B and fig. S3B). CRISPR-Cas9–mediated targeting of SLC19A2 in Jurkat, which decreased 13C-labeled thiamine uptake, showed that SLC19A2 was necessary for cells to proliferate optimally and maintain ASNsase resistance under human plasma thiamine levels relative to cells in standard culture (Fig. 4, C and D). Together, these data establish SLC19A2 expression as a determinant of (i) growth and (ii) ASNsase response at physiological thiamine levels.

Dietary thiamine intake influences ASNsase sensitivity of SLC19A2-low leukemia cells in vivo

To confirm that endogenously low expression of SLC19A2 in cell lines is not a tissue culture phenomenon, we probed for the existence of SLC19A2-low patient tumors. As ASNsase is a standard in the initial treatment phase of ALL, we explored RNA sequencing (RNA-seq) data of pediatric primary ALLs sampled from peripheral blood and bone marrow, as well as recurrent ALLs sampled from bone marrow. ASNs, TPK1, and SLC19A2 mRNAs were consistently detected in these three datasets (fig. S4A), and their expression was similar between primary and recurrent bone marrow datasets, suggesting that treatment generally did not influence mRNA levels of these genes. Notably, SLC19A3 mRNA was rarely detected, indicating that SLC19A2 is likely the primary thiamine transporter in these tumors. These data show a diverse range of SLC19A2 expression among patients and
confirm the existence of SLC19A2-low tumors in which ASNase response may depend on environmental thiamine.

We next asked whether extracellular thiamine availability also affects ASNase response of SLC19A2-low leukemia cells in vivo. To create a model of such tumors, we used the SLC19A2-low REH cell line and generated orthotopic tumors in nonobese diabetic severe combined immunodeficiency gamma (NSG) mice. First, mice that were on the conventional chow–based diet used in our barcoded competition experiment were placed on a purified-ingredients diet with either a high thiamine amount to mimic the supraphysiological plasma thiamine levels obtained with chow (Fig. 5A and fig. S4, B to D) or a low amount that resulted in levels resembling that of human serum (Fig. 5A and fig. S4D). Lowering plasma thiamine to human levels was well tolerated, as indicated by animal weights remaining unchanged after diet modification (Fig. 5B). We then engrafted REH cells in mice on either high- or low-thiamine diets and tested the efficacy of ASNase treatment in each of these cohorts. The ASNase regimen depleted plasma asparagine levels without changing the levels of abundant amino acids such as glutamine (fig. S4E). Compared to the high-thiamine diet group in which plasma thiamine was comparable to that seen with standard chow, neither ASNase treatment nor lowering dietary thiamine alone significantly affected survival from leukemia (Fig. 5C). Notably, proliferation of these leukemia cells slowed under low-thiamine conditions in vitro, raising the possibility that the in vivo microenvironment may not contain low enough thiamine to impair growth. However, combining ASNase treatment and low dietary thiamine significantly (Mantel-Cox ρ = 0.0034) extended survival from leukemia (Fig. 5C). Together, our results provide evidence that thiamine availability resulting from dietary intake can affect ASNase sensitivity of SLC19A2-low ALLs in vivo.

**DISCUSSION**

Our results suggest that extracellular thiamine availability influences ASNase response of a subset of leukemia cells. When combined with a change in dietary thiamine intake, ASNase treatment extends survival in a mouse leukemia model. It is important to note that the low-thiamine dietary intervention was equivalent to maintaining normal human serum levels and was sufficient to sensitize SLC19A2-low leukemia cells to ASNase. Lowering thiamine levels further than the normal physiological range for therapeutic purposes in experimental or clinical settings would likely have adverse consequences, because of the diverse functions of TPP in normal cell types. In addition to its role in the AKGDH complex, TPP serves as a cofactor for various other enzymes, including the pyruvate dehydrogenase (PDH) complex, the branched-chain alpha-ketoacid dehydrogenase complex, and transketolase (23). Thus, biochemical abnormalities, such as lactic acidosis resulting from diminished PDH activity, are among the symptoms observed in patients with nutritional thiamine deficiency (23–25).

Although high thiamine supplementation is warranted in cases of deficiency, prophylactic use has been suggested with limited evidence for other scenarios, including preventing potential side effects of adult...
Physiological thiamine

Thiamine supplementation

SLC19A2-high

SLC19A2-low

ASNase

This study was designed to investigate the metabolic determinants of leukemia cell response to ASNase treatment. To address this objective, we (i) performed unbiased genetic screens to find genes that are essential for proliferation under ASNase treatment; (ii) used loss-of-function studies and metabolite profiling to validate that TPK1 enables ASNase resistance by producing the cofactor TPP, which allows asparagine synthesis in glutamine-anaerobic leukemia cells; (iii) used a DNA-barcoded cell line competition assay, a genetic screen, and gain- and loss-of-function studies to identify that physiological thiamine is limiting for ASNase response of a subset of leukemia cell lines with low expression of SLC19A2; and (iv) orthotopically engrafted leukemia cells with endogenously low SLC19A2 in NSG mice to show that dietary thiamine can influence ASNase sensitivity of leukemia.

MATERIALS AND METHODS

Experimental design

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Fig. 5. Dietary thiamine intake influences ASNase sensitivity of SLC19A2-low leukemia cells in vivo. (A) Plasma thiamine profiling of mice on conventional chow (n = 3 mice), and mice on a modified AIN-93G purified diet of low thiamine content (n = 8 mice) (mean ± SD). Human serum was profiled simultaneously for relative comparison. Statistics: *P < 0.01 by two-tailed unpaired t test for unequal variances. n.s., not significant. (B) Weights of mice initially on conventional chow, then switched to standard AIN-3G purified diet for 1 week, and finally switched to a modified AIN-93G purified diet of low thiamine content (n = 8 mice) for indicated times, relative to initial weights on chow (mean ± SD). (C) Left: Kaplan-Meier survival curves of NSG mice on high- or low-thiamine AIN-93G diets engrafted with REH (endogenously low SLC19A2 cell line) by tail vein injection and treated with vehicle or ASNase (1000 U/kg, twice per week). Right: Box-and-whisker plots of survival data. Statistics: Left: n.s., Mantel-Cox P > 0.05/3 (Bonferroni correction); right: n.s., two-tailed unpaired t test for equal variances P > 0.05/3 (Bonferroni correction). For both analyses, **P < 0.005. n = 5 mice for untreated groups and n = 7 mice for ASNase groups. (D) Schematic depicting that environmental thiamine influences ASNase sensitivity of leukemia cells with low SLC19A2 expression.
Compounds, cell lines, cell culture, and constructs
Antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GTX627408) were from GenTex, and to TPK1 from Proteintech (10942-1-AP). Conjugated donkey anti-mouse IRDye 680LT and donkey anti-rabbit IRDye 800CW were from LI-COR Biosciences.

Asparagine, TPP, human serum, polybrene, and puromycin were from Sigma-Aldrich; blasticidin from Invivogen; d-glucose from VWR; l-glutamine from Gibco; thiamine hydrochloride from MP Biomedicals; Escherichia coli l-asparaginase from BioVendor.

For glutamine tracing, a powder base medium of RPMI without amino acids (R9010-01, US Biological) was supplemented with all individual amino acids (except for asparagine and glutamine) and glucose at RPMI concentrations. [U-13C]-glutamine [CLM-1822-H, Cambridge Isotope Laboratories (CIL)] was used for tracing, and asparagine was present or absent at RPMI concentration as detailed in experiment discussion. For thiamine uptake experiments, thiamine-(4-methyl-13C-thiazol-5-yl-13C3) hydrochloride (“13C-thiamine,” 731188, Sigma-Aldrich) was added to cells in Hank’s balanced salt solution (HBSS) containing calcium chloride and magnesium chloride (24020-117, Gibco). In tracing, uptake, and plasma profiling experiments, extraction solvents contained 15N and 13C fully labeled internal amino acid standards (MSK-A2-1.2, CIL) for normalization.

All human cell lines used were originally purchased from American Type Culture Collection or German Collection of Microorganisms and Cell Cultures (DZMZ) or obtained from the Sabatini and Weinberg laboratories (Whitehead Institute). Cell lines were verified to be free of mycoplasma and authenticated by short tandem repeat profiling. Cell lines were typically maintained in “standard RPMI”: RPMI 1640 medium supplemented with 10% “double-dialyzed FBS,” obtained by further in-house dialysis of Gibco’s dialyzed FBS.

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For manipulating thiamine levels in the REH EV and SLC19A2 OE experiments, thiamine-free RPMI was combined with 10% non-dialedyzed PBS. For achieving nanomolar thiamine levels that approximated physiological thiamine, thiamine-free RPMI was combined with 10% “double-dialyzed PBS,” obtained by further in-house dialysis of Gibco’s dialyzed FBS. In-house dialysis was performed at 4°C against a 20× volume of phosphate-buffered saline (PBS), using regenerated cellulose dialysis tubing (21-152-9, Fisherbrand), with a PBS change done after at least 8 hours of stirring, such that four total PBS volumes were spent.

Lentiviral sgTPK1 vector was generated via ligation of hybridized oligos (below) into lentiCRISPR-v2-puro, and lentiviral sgSLC19A2 vector was generated similarly (oligos below) but using lentiCRISPR-v1-RFP (red fluorescent protein). Both of these vectors were linearized with Bsm BI (New England BioLabs), and nonlinearized vectors were used for vector controls. sgRNA-resistant TPK1, or SLC19A2, cDNA gene blocks were cloned into pMXs-ires-blast by Gibson assembly, and an uncut vector was used as vector control.

Guide 5 in CRISPR-Cas9 screen, used to generate clonal KO1 and KO2 in proliferation assays:
sgTPK1_5F, 5′-caccGAGGCTTGCGCGAAAGACCGGT-3′;
sgTPK1_5R, 5′-aaaACGCGCTTCTTGCGGAGGCC-3′.

Guide 3 in CRISPR-Cas9 screen, referred to as sgSLC19A2_1 in proliferation assays:
sgSLC19A2_3F, 5′-caccGAGGCTGCGCGAAAGACCGGT-3′;
sgSLC19A2_3R, 5′-aaaACGCGCTTCTTGCGGAGGCC-3′.

Lentiviral sgTPK1 and sgSLC19A2 vectors generated as described above were transfected into human embryonic kidney (HEK) 293T cells with lentiviral packaging vectors vesicular stomatitis virus glycoprotein (VSV-G) and delta-viral protein R (dVPR). For the overexpression of TPK1 or SLC19A2, retroviral vector with cDNA generated as above was transfected into HEK293T cells with retroviral packaging vectors Gag-pol and VSV-G. X-tremeGENE reagent (Roche) was used for transfection. After 24 hours, cells were given fresh media. Forty-eight hours after transfection, virus-containing medium supernatants were filtered through a 0.45-µm filter to eliminate cells. Cells to be transduced were plated in six-well tissue culture plates with appropriate virus and polybrene (8 mg/ml). Cells were then spin-infected by plate centrifugation at 1100g for 1.5 hours at 32°C. After 24 hours, cells were given fresh media. At least 48 hours after infection, transduced cells were selected with puromycin or blasticidin or by bulk-sorting on a BD FACSaria (same gating for all samples, resulting in top 3 to 6% of GFP+ cells). Clonal TPK1-KO cells were generated from a single cell isolated by serial dilution of transduced cells into a 96-well plate, in 0.2 ml of 100 µM TPP-supplemented standard RPMI. Single-cell clones were grown for ~3 weeks, and the resultant clones were evaluated for TPK1 KO by immunoblotting. TPK1 KOs were then maintained in standard RPMI containing 20 nM TPP, as for the relevant vector control and cDNA-add-back controls, for at least 1 week before proliferation assays. Mixed-population cells transduced with sgSLC19A2s or vector control were maintained in standard RPMI, as this already oversupplements thiamine at 3 µM. Notably, REH and Jurkat cells expressing a luciferase reporter construct were used for generating the vector- or SLC19A2-overexpression pair, and the vector-, sgSLC19A2_1-, or sgSLC19A2_2-expressing lines, respectively.

Immunoblotting
Cells were first collected by centrifugation at 300g for 4 min and washed twice with PBS. For blotting TPK1, cells were then lysed in cold radioimmunoprecipitation assay (RIPA) buffer [20 mM tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate] supplemented with protease inhibitors (Roche) for 5 min on ice. Supernatants were then collected by centrifugation at 20,000g for 8 min at 4°C, and protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) with a bovine serum albumin protein standard series. Samples were resolved on 12% SDS–polyacrylamide gel electrophoresis gels and analyzed by immunoblotting using antibodies listed above and a LI-COR Odyssey CLx Imaging System coupled with Image Studio Lite.
RNA extraction, reverse transcription, and real-time quantitative polymerase chain reaction

RNA was extracted using the QIAGEN RNeasy Mini Kit, and 1 μg of RNA was reverse-transcribed using the SuperScript III Reverse Transcriptase Kit (Invitrogen), both according to the manufacturer’s instructions. Real-time quantitative polymerase chain reaction (PCR) (qPCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems), and GAPDH was used as a housekeeping control. qPCR primer sequences were as follows:

- GAPDH forward, 5′-TTGTATACGGAAGGACTC-3′;
- GAPDH reverse, 5′-ACAGCTTCCTGGTGCCAGT-3′;
- SLC19A2 forward, 5′-GGCGGACAAAGAACCT-3′;
- SLC19A2 reverse, 5′-ACACAGGAAACAGTAGCACCA-3′.

Proliferation assays

Cells were plated in 96-well plates at 1000 cells per well in triplicate in a final volume of 0.2 ml in indicated media and treatments. After 5 to 9 days of growth (depending on proliferation rate), 40 μl of CellTiter-Glo reagent (Promega) was added, and luminescence was read on a SpectraMax M3 plate reader (Molecular Devices). For each experiment, cells were also plated in triplicate in untreated medium for an initial luminescence time point for normalization. For each well, fold change in luminescence was calculated and reported on a log2 scale. In all proliferation assays, we used an AsNase dose ≤ 0.001 U/ml, which is a dose previously shown to deplete asparagine in medium within 24 hours, without affecting glutamine levels (34).

In assays that required achieving low thiamine levels, cells were first preincubated for 5 to 9 days in the same low-thiamine-base medium that was subsequently used for plating proliferation assays. Proliferation assays were plated multiple times within this preincubation period with similarly reproduced results.

Mouse studies

Animal studies were conducted according to a protocol approved by the Institutional Animal Care and Use Committee at the Rockefeller University. All experiments used age- and gender-matched NSG mice that were maintained on a standard light-dark cycle, with food and water provided ad libitum. Subcutaneous xenograft tumors for DNA-barcode competition assays were initiated by injecting 1.5 million of the pooled cells in 100 μl of Dulbecco’s Modified Eagle Medium (Gibco) with 30% Matrigel (Corning), once on each mouse flank. These mice were fed 5B1Q (TestDiet), a chow-based diet. Leukemia cells were injected directly onto the LC-MS column. For protein quantification, NaCl-washed cell pellets were instead stored at −20°C until RIPA lysis and protein quantification (as described above for TPK1 immunoblotting).

For thiamine uptake experiments, the three cell types were first taken from their maintenance medium (20 nM TPP) and washed twice with PBS. Cells were then plated at 500,000 cells/ml in medium containing 2.5 nM TPP, for ~1 day, to begin TPP depletion. To begin the experiment, cells were washed again and then plated in triplicate in condition in six-well plates at 1 million cells/ml, in a custom RPMI (described above) containing no asparagine, no TPP, and 2 mM [U-13C]-glutamine (supplemented with 10% dialyzed FBS). TPP (at RPMI thiamine concentration) and asparagine (at RPMI concentration) were then added to wells as appropriate. After 24 hours, 1 million cells from each well were washed twice with 1 ml of cold 0.9% NaCl; a separate tube of 1 M cells was also processed for each experimental condition to check protein content. After washing, polar metabolites were extracted in 1 ml of cold 80% methanol containing internal amino acid standards, by vortexing for 10 min at 4°C. Samples were then centrifuged at 20,000 g for 15 min at 4°C, and 900 μl of supernatants was stored overnight at −80°C and then nitrogen-dried. Dried extracts were kept at −80°C until LC-MS analysis. Dried samples were resuspended in 100 μl of 50:50 acetonitrile:water, and 2 μl was injected onto the LC-MS column. For protein quantification, NaCl-washed cell pellets were instead stored at −20°C until RIPA lysis and protein quantification (as described above for TPK1 immunoblotting).

For thiamine uptake experiments, the three cell types were first taken from their maintenance medium in standard RPMI and washed twice with PBS. Four million cells were then plated in triplicate per condition in six-well plates at 1 million cells/ml in warm HBSS. 13C-thiamine was then added to wells at a final concentration of 25 nM, for 5-min incubations at 37°C. The assay was stopped by pipetting each well into an ice-cold tube of HBSS containing unlabeled thiamine that came to 3 μM in the final mixture. Cells were then washed twice with cold 0.9% NaCl and processed as described above for polar metabolite extraction and LC-MS analysis. Dried samples were resuspended in 60 μl of 50:50 acetonitrile:water, and 5 μl was injected onto the LC-MS column.

For plasma profiling, polar metabolites were extracted by combining plasma with cold 75:25 acetonitrile:methanol containing internal amino acid standards. Plasma (5 μl) was combined with either 45 μl of solvent for the in vivo competition assay data or with 20 μl of solvent for custom thiamine diet experiments (for quantifying low-abundance thiamine in plasma). This was followed by vortexing for 5 min at 4°C and then centrifuging at 20,000 g for 10 min at 4°C. Supernatants were taken immediately for LC-MS analysis or first stored at −80°C for no longer than 24 hours (samples were never dried). Two microliters (for competition assay) or 5 μl (for custom thiamine diet experiments) was injected directly onto the LC-MS column.

LC-MS analysis was conducted on a Q Exactive benchtop Orbitrap mass spectrometer equipped with an Ion Max source and a
using lentiviruses generated with pLKO.1-puro vector, such that each seven–base pair sequences were transduced into each cancer cell line guide score for each screen flask was defined as the log2 fold change. After Illumina deep sequencing of initial and final screen pools, a library of chemical standards. For normalization, raw signal of each metabolite was first normalized to the appropriate labeled internal amino acid standard in that injection (except for the thiamine uptake data, where raw signal is presented because no signal was detected in sgSLC19A2 samples). Metabolite levels were also normalized by cell counting (per well) and by protein content (per condition, quantified by BCA Protein Assay) for glutamine tracing, by cell counting for thiamine uptake, and by plasma/serum volume as appropriate.

CRISPR-Cas9–based genetic screens
The metabolism-focused sgRNA library preparation and details on performing screens were previously described (36–38). sgRNA oligonucleotides were synthesized by Agilent and amplified by PCR. After Illumina deep sequencing of initial and final screen pools, a guide score for each screen flask was defined as the log2 fold change of sgRNA abundance from initial to final. Gene scores for each flask were defined as the median of the guide scores corresponding to a gene, and the complete lists of gene scores were used to make the gene score versus gene score linearity plots presented. For each sgRNA of a gene, the guide score for the control flask was subtracted from the guide score for the experimental flask, and the median of the resulting values was defined as the differential gene score. A gene lethality cutoff was used for differential gene score analysis: Genes that had a gene score less than or equal to −1 in the appropriate control condition were removed from the gene score list, and the updated gene list was ranked by differential gene score of experimental versus control to give the differential gene score graphs presented. Full screen results are available as supplementary data.

DNA-barcoded cell line competition assays
DNA-barcoded cell lines were generated, and competition assays were performed, as previously described (3, 39). In brief, three unique seven–base pair sequences were transduced into each cancer cell line using lentiviruses generated with pLKO.1-puro vector, such that each cell line had three data points used for statistical analysis. To perform cell competition assays, barcoded cell lines were mixed in approximately equal amounts, an initial pool sample was taken, and the remainder of the pool was grown for ~2 weeks under indicated in vitro or in vivo conditions. At end point, genomic DNA was extracted from the initial and final pool samples, DNA barcode regions were amplified by PCR, and PCR amplicons were processed for Illumina deep sequencing. Fold change of barcode abundance from initial to final pools was determined, and results are presented as detailed in appropriate figures.

Patient tumor and cell line RNA-seq
Patient RNA-seq data for SLC19A2, SLC19A3, TPK1, and ASNS were generated by the Therapeutically Applicable Research to Generate Effective Treatments (http://ocg.cancer.gov/programs/target) initiative, phs000218. These data were part of the Acute Lymphoblastic Leukemia (ALL) Expansion Phase 2 TARGET substudy, phs000464, and were downloaded from the TARGET Data Matrix (https://ocg.cancer.gov/programs/target/data-matrix) on 10 December 2019. All files used were in the BCCA (British Columbia Cancer Agency) subdirectory, and tumor sample details are indicated within the relevant figures. Cancer cell line RNA-seq data presented throughout this study were obtained from the CCLE (https://portals.broadinstitute.org/ccle/data), accessed on 27 January 2020 (file version: CCLE_ RNaseq_rsem:genes_tpm:20180929.txt.gz) (40).

Statistical analysis
Significance P values, sample sizes, and means are indicated in text or figures. Error bars represent SD from biological replicates or independent samples as indicated. Statistical analyses were performed with GraphPad Prism or Microsoft Excel as appropriate. P ≤ 0.05 was considered statistically significant, and the specific statistical test used and any adjustments for multiple comparisons are indicated in the relevant figure legends.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/41/eabc7120/DC1 View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES


Dietary thiamine influences l-asparaginase sensitivity in a subset of leukemia cells
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Sci Adv 6 (41), eabc7120.
DOI: 10.1126/sciadv.abc7120