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The Role of Nutrient Availability in Therapeutic Response of Leukemia

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THE ROLE OF NUTRIENT AVAILABILITY IN THERAPEUTIC RESPONSE OF LEUKEMIA

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Tumor environment influences the response to anti-cancer therapy, but which extracellular nutrients impact drug sensitivity is largely unknown. In this work, we used functional genomics to identify metabolic modifiers of the response to L-asparaginase (ASNase), a therapy that depletes plasma asparagine and targets leukemic cells with insufficient asparagine synthesis.

Our approach revealed thiamine pyrophosphate kinase 1 (TPK1), which converts vitamin B1 (thiamine) into the cofactor thiamine pyrophosphate (TPP), as a metabolic dependency under ASNase treatment. In glutamine-anaplerotic leukemia cells, we found that TPP availability enables asparagine synthesis from extracellular glutamine. Mechanistically, TPP is critical for the activity of alpha-ketoglutarate dehydrogenase (AKGDH), a TCA cycle enzyme that catalyzes a step in the overall conversion of glutamine to asparagine. When TPP availability is limiting for cell proliferation of TPK1 KOs, ASNase sensitivity is significantly increased.

Standard cell culture media formulations provide thiamine at a concentration that is ~100-fold higher than that observed in human plasma. While thiamine is generally not limiting for cell proliferation under standard culture conditions, a DNA-barcode competition assay identified a subset of leukemia cell lines that grow sub-optimally under lower, more physiological thiamine levels. These cell lines are characterized by low expression of SLC19A2, a high affinity thiamine transporter. Intriguingly, SLC19A2 expression was necessary for not only optimal growth, but also for maintaining ASNase resistance, when standard media thiamine was lowered to the concentration of human plasma.

Importantly, analyzing RNAseq data of pediatric acute lymphoblastic leukemia (ALL) tumor samples revealed that SLC19A2 is the primary thiamine transporter expressed in these cancers, and that SLC19A2-low tumors exist among patients. To model such tumors, we used a SLC19A2-low cell line to generate orthotopic tumors in NSG mice. Remarkably, humanizing blood thiamine content of mice through diet sensitized these leukemia cells to ASNase *in vivo*.

Altogether, our work reveals that utilization of thiamine is a determinant of ASNase response for some cancer cells, and that over-supplying vitamins may impact therapeutic response in leukemia. Additionally, our work adds to the recent literature that demonstrates how physiological levels of certain nutrients in cell culture can affect therapy. Specifically, our work provides the first proof of principle that humanizing the vitamin levels of both *in vitro* and *in vivo* models can affect drug sensitivity. This has broad implications for the screening and validation of new therapeutic candidates.

To my parents, my sister, Jill, and Olive

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CHAPTER 1. Introduction

1.1 Nutrient availability influences cellular metabolism and therapeutic response

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*). Indeed, 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 upregulate 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 cancer cells rely on (*4*). Interestingly, exogenous modulation of tumor nutrient supply can also affect 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 media concentrations of uric acid and cystine to physiological levels altered cancer cell responses to 5-fluorouracil and CB-839, respectively (*9, 10*). Taken together, these examples illustrate the need for further investigation into which nutrients impact therapeutically-relevant metabolic pathways. In particular, it is poorly understood whether vitamins, which act as cofactors for many metabolic reactions, can affect responses to anti-cancer drugs.

1.2 Specific vitamin dependencies in cancer and how they can be targeted

Cancer cells may demonstrate increased dependencies on vitamins due to their need for particular vitamin-dependent reactions or because of defects in vitamin transport or activation. Vitamin B9, or folate, is a precursor for tetrahydrofolate, a coenzyme involved in enzymatic transfer of one-carbon groups in various amino acid and nucleic acid synthesis pathways collectively referred to as one-carbon metabolism. As many cancers rely on one-carbon pathways for proliferation (*11*), targeting one-carbon metabolism pathways with drugs such as methotrexate and 5-fluorouracile (5-FU) has been a successful strategy since the 1940s (*12*).

Few reports have identified vitamin utilization defects such as impaired transport or inability to synthesize activated derivatives, defects that can be exploited therapeutically. In breast cancer cells, thiamine transporters (SLC19A2 and SLC19A3) have been found to be expressed at lower levels than their normal tissue counterparts. This led to the hypothesis that decreased thiamine transporter expression may make these cancers more dependent on exogenous thiamine, a vulnerability that can be targeted with acute thiamine starvation (*13, 14*). To circumvent the effects of chronic thiamine starvation, studies utilized a recombinant thiaminase enzyme that digests

thiamine and induces an acute thiamine depletion state (*15*). One subset of leukemias were more dependent on extracellular thiamine than other tested cell lines (*15, 16*). In follow-up studies, thiaminase was found to have *in vivo* efficacy against breast cancer and leukemia subcutaneous xenografts, as well as primary ALL cells injected intravenously (*17*). These thiaminase studies provide interesting examples of differential dependencies on extracellular vitamin levels possibly explained by varying degrees of vitamin transport capabilities.

Another vitamin with reported heterogeneity in transporter expression across normal and cancerous tissues is riboflavin. Some riboflavin transporters were observed to be overexpressed in melanoma, breast cancer, and squamous cell carcinoma samples relative to healthy tissues (*18*). While these expression patterns may be useful for designing targeted drug-delivery systems, these findings may suggest the existence of cancers with increased riboflavin dependencies. It is also possible for cancers to have vulnerabilities in vitamin utilization downstream of extracellular uptake, such as in the enzymatic conversion of vitamins to activated derivatives. Examples include vitamin B6, an umbrella term for the 6 different vitamers that require different enzymatic steps for interconversion before yielding the activated cofactor form, pyridoxal phosphate (PLP) (*19*). Dependencies on exogenous pyridoxal (PL) or PLP forms of vitamin B6 have been observed in some tumors (*20*). Overall, with the exception of folate, there are promising but few reports on unique vitamin dependencies in cancer. Studying these dependencies could lead to dietary interventions or new therapeutic targets that could be as impactful as anti-folates have been for the last several decades.

1.3 Acute lymphoblastic leukemia (ALL)

1.3.1 Biology and pathophysiology

Acute lymphoblastic leukemia (ALL) is a cancer of white blood cells (WBCs), primarily arising from cells of the B and T lineages. B-cell ALL represents ~85% of childhood and ~75% of adult ALL cases, and T-cell ALL makes up the majority of the remaining cases in both age groups (*21, 22*). As a hematologic malignancy, the disease affects blood and lymph nodes, as well as bone marrow, where WBCs are originally produced. The most common presenting symptoms reflect these disease sites, and include blood abnormalities such as anemia and bleeding, lymph node involvement appearing as lymphadenopathy or hepatosplenomegaly, and bone pain due to marrow colonization (*23*). Since the involvement of both blood and lymphoid organs can result from the same initial white blood cell cancer, recent classification guidelines recognize these leukemias and lymphomas as a single disease that may also be referred to as acute lymphoblastic leukemia/lymphoblastic lymphoma (ALL/LBL). For simplicity, the remainder of this thesis will use the more established and widespread term “ALL”.

The majority of ALL cases arise spontaneously with no known cause, but some environmental and genetic factors have been associated with an increased risk of ALL

occurrence (*22, 24*). Exposure to ionizing radiation has been linked with ALL, but most studies that examine the influence of environmental factors on ALL incidence have yielded inconsistent results (*25*). Among genetic syndromes, Down's Syndrome is most commonly associated with increased risk of ALL (*24, 26*). Additionally, germline mutations in ETV6, PAX5, and TP53 have been associated with rare familial ALL cases (*27-32*). Finally, genome-wide association studies have implicated multiple genes as having polymorphic variants that increase the risk of ALL, including ARID5B, CEBPE, and IKZF1, which are annotated as genes involved in B-cell progenitor transcriptional regulation and differentiation (*33, 34*). Some of these factors that increase ALL risk have prognostic and therapeutic significance. For instance, TP53 mutations and IKZF1 alterations are notable for their associations with ALL subtypes that have poor outcomes, and patients with abnormalities in these genes are assigned to high-risk chemotherapy regimens (*21, 24*).

In addition to the genetic factors that predispose individuals to ALL, there are many common genomic alterations that arise spontaneously during leukemic cell transformation. These alterations are also important for prognosis and are used for risk stratification and assigning therapy (*21, 22*). Among the most common cytogenetic changes are ETV6-RUNX1 rearrangements and hyperdiploidy, which each carries a favorable prognosis (*21, 35, 36*). In contrast, less common genomic features may be associated with poor prognosis, leading to augmented chemotherapy protocols, or targeted treatment if applicable. In patients with previously high-risk Philadelphia Chromosome-positive ALL (Ph-positive ALL), targeting BCR-ABL1 with the ABL-class tyrosine kinase inhibitor (TKI) imatinib led to improved outcomes (*37-41*). Additionally, a high-risk ALL subtype termed Ph-like ALL does not express BCR-ABL1 but does contain kinase-activating genomic rearrangements involving genes like ABL1, JAK2, and PDGFRB, and may benefit from ABL-class TKIs like imatinib as well as JAK inhibitors like ruxolitinib (*42-48*). Recently, the FLT3 overexpression observed in high-risk ALL with rearranged KMT2A (also referred to as MLL) has been targeted with FLT3 inhibitors, in preclinical and clinical settings (*49-51*). Of note, the use of targeted therapies in ALL is still in early stages, and the vast improvements in ALL outcomes in the last few decades are largely attributed to the fine-tuning of treatment regimens involving established chemotherapeutics (*24*). However, the positive clinical outcomes observed with targeted therapies thus far demonstrate the need for further investigation of whether ALLs of certain genotypes may benefit from specific medications.

1.3.2 Epidemiology and outcomes

ALL is the most common cancer in children, representing ~26% of new cancer diagnoses in patients of ages 0-14 (*52*). In the United States, this translates to ~3,000 new childhood ALL cases every year (*21, 24*). Fortunately, survival rates for childhood ALL have increased steadily over the last few decades (*24, 53, 54*), and five-year overall survival for childhood ALL is currently ~90% (*24, 55*). However, there are also ~3,000 adult ALL cases diagnosed annually in the United States, and adult ALL patients

are generally considered to be high risk cases with a worse prognosis (*22, 24, 56*). This is reflected in the 5-year overall survival for adult ALL, which has improved in the last decades but is still estimated to be ~40%, with lower survival rates observed in older populations (*57-60*).

The increase in 5-year survival for childhood ALL over the years has been attributed to the introduction of multi-drug, multi-phase regimens in the 1960s and 1970s (*61-63*), the inclusion of treatments aimed at preventing relapse within the central nervous system (CNS) (*64*), and the continuous improvement of risk stratification practices used to place patients on appropriate regimens (*24, 63*). Furthermore, the recent improvements in outcomes for adult ALL patients are associated with adapting successful pediatric protocols for use in adult patients (*65-67*). Although pediatric treatment regimens have proven effective in both children and adults, the survival rates for both childhood and adult ALL demonstrate that a significant number of patients in both age groups still fail these treatment protocols today. Thus, there is a need for continued research into not only new drug candidates, but also the improvement of existing ALL treatment protocols however possible.

1.3.3 Risk stratification and treatment

Although many factors have been evaluated for their importance in predicting ALL outcomes over the last few decades, only five are still routinely used for risk stratification: age, initial WBC count, leukemic cell cytogenetics and ploidy, immunologic subtype (B or T lineage), and initial treatment response (*24, 63*). Increased age, higher WBC count, certain cytogenetic features such as a BCR-ABL1 fusion, T-cell ALL, and persistence of disease after the first treatment phase are all indicators of a high-risk case (*24*). If a patient has high-risk characteristics, then standard multi-phase chemotherapy regimens may be augmented in terms of dose and frequency, or with additional medications (*68, 69*). Additionally, targeted treatments may be added for patients with specific cytogenetic characteristics. As stated above, patients with a BCR-ABL1 fusion have a survival benefit with addition of the tyrosine kinase inhibitor (TKI) imatinib to standard therapy (*39-41, 70*), and to circumvent resistance or poor CNS penetration with imatinib, second- and third-generation TKIs have also been used with positive outcomes (*71-77*). Specific treatments have also been used recently for each immunologic subtype in refractory and relapsed cases. Blinatumomab is an antibody therapy that has produced survival benefits in advanced B-ALL (*78-81*), and nelarabine is a T-cell specific chemotherapy that has had success in refractory and relapsed T-ALL patients (*82*). However, allogeneic stem cell transplantation remains the gold standard for all refractory and relapsed ALL cases (*22*), and is beneficial for high-risk patients after completion of the initial phase of chemotherapy (*83-86*).

Current treatment for standard-risk ALL patients involves multi-drug chemotherapy regimens, given as five phases of treatment that each last at least 1 month: induction, consolidation, interim maintenance, delayed intensification, and maintenance (*24, 68*).

Many of the medications and drug classes used in the initial induction and consolidation phases are used again in later phases, with possible substitution of certain drugs for another of the same class to further help eliminate residual drug-resistant cancer cells (Table 1). In standard pediatric regimens, the induction phase includes the chemotherapeutic vincristine, a corticosteroid such as prednisone, and the metabolism-focused drug asparaginase (ASNase). In most cases, the induction phase causes a complete remission, but additional treatment phases are needed to prevent relapse (*24*). In the next treatment phase, consolidation, patients receive multiple chemotherapeutics that act by causing DNA damage or preventing DNA synthesis in proliferating cells (Table 1). Additionally, throughout these first two treatment phases and some of the subsequent phases, chemotherapies targeting DNA synthesis are administered directly into the spinal canal (*24, 68*). This is to prevent relapse of leukemia within the CNS, which would previously occur in the majority of patients who achieved complete remission with systemic chemotherapy (*24, 64*).

Table 1.1. Components of standard ALL treatment.

Drug	Type	Target
cyclophosphamide	chemotherapy	DNA stability
daunorubicin	chemotherapy	DNA stability
doxorubicin	chemotherapy	DNA stability
cytarabine	chemotherapy	DNA synthesis
6-mercaptopurine	chemotherapy	DNA synthesis
6-thioguanine	chemotherapy	DNA synthesis
vincristine	chemotherapy	microtubule formation
dexamethasone	steroid	various signaling pathways in WBCs
prednisone	steroid	various signaling pathways in WBCs
methotrexate	DHFR inhibitor	one-carbon metabolism
L-asparaginase	enzyme	asparagine availability

Overall, the medications used in the various treatment phases of ALL are drugs that non-specifically target processes common to all proliferating cells, such as DNA synthesis. Interestingly, two of the therapies used in standard ALL treatment act by limiting the cellular availability of important metabolites. Methotrexate is a drug of the

antifolates class that inhibits dihydrofolate reductase (DHFR), the enzyme required for converting folate (vitamin B9) into tetrahydrofolate (THF), a folate derivative that is essential for DNA synthesis and other cellular processes (*11, 12*). L-asparaginase functions by depleting circulating levels of the amino acid asparagine, which forces leukemia cells to obtain sufficient intracellular asparagine for proliferation via mechanisms other than uptake (*87, 88*). Interestingly, the nutrient environment has been shown to influence leukemic cell responses to both methotrexate and ASNase.

1.3.4 Nutrient supplements affect components of ALL therapy

Folic acid, a synthetic conjugate base of folate, is readily available as a dietary supplement (*89*). For decades, some clinicians have advised against folic acid supplementation during methotrexate treatment of ALL (*89-91*). This advice stems from several observations. First, in the 1940s, Sidney Farber and his colleagues reported that administration of folic acid conjugates accelerates pediatric leukemia (*92*). This observation led to the seminal study that demonstrated the antifolate aminopterin (later replaced by methotrexate) could cause temporary remission of childhood leukemias (*93*). In addition to its growth-promoting effect in leukemia, supplemental folate has also been shown to interfere with leukemic cell uptake of methotrexate (*94, 95*). Finally, folate competes with methotrexate for the enzyme folypolyglutamate synthase that is required to produce polyglutamylated derivatives of these compounds, and polyglutamylated methotrexate has greater intracellular retention than methotrexate and contributes significantly to cytotoxicity (*90, 91, 96*).

In cells, folate is metabolized into various derivatives, including folinic acid (5-formyl-THF), a compound that is available as both a commercial vitamin supplement and as the chemoprotective medication leucovorin (*90*). Although folic acid supplements are not recommended during methotrexate treatment, folinic acid is used in the form of leucovorin to rescue toxicity in normal tissues by administration at adequate time-points following methotrexate treatment (*90*). Folinic acid is formed downstream from the DHFR reaction inhibited by methotrexate, and can be converted to THF for use in cellular metabolic reactions (*89, 97*). Thus, in multiple mouse and cell culture studies, folinic acid reversed the anti-cancer effects of methotrexate when administered simultaneously (*90*). As folinic acid is readily available as a nutritional supplement, patients must be advised against taking these supplements without clinician oversight, as dosage and timing may affect methotrexate treatment (*90*).

Recently, exogenous supplementation of the amino acid histidine was shown to increase methotrexate sensitivity of leukemic cancer cell lines *in vivo* (*6*). Histidine catabolism utilizes THF, and increasing the flux of histidine into this degradation pathway depletes the cellular THF pools that are already in limited supply during DHFR blockade by methotrexate (*6*). Thus, along with folic acid and folinic acid, histidine is now one of the known readily-available nutritional supplements that can influence methotrexate response of leukemia cells.

Of the other medications used in standard ALL treatment, ASNase stands out as another therapy whose efficacy can be influenced by environmental nutrient availability (*98, 99*). However, exactly which extracellular metabolites affect ASNase sensitivity remains poorly understood.

1.4 Leukemic cell response to L-Asparaginase (ASNase)

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 (*87, 88*). ASNase is unique among ALL therapies in that it exploits a specific metabolic vulnerability, observed several decades ago. The history of ASNase in cancer treatment dates back to the 1950s, when John Kidd first discovered that administering guinea pig serum to mice harboring transplanted lymphomas, or rats harboring transplanted lymphosarcomas, caused the cancers to regress (*100, 101*). In the early 1960s, J.D. Broome brought to light the observation that L-asparaginase is abundant in serum of guinea pigs but not other mammals, a phenomenon that had first been reported in the 1920s (*102, 103*). In a series of experiments, Broome then demonstrated that the L-asparaginase component of guinea pig serum was indeed responsible for its therapeutic effect (*102, 103*). ASNase was subsequently purified from *E. coli* and used in multiple clinical studies beginning in the late 1960s, in which it successfully treated ALL even as a monotherapy (*104-107*). Today, ASNase is a component of standard multi-agent treatment protocols for ALL. Unfortunately, as reflected in the current outcomes for childhood and adult ALL, not all patients respond to contemporary ASNase-containing regimens. Furthermore, the determinants of ASNase response in ALL remain incompletely understood.

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 upregulate ASNS through ZBTB1 and activating transcription factor 4 (ATF4) (*108, 109*). Recent work has identified alternative ways of increasing asparagine availability, such as through proteasome degradation (*110*) or aspartate uptake by solute carrier family 1 member 3 (SLC1A3) (*111*). Additionally, other cell types can secrete asparagine or its precursors, which ALLs can utilize, attenuating ASNase cytotoxicity (*98, 99*). These latter studies are particularly interesting because they exemplify that environmental nutrient availability can affect ASNase response of ALL cells. It remains unclear, however, whether extracellular nutrients that are not asparagine precursors can influence sensitivity to ASNase.

1.5 Overview and Significance of findings

Using a CRISPR/Cas9-based genetic screen, here, we interrogated the metabolic determinants of ASNase sensitivity in leukemia. Our analysis pinpointed thiamine pyrophosphate kinase 1 (TPK1) as essential for proliferation under ASNase treatment.

TPK1 converts thiamine to thiamine pyrophosphate (TPP), a cofactor necessary for the activity of the alpha-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 resistance 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. Altogether, our results suggest that SLC19A2 expression may be a determinant of ASNase response and that supra-physiological thiamine supplementation could potentially ablate such an association. Furthermore, our work provides a proof of concept that using human physiological vitamin levels can affect cancer cell sensitivity to therapies.

CHAPTER 2. Metabolic determinants of proliferation under ASNase treatment

2.1 *In vivo* ASNase responses of 62 cancer cell lines using a barcode-based competition assay

To simultaneously test the responses of several cell lines to ASNase treatment, I first worked on expanding a previously reported barcoded cell line library that allows for cell competition assays under nutrient deprivation conditions (*3*). By inserting one or more permanent DNA barcodes into a cell line, the response of that cell line can be tracked through an experiment in which a pool of barcoded cell lines are grown together in a single vessel. By comparing responses in a vessel with replete medium to responses in a vessel containing a single nutrient depletion, we can screen for cell lines that may be resistant or sensitive to that particular nutrient depletion. These results can then be validated using individual cell line proliferation assays. This technique was first used to test responses of 28 cell lines to low glucose (*3*). Since the initial publication of this technique, our lab has continued to barcode cell lines, and we now have over 60 cell lines that can be included in a single competition experiment.

Our expanded library of barcoded cell lines is largely composed of diverse subsets of leukemias and lymphomas, but also includes several myelomas, as well as smaller subsets of cell lines from epithelial malignancies such as breast, lung, and GI cancers. Each cell line has been infected with a lentiviral pool carrying 3 different DNA barcodes, and each barcode consists of a 7-nucleotide DNA sequence. When genomic DNA is extracted from a pool of barcoded lines, each of the 3 barcodes for a line is PCR amplified during preparation for deep sequencing, allowing for triplicate measures of each cell line in the competition assay. Barcoded cell lines are first cultured independently until the competition assay. To begin a competition experiment, the barcoded lines are pooled in approximately equal proportions. An initial aliquot is then taken, to be used for determining the initial distribution of barcodes in the pool. Another aliquot can then be placed into a vessel containing a control medium, or various vessels that each contain the same medium but with a particular nutrient missing (Figure 2.1). After ~2 weeks, aliquots are taken from the control and experimental vessels. Deep sequencing of the initial, control, and experimental samples allows us to see how the distribution of barcodes changed since the initial distribution, under both the control and experimental conditions.

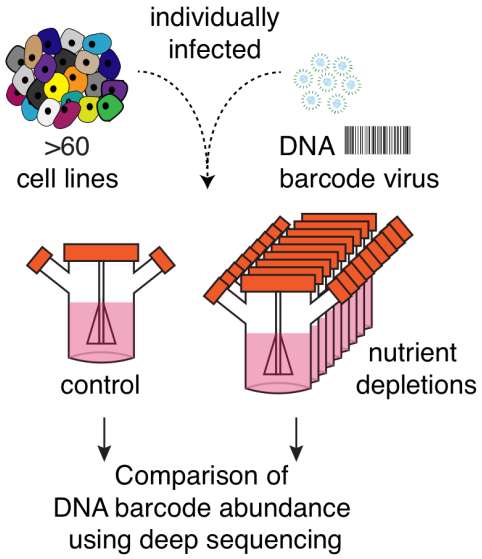


Figure 2.1. Barcode-based competition assays for systematically mapping cancer nutrient dependencies.

As multiple wild-type cell lines are known to be sensitive to asparagine depletion due to insufficient expression of ASNS (*108*), we hypothesized that cancers may have diverse responses to depletion of other non-essential amino acids as well. Thus, we attempted to validate our large barcoded cell line library *in vitro* in a set of custom RPMI medias that were each depleted in one non-essential amino acid. These flasks were cultured for 14-18 days, after which all flasks had undergone ~10 population doublings, and the experiment was then processed for deep sequencing.

The results of this experiment are presented in Figure 2.2. During analysis of our data, it was particularly informative to see which cell lines were represented in the sequencing reads of the initial pool but not in the control or experimental pools. The growth rates of these cell lines were likely too slow relative to other cell lines in these competitions, as they were not represented even in the control condition after ~10 population doublings. Thus, we could not obtain data for how these cell lines respond to nutrient depletions, and we filtered these cell lines out of our pipeline, leaving 49 cell lines with robust sequencing data for our final analysis. Notably, all of our ALL cell lines known to be sensitive to asparagine depletion were filtered out during our analysis due to this type of lack of representation in our control condition that suggested poor growth within competitions. However, analyzing the data for other cell lines passing our filters did show that cell lines respond quite differently to distinct amino acid depletions.

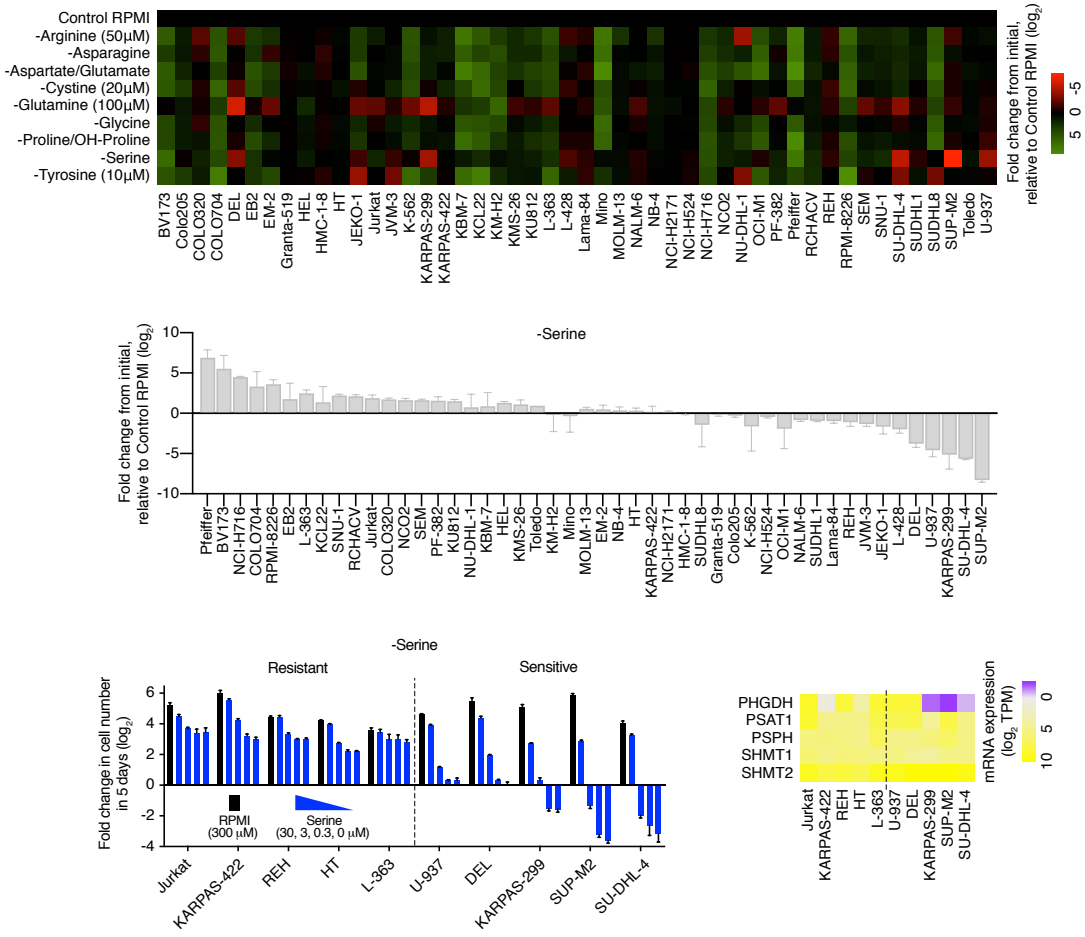


Figure 2.2. Validation of a blood and lymphoid cancer barcoded-based cell competition assay.

(Top) Heatmap of barcoded cell line responses to various amino acid deprivation conditions. Data presented is the Log2 fold change in abundance from initial pool, of barcodes (n=3) representing indicated cell lines in the competition assay, relative to the control media condition.

(Middle) Barcoded cell line responses to serine depletion (mean ± SD, n=3 barcodes).

(Bottom-Left) Fold change in cell numbers (Log2) of a panel of wild-type cell lines used in our barcoded competition experiment, after individual growth assays in various serine concentrations for 5 days (mean ± SD, n=3).

(Bottom-Right) CCLE RNAseq expression levels of indicated genes involved in serine synthesis.

For instance, simultaneous deprivation of aspartate and glutamate in one vessel, or of proline and hydroxyproline in a different vessel, both had no discernible effects on cell proliferation across all cell lines. In contrast, low glutamine, low tyrosine, and complete serine depletion all caused subsets of the barcoded lines to drop out of the cell

competitions. Interestingly, there have been multiple recent reports on using serine/glycine-depleted diets to slow the growth of cancers (*112*). We were intrigued by the possibility that such diets could be used to treat some of the cancers that were sensitive to serine depletion in our competition assay. Thus, we attempted to validate our competition assay data for serine depletion by conducting independent proliferation assays under serine depletion using a panel of cell lines.

Using a panel of 10 cell lines, we confirmed the existence of 5 cell lines that were resistant to serine depletion and 5 lymphoma cell lines that were sensitive (Figure 2.2). Whereas 2 sensitive cell lines arrested in serine-depleted media (U937 and DEL), 3 cell lines strikingly underwent substantial cell death (KARPAS299, SUPM2, and SUDHL4). We next looked at CCLE RNAseq data of these cell lines to determine whether low expression of any particular gene involved in serine synthesis could explain these growth responses to serine limitation. We found that KARPAS299, SUPM2, and SUDHL4 had very low PHGDH mRNA expression levels. Interestingly, the sensitivities of DEL and U937 to serine depletion were not explained by low CCLE expression of genes known to be involved in serine synthesis, including PHGDH, PSAT1, PSPH, SHMT1, or SHMT2. It is intriguing to consider why DEL and U937 are sensitive to extracellular serine depletion despite normal basal expression of serine synthesis pathway genes. One possibility is that these cell lines simply have a greater demand for serine than other cell lines and that this demand cannot be met by intracellular synthesis alone. If this is the case, then under serine limitation, the serine pools in these cells may be insufficient for carrying out processes required for cell proliferation. For example, if a cancer cell had a defect in glycine transport, then sufficient intracellular serine may be needed for not only incorporating serine into new proteins but also for maintaining glycine synthesis, as glycine is also required for protein synthesis as well as for synthesizing purines (*11*). Ultimately, further work is needed to determine whether DEL and U937 have specific metabolic vulnerabilities or demands that render them particularly sensitive to serine limitation.

Of translational interest, we found that the CCLE data actually had several other lymphoma cell lines with PHGDH levels that predict serine auxotrophy according to our results. Interestingly, recent evidence showed that progression of *in vivo* lymphoma models can be slowed using serine/glycine-depleted diets (*112*). We considered whether the cancers represented by our serine auxotroph lymphoma cell lines could be targeted with low serine/glycine diets, but noted that published serine/glycine-free diets result in a circulating plasma serine concentration of ~50 μM instead of a true serine-depleted environment. In our data, the lymphoma cell lines found to be serine auxotrophs can proliferate substantially at a media serine concentration of 30 μM. This suggests that targeting such lymphomas *in vivo* may require the use of alternative methods to achieve extracellular serine depletion, such as a serine-depleting enzyme analogous to L-asparaginase.

Our experiments involving serine depletion validated that our barcode-based competition assay allows us to identify cancers that are resistant and sensitive to

deprivation of a single amino acid. Furthermore, coupling this barcoding technique with transcriptomics can help identify major genetic determinants of these growth responses, as in the case of low PHGDH predicting serine auxotrophy. However, we were not able to obtain a complete cell line sensitivity spectrum for L-asparaginase using this technique *in vitro*, because many ALL cell lines appeared to grow too slowly *in vitro* for sufficient barcode representation to remain even under control conditions at the end of the competition assay. As *in vitro* growth rates of cell lines do not always predict *in vivo* growth rates, we wondered whether these ALL cell lines could grow sufficiently *in vivo* relative to our other barcoded cell lines such that an *in vivo* competition assay would be possible. Furthermore, although ASNS expression is known to be a major determinant of ASNase sensitivity, it remains unclear whether ASNS expression perfectly predicts *in vivo* response to ASNase (*113*).

We determined that *in vivo* competition assays with our large barcoded cell line library can be performed similarly to our *in vitro* competitions but with slight modification. Our pool of barcoded cell lines can be injected as multiple subcutaneous tumors in NSG mice, and mice can be divided into groups of control mice and mice treated with a drug of choice such as ASNase. After 2 weeks of tumor growth, we harvest the tumor xenografts, extract DNA from them, and process the DNA for sequencing as previously described for our *in vitro* competition assays. One important output of this type of experiment is the xenograft growth of these cell lines relative to each other. Some cell lines have low reads across all tumors, and these are filtered out from the final analysis, similarly to how we have to exclude slow growing cell lines in *in vitro* assays. However, in contrast to what we observed in control flasks of our *in vitro* experiments, many of the barcodes for ALL cell lines in our library are still represented in control tumors at experimental endpoint.

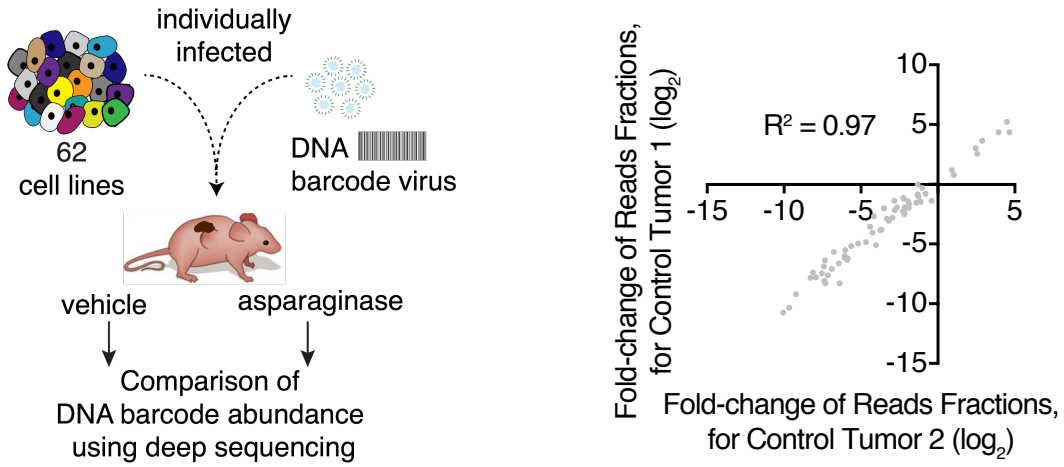


Figure 2.3. *In vivo* barcode-based cell competition assay for ASNase responses.
(Left) Schematic outlining cell line competition assay.
(Right) Inter-tumor correlation of individual cell line responses (gray dots) observed in two independent tumors from the vehicle-treated group of the *in vivo* competition assay.

Thus, to map *in vivo* ASNase responses, we used our pool of barcoded cell lines to generate subcutaneous xenografts in mice treated with either vehicle or ASNase (Figure 2.3). Our final analysis showed good inter-tumor correlation for the responses of 62 cell lines (Figure 2.3). During our experiment, we harvested plasma from both mice groups. LC/MS profiling of plasma suggested that asparagine was the only depleted plasma metabolite (Figure 2.4).

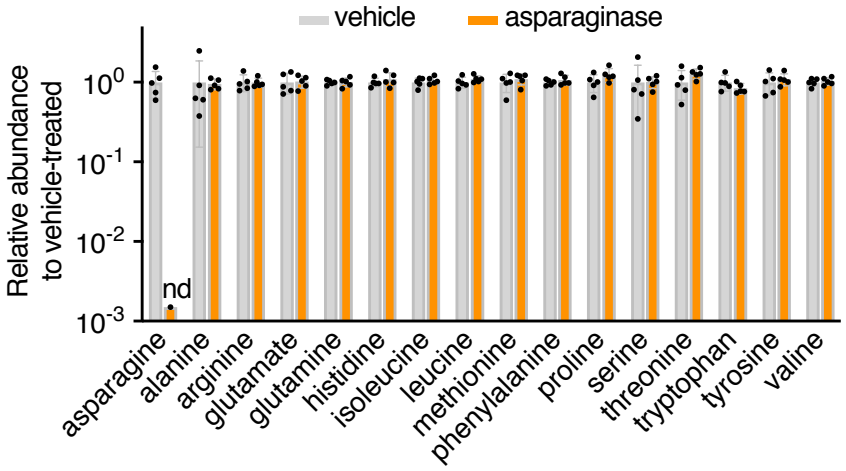
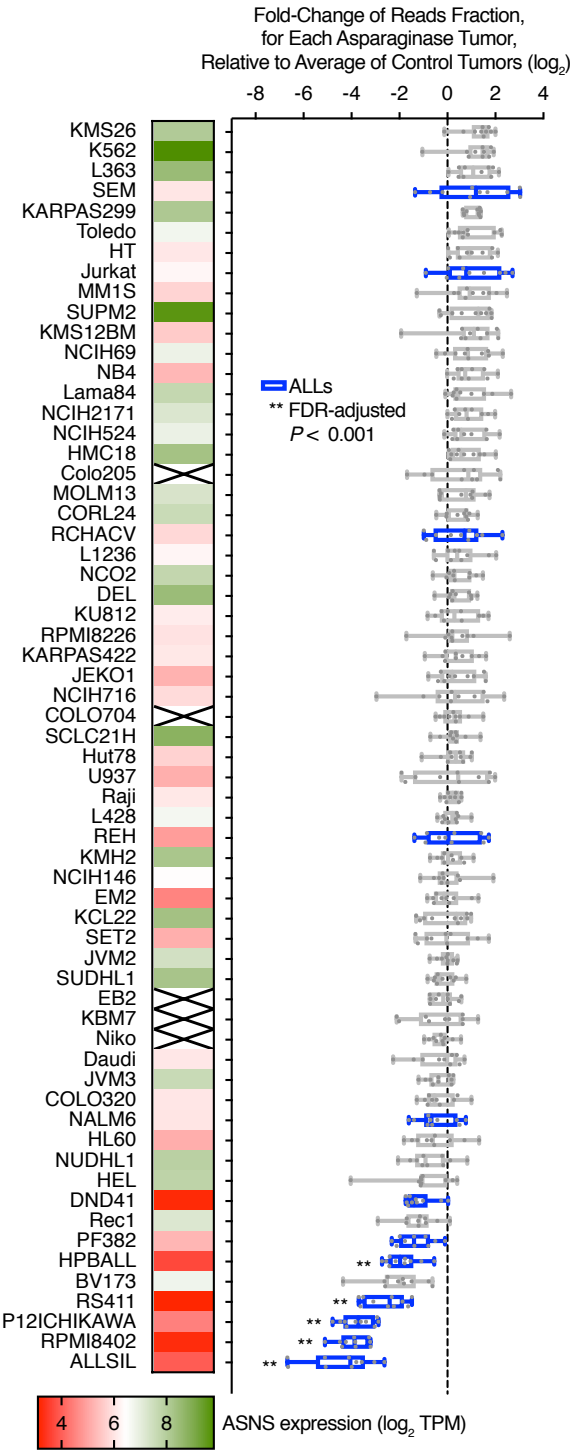


Figure 2.4. Plasma profiling of mice treated with ASNase regimen.
Relative abundance of indicated amino acids detected in plasma of mice on chow diet 5B1Q, treated with vehicle (gray) or 1000 U/kg ASNase (orange) daily during *in vivo* competition assay. Plasma was collected after the first 2 days of treatment, 24h after last dose (n=5 mice per treatment, mean ± SD). nd: asparagine was not detected in 4 of 5 ASNase-treated mice. Other measured amino acid levels, including that of glutamine, were unaffected by ASNase treatment.

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 (*114*), many of these lines belong to ALLs that express low levels of ASNS mRNA (Figure 2.5). Although it is known that ALLs can be extremely sensitive to ASNase, it was striking to see that across all the cancers tested in our barcoded library, ALL remains the only cancer enriched at the ASNase-sensitive end. 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 appeared resistant. For instance, the chronic myeloid leukemia (CML) cell line BV173 was one of the 6 most sensitive cell lines, despite having middle to high ASNS expression. To contrast, the ALL line REH and the CML line EM2 had quite low ASNS expression but were not sensitive to ASNase. Overall, these data demonstrate that our large library of barcoded cell lines can be applied towards *in vivo* competition assays in order to systematically map cancer responses to therapies or nutrient depletions. Furthermore, data from these experiments can also be coupled with mRNA expression data to interrogate determinants of assay response. Importantly, our results with this barcoded competition assay system suggest that there may be factors other than ASNS that impact *in vivo* ASNase response.

Figure 2.5. ASNS expression is a reasonable, but imperfect, predictor of *in vivo* ASNase sensitivity across hematopoietic and lymphoid cancer cell lines.
Log_2 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: FDR-adjusted *P*, by two-tailed unpaired t-test for unequal variances, of ASNase tumor group versus vehicle tumor group. Individual CCLE RNAseq ASNS expression levels of cell lines are also shown (x indicates no data available).


Fold-Change of Reads Fraction,
for Each Asparaginase Tumor,
Relative to Average of Control Tumors (log2)
-8
-6
-4
-2
0
2
4
KMS26
K562
L363
SEM
KARPAS299
Toledo
HT
Jurkat
MM1S
SUPM2
KMS12BM
NCIH69
NB4
Lama84
NCIH2171
NCIH524
HMC18
Colo205
MOLM13
CORL24
RCHACV
L1236
NCO2
DEL
KU812
RPMI8226
KARPAS422
JEKO1
NCIH716
COLO704
SCLC21H
Hut78
U937
Raji
L428
REH
KMH2
NCIH146
EM2
KCL22
SET2
JVM2
SUDHL1
EB2
KBM7
Niko
Daudi
JVM3
COLO320
NALM6
HL60
NUDHL1
HEL
DND41
Rec1
PF382
HPBALL
BV173
RS411
P12ICHIKAWA
RPMI8402
ALLSIL
ALLs
** FDR-adjusted
P < 0.001
**
**
**
**
**
4
6
8
ASNS expression (log2 TPM)

2.2 CRISPR-based screen identifies metabolic genes whose loss sensitizes cells to ASNase

To further investigate the metabolic determinants of ASNase response, we performed metabolism-focused CRISPR/Cas9-based screens in Jurkat, a T-ALL cell line resistant in our *in vivo* competition assay (Figure 2.6). Consistent with the essential role of asparagine synthesis under asparagine depletion, our screens yielded ASNS as the top scoring gene, with 6 out of 8 ASNS guides differentially depleted under ASNase treatment (Figures 2.7 and 2.8).

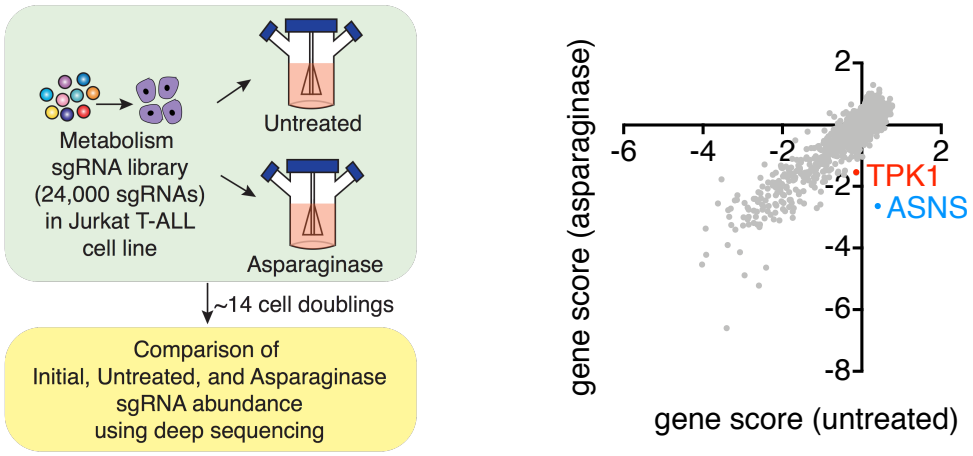


Figure 2.6. CRISPR-based screen for metabolic determinants of ASNase response.
(Left) Schematic depicting pooled CRISPR screen under ASNase treatment (0.25 U/mL) using a metabolism-focused sgRNA library.
(Right) Gene scores for Jurkat cells grown in untreated versus ASNase-treated vessels. Most genes, as well as non-targeting control sgRNAs, gave similar scores in untreated and treated vessels.

In addition to ASNS, we noted scoring of TCA cycle genes fumarate hydratase (FH) 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 (Figures 2.7, 2.8, and 2.9). Furthermore, sgRNAs targeting solute carrier family 1 member 5 (SLC1A5), the major glutamine transporter, and solute carrier family 25 member 12 (SLC25A12), a mitochondrial glutamate/aspartate exchanger, were also significantly depleted under ASNase treatment (Figure 2.6). Since many cancers use glutamine as a major anaplerotic source (*115*), 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 (Figure 2.9).

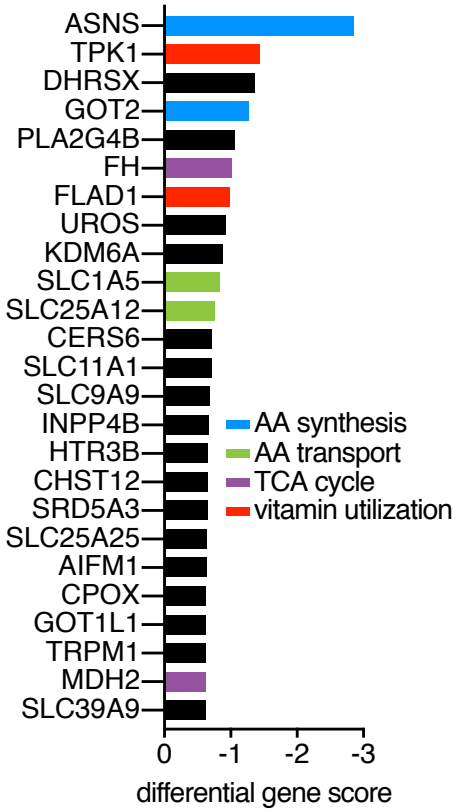


Figure 2.7. Top 25 metabolic genes differentially required under ASNase treatment.

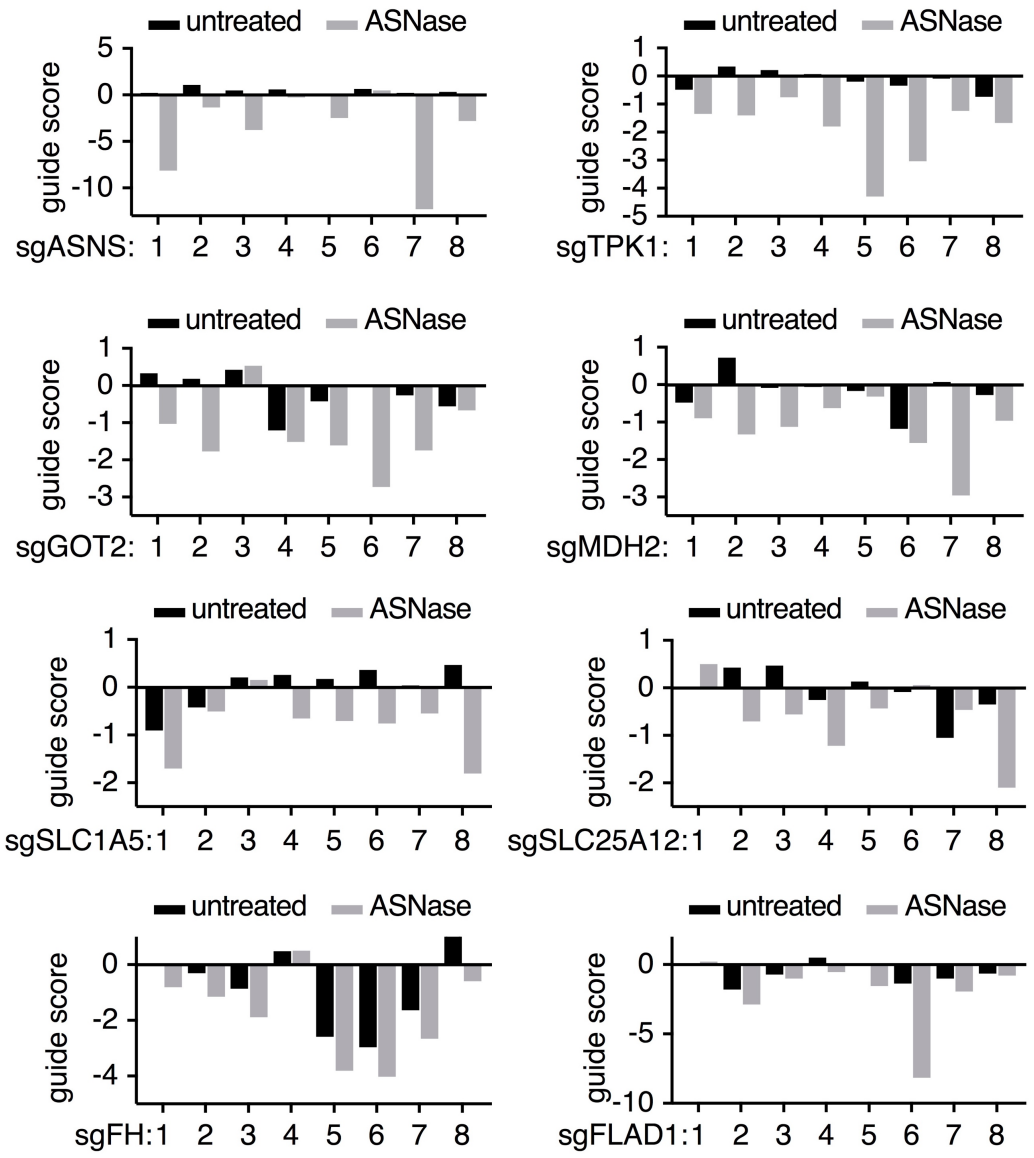


Figure 2.8. CRISPR screen guide scores for top scoring genes.
Log_2 fold change in the abundance of individual sgRNAs in untreated (black) or ASNase-treated (gray) for the following top scoring genes in our CRISPR screen: ASNS, TPK1, GOT2, MDH2, SLC1A5, SLC25A12, FH, and FLAD1.

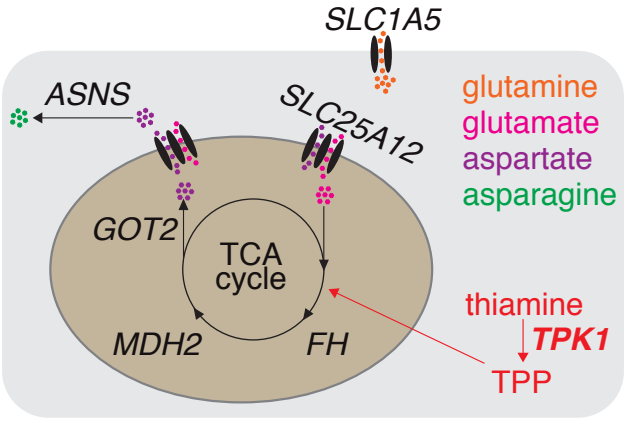


Figure 2.9. Top scoring genes in CRISPR screen highlight a specific route from glutamine to asparagine as essential under ASNase treatment.

Interestingly, the second top-scoring gene in our screens was TPK1, for which all 8 guides were differentially depleted when we treated cells with ASNase (Figures 2.7 and 2.8). TPK1 is a ubiquitously expressed cytosolic kinase that uses imported thiamine and ATP to produce TPP, the thiamine derivative that serves as a cofactor for many enzymes, including AKGDH in the TCA cycle (*116*). 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.

CHAPTER 3. TPP enables de novo asparagine synthesis and proliferation under ASNase treatment

3.1 TPK1-null cells are sensitized to ASNase treatment when supplemental TPP is limiting

To understand how TPK1 loss sensitizes leukemia cells to ASNase, we generated CRISPR/Cas9-mediated clonal knockouts of TPK1, in which TPK1 protein levels were undetectable (Figure 3.1). Interestingly, unlike in our genetic screening conditions, TPK1 null Jurkat cells die in culture unless supplemented with TPP. This suggests that TPP is indeed 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 sub-optimal proliferation of TPK1 null cells under standard culture conditions (2.5 nM). At this dose, TPK1 null cells were significantly more sensitive to ASNase treatment compared to parental controls (Figure 3.1), consistent with our screen results.

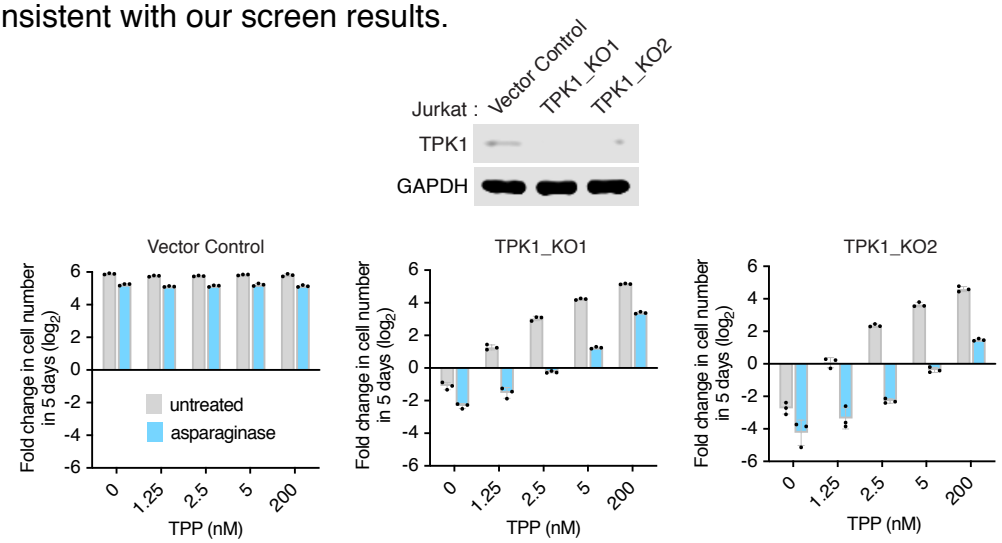


Figure 3.1. Effect of TPP availability on ASNase sensitivity in TPK1 KOs.
(Top) Immunoblot analysis of Vector Control and two clonal TPK1 knockouts made from Jurkat cells. GAPDH was used as a loading control.
(Bottom) Fold change in cell number (log_2) of Vector Control, TPK1_KO1, and TPK1_KO2 Jurkat 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.

Overexpressing sgRNA-resistant TPK1 cDNA rescued both TPP-dependency and the TPP-dependent ASNase sensitivity of TPK1 null cells (Figure 3.2). Altogether, these results show that TPP availability enables proliferation of leukemia cells upon ASNase treatment.

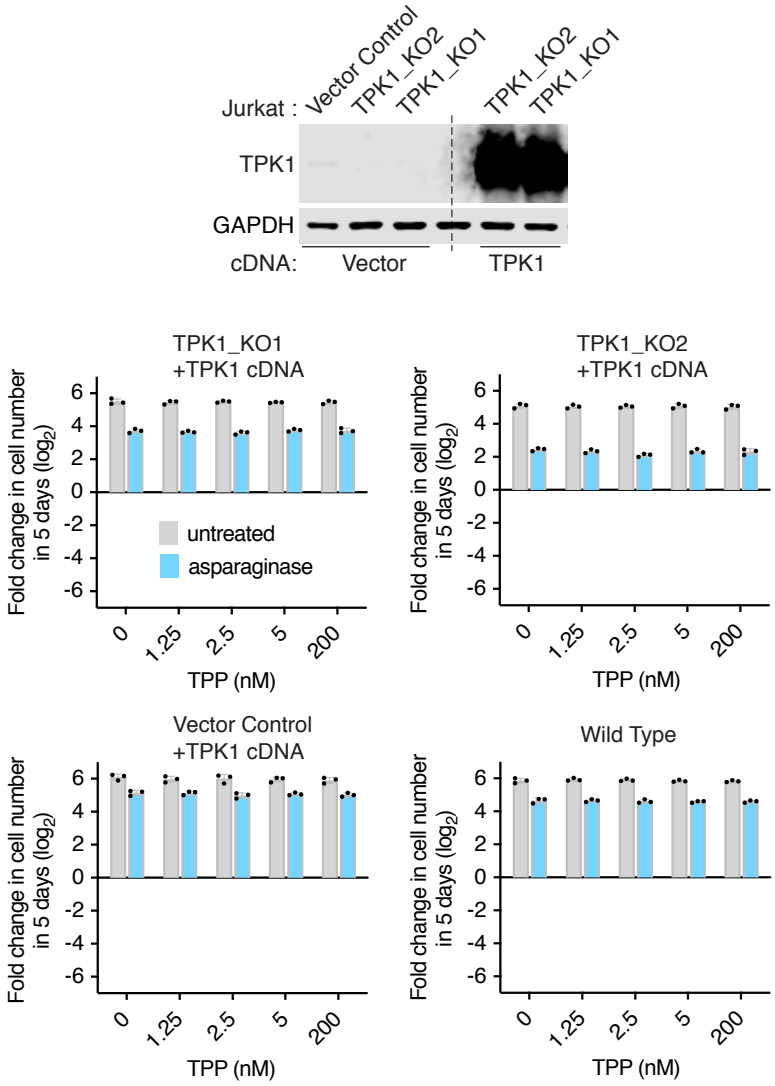


Figure 3.2. Overexpression of sgRNA-resistant TPK1 cDNA rescues TPP-dependence and TPP-dependent ASNase sensitivity of TPK1 KOs.
(Top) Immunoblot analysis of Vector Control, TPK1 knockout, and cDNA-rescued TPK1 knockout Jurkat cells. GAPDH was used as a loading control.
(Bottom) Fold change in cell number (log_2) of cDNA-rescued TPK1 knockout Jurkat cells, compared to that of cDNA-expressing Vector Control cells generated in parallel and that of wild-type 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 20 untreated-treated pairs.

3.2 TPP allows asparagine synthesis from glutamine under extracellular asparagine depletion

TPP is used as a cofactor by various enzyme complexes, including the a-ketoglutarate dehydrogenase complex (AKGDH), which catalyzes the oxidative decarboxylation of a-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), 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 (Figure 3.3).

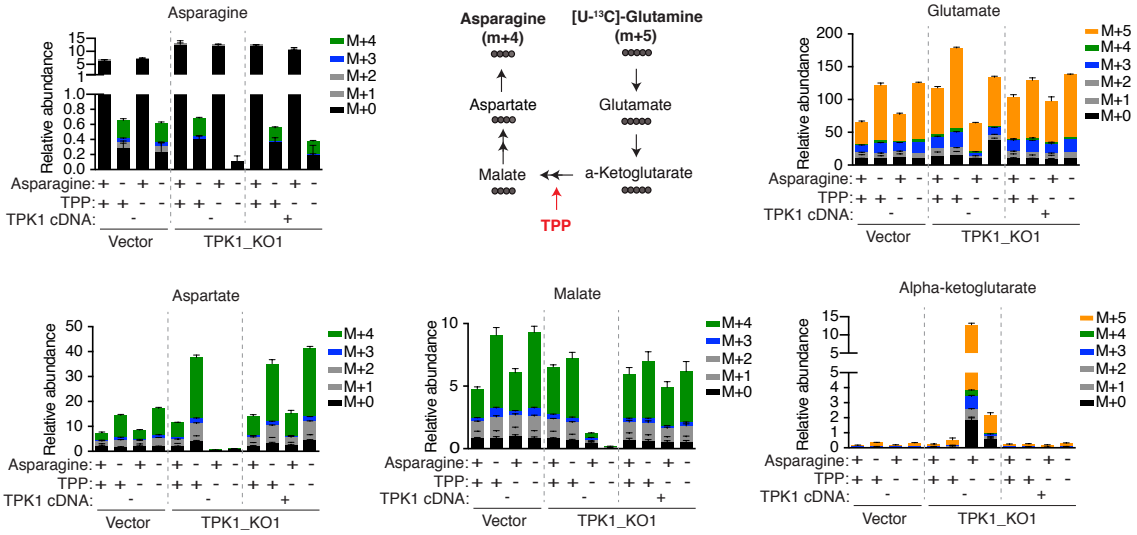


Figure 3.3. [U-^{13}C]-L-glutamine tracing in TPK1 KO cells and cDNA-rescued counterparts in the presence and absence of TPP and asparagine.
(Top-Middle) Schematic depicting a metabolic route of asparagine synthesis from glutamine. Filled circles represent 13C atoms derived from [U-^{13}C]-glutamine.
(Rest) Abundance of indicated metabolites derived from labeled glutamine in Vector Control, TPK1_KO1, and cDNA-rescued TPK1_KO1 Jurkats. Cells were incubated for 24 hours in media containing [U-^{13}C]-glutamine (2 mM) in the presence or absence of asparagine (378 μM) and TPP (3 μM). Colors indicate mass isotopomers (mean ± SD, n=3).

In asparagine replete conditions, we did not detect any asparagine labeling, indicating the lack of asparagine synthesis. 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, in line with ATF4-mediated upregulation of ASNS. Remarkably, in the absence of both asparagine

and TPP, we observed a substantial decrease in total as well as oxidatively labeled (m+4) malate, aspartate, and asparagine in TPK1 null cells. Addition of TPP or expression of TPK1 cDNA rescued all decreases in these metabolite levels. Strikingly, TPK1 loss also led to the massive accumulation of alpha-ketoglutarate. Together with the depletion of metabolites downstream from alpha-ketoglutarate, this is consistent with the cofactor role of TPP for the AKGDH complex. Altogether, these metabolite profiling data 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 the extracellular asparagine depletion.

CHAPTER 4. Physiological thiamine is limiting for growth and ASNase response of low SLC19A2 leukemia cells

4.1 Barcode-based competition assay identifies cell lines that grow sub-optimally at low, physiologically-relevant thiamine concentrations

Given that thiamine-derived TPP availability enables growth under ASNase treatment, we considered that conventional RPMI 1640 (RPMI) medium provides supra-physiological thiamine by supplying it at 3 μM. This is approximately 100-fold the content of normal human plasma, which ranges from 6.6 to 43 nM (*117*). 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 impact ASNase responses. To address our first question, we decided to perform cell line competition assays in RPMI, with and without added thiamine (supplemented with 10% dialyzed FBS). Notably, the trace thiamine in dialyzed FBS leads to ~1 nM total thiamine in culture, which approximates the content of human plasma.

As noted in Chapter 2, once individually-cultured barcoded cell lines have been pooled together, this pool can be divided into many different conditions of interest. Although we intended to focus on finding cancer cell lines for which the lower thiamine concentrations of human plasma may be limiting, we decided to simultaneously test responses of our barcoded cells to other vitamin limitations. Our rationale was an unbiased one: we did not know beforehand whether any vitamin deprivation condition could be limiting for some cancers while not affecting others, and testing multiple conditions gave us an answer this question. Thus, rather than make only a control medium and a low thiamine medium to grow barcoded pools in, I systematically made 7 custom RPMI medias that were each missing a single water-soluble vitamin normally contained in RPMI. This was done by using the same base medium for each condition, and by adding all but one vitamin to this base medium.

Although vitamins are essential nutrients for cell viability and growth, circulating human blood contains very low levels of some vitamins, as exemplified by human plasma having thiamine in the nanomolar range. These low levels of vitamins are still sufficient for cellular metabolism and growth, likely because most vitamins are metabolized into derivatives that serve as enzymatic cofactors rather than as cellular building blocks. When we conducted barcoded competition assays in amino acid depletion conditions, we had to note which amino acids were essential in culture. Conditions in which an essential amino acid was depleted had to contain supplemental levels of the amino acid that permitted mildly-inhibited cell proliferation. These supplemental amino acid amounts were in the micromolar range. In contrast, for our vitamin limitation competitions, we hypothesized that supplementation of media with even a dialyzed form

of FBS would provide the minuscule amounts of a depleted vitamin needed for cells to grow. We tested this in pilot experiments beforehand. Indeed, for most vitamins in RPMI, it is possible to completely remove the vitamin from RPMI and cells will still grow using the vitamin amount supplied solely by the supplemental dialyzed FBS. Thus, the 7 custom RPMIs I made that were each missing a vitamin were each simply combined with 10% dialyzed FBS, and these were the final mediums used for cell competitions. As a control, competitions were also grown in complete RPMI with 10% dialyzed FBS.

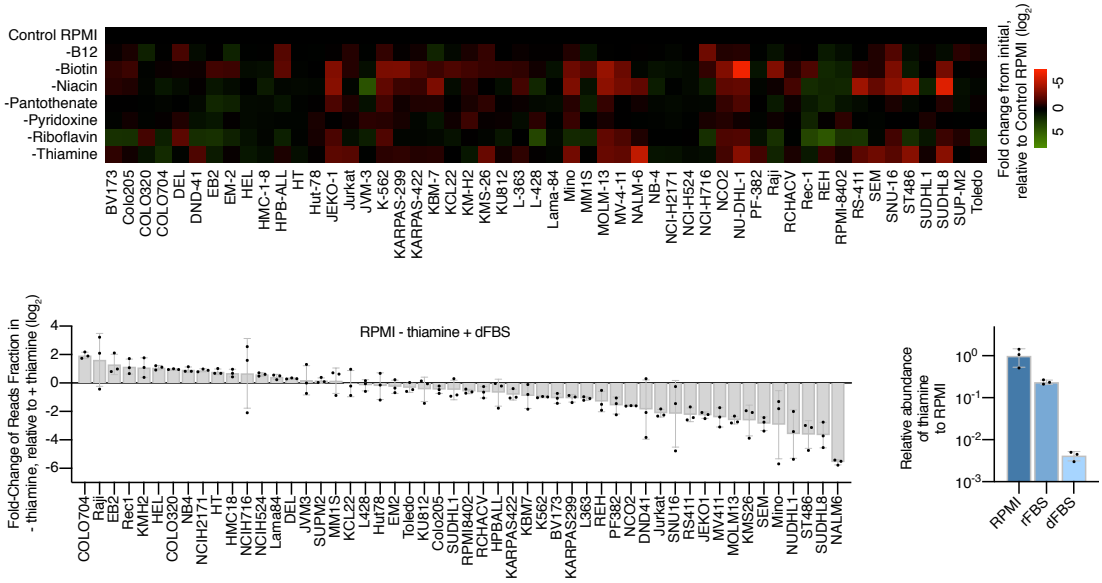


Figure 4.1. Barcode-based competition assay at conventional and lower, physiologically-relevant thiamine concentrations and under other vitamin depletions.
(Top) Heatmap of barcoded cell line responses to various vitamin deprivation conditions. Data presented is the Log2 fold change in abundance from initial pool, of barcodes (n=3) representing indicated cell lines in the competition assay, relative to the control media condition.
(Bottom-Left) Barcoded cell line responses to thiamine depletion (mean ± SD, n=3 barcodes). Of note, cells were grown in the absence of thiamine (-thiamine), or in the RPMI concentration of 3 μM thiamine (+thiamine). Media were supplemented with 10% dialyzed FBS, which contributes ~1 nM thiamine.
(Bottom-Right) Relative abundance of thiamine in RPMI (containing 3 μM thiamine), regular FBS (rFBS), and dialyzed FBS (dFBS) (mean ± SD, n=3), used to estimate the thiamine contribution of each FBS when combined with thiamine-free RPMI.

The data for this systematic characterization of vitamin dependencies is presented in Figure 4.1. Interestingly, for many vitamin depletion conditions, no cell lines seemed to be affected by limitation of the vitamin. In fact, in low B12, low pantothenate, and low pyridoxine, cells behaved almost identically to how they grew in control medium. In low riboflavin, a few cell lines appear to have been actually mildly selected for, perhaps

these cells had a relative growth advantage in this condition. In contrast, in low biotin, low niacin, and low thiamine, we observed that subsets of cell lines seemed to drop out of the cell competitions. This was intriguing, as it generally suggested a remarkable heterogeneity among cancers in responding to low levels of these vitamins but not others in our experiment. It is possible that in conditions like low B12, low pantothenate, and low pyridoxine, the vitamin level was not low enough to affect cell proliferation. These vitamins are all essential for human cells, and a low enough extracellular concentration of these vitamins would likely slow proliferation. Our results suggest that the trace amounts of these vitamins supplied by dialyzed FBS in our experiments was actually sufficient to enable maximal cancer cell proliferation of all cell lines tested. It is interesting to consider that cancer cell lines had heterogeneous responses to low levels of certain vitamins. This could potentially be due to different vitamin transport capabilities among cancer cell lines. For instance, it is possible that the cell lines that were depleted in these competitions have low expression of high-affinity transporters of these vitamins. If this is the case, it's possible these vitamins can enter cells through various low-affinity transporters under the excessive vitamin concentrations of RPMI, whereas a high-affinity transporter may be required for optimal proliferation in lower vitamin concentrations. Alternatively, it is possible that cell lines depleted in competitions rely more on a specific vitamin-utilizing pathway for proliferation compared to other lines. We know that inhibiting folate metabolism interrupts DNA synthesis, which is essential for cell proliferation. Similarly, it is possible that depleting biotin, niacin, or thiamine limits flux through one particular metabolic pathway that some cancers greatly depend on for proliferation.

Of particular interest to us, many cancer cell lines grew similarly in control and low thiamine conditions, but the fact that a small subset was depleted in the competition assay specifically under low thiamine suggested that low thiamine content could limit proliferation of some cancers as we had hoped to find.

We next determined whether the expression of any particular thiamine utilization gene is predictive of growth responses to thiamine limitation. Correlating growth in low thiamine with mRNA expression data of metabolic genes from CCLE revealed the plasma membrane thiamine transporter SLC19A2 as a top scoring gene (Figure 4.2). 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 of 0.1 vs 6.6) suggested SLC19A2 may be the primary transporter expressed in cancer lines.

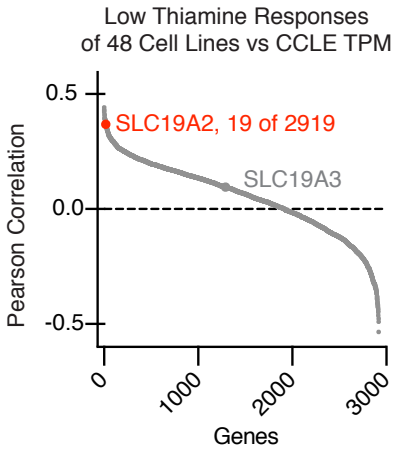


Figure 4.2. Expression of SLC19A2, but not SLC19A3, is a predictor of proliferation capacity at low thiamine.
Low thiamine cell competition responses were correlated with CCLE mRNA expression data of metabolic genes, and thiamine transporter 1 (SLC19A2), but not thiamine transporter 2 (SLC19A3), had one of the top scoring Pearson's correlation coefficients.

4.2 CRISPR-based screen identifies metabolic genes essential for proliferation in low thiamine

To further support our 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 5 of 8 guides being selectively depleted in low thiamine media (Figure 4.3). Notably, additional genes with no known connections to thiamine uptake scored in our screen, suggesting that other metabolic dependencies exist when thiamine is low. Altogether, our unbiased competition assay and genetic screen approaches both pinpoint that SLC19A2 expression may be a determinant of growth at physiologically relevant thiamine concentrations.

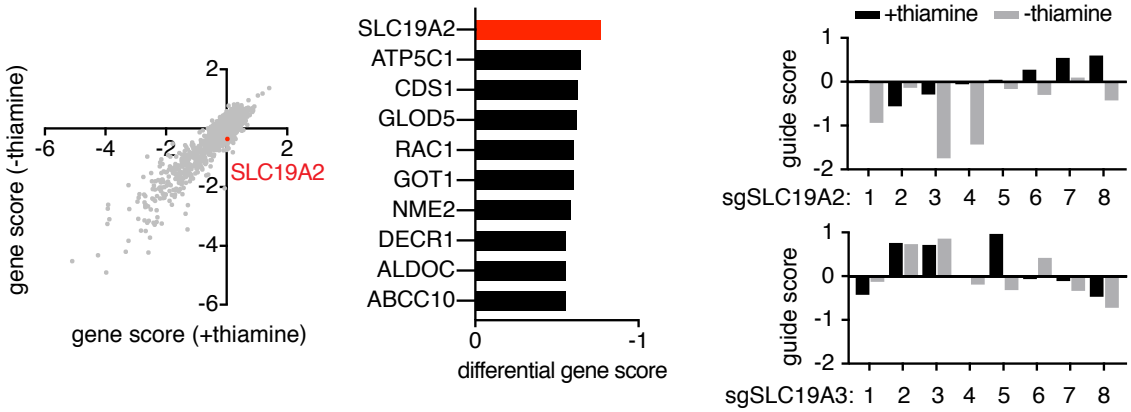


Figure 4.3. CRISPR-based screen for metabolic determinants of growth in low thiamine.

(Left) Gene scores for a CRISPR screen done with Jurkat cells in the presence (+thiamine) or absence (-thiamine) of 3 μM thiamine. Media were supplemented with 10% dialyzed FBS, which contributes ~1 nM thiamine. Most genes gave similar scores in the two conditions.

(Middle) The top 10 genes differentially required in low thiamine are shown.

(Right) Log_2 fold change in the abundance of individual sgRNAs in our CRISPR screen, in the presence (+thiamine) or absence (-thiamine) of 3 μM thiamine, for SLC19A2 and SLC19A3.

4.3 SLC19A2 expression is a determinant of not only growth, but also ASNase response, at physiological thiamine levels

Given the connection between thiamine utilization and ASNase response, we next asked whether cell lines with low SLC19A2 are more sensitive to ASNase in limiting physiological thiamine than in the high thiamine of conventional media. To address this, we identified three B-ALL cell lines, KOPN8, REH, and NALM6, in which SLC19A2 mRNA was undetectable, consistent with CCLE data (Figure 4.4). We had previously noted REH and NALM6 were ASNase-resistant in barcoded competitions in mice, but we considered that the standard mouse chow we used may have supplied supra-physiological thiamine.

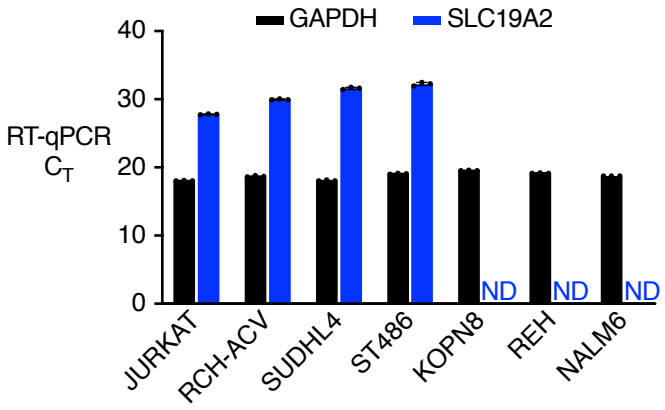


Figure 4.4. Baseline mRNA levels by RT-qPCR of SLC19A2 in a panel of leukemia and lymphoma cell lines.
Baseline mRNA levels by RT-qPCR of indicated genes for cell lines representing the following cancers: T-ALL (JURKAT), B-ALL (RCH-ACV, KOPN8, REH, NALM6), diffuse large B-cell lymphoma (SUDHL4), and Burkitt lymphoma (ST486). ND: not detected (mean ± SD, n=3).

Consistent with the *in vivo* competition results, REH cells proliferated similarly in the presence and absence of ASNase under standard culture conditions (3 μM thiamine). However, under low thiamine (~60 nM) conditions, untreated cells indeed grew slightly less than at 3 μM (2 versus 4 doublings in 6 days) but now, strikingly, ASNase 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 ASNase response at ~60 nM thiamine (Figure 4.5). This suggests that SLC19A2 expression is a determinant of both growth and ASNase sensitivity in low thiamine.

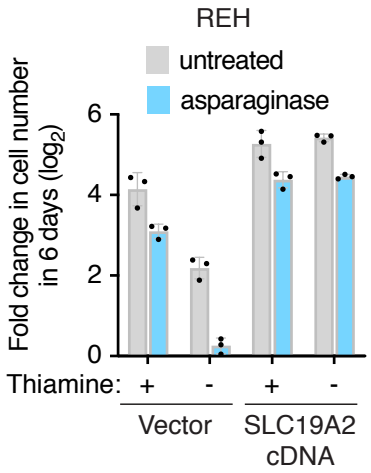


Figure 4.5. Lowering media thiamine sensitizes SLC19A2-low cells to ASNase, and this can be rescued by overexpressing SLC19A2 cDNA.
Fold change in cell number (log_2) of Vector Control and SLC19A2-cDNA-expressing REH cells, after untreated or 0.001 U/ml ASNase conditions for 6 days, in the presence (+) or absence (-) of 3 μM thiamine (mean ± SD, n=3). Media were supplemented with 10% regular FBS, which contributes ~60 nM thiamine. $P < 0.05$ for all 4 untreated-treated pairs.
Statistics: two-tailed unpaired t-test for equal variances.

Consistent with this, as we further lowered thiamine towards 10 to 40 nM to be within the human plasma range, we found that for SLC19A2-low REH and NALM6 cells, thiamine levels constrained growth and ASNase had a greater effect, compared to cells cultured in supra-physiological thiamine (Figures 4.6 and 4.7). At 10 nM thiamine, live REH cells were still visualized in ASNase-treated conditions (Figure 4.7). This is consistent with the REH data shown in Figure 4.6 in which cells appeared to arrest rather than die at 10 nM thiamine when ASNase-treated. Thus, at 10 nM thiamine, REH cells can proliferate when untreated but appear to undergo stasis when treated with ASNase. In contrast, SLC19A2-high Jurkat, RCH-ACV, and ST486 cells were unaffected by physiological thiamine concentrations, and their ASNase response was similar at high and low thiamine (Figure 4.6).

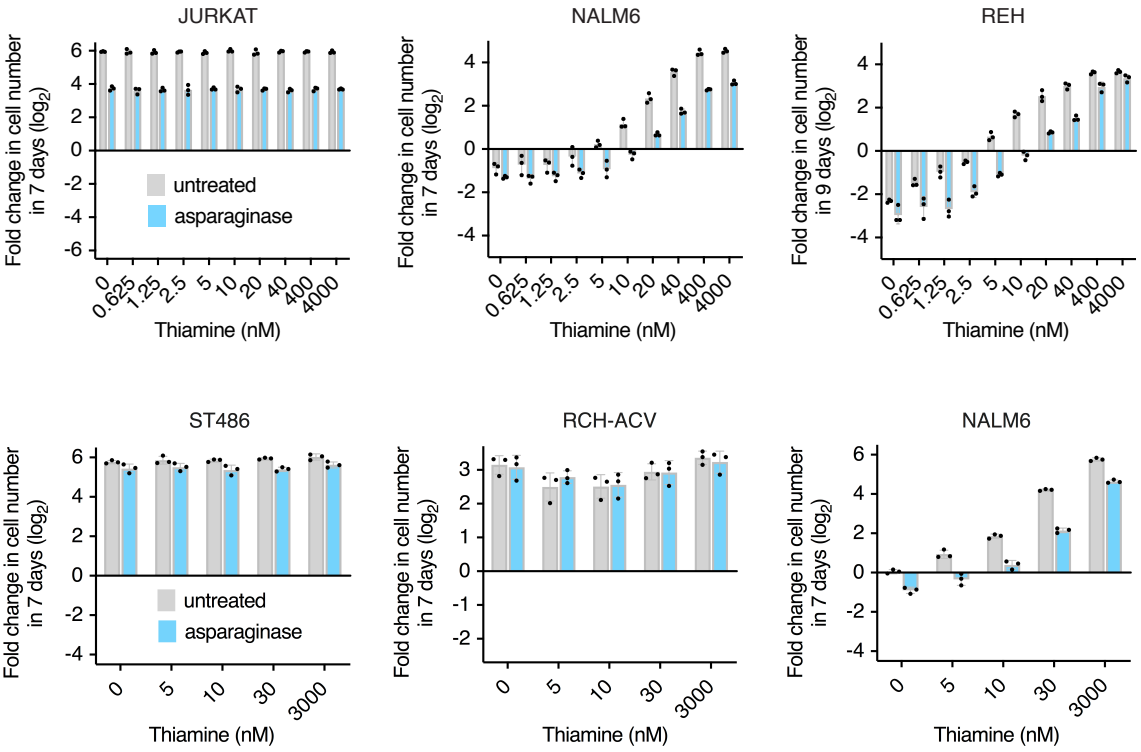


Figure 4.6. SLC19A2 expression is a determinant of both growth and ASNase response at physiological thiamine in a panel of cell lines.
(Top) Fold change in cell number (log_2) of SLC19A2-high (Jurkat) and -low (NALM6, REH) wild-type cell lines, after untreated or 0.001 U/ml ASNase conditions for 7-9 days, at different thiamine concentrations added to thiamine-free RPMI supplemented with 10% double-dialyzed FBS (mean ± SD, n=3). $P < 0.05$ for untreated-treated pairs at these thiamine concentrations: JURKAT- all concentrations, NALM6- all above 1.25 nM, REH- all above 0 nM.
(Bottom) Independent experiment similar to that in (Top), showing fold change in cell number (log_2) of additional SLC19A2-high (ST486, RCH-ACV) wild-type cell lines compared to SLC19A2-low NALM6, after untreated or 0.001 U/ml ASNase conditions for 7 days, at different thiamine concentrations added to thiamine-free RPMI supplemented with 10% double-dialyzed FBS (mean ± SD, n=3). Statistics: For NALM6 only, $P < 0.05$ by two-tailed unpaired t-test for equal variances, for all 5 untreated-treated pairs.
Statistics: two-tailed unpaired t-test for equal variances.

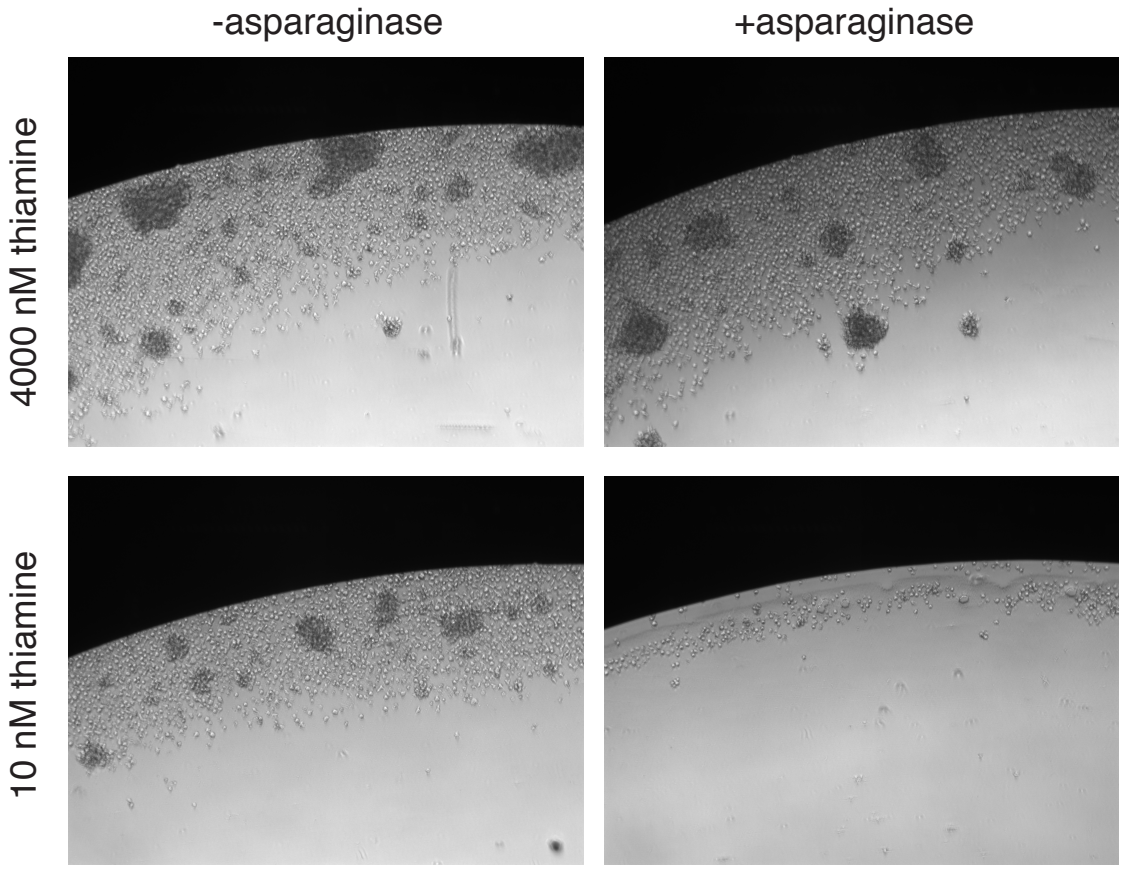


Figure 4.7. Bright-field micrographs of a SLC19A2-low cell line in the presence and absence of ASNase at high and low thiamine.
Representative bright-field micrographs of the wild-type REH experiment in Figure 4.6, after untreated or 0.001 U/ml ASNase conditions for 9 days at the indicated thiamine concentrations added to thiamine-free RPMI supplemented with 10% double-dialyzed FBS (mean ± SD, n=3).

Importantly, 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 ASNase resistance under human plasma thiamine levels relative to cells in standard culture (Figure 4.8).

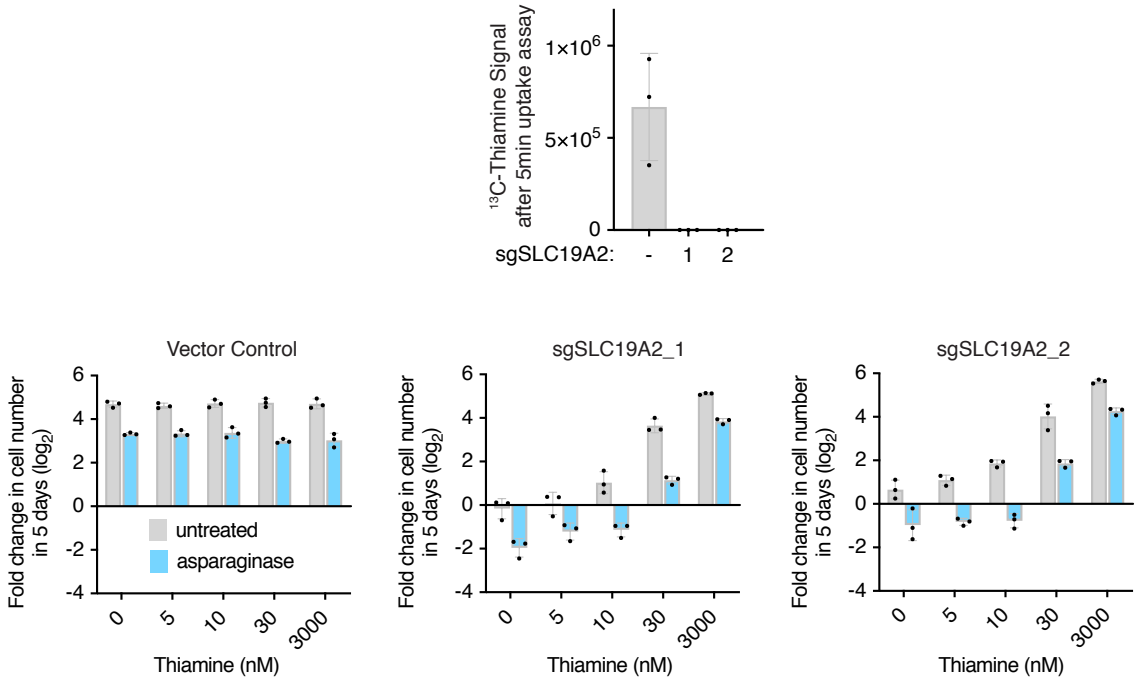


Figure 4.8. Effects of CRISPR-mediated targeting of SLC19A2 on ^{13}C-Thiamine uptake and ASNase sensitivity at physiological thiamine.
(Top) ^{13}C-thiamine uptake in Vector Control, sgSLC19A2_1, and sgSLC19A2_2 expressing Jurkat cells (mean ± SD, n=3).
(Bottom) Fold change in cell number ($\log_2$) of Vector Control, sgSLC19A2_1, and sgSLC19A2_2 expressing Jurkat cells, after untreated or 0.001 U/ml ASNase conditions for 5 days, at different thiamine concentrations added to thiamine-free RPMI supplemented with 10% double-dialyzed FBS (mean ± SD, n=3). $P < 0.05$ for all 15 untreated-treated pairs.
Statistics: two-tailed unpaired t-test for equal variances.

Taken together, these data establish SLC19A2 expression as a determinant of (1) growth and (2) ASNase response at physiological thiamine levels.

CHAPTER 5. Dietary thiamine intake influences ASNase sensitivity of low SLC19A2 leukemia cells *in vivo*

5.1 SLC19A2 is the only detectable thiamine transporter in patient ALL samples, and a subset of patients have low SLC19A2 tumors

To confirm that endogenously-low expression of SLC19A2 in cell lines is not a tissue culture artifact, we probed for the existence of SLC19A2-low patient tumors. As ASNase is a standard in the initial treatment phase of ALL, we explored RNAseq data of pediatric primary ALLs sampled from peripheral blood and bone marrow, as well as recurrent ALLs sampled from bone marrow (Figure 5.1).

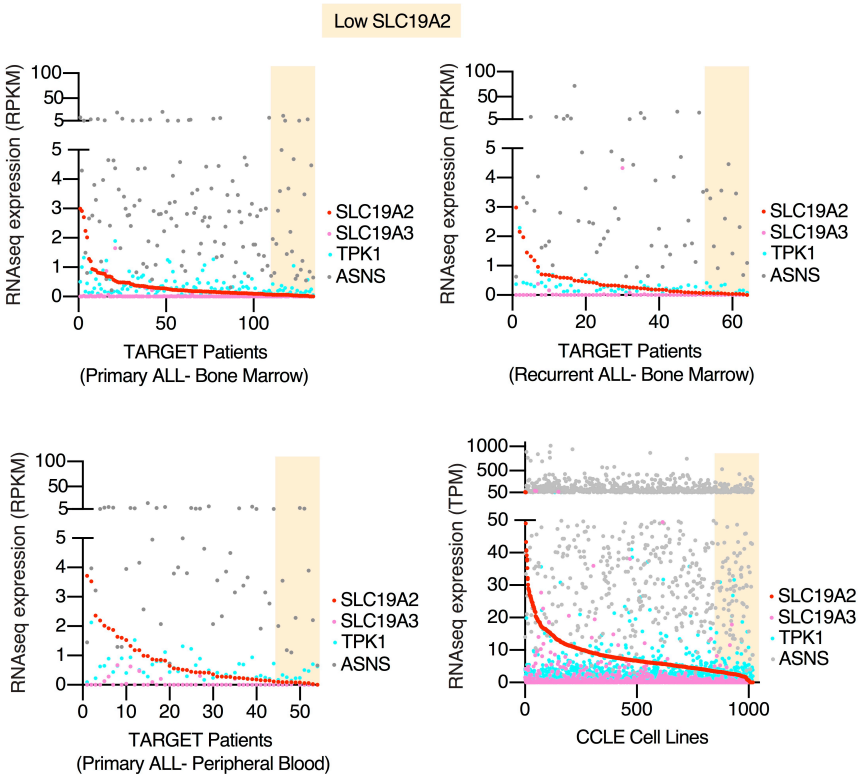


Figure 5.1. Patient RNAseq data from TARGET ALL Sub-study.
RNAseq expression data for indicated genes, for various patient tumor samples from ALL Expansion Phase 2 Sub-study of TARGET, and for every cell line in CCLE (representing diverse tissue origins) for comparison.

ASNS, TPK1, and SLC19A2 mRNAs were consistently detected in these 3 datasets (Figure 5.1), and their expression was similar between primary and recurrent bone marrow datasets, suggesting that treatment generally did not influence mRNA levels of these genes. Of note, SLC19A3 mRNA was very rarely detected, indicating that SLC19A2 is likely the primary thiamine transporter expressed in these tumors, similar to that seen across CCLE cell lines. Importantly, these data show a diverse range of SLC19A2 expression among patients and confirm the existence of low SLC19A2 tumors in which ASNase response may depend on environmental thiamine.

5.2 Humanizing blood thiamine of mice through diet sensitizes low SLC19A2 ALL cells to ASNase *in vivo*

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 NOD-SCID 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 supra-physiological plasma thiamine levels obtained with chow, or a low amount that resulted in levels resembling that of human serum (Figure 5.2, Table 5.1).

Importantly, it is also possible to maintain supra-physiological blood thiamine levels in humans. In the clinic, supplemental thiamine is usually administered when thiamine deficiency is suspected. However, prophylactic use has been recommended for other scenarios, such as for preventing malignancy-associated thiamine deficiency (*118, 119*) or for cases of malnutrition such as patients struggling with alcohol dependency (*120*). There is no standard regimen for clinical thiamine supplementation even for cases of deficiency (*120-122*). In a study that evaluated thiamine supplementation regimens used in out-patient management of alcohol dependency, a total daily administration of 500 mg thiamine by intramuscular or oral routes led to plasma thiamine levels ~10-fold and ~8-fold greater than normal levels, respectively (*123*). These regimens were given for 11 days, and these supra-physiological levels were maintained over the majority of this timeframe. Even higher plasma thiamine levels (~100-fold of normal) have been observed transiently after fast intravenous administration of less than 500 mg thiamine (*117, 124*), and intravenous regimens are recommended when deficiency is suspected (*120-122*). Of note, 500 mg thiamine tablets are commercially available for dietary supplementation, with suppliers recommending daily intake of these tablets. Thus, it is plausible to consider that supra-physiological plasma thiamine could be reached in a cancer patient not only through clinical prophylactic regimens aimed at preventing malnutrition, but also through consumption of readily-available dietary supplements.

Lowering plasma thiamine to human levels was well-tolerated, as indicated by animal weights remaining unchanged after diet modification (Figure 5.2). 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. Importantly, our ASNase regimen depleted plasma asparagine levels without affecting the levels of abundant amino acids such as glutamine (Figure 5.3).

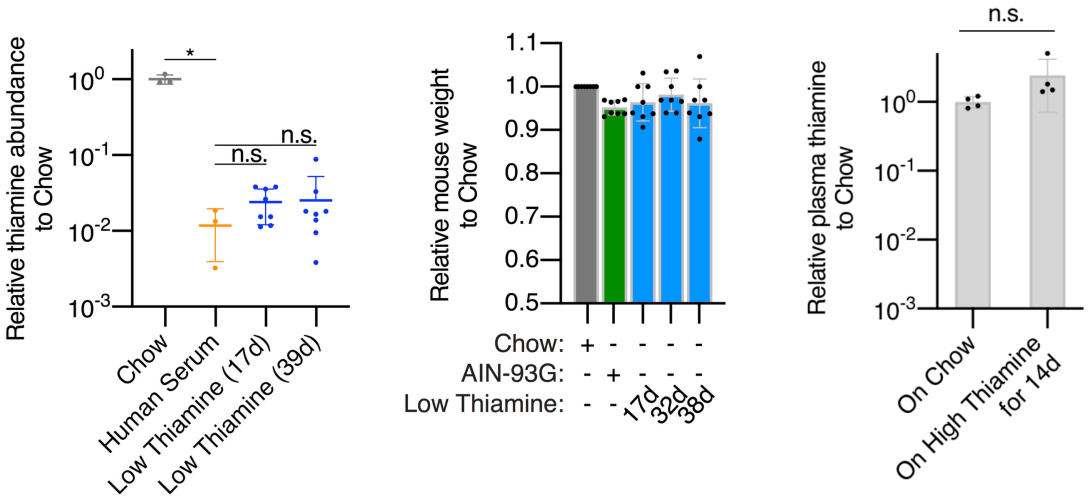


Figure 5.2. Humanizing plasma thiamine levels in mice by lowering thiamine content of diet.
(Left) Plasma thiamine profiling of mice on conventional chow (n=3 mice), and mice on a modified AIN-93G purified diet of low thiamine content (n=8 mice) (mean ± SD). Human serum was profiled simultaneously for relative comparison. Statistics: $*P < 0.01$ by two-tailed unpaired t-test for unequal variances.
(Middle) 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).
(Right) Plasma thiamine profiling of mice on conventional chow (n=4 mice), and mice on a modified AIN-93G purified diet of high thiamine content (n=4 mice) (mean ± SD). Statistics: two-tailed unpaired t-test for unequal variances.

Table 5.1. Summary of mouse diets used.

Diet Name	Feed	Feed type	Thiamine in Feed	Thiamine in Water
Chow	5B1Q (TestDiet)	chow	14 mg/kg diet	none
High Thiamine	Thiamine-free AIN-93G (Dyets, Inc.)	defined purified ingredients	none	125 μM
Low Thiamine	Thiamine-free AIN-93G (Dyets, Inc.)	defined purified ingredients	none	5 μM

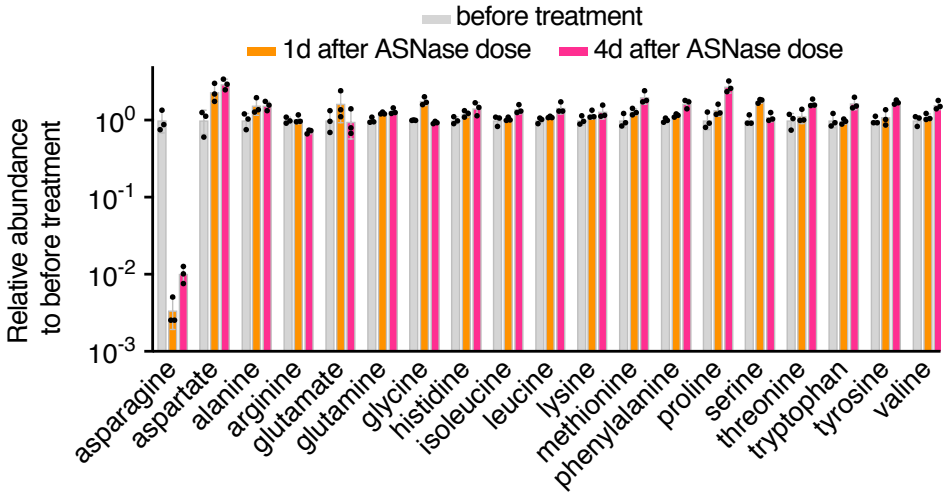


Figure 5.3. Alternative ASNase regimen in mice with less frequent administration. Relative abundance of indicated amino acids detected in plasma of mice on purified diet AIN-93G containing high thiamine, before treatment (gray) and after 1000 U/kg ASNase. Plasma was collected after a single dose, at days 1 (orange) and 4 (pink) after administration (mean ± SD, n=3 mice), and because asparagine remained depleted after 4 days, mice were treated only twice weekly in the xenograft survival experiments. Of note, glutamine was not depleted at either time-point.

Compared to the high thiamine diet group in which plasma thiamine was comparable to that seen with standard chow, neither ASNase treatment or lowering dietary thiamine alone significantly affected survival from leukemia (Figure 5.4). Of note, proliferation of these leukemia cells slowed under low thiamine conditions *in vitro*, raising the possibility that the *in vivo* microenvironment that cells were directly exposed to may not contain low enough thiamine to impair growth. Remarkably, however, combining ASNase

treatment and low dietary thiamine did indeed significantly (Mantel-Cox $P = 0.0034$) extend survival from leukemia (Figure 5.4).

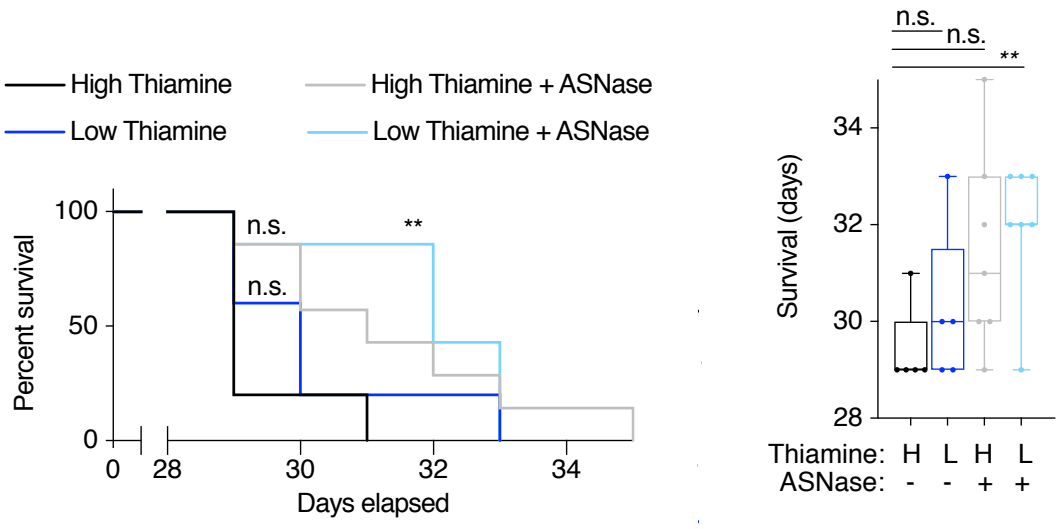


Figure 5.4. ASNase significantly increases survival of mice bearing orthotopic xenografts of an SLC19A2-low cell line, only when dietary thiamine is lowered.
(Left) Kaplan-Meier survival curves of NSG mice on high or low thiamine AIN-93G diets engrafted with REH (endogenously-low SLC19A2 cell line) by 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), and (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.

Altogether, our results provide evidence that thiamine availability resulting from dietary intake can impact ASNase sensitivity of low SLC19A2 ALLs *in vivo*.

CHAPTER 6. Discussion

In previous studies, a cell competition assay of 28 barcoded cell lines representing various cancer types had shown that growth of different cancers can vary greatly under nutrient limitations such as low glucose and low cholesterol (*3, 125*). In this work, I helped expand this library of barcoded cell lines by both lentivirally infecting more cell lines with barcodes and by validating that all cell lines in our final library had correctly annotated barcodes. This resulted in a comprehensive list of more than 60 barcoded cell lines, mainly representing cancers of hematopoietic and lymphoid tissues. Cancers of these origins were of particular interest to us, as leukemias such as ALLs have already been treated successfully in the past through the depletion of circulating nutrients. This is best exemplified by the clinical success of ASNase even as a monotherapy for ALL, and also by the more recent use of arginase for certain AMLs (*126*). Thus, it is possible that there may be other unknown, clinically-relevant nutrient dependencies still to be mapped among cancers of blood and lymphoid origins. Importantly, we validated that our large barcoded cell line library could indeed be used for *in vitro* cell competitions, but also for *in vivo* competitions by injection of barcoded pools as subcutaneous xenografts in mice. This methodology can now be applied not only towards mapping responses to nutrient deprivation conditions, but also towards testing novel drug treatments across these cancer cell lines.

Here, we first applied our barcoded cell line assay towards a systematic mapping of cancer cell responses to the depletion of individual non-essential amino acids (NEAAs) *in vitro*. To do this, we simultaneously made 9 custom RPMI medias, where each was missing only 1 or 2 NEAAs, and grew barcoded pools under these different conditions. In our assays, there were some conditions in which no cell lines were affected, such as in aspartate/glutamate-free media, as well as in proline/hydroxyproline-free media. In contrast, we observed that in low tyrosine, low glutamine, and complete serine depletion, a subset of barcoded cell lines proliferated less than in control conditions. This raised the possibility that some of these cancers could be targeted by limiting the extracellular availability of one of these amino acids. Serine/glycine-depleted diets have already been shown to slow the growth of certain cancers (*112*), and we were intrigued by the possibility of identifying additional cancers that could benefit from this dietary intervention. Thus, we turned towards validating our low serine competition assay results as a means of determining whether our large barcoded cell line technique could indeed yield robust data on cellular nutrient dependencies.

We attempted to validate the results of our *in vitro* serine depletion competition by conducting individual growth assays of cell lines that had been used in our barcoded experiment. Remarkably, we confirmed that five lymphoma cell lines that had dropped out of the cell competitions in low serine were in fact incredibly sensitive to serine restriction compared to five control cell lines. Among these cell lines sensitive to serine restriction were two cell lines that arrested at low serine and three cell lines that appeared to be complete serine auxotrophs incapable of surviving without extracellular

serine availability. These three serine auxotrophic cell lines had very low basal PHGDH expression in CCLE RNAseq data. However, the other two lymphoma cell lines that arrested at low serine did not have low expression of PHGDH or other genes known to be involved in serine synthesis. Further experiments are needed to truly rule out a defect in serine synthesis in these lines, such as a probing for the ability to upregulate serine synthesis genes in these cancer cells. If these cells do not have a defect in serine synthesis, it is possible that they have a substantially greater demand for serine than other cell lines. Another interesting observation was that 4 out of 5 of the cell lines we identified as sensitive to serine depletion were also previously confirmed to be cholesterol auxotrophs by our lab (*125*). Cholesterol auxotrophy in some of these cell lines was associated with promoter hypermethylation of a cholesterol synthesis pathway gene, and it is possible that serine synthesis gene promoters are similarly methylated in these cancer cells.

Although we thought these cancers could potentially be targeted with serine/glycine-depleted diets *in vivo*, we noted that these diets do not fully deplete circulating serine but instead bring it down to ~50 μM in serum (*112*). However, even at ~30 μM serine *in vitro*, these lymphoma cell lines still undergo multiple population doublings in a matter of days. It appears that a method of completely depleting circulating serine, down to levels below 3 μM based on our data, is needed to target these serine auxotrophic cancer cells. Such depletion could perhaps be achieved by a serine-depleting enzyme analogous to ASNase, which has been previously proposed (*127*). A method for depleting serine is indeed warranted, as further analysis revealed that there's as many lymphoma cell lines with CCLE PHGDH levels that predict serine auxotrophy as there are ALL cell lines with CCLE ASNS levels that predict ASNase sensitivity.

Through our work with serine depletion, we validated that our large barcoded competition assay can indeed pinpoint cancers that are resistant and sensitive to a particular nutrient depletion. Furthermore, coupling our competition assay responses with gene expression data proved to be very valuable in elucidating determinants of growth in nutrient deprivation. As ASNase is an established nutrient-depleting therapy already in use for ALLs, we were interested in mapping responses of other blood and lymphoid cancers to ASNase and also in determining pathways essential for ASNase resistance. We noted that many ALL cell lines in our barcoded library had been previously reported to be ASNase-sensitive and could serve as useful control cell lines in a competition grown under ASNase treatment. One challenge we encountered, however, was that these ALL cell lines grew too slowly *in vitro* to be represented even in control competition pools after several population doublings. However, we hypothesized that most cell lines in our barcoded library may undergo some change in doubling time when grown *in vivo*, which would perhaps permit ALL cells to persist through the course of the assay.

Thus, we grew barcoded cell line pools as subcutaneous xenografts in mice that were divided into control and ASNase treated groups. This resulted in a comprehensive

mapping of the *in vivo* responses of 62 cell lines to ASNase. We had thought there may be other cancers besides ALLs that may benefit from ASNase therapy. To our surprise, across all of the different blood and lymphoid cancer types represented in our barcoded library, the ASNase-sensitive end of the response spectrum contained almost exclusively ALL cell lines. Thus, our data did not identify other cancer types that may benefit from ASNase monotherapy, but instead further emphasized that asparagine auxotrophy is a phenomenom that is intriguingly unique to ALLs. Importantly, this compendium of cell line response to ASNase allowed us to interrogate potential determinants of ASNase sensitivity in an effort to identify pathways that may be essential under ASNase treatment. Using CCLE mRNA expression data, we found that 5 of the 6 most sensitive cell lines in our experiment were ALLs that expressed low basal ASNS levels. This was consistent with basal ASNS expression being a known major determinant of the cellular response to asparagine depletion. Although basal ASNS expression did appear to predict many of the growth responses in our data, it was not a perfect predictor, and our data suggested there may be additional determinants of ASNase sensitivity.

In order to further elucidate determinants of ASNase response, we performed a negative selection genetic screen to identify genes that are required for maintaining ASNase resistance in the Jurkat T-ALL cell line. This cell line was appropriate for this screen because it was one of the few ALLs in our *in vivo* competition assay that was not sensitive to ASNase. As discussed previously, there is already evidence that increasing the availability of certain nutrients through diet can affect response to a different component of ALL therapy, the DHFR inhibitor methotrexate. Thus, we were particularly interested in seeing whether utilization of any particular nutrients affects response to ASNase in ALLs. Our metabolism-focused sgRNA library was well-suited to investigate this question, as it targets many transporters and enzymes involved in nutrient uptake and processing.

Consistent with our barcoded competition assay highlighting the importance of ASNS expression under ASNase treatment, ASNS was the top hit of our genetic screen. ASNS is the final step in de novo asparagine synthesis, and as discussed in detail in Chapter 2, several other scoring genes appeared to delineate a particular metabolic route as essential for providing the aspartate substrates used by ASNS to make asparagine. In brief, these genes were SLC1A5, SLC25A12, FH, MDH2, and GOT2. The scoring of these genes suggested that glutamine is taken up by Jurkat cells, metabolized through the forward TCA cycle to produce oxaloacetate, and that oxaloacetate is then converted to aspartate. The results of our genetic screen were intriguing for multiple reasons.

Firstly, many cancer types have been shown to utilize glutamine for filling their TCA cycle (a process termed glutamine anaplerosis) (*115*). Thus, the results of our genetic screen may delineate a pathway towards asparagine used by other cancer types. Secondly, although the aforementioned genes along this pathway all scored strongly,

the second top hit in our screen was actually a gene that does not directly interact with an asparagine precursor. This gene was TPK1, which encodes the kinase that converts the vitamin thiamine into its activated derivative TPP.

TPP is used as a cofactor in several metabolic enzyme complexes, but our screen suggested that its role under ASNase treatment was likely to enable the activity of AKGDH in the TCA cycle. As TPP is derived from thiamine, an essential nutrient that must be obtained from the environment, we became interested in determining whether extracellular availability of thiamine may influence ASNase sensitivity of glutamine-anaplerotic cells. We felt this would be a significant finding, as there are only a few reported cases in which a nutrient affects therapy. Furthermore, it would mean that more than one component of ALL therapy could be influenced by a nutrient obtained through diet. To begin to answer whether thiamine availability affects ASNase response, we first aimed to confirm that maintaining TPP levels through TPK1 is essential for growth under ASNase.

Thiamine, as a vitamin, is a dietary requirement in humans, and its only established physiological role is to become TPP for use as a cofactor (*116*). TPK1 is the only enzyme known to convert thiamine to TPP in humans. Thus, we anticipated that TPK1 would be an essential gene in cultured cells, and to our knowledge, full TPK1 knockouts in a human cell line have never been reported. Intriguingly, TPK1 did not appear to be essential based on the sgRNA readouts of our genetic screen. Abundance of sgRNAs targeting TPK1 did not change in the untreated condition of our genetic screen after several doublings. We hypothesized that this may be due to multiple reasons. Free TPP may have been present in the screening media due to release from dead or live cells. In later experiments, we ruled out that FBS contributed significant TPP in our screens, as even mixed population TPK1 KOs cannot grow in cell culture media supplemented with regular FBS. An additional consideration is that the half-life of TPP in cells may be on the order of several days (*128*). Regardless of its source, if free TPP was present in the media, then TPK1 KOs would need to have a TPP transporter to utilize this source, which to date appears to have only been described in colonocytes (*129*). Overall, the observation of TPK1 appearing non-lethal in our screen was puzzling, but had important implications for interpretation of gene essentiality screens. In large datasets of essentiality screens, it is possible that some metabolic genes will not appear as essential if the product of this gene is present in sufficient quantity in the media due to release from other cells or as a component of the cell culture media itself. To us, the most intriguing aspect of these TPK1 screen results were that a therapeutic window appeared to exist where cells could have enough TPP to proliferate, unless treated with ASNase.

By supplementing TPP in cell culture media, I was able to successfully generate CRISPR/Cas9-mediated clonal knockouts of TPK1 using the Jurkat cell line. We found that loss of TPK1 is indeed lethal but that with sufficient exogenous TPP, TPK1 KO cells could be made to proliferate at the same level as their WT counterparts. Only

nanomolar amounts of TPP were necessary to rescue TPK1 KOs from death, suggesting the presence of a high-affinity TPP transporter in Jurkat cells. Our TPK1 KOs could perhaps be used to identify such a transporter using a secondary genetic screen for genes required for these KOs to proliferate under minimal supplemental TPP. Furthermore, these KOs can be used by others for studying the various metabolic processes dependent on TPP. In our work, we focused on validating that TPP availability enables de novo asparagine synthesis and ASNase resistance.

To perform cell proliferation assays with TPK1 KOs, I found a minimum media concentration of supplemental TPP that would allow for routine culture of these cells, which resulted in similar doubling times as parental cells. Importantly, this minimum concentration allows for plating growth assays in which TPK1 KOs die within 5 days, which is a typical length for a cell proliferation experiment. Thus, these cell culture practices can now be easily used by others to determine the influence of TPP availability on cellular processes of interest. To determine whether TPP availability affects ASNase response, I then established a range of TPP concentrations in which TPK1 KOs would go from death, to mild proliferation, to a proliferation rate that was only mildly growth-limiting relative to the growth of both KO clones in high-dose TPP and parental controls. This range was narrow and on the nanomolar level (0 to 5 nM), demonstrating the potent effect of TPP on the viability of cells, and further illustrating the presence of an unknown high-affinity TPP transporter. Furthermore, this range was near the human plasma range of thiamine (6.6 to 43 nM), which suggested that our experiments within these conditions may have physiological relevance. To validate the results of our ASNase genetic screen, we performed proliferation assays of TPK1 KOs in 0 to 5 nM TPP in the presence and absence of ASNase. Interestingly, when supplemental TPP was limiting for proliferation, cells became significantly sensitized to ASNase. Finally, we confirmed that the effects of TPP limitation on both general growth and ASNase response of KO clones could be rescued by overexpressing a sgRNA-resistant TPK1 cDNA, validating the results of our genetic screen.

Having established that TPP does indeed enable growth under ASNase treatment, we next aimed to test whether this is due to TPP being required for AKGDH activity during utilization of extracellular glutamine to make asparagine. As detailed in Chapter 3, we performed glutamine tracing studies to answer this question. The results of this were consistent with our genetic screen results that suggested a metabolic pathway from extracellular glutamine to intracellular asparagine is essential under asparagine depletion by ASNase. Furthermore, our data pinpointed that the role of TPP during this response to ASNase treatment is indeed to enable the activity of AKGDH. In some cancers, alpha-ketoglutarate derived from glutamine can proceed in the reverse direction of the TCA cycle to make citrate, in a process called reductive carboxylation (*130*). Citrate could then be exported to the cytoplasm to be used to generate oxaloacetate via ACLY, which could then proceed through GOT1 and ASNS to generate asparagine as an adaptation to asparagine depletion. However, in our experiments, labeling suggested that citrate is primarily derived through forward TCA in Jurkat cells.

Furthermore, the minimal M+3 asparagine labeling in our data suggested that Jurkat cells do not generate substantial asparagine through a reverse TCA route. Thus, our data suggests that TPP is essential for the primary metabolic pathway that these cells can utilize to make asparagine from glutamine. This could potentially be the case in other glutamine-anaplerotic cancers as well.

Overall, our studies with TPK1 KOs demonstrated that TPP enables asparagine synthesis under extracellular asparagine depletion, and consequently, that sufficient TPP is needed for maximal ASNase resistance. Supplemental TPP was mildly growth-limiting for TPK1 KOs at extracellular concentrations that were similar to the normal concentration of thiamine in human plasma, and ASNase sensitivity was exacerbated when TPP was limiting. We hypothesized that there may be some cancers for which physiological thiamine levels are limiting, and that these cancers may have increased ASNase sensitivity at these limiting concentrations relative to when in supra-physiological thiamine concentrations like that found in conventional media. To first test whether physiologically-relevant thiamine levels could be growth-limiting for some cancers, we decided to apply our barcoded competition assay technique. Whether a vitamin can be growth-limiting at physiological concentrations, such that excess supplementation could exacerbate its growth, was an interesting concept that appeared to be largely understudied. Thus, we decided to study barcoded cell line responses to not only *in vitro* limitation of thiamine, but also of other vitamins.

Interestingly, we found that in low thiamine, low biotin, and low niacin, subsets of cancer cell lines were particularly sensitive to the vitamin limitation. These cancer types may have defects in vitamin utilization processes such as import, causing them to require greater extracellular availability of a particular vitamin to obtain sufficient intracellular pools for maximal proliferation. Alternatively, it is possible that these cancers have a greater dependence on particular metabolic pathways that require a vitamin-derived cofactor. Overall, this dataset provided a mapping of how several cancer types respond to fluctuating vitamin levels, and demonstrated that some cancers can be more dependent on extracellular vitamin levels than others. Further study of why some cancers have increased dependencies on biotin and niacin is needed, but we next focused on investigating why certain cancers exhibited sub-optimal proliferation when media thiamine was lowered to physiologically-relevant concentrations.

Correlating the barcoded competition assay growth responses in low thiamine with CCLE mRNA expression data of metabolic genes gave thiamine transporter SLC19A2 as a top hit. To further support this data, we also performed a CRISPR/Cas9-based metabolism-focused genetic screen for genes essential for growth in low thiamine. SLC19A2 also scored in this functional screen, suggesting SLC19A2 expression does indeed influence growth capacity at low, physiologically-relevant thiamine levels. Interestingly, previous work had shown that the thiamine transporters SLC19A2 and SLC19A3 were expressed in lower levels in some breast and lung cancers relative to normal tissue counterparts (*13, 131*). Follow-up work showed that breast cancers with

low SLC19A3 may have increased dependence on extracellular thiamine, a dependency that can potentially be exploited by thiamine depletion with the drug thiaminase (*14*). Thiaminase was also found to have a cytotoxic effect in other cancer types, including leukemias, likely due to the general essentiality of thiamine for cell viability (*16, 132*). Interestingly, our analysis of the ~1,000 cell lines in CCLE suggested that SLC19A3 is not expressed in most cancer cell lines. This suggests that SLC19A2 is the primary known thiamine transporter in most cancers. Additionally, our barcoded competition assay and genetic screen approaches both suggested that expression of SLC19A2 is a major determinant of cellular growth under thiamine limitation. Thiaminase-induced depletion can be toxic over time (*14*), but our data suggested that physiologically-relevant thiamine concentrations could already be growth-limiting for some cancers with low SLC19A2. As mildly-limiting TPP availability was sufficient to sensitize TPK1 KOs to ASNase, we next aimed to test whether physiological thiamine could be limiting for both growth and ASNase response of SLC19A2-low cancer cells.

We next identified multiple SLC19A2-low ALL cancer cell lines. Two of the SLC19A2-low cell lines identified, REH and NALM6, were notable as being some of the few ASNase-resistant ALL cell lines in our *in vivo* barcoded competition. We hypothesized that lowering extracellular thiamine to physiological levels could be limiting for these SLC19A2-low cell lines, and would sensitize them to ASNase. Indeed, simply lowering media thiamine ~100-fold from standard RPMI concentration to a more physiological level led to thiamine becoming slightly growth-limiting for REH, which was associated with a greater ASNase response. Furthermore, expressing a SLC19A2 cDNA in REH was sufficient to rescue both the sub-optimal growth and increased ASNase sensitivity seen at lower thiamine. As detailed in Chapter 4, we went on to confirm in a panel of wild-type cell lines that SLC19A2 was in fact a determinant of growth and ASNase response at human plasma thiamine concentrations. Additionally, SLC19A2 was necessary for SLC19A2-high cells to maintain maximal growth and ASNase resistance at physiological thiamine. Of translational importance, we also found that low expression of SLC19A2 was not a phenomenon solely observed in cultured cells. By analyzing human ALL tumor mRNA expression data, we first confirmed that SLC19A3 is largely not expressed in these human tumors, suggesting that SLC19A2 is the primary thiamine transporter in these ALLs. Finally, we confirmed that patient tumors exhibit a range of SLC19A2 expression and that SLC19A2-low tumors do in fact exist, consistent with CCLE cell line data.

We next aimed to test whether extracellular thiamine affects ASNase response of SLC19A2-low cells *in vivo*. To do this, we first had to determine how to modulate circulating plasma thiamine in mice through diet. This posed a challenge, as it is well known that dietary thiamine deficiency is toxic in humans, and thiaminase administration in mice had been shown to have some toxicity over time. However, our cell culture experiments suggested that we did not have to cause a thiamine deficient state to sensitize cancers to ASNase. Instead, our *in vitro* data suggested that SLC19A2-low cells intriguingly already have an inherent ASNase sensitivity at physiological thiamine

levels, which can be inadvertently rescued through excess thiamine supplementation. Thus, we aimed to determine whether physiological human thiamine plasma concentrations are already sufficient to sensitize SLC19A2-low leukemia cells to ASNase in an *in vivo* model. We had observed that SLC19A2-low REH cells were ASNase-resistant when engrafted in mice fed standard chow and when grown in media containing supra-physiological thiamine, but that humanizing thiamine levels *in vitro* sensitized these cells to ASNase. Thus, we hypothesized that standard mouse chow contained supra-physiological thiamine levels and that SLC19A2-low cells would be more sensitive to ASNase if circulating thiamine was humanized. Plasma profiling revealed that standard mouse chow did indeed cause plasma of mice to have orders of magnitude more thiamine than human serum. This suggested that many research facilities may be using standard mouse diets that inadvertently over-supplement thiamine. As thiamine-derived TPP is involved in many metabolic processes, further work is perhaps warranted to determine the effects of such practices on organismal and cellular metabolism.

To determine whether I could humanize plasma thiamine levels of mice, I conducted several experiments using purified ingredients diet pellets that were custom-made to be thiamine-free. While mice groups were fed this diet, I supplied various amounts of thiamine in their water bottles to determine whether I could achieve human plasma thiamine levels without adverse effects. I found that it was indeed possible to achieve a human thiamine plasma level of ~10 nM in mice without causing any weight loss or other observable adverse reactions. The diet used to achieve these plasma levels consisted of thiamine-free purified pellets combined with a low thiamine amount within mouse water bottles, and the amount provided in water could be raised to obtain an appropriate high-thiamine control diet. In the end, I had a Low Thiamine diet that resulted in a circulating thiamine content comparable to that of human serum, and a High Thiamine diet that differed only in water thiamine content. Furthermore, this High Thiamine diet was designed to mimic plasma thiamine levels obtained with standard mouse chow. To our knowledge, no other mouse diets currently exist for providing circulating thiamine at the level of human plasma. This physiological low thiamine diet, and its high thiamine diet control, may be of value to others studying the effects of thiamine availability on biological processes *in vivo*.

After developing these diets that allowed us to modulate plasma thiamine levels, our Low Thiamine and High Thiamine diets were fed to NSG mice. We subsequently administered SLC19A2-low REH cells by tail-vein to these mice to form orthotopic ALL xenografts. Compared to the High Thiamine group that had circulating plasma thiamine levels comparable to that obtained with standard chow, neither lowering dietary thiamine or treatment with ASNase caused a significant effect on survival from leukemia. Remarkably, combining a lower dietary thiamine intake with ASNase treatment did significantly extend survival. Together with our *in vitro* experiments, our results demonstrate that lowering thiamine to human physiological levels in culture media, and in mouse plasma through diet, increases the response of a subset of cancer

cell lines to ASNase, an important chemotherapeutic in the treatment of ALL (Figure 6.1). This subset is defined by low expression of SLC19A2, the primary thiamine transporter in ALL tumor samples.

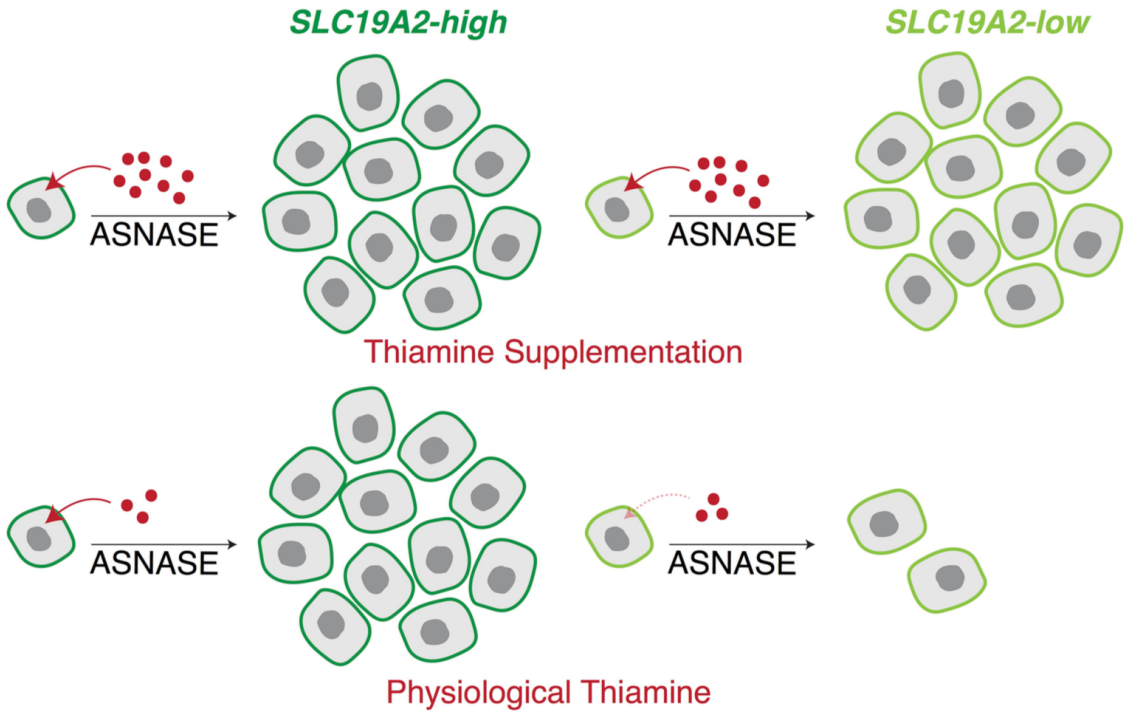


Figure 6.1. Environmental thiamine influences ASNase sensitivity in a subset of leukemia cells with low SLC19A2 expression.

This work raises the possibility that low SLC19A2 expression may be associated with greater patient response to ASNase. Importantly, however, increasing blood thiamine concentrations through excess supplementation could diminish ASNase efficacy in such cancers. More broadly, this thesis provides a proof of principle that humanizing the vitamin levels of both *in vitro* and *in vivo* models can affect therapeutic sensitivity of cancers that have specific vitamin utilization deficiencies.

CHAPTER 7. Future directions and perspectives

7.1 Relevance to vitamin supplementation during cancer treatment

Our results suggest that extracellular thiamine availability influences ASNase response of a subset of leukemia cells. Indeed, 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 used here was equivalent to simply 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, due to 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 (*133*). Thus, biochemical abnormalities, such as lactic acidosis resulting from diminished PDH activity, are among the symptoms observed in patients with nutritional thiamine deficiency (*133-135*).

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 ASNase administration (*136, 137*). Such use in ALL patients should therefore be weighed with the possibility that excess blood thiamine may negatively impact ASNase response of a subset of cancers. However, human studies are needed to further elucidate the effect of excess thiamine on therapeutic response. To determine whether SLC19A2 expression is predictive of responses to ASNase-containing regimens and patient survival, it would be necessary to measure patient plasma thiamine levels during treatment since having supra-physiological levels could affect response. Another possibility is to document vitamin supplementation during clinical trials, which has been done for those with antioxidant properties like vitamin E, and their use is now discouraged while undergoing chemotherapy and radiotherapy for various cancers (*138-140*). Collectively, our work adds to the growing list of studies available that probe whether excess vitamin supplementation may be detrimental during chemotherapy (*141*). Unfortunately, the lack of clinical reports on this topic has translated to non-uniform physician advice with regards to taking vitamin regimens during cancer treatment (*142, 143*).

7.2 Implications for vitamin use in pre-clinical *in vitro* and *in vivo* studies

Our work emphasizes the need to better recapitulate tumor nutrient environment *in vitro*. Conventional cell culture media are complex, containing both proteinogenic and non-proteinogenic amino acids, important polar metabolites such as glucose and glutathione, inorganic salts such as NaCl, trace elements, and of course, essential

vitamins. Recently, custom media have been designed to contain physiologically-relevant concentrations of these components. However, it is important to note some of the limitations of these physiological media, and what can be done to better simulate nutrient environments in the future.

Physiological environments, such as that of blood plasma, contain many more metabolites than that found in synthetic culture media. Thus, quite reasonably, recent reports of plasma-like media focused on manipulating only a portion of the metabolome found in human plasma. This is partly due to the fact that nutrients in cell culture systems are provided not only through the synthetic media itself, but also through the supplementation of media with various percentages of fetal bovine serum (FBS). FBS provides compounds essential for cell proliferation that are not present in chemically defined media, such as lipids and additional micronutrients. FBS can also be treated to yield variations with distinct metabolite profiles such as lipid-depleted FBS obtained by charcoal-stripping, or small-molecule-depleted FBS resulting from extensive dialysis (as used in this work). To date, cell culture media designed to mimic human plasma have been supplemented with either a low percentage (2.5%) of regular FBS, or a conventional percentage (10%) of dialyzed FBS (*9, 144*). These FBS practices contribute low amounts of the small molecule metabolites that are already present at defined, desired concentrations in the synthetic media. Thus, the final complete media that results does indeed contain human plasma-like levels of the metabolites considered in the synthetic media design. However, it remains unclear how the final media concentrations of other metabolites, such as lipids, compare to that of human plasma. Additionally, these recent plasma-like media studies did not attempt to mimic the vitamin concentrations of human plasma, and vitamin levels in these media remain at the supra-physiological concentrations found in conventional media. To contrast, the work described here only manipulated cell culture media so that it contained human plasma levels of thiamine, and the levels of other vitamins and metabolites in our experiments remained at conventional cell culture concentrations. However, manipulating the levels of thiamine alone caused significant changes in leukemia cell responses to an important therapeutic, demonstrating a need for considering vitamins during physiological media design. Future development of plasma-like media should improve upon the important recent variations, by attempting to humanize the levels of additional metabolites such as lipids and vitamins. Such media formulations may prove valuable for studying the biology and therapeutic responses of blood cancers *in vitro*.

Different tissues may provide metabolically distinct environments in the body, and plasma-like media may not be suitable for the study of all cell types and cancers. Thus, it may be useful to consider designing custom media on a case-by-case basis, so as to best recapitulate the relevant tissue environment. This concept was exemplified by a recent study that developed a cell culture medium that mimics the metabolic environment of the brain, which can be used for studying neuronal biology *in vitro* (*145*). However, it is important to note that the metabolic environments of tumors can differ from that of blood and normal tissues (*146*). Thus, if it is possible to characterize a

tumor environment of interest, then it may be useful to do *in vitro* studies of this cancer type in a medium that metabolically mimics the native tumor environment.

In vivo nutrient environments, particularly those experienced by cancer cells in orthotopic xenografts and autochthonous tumors, may be thought of as more physiologically-relevant than that of cell culture systems. Importantly, there has been an abundance of recent evidence demonstrating that manipulation of these nutrient environments through diet can influence tumor growth (*1, 5*). In our work, we have demonstrated that conventional mouse chow can lead to supra-physiological levels of a vitamin in mouse plasma. Thus, it is possible that other *in vivo* cancer studies are currently utilizing animal diets that cause non-physiological levels of metabolites to circulate in blood, and as a result, through tumor microenvironments. Furthermore, we showed that bringing the plasma level of thiamine down to normal human levels in mice affected response of orthotopic leukemia xenografts to an established therapeutic. It remains to be determined whether humanizing the plasma levels of other nutrients in mouse cancer models could affect tumor growth or response to anti-cancer therapy, but our results suggest such studies are warranted.

7.3 Further characterization of cancer nutrient dependencies is warranted

Recently, compounds have been designed for plasma depletion of amino acids other than asparagine. These include an arginase, a cyst(e)inase, and a methioninase (*147-149*). However, these compounds have only been tested on a subset of cancer models. Custom diets have also been used to deplete certain amino acids *in vivo*, such as serine and glycine (*112*). These dietary depletions have slowed tumor growth in some cancer models, but also remain unstudied in many cancers. The techniques used in this thesis, particularly barcode-based cell competition assays, can be used for a systematic mapping of the responses of several cancers to various nutrient depletions. Although we conducted some amino acid and vitamin deprivation studies in our work, it would be interesting to see how barcoded libraries consisting of other cancer types may fare under the conditions studied here or in other nutrient depletions. This would, at the very least, pinpoint cancers that could be therapeutically targeted with recently described compounds and dietary interventions.

Identifying cancers that are resistant and sensitive to particular nutrient depletions can also lead us towards pinpointing biological pathways that are essential for maintaining intracellular nutrient levels in specific cancer subtypes. This can lead to additional translational findings, such as alternative ways to target cancers with a particular nutrient dependency besides simply limiting dietary intake of the nutrient or depleting it from plasma with an enzyme. For instance, mapping cell line responses to cholesterol limitation identified a subset of cancers that cannot synthesize cholesterol and depend on its uptake from the environment for proliferation (*125*). Thus, the LDLR gene required for cholesterol uptake was found to be a potential therapeutic target in this subset of cancers.

In this work, we investigated the responses of several barcoded cancer cell lines, primarily of blood and lymphoid origin, to depletion of non-essential amino acids and vitamins. However, further work is needed to validate whether some of the cancer subsets in our assays may be targetable with a nutrient-depleting drug or dietary intervention. It will also be interesting to investigate the genetic determinants of growth under the nutrient limitations that we have not yet followed up. In particular, future work on vitamin depletions is warranted, as vitamins are relatively understudied in the cancer metabolism field. Further work could determine whether subsets of cancers that proliferate less under particular vitamin limitations may have targetable vitamin utilization deficiencies, as exemplified by SLC19A2-low cells being targetable with ASNase under physiological thiamine concentrations.

CHAPTER 8. Materials and Methods

8.1 Experimental design

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-anaplerotic 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 the thiamine transporter SLC19A2, and (iv) orthotopically engrafted leukemia cells with endogenously-low SLC19A2 in NSG mice to show that dietary thiamine can influence ASNase sensitivity in leukemia.

8.2 Compounds, Cell lines, Cell Culture, and Constructs

Antibodies to GAPDH (GTX627408) were from GeneTex, and to TPK1 from Proteintech (10942-1-AP). Conjugated Donkey Anti-Mouse IR-Dye 680LT and Donkey Anti-Rabbit IR-Dye 800CW were from LI-COR Biosciences.

Asparagine, TPP, polybrene, and puromycin were from Sigma; blasticidin from Invivogen; D-glucose from VWR; L-glutamine from Gibco; thiamine hydrochloride from MP Biomedicals; *E. coli* L-asparaginase from BioVendor.

For glutamine tracing, a powder base media 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-^{13}C]-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-^{13}C-thiazol-5-yl-$^{13}C_3$*) hydrochloride ("^{13}C-thiamine", 731188, Sigma) was added to cells in Hank's Balanced Salt Solution containing calcium chloride and magnesium chloride ("HBSS", 24020-117, Gibco). In tracing, uptake, and plasma profiling experiments, extraction solvents contained ^{15}N and ^{13}C fully-labeled internal amino acid standards (MSK-A2-1.2, CIL) for normalization.

All human cell lines used were originally purchased from ATCC or DSMZ, or obtained from the Sabatini and Weinberg labs (Whitehead Institute). Cell lines were verified to be free of mycoplasma, and authenticated by STR profiling. Cell lines were typically maintained in "standard RPMI": RPMI-1640 medium containing 2 mM glutamine (Gibco), 10% fetal bovine serum ("FBS", Sigma), and penicillin-streptomycin (Gibco), at

37 C and 5% CO_2, unless otherwise noted. For all experiments, cells were counted with a Beckman Z2 Coulter Counter.

The DNA-barcoded cell line competition assay and CRISPR-based screen done in low thiamine media used thiamine-free RPMIs supplemented with 10% dialyzed FBS ("dialyzed FBS", 26400044, Gibco). All thiamine-free RPMIs were made with glucose and glutamine at standard RPMI concentrations (11 and 2 mM, respectively). For the competition assay, a powder base media of RPMI without vitamins (US Biological) was supplemented with all individual vitamins (except for thiamine) at RPMI concentrations. The CRISPR screen used a different powder base media of RPMI made without thiamine (R9011-01, US Biological), which was used for all other experiments that utilized thiamine-free RPMI.

For manipulating thiamine levels in the REH EV and SLC19A2 OE experiments, thiamine-free RPMI was combined with 10% non-dialyzed FBS. For achieving nanomolar thiamine levels that approximated physiological thiamine, thiamine-free RPMI was combined with 10% "double-dialyzed FBS", obtained by further in-house dialysis of Gibco's dialyzed FBS. In-house dialysis was performed at 4 C against a 20X volume of PBS, using regenerated cellulose dialysis tubing (21-152-9, Fisherbrand), with a PBS change done after at least 8 hours of stirring, such that 4 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. Both of these vectors were linearized with BsmBI (New England BioLabs), and non-linearized vectors were used for Vector Controls. sgRNA-resistant TPK1, or SLC19A2, cDNA gene blocks were cloned into pMXs-IRES-blast by Gibson assembly, and uncut vector used as Vector Control.

Guide 5 in CRISPR/Cas9 screen, used to generate clonal KO1 and KO2 in proliferation assays:
sgTPK1_5F, 5'-caccGGTGATATCATATAAGCGGT-3';
sgTPK1_5R, 5'-aaacACCGCTTATATGATATCACC-3'.

Guide 3 in CRISPR/Cas9 screen, referred to as sgSLC19A2_1 in proliferation assays:
sgSLC19A2_3F, 5'-caccGAGGCTGGCGAAGAAGCCGT-3';
sgSLC19A2_3R, 5'-aaacACGGCTTCTTCGCCAGCCTC-3'.

Guide 4 in CRISPR/Cas9 screen, referred to as sgSLC19A2_2 in proliferation assays:
sgSLC19A2_4F, 5'-caccGGACAAGAACCTGACCGAGA-3';
sgSLC19A2_4R, 5'-aaacTCTCGGTCAGGTTCTTGTCC-3'.

8.3 Generation of Knockout and cDNA Overexpression Cell Lines

Lentiviral sgTPK1 and sgSLC19A2 vectors generated as described above were transfected into HEK293T cells with lentiviral packaging vectors VSV-G and Delta-VPR. 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 h, cells were given fresh media. 48 hours after transfection, virus-containing media supernatants were filtered through a 0.45 μm filter to eliminate cells. Cells to be transduced were plated in 6-well tissue culture plates with appropriate virus and 8 mg/ml of polybrene. Cells were then spin-infected by plate centrifugation at 1,100 g for 1.5 h at 32 C. After 24 h, cells were given fresh media. At least 48 h after infection, transduced cells were selected with puromycin, blasticidin, or by bulk-sorting on a BD FACSAria (same gating for all samples, resulting in top 3-6% of RFP^+ cells). Clonal TPK1-knockout 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 knockout by immunoblotting. TPK1 KOs were then maintained in standard RPMI containing 20 nM TPP, as were the relevant Vector Control and cDNA-add-back controls, for at least 1 week prior to proliferation assays. Mixed population cells transduced with sgSLC19A2s or Vector Control were maintained in standard RPMI, as this already over-supplements thiamine at 3 μM. Of note, 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.

8.4 Immunoblotting

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

8.5 RNA Extraction, Reverse Transcription, and Real-time Quantitative PCR

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 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'-TTGGTATCGTGGAAGGACTC-3'
GAPDH reverse, 5'-ACAGTCTTCTGGGTGGCAGT-3'
SLC19A2 forward, 5'-GGCCGGACAAGAACCTGAC-3'
SLC19A2 reverse, 5'-ACACAGGAAACAGTAGCACCA-3'

8.6 Proliferation Assays

Cells were plated in 96-well plates at 1,000 cells per well in triplicate in a final volume of 0.2 ml in indicated medias and treatments. After 5-9 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 media for an initial luminescence time point for normalization. For each well, fold change in luminescence was calculated and reported on a $\log_2$ 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 h, without affecting glutamine levels (*150*). In assays that required achieving low thiamine levels, cells were first pre-incubated for 5-9 days in the same low thiamine base media that was subsequently used for plating proliferation assays. Proliferation assays were plated multiple times within this pre-incubation period with similarly reproduced results.

8.7 Mouse studies

Animal studies were conducted according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) 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-barcoded competition assays were initiated by injecting 1.5 million of the pooled cells in 100 μl of DMEM (Gibco) with 30% Matrigel (Corning), once on each mouse flank. These mice were fed 5B1Q (TestDiet), a chow-based diet. Leukemia xenografts were initiated by injecting 750,000 REH cells in 100 μl PBS through the tail vein. 2 weeks prior to tail-vein injections, these mice were switched from 5B1Q to a purified diet of thiamine-deficient AIN-93G pellets (Dyets, Inc.) coupled with high or low thiamine supplementation in water as described below. 1 week prior to tail-veins, REH cells were switched from standard RPMI to a low thiamine media (thiamine-free RPMI + dialyzed FBS) to remove excess thiamine from cells. To determine survival after leukemia engraftment, weights were first checked weekly. After the first signs of progressive disease, mice were monitored daily for deaths or signs of endpoint, and weights taken at a minimum of twice weekly. Humane endpoints were 1) 20% weight loss from initial weight, and 2) other obvious signs of disease such as hind limb dysfunction or significant distress.

Low Thiamine groups were maintained by combining thiamine-deficient AIN-93G pellets with water bottles containing 5 μM thiamine. High Thiamine groups were maintained by combining the same thiamine-deficient pellets with 125 μM thiamine in water. Mice were treated with L-asparaginase (1000 U/kg) daily for cell competition assays (2 weeks), and twice weekly for leukemia xenograft experiments (~4 weeks). For all plasma profiling, whole blood was collected using EDTA-coated capillaries or tubes by tail clip (except for the 39d Low Thiamine time-point and the Chow vs High Thiamine data of Figure 5.2, for which submandibular bleeds were done), then centrifuged at 20,000 g for 5 min at 4 C to yield the plasma supernatants used for LC-MS analysis.

8.8 Metabolite Profiling: Isotope Tracing, Isotope Uptake, Plasma Profiling

For glutamine tracing, the 3 cell types were first taken from their maintenance media (20 nM TPP), and washed twice with PBS. Cells were then plated at 500,000 cells/ml in media containing 2.5 nM TPP, for ~1 day, to begin TPP depletion. To begin the experiment, cells were washed again then plated in triplicate per condition in 6-well plates at 1 million cells/ml, in a custom RPMI (described above) containing no asparagine, no TPP, and 2 mM [U-^{13}C]-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 h, 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 stored overnight at -80 C, then nitrogen-dried. Dried extracts were kept at -80 C until liquid chromatography / mass spectrometry ("LC/MS") analysis. Dried samples were resuspended in 100 μl 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 3 cell types were first taken from their maintenance in standard RPMI and washed twice with PBS. 4 million cells were then plated in triplicate per condition in 6-well plates at 1 million cells/ml in warm HBSS. ^{13}C-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 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. 5 μl plasma was combined with either 45 μl solvent for the *in vivo* competition assay data, or with 20 μl

solvent for custom thiamine diet experiments (for quantifying low abundance thiamine in plasma). This was followed by vortexing for 5 min at 4 C, 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 h (samples were never dried). 2 μl (for competition assay) or 5 μl (for custom thiamine diet experiments) were 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 HESI II probe, which was coupled to a Vanquish UPLC system (Thermo Fisher Scientific). External mass calibration was performed using the standard calibration mixture every 4-6 days. Samples were injected onto a ZIC-pHILIC 150 × 2.1 mm (5 μm particle size) column (EMD Millipore). Chromatographic separation was achieved using the following conditions: buffer A was 20 mM ammonium carbonate, 0.1% ammonium hydroxide; buffer B was acetonitrile. The pH for buffer was adjusted to 9.3 using formic acid. The column oven and autosampler tray were held at 25 C and 4 C, respectively. The chromatographic gradient was run at a flow rate of 0.150 ml min^{-1} as follows: 0–22 min: gradient from 90% to 40% B; 22-24 min: held at 40% B; 24–24.1 min: return to 90% B; 24.1–30 min: hold at 90% B. The mass spectrometer was operated in full-scan, polarity switching mode with the spray voltage set to 3.0 kV, the heated capillary held at 275 C, and the HESI probe held at 250 C. The sheath gas flow was set to 40 units, the auxiliary gas flow was set to 15 units. The MS data acquisition was performed in a range of 55–825 *m*/*z*, with the resolution set at 70,000, the AGC target at 10×10^6, and the maximum injection time at 80 msec. Relative quantitation of polar metabolites was performed with Skyline Daily from MacCoss Lab Software (*151*), using a 2 ppm mass tolerance. For all experiments, total signal for a metabolite was first quantified in Skyline, in which metabolite identification was based on mass, retention time, and the signal observed for an in-house chemical standard of the metabolite. The chemical standard was present in a separate injection of the same LC/MS run. For glutamine tracing data, natural abundance corrections were not performed in Skyline, as the primary mass isotopologues of interest were M+4 and M+5 species, which were deemed to have negligible natural abundance for the metabolites analyzed. For instance, naturally-occurring asparagine is ~0.001% M+4 asparagine and naturally-occurring glutamate is ~0.0001% M+5 glutamate. For normalization, raw signal of each metabolite was first normalized to the appropriate labeled internal AA 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 BCA protein quantification (per condition) for glutamine tracing, by cell counting for thiamine uptake, and by plasma/serum volume as appropriate.

8.9 CRISPR/Cas9-based genetic screens

The metabolism-focused sgRNA library preparation, and details on performing screens, were previously described (*152-154*). 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 vs. 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 cut-off 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 vs. control to give the differential gene score graphs presented. Full screen results are available as supplementary data.

8.10 DNA-barcoded cell line competition assays

DNA-barcoded cell lines were generated, and competition assays performed, as previously described (*3, 125*). In brief, three unique 7-bp sequences were transduced into each cancer cell line using lentiviruses generated with pLKO.1-puro vector, such that each cell line had 3 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 endpoint, 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 presented as detailed in appropriate figures.

8.11 Patient tumor and cell line RNA sequencing

Patient RNAseq data for SLC19A2, SLC19A3, TPK1, and ASNS were generated by the Therapeutically Applicable Research to Generate Effective Treatments (https://ocg.cancer.gov/programs/target) initiative, phs000218. These data were part of the Acute Lymphoblastic Leukemia (ALL) Expansion Phase 2 TARGET Sub-study, phs000464, and were downloaded from the TARGET Data Matrix (https://ocg.cancer.gov/programs/target/data-matrix) on December 10, 2019. All files used were in the BCCA subdirectory and tumor sample details are indicated within the relevant figures. Cancer cell line RNAseq data presented throughout this study were obtained from the CCLE (https://portals.broadinstitute.org/ccle/data), accessed on January 27, 2020 (file version: CCLE_RNAseq_rsem_genes_tpm_20180929.txt.gz) (*155*).

8.12 Statistical Analysis

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

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R. Guarecuco, R. T. Williams, L. Baudrier, K. La, M. C. Passarelli, N. Ekizoglu, M. Mestanoglu, H. Alwaseem, B. Rostandy, J. Fidelin, J. Garcia-Bermudez, H. Molina, K. Birsoy, Dietary thiamine influences L-asparaginase sensitivity in a subset of leukemia cells. Sci Adv, in press (2020).

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Other contributions to publications:

R. T. Williams, **R. Guarecuco**, L. A. Gates, D. Barrows, M. C. Passarelli, B. Carey, L. Baudrier, S. Jeewajee, K. La, B. Prizer, S. Malik, J. Garcia-Bermudez, X. G. Zhu, J. Cantor, H. Molina, T. Carroll, R. G. Roeder, O. Abdel-Wahab, C. D. Allis, K. Birsoy, ZBTB1 Regulates Asparagine Synthesis and Leukemia Cell Response to L-Asparaginase. Cell Metab 31, 852-861 e856 (2020).

J. Garcia-Bermudez, L. Baudrier, E. C. Bayraktar, Y. Shen, K. La, **R. Guarecuco**, B. Yucel, D. Fiore, B. Tavora, E. Freinkman, S. H. Chan, C. Lewis, W. Min, G. Inghirami, D. M. Sabatini, K. Birsoy, Squalene accumulation in cholesterol auxotrophic lymphomas prevents oxidative cell death. Nature 567, 118-122 (2019).

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