ABSTRACT

The LKB1/STK11 tumor suppressor encodes a serine/threonine kinase, which coordinates cell growth, polarity, motility, and metabolism. In non-small cell lung carcinoma, LKB1 is somatically inactivated in 25% to 30% of cases, often concurrently with activating KRAS mutations. Here, we used an integrative approach to define novel therapeutic targets in KRAS-driven LKB1-mutant lung cancers. High-throughput RNA interference screens in lung cancer cell lines from genetically engineered mouse models driven by activated KRAS with or without coincident Lkb1 deletion led to the identification of Dtymk, encoding deoxythymidylate kinase (DTYMK), which catalyzes dTTP biosynthesis, as synthetically lethal with Lkb1 deficiency in mouse and human lung cancer lines. Global metabolite profiling showed that Lkb1-null cells had a striking decrease in multiple nucleotide metabolites as compared with the Lkb1–wild-type cells. Thus, LKB1-mutant lung cancers have deficits in nucleotide metabolism that confer hypersensitivity to DTYMK inhibition, suggesting that DTYMK is a potential therapeutic target in this aggressive subset of tumors.

SIGNIFICANCE: Using cell lines derived from the lung cancers occurring in genetically engineered mice, we conducted an integrative genome-wide short hairpin RNA and metabolite screen to identify DTYMK as a potential therapeutic target in Kras/Lkb1–mutant lung cancer. We believe that DTYMK is tractable for the development of novel therapeutics, and show an integrative approach to target identification that reduces false-positive candidates and should have broad applicability for the development of targeted therapeutics. Cancer Discov; 3(8): 1–10. © 2013 AACR.

See related commentary by Marcus and Khuri, p. 843.
Identifying Lkb1-Mutant Synthetic Lethal Targets

INTRODUCTION

LKB1/STK11 functions as a master regulator of cell metabolism and energy stress responses (1, 2). Its best-characterized target is AMP-activated protein kinase (AMPK), which is directly phosphorylated and activated by LKB1 in the context of low cellular ATP levels (2). AMPK in turn modulates nutrient use to restore energy homeostasis through phosphorylation of multiple substrates controlling nutrient uptake and metabolism (1, 2). LKB1 also activates other members of the AMPK-related family of kinases, which regulate diverse aspects of cell metabolism, growth, and polarity (1, 2). LKB1/STK11 deficiency results in broad defects in metabolic control, as evidenced by primary cells and cancer cell lines lacking LKB1 being sensitized to nutrient deprivation and other types of metabolic stress (3–5). LKB1 is also a major tumor suppressor gene that is somatically inactivated in many common types of cancer (3, 4). Human tumor data and genetic studies in mice suggest that LKB1-mutant cancers are biologically distinct from those with LKB1 intact (6). Notably, LKB1 inactivation is the single most prominent biomarker for poor outcome in cervical cancer, predicting survival of 1 year, as compared with 10-year survival for LKB1 wild-type (WT) tumors (7). In mouse models of lung cancer and melanoma, Lkb1 loss synergizes with active KRAS to drive a highly metastatic phenotype not seen in the context of other combinations of mutations (6, 8).

Unfortunately, there are currently few drugs available for clinical use that target LKB1 loss specifically, and recent human cancer cell line screens using more than 130 drugs under clinical and preclinical investigation failed to identify known cancer cell line screens using more than 130 drugs under clinical and preclinical investigation failed to identify known anticancer agents with strong selective activity in this subset of tumors (data not shown; ref. 9).

Here, we sought to use an integrative program to systematically identify novel drug targets in LKB1-mutant lung cancer using a synthetic lethal RNA interference (RNAi) screen and comprehensive metabolomics analysis. For these studies, we took advantage of a series of low-passage lung cancer cell lines derived from genetically engineered mouse models (GEMM) programmed with common mutations in Kras and Trp53, alone or in combination with Lkb1. Although the heterogeneity of human cancer cell lines can obscure synthetic lethal associations, we predicted that this murine cell line panel, developed in the context of a well-defined model system, would effectively enable the discovery of genotype-driven sensitivities.

RESULTS

Generation of Lung Cancer Cell Lines from GEMMs

To generate isogenic lung cancer cell lines, somatic KRAS activation and Trp53 loss with or without Lkb1 inactivation were induced in the lungs of genetically engineered mice (KrasLSL-G12D/Trp53+/− or KrasLSL-G12D/Trp53+/−; Lkb1+/−) by intranasal administration of Adenovirus-Cre as previously described (6). Inactivation of Trp53 was included in these models, as inactivation of Trp53 is common in human nonsmall cell lung carcinoma (NSCLC; >50%; ref. 10). Tumor nodules from mice of defined genotypes were dissected, minced, and cultured, resulting in the derivation of the 634, 855, and 857 lines from KrasLSL-G12D/Trp53+/− mice (Lkb1-WT) and the t2, t4, and t5 lines from KrasLSL-G12D/Trp53+/−; Lkb1−/− mice (Lkb1-null; Supplementary Fig. S1A). Genotype, LKB1 expression, and epithelial origin of the lines were confirmed by PCR, Western blot analysis, and pan-cytokeratin immunostaining (Supplementary Fig. S1B–S1D). These six lines showed similar growth rates (Supplementary Fig. S1E), and the Lkb1-null lines exhibited lower cellular ATP levels compared with Lkb1-WT cells (Supplementary Fig. S1F).

Identification of Selective Essential Genes in Kras/Trp53/Lkb1 GEMM-Derived Cell Lines

To identify genes that induce cell death selectively in Lkb1-null lung cancers, a synthetic lethal screen was conducted using a pooled 40K murine short hairpin RNA (shRNA) lentiviral library with each of the Lkb1-WT and Lkb1-null cell lines. The relative abundance of shRNAs in each cell line sample was determined by deep-sequencing, and for every shRNA, a log2 fold change (log2FC) value was calculated from the difference in relative abundance at a late time point after infection versus the initial shRNA-infected sample. Unsupervised hierarchical clustering analysis of the ranked hairpins from the triplicate pooled shRNA library screens revealed clear clustering of the Lkb1-WT and Lkb1-null cells into distinct groups, and the blue color in the top right corner represents genes for which the abundance of shRNAs is significantly reduced in all Lkb1-null cultures, suggesting a specific effect in the inhibition of Lkb1-null cell growth (Fig. 1A). We collapsed the ranked hairpins using two methods, a RIGER analysis (Kolmogorov–Smirnov test–based statistics) and a weighted second-best analysis to rank genes that selectively impaired proliferation/viability in Lkb1-null cells. We nominated a union of 344 genes, identified by the top 100 individual hairpins for 88 genes (Supplementary Table S1.1) and the top 200 genes from both the Kolmogorov–Smirnov (Supplementary Table S1.2) and weighted second-best analysis (Supplementary Table S1.3), as our initial prioritized list (Fig. 1B). Of note, 340 shRNAs, targeting 70 candidate genes from this prioritized list, were chosen for validation (Supplementary Table S1.4). These 70 genes consisted of the top 10 candidates from the Kolmogorov–Smirnov analysis, as well as 60 others involved in a range of biologic processes in an attempt to represent all biologic categories in the validation process. Validation was conducted in an array format and identified 13 genes that displayed two or more hairpins with a significant growth disadvantage in the Lkb1-null cells (Supplementary Table S1.5). Deoxothymydylate kinase (D tymk), Chek1, Pbb1, and Cmgp1 were the top four candidates, each with two or more hairpins that scored in the validation assay (Fig. 1C and Supplementary Table S1.5).

Metabolomics Analysis Implicates D tymk as a Critical Gene in Lkb1-Null Cells

LKB1 is reported to be involved in metabolic reprogramming (4, 11); therefore, we assessed the metabolic profile of Lkb1-WT and Lkb1-null cells and discovered a set of 58 metabolites, including the nucleotide metabolites IMP, AMP, ADP, GMP, dGMP, UMP, UDP, CDP, dCDP, and dTDP, which were present at consistently lower levels in Lkb1-null cells (Fig. 1D). Pathway enrichment analysis showed that metabolites in both purine and pyrimidine metabolism were significantly reduced in Lkb1-null compared with Lkb1-WT cells (Fig. 1D; P = 3.5 × 10−7 and 3.4 × 10−3, respectively), including multiple metabolites involved in dTTP synthesis, such as dTDP.
Figure 1. Identifying Dtymk. A, unsupervised hierarchical clustering analysis of results from triplicate pooled shRNA library screens of Lkb1-WT and Lkb1-null mouse cancer cell lines based on log.FC. Negative numbers (blue) reflect relative depletion of shRNAs at late time points. B, two-class comparison of Lkb1-null versus Lkb1-WT cell lines was used to generate a ranked hairpin list of selectively essential hairpins in an Lkb1-null background. Hairpins were collapsed to gene values using either the weighted second-best or the Kolmogorov–Smirnov (KS) statistic in GENE-E. Venn diagram depicts the overlap of most essential genes in the Lkb1-null background nominated by the top 100 independent hairpins, and the top 200 genes from both weighted second-best and KS. C, validation study. Relative viability of Lkb1-WT and Lkb1-null cells infected with 340 individual hairpins for 5 days. Genes of interest are highlighted by the colors indicated. D, metabolic signature of Lkb1-null lung cancer cells. Unsupervised clustering analysis of metabolomic data from Lkb1-WT and Lkb1-null cells. The heatmap displays those metabolites with the greatest difference between Lkb1-WT and Lkb1-null cell lines, along with compound name (ID), description (KEGG identification number), and P value, etc., for comparison between the two sets of lines. Bottom, significantly enriched metabolic pathways in downregulated components of the Lkb1-null metabolic signature using the pathway analysis module from the MetaboAnalyst tool (http://www.metaboanalyst.ca). E, a comprehensive metabolic map of the de novo (solid line) and the salvage (dashed line) pyrimidine deoxyribonucleotide biosynthetic pathway. This map was created with CellDesigner version 4.2 using a template from the Panther Classification System Database (www.pantherdb.org). DTYNMK is highlighted in bold. Metabolites depicted in light blue were significantly downregulated in Lkb1-null cells.
Identifying Lkb1-Mutant Synthetic Lethal Targets

Dtymk Is a Synthetic Lethal Gene Selectively Required for Lkb1-Null Cell Proliferation

To further examine the role of DTYMK in lung tumorigenesis, we screened five shDtymk and identified shDtymk-1 and shDtymk-3, which knocked down DTYMK to nearly undetectable levels (Supplementary Fig. S2A and Supplementary Table S2). Compared with shGFP, both shDtymk-1 and shDtymk-3 strongly inhibited the growth of the Lkb1-null cells (t2, t4, and t5), while producing a weaker effect in the Lkb1-WT (634, 855, and 857) cell lines (Fig. 2A and Supplementary Fig. S2B). To see if overexpression of shRNA-resistant Dtymk can rescue the shDtymk effect, Dtymk-R1 and Dtymk-R3 were cloned into the pLenti6 vector and then transduced into Lkb1-null t4 cells. Blasticidin-resistant cells were pooled and further transduced with shGFP, shDtymk-1, or shDtymk-3, respectively. Consistently, shDtymk-1 and shDtymk-3 killed Lkb1-null t4 cells within 3 days, whereas Dtymk-R1 and Dtymk-R3 expression largely restored the growth of shDtymk-1- and shDtymk-3-transduced t4 cells (Fig. 2B). Western blot analysis revealed lower DTYMK signals in t4-Dtymk-R1/shDtymk-1 and t4-Dtymk-R3/shDtymk-3 cells, suggesting that some of the blasticidin-resistant cells were not DTYMK-R–positive and thus were killed by shDtymk (Fig. 2B), which likely accounted for the significant but incomplete rescue by Dtymk-R1 or Dtymk-R3. To extend these findings to tumorigenesis in vivo, Lkb1-WT (634 and 857) and Lkb1-null (t2 and t4) cells were transduced with doxycycline-inducible (TetOn) shGFP or shDtymk and then implanted into athymic nude mice. Consistent with the in vitro proliferation assay, doxycycline-induced expression of shDtymk-3 for 3 weeks resulted in a marked impairment in the growth of Lkb1-null tumors while producing more modest effects in the Lkb1-WT tumors (Fig. 2C).

Dtymk Knockdown Alters Pyrimidine Metabolism

DTYMK catalyzes the phosphorylation of dTMP to form dTDP, and it is the first merged step of both the de novo and salvage pathways in the production of dTTP. We expected that knockdown of Dtymk would inhibit this pathway and lead to accumulation of the substrate dTMP and a decrease in the product dTTP. Corresponding metabolite analysis of Lkb1-WT 634 and Lkb1-null t4 cells transduced with shDtymk-1 revealed the expected significant increase in dTMP and moderate decrease in dTTP levels in both cell lines (Fig. 2D), indicating that DTYMK is a major source of dTTP in the cells and underscores the importance of this gene in cancer cell proliferation, as dTTP is required for production of dTTP for DNA synthesis.

dTTP Rescues the shDtymk Growth Phenotype

To investigate whether adding dTTP to the medium can rescue shDtymk-induced cell death, Lkb1-WT 634 and Lkb1-null t4 cell lines were transduced with shGFP, shDtymk-1, or shDtymk-3 and cultured in the presence or absence of 100 μmol/L dTTP for 4 days (14). Consistently, shDtymk-1 and shDtymk-3 killed more Lkb1-null t4 cells than Lkb1-WT 634 cells, but not the cells cultured in medium containing exogenous dTTP (Fig. 2E; confirmation of Dtymk knockdown and incorporation of the exogenous dTTP into DNA are shown in Supplementary Fig. S3A and S3B). Collectively, our data indicate that dNTP metabolism is impaired in Lkb1-deficient lung cancer cells, and that targeting Dtymk is synthetically lethal in this setting.

Lkb1-Null Cells Are More Prone to DNA Damage than Lkb1-WT Cells

Knockdown of Dtymk will consequently reduce dTTP but also increase dUTP levels. Such changes have been previously linked to dUTP misincorporation and DNA damage, when high expression levels of ribonucleotide reductase R2 subunit activate nucleotide excision repair (15–18). We noted that, although Lkb1-null and Lkb1-WT cells have similar R2 expression, Lkb1-null cells have much lower DTYMK expression (Fig. 3A), potentially creating a cellular state favorable for dUTP misincorporation. Supportively, Lkb1-null cells have a large 4N peak in DNA content (Fig. 3B), are more sensitive to CHEK1 inhibition (Fig. 3C and Supplementary Fig. S4A), and have slightly increased basal phospho-CHEK1 (Fig. 3A), consistent with the activation of a G2 DNA damage checkpoint during replication in Lkb1-null cells (19–22). This pathway seems relevant in vivo. Lkb1-null tumors exhibited increased γHAX and phospho-CHEK1 signals as compared with Lkb1-WT tumors (Supplementary Fig. S5A–S5C), suggesting there is more DNA damage in vivo than under in vitro culture conditions. In line with this evidence for more DNA damage in Lkb1-null cells, it is notable that Chek1 ranked second in our screen (Fig. 1C and Supplementary Table S1.5), suggesting a dependence of Lkb1-null cell survival on CHEK1 function. Knockdown of Dtymk over a short period (i.e., 2.5 days after shDtymk-transduction) resulted in comparable increases in the phosphorylation of CHEK1 and H2AX in both cell types, whereas the phosphorylation of replication protein A 32 kDa subunit (RPA32) was much more pronounced in Lkb1-null cells (Fig. 3A), suggesting more DNA damage and elevation in nucleotide excision repair in Lkb1-null cells (23). Interestingly, the expression of total RPA32 was increased in Lkb1-WT cells (Fig. 3A), suggesting that LKB1 may positively regulate RPA32 expression following Dtymk knockdown and more DNA damage. Collectively, these data suggest that Lkb1 loss sensitizes cells to DTYMK-depletion–induced DNA damage and replication stress, as equivalent knockdown of Dtymk in Lkb1-null and Lkb1-WT cells leads to more robust DNA damage in the Lkb1-null cell lines.

DNA Replication Is More Sensitive to Dtymk Knockdown in Lkb1-Null Cells than in Lkb1-WT Cells

To examine how the knockdown of Dtymk affects DNA synthesis, Lkb1-WT and Lkb1-null cells were pulse-labeled with 5-iododeoxyuridine (IdU) for 20 minutes at 0, 2.5, and 3.5 days posttransduction with shDtymk-1. As shown in Fig. 3D,
**Figure 2.** *Dtymk* is a synthetic lethal target of Lkb1 loss. 

**A,** Lkb1-WT (634, 855, and 857) and Lkb1-null (t2, t4, and t5) cells were transduced with the indicated shRNA for 2 days and then plated into 96-well plates at 2,000 cells per well in 150 μL medium with 3 μg/mL puromycin (puro). Viable cells were measured daily using Promega’s CellTiter-Glo Assay. The data represent mean ± SD for three replicates.

**B,** Lkb1-null t4 cells were first transduced with pLenti6-*Dtymk*, pLenti6-*Dtymk-R1*, or pLenti6-*Dtymk-R3*, and selected with blasticidin. The blasticidin-resistant cells were pooled and further transduced with the indicated shRNA for 2 days and then plated for proliferation assay as described in **A**. The data represent mean ± SD for three replicates.

**C,** a total of 1 × 10⁶ Lkb1-WT (634 and 857) and Lkb1-null (t2 and t4) cells transduced with the indicated shRNA were implanted into athymic nude mice for 3 weeks. Tumor volume (mm³) was calculated as (length × width²)/2. The data represent mean ± SD for four mice. Lkb1-WT 634 and Lkb1-null t4 tumors with the indicated shRNAs are shown.

**D,** graph of dTMP and dTDP levels in Lkb1-WT 634 and Lkb1-null t4 cells transduced with the indicated shRNA for 3 days. The data represent mean ± SD for six replicates. Expression of DTYMK in these cells at the time of metabolite extraction was determined by Western blotting.

**E,** morphology of Lkb1-WT 634 and Lkb1-null t4 cells transduced with the indicated shRNA and then cultured in medium with or without additional 100 μmol/L dTTP for 4 days.
Figure 3. Characterization of Lkb1-WT and Lkb1-null cell lines. A, Western blot analyses of the indicated protein expression in Lkb1-WT (634, 855, and 857) and Lkb1-null (t2, t4, and t5) cell lines after Dtymk-1 knockdown. Phospho-CHEK1 Western blot analysis bands were quantified by ImageJ. B, Lkb1-WT (634, 855, and 857) and Lkb1-null (t2, t4, and t5) cell lines in log-phase growth were fixed with cold 70% ethanol, stained with propidium iodide (PI), and then analyzed with flow cytometry; 20,000 cells per line were analyzed. C, Lkb1-WT (634, 855, and 857) and Lkb1-null (t2, t4, and t5) cell lines were plated into 96-well plates at 2,000 cells per well in 150 μL medium containing the indicated concentrations of AZD7762 or CHIR124 for 3 days. Viable cells were then counted with Dojindo's Cell Counting Kit-8 Assay. The data represent mean ± SD for three repeats. Right: GI50 was calculated with GraphPad. D, Lkb1-WT and Lkb1-null cells in six-well plates were transduced with shDtymk-1. Two sets of the cells were plated into multiple chamber slides: one was 2 days and the other was 3 days posttransduction. After overnight culturing, the cells were labeled with 100 μmol/L IdU for 20 minutes, then fixed for indirect immunofluorescence staining with anti-bromodeoxyuridine (BrdU). The data represent mean ± SD for 200 to 300 cells. E, representative merged images from the cells stained with IdU (red) and 4′,6-diamidino-2-phenylindole (DAPI; blue) as described in D are shown.
the proportion of IdU-labeled cells decreased upon Dtrymk knockdown regardless of Lkb1 status, although the decrease was much greater in the Lkb1-null cells (dropping from 43.1% to 5.8% in 3.5 days, a decrease of 86.5%) as compared with those with Lkb1-WT (decreasing from 57.7% to 22.3%, a decrease of 61.2%). The lower degree of labeling of Lkb1-null cells compared with Lkb1-WT cells observed under basal conditions (43.1% vs. 57.7%) may be related to the broad reductions in dTTP metabolism in Lkb1-null cells. After shDtymk transduction, Lkb1-null cells appeared normal for 3 days, but by day 4 there was massive cell death leaving virtually no surviving cells, although there was no evidence of apoptosis (data not shown). After 3.5-day knockdown of Dtrymk, the remaining Lkb1-null t4 cells showed deformed and fragmented nuclei, indicative of thymineless death (Fig. 3E; refs. 24–27).

**LKB1-Mutant Human NSCLC Cell Lines Are Hypersensitive to DTYMK Knockdown**

We further sought to determine whether our observations in mouse lung cancer cells could be recapitulated in human LKB1-deficient NSCLC cell lines. We first screened five shRNAs targeting DTYMK and identified two, shDTYMK-D8 and shDTYMK-D10, that gave efficient knockdown (Supplementary Fig. S6A and Supplementary Table S2). Next, we screened Lkb1-WT and LKB1-deficient NSCLC cell lines (Fig. 4A) for proliferation in response to DTYMK knockdown and found that LKB1-deficient H2122 and A549 cell lines had heightened sensitivity as compared with LKB1-WT H358 and Calu-1 cell lines (Fig. 4B). Knockdown of DTYMK was confirmed by Western blot analysis (Fig. 4C). ShDTYMK-D10 transduction showed an increased lethality in LKB1-WT H358 and Calu-1 cells, trending toward that of the LKB1-deficient cell lines (Fig. 4B). One possible explanation could involve a differential threshold of DTYMK knockdown, as the remaining DTYMK protein levels after shDTYMK-D10 transduction were lower than those of shDTYMK-D8 (Fig. 4C). This suggests that there may be a differential sensitivity to absolute DTYMK reduction between LKB1-deficient and LKB1-WT cells. DTYMK was reported to be synthetic lethal with doxorubicin in colon cancer cells independent of p53 status, as LKB1-deficient cell lines with (A549) or without (H2122) functional p53 behaved similarly (Fig. 4B).

Next, we showed that knockdown of DTYMK in A549 cells reduced dTDP levels (Fig. 4D), suggesting that DTYMK is a major source of dTDP in human lung cancer cells. We further showed that LKB1-deficient cells H2122 and A549 were more sensitive than LKB1-WT H358 and Calu-1 cell lines to treatment with the selected CHEK1 inhibitors (Fig. 4E and Supplementary Fig. S4B), suggesting more DNA damage in LKB1-deficient cells than in LKB1-WT cell lines. This pathway seems relevant in vivo as LKB1 loss was associated with elevated CHEK1 expression in KRAS-mutant NSCLCs (Supplementary Fig. S6B).

**DISCUSSION**

In the current study, we created cell lines using Lkb1-null lung tumor nodules and conducted multiple screens that identified Dtrymk as a putative synthetic lethal candidate with Lkb1 loss. Furthermore, we showed that depletion of DTYMK in mouse and human NSCLC cells diminished the dTDP pool and led to greater growth inhibition in Lkb1/LKB1-deficient cells; and that LKB1 loss in mouse and human cells was linked to more DNA damage. These results suggest that DTYMK is a potential therapeutic target in LKB1-mutant human cancer. In addition, the parallel results observed in both mouse and human cell lines suggest that GEMM-derived tumor cell lines can be used successfully for in vitro synthetic lethal screening.

One possible explanation for the synthetic lethality of Lkb1 loss and Dtrymk knockdown is partly because of the lower expression of DTYMK in Lkb1-null cell lines, leading them to be more dependent on the dTTP synthetase pathway. ShDtrymk depletes the absolute amount of DTYMK protein below a critical threshold, resulting in thymineless death in Lkb1-null cells but not in Lkb1-WT cells (Supplementary Fig. S7). In addition, unlike Lkb1-WT cell lines, Lkb1-null cell lines lack feedback upregulation of RPA32 expression upon Dtrymk knockdown (Supplementary Fig. S7). Because RPA32 is involved in binding and stabilizing ssDNA during repair and replication, the lack of feedback upregulation of RPA32 expression may hinder DNA repair. A preliminary study revealed lower Dtrymk and Chek1 transcripts in Lkb1-null cell lines (Supplementary Fig. S8A and S8B), suggesting that transcriptional regulation contributes to the lower DTYMK and CHEK1 protein levels in Lkb1-null cell lines. More work will be needed to decipher the roles of LKB1 in the regulation of DTYMK, CHEK1, and RPA32 expression. Although LKB1-deficient NSCLC cell lines did not apparently show less DTYMK expression, the shDTYMK data still suggested a differential sensitivity to absolute DTYMK reduction between LKB1-deficient and LKB1-WT cells. As an essential gene governing dTTP biosynthesis and DNA replication, DTYMK is necessary to all dividing cells, and overdepletion of DTYMK below the threshold is lethal to all dividing cells, especially to the tumor cells carrying low levels of deoxynucleotide pools yet maintaining a fast growth rate. This may explain the eventual death of Lkb1/LKB1-WT cells after Dtrymk/DTYMK knockdown.

Human DTYMK was cloned by functional complementation of a *Saccharomyces cerevisiae* cell-cycle mutant cdc8, an essential gene for DNA synthesis (29). DTYMK is the first enzymatic step following the convergence of the *de novo* and salvage pathways in dTTP biosynthesis. In the *de novo* pathway, the DTYMK substrate dTMP is synthesized from methylthionyl dUMP by thymidylate synthase. In the salvage pathway, dTMP is produced from phosphorylation of thymidine by thymidine kinase. The next step in both pathways is the DTYMK-mediated phosphorylation of dTMP to form dTDP (30, 31). The production of dTDP is in contrast to that of the other deoxynucleotides used in DNA synthesis—dADP, dGDP, dCDP, and dUDP, which are synthesized from ADP, GDP, CDP, and UDP by ribonucleotide reductase (12, 32). Therefore, the unique dTTP biosynthesis pathway is a good target for drugs. There are multiple precedents of inhibition of the key enzymes in the *de novo* dTTP synthesis pathway, including thymidylate synthase by 5-fluorouracil or pemetrexed (15) and ribonucleotide reductase by hydroxyurea (33). We have targeted thymidylate synthase and ribonucleotide reductase in both the mouse and human.
Identifying Lkb1-Mutant Synthetic Lethal Targets

**Figure 4.** Knockdown of DTYMK in LKB1-WT and LKB1-mutant NSCLC cell lines. A, Western blot analyses of LKB1 expression in LKB1-WT (H358 and Calu-1) and LKB1-deficient (H2122 and A549) NSCLC cell lines. **B**, LKB1-WT (H358 and Calu-1) and LKB1-deficient (H2122 and A549) NSCLC cell lines were transduced with the indicated shRNAs for 1 day and then selected with 5 μg/mL puromycin (puro) for 2 days in six-well plates. The cells were collected by trypsin and replated into 96-well plates at 2,000 cells per well in 150 μL medium containing 5 μg/mL puromycin and measured daily using Promega’s CellTiter-Glo Assay. The data represent mean ± SD for three replicates. **C**, the cells left from the replating were lysed for Western blot analysis of DTYMK expression. **D**, graph of dTDP levels in A549 cells transduced with the indicated shRNA for 4 days. The data represent mean ± SD for six replicates. Expression of DTYMK in these cells at the time of metabolite extraction was determined by Western blotting. **E**, LKB1-WT (H358 and Calu-1) and LKB1-deficient (H2122 and A549) NSCLC cell lines were plated into 96-well plates at 2,000 cells per well in 150 μL medium containing the indicated concentrations of AZD7762 or CHIR124 for 3 days. Viable cells were counted daily using Dojindo’s Cell Counting Kit-8 Assay. The data represent mean ± SD for three repeats. Bottom: GI50 was calculated with GraphPad.

NSCLC Lkb1/LKB1-mutant cell lines using the same drugs and have not seen any selective effect on Lkb1/LKB1–deficient cell growth, likely because of an escape mechanism from the salvage pathway (Supplementary Fig. S9). In summary, the lack of redundant pathways for dTTP biosynthesis and the vital role of DTYMK in this process together make DTYMK a new anticancer target. In this regard, expression of DTYMK is increased in the majority of lung adenocarcinomas in comparison with normal lung (Supplementary Fig. S10A), and elevated DTYMK levels are correlated with poor survival.
In Vivo Studies

Lkb1-WT and lkb1-null cells were transduced with pTetOn-shGFP (puromycin) or pTetOn-shDytk-3 (puromycin), and then 1 million puromycin-resistant cells per transduction were implanted into athymic nude mice. When tumors grew to a diameter of 3 mm, the mice were maintained on doxycycline diet for 3 weeks to allow 634/shGFP tumors reach about 1,000 mm³.

Disclosure of Potential Conflicts of Interest

K. Marks is employed as Associate Director at Agios Pharmaceuticals and has ownership interest (including patents) in the same. E.M. Driggers has commercial research support from Agios Pharmaceuticals. P.A. Janne has received commercial research grants from Pfizer, Boehringer Ingelheim, Sanofi-Aventis, AstraZeneca, Roche, and Genentech. J.A. Engelman has ownership interest (including patents) in Agios Pharmaceuticals and is a consultant/advisory board member of the same. R. Scully has ownership interest (including patents) in Dana-Farber Cancer Institute. A. Kimmelman is a consultant/advisory board member of Forma Therapeutics. L.C. Cantley is on the Board of Directors of Agios Pharmaceuticals, has ownership interest (including patents) in Agios Pharmaceuticals, and is a consultant/advisory board member of the same. K.-K. Wong has commercial research support from Millennium, AstraZeneca, and Infinity, has ownership interest (including patents) in G1 Therapeutics, and is a consultant/advisory board member of MolecularMD. No potential conflicts of interest were disclosed by the other authors.

The Editor-in-Chief of Cancer Discovery (Lewis C. Cantley) is an author of this article. In keeping with the AACR’s Editorial Policy, the article was peer reviewed and a member of the AACR’s Publications Committee rendered the decision about acceptability.

Methods

Detailed protocols for all sections are described in the Supplementary Methods.

RNAi Screening and Metabolite Profiling

Large-scale pooled screening and data analysis were conducted at the Broad Institute’s RNAi Platform as recommended previously (34), and metabolite extraction and targeted mass spectrometry analysis were conducted as reported previously (35, 36).

Cell Lines and Cell Culture

Fresh murine lung tumor nodules were minced and cultured in 100-mm dishes with RPMI-1640/10% FBS/1% penicillin-streptomycin. Calu-1, H358, H2122, and A549 (obtained from American Type Culture Collection) were cultured in RPMI-1640/10% FBS/1% penicillin-streptomycin and 293ft (Invitrogen) was cultured in Dulbecco’s Modified Eagle Medium (DMEM)/10% FBS/1% penicillin-streptomycin. All cells were cultured at 37°C in a humidified incubator with 5% CO₂.

Plasmid Constructs and Mutagenesis

pLKO.1-shRNAs were purchased from the Broad Institute (Cambridge, MA). DTYMK (BC030178) DNAs were purchased from Thermo Scientific. shRNA-resistant DTYMKs were made by mutagenesis PCR and subcloned into the BamHI and XhoI sites of pLent6 vector (Invitrogen). All mutated cDNAs were confirmed by sequencing.

Multiple Routine In Vitro Studies

Lentiviral production and target cell transduction, proliferation assay, quantitative real-time PCR, Western blot analysis, flow cytometry, and immunofluorescence microscopy were conducted as described in the Supplementary Methods.

In Vivo Study

Lkb1-WT and lkb1-null cells were transduced with pTetOn-shGFP (puromycin) or pTetOn-shDytk-3 (puromycin), and then 1 million puromycin-resistant cells per transduction were implanted into athymic nude mice. When tumors grew to a diameter of 3 mm, the mice were maintained on doxycycline diet for 3 weeks to allow 634/shGFP tumors reach about 1,000 mm³.

Disclosure of Potential Conflicts of Interest

K. Marks is employed as Associate Director at Agios Pharmaceuticals and has ownership interest (including patents) in the same. E.M. Driggers has commercial research support from Agios Pharmaceuticals. P.A. Janne has received commercial research grants from Pfizer, Boehringer Ingelheim, Sanofi-Aventis, AstraZeneca, Roche, and Genentech. J.A. Engelman has ownership interest (including patents) in Agios Pharmaceuticals and is a consultant/advisory board member of the same. R. Scully has ownership interest (including patents) in Dana-Farber Cancer Institute. A. Kimmelman is a consultant/advisory board member of Forma Therapeutics. L.C. Cantley is on the Board of Directors of Agios Pharmaceuticals, has ownership interest (including patents) in Agios Pharmaceuticals, and is a consultant/advisory board member of the same. K.-K. Wong has commercial research support from Millennium, AstraZeneca, and Infinity, has ownership interest (including patents) in G1 Therapeutics, and is a consultant/advisory board member of MolecularMD. No potential conflicts of interest were disclosed by the other authors.

The Editor-in-Chief of Cancer Discovery (Lewis C. Cantley) is an author of this article. In keeping with the AACR’s Editorial Policy, the article was peer reviewed and a member of the AACR’s Publications Committee rendered the decision about acceptability.
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