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Mouse Genetics Suggests Cell-Context Dependency for Myc-Regulated Metabolic Enzymes during Tumorigenesis

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Abstract

c-Myc (hereafter called Myc) belongs to a family of transcription factors that regulates cell growth, cell proliferation, and differentiation. Myc initiates the transcription of a large cast of genes involved in cell growth by stimulating metabolism and protein synthesis. Some of these, like those involved in glycolysis, may be part of the Warburg effect, which is defined as increased glucose uptake and lactate production in the presence of adequate oxygen supply. In this study, we have taken a mouse-genetics approach to challenge the role of select Myc-regulated metabolic enzymes in tumorigenesis in vivo. By breeding λ-Myc transgenic mice, Apcmin mice, and p53 knockout mice with mouse models carrying inactivating alleles of Lactate dehydrogenase A (Ldha), 3-Phosphoglycerate dehydrogenase (Phgdh) and Serine hydroxymethyltransferase 1 (Shmt1), we obtained offspring that were monitored for tumor development. Very surprisingly, we found that these genes are dispensable for tumorigenesis in these genetic settings. However, experiments in fibroblasts and colon carcinoma cells expressing oncogenic Ras show that these cells are sensitive to Ldha knockdown. Our genetic models reveal cell context dependency and a remarkable ability of tumor cells to adapt to alterations in critical metabolic pathways. Thus, to achieve clinical success, it will be of importance to correctly stratify patients and to find synthetic lethal combinations of inhibitors targeting metabolic enzymes.

Introduction

Activation of one of the three MYC oncogenes is frequently selected for during tumorigenesis. These genes encode the transcription factors c-Myc, N-Myc and L-Myc that regulate a large number of downstream target genes. Although most of the work on MYC oncogenes has involved their role in cell proliferation, it is becoming clear that they may be involved in most aspects of oncogenic transformation [1]. As such, unravelling the mechanisms by which Myc proteins activate genes, and which are the essential genes, is paramount as studies resolving these mechanisms may open up new avenues of targeted intervention against various cancers.

Some of Myc’s earliest discovered transcriptional targets were genes encoding metabolic enzymes such as Ornithine decarboxylase [Odc] [2,3], Lactate dehydrogenase A [Ldha] [4] and Carbamoyl-phosphate synthase/aspartate carbamoyltransferase/dihydroorotase [Cad] [5]. Later studies using expression profiling identified even more of these genes, indicating that Myc is a master regulator of cellular metabolism and cell growth [6,7]. Interestingly, inhibition of polyamine biosynthetic enzymes Odc and Spermidine synthase have shown efficacy in chemoprevention of several cancers in experimental models [8–14] and in colon cancer patients [15]. Furthermore, Myc-regulated Ldha, Pyruvate kinase M2 and Glutaminase have also emerged as promising targets based on experimental models of human cancer [16–23], suggesting that targeting various metabolic pathways regulated by Myc may prove beneficial in cancer therapies of patients. To gain in vivo support for this notion we performed genetic ablation experiments in mice to determine the individual contribution to tumorigenesis of three different Myc-regulated metabolic enzymes.

Results

To identify critical Myc-regulated metabolic enzymes, we performed Illumina bead chip arrays on RNA isolated from 4–6 week old wildtype or precancerous, B cell lymphoma-prone λ-Myc transgenic mice, where the human MYC gene is under the control of the immunoglobulin [Ig] λ enhancer [24]. Interestingly, when we performed unsupervised Hierarchical clustering on 153 genes (Table S1) encoding metabolic enzymes involved in glycolysis, the Kreb’s cycle, oxidative phosphorylation, serine synthesis and one-carbon
Cancer occurs when cells change their behavior and start to divide in an uncontrolled manner. To achieve this altered behavior, cells need to change their metabolism to be able to grow even when nutrient and oxygen supplies are limiting. Therefore, targeting metabolic pathways could be used to treat patients suffering from cancer. Here we studied a gene called MYC, which can regulate many metabolic pathways. By using genetically modified mice we can show that tumors have a remarkable ability to change their metabolism, even if key enzymes are removed. Taken together, our data suggest that metabolic disturbance by drugs in the clinic may present a future challenge.

Serine and folate metabolites can also be made via pathways involving Shmt2 and Phgdh. However, to assess the role of Shmt2, we were forced to take a different approach, as Shmt2 gene-trap clones or knockout mice were not available when initiating this project. We hence infected Colon 26 cells, which carry an NMLI-induced Evis mutation [29], with lentiviruses expressing shRNA directed against Shmt2 and Phgdh. Despite achieving potent knockdown levels, we did not observe any effect on viability or ability to form subcutaneous tumors when injected into syngenic Balb/c mice, as compared to cells infected with a control lentivirus (Figure S3A and S3B).

To further assess the effect of Phgdh loss in different tumor models, we obtained a Phgdh knockout mouse. Since Phgdh is essential for neurogenesis [30], Phgdh null embryos die at around embryonic day (E) 13.5, which prevented us from analyzing the effects of loss of Phgdh by conventional breeding to our tumor models. We therefore started out by assessing the impact of removal of just one allele of Phgdh on tumorigenesis in λ-Myc transgenic mice and in Apemice. At variance with Odc, which is haploinsufficient for tumor progression in these models, Phgdh heterozygosity did not impact tumorigenesis in these tumor models (Figure 3A and 3B), despite the 50% reduction in Phgdh activity (Figure 3C). As an alternative approach, we crossed λ-Myc;Phgdh+/− mice with Phgdh+/− mice and isolated hematopoietic stem cells from E13.5 fetal livers. These cells were then used to reconstitute lethally irradiated syngenic recipients, creating lymphoma-prone mice with varying expression of Phgdh (Figure 3D). Even in this setting, Phgdh was dispensable for Myc-induced tumorigenesis (Figure 3E), suggesting that hematopoiesis and Myc-driven tumorigenesis can occur in the absence of Phgdh.

Phgdh is linked to glycolysis and could potentially divert metabolites away from pyruvate usage in the TCA cycle in the mitochondrion. Pyruvate is also kept from entering the TCA cycle via the action of Ldha, encoded by another Myc-regulated gene [23]. Except for RNAi or antisense studies in established tumors, it is not known whether Ldha is needed for the actual transformation event in vivo. To assess this we used a mouse model carrying a procarbazine-induced homozygous germline mutation of Ldha [31]. The mutation has been mapped to an aspartate 223 to histidine exchange which results in a very strong phenotype in erythrocytes causing anemia that is counteracted by extra-medullary hematopoiesis with an associated splenomegaly (Figure 4A). We crossed Ldha mutant mice with λ-Myc mice to generate mice of all relevant genotypes. Some of these mice were sacrificed before they developed tumors to allow analysis of Ldha activity in splenic B cells. Other mice were aged and monitored for tumor development. As seen in Figure 4B, splenic B cells from λ-Myc mice exhibited an elevated level of Ldha activity - consistent with the expression analysis in Figure 1 - whereas the Ldha mutation severely diminished Ldha activity in B cells from both non-transgenic and Myc transgenic mice (Figure 4B). Unexpectedly, the Ldha mutation did not affect Myc-induced B-cell lymphogenesis. In two independent survival curves generated at Umeå University and Helmholtz Center Munich the median survival time for λ-Myc;Ldha+/−/− was similar to that of λ-Myc;Ldha+/−, with no statistical difference (Figure 4A for the Umeå-generated survival curve; Munich curve is shown in Figure S4C).

Our unexpected results suggest several possibilities: either Ldha is dispensable for Myc-induced lymphogenesis; or a compensatory mechanism is occurring; or Ldha deficiency alters the route of transformation. Firstly, a compensation by another Ldh form is improbable since Ldha activity and Ldha expression were very low or absent in tumors arising in λ-Myc;Ldha+/−/− mice (Figure S4D and S4E).

Metabolic Pathways Can Be Redundant in Lymphomas
Figure 1. Myc regulates the metabolic transcriptome. (A) Unsupervised hierarchical clustering of Illumina bead arrays made from RNA of splenic B cells from three wildtype and four precancerous $\lambda$-Myc transgenic mice. See Table S1 for genes used in the clustering. (B) qRT-PCR confirmation of 4 of the 20 most significantly, or most elevated expressed genes, in B220-sorted B cells from $\lambda$-Myc transgenic mice. *indicates $p<0.05$ and ** indicates $p<0.01$.
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is known to involve spontaneously arising, cooperating oncogenic mutations of tumor suppressors and other oncogenes to block the oncogenic stress response of Myc [32,33]. Hence, overexpression of anti-apoptotic proteins such as Bcl-2 or genetic deletion of one tumor suppressor allele such as Arf or p53 dramatically accelerates lymphomagenesis [34–36]. To neutralize the genetic heterogeneity in the cooperating oncogenic lesion during lymphomagenesis we interbred the p53 knockout mouse with the Ldhα mutant mouse and the λ-Myc mouse. λ-Myc/wildtype, λ-Myc/Ldhαmut/mut or λ-Myc/Ldhαmut/wildtype were made heterozygous for p53 by interbreeding. All mice developed disease at an accelerated rate as compared to λ-Myc mice (Figure 4B). The tumors that developed lost the wildtype p53 allele (data not shown). Moreover, the frequency of p53 mutation in the tumors that developed in the first cross (Figure 4A) was not different between Ldhα genotypes (data not shown). We conclude that Myc-induced lymphomagenesis can occur normally in mice lacking fully functional Ldhα.

Studies using antisense or RNAi have shown that Ldhα is important for breast carcinoma, neuroblastoma, fumarase-deficient renal cell cancers, as well as fibroblast and B-cell tumor cells in vitro [17,18,20,23,37]. Although the specific combination of Myc overexpression with loss of p53 was previously unexplored, other explanations to the differences between our findings and those of others, like experimental methods, culture conditions, oxygen supply or oncogenic pathway, could be at play. To test if a dependency of Ldhα could be revealed in settings where Myc is downstream rather than the primary oncogenic instigator, we interbred Apcmut mice and p53 knockout mice with the Ldhα mutant mouse. As seen in Figure 4C, Ldhα deficiency did not impact adenomagenesis in the Apcmut mice. However, we also created p53−/−:Ldhαmut/mut or Ldhαmut mouse embryo fibroblasts (MEFs) which were transduced with an oncogenic H-Ras (pBabe-HrasG12V-puro) retrovirus. Interestingly, whereas no growth defect could be observed in vitro, the Ldhα mutant Ras-transformed p53 knockout MEFs generated significantly smaller tumors in vivo that appeared less vascularized (Figure 4D and Figure S5A). This effect was not a result of varying expression of oncogenic H-Ras in the different tumors (Figure S5B). Moreover, Colon 26 cells with an endogenous oncogenic Kras allele could not be propagated when transduced with lentiviruses expressing two different Ldhα shRNAs despite the fact that these constructs were not lethal in NIH 3T3 cells (data not shown). To confirm the dependency on Ldhα we also co-transfected a GFP expressing plasmid with the lentiviral expression constructs expressing Ldhα shRNA into Colon 26. As seen in Figure S3C and S3D, we were able to knockdown expression of Ldhα in the cells, which resulted in a progressive loss of cells from 48 h post-transfection.

To investigate the ability of Ldhα-deficient fibroblasts to proliferate in a hypoxic environment, NIH 3T3 cells infected with Myc (pWBL-Asc-Myc) or Ras (pBabe-HrasG12V-hygro) retroviruses and control or Ldhα shRNA lentiviruses were exposed to hypoxia. As expected, hypoxia resulted in the induction of Ldhα and Pdk1, both downstream targets of Hif1α (Figure S5C). Interestingly, cells infected with the Ldhα shRNA incorporated less 3H-thymidine than cells infected with a control lentivirus (Figure S5D). Thus, Ldhα is required under defined conditions such as hypoxia and/or in cells with a deregulated Ras pathway. Therefore an Ldhα dependency may not be manifested in a Myc-induced lymphomagenesis setting.

In agreement with this notion, the λ820 mouse B-cell lymphoma line established from λ-Myc mice [38] succumbed to apoptosis when exposed to hypoxia (Figure S6A and S6B), regardless of whether or not Ldhα was knocked down (Figure S6C). In addition, Ldhα knockdown did not impact lymphomagenesis in vivo (Figure S6D), although knockdown still left a substantial amount of Ldhα transcript and activity in this highly Ldhα-expressing cell line (Figure S6C and S6E). Nevertheless, given the
sensitivity of λ-Myc lymphoma cells to hypoxia (Figure S6A and S6B), it is unlikely that tumorigenesis in this model contains a hypoxic component and thereby dependency on elevated Ldha activity. Indeed, immunohistochemistry showed that Ldha wildtype or mutant lymphomas from λ-Myc mice exhibited a remarkable sparse expression of angiogenic markers CD34 and SMA (Figure S7A).

Figure 3. Phgdh is dispensable for lymphomagenesis in λ-Myc transgenic mice. (A) Survival curve of λ-Myc mice generated from interbreedings between Phgdh+/+ and λ-Myc transgenic mice. λ-Myc; Phgdh+/+ n = 21, λ-Myc; Phgdh+/− n = 23. (B) Amount of adenomas in ApcMin mice with different Phgdh genotypes. (C) Enzymatic activity of Phgdh analyzed in sixth λ-Myc; Phgdh+/+ and in sixth λ-Myc; Phgdh+/− lymphomas. (D) Western blot analysis confirming that Phgdh is absent in tumors arising in recipient mice from λ-Myc Phgdh−/− embryos. (E) Survival curve of C57BL/6 mice transplanted with I-Myc transgenic E13.5 FLi of indicated Phgdh genotype. λ-Myc; Phgdh+/+ n = 5, λ-Myc; Phgdh+/− n = 14, λ-Myc; Phgdh−/− n = 7. doi:10.1371/journal.pgen.1002573.g003
Despite this there were no signs of obvious necrotic areas, suggesting that nutrients and oxygen can diffuse in these non-solid tumors. The staining results were not due to non-functional antibodies as they readily detected the angiogenic markers in normal spleen and in lymphomas that had disseminated in spleens of \( \text{l-Myc} \) mice (Figure S7B). It therefore appears as if lymphomas arising in lymph nodes of \( \text{l-Myc} \) mice are neither angiogenic, hypoxic or dependent on Ldha activity.

**Discussion**

We are today beginning to appreciate the fact that oncogenes and tumor suppressor genes not only regulate cell proliferation, immortalization, apoptosis, metastasis and angiogenesis [39] but also cellular metabolism. The change in metabolism and the Warburg effect were for a long time believed to be self-evident and secondary to transformation. It is now known that the metabolic changes occur simultaneously and are governed by the same signal transduction pathways as those governing cell proliferation [40]. Since different tumor cells transform in response to variations of oncogenic mutations it is therefore likely that tumor cells can, or even have to, make different metabolic adaptations as well. Some of these adaptations may make the cells dependent on a certain enzyme, whereas others do not. Our study highlights the Ras oncogene as a potential pathway that requires Ldha, illustrated in Ras-transformed fibroblasts and Colon 26 cells, which carry an endogenous \( \text{kras} \) mutation. Indeed, it has previously been shown that neu-transformed breast cancer cells are sensitive to Ldha inhibition by RNAi [37]. Since these cells have an activated Ras pathway [41], this may explain why knockdown of Ldha sensitizes these cells. The potential explanation why Ras-induced fibrosarcomas are sensitive to the Ldha mutation in vivo is that they are not inherently angiogenic, making them sensitive to metabolic perturbation before angiogenesis has occurred. The fact that Myc can stimulate angiogenesis independently of hypoxia-inducible factors [42–44] may account for the lack of impact on lymphogenesis upon mutation of e.g. Ldha. Indeed, we show here that lymphomas from \( \text{l-Myc} \) mice are very sensitive to hypoxia,
most likely since they are Myc-driven and therefore rely on the TCA cycle and oxidative phosphorylation [45].

Folate biosynthesis has been linked to cancer both from studies on dietary supplements and by the identification of polymorphisms in genes encoding enzymes in folate biosynthesis like Shmt1, MTHFR and TS [46]. In addition, certain drugs like methotrexate target the folate biosynthetic pathway suggesting that this pathway is of critical importance for tumor cell survival. Interestingly, Shmt2 was first identified as a target in a screen for genes that can rescue the growth defect of Myc null rat fibroblasts [47]. In the same study Shmt2 was also shown to be a Myc transcriptional target gene but it was not further functionally characterized. Herein, we provide evidence that Shmt1 is dispensable for Myc-induced lymphomagenesis and that its deletion even accelerates tumorigenesis. The reason for this acceleration is unknown. It could involve effects on senescence or B-cell development as deletion of genes like Suv39h1 and E2f2 accelerates tumorigenesis by these mechanisms, respectively [48,49]. However, our data corroborate other very recent studies. Using an Shmt1 knockout mouse [50], the Stover group showed that deletion of one allele of Shmt1 promotes adenomagenesis in the ApcMin mice when administered a special diet [26]. In our study the Shmt1 heterozygous ApcMin mice also have the largest mean amount of adenomas, albeit we did not investigate the impact of diet on this model. Interestingly, homozygous deletion of Shmt1 did not impact adenoma formation in ApcMin mice since there was a compensatory increase in thymidylate kinase (TK1) expression [47]; a salvage pathway for thymidine synthesis. We observed a stronger effect on acceleration of Myc-induced lymphomagenesis in homozygous mutant Shmt1 mice, suggesting that the salvage pathway is not completely penetrant. Taken together, we would argue that Shmt1 is a tumor-suppressing modifier in the context of B-cell lymphomas and colorectal adenomas.

One of the most important reasons for the systematic analysis of Myc target genes in tumorigenesis is the potential of identifying or validating future drug targets. Our lymphoma and adenoma data cast doubt on the utility of developing targeted interventions against Ldha, Phgdh and Shmt1. On the other hand, in these models, tumors arise in mice carrying germline mutations of both the oncogenic lesion and the genes encoding the metabolic enzymes. It is thus plausible that adaptations have occurred during development that would not have occurred in cells acutely exposed to an inhibitor. Nevertheless, our data suggest that tumor cells eventually will develop resistance to putative treatments directed against metabolic enzymes since tumor growth undoubtedly can occur in the absence of Shmt1, Phgdh or Ldha. Therefore, a correct stratification is needed to identify patients whose tumors would be sensitive to inhibitors that are under development, for instance against Ldha [6,18]. Such stratification can be performed based on which oncogenic driver mutation the tumor has acquired. As shown here, oncogenic Ras or pathways utilizing Ldha can contribute to Myc-driven tumorigenesis [10]. As shown here and in these two studies, Phgdh is important in some but not all contexts.

To date, very few inhibitors against Ldha have been identified and those known are either poorly bioavailable and/or have other targets. For instance, sodium oxamate is used in high millimolar concentrations but inhibits aspartate aminotransferase at concentrations where lactate production is not even affected when tumor cell growth is [53]. Gossypol, a natural compound from cottonseed first identified as a male contraceptive, also inhibits anti-apoptotic proteins of the Bcl-2 family making interpretation of anti-cancer activity difficult [54]. Even if improved inhibitors are developed the issue whether or not Ldha is a good target is unresolved. Our genetic study shows that cells carrying a defective Ldha are capable of forming tumors, albeit hindered by hypoxia. We and others also show that ablation of Ldha by RNAi can be detrimental for the cell. It is thus possible that either cell context determines sensitivity, or that the ablation of Ldha protein (RNAi) is more severe than inhibition of its activity (D223H mutation). This notion would lend support to the idea that Ldha may have other functions, potentially disconnected from its activity [55]. For instance, Ldha can be phosphorylated (Ty238) and localized to the nucleus [56] and has recently been shown to exist in transcription complexes in ES cells [57]. Future studies should address if glycolysis-independent functions of Ldha, as suggested in transcription [58,59], are the most important functions in some tumors. If so, focus on the development of new therapies should aim at blocking all activities of Ldha.

Materials and Methods

Ethics statement

All animal experiments were performed in accordance with the Regional Animal Ethic Committee Approval no. A6-08 or no. A18-08.

Mouse colonies

All transgenic mice in the study were on pure C57BL/6 background. The λ-Myc-mice and the Ldhmut/mut mice have been previously described [24,31]. The p53 knockout mice and the ApcMin mice were from Jackson Labs, the C57BL/6 and Balb/c mice used as recipients were from Taconics, and the Phgdh knockout mice were from RIKEN BRC, Japan. Shmt1 knockout mice were generated by blastocyst injection of gene-trapped ES cells (clone AD0236, Sanger Institute Gene-trap Resources) at Umeå Transgene Core Facility. After confirmation of germline transmission, mice were backcrossed to C57BL/6 for at least ten generations. Illumina SNP genotyping confirmed that the mice were at least 96% C57BL/6 before starting interbreeding with λ-Myc transgenic mice, p53 knockout mice and ApcMin mice.

Tumor monitoring and analyses

All mice used in the study were monitored by group members and personnel at the animal facilities (Umeå Transgene Core Facility or Helmholtz Centre, Munich). When showing signs of disease, λ-Myc mice were sacrificed and lymphomas were collected for analyses. Dates of sacrifice were entered into GraphPad Prizm software for the generation of survival curves. Lymphomas were either snap frozen for RNA and protein analyses, or formalin-fixed and embedded in paraffin. Paraffin blocks were sectioned and processed by standard immunohistochemistry methodology using antibodies directed against smooth muscle actin (SMA; Sigma) or CD34 (Abcam) at the Histocenter core facility (Göteborg, Sweden).

The ApcMin mice used for adenoma formation studies were sacrificed and analyzed between 120–140 days of age, or when showing signs of disease. The small intestine and colon were dissected out, washed with phosphate-buffered saline and cut length-wise at which point adenomas were counted and tissues were harvested for analyses. Adenomas were counted using dissection microscope as well as by eye by two independent observers. The adenomas were scored irrespective of size and numbers per mouse were entered into GraphPad Prizm software for generation of graphs.
Microarray analysis
The analysis of gene expression changes between magnetically sorted B cells from wildtype or λ-Myc transgenic mice was performed using the Illumina BeadChip system. For in vitro transcription amplification, 200 ng of RNA was used with the Illumina RNA Amplification Kit (Ambion). Amplified RNA (1.5 μg) was hybridized to the Sentrix MouseRef-8 Expression BeadChip (Illumina). The primary data were collected from the BeadChips using the manufacturer’s BeadArray Reader and analyzed using the supplied scanner software. Data normalization was performed by cubic spline normalization using Illumina’s Beadstudio v3 software. Clustering and visualization of genes encoding metabolic enzymes was done using the Spotfire software.

Cell culture
MEFs were generated by mechanical disruption and trypsin-digestion of E13.5 embryos from which the fetal liver and the head had been removed. The single-cell suspension was grown in DMEM supplemented with 10% fetal bovine serum (FBS), 50 μM β-mercaptoethanol, 1 x glutamine, pyruvate, non-essential amino acids and antibiotics (Invitrogen). 293T cells and NIH 3T3 (from ATCC) were routinely maintained in DMEM supplemented with 10% FBS, glutamine and antibiotics. Colon 26 cells (from Cell Line Services) were cultured in RPMII supplemented with 10% FBS, glutamine and antibiotics. The λ200 cell line was established from λ-Myc transgenic mice and cultured as previously described [38].

Viral production and transductions
Retroviruses and lentiviruses were produced by calcium phosphate-mediated transfection of 293T cells. For retroviruses the following plasmids from Addgene were used: pBABE-HrasG12V-puro, pBABE-HrasG12V-hygro, MSCV-Myc-IRES-GFP, pWZL-Myc-blasticidin, pBABE-puro, pBABE-hygro together with pCL-Eco (encoding gag, pol and ecotropic envelope). Lentiviruses for RNAi were made using plKO.1 puro vectors expressing shRNAs (Sigma Mission RNAi), together with packaging plasmids pCMV R8.2dvpr and pHCMV-EcoEnv (both from Addgene). Two or three different shRNAs were used per gene (Table S2) and they were compared to a control plKO vector expressing a control shRNA with no known target in the mouse genome (non-target vector from Sigma). Thirty-six hours post-transfection, the media was harvested four times during an additional 36 h. The virus was filtered and either frozen down in aliquots or applied on target cells in the presence of 4–8 μg/ml polybrene. Following antibiotics selection for 48 h (or GFP analysis of FACs to confirm at least 90% positive cells) cells were expanded and used for experiments. The shRNA-containing viruses were always introduced into NIH 3T3 cells after the transduction with control, Myc or Ras retroviruses.

Subcutaneous tumor formation and analyses
MEFs used for sarcoma formation studies were infected with retroviruses encoding oncogenic Hras, whereas Colon 26 cells were infected with lentiviruses expressing shRNA against Shmt2, Pigdh and Ldh. For MEFs, 1x10^5 were injected subcutaneously into C57BL/6 recipients, whereas 5x10^5 Colon 26 cells were mixed with Matrigel (1:1) and injected into Balb/c mice. When tumors appeared, the mice were sacrificed and tumors were weighed and material was harvested for analyses. For immunofluorescence, formalin-fixed tumors were embedded in paraffin and sectioned (8 μm) onto glass slides. Following deparaffinization and rehydration, slides were either stained with H&E or subjected to Hoechst and antibody staining using Cy3-conjugated control or anti-smooth muscle actin antibody (Sigma) according to standard methodology. Following mounting the sections they were analyzed in a fluorescence microscope.

Fetal liver transplants
Fetal livers of E13.5 embryos were dissected out of embryos from timed pregnancies between λ-Myc, Pigdh–/– males and Pigdh–/– females. Each individual liver was dissociated through a cell strainer and injected via the tail-vein into one lethally gamma-irradiated (9.25 Gy) C57BL/6 recipient. Tissue from each embryo was taken for genotyping and the mice positive for the λ-Myc transgene were followed for lymphoma development and treated as previously described in Mouse colonies and Tumor monitoring and analyses.

Protein and RNA expression
For protein expression analyses by Western blot, cells and tumors were lysed in an appropriate amount of lysis buffer on ice for 30 min. Following sonication, clearing by centrifugation and protein determination, an equal amount of protein per well was loaded on SDS-PAGE gels and separated by electrophoresis. The proteins were transferred to a nitrocellulose membrane, which was subsequently blocked with TBST containing 5% non-fat dried milk. The membranes were then blotted with primary and horseradish peroxidase-conjugated secondary antibodies dissolved in blocking solution. After washing with TBST, the bound proteins were visualized by enhanced chemoluminescence. The primary antibodies used were from BD Biosciences (H-Ras), Cell signalling (c-Myc and Pdk1), Sigma-Aldrich (Ltha, Ldhb, Shmt1, Shmt2 and β-actin) and Atlas Antibodies (Shmt1 and Pigdh).

RNA expression was measured by quantitative reverse transcriptase PCR (qRT-PCR). Briefly, RNA was prepared using the NucléoSpin RNA II kit (Macherey-Nagel). cDNA was prepared using the First strand synthesis kit (Fermentas) and the PCR was run using the KAPA mastermix (Biotools) on an iQ real-time PCR machine (Bio-Rad). Primer sequences can be found in Table S3.

Hypoxia experiment
NIH 3T3 cells expressing either HrasV12, c-Myc or both were used for parallel infections of lentiviruses encoding shRNAs against Ldha. The same amount of cells were seeded in 24-well format and subsequently infected. 72 hours post infection, each well was split into 3x96 well format in duplicate plates. One set of plates was placed in a hypoxic environment (using the Modular incubator chamber, Billups-Rothenberg Inc.) for 40 hours after which the hypoxic treatment was terminated and 3H-thymidine was added to all wells. After two hours, the plates were freeze-thawed and the cells harvested onto glass fibre filters. Microscint scintillation solution was administered to the dried filter, which were subsequently counted on a TopCount scintillation counter. λ820 cells were subjected to hypoxia in 24 well plates or 25 cm² flasks (2x10^5 cells/ml) and were harvested for cell counting and apoptosis analyses or RNA analyses, respectively. For apoptosis analyses, cells were stained with Vindelovs reagent (10 mM Tris, 10 mM NaCl, 75 μM propidium iodine, 0.1% Igepal, and 700 units/liter RNase adjusted to pH 8.0) and then analyzed with a FACScalibur flow cytometer (BD Biosciences). Apoptosis was determined using DNA histograms and was based on the number of cells that carried less than diploid DNA content (sub-G1) in a logarithmic FL2 channel.

Enzyme activity assay
Total protein lysates were prepared as described above and the same amount of protein was assayed for LDH activity using the
Cytotoxicity detection kit (Roche Applied Science). The reactions were read using the Tecan Infinite200 plate reader at 492 nm. Lysates were also used to assess Shmt activity and Phgdh in accordance with published methods [60,61].

Supporting Information

Figure S1 Metabolic pathways linking glycolysis and serine/folate metabolism. The enzymes: LDH (lactate dehydrogenase); PHGDH (3-phosphoglycerate dehydrogenase); PSAT (phosphoserine aminotransferase); PSPH (phosphoserine phosphatase); SHMT (serine hydroxymethyltransferase 1 and 2); DHFR (Dihydrofolate reductase); TYMS (Thymidylate synthase).

Figure S2 Further characterization of Shmt1 mutant mice. (A) Protein expression of Shmt1, Shmt2 and Phgdh in λ-Myc cells and tumors. (B) Shmt activity in B cells from three 4-weeks old λ-Myc and three wildtype littersates. (C) Genomic organisation of the mouse Shmt1 locus indicating insertion of the gene-trap cassette. (D) Typical PCR genotyping results of tail DNA from offspring of matings between heterozygous Shmt1 mutant mice. (E) Western blot analysis showing absence of detectable protein in tissues from Shmt1 mutant mice.

Figure S3 Ldha is essential for Colon 26 cells. (A) Subcutaneous tumors arising in syngenic Balb/c mice by injecting Colon 26 cells infected with lentiviruses expressing shRNAs against Shmt2 and Phgdh (three separate hairpins of each). The symbols correspond to the different hairpins used in the experiment. In the control group, black symbol represents uninfected (UT) cells and white refers to the control non-targeting shRNA (NT). In the Phgdh shRNA group, white symbol represents shRNA#2, grey symbol shRNA#3 and black symbol shRNA#5. In the Shmt2 group, white symbol represents shRNA#3, grey symbol shRNA#4 and black symbol shRNA#5. See Table S2 for details on the specific shRNA constructs. * Colon 26 cells expressing Ldha shRNA#2 and shRNA#5 were depleted in culture and could not be transplanted (B) The level of knockdown in the tumors from panel C was analyzed by qRT-PCR. (C) Colon 26 cells were transiently co-transfected in 6-well plates with a GFP-expressing plasmid and a plasmid expressing either a non-targeting (NT) shRNA or Ldha shRNA#5. Cells were analyzed for GFP and found to be 50–60% of the original cells. (D) The same cells as in panel B were analyzed by qRT-PCR to confirm Ldha knockout.

Figure S4 Further characterization of Ldha mutant mice. (A) Splenomegaly in Ldha mutant mice. (B) Ldha activity in magnetically sorted splenic B cells derived from mice of indicated genotypes. (C) Survival curve of λ-Myc mice generated from interbreedings between Ldhamut/mut and λ-Myc transgenic mice generated at Helmholtz Center in Munich. (D) Ldha activity in some of the tumors developed in mice described in Figure 4A. (E) Western blot analysis of Ldha and Ldhb in some of the tumors developed in mice described in Figure 4A and 4B.

Figure S5 Hypoxia sensitizes fibroblasts to Ldha inhibition. (A) Immunofluorescence analysis of α-Smooth muscle actin in representative sarcomas from Figure 4D. Hoechst staining was used to stain the nuclei. (B) Western blot analysis for Ha-Ras on representative sarcomas from Figure 4D. (C) NIH 3T3 cells were infected with Myc, Ras or MycRas retroviruses, followed by lentiviral infection with a control or an Ldha-targeting shRNA. The cells were subjected to hypoxia and cells were either analyzed by Western blot using antibodies against Ldha or Pdk1 (Hif target) (E) or incubated with radio-labelled thymidine to measure cell proliferation.

Figure S6 Myc-induced lymphoma cells are sensitive to hypoxia. (A-B) λ820 cells infected with lentiviruses expressing a non-target control shRNA (NT) or shRNAs against Ldha were subjected to hypoxic conditions. Quantification of apoptosis was performed by measuring the sub-G1 content using the gate shown in A and cell numbers were collected by counting viable cells. (C) qRT-PCR analysis for Ldha and Pdk1 in the cells analyzed in B. (D) λ820 cells carrying an shRNA against Ldha or a control shRNA were transplanted into syngenic C57BL/6 recipients via the tail vein and monitored for tumor growth. (E) Ldh activities of λ820 cells infected with indicated lentiviruses.

Figure S7 Nodal lymphomas from λ-Myc transgenic mice express less angiogenic markers than splenic lymphomas. A) Paraffin-embedded lymphomas developed in lymph nodes of λ-Myc or λ-MycLdhamut/mut mice were sectioned and stained with antibodies directed against angiogenic markers CD34 (brown staining) and SMA (red staining). Shown is one representative field of view encompassing most of the lymphoma (40×) or a larger magnification of the dashed square (200×). B) Paraffin-embedded spleens from wildtype mice or lymphomas developed in spleens of λ-Myc or λ-MycLdhamut/mut mice were sectioned and stained with antibodies directed against angiogenic markers CD34 (brown staining) and SMA (red staining). Shown is one representative field of view at 200×.

Table S1 Expression of genes used in the unsupervised hierarchical clustering shown in Figure 1. Gene list is sorted based on the average fold-change between RNA expression in wildtype (wt) and λ-Myc splenic B cells.

Table S2 List of shRNAs used in the study. The shRNAs came from the TRC1.0 mouse lentiviral library and their library number is indicated.

Table S3 Sequences of qRT-PCR primers used in the study. Primers were designed using the SciTools at www.idtdna.com.

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Author Contributions

Conceived and designed the experiments: LMN GWB JAN. Performed the experiments: LMN TZPF SR CK JAN. Analyzed the data: LMN TZPF SR CK GWB JAN. Contributed reagents/materials/analysis tools: WP. Wrote the paper: LMN GWB JAN.
References


