The miR-17～92 microRNA Cluster Is a Global Regulator of Tumor Metabolism

Highlights

- miR-17～92 is required for metabolic reprogramming of Myc+ tumor cells
- miR-17/20 is the primary metabolic regulatory element of miR-17～92
- miR-17 negatively regulates the tumor suppressor LKB1
- miR-17-dependent silencing of LKB1 dictates metabolic and tumorigenic potential

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In Brief

Cancer cells re-wire cellular metabolic pathways to help fuel the increased bioenergetic and biosynthetic demands of malignant growth. Work by Izreig et al. demonstrate that this process is controlled in lymphoma cells by the miR-17～92 microRNA cluster, which coordinates tumor metabolism by silencing the LKB1 tumor suppressor pathway.

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The miR-17–92 microRNA Cluster Is a Global Regulator of Tumor Metabolism

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SUMMARY

A central hallmark of cancer cells is the reprogramming of cellular metabolism to meet the bioenergetic and biosynthetic demands of malignant growth. Here, we report that the miR-17–92 microRNA (miRNA) cluster is an oncogenic driver of tumor metabolic reprogramming. Loss of miR-17–92 in Myc+ tumor cells leads to a global decrease in tumor cell metabolism, affecting both glycolytic and mitochondrial metabolism, whereas increased miR-17–92 expression is sufficient to drive increased nutrient usage by tumor cells. We mapped the metabolic control element of miR-17–92 to the miR-17 seed family, which influences cellular metabolism and mammalian target of rapamycin complex 1 (mTORC1) signaling through negative regulation of the LKB1 tumor suppressor. miR-17–dependent tuning of LKB1 levels regulates both the metabolic potential of Myc+ lymphomas and tumor growth in vivo. Our results establish metabolic reprogramming as a central function of the oncogenic miR-17–92 miRNA cluster that drives the progression of MYC-dependent tumors.

INTRODUCTION

Tumor cells must manage their energetic resources to grow and survive. This involves coordinating metabolic activities to produce ATP, and the acquisition or synthesis of macromolecules (i.e., proteins, lipids, and nucleotides) at sufficient rates to meet the demands of malignant growth (Lunt and Vander Heiden, 2011). A common characteristic of cancer cells is the reprogramming of cellular metabolism to favor metabolic pathways that fuel aberrant cell growth such as aerobic glycolysis (i.e., the Warburg effect) and mitochondrial metabolism (Vander Heiden et al., 2009; Weinberg and Chandel, 2015). Many of the predominant oncogenic mutations observed in cancer also control tumor cell metabolism as part of their mode of action (Deberardinis et al., 2008; Jones and Thompson, 2009), linking metabolic dysregulation to tumor progression.

One of the major drivers of metabolic reprogramming in tumor cells is the c-Myc proto-oncogene (hereafter referred to as Myc), a transcription factor overexpressed or deregulated in over 50% of human cancers including hematopoietic, brain, breast, colorectal, and lung malignancies (Bredel et al., 2009; Cancer Genome Research Atlas, 2012, 2014; Li et al., 2013; Schmitz et al., 2012). Increased MYC expression is associated with poor prognosis in Burkitt Lymphoma (BL) (Lin et al., 2012b) and diffuse large B cell lymphoma (DLBCL) (Barrans et al., 2010; Savage et al., 2009; Zhou et al., 2014), with an overall 5-year survival rate of only ~30% (Savage et al., 2009).

Myc promotes the re-wiring of tumor cell metabolism through the transcriptional regulation of metabolic pathway genes, including enzymes that regulate glycolysis, tricarboxylic acid (TCA) cycle metabolism, oxidative phosphorylation (OXPHOS), and mitochondrial biogenesis (Stine et al., 2015). Oncogenic Myc can also function as an amplifier of cellular gene expression programs (Lin et al., 2012a; Nie et al., 2012), which may occur through non-specific “invading” of enhancer and promoter regions, or recruitment of specific cofactors, such as Miz1 and Max, that help shape transcriptional responses triggered by supraphysiological levels of oncogenic Myc (Wolf et al., 2015). Myc also regulates the transcription of microRNAs (miRNAs), which are small, non-coding RNAs that negatively regulate mRNA stability and/or translation through partial complementary base pairing to the 3’ UTR of target mRNAs (Bartel, 2009). Myc has been associated with widespread repression of miRNA expression (Chang et al., 2008), but is also found to induce expression of the polycistronic miRNA cluster miR-17–92. Originally identified as a candidate gene in the 13q31-q32 amplification observed in lymphoma (Ota et al., 2004), miR-17–92 is a direct transcriptional target of Myc (O’Donnell et al., 2005) and cooperates with Myc to promote lymphomagenesis in animal models (He et al., 2005). miR-17–92 expression is elevated in a number of human tumors, including cancers of the colon (He
et al., 2005), lung (Hayashita et al., 2005), and DLBCL (Cerami et al., 2012; Olive et al., 2010; Pei et al., 2013).

Recent work indicated that elevated miR-17–92 expression can maintain tumor growth even when Myc is inactivated in tumors (Li et al., 2014), suggesting that miR-17–92 may mediate several of the pro-tumorigenic effects ascribed to Myc. Here, we describe an essential function for the Myc-regulated miRNA cluster miR-17–92 in mediating Myc-driven metabolic reprogramming, implicating control of tumor metabolism as a central part of miR-17–92 oncogenic activity.

RESULTS

miR-17-92 Regulates Glycolytic and Oxidative Metabolism in Lymphoma Cells

Myc has been characterized as a master regulator of tumor metabolism through the direct transcriptional regulation of metabolic enzymes involved in intermediary metabolism (Stine et al., 2015). However, Myc also induces the expression of miR-17–92, a polycistronic miRNA cluster which encodes six individual miRNAs from a single RNA precursor (Figure 1A) (Olive et al., 2013). To investigate the influence of miR-17–92 on the metabolism of Myc+ tumor cells, we used established Eµ-Myc B cell lymphomas harboring conditional floxed alleles of miR-17–92 (miR-17–92fl/fl, denoted hereafter as fl/fl) (Mu et al., 2009). Examination of miRNA expression revealed miR-17, miR-20, and miR-92a as the most abundant mature miRNAs of the cluster expressed in Eµ-Myc lymphoma cells (Figure 1B). Parental fl/fl Eµ-Myc lymphoma cells were cultured with 4-OHT to generate isogenic lymphoma cells lacking (miR-17–92fl/fl), denoted as Δ/Δ (Figures S1A and S1B). We did not observe compensation of mature miRNA expression from the paralogous clusters miR-16-5p–15a–92a (Olive et al., 2013) or lacking (Δ/Δ) (Figures S1A and S1B). Deletion of miR-17–92 led to a slight reduction in Eµ-Myc lymphoma cell proliferation as previously described (Mu et al., 2009) but did not significantly alter tumor cell viability (Figures S1D and S1E).

We next assessed the impact of miR-17–92 deletion on the metabolism of Myc+ lymphomas by measuring their extracellular acidification rate (ECAR), a measure of aerobic glycolysis, and oxygen consumption rate (OCR), a measure of OXPHOS (Wu et al., 2007). Δ/Δ lymphomas displayed an ~50% reduction in both their ECAR and OCR compared to parental fl/fl lymphoma cells (Figure 1C). Consistent with the reduction in aerobic glycolysis, Δ/Δ lymphomas displayed lower glucose uptake and lactate production compared to lymphomas expressing...
miR-17–92 (Figure 1D). Glutamine consumption and ammonia production (a measure of glutaminolysis) were similarly reduced in $\Delta\Delta$ lymphoma cells (Figure 1E). The expression of key metabolic enzymes involved in glycolysis (HK2, Aldolase, LDha) and glutaminolysis (GLs1, GLs2) were reduced in $\Delta\Delta$ lymphoma cells compared to f/f/f controls (Figure S1F). Proliferating $\Delta\Delta$ lymphoma cells also displayed reduced cell size relative to parental tumor cells (Figure S1G).

miR-17–92 cooperates with Myc to promote lymphomagenesis and tumor progression in animal models (He et al., 2005). To assess whether overexpression of miR-17–92 was sufficient to alter the metabolic activity of lymphoma cells, we ectopically expressed miR-17–92 in Raji cells (Figure S1H), a human BL cell line harboring a t(8;14)(q24;q32) MYC-IGH translocation (Hamlyn and Rabbits, 1983). Ectopic expression of miR-17–92 increased the proliferative capacity but not the viability of Raji cells in culture (Figures S1I and S1J). In contrast to miR-17–92 deletion, ectopic expression of miR-17–92 increased both the ECAR and OCR of Raji cells (Figure 1F). We observed similar increases in both glucose consumption and lactate production (Figure 1G) and glutaminolysis (Figure 1H) in Raji cells overexpressing miR-17–92. Together these data indicate that miR-17–92 is both sufficient to drive enhanced metabolism and required for global maintenance of glycolytic and oxidative metabolism in Myc-dependent lymphoma cells.

miR-17–92 Is a Global Regulator of Myc-Dependent Metabolic Reprogramming

We next set out to identify pathways under miR-17–92 control by conducting RNA sequencing (RNA-seq) analysis of isogenic lymphoma cells expressing f/f/f or lacking $\Delta\Delta$ miR-17–92. Deletion of miR-17–92 promoted widespread changes in gene expression in Myc$^+$ lymphoma, with the expression of >5,700 genes significantly altered in $\Delta\Delta$ lymphomas relative to control cells (Figure S2A; Table S1). Analysis of KEGG pathways significantly decreased in $\Delta\Delta$ lymphomas revealed enrichment in metabolic pathway genes, mRNA transport and translation, and proteasome and peroxisome pathway components (Figure 2A). Further analysis of the metabolic pathways genes influenced by miR-17–92 revealed a global decrease in metabolic pathways including glycolysis, the TCA cycle (Figure S2B), components of the electron transport chain (Figure S2C), amino acid metabolism, the pentose phosphate pathway, serine biosynthesis, and nucleotide biosynthesis (Figure 2A). We next assessed overlap between the miR-17–92-regulated transcriptome and known Myc target genes (Kim et al., 2010). This analysis revealed that ~45% of defined Myc target genes were significantly influenced by loss of miR-17–92 (Figure 2B). Enriched in this group of miR-17–92-dependent Myc-target genes were metabolic pathway genes involved in central carbon metabolism and cellular biosynthesis (i.e., amino acid, purine, and pyrimidine biosynthesis). Details of differentially expressed miR-17–92-dependent Myc-target genes are summarized in Figure S2D and Table S2.

We next assessed the impact of miR-17–92 loss on metabolic network connectivity in Myc-dependent lymphoma cells. This analysis, which is based on connectivity between metabolite abundance and metabolic enzyme gene expression (Jha et al., 2015; Vincent et al., 2015b), revealed several metabolic sub-networks dependent on miR-17–92 expression (Figure 2C). This network-based analysis confirmed a global downregulation of cellular metabolic pathways at the transcriptional level in $\Delta\Delta$ lymphomas. Pathways decreased in miR-17–92-deficient Eµ-Myc lymphoma cells included glycolysis, and key branching pathways from the glycolytic pathway supporting nucleotide biosynthesis (i.e., pentose phosphate and serine biosynthesis pathways). The network also revealed lower TCA cycle metabolism and glutathione biosynthesis in miR-17–92-deficient lymphomas, while highlighting increases in genes associated with inositol biosynthesis and acetate metabolism (AcSs1 and Aldh3b1). Taken together, these data suggest an active role for miR-17–92 in enforcing the Myc-dependent metabolic transcriptome.

Myc-Dependent Regulation of Central Carbon Metabolism Requires miR-17–92

Given the decreased glycolytic and oxidative metabolism of Myc$^+$ lymphoma cells lacking miR-17–92 (Figure 1), we conducted stable isotope tracer analysis (SITA) on f/f/f and $\Delta\Delta$ Eµ-Myc lymphoma cells by culturing them with either U-[13C]-glucose or -glutamine. $\Delta\Delta$ lymphoma cells displayed decreased overall abundance of intracellular metabolites involved in central carbon metabolism (Figure 3A). Total intracellular pyruvate and lactate levels were decreased by ~50% in $\Delta\Delta$ Eµ-Myc cells (Figure 3A), due largely to reduced production of $^{13}$C$_2$-pyruvate and $^{13}$C$_3$-lactate isotopologues from glucose (Figure 3B). Similarly, the total abundance of TCA cycle metabolites including citrate and fumarate, as well as the incorporation of $^{13}$C-glucose-derived carbon into these metabolite pools, were decreased in $\Delta\Delta$ cells relative to control lymphoma cells (Figures 3A and 3B). The synthesis of glutamate from $\alpha$-ketoglutarate, which is mediated by glutamate dehydrogenase (GLDH) in Myc-driven cancer cells (Bott et al., 2015), was similarly decreased in Myc$^+$ lymphoma cells lacking miR-17–92 (Figure 3A). We observed a significant abundance of $^{13}$C$_3$ isotopologues of citrate, fumarate, and glutamate (blue bar, m/z3), suggesting contribution of pyruvate carboxylase (PC) activity to TCA cycle anaplerosis in Eµ-Myc lymphoma cells (Figure 3B). Decreased mRNA levels for the glucose transporters Glut1 (Slc2a1) and Glut3 (Slc2a3) were observed in $\Delta\Delta$ Eµ-Myc cells (Figure S3), which may contribute to lower glucose utilization by these cells.

Myc is a major regulator of glutaminolysis (Gao et al., 2009; Wise et al., 2008) and regulates glutamine-dependent TCA cycle activity under basal and hypoxic conditions (Le et al., 2012). However, we found that miR-17–92 was required to support glutamine-dependent TCA cycle anaplerosis in Eµ-Myc lymphoma cells (Figure 3C). Incorporation of U-[13C]$^3$-glutamine carbon into TCA intermediates was significantly reduced in $\Delta\Delta$ Eµ-Myc lymphoma cells, both in terms of total abundance (Figure 3C) and isotopomer distribution (Figure 3D). Of note, glutamine-dependent production of $^{13}$C$_2$-citrate and $^{13}$C-aspartate, which play essential roles in supporting cancer cell proliferation (Birsoy et al., 2015; Hatzivassiliou et al., 2005; Sullivan et al., 2015), were significantly reduced in $\Delta\Delta$ Eµ-Myc lymphoma cells.
Figure 2. miR-17–92 Is a Global Regulator of Metabolism Downstream of Myc

(A) List of KEGG pathways significantly enriched in parental control (fl/fl) versus miR-17–92-deficient (D/D) Eμ-Myc lymphoma cells (q value <0.1) using the Benjamini-Hochberg method.

(B) Analysis of Myc target genes differentially expressed in Eμ-Myc lymphoma cells lacking miR-17–92. Top, Venn diagram depicting all differentially expressed genes enriched in fl/fl versus D/D Eμ-Myc lymphoma cells (5779) and overlap with Myc target genes (1610). Bottom, KEGG pathway analysis of miR-17–92-dependent Myc target genes.

(legend continued on next page)
miR-17 and -20 Drive Metabolic Reprogramming Downstream of Myc

Given that the miR-17~92 gene encodes six mature miRNAs comprising four seed families (Figure 1A), we next sought to determine which miRNAs contribute to the metabolic regulatory activity of the cluster. We engineered a series of miR-17/C24-92-deficient Eµ-Myc lymphoma cell lines re-expressing the full miRNA cluster (+17~92) or mutant miR-17~92 alleles lacking specific seed family members (Figure 4A). The relative expression of each mature miRNA in control (fl/fl) and miR-17~92 mutant cell lines was verified by qPCR (Figure S4A). Seahorse analysis of these lymphoma cell lines revealed differential contributions of each miR-17~92 seed family to the bioenergetic profiles of Eµ-Myc lymphoma cells (Figure 4B). Addback of the full miR-17~92 cluster rescued the OCR and enhanced the ECAR of Δ/Δ cells, confirming our earlier results that elevated miR-17~92 expression can enhance glycolysis (Figures 1F and 1G). Addback of the cluster lacking only miR-92a (Δ92) or miR-19a and miR-19b (Δ19a,b) increased both OCR and ECAR above levels seen in fl/fl cells, whereas addback of a mutant miR-17~92 allele lacking the miR-17 family members miR-17 and miR-20a (Δ17,20) failed to rescue the ECAR and OCR of Δ/Δ cells (Figure 4B).

Further metabolic profiling was conducted using individual Δ/Δ lymphoma cell clones expressing all components of
Figure 4. The miR-17 Family Members miR-17 and miR-20a Are Required for miR-17–92-Dependent Metabolic Reprograming
(A) Schematic of miR-17–92 constructs lacking individual seed families used in this study.
(B) OCR versus ECAR plot for fl/fl and Δ/Δ Eμ-Myc lymphoma cells, as well as Δ/Δ lymphomas expressing miR-17–92 addback constructs described in (A).
(C and D) Relative glucose consumption (C) and lactate production (D) of control (fl/fl, white bar), miR-17–92-deficient (Δ/Δ, black bar), or miR-17/20-deficient (+17/20, blue bars) Eμ-Myc lymphoma cells after 48 hr of culture. Δ/Δ lymphoma cells re-expressing the entire miR-17–92 polycistron (+Δ17/92, gray bar) are included.
(E) Immunoblot of metabolic enzyme expression from lysates of Eμ-Myc lymphoma cells described in (B). Actin levels are shown as a control for protein loading. HK2, hexokinase-2; LDHA, lactate dehydrogenase A; GLS2, glutaminase-2.

(legend continued on next page)
miR-17–92 except miR-17 and miR-20a (Δ17,20). Lymphoma cells lacking miR-17/20 displayed similar reductions in glucose consumption (Figure 4C), lactate production (Figure 4D), glutamine consumption (Figure S4B), and ammonia production (Figure S4C) compared to Δ/Δ lymphoma cells, which was fully rescued only through addback of the full miR-17–92 cluster. The reduction in glycolysis and glutaminolysis correlated with reduced levels of key glycolysis (Hk2, Aldolase, Ldha) and glutaminolysis (Gls2) enzymes in Δ17,20 lymphoma cells relative to control cells (either fl/fl or full miR-17–92 addback) (Figure 4E).

We next used SITA to assess whether the miR-17 seed family influences nutrient utilization by Myc+ lymphoma cells. Control (fl/fl), miR-17–92-deficient (Δ/Δ), miR-17–92 addback, or Δ17,20 lymphoma cells were cultured in medium containing either U-[13C]-glucose or -glutamine, and metabolite abundances determined by gas chromatography-mass spectrometry (GC-MS). Re-expression of miR-17–92 was sufficient to restore both glucose- and glutamine-dependent metabolic flux in Δ/Δ lymphoma cells (Figures 4F, 4G, and S4D–S4G). Ectopic miR-17–92 expression actually enhanced metabolic flux in Δ/Δ lymphoma cells beyond control levels, marked by increased production of lactate from 13C-glucose (Figure 4F) and TCA cycle metabolites from 13C-glutamine (Figures 4G and S4G). In contrast, Δ17,20 lymphoma cells displayed similar 13C-labeling patterns to Δ/Δ lymphoma cells (Figures 4F and 4G).

Finally, we assessed the contribution of miR-17/20 to the tumorigenic potential of Eµ-Myc lymphomas. While re-expression of full-length miR-17–92 restored the proliferative capacity of Δ/Δ lymphoma cells to control levels, Δ17,20 failed to rescue lymphoma cell proliferation in vitro (Figure 4H). Next, we injected nude mice with control (fl/fl) or miR-17–92-deficient Eµ-Myc lymphoma cells expressing full-length miR-17–92 or Δ17,20, and monitored palpable tumor formation over time. While control lymphomas and Δ/Δ lymphomas re-expressing miR-17–92 formed tumors rapidly (median onset of ~2 weeks), the development of tumors lacking miR-17 and ~20 (Δ17,20) was significantly slower, with an average latency of 4 weeks (Figure 4I).

For one clone in particular (Δ17,20–2), >30% of animals remained tumor-free for up to 8 weeks, compared to 50% for animals who had received Δ/Δ lymphoma cells (Figure 4I).

**LKB1 Is a Direct Target of the miR-17 Seed Family**

miRNAs act to repress mRNA translation or promote mRNA degradation via partial complementary binding to the 3’ UTR of target mRNAs (Fabian et al., 2010). Previous work has linked degradation of the 3’ UTR of target mRNAs with either U-[13C]-glucose or -glutamine, and the proportion of 13C-labeled (black bar) or unlabeled (12C, white bar) metabolites was determined by GC-MS.

(G) Relative abundance of U-[13C]-glutamine-derived metabolites in Eµ-Myc lymphoma cells. Cells were cultured for 2 hr with U-[13C]-glutamine, and the proportion of 13C-labeled (black bar) or unlabeled (12C, white bar) metabolites was determined by GC-MS.

(H) Growth curves of control (fl/fl), miR-17–92-deficient (Δ/Δ), and miR-17–92-expressing (Δ/Δ + Δ17,20) Eµ-Myc lymphoma cells.

(1) Kaplan-Meier curve showing latency to tumor onset for Eµ-Myc lymphoma cells described in (H). The number of mice analyzed per genotype was as follows: fl/fl, n = 15; Δ/Δ + Ctrl, n = 12; Δ/Δ + Δ17,20, n = 14; Δ/Δ + Δ17,20–2, n = 14; Δ/Δ + Δ17–92, n = 15.

*p < 0.05; **p < 0.01.

We used the miRNA prediction algorithm Targetscan (Agarwal et al., 2015) and curated miRNA-mRNA interactions (Helwak et al., 2013) to identify Stk11, which encodes the serine-threonine kinase Liver Kinase B1 (LKB1), as a putative miR-17 family target. Previous work had identified LKB1 as a potential target of miR-17 in ovarian cancer cells (Liu et al., 2015). The seed region of miR-17/20 was predicted to bind to one site (base pairs 122–130) in the mouse Stk11 3’ UTR sequence (Figure 5A), and that this 3’ UTR target site was retained across several mammalian species including humans (Figure S5B). This was intriguing given that haploinsufficiency of LKB1, rather than biallelic inactivation of the gene, is commonly associated with tumor development (Vaahtomeri and Mäkelä, 2011). LKB1 is also a negative regulator of tumor metabolism whose loss promotes the Warburg effect in tumors (Faubert et al., 2014; Shackleford et al., 2009). We conducted 3’ rapid amplification of cDNA end (RACE) using mRNA isolated from Eµ-Myc lymphoma cells and, consistent with previous reports (Smith et al., 1999), found that Stk11 possesses an alternative poly-adenylation sequence that gives rise to both short and long 3’ UTR isoforms (denoted S1 and L1, for short and long, respectively) that contain the miR-17/20 targeting sequence (Figure S5C).

We next assessed whether miR-17 directly acts on the 3’ UTR of Stk11 mRNA to regulate its expression. We first used a reporter assay in which the Stk11 3’ UTR was cloned downstream of a luciferase reporter gene to assess miRNA-dependent suppression of the 3’ UTR sequence. Normalized luciferase activity was reduced in 293T cells expressing either the S1 or L1 form of the Stk11 3’ UTR (Figure S5D), indicating suppression of this 3’ UTR by endogenous miRNAs or other RNA binding proteins. The Stk11 3’ UTRs were suppressed further by ectopic expression of miR-17 (Figure 5B). Mutating the complementary miR-17 target site (Figure 5A) abolished miR-17-dependent regulation of the Stk11 3’ UTR in this assay (Figures S5B and S5D).

We next expressed FLAG-tagged versions of LKB1 in 293T cells using expression constructs encoding Stk11 mRNA with no 3’ UTR (Δ), wild-type 3’ UTR (WT), or a mutated 3’ UTR lacking complementarity with the miR-17 seed region (MUT) (Figure S5E). Protein levels of FLAG-LKB1 were reduced in cells expressing the wild-type Stk11 3’ UTR when co-transfected with a miR-17 expression vector (Figure 5C). Conversely, FLAG-LKB1 with the mutant Stk11 3’ UTR was refractory to miR-17 expression (Figure 5C).
We next examined the impact of miR-17/20 on LKB1 expression in lymphoma cells. LKB1 protein levels were elevated in Δ/Δ Eμ-Myc lymphoma cells compared to control cells (Figure 5D). Stk11 mRNA levels were unaffected by deletion of miR-17–92 (Figure 5E), suggesting a mechanism of translational suppression of LKB1 mRNA by miR-17. In contrast, LKB1 protein levels were reduced in Raji cells ectopically expressing miR-17–92 (Figure 5F). Lymphoma cells lacking miR-17 and -20 expression (Δ17,20) displayed elevated LKB1 expression similar to miR-17–92-deficient lymphomas, whereas addback of the entire miR-17–92 cluster potently reduced LKB1 protein levels in Eμ-Myc lymphoma cells (Figure 5G).

miR-17–92 Regulates mTORC1 Signaling in Lymphoma through LKB1 Silencing

LKB1 is a tumor suppressor mutated in a number of human cancers and plays a central regulatory role in tumor metabolic and cell growth control through downstream effects on AMPK and mTOR signaling (Shackelford and Shaw, 2009). One prediction of our findings was that miR-17–92 may influence these pathways through suppression of LKB1-dependent signaling. Deletion of miR-17–92 promoted an increase in basal AMPK signaling, as determined by increased AMPKα phosphorylation at Thr-172 and increased phosphorylation of the AMPK targets ULK1 (Ser-555) and Raptor (Ser-792) in Δ/Δ lymphoma cells (Figure 6A). mTORC1 pathway activity, as determined by rS6
and 4EBP phosphorylation, was decreased in Δ/Δ lymphoma cells (Figure 6B). Similar patterns of increased AMPK activity and decreased mTORC1 activity were observed in lymphoma cells specifically lacking miR-17 and -20 (Δ17/20, Figure 6C), implicating the miR-17 seed family as a mediator of these signaling changes. Reducing LKB1 levels in Δ/Δ lymphoma cells by stable shRNA expression (Figure 6D) reversed the effects of miR-17/20 deletion on AMPK and mTORC1 pathway activity (Figure 6E).

LKB1 Suppression by miR-17 Dictates Metabolic Reprogramming and Tumorigenic Potential of Myc+ Lymphomas

We next examined the contribution of miR-17/20-dependent suppression of LKB1 to the metabolic reprogramming that drives Myc+ lymphoma growth. Silencing LKB1 increased metabolic enzyme expression in Δ/Δ lymphoma cells compared to Δ/Δ cells transduced with a control shRNA hairpin (Figure 7A). The recovery of metabolic gene expression in LKB1 shRNA-expressing Δ/Δ lymphoma cells correlated with increased ECAR and OCR levels (Figure 7B), increased glucose consumption and lactate production (Figure S6A), and increased gluconeogenesis (Figure S6B) in these cells. We next cultured Δ/Δ lymphoma cells expressing control or LKB1 shRNAs with either U-[13C]-glucose (Figures 7C and S6C) or U-[13C]-glutamine (Figures 7D and S6D). Silencing LKB1 in Δ/Δ lymphomas restored the defects in central carbon metabolism normally seen in these cells and was characterized by a re-emergence of Warburg metabolism ([13C]-glucose to lactate conversion, Figure 7C) and increased contribution of both glucose and glutamine to the TCA cycle (Figures 7C, 7D, S6C, and S6D).

Finally, we examined whether silencing LKB1 was sufficient to rescue the tumorigenic potential of lymphoma cells lacking miR-17/20. Expression of shRNAs targeting LKB1 reversed the proliferative defect of Δ/Δ lymphoma cells in vitro (Figure 7E). When injected into nude mice, Δ/Δ lymphoma cells expressing LKB1 shRNA (Δ/Δ+shLKB1) formed palpable lymph node tumors with latency similar to wild-type E1-Myc lymphomas expressing miR-17–92 (fl/fl) (Figure 7F), despite the fact that these cells lacked expression of any mature miR-17–92 derived miRNAs. These data indicate that the effects of miR-17–92 loss on cell metabolism and tumor growth can be overcome simply by disrupting LKB1 signaling, highlighting the miR-17-LKB1 circuit as a key regulator of tumor metabolism and growth.

DISCUSSION

Myc is among the most implicated genes in human cancer (Zack et al., 2013) and has been shown to coordinate multiple cellular behaviors in support of malignancy (Dang, 2012). Here, we describe a new paradigm for Myc-dependent metabolic reprogramming involving the polycistronic miRNA miR-17–92. Using isogenic Myc-dependent lymphoma cells lacking miR-17–92, we demonstrate that miR-17–92 is a critical regulator of metabolic reprogramming in Myc+ tumors. We show that miR-17–92 is required to sustain both glycolytic and oxidative metabolism and promote multiple anabolic pathways required for tumor growth, while amplified miR-17–92 expression, which is observed in many tumor types, is sufficient for cell autonomous upregulation of tumor metabolic activity. Our data implicate the miR-17 seed family (comprising miR-17 and miR-20) as the key regulatory element of miR-17–92 that influences both metabolic reprogramming and the oncogenic potential of Myc-dependent lymphomas through negative regulation of the LKB1 tumor suppressor. Our results establish metabolic reprogramming as a central function for miR-17–92 and implicate metabolic control as an essential part of the oncogenic activity of this miRNA cluster.

Previous work has established miR-17–92 as an oncogenic miRNA cluster through its ability to promote tumor cell...
proliferation, cell viability, and angiogenesis while inhibiting cellular differentiation and senescence (Dews et al., 2006; Olive et al., 2010, 2013). Our data extend the regulatory repertoire of miR-17–92 to include many of the core metabolic processes in cancer. Although Myc can influence specific metabolic nodes such as glutaminolysis through suppression of miRNAs targeting metabolic enzymes (Gao et al., 2009), our data indicate that miR-17–92 functions broadly to support Myc-dependent metabolic programs. Loss of miR-17–92 in Eμ-Myc lymphoma cells leads to reduced transcription of core bioenergetic pathway genes (glycolysis; TCA cycle; OXPHOS) while simultaneously altering anabolic pathways (pentose phosphate pathway; biosynthesis of serine, aspartate, and glutamine; purine and pyrimidine metabolism) and ribosome biogenesis. This global reduction in metabolic pathway gene expression is coupled to reduced nutrient consumption and processing, including glucose and glutamine, which serve as two major carbon sources for lymphoma growth.

Although miR-17–92 cooperates with Myc to promote tumor aggressiveness (He et al., 2005; Mu et al., 2009; Olive et al., 2009), recent work has demonstrated Myc-independent roles for miR-17–92 in tumor maintenance (Li et al., 2014). Our data indicate that miR-17–92 expression influences almost 50% of the Myc-regulated transcriptome in Myc+/− lymphomas. Included in this regulation are a majority of metabolic pathway genes previously established as Myc targets (Lin et al., 2012a; Wang et al., 2011), suggesting that many of the effects on metabolism of miR-17–92-deficient (ΔΔ) lymphoma cells is simply a by-product of reduced cell size and/or proliferation but rather reflects the action of the clus-

Figure 7. miR-17/20-Dependent Control of LKB1 Dictates Metabolic and Tumorigenic Potential of Myc+ Lymphoma

(A) Immunoblot for metabolic enzyme expression in control (fl/fl) or ΔΔ Eμ-Myc lymphoma cells expressing control (Ctrl) or LKB1-specific shRNAs (LKB1). (B) ECAR and OCR of control (fl/fl, white) or ΔΔ Eμ-Myc lymphoma cells expressing control (Ctrl, black) or LKB1-specific shRNAs (LKB1) as in (A). Shown are two independent LKB1 shRNA clones (dark gray, shLKB1-1; light gray, shLKB1-2). (C) Relative abundance of lactate, citrate, and glutamate in control (fl/fl) or ΔΔ Eμ-Myc lymphoma cells expressing the indicated shRNAs. Cells were cultured for 2 hr in medium containing U-[13C]-glucose and unlabeled glutamine, and the proportion of 13C-labeled (black bar) or unlabeled (12C, white bar) metabolites as determined by GC-MS is shown. (D) Relative abundance of glutamate, citrate, and aspartate in ΔΔ Eμ-Myc lymphoma cells cultured with U-[13C]-glutamine. The proportion of 13C-labeled (gray bar) or unlabeled (12C, white bar) metabolites is shown. (E) Growth curve of control (fl/fl) or ΔΔ Eμ-Myc lymphoma cells expressing control (Ctrl, black) or LKB1-specific shRNAs (shLKB1-1, dashed line; shLKB1-2, green). (F) Kaplan-Meier curve showing latency to tumor onset for control (fl/fl, n = 14) and miR-17–92-deficient (ΔΔ) Eμ-Myc lymphoma cells (ΔΔ, n = 9), or ΔΔ lymphomas expressing control (Ctrl, n = 10) or LKB1-specific (LKB1-1, n = 13; LKB1-2, n = 12) shRNAs.

**p < 0.01; ***p < 0.001.
Together our data highlight a central role for LKB1, and the LKB1 tumor suppressor pathway. LKB1 has widespread impact on tumor growth and metabolism, in part by modulating AMPK and mTORC1 activity (Shackelford and Shaw, 2009). Reducing LKB1 signaling promotes the development of tumors with high mTORC1 activity and an aggressive metabolic phenotype (Dupuy et al., 2013; Faubert et al., 2014; Shaw et al., 2004). Somatic inactivating mutations in LKB1 are observed in many cancers, including ~30% of non-small cell lung cancer (NSCLC) cases (Gill et al., 2011). However, biallelic loss of LKB1 is rare among tumors with inactivating LKB1 mutations. Rather, haploinsufficiency of LKB1, similar to other tumor suppressors including Tsc2, Pten, and Smad4, is sufficient to promote tumorigenesis (Yoo et al., 2002), arguing that gene dosage is critical for LKB1-dependent tumor suppression. In this regard, miR-17~2~92-dependent post-transcriptional regulation of LKB1 may help fine-tune its tumor suppressor functions. This has clear implications for the tumorigenic potential of Myc+ tumors, as silencing LKB1 reverses the severe metabolic and growth defects of lymphoma cells lacking miR-17~2~92.

Our results indicate that post-transcriptional regulation of LKB1 by miRNAs can allow tumor cells to bypass LKB1-mediated growth suppression without the need for inactivating STK11 mutations. This raises the possibility that miRNA-dependent tuning of LKB1 levels may be a general mechanism for balancing cellular bioenergetics and cell growth in cancer, and may contribute to the pathophysiology of LKB1-regulated diseases such as Peutz-Jeghers syndrome. It is possible that endogenous miRNAs in addition to miR-17 are capable of silencing LKB1 in tumor cells and that miRNAs targeting other components of the LKB1 complex, such as miR-451 (Godlewski et al., 2010), may exert similar effects on tumor metabolism by influencing LKB1-dependent signaling. miR-17~2~20 belong to a larger miRNA family that includes miR-93, -106a, and -106b, all sharing a common seed sequence (5’-AAA GUG-3’). Interestingly, miR-106a and -106b, which are also encoded by miRNA clusters (miR-106a~363 and miR-106b~25, respectively), are also associated with oncogenesis (Conkrite et al., 2011; Landais et al., 2007), although our data suggest that these miRNAs do not compensate for the loss of miR-17~2~92 in the lymphoma cells examined here. Whether related miR-17 family members also influence LKB1 expression and/or tumor metabolism remains to be determined. Together our data highlight a central role for miR-17~2~92 in balancing both metabolic advantage and growth potential in cancer.

EXPERIMENTAL PROCEDURES

Cell Lines, DNA Constructs, and Cell Culture

The generation of E16-Myc Cre-ERT2; miR-17~2~92+ lymphoma cells has been described previously (Mu et al., 2009). E16-Myc cells were cultured on a layer of irradiated Ink4a-null MEF feeder cells in DMEM and IMDM medium (50:50 mix) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and β-mercaptoethanol. Raji cells were cultured in RPMI medium supplemented with 10% FBS and 2 mM glutamine. Retroviral-mediated gene transfer into lymphoma cells was conducted as previously described (Faubert et al., 2013).

For growth assays, cells were seeded at a density of 1 × 10^6 cells/ml in 3.5-cm dishes, and cell counts were determined via trypan blue exclusion using a TC20 Automated Cell Counter (Bio-Rad). For viability measurements, cells were stained with Fixable Viability Dye eFluor 780 (eBioscience) and analyzed using a Gallios flow cytometer (Beckman Coulter) and FlowJo software (Tree Star). Cell size was determined by forward scatter using flow cytometry.

Seahorse Analysis and Metabolic Assays

OCR and ECAR were determined using an XF96 Extracellular Flux Analyzer (Seahorse Bioscience) using established protocols (Faubert et al., 2014; Vincent et al., 2015a). For media metabolite determination, cells were cultured for 2 days, and culture medium was analyzed for extracellular metabolites (glucose, glutamine, lactate, and ammonia) using a BioProfile Analyzer (NOVA Biomedical) as previously described (Vincent et al., 2015a). Metabolic parameters were assessed in lymphoma cells undergoing logarithmic growth and standardized to cell number.

For metabolomics experiments using 13C-labeled glucose or glutamine, lymphoma cells (~3 × 10^6 per 3.5-cm dish) were cultured for 2 hr in glucose- and glutamine-free DMEM/IMDM (50:50 mix) containing 10% dialysed FBS (Wisent Bioproducts) and either uniformly labeled [1-13C]-glucose or [1-13C]-glutamine (Cambridge Isotope Laboratories). 13C-glutamine (2 mM) or -glucose (25 mM) were added back to the culture medium depending on the tracer used. Cells were washed twice with saline and then lysed in ice-cold 80% methanol, sonicated, derivatized as tert-butyl(dimethyl)silyl (TBDMS) esters, and analyzed by GC-MS using previously described protocols (Dupuy et al., 2013; Faubert et al., 2014; McGurk et al., 2013). Metabolite abundance was expressed relative to an internal standard (D-glucaric acid) and normalized to cell number. Additional experimental details are described in Supplemental Experimental Procedures.

Immunoblotting, Quantitative Real-Time PCR, and RNA Sequencing

Lymphoma cell lines were subjected to SDS-PAGE and immunoblotting using CHAPS and AMPK lysis buffers as previously described (Faubert et al., 2013). Primary antibodies against hexokinase 2, aldolase, LDHA, GLS2, and GAPDH were obtained from Santa Cruz Biotechnology. For qPCR quantification of mature miRNAs, Qiazol was used to isolate RNA, miRNAeasy Mini kit was used to purify miRNAs and total mRNA, and cDNA synthesized using the miScript II RT kit (QIAGEN). qPCR was performed using the SensiFAST SYBR HI-ROX kit (Bioline) and an AriaMX Real Time Pcr system (Agilent Technologies), miScript primer assays (QIAGEN) were used to detect mature miRNAs of the miR-17~2~92 cluster, with miRNA expression normalized relative to U6 RNA levels. RNA preparation and library construction for RNA sequencing was conducted as previously described (Jha et al., 2015). Libraries were sequenced using a Hiseq 2500 (illumina) using 40 bp by 10 bp pair-end sequencing. Gene set enrichment analysis (GSEA) on RNA-seq data was conducted using the gage function and non-parametric Kolmogorov-Smirnov test from the GAGE R Biocouporter package (Luo et al., 2009).

For miRNA quantification, cDNA was amplified using previously described (Jha et al., 2015). Additional experimental details are described in Supplemental Experimental Procedures.

3 UTR Cloning and Validation

3’ UTR isoforms for mouse Stk11 were determined by 3’ RACE as previously described (Wu et al., 2019). GeneArt DNA fragments (Life Technologies) for
either the wild-type Stk11 3’ UTR or harboring a mutated miR-17 seed region (bases 2–8 in the seed region) were cloned into the Xhol/XbaI sites of the pmirGLO vector (Promega). 293T cells (10^5 cells/well) stably expressing FLAG-Ago2 (Valdmanis et al., 2012) were transfected with individual pmirGLO-3’ UTR constructs with or without co-transfection of a miR-17 expression plasmid (miR-17x4) (Hong et al., 2010). Cells were lysed 48 hr post-transfection, and the ratio of luciferase to renilla luciferase activity determined using a Dual-Glo Luciferase assay kit (Promega) and a FLUOstar Omega plate reader. FLAG-tagged LkB1 expression constructs were generated by PCR amplification of the full-length LkB1 coding sequence from Eμ–Myc lymphoma RNA, using sequence specific primers to amplify inserts with short or long forms of the Stk11 3’ UTR.

Tumor Xenograft Assays

Lymphoma cells were resuspended in Hank’s balanced salt solution (HBSS) and injected intravenously (10^6 cells/mouse in 200 μl) into CD-1 nude mice (Charles River Laboratories). Tumor onset was monitored by palpation of inguinal and axillary lymph nodes, and tumor-free survival scored as the time elapsed between injection and first detection of palpable tumors as previously described (Faubert et al., 2013). All procedures were carried out in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University.

Statistical Analysis

Statistics were determined using paired Student’s t test, ANOVA, or log-rank (Mantel-Cox) using Prism software (GraphPad) unless otherwise stated. Data are calculated as the mean ± SEM for biological triplicates and the mean ± SD for technical replicates unless otherwise stated. Statistical significance is represented in figures by *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

ACCESSION NUMBERS

The accession number for the RNA-seq data reported in this paper is GEO: GSE77010.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.07.036.

AUTHOR CONTRIBUTIONS

Experimental design was conducted by S.I., B.S., S.M.S., T.F.D., and R.G.J., and the majority of experiments were executed by S.I. and B.S. Data interpretation was performed by S.I., B.S., E.H.M., T.F.D., and R.G.J. RNA-seq and metabolic network analysis were conducted by R.M.J., A.S., and M.N.A. The tumor xenograft assay was performed by S.I., B.S., E.H.M., T.F.D., and R.G.J. RNA-seq and the majority of experiments were executed by S.I. and B.S. Data interpretation was performed by S.I., B.S., E.H.M., T.F.D., and R.G.J. Statistical analysis was conducted by S.I., B.S., E.H.M., T.F.D., and R.G.J. The manuscript was written and edited by S.I., B.S., E.E.V., T.F.D., and R.G.J.

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