S6K1 determines the metabolic requirements for BCR-ABL survival

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Abstract

In Chronic Myelogenous Leukemia, the constitutive activation of the BCR-ABL kinase transforms cells to an "addicted" state that requires glucose metabolism for survival. We investigated S6K1, a protein kinase that drives glycolysis in leukemia cells, as a target for counteracting glucose-dependent survival induced by BCR-ABL. BCR-ABL potently activated S6K1-dependent signaling and glycolysis. Although S6K1 knockdown or rapamycin treatment suppressed glycolysis in BCR-ABL transformed cells, these treatments did not induce cell death. Instead, loss of S6K1 triggered compensatory activation of fatty acid oxidation, a metabolic program that can support glucose-independent cell survival. Fatty acid oxidation in response to S6K1-inactivation required the expression of the fatty acid transporter Cpt1c, which was recently linked to rapamycin resistance in cancer. Finally, addition of an inhibitor of fatty acid oxidation significantly enhanced cytotoxicity in response to S6K1 inactivation. These data indicate that S6K1 dictates the metabolic requirements mediating BCR-ABL survival and provide a rationale for combining targeted inhibitors of signal transduction with strategies to interrupt oncogene-induced metabolism.

Keywords

S6K1; glycolysis; fatty acid oxidation; rapamycin; leukemia; Cpt1c

Introduction

Oncogenic signaling pathways, such as Ras, Akt and myc, reprogram the metabolism of transformed cells to promote cell survival (1). In particular, Akt signaling can trigger increased cell survival that depends on continued glycolytic metabolism (2, 3). Beyond glycolysis, it is now recognized that oncogenes such as myc and ErbB2 induce glutaminolysis and fatty acid oxidation (FAO) to fuel proliferation and survival (4-6). Oncogene-induced metabolic programs have been investigated as targets for

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chemotherapeutics. Compounds that inhibit or redirect glucose metabolism such as dichloroacetate, 2-deoxyglucose and 3-bromopyruvate have demonstrated substantial therapeutic potential in animal models, but are still at early stages of application in the clinic (7-9). Because specific oncogenic signaling pathways propel specific metabolic programs, an alternative approach to counteracting oncogenic metabolism is to target the signaling pathways responsible for metabolic reprogramming.

In Chronic Myelogenous Leukemia (CML), the BCR-ABL oncogene activates glucose metabolism as part of its transforming activity. Activation of glycolysis in BCR-ABL^{+} cells is associated with an increase in GLUT-1 glucose transporter molecules at the membrane and suppression of p53 (10-12). In response to the BCR-ABL inhibitor imatinib, reduction in surface localization of GLUT-1 correlates with decreased glucose uptake and lactate production. Importantly, inhibition of glycolysis can enhance the imatinib cytotoxicity (10). Thus glycolytic metabolism is suggested to play a key role in determining imatinib efficacy and provides a rationale for targeting glycolytic metabolism therapeutically (13, 14).

While inhibition of BCR-ABL kinase activity enables long-term suppression of disease for a majority of patients, imatinib resistance is observed in advanced stages of disease or with the outgrowth of clones containing specific imatinib resistance mutations (15). Resistance may be a result of mutations in the BCR-ABL kinase domain, or with gene amplification or activation of alternative signaling pathways such as the PI3K/Akt pathway (16-18). The PI3K/Akt pathway activates glycolysis and is required for transformation of BCR-ABL^{+} leukemia cells (19, 20).

We previously found that targeting S6K1 reduces glycolysis in PTEN-deficient leukemia cells (21). Decreased glycolysis enhanced apoptosis and reduced the leukemic potential of PTEN-deficient cells. S6K1 inactivation recapitulated the effects of rapamycin, suggesting that the majority of the chemotherapeutic benefits of rapamycin may be mediated by S6K1 inactivation. We therefore investigated the metabolic effects of rapamycin and S6K1 inactivation in BCR-ABL^{+} cells. Although S6K1 was required for glycolysis, S6K1 inactivation did not trigger programmed cell death. Instead, inactivation of S6K1 triggered a compensatory metabolic pathway that supported cell survival in BCR-ABL^{+} cells. Targeting this compensatory metabolic response in conjunction with mTORC1/S6K1 significantly enhanced the cytotoxic efficacy of rapamycin and S6K1 inactivation.

Results

BCR-ABL activates glycolysis through S6K1

To determine the effect of BCR-ABL on the regulation of glycolysis, we measured glycolysis in IL-3 dependent FL5.12 immortalized murine hematopoietic progenitor cells that were transduced with the p210 isoform of BCR-ABL. BCR-ABL substantially increased glycolysis in cells cultured in either the presence or absence of cytokine (Figure 1A). Increased glycolysis was associated with cytokine-independent activation of S6K1 (Figure 1B). S6K1 knockdown reduced glycolysis in BCR-ABL cells, indicating a requirement for S6K1 signaling to maintain glycolysis (Figure 1C), similar to the effect of S6K1 knockdown in PTEN-deficient cells (21). Rapamycin and its analogs (such as

everolimus and temsirolimus) are approved cancer chemotherapeutics that can prevent the activation of S6K1 by mTORC1 (22). Similar to S6K1 knockdown, rapamycin suppressed glycolysis in BCR-ABL transformed FL5.12 cells and also the human BCR-ABL[+] cell lines KBM7 and K562 (Figures 1D,F, G). The BCR-ABL tyrosine kinase inhibitor imatinib also reduced glycolysis, indicating that elevated glycolysis in these cells was induced by the transforming oncogene (Figures 1E and G). Altogether, the data indicate that mTORC1-S6K1 signaling mediates the induction of glycolysis downstream of BCR-ABL.

BCR-ABL survival is glucose-dependent

There is increasing interest in exploiting oncogenic cellular metabolism to induce or enhance cytotoxicity in cancer cells. Glucose-dependent survival is associated with the activation of S6K1 (21). As BCR-ABL is a strong activator of S6K1 (23) (Figure 1B), we determined the requirement for glycolysis in BCR-ABL transformed cells. The data showed that BCR-ABL-dependent survival is severely compromised in media containing 0.02 mM glucose, a concentration that is known to be rate-limiting for cultured cells (24) (Figure 2A). Cell death of BCR-ABL cells exceeded the of cells expressing constitutively active myristoylated Akt (myrAkt), which is a well-recognized mediator of glucose-dependent survival (2, 3). In contrast, Bcl-xL-dependent survival, which depends more on autophagy than glycolysis for survival, exhibited minor cytotoxicity in response to reduced glucose availability (2, 25). Similar to BCR-ABL[+] FL5.12 cells, K562 cells were also glucose-dependent (Figure 2B). Glucose limitation induced cell death at a rate that was comparable to the effect of the BCR-ABL tyrosine kinase inhibitor imatinib (Figure 2B). These data indicate that BCR-ABL signaling engages a glucose-dependent survival program. We therefore investigated the effect of inactivating S6K1 on BCR-ABL-dependent survival.

S6K1 is not required for the BCR-ABL survival program

Because S6K1 inactivation suppressed glycolysis (Figure 1), and glucose was required for the survival of BCR-ABL transformed cells (Figure 2), we hypothesized that S6K1 inactivation would trigger cell death in BCR-ABL cells. Loss of S6K1 was sufficient to reduce survival in glucose-dependent cells expressing an activated form of Akt (myrAkt) (Figure 3A), in agreement with our previous findings (21). However, loss of S6K1 did not trigger cell death in BCR-ABL cells, despite effective S6K1 knockdown (Figure 3A, B).

To understand how the inactivation of S6K1 could reduce glycolysis without triggering cell death in BCR-ABL cells, we determined the requirement for glucose in cells transfected with control and S6K1 siRNAs. In vector control cells, the presence or absence of S6K1 had no impact on the ability of cells to survive in the presence or absence of glucose (Figure 3C). In BCR-ABL expressing cells, survival was compromised in low glucose conditions, as shown in Figure 2. Interestingly, after knockdown of S6K1, BCR-ABL-expressing cells acquired a survival advantage, sustaining a significant increase in viability in low glucose (Figure 3C). This result was confirmed with a separate S6K1 targeting sequence in shRNA-transduced cells (Supplemental Figure 1). S6K1-knockdown cells sustained higher levels of ATP compared to control cells (Figure 3D) when measured at a timepoint prior to commitment to apoptosis (no cleavage of Caspase 3). This suggests that BCR-ABL cells activate a metabolic pathway that can confer a bioenergetic advantage (Figure 3D).

Rapamycin triggers S6K1 inactivation by preventing its phosphorylation by mTORC1. In BCR-ABL+ FL5.12 cells, rapamycin did not induce cell death in glucose-containing media, and conferred a survival advantage under low glucose conditions (Figure 3E). Similarly, rapamycin enhanced survival in BCR-ABL+ primary mouse hematopoietic cells cultured under low glucose conditions (Figure 3F). In the human K562 line, rapamycin also enhanced cell survival under low glucose conditions (Figure 3G). These data indicate that inactivation of mTORC1-S6K1 permits BCR-ABL cells to induce a glucose-independent survival program that can partially substitute for the survival signals transduced by S6K1.

Previous work demonstrated that under nutrient starvation conditions, mTORC1-S6K1 signaling is acutely extinguished (26-29). However, S6K1 activity can recover as cells engage alternative metabolic programs (28). The knockdown of S6K1 enhanced survival under low glucose conditions, suggesting that perhaps BCR-ABL elevated S6K1 activity even under nutrient-limiting conditions. To determine the relative activity of S6K1 downstream of BCR-ABL under nutrient limiting conditions, we examined S6K1 phosphorylation and the phosphorylation of the ribosomal protein S6 over time after switching cells to low glucose conditions. We found elevated S6K1 and S6 phosphorylation in BCR-ABL cells at all timepoints examined, relative to control cells (Supplemental Figure 2). At later timepoints, S6K1 and S6 phosphorylation exhibited a modest recovery. These data indicate that downstream of BCR-ABL, S6K1 signaling can continue to regulate metabolism and survival under nutrient-limiting conditions.

Inactivtion of mTORC1 and S6K1 can trigger increases in the activity of upstream kinases such as Akt, due to loss of feedback regulation. This effect, often described as "rebound activation" of Akt, has been proposed to mediate cell survival in transformed cells (30). Although Akt activation has been previously associated with glucose-dependent survival (Figure 3A and (2)), we tested whether rebound activation of Akt was associated with increased survival in BCR-ABL+ cells cultured under low glucose conditions. In BCR-ABL + FL5.12 cells, S6K1 knockdown triggered increased Akt phosphorylation at serine 473, in agreement with these previous findings (Supplemental Figure 3A). The PI3K inhibitor BEZ235 prevented this increase in Akt activation (Supplemental Figure 3A), but did not prevent glucose-independent survival in response to S6K1 inactivation (Supplemental Figure 3B). Rapamycin treatment can induce rebound Akt activation in short-term cultures, but long term treatment can suppress Akt activation by the upstream kinase mTORC2 (31). Consistent with this observation, rapamycin did not trigger increased Akt phosphorylation even though rapamycin sustained glucose-independent survival (Supplemental Figure 3C, Figure 3E). Related to these effects, S6K2 has been shown to functionally compensate for S6K1 inactivation in cellular transformation (32), but we observed no alteration in S6K2 expression levels in response to S6K1 knockdown or rapamycin (Supplemental Figure 3C). Altogether these results suggest that rebound activation of Akt is not required for glucose-independent survival.

S6K1 is not required for BCR-ABL leukemogenesis

Activation of the PI3K/Akt pathway is important for BCR-ABL transformation and may play a role in therapeutic resistance (20, 33). Chemotherapeutic treatment with rapamycin

can interfere with signaling downstream of PI3K/Akt by preventing mTORC1 phosphorylation of substrates. In mice transplanted with BCR-ABL[+] bone marrow cells, rapamycin can delay the development of fatal myeloproliferative disease (34). These results established the benefits of interfering with mTORC1 signaling downstream of BCR-ABL with rapamycin, although it is not clear if this was due to cytostatic or cytotoxic effects. To determine the consequences of S6K1 inactivation in BCR-ABL[+] myeloproliferative disease, we transplanted BCR-ABL[+] S6K1^{+/+} or BCR-ABL+ S6K1^{−/−} bone marrow cells into recipient mice. We observed a trend towards a more aggressive disease in mice transplanted with BCR-ABL-transduced S6K1^{−/−} bone marrow cells, compared to S6K1^{+/+} (Figure 4). Despite the acceleration of disease kinetics, there were no significant differences in disease characteristics at the endpoint of the experiment, including splenomegaly, accumulation of myeloid cells and accumulation of GFP[+] (BCR-ABL^{+}) cells in the bone marrow (Figure 4B). That the loss of S6K1 did not slow the progression of myeloproliferative disease suggests that an alternative metabolic program substituted to support BCR-ABL oncogenesis despite decreased glycolysis in S6K1-deficient cells. Therefore we determined the requirement for metabolic programs that can substitute for glycolysis in transformed cells.

Loss of S6K1 activates fatty acid oxidation and glucose-independent survival in BCR-ABL^{+} cells

Activation of fatty acid oxidation (FAO) can substitute for decreased glycolysis to promote cell survival (4, 5). Recent studies showed that inactivation of mTORC1 can trigger a switch from glycolytic to oxidative forms of metabolism in immortalized fibroblast cells (35). Therefore, we determined whether the rate of FAO in BCR-ABL^{+} cells responded to S6K1-knockdown. Using the rate of ^{3}H release from ^{3}H-palmitate to measure FAO, we found significantly increased FAO in both human and murine BCR-ABL expressing cells upon knockdown of S6K1 or upon treatment with rapamycin (Figure 5A-C, Supplemental Figure 4). ^{3}H release from palmitate was mediated by mitochondrial FAO because treatment with etomoxir, an inhibitor of the mitochondrial carnitine palmitoyl transferase (CPT) system, which is essential for FAO (36), reduced ^{3}H release (Figure 5A-C).

Inactivation of S6K1 increased FAO, but it was not known whether FAO mediated glucose-independent survival. We cultured S6K1 knockdown/BCR-ABL^{+} cells in low glucose and in the presence and absence of etomoxir (Figure 6A). Etomoxir prevented the survival of S6K1-deficient cells, restoring glucose-dependence to BCR-ABL^{+} cells despite the reduction in S6K1. To test whether activation of FAO is sufficient for glucose-independent survival of BCR-ABL^{+} cells, we cultured cells with 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, an agonist for the AMP-activated protein kinase (AMPK), which can activate FAO. AMPK activation triggered glucose-independent survival to a similar level as S6K1 knockdown (Figure 6B). Importantly, treatment with the FAO inhibitor etomoxir prevented cell survival mediated by both AICAR and S6K1 knockdown (Figure 6B). The ability of AICAR to promote survival in an etomoxir-sensitive manner suggests that FAO is necessary and sufficient for glucose-independent survival in BCR-ABL-transformed cells.

Etomoxir targets the import of fatty acids into mitochondria by carnitine palmitoyltransferase 1 (Cpt1). There are three isoforms of Cpt1, each coded by a distinct genomic locus, designated *Cpt1a, Cpt1b*, and *Cpt1c* (37). Increased expression of *CPT1C* was recently described in human lung cancers, and expression correlated with increased FAO in these tumors (38). Interestingly, *CPT1C* expression was sufficient to overcome rapamycin repression of cell growth in a breast cancer cell line. However, it was not known which of the effectors downstream of mTORC1 determined the requirement for Cpt1c.

We investigated the effect of depleting Cpt1a or Cpt1c on glucose-independent survival in BCR-ABL^{+} cells lacking S6K1. Knockdown of *Cpt1c* reduced the survival of S6K1-deficient cells in low glucose conditions (Figure 6C, Supplemental Figure 5). The requirement for *Cpt1c* was specific, as knockdown of *Cpt1c*, but not *Cpt1a*, reduced the survival of BCR-ABL^{+} cells lacking S6K1. FAO can be induced by AMPK activation in fibroblasts. Although AMPK can mediate induction of FAO and *Cpt1c* mRNA, we did not observe a requirement for AMPK catalytic subunits (*Prkaa1* and *Prkaa2*) for cell survival in response to S6K1 knockdown (data not shown). Nevertheless, reduced viability of cells transfected with siCpt1c confirms that FAO is required for S6K1-independent survival.

The induction of glucose-independent survival may explain why siS6K1 failed to induce cytotoxicity despite a strong effect in suppressing glycolysis (Figures 1C, 3A). Because FAO is required for glucose-independent survival, we tested the combination of siS6K1 with etomoxir under full glucose conditions. Again, siS6K1 was unable to induce cell death as a single agent, while etomoxir triggered only a mild cytotoxic effect as a single agent (Figure 7A). However, S6K1 knockdown in combination with etomoxir induced significant apoptosis in cells cultured in full glucose (Figure 7A). A similar effect was observed in rapamycin-treated cells, though combination of rapamycin with etomoxir was not as strong as S6K1 inactivation (Supplemental Figure 6). In dose curve analysis, S6K1 knockdown enhanced cell death in response to etomoxir at doses ranging from 200-300 μM, suggesting a synergistic effect (Figure 7B).

Altogether these data demonstrate that S6K1-inactivation triggered the compensatory activation of FAO, a pro-survival metabolic program that was not previously available to cells expressing S6K1. Furthermore, counteracting FAO resulted in improved cell death in response to the S6K1 inactivation, suggesting a new approach based on inactivation of the pro-glycolytic signaling pathway in conjunction with the inactivation of a metabolic adaptive response.

Discussion

In this study we demonstrated that BCR-ABL requires glucose to mediate cell survival. Increased glucose transporter expression, glucose uptake and lactate production have been previously reported in BCR-ABL^{+} cells (13, 14), and resistance to tyrosine kinase inhibitors is correlated with sustained glucose metabolism. Increased glycolysis coupled with a requirement for cell survival is associated with loss-of-function mutations in the PTEN tumor suppressor, or activating mutations in PI3K subunits in multiple cancers (39). These

mutations trigger increased signaling through key downstream protein kinases, including Akt, mTORC1, and S6K1, which mediate increased glycolysis (2, 21, 40).

Although S6K1 was required for BCR-ABL to induce glycolysis, the metabolic requirements for survival were altered when S6K1 was inactivated (Supplemental Figure 7). Under low-glucose conditions, S6K1 inactivation paradoxically conferred a survival advantage to BCR-ABL cells, which was recapitulated by rapamycin treatment. Thus an alternative metabolic program that provides a cell survival advantage compensated for loss of S6K1. In bone marrow transplant experiments using BCR-ABL$^+$ S6K1$^{-/-}$ cells, the oncogenic potential of BCR-ABL was not compromised and trended towards more aggressive disease, indicating that the oncogene can function independent of the mTORC1-S6K1 pathway. This differed from the delay in leukemia that we observed in PTEN-deficient S6K1$^{-/-}$ leukemias (21), but is consistent with the previously published lack of a requirement for S6K1 in mediating neuronal hypertrophy (41). We propose that the requirement for S6K1 in oncogenesis varies depending on the transforming mutations and cellular background.

Rapamycin can delay leukemia progression in mice transplanted with BCR-ABL$^+$ bone marrow cells (34). One difference between the effects of rapamycin and S6K1 inactivation may be related to the potential for rapamycin to reduce mTORC1 phosphorylation of additional targets that regulate cell cycle progression, such as 4EBP-1, S6K2 and/or ULK1. Importantly, 4EBP1 was recently shown to mediate cytostatic responses, while S6K1 regulates growth and metabolism (21, 42, 43). It is possible that rapamycin has mixed effects in BCR-ABL leukemogenesis, inducing a cytostatic response, but also activating S6K1-independent metabolic and survival effects. Indeed, there is evidence that rapamycin can preserve BCR-ABL$^+$ leukemia-initiating cells in serial transplant experiments (44). Similar issues involving cytostatic and pro-survival effects have been raised with the application of rapamycin and its analogs in solid tumor settings (45).

Our results indicate that an inhibitor of FAO could tip responses to inactivation of mTORC1-S6K1 towards induction of programmed cell death (Supplemental Figure 7). FAO was previously shown to compensate for the loss of glycolysis in glioblastoma cell lines and 3-dimensional cultures of breast epithelial cells (4, 5). Interestingly, glucose-independent survival in BCR-ABL-transformed cells required Cpt1c, the CPT enzyme that was recently identified to correlate with rapamycin resistance in human lung tumors (38). Our results suggest that expression of Cpt1c may be an important determinant of cell death responses to mTORC1-S6K1 inactivation.

The mTORC1-S6K1 pathway has been previously linked to the regulation of FAO. In mice with liver-specific deletion of the TSC1 tumor suppressor protein, which results in elevated mTORC1 activation, liver FAO is decreased (46). Conversely, inactivation of S6K1 in muscle triggers increased expression of CPT1 mRNA (47). Together, these reports suggest that at least in insulin-responsive tissues, FAO is inversely correlated with signal transduction through the mTORC1-S6K1 pathway. However, increased FAO is not a necessary response to inactivation of mTORC1-S6K1, as suggested by our earlier studies in PTEN-deficient cells, in which inactivation of mTORC1-S6K1 reduced glycolysis and

induced programmed cell death. This suggests that oncogene or cell-type specific factors govern the metabolic response to mTORC1-S6K1 inactivation.

Unsatisfactory clinical trials with rapamycin and its analogs have led many to propose that feedback loops in signal transduction pathways induce compensatory survival signals. To overcome feedback effects in signal transduction, inhibitors that target multiple points of the PI3K-Akt-mTORC1-S6K1 pathway are under intense investigation (30, 48). Our data indicate that metabolic adaptive responses can also play a role in suppressing chemotherapy cytotoxicity. Thus it may be necessary to target the compensatory metabolic programs that contribute to cell survival in order to achieve the full therapeutic potential of agents that target PI3K-Akt-mTORC1-S6K1.

Materials and Methods

Cell analysis

IL-3 dependent FL5.12 cells were cultured as previously described (21). Human BCR-ABL[+] cell lines were cultured in RPMI containing 20% FBS, HEPES, 2-ME, penicillin and streptomycin. Low glucose media contains glucose-free RPMI (Mediatech Inc, Manassas, VA, USA), 10% dialyzed FBS (Sigma Aldrich, St. Louis, MO, USA), HEPES, 2-ME, penicillin and streptomycin and was supplemented with 10 mM or 0.02 mM Glucose (Thermo Fisher). 1×10^6 cells were transfected using 1μg Accell siRNA pools (Dharmacon, Thermo Fisher, Waltham, MA USA) using the G-016 program on the Nucleofector II (Amaxa Biosystems, Lonza, Walkersville, MD, USA) for both FL5.12 and human BCR-ABL[+] cell lines. siNon-targeting (siNT or NT) was incorporated as a negative control in all experiments involving siRNA. The shRNA construct targeting S6K1 was described previously (21). Viability was assessed using propidium iodide exclusion in a FACSAria flow cytometer (BD Franklin Lakes, NJ, USA). Cells were washed 3x in PBS and resuspended at a concentration of 2×10^5/mL in media supplemented with 2μg/mL of propidium iodide (Molecular Probes) prior to analysis. For the viability analysis of BCR-ABL[+] primary bone marrow cells, viability was measured after withdrawal from cytokines for 5 days in media containing full levels of glucose (10 mM) or low glucose (0.02 mM glucose).

Reagents

Rapamycin, BEZ235, and Imatinib were obtained from LC Labs. Etomoxir was from Sigma Aldrich (St Louis, MO, USA) and 5-Aminoimidazole-4-carboxyamide ribonucleoside (AICAR) was from Cell Signaling Technology (Danvers, MA, USA). The following antibodies were used for immunoblots: Akt p473, S6K1 p389, S6 p235/236, AMPK p172, Akt, S6k1, S6 and AMPK from Cell Signaling Technology (Danvers, MA, USA). ACC and ACC p79 were obtained from Millipore and GAPDH from Abcam (Cambridge, MA, USA). siRNA experiments used Accell pools of siRNA duplexes (Dharmacon, Lafayette, CO, USA) targeting each gene.

Quantitative Real-time PCR

RNA was isolated using Qiagen RNeasy mini kit. 1μg of RNA was reverse transcribed using TaqMan Reverse Transcription reagents (Applied Biosystems). Quantitative PCR was performed using TaqMan Gene Expression Master Mix and S6K1 and Actin TaqMan probes (Applied Biosystems).

Glycolysis

Glycolysis was measured using ^{3}H-glucose as described previously (2). Briefly, 1×10^6 cells were cultured with 5 μCi of 5-^{3}H-glucose for up to 2 hours at 37°C. Following incubation, 0.2M HCl was added to the mixture and the mixture was put into an eppendorf tube inside of a closed system to separate ^{3}H-water from ^{3}H-glucose. After 24-48 hours at room temperature, ^{3}H-water was equilibrated between the inner and outer chambers and the ^{3}H-water was measured in both chambers using a scintillation counter and standardized to controls. ^{3}H-H_2O was used as a standard to determine the efficiency of equilibration.

Fatty Acid Oxidation

Fatty acid oxidation was measured using a ^{3}H-palmitate adapted from a previous publication (49). Briefly, 0.5×10^6– 1×10^6 were washed with PBS, then cultured with 400μl of (9,10-^{3}H) Palmitate:albumin for 4 hours at 37°C. After incubation, 10% TCA was added to each tube and centrifuged at 3300rpm for 10 min at 4°C and then mixed with 6N NaOH and applied to ion-exchange columns. The column was washed with 1mL of water and the eluates were counted using a scintillation counter.

Mice

S6K1$^{+/+}$ and S6K1$^{-/-}$ mice were generously provided by G. Thomas and S. Kozma (50). C57/Bl6 mice were obtained from Jackson Laboratories. Lineage negative, Sca1$^+$, cKit$^+$ (LSK) hematopoietic cells from S6K1$^{+/+}$ or S6K1$^{-/-}$ mice were isolated using a FACSAria cell sorter. LSK cells were then transduced with BCR-ABL-GFP retrovirus. Recipient C57/Bl6 mice were lethally irradiated and 10,000 GFP$^+$ LSK cells supplemented with 300,000 whole bone marrow cells were injected via the tail vein. Survival of recipient mice that exhibited symptoms of a lethal myeloproliferative disease was measured from the day of bone marrow transplant and the log rank test was used to determine significance.

Statistics and Image Software

Statistical analysis using the log-rank test was performed in GraphPad Prism software. Error bars were calculated using standard deviation in Excel, except for qRT-PCR results, where standard error was employed. Figures were prepared using Adobe Photoshop, Adobe Illustrator, GraphPad Prism and Microsoft Excel, using Crop, Levels, Contrast, and Brightness controls applied to whole images.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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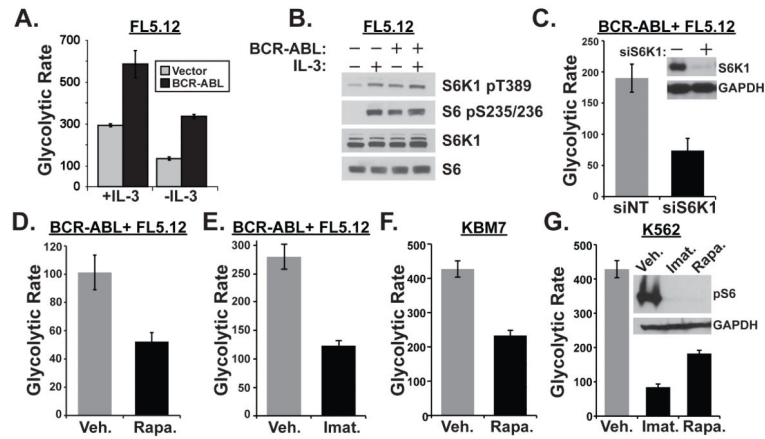
Figure 1.
BCR-ABL activates growth factor-independent glycolysis through S6K1. A. IL-3-dependent
FL5.12 cells were transduced with vector control or BCR-ABL-expressing retrovirus, then
cultured in the presence or absence of IL-3 for 3 hours. Glycolytic release of ^3H_2O from
5-^{3}H-glucose in the absence of growth factor was increased by BCR-ABL. The mean
±standard deviation is plotted. B. BCR-ABL strongly activated S6K1. Vector control and
BCR-ABL cells were cultured for 3 hours in the absence of growth factor, then restimulated
for 30 minutes where indicated for analysis of S6K1 phosphorylation at T389 and the
phosphorylation of ribosomal protein S6 at serines 235/236. C. S6K1 knockdown (inset)
impaired glycolysis in BCR-ABL$^+$ FL5.12 cells cultured in the absence of IL-3. siRNA
pools targeting S6K1 or Non-targeting siRNA were transfected where indicated. D - F.
Rapamycin (Rapa., 20 nM) and imatinib (1μM) suppressed glycolysis in BCR-ABL$^+$ FL5.12
cells (D and E), human BCR-ABL$^+$ KBM7 cells (F) and human BCR-ABL$^+$ K562 cells (G).
Inset, Both imatinib and rapamycin reduced S6K1 activity as indicated by reduced
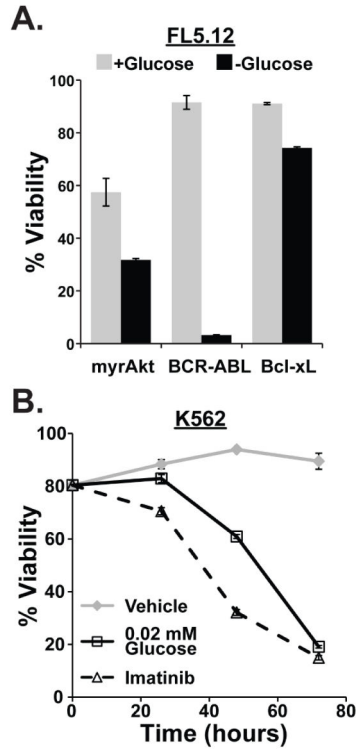phosphorylation of small ribosomal protein S6 (pS6).

Figure 2.
BCR-ABL-activated survival is glucose-dependent. A. Viability of BCR-ABL[+] FL5.12 cells cultured in cytokine-free medium containing 0.02mM glucose (-Glucose) for 48 hours was significantly reduced compared to cells cultured in 10mM glucose (+Glucose). Survival of cells expressing an activated mutant of Akt (myrAkt), which mediates a glucose-dependent form of survival is shown for comparison. For reference, the glucose-independent survival of cells expressing Bcl-xL is also shown. The mean of triplicate viability measurements ±standard deviation were determined by propidium iodide exclusion in a flow cytometer. B. BCR-ABL^{+} K562 cells require glucose for long-term survival. Cell death in response to low glucose and imatinib were comparable.
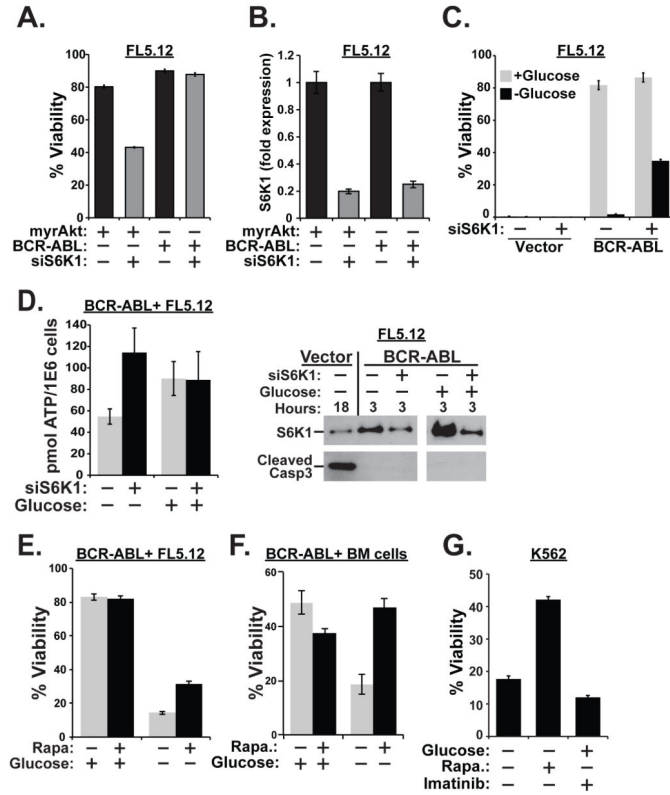
Figure 3.
Glucose independent survival upon loss of S6K1. A. S6K1 is not required for cytokine-independent survival in BCR-ABL[+] FL5.12 cells. Decreased viability in myrAkt[+] FL5.12 cells upon loss of S6K1 is shown for comparison. The siRNA pool targeting S6K1 is indicated by (+); Non-targeting siRNA is indicated by (-). B. Efficacy of S6K1 knockdown in treatments shown in A. C. S6K1 knockdown BCR-ABL^{+} cells exhibited a survival advantage when cultured under low glucose conditions (0.02 mM Glucose, indicated as -Glucose). D. Increased ATP in S6K1-knockdown cells cultured under low glucose conditions for 3 hours (left). Cells were not yet committed to programmed cell death at the time point measured, as illustrated by the absence of cleaved Caspase 3 at 3 hours (right). E-G. Rapamycin inactivation of S6K1 triggered a survival advantage in low glucose conditions in BCR-ABL[+] FL5.12 (E), BCR-ABL[+] primary murine bone marrow cells (F), and K562 cells (G).
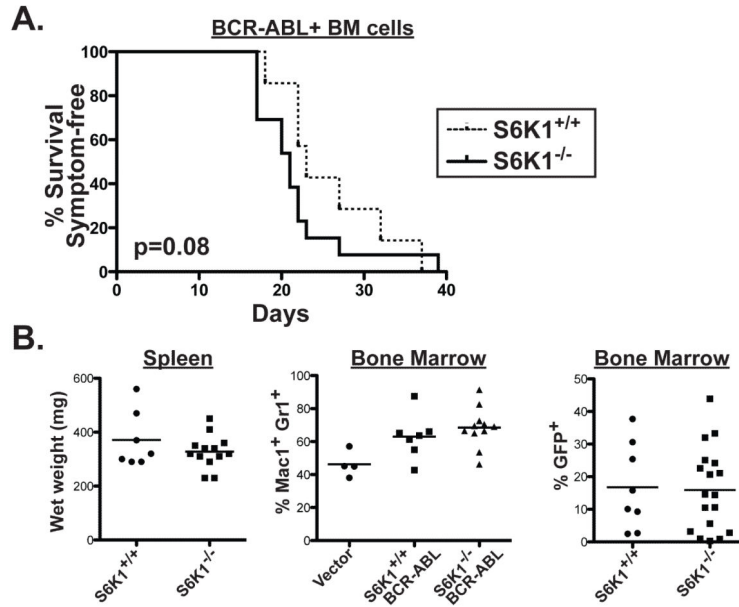
Figure 4.
S6K1 deficiency does not impair BCR-ABL leukemogenesis. A. Bone marrow cells from S6K1$^{+/+}$ or S6K1$^{-/-}$ mice were transduced with BCR-ABL then transplanted into lethally irradiated recipients. Symptom-free survival is plotted. A trend towards more aggressive disease was observed in mice transplanted with BCR-ABL$^+$ S6K1$^{-/-}$ cells, although the trend is not statistically significant (log-rank test). B. Little change in disease characteristics in mice that received S6K1$^{-/-}$ vs. S6K1$^{+/+}$ BCR-ABL transformed cells. Shown are spleen wet weight (left), frequency of myeloid cells in the spleen (center), and frequency of BCR-ABL$^+$ (GFP$^+$) cells in the bone marrow (right) at the time of organ harvest.
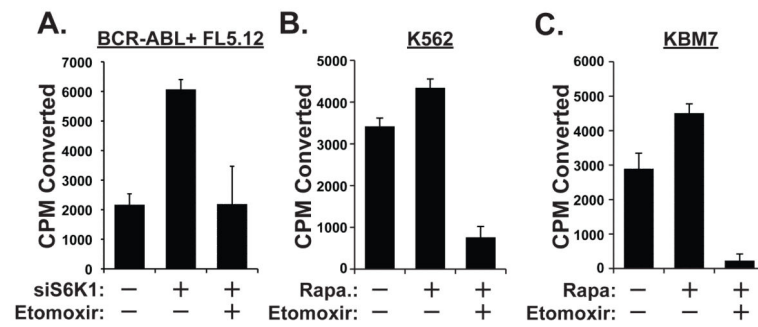
Figure 5.
FAO is activated in BCR-ABL$^+$ cells upon S6K1 inactivation. A. BCR-ABL$^+$ FL5.12 cells were cultured in the absence of cytokine and under low glucose conditions in the presence of ^{3}H palmitate. S6K1 knockdown increased ^{3}H$_2$O release from ^{3}H-palmitate in BCR-ABL$^+$ FL5.12 cells. The CPT1 inhibitor etomoxir, which blocks mitochondrial FAO, counteracted this effect. The mean ±standard deviation of triplicate measurements is shown. B,C. Rapamycin increased FAO release of ^{3}H from ^{3}H-palmitate, which was inhibited by etomoxir in human BCR-ABL$^+$ cell lines K562 (B) and KBM7 (C).
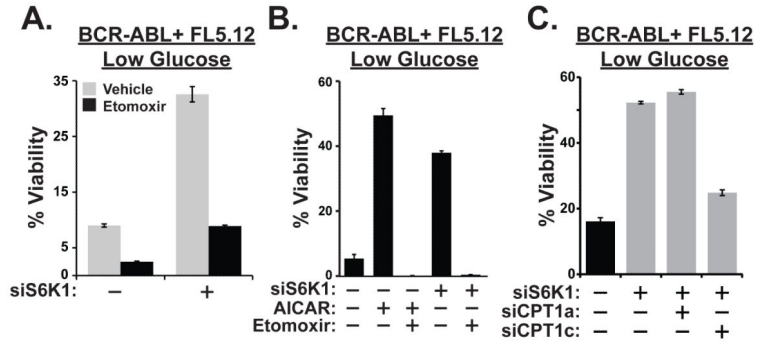
Figure 6.
Requirement for FAO to mediate glucose-independent survival upon S6K1 inactivation. A.
S6K1 knockdown improved survival under low glucose conditions and the FAO inhibitor
etomoxir prevented this survival advantage in BCR-ABL[+] FL5.12 cells. B. The AMPK
agonist AICAR induced FAO-dependent survival in low glucose conditions, which was
comparable to the survival observed in response to S6K1 knockdown. The FAO inhibitor
etomoxir prevented survival in response to either AICAR or S6K1 knockdown. C. Cpt1c
knockdown specifically reduced glucose-independent survival in S6K1-knockdown cells.
siRNA targeting Cpt1a and Cpt1c (indicated by 1a and 1c, respectively) was transfected,
followed by measurement of cell viability after 48 hours of culture in low glucose cytokine-
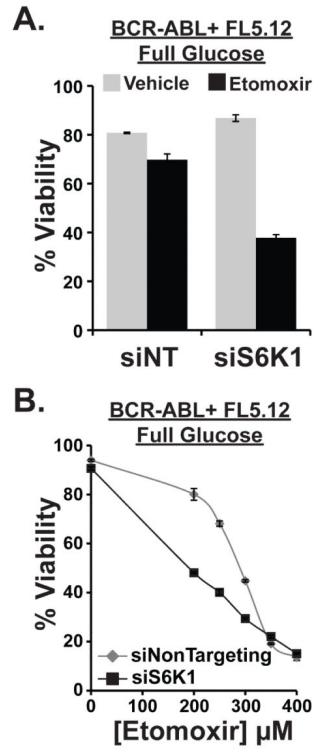free medium.

Figure 7.
Coordinated metabolic inactivation enhances cytotoxicity in response to rapamycin. A. In the presence of full concentrations of glucose, neither S6K1 knockdown nor etomoxir induced significant cytotoxicity. Combination of S6K1 knockdown with 200 μM etomoxir was markedly cytotoxic for BCR-ABL[+] FL5.12 cells measured at 48 hours. B. siS6K1 enhanced the sensitivity to a range of doses of etomoxir in BCR-ABL[+] FL5.12 cells cultured in full glucose, suggesting a synergistic effect. Viability was measured at 48 hours.