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AMPK protects leukemia-initiating cells in myeloid leukemias from metabolic stress in the bone marrow

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SUMMARY

How cancer cells adapt to metabolically adverse conditions in patients and strive to proliferate is a fundamental question in cancer biology. Here we show that AMP-activated protein kinase (AMPK), a metabolic checkpoint kinase, confers metabolic stress resistance to leukemia-initiating cells (LICs) and promotes leukemogenesis. Upon dietary restriction, MLL-AF9-induced murine AML activated AMPK and maintained leukemogenic potential. *AMPK* deletion significantly delayed leukemogenesis and depleted LICs by reducing the expression of glucose transporter 1 (Glut1), compromising glucose flux, and increasing oxidative stress and DNA damage. LICs were particularly dependent on AMPK to suppress oxidative stress in the hypoglycemic bone marrow environment. Strikingly, AMPK inhibition synergized with physiological metabolic stress caused by dietary restriction and profoundly suppressed leukemogenesis. Our results indicate that AMPK protects LICs from metabolic stress, and that combining AMPK inhibition with physiological metabolic stress potently suppresses AML by inducing oxidative stress and DNA damage.

Abstract

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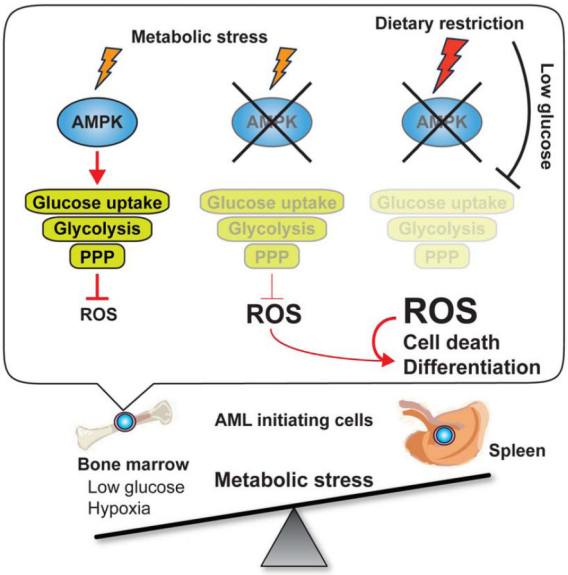
AUTHOR CONTRIBUTIONS

Y.S. performed most experiments with help from R.C., A.L., and A.K.. R.C. and A.L. contributed equally. Y.S. and D.N. designed the experiments, analyzed the results, and wrote the manuscript.

The authors declare no competing financial interests.



Keywords

AMPK; AML; leukemia-initiating cells; metabolic stress; dietary restriction; glycolysis; ROS; DNA damage

INTRODUCTION

A fundamental question in cancer biology concerns how cancer cells residing in metabolically adverse conditions with low oxygen, glucose, and nutrient levels strive to proliferate, and whether this feature of cancer cells can be turned into vulnerability by interventions (Cairns et al., 2011; Schulze and Harris, 2012). Dietary restriction (DR) causes systemic changes in metabolic parameters such as reduced insulin, insulin-like growth factor 1 (IGF1), and glucose levels (Masoro, 2005), and delays the incidence and growth of various tumor models (Colman et al., 2009; Curry et al., 2013; Kalaany and Sabatini, 2009; Longo and Fontana, 2010; Mattison et al., 2012; Mihaylova et al., 2014), demonstrating that cancer cells can be targeted by modulating systemic metabolic parameters. Nonetheless, some cancer cells acquire adaptive mutations, such as activating mutations in the phosphoinositide 3-kinase (PI3K) pathway, and become insensitive to insulin/IGF1 lowering effects of DR (Curry et al., 2013; Kalaany and Sabatini, 2009). It is, however, still unknown whether the PI3K pathway mutations are the sole determinant of DR sensitivity, or whether cancer cells are equipped with other protective mechanisms independent of their mutation status. Moreover, how DR affects expansion of cancer cells by regulating cancer-initiating cells is unexplored.

AMP-activated protein kinase (AMPK) orchestrates cellular metabolic state with proliferation by promoting catabolism and inhibiting anabolism (Hardie et al., 2012). AMPK is a heterotrimeric complex consisted of a catalytic α subunit and two regulatory subunits (β and γ). Energetic stress increases AMP:ATP ratio, upon which AMPK is activated in a manner dependent on the phosphorylation by the tumor suppressor kinase Lkb1

(Shackelford and Shaw, 2009). Activated AMPK promotes multiple catabolic processes to maintain metabolic homeostasis, such glucose uptake via glucose transporters Glut1 and Glut4 (Barnes et al., 2002; Kurth-Kraczek et al., 1999), glycolysis (Almeida et al., 2004), fatty acid uptake and oxidation (Hardie et al., 2012), and mitochondrial biogenesis (Jager et al., 2007; Zong et al., 2002). Furthermore, AMPK activation suppresses anabolic processes such as protein and lipid synthesis. Of note, AMPK inhibits the mammalian target of rapamycin (mTOR) pathway, which promotes protein translation and cell growth, and is thus frequently over-activated in many cancers (Laplante and Sabatini, 2012). Consistent with the tumor suppressive role of the Lkb1-AMPK pathway, AMPK activating drugs suppress cancer cell proliferation and tumor formation (Shackelford and Shaw, 2009), while disruption of AMPK promotes some tumors (Faubert et al., 2013). On the contrary, AMPK can promote cancer cell maintenance *in vitro* and in xenograft models by regulating redox homeostasis (Jeon et al., 2012). Thus, it remains elusive how AMPK function affects cancer in a physiological setting (Faubert et al., 2015; Liang and Mills, 2013; Saito and Nakada, 2014).

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, and it appears increasingly with age with devastating prognosis for elder patients (Ferrara and Schiffer, 2013). AML is a heterogeneous disease caused by various genetic lesions, among which a translocation between the mixed lineage leukemia (MLL) and AF9 genes (producing MLL-AF9) is often found and have poor prognosis (Krivtsov and Armstrong, 2007; Muntean and Hess, 2012). Leukemia-initiating cells (LICs), a cell population capable of initiating leukemias, have been functionally identified in murine AML models as well as in human AML specimens through transplantation assays, and have been postulated to be involved in disease initiation, progression, and relapse (Bonnet and Dick, 1997; Huntly and Gilliland, 2005; Kreso and Dick, 2014; Lapidot et al., 1994). Similar to normal hematopoietic progenitors, LICs demand tightly regulated metabolism, since disruption of either glycolysis or mitochondrial respiration impairs leukemogenesis (Lagadinou et al., 2013; Wang et al., 2014b). LICs of human AML maintain low oxidative stress compared to the bulk of the leukemia and utilize mitochondrial respiration to support metabolic homeostasis (Lagadinou et al., 2013). Since both LICs of AML and normal hematopoietic stem cells (HSCs) reside in the hypoxic bone marrow environment (Ishikawa et al., 2007; Morrison and Scadden, 2014; Nombela-Arrieta et al., 2013; Spencer et al., 2014; Suda et al., 2011), this raises a question of whether LICs share metabolic regulation with HSCs to meet the bioenergetic demands in the hypoxic environment, or whether maintenance of LICs in hypoxia is regulated by leukemia specific mechanisms, potentially providing a therapeutic target.

Here, we demonstrate that LICs activate AMPK upon systemic metabolic stress caused by DR, and that *AMPK* deletion profoundly depletes LICs residing in the hypoxic bone marrow environment by attenuating glucose metabolism. Interrupting the AMPK pathway rendered LICs sensitive to physiological metabolic stress caused by DR, and this combination profoundly suppressed AML. Since *AMPK* deletion does not impair normal HSC function (Nakada et al., 2010), our results indicate that LIC metabolism can be targeted to make them vulnerable to metabolic stress in the bone marrow, without affecting normal HSCs.

RESULTS

Dietary restriction activates AMPK in MLL-AF9-induced AML

To examine how metabolic stress caused by diet affects LICs, we used a murine AML model driven by MLL-AF9 oncogene (Krivtsov et al., 2006; Somervaille and Cleary, 2006). In this model, LICs have been immunophenotypically identified as lineage^{low}Sca-1^{-}c-kit^{+}CD16/32^{+}CD34^{+} cells, which shares the same immunophenotype as granulocyte-macrophage progenitors (GMPs) and are thus termed GMP-like leukemic cells (L-GMPs) (Krivtsov et al., 2006). Murine hematopoietic progenitor cells were transduced with retrovirus encoding both MLL-AF9 and GFP, and transplanted into irradiated syngeneic mice. Upon development of AML, AML cells were transplanted into secondary recipients, which were either fed ad libitum (AL) or subjected to dietary restriction (DR, 70% caloric uptake (Ertl et al., 2008)). Blood glucose levels dropped from 148±17 mg/dl (AL) to 104±14 mg/dl (DR, $p<0.01$, Figure S1A) within 14 days of DR. However, all recipient mice died within 60 days regardless of the dietary manipulation, indicating that MLL-AF9-induced AML are not significantly affected by this DR regimen (Figure 1A). Consistent with a previous report (Sykes et al., 2011), L-GMPs had low levels of phosphorylated Akt (Figure 1B), unlike other tumors in which hyperactivated PI3K-Akt pathway rendered tumors DR resistant (Curry et al., 2013; Kalaany and Sabatini, 2009).

To examine how MLL-AF9-induced AML responds to DR, we isolated AML cells from the bone marrow and spleens of AL and DR mice, and examined the activation status of several metabolic pathways. Interestingly, we found that AMPK is highly activated in AML cells from the bone marrow of DR mice compared to AL mice, whereas cells from the spleens showed little change (Figure 1C, D). Activation of the PI3K/Akt/mTOR pathway did not change upon DR (Figure 1C and Figure S1B). These results raised a possibility that AMPK activation is an adaptive mechanism that protects AML cells from metabolic stress caused by diet, and that the levels of stress DR causes is much higher in the bone marrow compared to that in the spleen.

AMPK is required for the maintenance of LICs in myeloid leukemias

We next tested whether AMPK regulates AML. We treated *Mx1-Cre*; *Prkaa1*$^{fl/fl}$; *Prkaa2*$^{fl/fl}$ and control *Prkaa1*$^{fl/fl}$; *Prkaa2*$^{fl/fl}$ mice (Nakada et al., 2010) with polyinosinic:polycytidylic acid (poly(I:C)) to delete *Prkaa1* and *Prkaa2*, two genes encoding the catalytic α subunits of AMPK, from hematopoietic cells. *Prkaa1*$^{fl/fl}$; *Prkaa2*$^{fl/fl}$ and *Mx1-Cre*; *Prkaa1*$^{fl/fl}$; *Prkaa2*$^{fl/fl}$ mice treated with poly(I:C) are hereafter described as *AMPKα*$^{fl/fl}$ and *AMPKα*$^{\Delta/\Delta}$ mice, respectively. All mice transplanted with MLL-AF9 transduced *AMPKα*$^{fl/fl}$ cells developed AML and succumbed within 130 days post transplantation (Figure 1E). In contrast, recipients of MLL-AF9 transduced *AMPKα*$^{\Delta/\Delta}$ cells had significantly extended survival (Figure 1E). Moreover, *AMPKα*$^{\Delta/\Delta}$ AML cells proliferated less extensively than *AMPKα*$^{fl/fl}$ cells *in vitro* and exhibited differentiated morphologies (Figure S2A and S2B). *AMPKα*$^{\Delta/\Delta}$ MLL-AF9 transduced cells caused AML in some of the recipients, exhibiting increased GFP^{+} AML cells in the bone marrow and the spleens (Figure 1F) and increased blast cells in peripheral blood and in the bone marrow (Figure 1G, H), although the frequencies of AML cells and blasts were lower than that in the recipients of *AMPKα*$^{fl/fl}$ cells. We also used two

additional murine leukemia models to examine the role of AMPK in other leukemias. Hematopoietic progenitor cells infected with MOZ-TIF2 (Deguchi et al., 2003) or BCR-ABL (Pear et al., 1998) expressing retrovirus were transplanted into irradiated recipient mice. Although MOZ-TIF2 expression in *AMPKα*$^{fl/fl}$ cells induced AML in most of the recipient mice as reported before (Deguchi et al., 2003), MOZ-TIF2 expressing *AMPKα*$^{\Delta/\Delta}$ cells developed AML in only one out of 9 recipients (Figure S1C). Expression of BCR-ABL caused fully penetrant chronic myelogenous leukemia (CML)-like disease as reported before (Figure S1D) (Pear et al., 1998). *AMPK* deletion significantly delayed the onset of the disease (Figure S1D). These results indicate that AMPK, which is phosphorylated at a basal level in steady state (Figure 1C and S2F), is required to maintain the full leukemogenic potential of multiple leukemia models, including those induced by MLL-AF9, MOZ-TIF2, and BCR-ABL.

To examine the function of AMPK in LICs in the MLL-AF9 model, we performed secondary transplantation with 10^3 to 10^5 AML cells isolated from primary recipient mice. *AMPKα*$^{fl/fl}$ cells, regardless of the cell dose infused, caused AML in secondary recipients within 60 days of transplantation, whereas *AMPKα*$^{\Delta/\Delta}$ cells were severely compromised in their ability to develop AML, suggesting that the LIC fraction is negatively affected by *AMPK* deletion (Figure 2A). The expansion of *AMPKα*$^{\Delta/\Delta}$ AML cells in the bone marrow, but not in the spleen, was significantly lower than that of *AMPKα*$^{fl/fl}$ AML cells (Figure 2B). Tertiary transplantation of bone marrow cells from moribund secondary recipients of *AMPKα*$^{\Delta/\Delta}$ cells caused AML, but again with delayed onset compared to *AMPKα*$^{fl/fl}$ cells (Figure S2C). Consistent with the previous report (Krivtsov et al., 2006), we confirmed that clonogenic cells reside in the L-GMP fraction, and that L-GMPs are highly enriched for LIC activity (Figure S2D, E). Flow cytometry revealed that L-GMP fraction was markedly depleted in the bone marrow of *AMPKα*$^{\Delta/\Delta}$ primary recipients compared to *AMPKα*$^{fl/fl}$ recipients (Figure 2C-E), as well as in the secondary recipients of *AMPKα*$^{\Delta/\Delta}$ AML cells (Figure 2F-G). The defective leukemogenic potential of *AMPKα*$^{\Delta/\Delta}$ cells (Figure 2A) were not solely due to the depletion of LICs, since transplantation of 10^3 purified *AMPKα*$^{\Delta/\Delta}$ L-GMPs had significantly delayed onset of AML compared to *AMPKα*$^{fl/fl}$ L-GMPs (Figure 2H). Western blotting with purified L-GMPs and whole AML cells (sorted GFP$^+$ cells) confirmed efficient deletion of *AMPK* accompanied by reduced phosphorylation of the AMPK target ACC, without any changes in the expression of MLL-AF9 (Figure S2F). Gene expression profiling of *AMPKα*$^{fl/fl}$ and *AMPKα*$^{\Delta/\Delta}$ L-GMPs revealed that *AMPKα*$^{\Delta/\Delta}$ L-GMPs have increased expression of genes regulating myeloid differentiation (Figure S2G-H). Freshly isolated *AMPKα*$^{\Delta/\Delta}$ L-GMPs also displayed differentiated morphologies compared to *AMPKα*$^{fl/fl}$ L-GMPs, suggesting that AMPK suppresses differentiation of L-GMPs (Figure S2I). These data indicate that AMPK promotes leukemogenesis by both maintaining the LIC pool size and the function in the MLL-AF9 model.

To determine whether AMPK is continuously required for the maintenance of leukemia after its development, we conditionally deleted *AMPK* from established AML using a tamoxifen inducible *Ubc-Cre-ERT2* model. After establishing primary MLL-AF9-induced AML with *Ubc-Cre-ERT2*; *AMPKα*$^{fl/fl}$ cells, we performed serial transplantation, and secondary recipients were treated with control oil or tamoxifen 14 days after transplantation to delete

AMPK. Efficient deletion of *AMPKα* by tamoxifen treatment was confirmed by PCR with sorted AML cells (Figure S3A). Acute deletion of *AMPK* from fully established AML significantly extended the survival of recipient mice, although it did not completely block leukemia progression (Figure 3A, Figure S3B). Interestingly, this acute deletion of *AMPK* led to rapid reduction in the frequencies of L-GMPs residing in the bone marrow but not in the spleens (Figure 3B), without significantly affecting the frequencies of GFP^+ AML cells in either tissue (Figure 3C). Acute *AMPK* deletion also led to rapid reduction in the total numbers of L-GMPs in the bone marrow (Figure 3D and 3E) and reduced the white blood cell (WBC) counts that were increased by AML progression (Figure 3F). The depletion of L-GMPs in the bone marrow paralleled the reduction of whole AML cell numbers in the bone marrow and spleens (Figure 3D and 3E). These results indicate that AMPK is required within established leukemias to maintain L-GMPs, particularly those that reside in the bone marrow.

The LIC population of the BCR-ABL-induced CML-like disease has been identified as $lineage^-Sca\text{-}1^+c\text{-}kit^+$ (LSK) cells (Hu et al., 2006; Naka et al., 2010; Neering et al., 2007). We found that the leukemic LSK cells were reduced by *AMPK* deletion in the bone marrow but not in the spleen (Figure S1F and G). These results further support our notion that LICs residing in the bone marrow depend more upon AMPK than those in the spleen.

AMPK suppresses ROS and DNA damage in AML

To determine how AMPK regulates leukemogenesis, we examined cell death in L-GMPs. *Mx1-Cre; AMPKα*$^{Δ/Δ}$ L-GMPs in the bone marrow had significantly increased frequencies of Annexin-V^+ cells and increased level of cleaved caspase-3, indicating that AMPK suppresses cell death of L-GMPs (Figure 4A and 4B). Increased levels of reactive oxygen species (ROS) in hematopoietic progenitors and leukemia cells are associated with myeloid differentiation, and suppression of ROS is crucial to maintain LICs (Lagadinou et al., 2013; Tothova et al., 2007). We found that *AMPK* deletion significantly increased the level of ROS (Figure 4C and 4D) and mitochondrial superoxide (Figure 4G and 4H) in whole AML cells and L-GMPs in the bone marrow. Interestingly, induction of ROS or superoxide was not observed in the splenic GFP^+ AML cells or L-GMPs after *AMPK* deletion (Figure 4D and 4H), suggesting that AML cells are more dependent upon AMPK to suppress oxidative stress in the bone marrow environment. Consistent with the increased oxidative stress, reducing agents in *AMPKα*$^{Δ/Δ}$ AML cells and L-GMPs in the bone marrow were depleted as indicated by the increased $NADP^+$/NADPH and reduced GSH/GSSG ratios (Figure 4E and 4F). The induction of ROS in the bone marrow AML cells correlated with increased DNA damage, shown by γH2AX immunoblotting and immunofluorescence of freshly isolated bone marrow L-GMPs and whole AML cells (Figure 4B, 4I and 4J).

To test whether the oxidative stress caused by *AMPK* deletion induced cell death of *AMPKα*$^{Δ/Δ}$ AML cells, AML recipient mice were given a superoxide scavenger TEMPOL (Wang et al., 2010) in their drinking water. TEMPOL treatment of *AMPKα*$^{Δ/Δ}$ AML recipient mice significantly increased the total numbers of AML cells in the bone marrow (Figure 4M) and partially reduced the levels of ROS, DNA damage, and apoptosis of AML cells and L-GMPs in the bone marrow (Figure 4I-4L, 4N-P). TEMPOL treatment also

slightly accelerated the onset of leukemogenesis by *AMPKα*$^{\Delta/\Delta}$ AML cells (Figure 4Q), consistent with the partial suppression of ROS, DNA damage, and apoptosis by TEMPOL treatment. These results indicate that loss of AMPK compromises leukemogenesis, at least partially, by inducing oxidative stress, DNA damage, and cell death.

AMPK promotes glucose uptake and metabolism in AML

We next examined whether AMPK regulates energy metabolism in AML cells. A metabolic phenotyping microarray analysis (Bochner et al., 2011) was performed to identify metabolites whose consumption is regulated by AMPK. We found that immature c-kit^{+} AML cells metabolized glucose at an extremely high level compared to normal GMPs consistent with the Warburg effect (Cairns et al., 2011; Warburg, 1956), and that *AMPK* deletion attenuated glucose metabolism of AML cells (Figure S4). We then tested whether AMPK regulates glucose uptake *in vivo*. Leukemic mice were injected with a fluorescent glucose analog 2-NBDG, and 2-NBDG uptake was analyzed by flow cytometry as previously described (Warr et al., 2013). *AMPKα*$^{\Delta/\Delta}$ whole AML cells and L-GMPs in the bone marrow, but not L-GMPs in the spleen, had significantly reduced 2-NBDG uptake compared to *AMPKα*$^{fl/fl}$ cells (Figure 5A and 5B). Furthermore, we found that the mRNA and protein levels of a glucose transporter Glut1, but not Glut4, were significantly reduced in whole AML cells and L-GMPs upon *AMPK* deletion (Figure 5C and 5D, Figure S5B). AMPK phosphorylates and destabilizes Txnip1, which suppresses *Glut1* expression transcriptionally and by preventing plasma membrane translocation of Glut1 protein (Wu et al., 2013). Consistent with this finding, *AMPKα*$^{\Delta/\Delta}$ whole AML cells and L-GMPs from the bone marrow had increased protein levels of Txnip1 (Figure 5C), suggesting that AMPK may regulate Glut1 levels through Txnip1. We then tested whether overexpression of Glut1 can rescue the reduced proliferative potential of *AMPKα*$^{\Delta/\Delta}$ AML cells. Overexpression of Glut1 in *AMPKα*$^{\Delta/\Delta}$ AML cells substantially rescued the defective clonogenic potential, indicating that the defective Glut1-mediated glucose uptake is at least partly responsible for the impaired AML cell proliferation *in vitro* (Figure 5E). We also tested whether Glut1 is required for the proliferative potential of AML cells, by deleting *Glut1* from AML cells. We derived AML clones that express doxycycline-inducible Cas9 endonuclease, and introduced short guide RNAs (sgRNAs) against *Rosa26* or *Glut1*. We found two sgRNA clones against *Glut1* (clones 1 and 2) that reduced Glut1 protein levels and reduced clonogenicity of AML cells (Figure 5F and 5G). Transplantation of these *Glut1*-deleted AML cell revealed that *Glut1* deletion significantly suppressed AML, with only one out of ten recipients developing AML (Figure 5H). Collectively, these results indicate that AMPK maintains glucose metabolism and leukemogenic potential of AML cells at least partly through Glut1.

To better understand how AMPK regulates glucose metabolism in AML, we performed metabolic flux analysis using ^{13}C-glucose to trace cellular carbon flux. MLL-AF9-induced AML cells were isolated from recipient mice, briefly exposed to ^{13}C-labeled glucose tracer, and ^{13}C incorporation into metabolites was analyzed by mass spectrometry. We found that *AMPKα*$^{\Delta/\Delta}$ AML cells have significantly reduced ^{13}C incorporation into glycolytic intermediates 3-phosphoglycerate/2-phosphoglycerate and lactate, as well as ATP and NADH (Figure 6A). To directly access glycolysis, we analyzed extracellular acidification rates (ECAR) of freshly isolated AML cells from the bone marrow. *AMPKα*$^{\Delta/\Delta}$ AML cells

had significantly reduced basal ECAR and the glycolytic capacity, as determined by the ECAR after oligomycin treatment (Figure 6C), indicating that AMPK is required for glycolysis of AML cells. Consistently, GSEA and qPCR analyses revealed decreased expression of genes involved in glycolysis in *AMPKα*$^{\Delta/\Delta}$ L-GMPs (Figure S5A-B). *AMPKα*$^{\Delta/\Delta}$ AML cells also exhibited significantly reduced oxygen consumption rates (OCR), indicating that mitochondrial respiration is compromised by *AMPK* deletion (Figure 6D). The reduced OCR of *AMPKα*$^{\Delta/\Delta}$ AML cells is reminiscent of *Lkb1*-deficient hematopoietic cells (Gurumurthy et al., 2010), and suggests that the increased ROS of *AMPKα*$^{\Delta/\Delta}$ AML cells is unlikely to be caused by increased oxidative phosphorylation. Collectively, these data indicate that AMPK promotes glucose uptake and subsequent glucose metabolism.

Since *AMPK* deletion significantly increased ROS (Figure 4C, D, G, H) and reduced the amount of antioxidants (Figure 4E, F), we tested whether AMPK regulates the pentose phosphate pathway (PPP), a metabolic pathway that branches from glycolysis and produces antioxidants (Patra and Hay, 2014). ^{13}C-glucose metabolic flux analysis using 2.0 g/L glucose only revealed a trend towards reduced glucose flux through PPP by *AMPK* deletion (Figure 6B, left). Interestingly, a similar flux analysis in the presence of low glucose (0.2 g/L) revealed significantly reduced flux into the PPP by *AMPK* deletion, as shown by reduced ^{13}C incorporation into ribose-5-phosphate (R5P)/xylulose-5-phosphate (X5P) and sedoheptulose-7-phosphate (S7P, Figure 6B, right). These results indicate that AMPK is important for carbon flux through the PPP in AML cells, particularly under low glucose conditions.

Dietary restriction suppresses *AMPK*-deficient AML

AMPK was hyper-activated in the bone marrow but not spleens of DR mice (Figure 1C), and L-GMPs and AML cells residing in the bone marrow were more dependent upon AMPK than those in the spleens for their maintenance and to suppress ROS (Figure 3B, 4D, and 4H). We found that the glucose levels in the bone marrow were much lower than that in the spleens or peripheral blood, and that DR further reduced the glucose levels in the bone marrow (Figure S1A). This raised a possibility that AML cells become acutely depleted in the bone marrow upon *AMPK* deletion (Figure 3B) due to the lower glucose levels in the bone marrow that in the spleens. Consistently, exposure of *AMPKα*$^{\Delta/\Delta}$ AML cells *in vitro* with no or low glucose conditions, but not cytokine deprivation, was sufficient to reduce cell survival (Figure 7A and S6A). Interestingly, upon glucose deprivation, *AMPKα*$^{\Delta/\Delta}$ AML cells upregulated ROS (Figure 7B), consistent with our observation that the PPP flux is reduced in *AMPKα*$^{\Delta/\Delta}$ AML cells under low glucose condition (Figure 6B). These results suggest that AMPK is particularly important to suppress ROS in a hypoglycemic condition such as in the bone marrow.

We then tested whether AMPK inhibition sensitizes AML cells to metabolic stress caused by DR by subjecting secondary recipient mice of *AMPKα*$^{fl/fl}$ and *AMPKα*$^{\Delta/\Delta}$ AML cells to AL or DR. Strikingly, although DR did not extend the survival of mice transplanted with *AMPKα*$^{fl/fl}$ AML cells (as in Figure 1A), it significantly extended the survival of mice transplanted with *AMPKα*$^{\Delta/\Delta}$ AML cells (Figure 7C). Additionally, *AMPKα*$^{\Delta/\Delta}$ L-GMPs

from mice subjected to DR had significantly increased levels of ROS, DNA damage, and apoptosis compared to mice subjected to AL, consistent with our findings that glucose deprivation increases oxidative stress in *AMPK*$\alpha^{\Delta/\Delta}$ AML cells (Figure 7D-F). DR did not affect the frequencies of normal hematopoietic progenitors including HSCs, multipotent progenitors (MPPs), and GMPs of *AMPK*-deficient mice (Nakada et al., 2010) even though DR activated the AMPK pathway in bone marrow cells, indicating that normal hematopoietic progenitors do not depend upon AMPK during DR (Figure S6B-D). *AMPK*-deficient hematopoietic progenitor cells including HSCs, MPPs, MEPs, CMPs, and GMPs were not sensitive to metabolic stress caused by metformin, even though metformin treatment activated AMPK in bone marrow cells (Figure S6E-F). These results indicate that although both normal hematopoietic cells and AML cells activate AMPK upon metabolic stress, AML cells are particularly dependent on *AMPK* to suppress oxidative stress, DNA damage, and apoptosis upon metabolic stress.

Finally, we tested whether pharmacological inhibition of AMPK by an AMPK inhibitor compound C suppresses AML. Treatment of MLL-AF9 transduced AML cells with compound C *in vitro* reduced AMPK activation and induced apoptosis (Figure 7G). Moreover, L-GMPs were more sensitive to compound C than normal GMPs (Figure S6H). Next, we examined whether compound C inhibits murine AML *in vivo*. We treated secondary recipients of MLL-AF9 AML cells with compound C (4mg/kg/day) or control vehicle starting from 7 days after transplantation. Compound C treatment significantly extended the survival of recipient mice (Figure 7H) and reduced the number of circulating GFP^+ AML cells in peripheral blood (Figure 7I and J). Interestingly, the combination of compound C with DR markedly prolonged the survival compared to compound C treatment alone (Figure 7H) similar to the results obtained by genetically deleting *AMPK*, suggesting that AMPK can be manipulated pharmacologically to sensitize AML to physiological metabolic stress. To test whether human AML cells can be targeted by this combination therapy we transplanted MOLM13 cells, which carry MLL-AF9 rearrangements, into NOD-SCID mice and treated them with compound C and DR. The survival of recipient mice was significantly extended by both compound C and DR regimen, and further extended by the combination of compound C and DR (Figure S6I). Together with the genetic data, these results suggest that AMPK inhibition renders both murine and human AML cells sensitive to physiological metabolic stress, which can be exacerbated by dietary manipulation.

DISCUSSION

Maintaining metabolic homeostasis under stressful conditions is essential for cancer cells that propagate in metabolically adverse environment, such as in the bone marrow where AML cells expand. Our study shows that AMPK is critical for the maintenance of leukemic cells within the bone marrow, and that deletion of AMPK renders leukemias susceptible to physiological metabolic stress caused by dietary restriction. AMPK has been shown to have both tumor suppressor roles and tumor supportive roles. Supporting the tumor suppressive roles of AMPK, AMPK activator such as metformin has been shown to suppress tumor cell proliferation *in vitro* and *in vivo* (Faubert et al., 2015; Jeon and Hay, 2015; Liang and Mills, 2013; Saito and Nakada, 2014). Deletion of the α1 catalytic subunit of AMPK accelerated Eμ-Myc-induced B-cell lymphomagenesis by promoting aerobic glycolysis (Faubert et al.,

2013). The difference between our results showing that multiple AML and CML models depend upon AMPK and the results from Faubert et al. showing that B-cell lymphomas are suppressed by AMPK might be due to the different cell types in which the cancer was initiated. The metabolism of myeloid cells and lymphoid cells are known to be different (Fox et al., 2005; O'Neill and Hardie, 2013). AMPK activation in monocytes promoted glycolysis (Marsin et al., 2002), whereas in T-lymphocytes AMPK suppressed glycolysis (MacIver et al., 2011). The functions of FoxO transcription factors are also different between AML and B-lymphomas. MLL-AF9 driven AML maintain low Akt signaling in order to allow FoxO transcription factors to suppress differentiation (Sykes et al., 2011), whereas Eμ-Myc-induced B-cell lymphomagenesis was promoted by ablation of FoxO (Bouchard et al., 2007). Regardless of whether different tumors use AMPK to promote or suppress glycolysis, our results are overall consistent with the idea that AMPK inhibition sensitizes cancer cells to metabolic stress by increasing oxidative stress (Faubert et al., 2013; Jeon et al., 2012). Our results are also consistent with the findings from non-small cell lung cancer in which *Lkb1*-deficient tumors with attenuated AMPK activity were sensitive to metabolic stress caused by phenformin and exhibited increased apoptosis and ROS (Shackelford et al., 2013). Our results further demonstrate that AMPK inhibition can make LICs residing in the hypoxic bone marrow environment vulnerable to physiological metabolic stress caused by changes in diet, pointing to differential vulnerability of cancer cells on AMPK inhibition depending upon their tumor microenvironment.

AMPK was required to maintain the expression of Glut1, the predominant glucose transporter in hematopoietic cells (Rathmell et al., 2003), and to uptake glucose. AMPK deletion led to impaired glycolytic flux, reduced synthesis of ATP and reducing agents NADPH and GSH. Consistent with our results, AMPK deletion in cancer cell lines and mouse embryonic fibroblasts has been shown to decrease the levels of NADPH and GSH and increase ROS upon glucose deprivation (Jeon et al., 2012). Other regulators of glycolysis such as PKM2 and LDHA have also been shown to be critical for LIC maintenance by promoting glycolysis (Wang et al., 2014b). Additionally, Glut1 has been identified in a screen as important for cancer cell proliferation in low glucose culture condition (Birsoy et al., 2014). Consistent with a previous report (Wu et al., 2013), we found that *AMPK* deletion increases the protein levels of Txnip1, which reduces Glut1 expression. AMPK has been shown to phosphorylate and destabilize Txnip1, thereby increasing Glut1 expression. Our results therefore suggest that the previously documented AMPK-Txnip1-Glut1 module is functionally important for LIC maintenance. We note, however, that it is still unclear whether AMPK phosphorylates and destabilizes Txnip1 in AML cells, and whether increased Txnip1 is the cause of Glut1 depletion upon *AMPK* deletion in AML cells. Future studies will be needed to fully elucidate the role of Txnip1 in AMPK-dependent glucose metabolism of AML cells.

We found that LICs residing in the bone marrow were more dependent on AMPK to suppress oxidative stress than those residing in the spleen, indicating that the cancer microenvironment determines the extent to which cancer cells depends upon AMPK. Little is know about the difference in the metabolic milieu of the bone marrow and the spleens. We found that the glucose levels in the bone marrow were significantly lower that those in

the spleen and in the peripheral blood (Figure S1A). Consistent with the idea that the low glucose concentration in the bone marrow renders LICs in the bone marrow particularly dependent upon AMPK, *AMPK*-deficient AML cells cultured briefly in low or glucose free medium had reduced glucose flux through PPP, a metabolic pathway that branches from glycolysis and produces NADPH, leading to increased ROS and induced cell death. Thus, low glucose levels in the bone marrow at least partially contribute to the higher dependence of LICs upon AMPK. In addition to the lower glucose level in the bone marrow, other factors such as the oxygen tension (Nombela-Arrieta et al., 2013; Spencer et al., 2014) or IGF-1 (Kalaany and Sabatini, 2009; Longo and Fontana, 2010) may also contribute to increased dependence on AMPK in the bone marrow.

In contrast to myeloid leukemias that were dependent on AMPK, normal HSCs were not dependent upon AMPK during homeostasis, upon transplantation (Nakada et al., 2010), and even under metabolic stress by DR or metformin treatment (Figure S6). This raises a possibility that AMPK inhibition provides a therapeutic window to treat leukemias without harming normal hematopoiesis. Consistent with this hypothesis, we found that L-GMPs were more sensitive to an AMPK inhibitor, compound C, than normal GMPs. In both murine and human AML transplantation models, compound C single treatment delayed the onset of leukemogenesis and the combination of compound C with DR further delayed leukemogenesis. Although compound C may have AMPK-independent targets (Hao et al., 2010; Yu et al., 2008), our genetic analyses using three different myeloid leukemia models provide strong support for the idea that AMPK inhibition may suppress multiple myeloid malignancies.

In conclusion, our results demonstrate that targeting AMPK suppresses myeloid leukemias by interrupting glucose metabolism, and reveal different metabolic requirements of leukemias residing in the bone marrow and in the spleen. AMPK inhibition combined with dietary manipulation or its mimetics may also suppress other tumors that reside in hypoglycemic, hypoxic, nutrient poor conditions that are difficult to target with conventional therapies (Wilson and Hay, 2011).

EXPERIMENTAL PROCEDURES

Mice and treatments

Prkaa1^{fl} and *Prkaa2*^{fl} mice were reported before (Nakada et al., 2010). C57BL/Ka-Thy-1.2 (CD45.1), C57BL/Ka-Thy-1.1 (CD45.2), and NOD-SCID mice (8-12 weeks of age) were used for AML transplantation assays. Poly(I:C) (Amersham, Piscataway, NJ) was injected intraperitoneally with 0.5 μg/gram of body mass every other days for 6 days. 5 mg of tamoxifen (Sigma, St. Louis, MO) was administered daily by oral gavage for 5 days. 1 mM Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy) (Enzo Life Science, Farmingdale, NY) was given to mice in the drinking water. 4 mg/kg of compound C (Cayman Chemical, Ann Arbor, MI) diluted in DMSO was intraperitoneally injected for 4 weeks. Metformin (1.5 mg/ml) was given to mice in the drinking water. All mice were fed Harlan Rodent Diet 2920X, either ad libitum (AL) or with DR regimen (70% of the normal food intake). Mice were housed in AAALAC-accredited, specific-pathogen-free animal care facilities at Baylor

College of Medicine (BCM). All procedures were approved by BCM Institutional Animal Care and Use Committees.

Retroviral bone marrow transduction assays

5-FU primed bone marrow cells were incubated in X-Vivo 15 (Lonza, Allendale, NJ) supplemented with 50 ng/ml SCF, 50 ng/ml TPO, 10 ng/ml IL-3 and 10 ng/ml IL-6 (all from Peprotech, Rocky Hill, NJ) for 24 hours. After incubation, cells were spin-infected with retroviral supernatant supplemented.

CRISPR/Cas9-mediated gene editing in AML cells

AML cells were infected with doxycycline-inducible Cas9 expressing lentivirus (pCW-Cas9: Addgene 50661) (Wang et al., 2014a) and sgRNA expressing lentivirus (pKLV-U6gRNA(BbsI)-PGKpuro2ABFP: Addgene 50946) (Koike-Yusa et al., 2014). Infected clones were selected with puromycin (0.5 μg/ml, Life Technologies) for 5 days, and BFP^+ cells were sorted for further analysis.

Flow cytometry

ROS was measured using CellROX Deep Red Reagent (Life Technologies, Carlsbad, CA) or MitoSOX Red Mitochondrial Superoxide Indicator (Life Technologies). To analyze glucose uptake *in vivo*, 100 μl of 2mM 2-(n-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) (Life Technologies) was injected intravenously into mice transplanted with pMIR-MLL-AF9. One hour later, mice were sacrificed and bone marrow cells were collected and stained with antibodies to identify L-GMPs.

^{13}C-glucose tracing experiments

Freshly isolated cells from mice were incubated with RPMI-1640 containing 2% dialyzed FBS (Thermo Scientific, Waltham, MA), 2.0 g/L glucose and cytokines for 16 hours, then replaced in pre-warmed labeling medium containing 2.0 g/L or 0.2 g/L 1,2-$^{13}C_2$-glucose (Cambridge Isotope Laboratories, Andover, MA) with cytokines and incubated for 5 or 60 min at 37°C. Metabolites were extracted with an organic solvent (ice cold 90% 9:1 MeOH: $CHCl_3$) and separated on a 1mm ×150mm HILIC column in a 35 min cycle. All analytes and internal standards were measured by ESI^- ionization on an Agilent 6520 QTOF mass spectrometer equipped with a 1260 LC unit (Agilent, Santa Clara, CA).

Measurement of extracellular acidification rates (ECAR) and oxygen consumption rates (OCR)

ECAR and OCR were analyzed with a XF24 extracellular flux analyzer (Seahorse Bioscience, Chicopee, MA) following the manufacturer's protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- AMPK is activated upon metabolic stress and promotes survival of AML
- AMPK deletion disrupts glucose metabolism of AML cells and increases ROS
- AMPK is more important for LICs in the bone marrow compared to those in the spleen
- AMPK deletion sensitizes AML to metabolic stress caused by dietary restriction

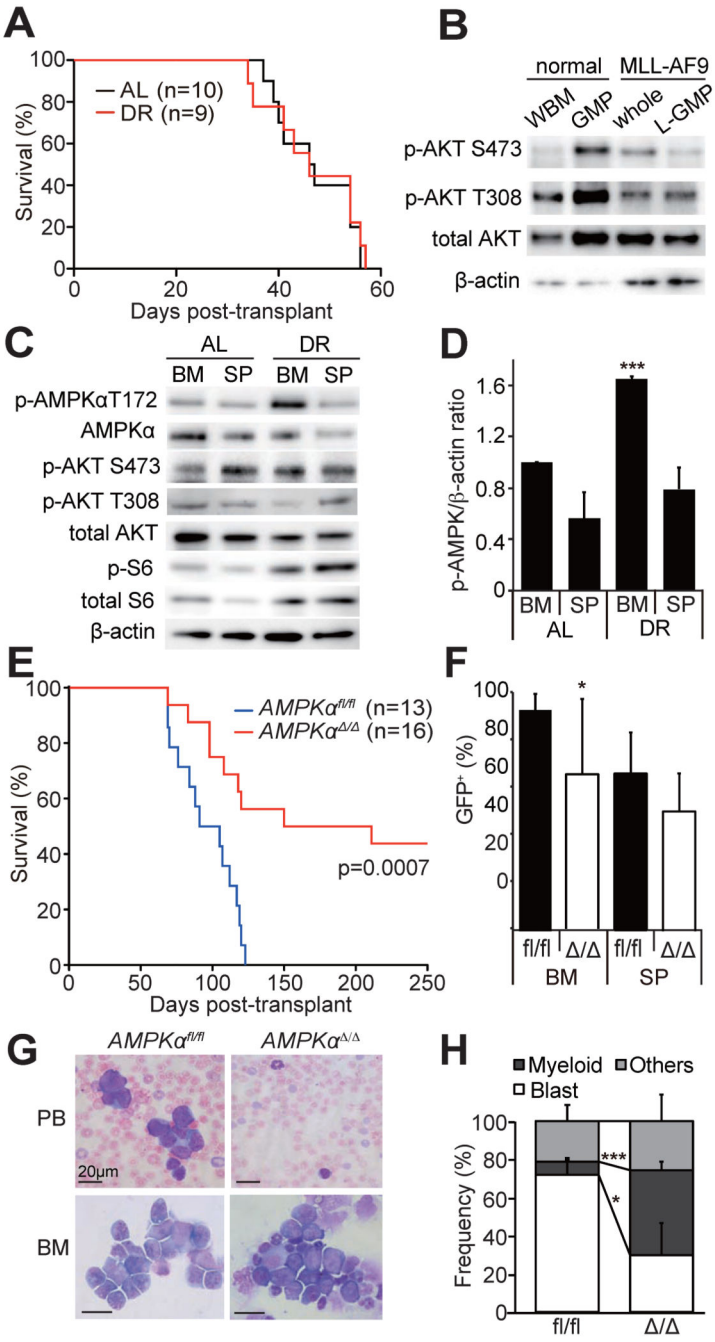

Figure 1. AMPK is activated in AML cells upon DR and promotes leukemogenesis

(**A**) Secondary recipients of 1,000 MLL-AF9-induced AML cells were either fed ad libitum (AL, n=10) or subjected to dietary restriction (DR, n=9). DR did not extend the survival of AML recipient mice. (**B**) Immunoblotting was performed on freshly isolated whole bone marrow cells (WBM) and GMPs from non-leukemic mice, and whole AML cells (sorted GFP^+ cells) and L-GMPs from leukemic mice, all fed AL. PI3K pathway, as determined by phosphorylation of Akt, was not activated in AML cells compared to normal progenitors. (**C**) Immunoblotting of freshly isolated AML cells from AL or DR mice revealed that

AMPK was highly activated in the bone marrow (BM), but not the spleens (SP), of DR mice. (**D**) Quantification of the results shown in (**C**). p-AMPK signals were normalized to the signals from β-actin. (**E**) Survival of mice after transplanting MLL-AF9 transduced *AMPKα*$^{fl/fl}$ (n=13) or *AMPKα*$^{\Delta/\Delta}$ cells (n=16, three independent experiments). (**F**) Frequencies of GFP^{+} AML cells in the bone marrow or the spleens of leukemic mice shown in (**E**). (**G**) Wright-giemsa staining of peripheral blood (PB) or the bone marrow (BM) samples from AML mice revealed fewer blasts in the recipients of *AMPKα*$^{\Delta/\Delta}$ AML. (**H**) Quantification of the cell types in the bone marrow. In all figures, data represent mean ±standard deviation; *, $p<0.05$; **, $p<0.005$; and ***, $p<0.0005$ by Student's t-test, except for comparison of the survival curves in which the significance was accessed by a log-rank test. See also Figure S1.

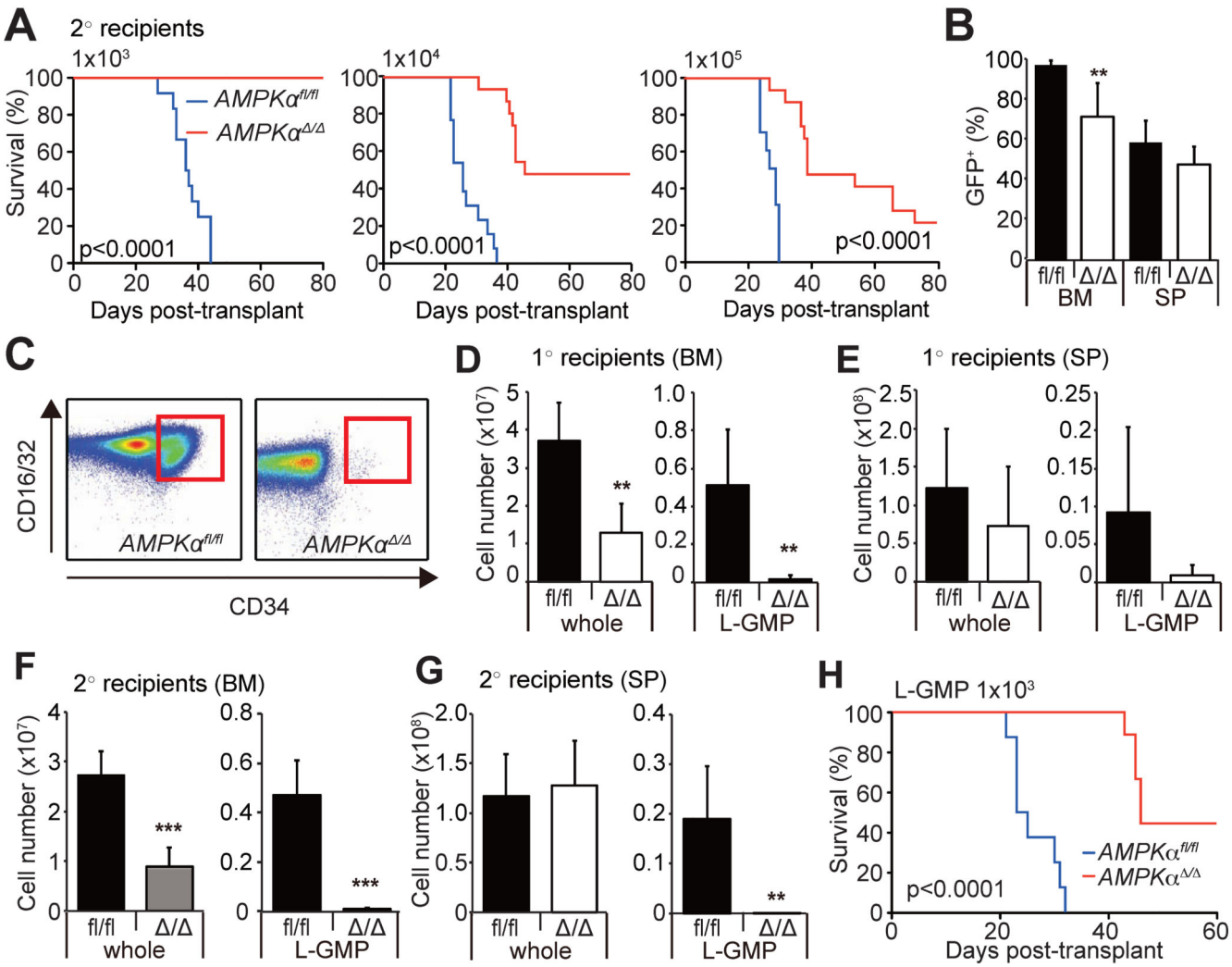

Figure 2. *AMPK* deletion suppresses leukemogenesis and depletes LICs

(**A**) Secondary transplantation of 10^3, 10^4, 10^5 GFP$^+$ AML cells revealed significantly delayed onset of leukemogenesis by *AMPKα*$^{Δ/Δ}$ cells. (**B**) Frequencies of GFP$^+$ AML cells in the bone marrow or the spleens of leukemic mice shown in (**A**). (**C**) Representative FACS profiles gated on lineage$^-$ Sca-1$^-$c-kit$^+$cells show reduced CD34$^+$CD16/32$^+$ L-GMPs (red box) after *AMPK* deletion. (**D** and **E**) Total numbers of L-GMPs and whole AML cells in the bone marrow and spleens of primary recipients. (**F** and **G**) Secondary recipients of *AMPKα*$^{Δ/Δ}$ AML cells had significantly reduced total numbers of whole AML cells and L-GMPs in the bone marrow (**F**), and L-GMPs in the spleens (**G**). (**H**) 10^3 L-GMPs isolated from primary recipients of *AMPKα*$^{fl/fl}$ and *AMPKα*$^{Δ/Δ}$ AML were transplanted into secondary recipient mice. *AMPKα*$^{Δ/Δ}$ L-GMPs had delayed onset of leukemogenesis compared to *AMPKα*$^{fl/fl}$ L-GMPs, indicating that *AMPK* deletion impairs L-GMP function. See also Figure S2.

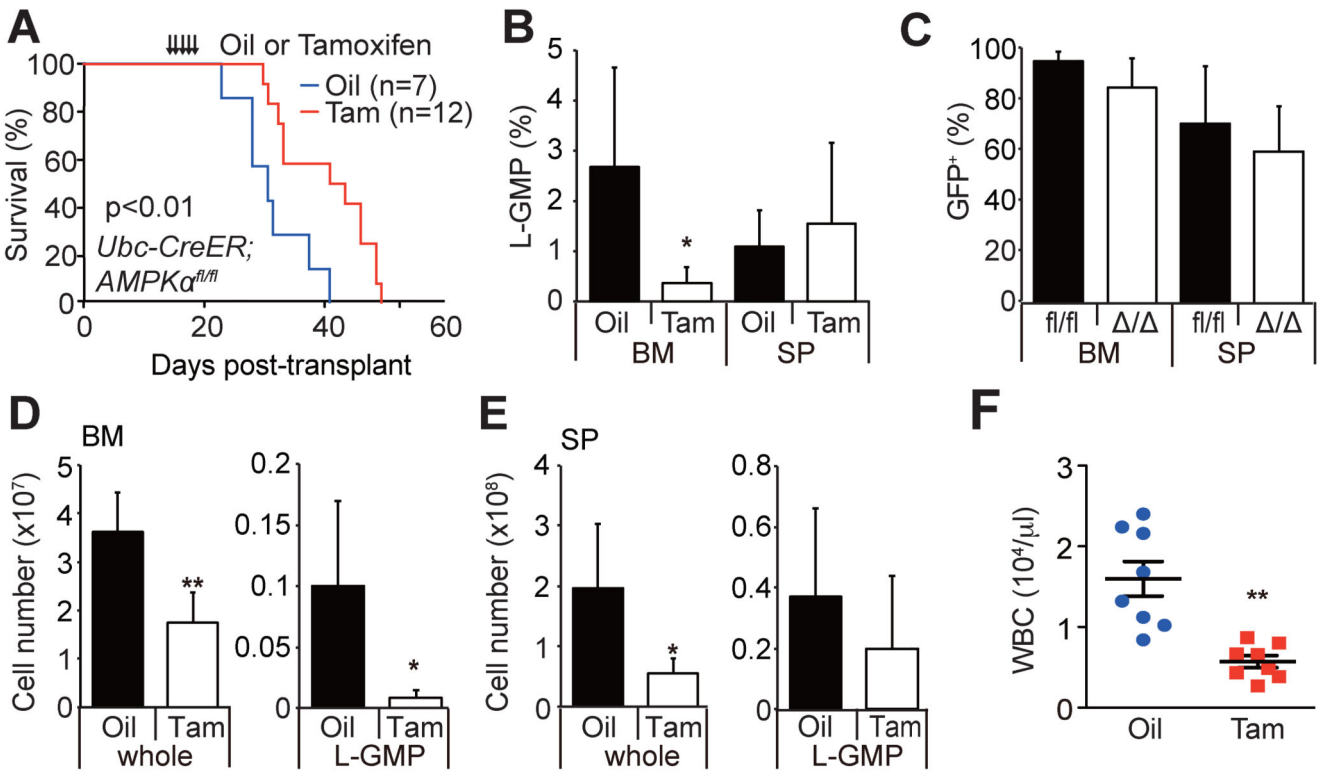

Figure 3. AMPK is required within established leukemias to maintain LICs

(**A** and **B**) Conditional deletion of *AMPK* from established AMLs by treating mice carrying MLL-AF9-transduced *Ubc-Cre-ERT2*; *AMPKα*$^{fl/fl}$ AML cells with tamoxifen led to significant extension of survival (**A**) and reduced frequency of L-GMP in the bone marrow but not in the spleens (**B**). (**C**) Acute *AMPK* deletion did not affect the frequencies of GFP^{+} AML cells in the bone marrow or in the spleens. (**D** and **E**) Acute *AMPK* deletion significantly reduced the numbers of L-GMPs in the bone marrow but not in the spleens, and reduced the numbers of whole AML cells in these two tissues (n=7). (**F**) *AMPK* deletion by tamoxifen treatment reduced WBC in the peripheral blood compared to oil-treated mice (n=8). See also Figure S3.

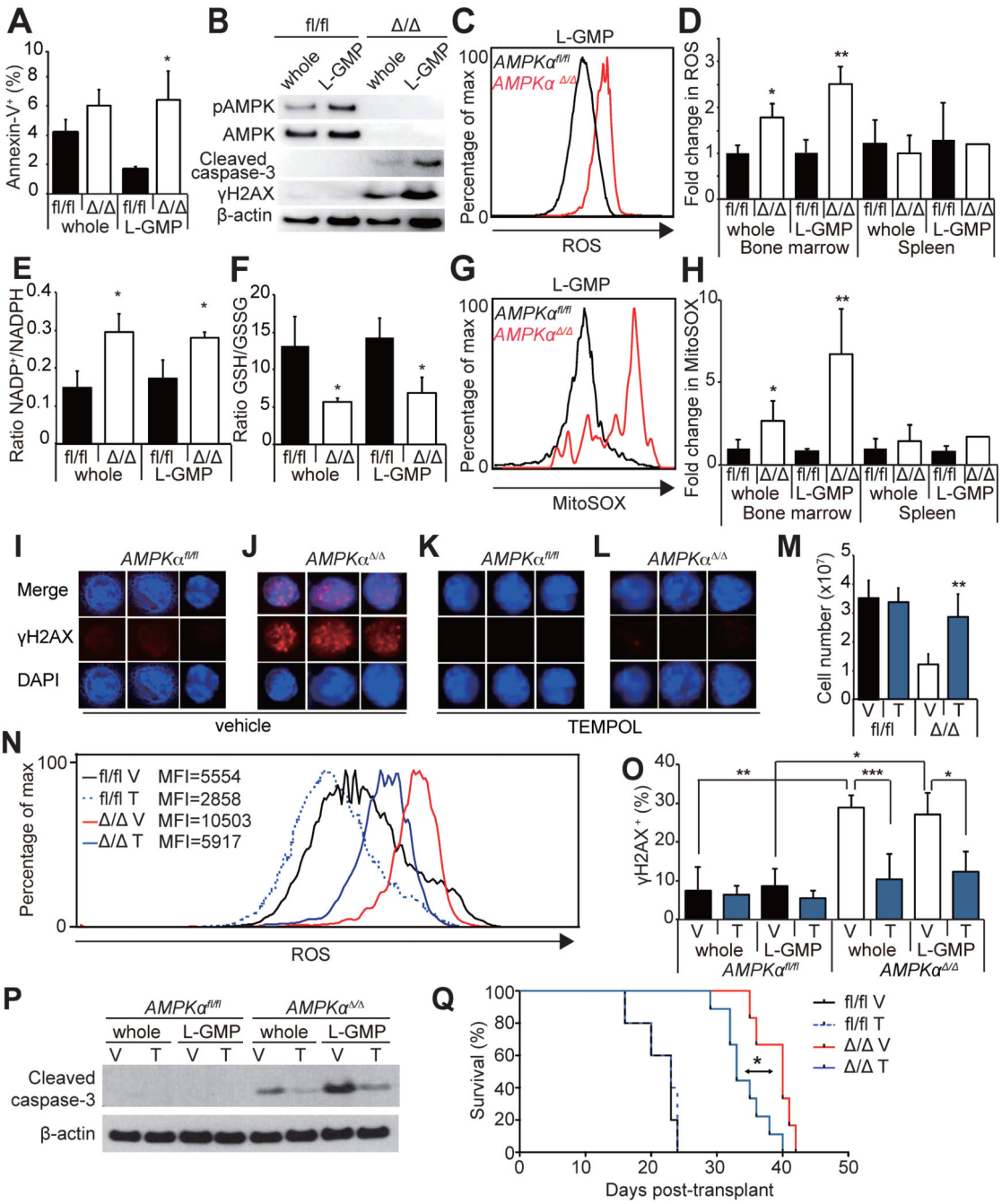

Figure 4. *AMPK* deletion induces oxidative stress and DNA damage in LICs

(**A**) *AMPK* deletion significantly increased the frequencies of Annexin-V$^+$ L-GMPs in the bone marrow (n=3). (**B**) Western blotting of *AMPKα*$^{fl/fl}$ and *AMPKα*$^{Δ/Δ}$ AML cells and L-GMPs from the bone marrow revealed increased cleaved caspase-3 consistent with the increased cell death, as well as increased γH2AX indicating increased DNA damage. (**C** and **D**) *AMPK* deletion increased the levels of ROS in whole AML cells and L-GMPs in the bone marrow but not in the spleens (n=5). (**E** and **F**) Deletion of *AMPK* from L-GMPs and whole AML cells from the bone marrow increased the NADP$^+$/NADPH ratio and reduced

the GSH/GSSG ratio consistent with the increased oxidative stress (n=3). (**G** and **H**) *AMPK* deletion also increased the levels of mitochondrial superoxide (as indicated by the MitoSOX staining) in whole AML cells and L-GMPs in the bone marrow but not in the spleens (n=5). (**I-L**) Immunofluorescence staining of freshly isolated bone marrow L-GMPs with anti-γH2AX antibodies revealed that *AMPK* deletion (**J**) increased DNA damage, which was significantly suppressed by treating the mice with an antioxidant TEMPOL (**L**). Scale bars indicate 5 μm. Quantification of **I-L** is shown in **O** (V: vehicle, T: TEMPOL, n=5). (**M**) TEMPOL treatment increased the numbers of $AMPK\alpha^{\Delta/\Delta}$ AML cells in the bone marrow, and reduced ROS levels in bone marrow L-GMPs (**N**). MFI indicates the mean fluorescence intensity of each sample. (**P**) Western blotting of $AMPK\alpha^{fl/fl}$ and $AMPK\alpha^{\Delta/\Delta}$ AML cells and L-GMPs isolated from the bone marrow of mice subjected to TEMPOL or vehicle treatment revealed that the levels of cleaved caspase-3 in $AMPK\alpha^{\Delta/\Delta}$ AML cells and L-GMPs were partially reduced by TEMPOL treatment. (Q) 10^4 GFP^+ AML cells from secondary recipients were transplanted and recipients treated with either control vehicle or TEMPOL. TEMPOL treatment slightly but significantly accelerated the onset of leukemogenesis by $AMPK\alpha^{\Delta/\Delta}$ AML cells.

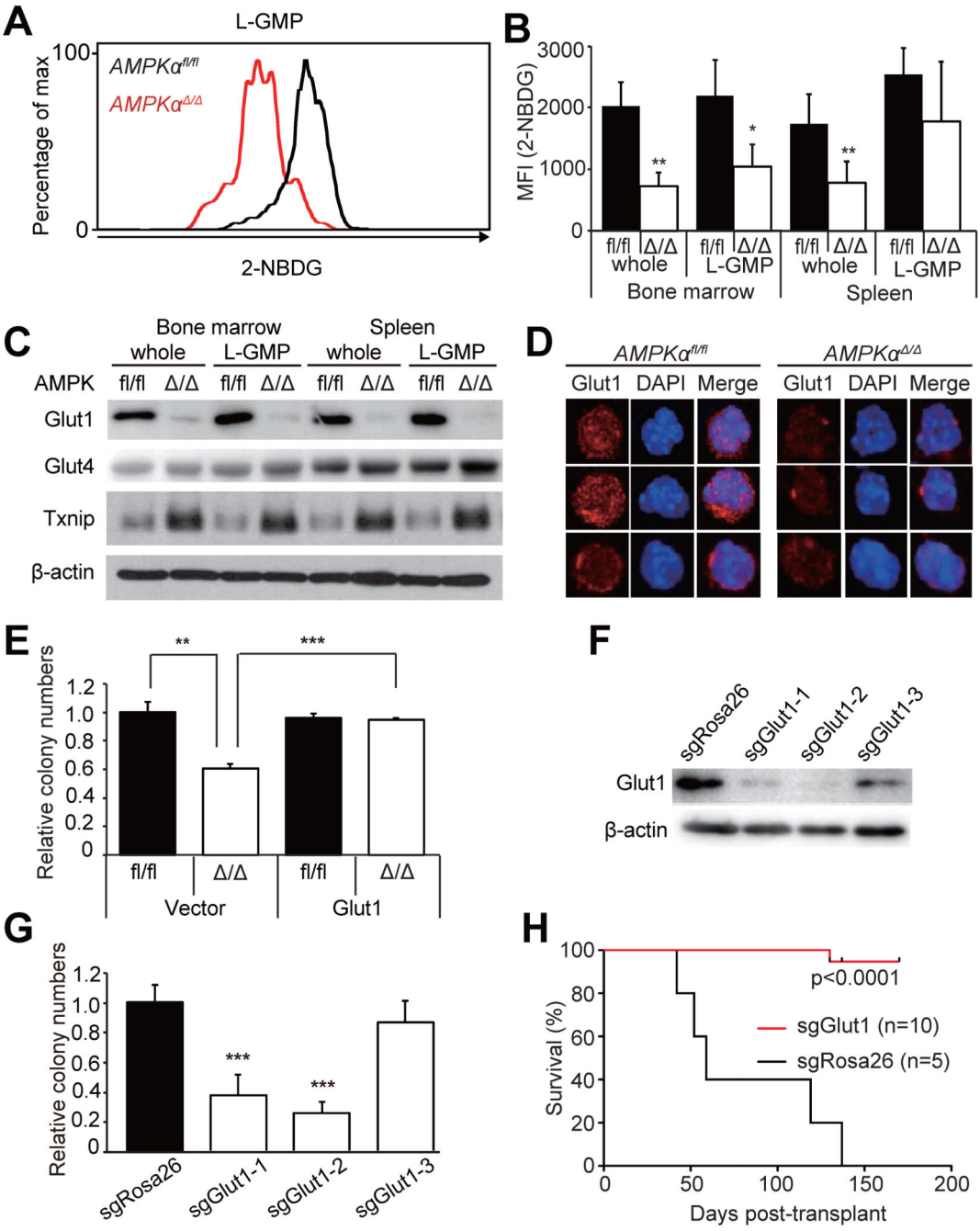

Figure 5. AMPK regulates glucose uptake through Glut1

(**A**) A representative histogram showing reduced 2-NBDG uptake by *AMPKα*$^{Δ/Δ}$ bone marrow L-GMPs *in vivo*. (**B**) *AMPK* deletion significantly reduced glucose uptake of whole AML cells in the bone marrow and spleens, and L-GMPs in the bone marrow but not in the spleens (n=5). Immunoblotting (**C**) and immunostaining (**D**) assays revealed that *AMPK* deletion reduced the protein levels of Glut1, but not Glut4, in whole AML cells and L-GMPs, concomitant with increased Txnip1 levels. Scale bars indicate 5 μm. (**E**) Overexpression of Glut1 substantially rescued the defective clonogenicity of *AMPKα*$^{Δ/Δ}$

AML cells (n=4). (**F**) Immunoblotting against Glut1 using gene-edited AML cells revealed that Glut1 sgRNA clones 1 and 2 reduced Glut1 protein levels, whereas clone 3 had little effects. (**G**) Clonogenecity of the gene-edited cells as in (**F**). Glut1 sgRNA clones 1 and 2 significantly reduced clonogenicity, whereas clone 3 did not (n=4). (**H**) AML cells expressing sgRosa26 (n=5) or sgGlut1 (clone 1, n=5; clone 2, n=5) were transplanted. Deletion of Glut1 by sgRNAs significantly attenuated leukemogenesis. See also Figure S4.

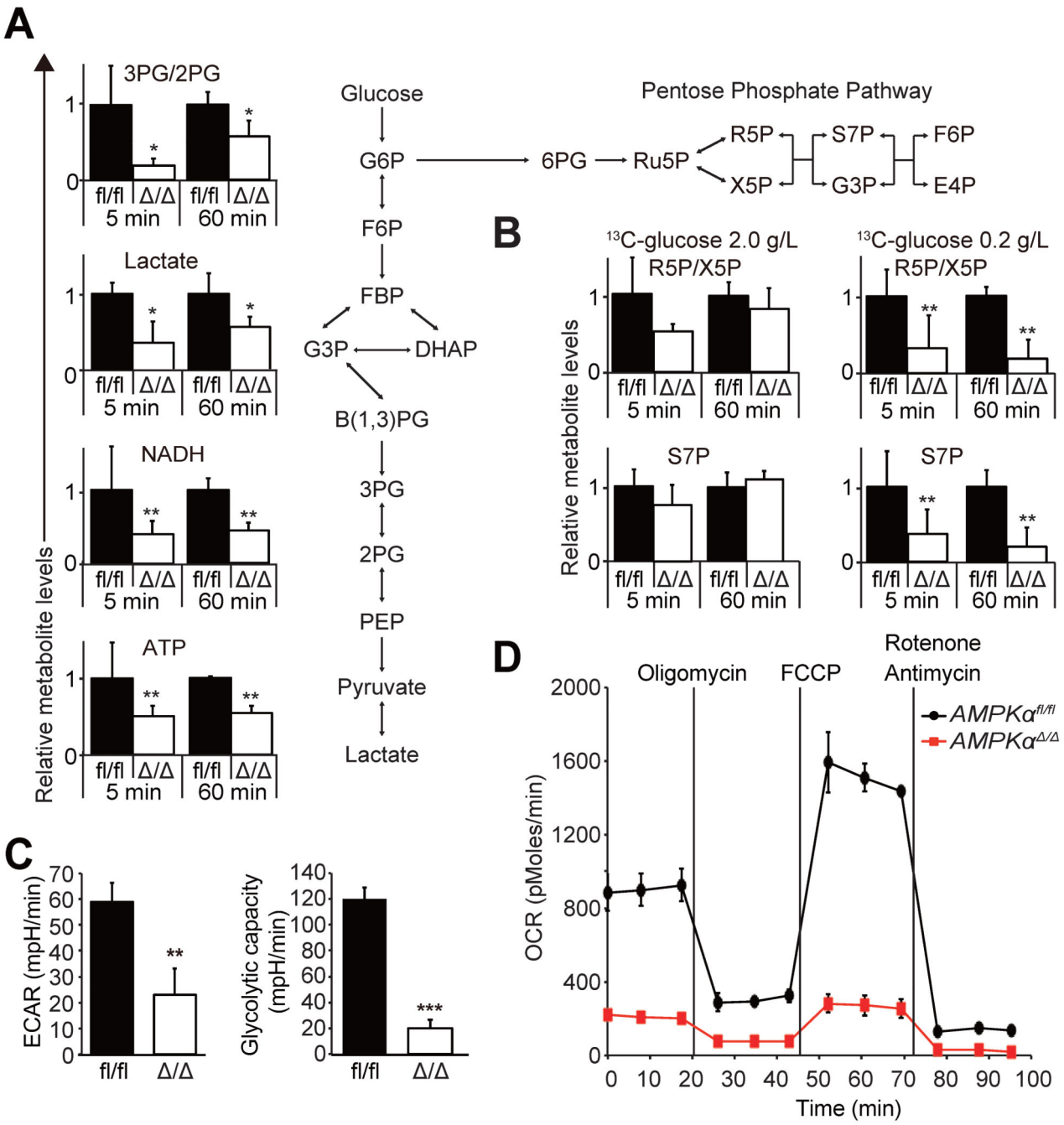

Figure 6. AMPK regulates glucose metabolism

(**A**) ^{13}C-glucose flux analysis performed on freshly isolated *AMPKα*$^{fl/fl}$ and *AMPKα*$^{\Delta/\Delta}$ AML cells exposed to 2.0 g/L ^{13}C-glucose for the indicated time revealed significantly reduced ^{13}C incorporation into glycolysis intermediates 3PG/2PG and lactate, in addition to NADH and ATP. The amounts of ^{13}C-labeled metabolites were normalized to the values of *AMPKα*$^{fl/fl}$ cells (n=3). Schematic of glycolysis and the pentose phosphate pathway (PPP) is shown on right. (**B**) ^{13}C-glucose flux analysis was performed as in (A) with either 2.0 g/L (left panels) or 0.2 g/L glucose (right panels) to access the glucose flux through PPP (n=3).

(**C**) Basal ECAR and ECAR after oligomycin treatment were significantly reduced in freshly isolated *AMPKα*$^{\Delta/\Delta}$ AML cells compared to *AMPKα*$^{fl/fl}$ AML cells (n=3). (**D**) Extracellular Flux analysis revealed that *AMPKα*$^{fl/fl}$ AML cells have significantly reduced OCR (n=3). See also Figure S5.

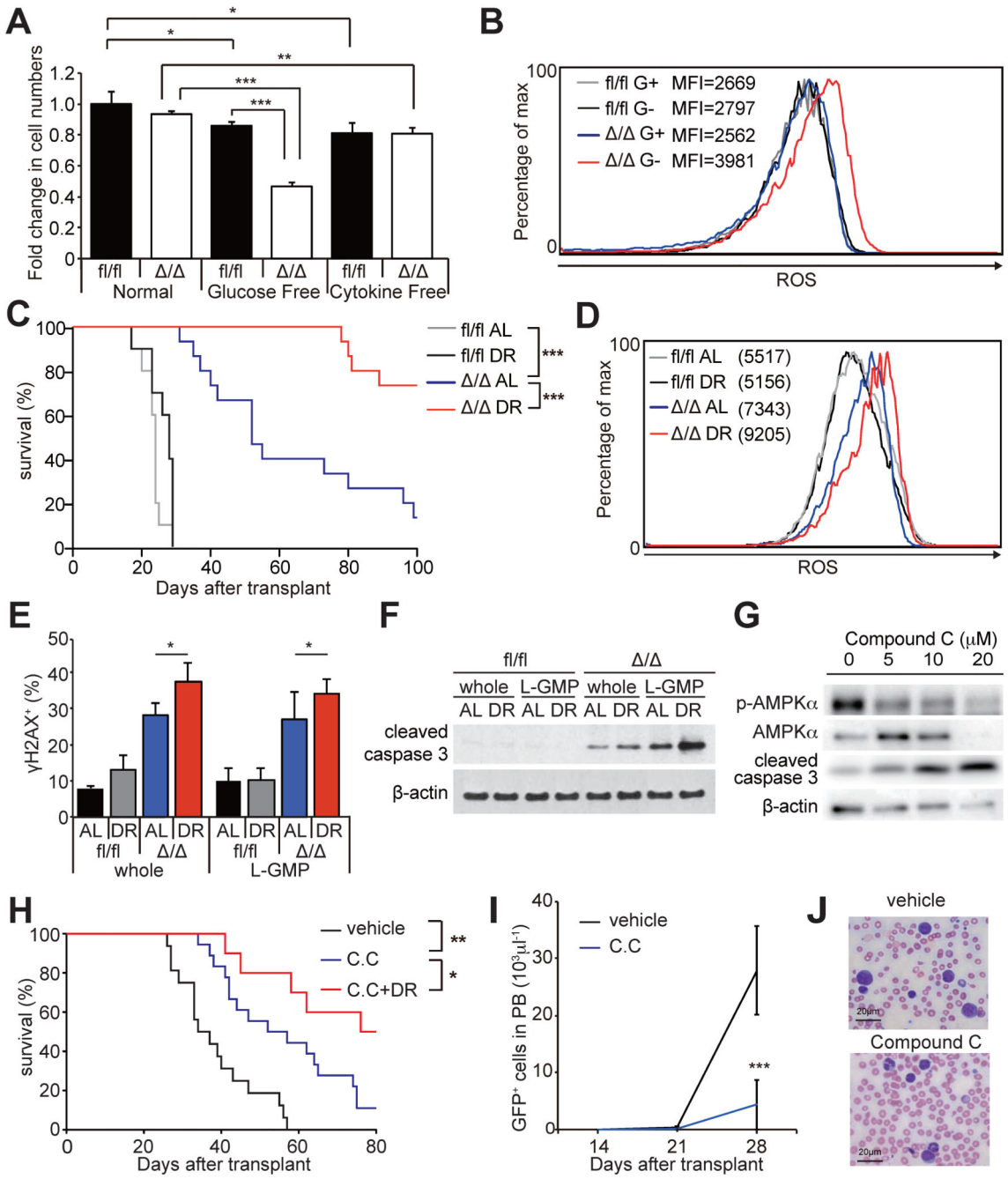

Figure 7. AMPK inhibition sensitized AML to dietary restriction

(**A**) *In vitro* glucose deprivation, but not cytokine deprivation, significantly reduced survival of *AMPKα*$^{Δ/Δ}$ AML cells during a 24-hour culture (n=4). (**B**) Representative histograms showing increased ROS levels in *AMPKα*$^{Δ/Δ}$ AML cells after glucose deprivation *in vitro*. G + and G− indicates cultured with or without glucose, respectively. (**C**) Secondary recipients of 10^4 *AMPKα*$^{fl/fl}$ (n=10 per group) and *AMPKα*$^{Δ/Δ}$ AML cells (n=15 per group) were either fed AL or subjected to DR and their survival monitored. DR did not extend the survival of *AMPKα*$^{fl/fl}$ AML recipients, but did significantly extend the survival of *AMPKα*$^{Δ/Δ}$ AML

recipients. (**D**) Representative histograms showing that DR further increased the higher ROS levels of *AMPKα*$^{\Delta/\Delta}$ L-GMPs (n=3). (**E**) The frequencies of whole AML cells and L-GMPs positive for γH2AX examined by immunofluorescence assay. (**F**) Immunoblotting assay using *AMPKα*$^{fl/fl}$ and *AMPKα*$^{\Delta/\Delta}$ whole AML cells and L-GMPs isolated from the bone marrow of the recipient mice subjected to AL or DR. (**G**) Immunoblotting assay of primary AML cells treated with compound C. (**H**) Secondary recipient mice of 10^3 MLL-AF9 AML cells were fed AL or subjected to DR after transplantation. 7 days after transplantation, mice were treated with 4 mg/kg of compound C (C.C) or control vehicle. Compound C treatment significantly extended the survival, and this was further extended when combined with DR (vehicle; n=16, C.C; n=18, C.C+DR; n=10). (**I** and **J**) Compound C treatment significantly reduced the total numbers of GFP$^+$ AML cells (**I**) and the frequencies of blasts (**J**) in peripheral blood (vehicle; n=5, C.C; n=8). Scale bars indicate 20 μm. See also Figure S6.