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14. ABSTRACT LKB1 is a master kinase that is mutated or underexpressed in 20-30% of non-small cell lung cancer (NSCLC) patient tumors. Because wild-type LKB1 functions as a sensor of energetic stress, the response to metabolic inhibition or nutrient depletion becomes dysregulated in LKB1-deficient cells. We have assessed the metabolic response to treatment of LKB1-deficient NSCLC cells with the clinically relevant EGFR inhibitor erlotinib. LKB1-deficient cells exhibited enhanced sensitivity to erlotinib treatment despite having wild-type EGFR. We have found that this enhanced response is due to mitochondrial dysfunction and altered energetic metabolism in LKB1-deficient cells, which prevents homeostatic maintenance of ATP and reactive oxygen species (ROS) levels in response to erlotinib treatment. This subsequently results in reductions in mTOR signaling and cell growth, as well as activation of apoptosis. These findings will be important for designing targeted treatments for LKB1-deficient NSCLC patient tumors, which disrupt metabolic and signaling pathways known to regulate energy metabolism in these cells.					
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INTRODUCTION

The overall objective of the current application is to apply metabolic flux analysis and quantitative metabolic profiling to identify critical nodes that regulate energy metabolism in LKB1-deficient NSCLC cells. LKB1 is a tumor suppressive serine/threonine kinase that activates diverse downstream kinases, thus regulating a variety of cellular phenotypes including metabolism, invasion, proliferation, and polarity [1]. LKB1 is lost in 20-30 percent of lung adenocarcinomas and 10-20 percent of lung squamous cell carcinomas, as well as in other primary tumor types at a lower prevalence. Our central hypothesis is that LKB1-deficient NSCLC cells have increased sensitivity to treatments that interfere with energy metabolism. Our long-term goal is to understand mechanisms of metabolic reprogramming in lung tumor cells so that these processes can be targeted by molecular therapeutics.

BODY

1. Determine the metabolic response of LKB1-deficient NSCLC cells to inhibiting mitochondrial oxidative metabolism.

Task 1.1: Identify mitochondrial inhibitors that differentially impact the growth, viability, and metabolic phenotypes of LKB1-deficient and LKB1-wildtype cells.

LKB1-deficient cells are more sensitive to inhibition of EGFR-PI3K-mTOR signaling. LKB1 mutation confers activation of the PI3K-mTOR signaling pathway and alters the cellular response to energy stress. We asked whether inhibitors of EGFR-PI3K-mTOR signaling could enhance growth inhibition in LKB1 mutant cells harboring KRAS mutations but with wild-type EGFR. LKB1-deficient cells were more sensitive to erlotinib on average, independent of EGFR mutation status (Fig. 1A). Furthermore, 25 μ M of the PI3K inhibitor LY294002 reduced growth by 30% in LKB1-wt cells, whereas 50-80% growth inhibition was observed in LKB1-deficient cells (Fig. 1B). Treatment with rapamycin also induced more growth inhibition in LKB1-deficient cells than in LKB1-wt cells (Fig. 1C). These results suggest that deficiency of LKB1 confers sensitivity to inhibition of EGFR-PI3K-mTOR signaling in NSCLC cells harboring KRAS activating mutations.

LKB1-deficient transgenic cell lines exhibit increased sensitivity to erlotinib treatment. KRAS and LKB1 mutant A549 and H460 cells were engineered with wild-type LKB1 overexpression constructs or vector controls. We first verified expression levels of LKB1 and LKB1-related downstream proteins in these stable transgenic cells lines (Fig. 2A). LKB1 directly phosphorylates Thr172 in the activation loop of AMPK [2,3] and activates AMPK under elevated AMP levels [4]. Activated AMPK subsequently phosphorylates and inhibits acetyl-CoA carboxylase (ACC). As expected, LKB1overexpressing A549 and H460 cells showed highly expressed LKB1 and phosphorylated AMPK α in comparison to vector controls. Consequently, activated AMPK α increased phosphorylation of ACC (Fig. 2A). Exposure of cells to varying concentrations of erlotinib from 12.5-25 μ M led to significantly more growth inhibition in LKB1 non-expressing vector control lines derived from both A549 ($p < 0.0001$) and H460 cells ($p < 0.005$) than in LKB1 overexpressing cells (Fig. 2B). In a converse experiment, we generated stable LKB1-knockdown lines from LKB1-wt Calu-6 cells using lentiviral transduction of LKB1 shRNA. As shown in Fig. 2C, levels of LKB1 protein expression were highly reduced in stable shLKB1 knockdown pools of Calu-6 cells as compared to control (non-targeted empty vector; pLKO.1). As expected, 10 μ M erlotinib induced a more substantial reduction in viability of

stable LKB1 knock down cells than the vector control (Fig. 2C, lower panel). Taken together, these results suggest that LKB1-deficiency enhances sensitivity to erlotinib in suppressing viability and growth rate of NSCLC cells.

Task 1.2: Construct metabolic flux maps depicting how NSCLC metabolic networks respond to treatments that selectively inhibit LKB1-deficient cells.

Erlotinib treatment inhibits glycolytic metabolism of LKB1-deficient cells. Because the LKB1-AMPK axis is involved in maintaining energy homeostasis, we next asked whether erlotinib treatment interferes with cellular energy metabolism by analyzing glucose consumption and lactate production. Erlotinib induced a >30% decrease in growth rate of non-expressing LKB1 A549 cells but had no effect on the growth of wild-type LKB1 expressing cells (Fig. 3A). In addition to reduced growth rate, erlotinib induced reduction of specific glucose consumption and lactate production in non-expressing LKB1 A549 cells. Furthermore, shLKB1 Calu-6 cells showed >80% decrease in growth rate by 25 μ M erlotinib compared to an insignificant reduction in control cells, which was accompanied by reduction of both glucose consumption and lactate production (Fig. 3B). Shaw *et al.* showed that LKB1-deficient cells are more prone to metabolic stress due to dysfunction of homeostatic energy maintenance regulated by the LKB1-AMPK axis [3]. In our data, LKB1 non-expressing A549 and shLKB1 Calu-6 cells showed basally high levels of glycolytic index, which was inhibited by erlotinib treatment.

Erlotinib treatment unmasks mitochondrial defects in LKB1-deficient cells. In a previous study, erlotinib induced apoptotic signaling pathways by loss of mitochondrial membrane potential in H3255 NSCLC cells harboring the EGFR(L858R) mutation [5]. We asked whether EGFR-wt NSCLC cells bearing loss-of-function mutations in LKB1 would show similar mitochondrial defects and apoptosis following metabolic stress by erlotinib treatment. As expected, erlotinib caused a greater depletion of ATP in shLKB1 Calu-6 cells compared to control cells (Fig. 4A). To investigate the mitochondrial defect, we measured the effect of erlotinib treatment on mitochondrial potential by using the cationic dye JC-1. As shown in Fig. 4B, following treatment with erlotinib or phenformin, shLKB1 H358 and Calu-6 cells had disruption of $\Delta\psi_m$ compared to control cells, reflecting impaired mitochondrial membrane integrity (red to green ratio of JC-1). shLKB1 Calu-6 cells also exhibited increased mitochondrial production of reactive oxygen species (ROS) in response to erlotinib treatment (Fig. 4C). We hypothesized that ATP depletion and loss of mitochondrial membrane potential might promote apoptosis in LKB1-deficient cells treated with erlotinib. As shown in Fig. 4D, erlotinib at 10 μ M induced cleavage of PARP in shLKB1 Calu-6, suggesting that erlotinib enhances mitochondrial-mediated apoptosis in LKB1-deficient cells.

2. Assess the roles of NADPH-producing pathways in regulating redox homeostasis of LKB1-deficient NSCLC cells.

Task 2.1: Determine the susceptibility of LKB1-deficient cells to treatments that block specific metabolic pathways required for NADPH production.

Erlotinib activates AMPK α and blocks mTOR signaling pathway in LKB1-deficient cells. Next, we evaluated the effect of erlotinib on phosphorylation of AMPK α at times 3 to 24 h post-treatment because AMPK is activated by AMP:ATP ratio. Interestingly, erlotinib induced phosphorylation of AMPK after 6 h treatment in shLKB1 cells (Fig. 5A), which preceded inhibition of ACC at 24 h. We thought that wild-type LKB1 might confer a protective effect to

prevent cell death in response to metabolic stress, whereas LKB1-deficient cells might have increased sensitivity to erlotinib due to dysfunctional LKB1-AMPK signaling axis. Therefore, we analyzed whether activation of AMPK suppresses mammalian target of rapamycin (mTOR) signaling and also the 70-kDa ribosomal protein S6 kinase (p70S6K1) as downstream effectors of mTOR. Calu-6 cells expressing shLKB1 cells showed complete disruption of mTOR pathway signaling by erlotinib treatment (Fig. 5B), and also LKB1 non-expressing A549 cells showed reduction of phosphorylated mTOR (Fig. 5C). These results indicate that sensitivity to erlotinib can be enhanced by AMPK α activation, independent of LKB1 expression.

Task 2.2: Quantify the relative contributions of G6PD/6PGD, ME1 and IDH1 pathways toward NADPH production and redox homeostasis under normal and stress conditions.

ROS production correlates with Nrf2-associated gene expression signature. Gene expression studies in Dr. Carbone's lab have revealed that a major component of the LKB1-deficient transcriptional signature is comprised of Nrf2 target genes involved in ROS detoxification, including enzymes involved in NADPH production. Nrf2 activation is observed in approximately half of tumors that have lost LKB1 function, but only 6% of LKB1 wild-type tumors. Analysis of mutation data reveals that this Nrf2-associated gene expression phenotype is driven primarily by somatic mutations in Keap1, of which 80% occur in tumors with LKB1 loss. LKB1-deficient tumors have been shown to be susceptible to oxidative stress, as they are unable to make the appropriate adaptive responses in metabolism and biosynthesis [6]. Thus, the high frequency of Nrf2 activation among LKB1-deficient tumors suggests that selective pressure exists for these mutations as a secondary protective mechanism. We have profiled endogenous ROS production in several LKB1 wild-type and mutant cell lines, which revealed significantly lower baseline ROS levels in LKB1-mutant NSCLC cells (Fig. 6). Taken together, these results indicate that Nrf2-activating mutations are closely associated with LKB1 deficiency and that Nrf2 activation functions to detoxify endogenous ROS in NSCLC cells.

KEY RESEARCH ACCOMPLISHMENTS

Major findings of this research to date:

- LKB1-deficient cells are more sensitive to inhibition of EGFR-PI3K-mTOR signaling.
- Erlotinib treatment inhibits glycolytic metabolism and induces energetic stress in LKB1-deficient cells.
- The enhanced susceptibility of LKB1-deficient cells is associated with defective mitochondrial metabolism.
- AMPK α activation by erlotinib blocks mTOR signaling in LKB1-deficient cells.
- ROS production correlates with Nrf2-associated gene expression signature in LKB1-deficient cells.

REPORTABLE OUTCOMES

Manuscript in preparation: Y-M. Whang, S.-I. Park, D. P. Carbone, J. D. Young*. Role of LKB1 in regulating the oxidative metabolism and drug response of lung cancer cells.

Invited talk: Uniting Against Lung Cancer, Investigator Meeting. New York, NY. Nov 15, 2012.

Cell lines: shLKB1 knockdown derivatives of Calu-6 and H358, stable LKB1-overexpressing derivatives of A549 and H460.

Training support for postdoctoral researcher, Dr. Young-Mi Whang.

CONCLUSION

EGFR inhibitors such as erlotinib are used clinically to treat EGFR-mutant NSCLC tumors but are not indicated for EGFR wild-type tumors. Our findings suggest that erlotinib (and possibly other inhibitors that block signaling through the EGFR-PI3K-mTOR signaling axis) may be successful in treating EGFR wild-type tumors that harbor LKB1 mutations. This appears to be due to dysregulation of LKB1-AMPK signaling in these LKB1-deficient tumors that disrupts the normal cellular response to energetic stress. In particular, erlotinib treatment leads to suppression of glycolytic metabolism that is associated with significant increases in ADP/ATP ratio, reductions in mitochondrial potential, and mitochondrial ROS accumulation in LKB1-deficient cells. These metabolic alterations are associated with reductions in mTOR signaling, inhibition of cell growth, and apoptosis activation. We expect that these findings may lead to novel lung cancer treatments that target specific metabolic pathways that are dysregulated in LKB1-deficient patient tumors.

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6. Jeon SM, Chandel NS, Hay N (2012) AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress. *Nature* 485: 661-665.

SUPPORTING DATA

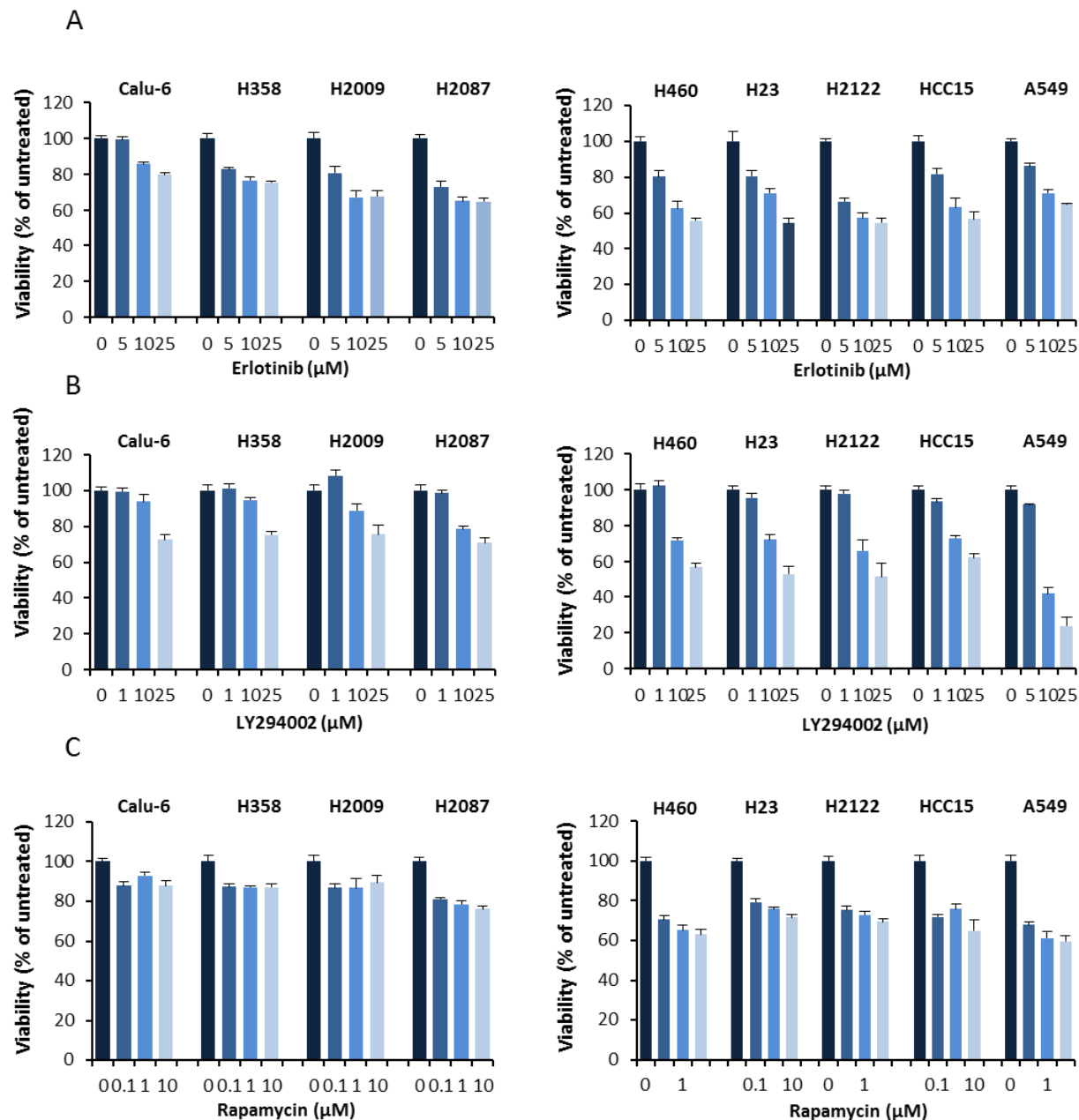


Figure 1. Erlotinib and inhibitors of PI3K-mTOR pathway enhance growth inhibition in LKB1-deficient NSCLC cells. Cells were seeded in 96 well plates. After 24 h, the cells were treated with indicated concentrations of erlotinib, LY294002, or rapamycin for 48 h. The viability was determined by using MTT assay. Previous reports show that Calu-6, H358, H2009, and H2087 cells (right panel) have normal LKB1 expression, whereas H460, H23, H2122, HCC15, and A549 cells are deficient in LKB1 expression.

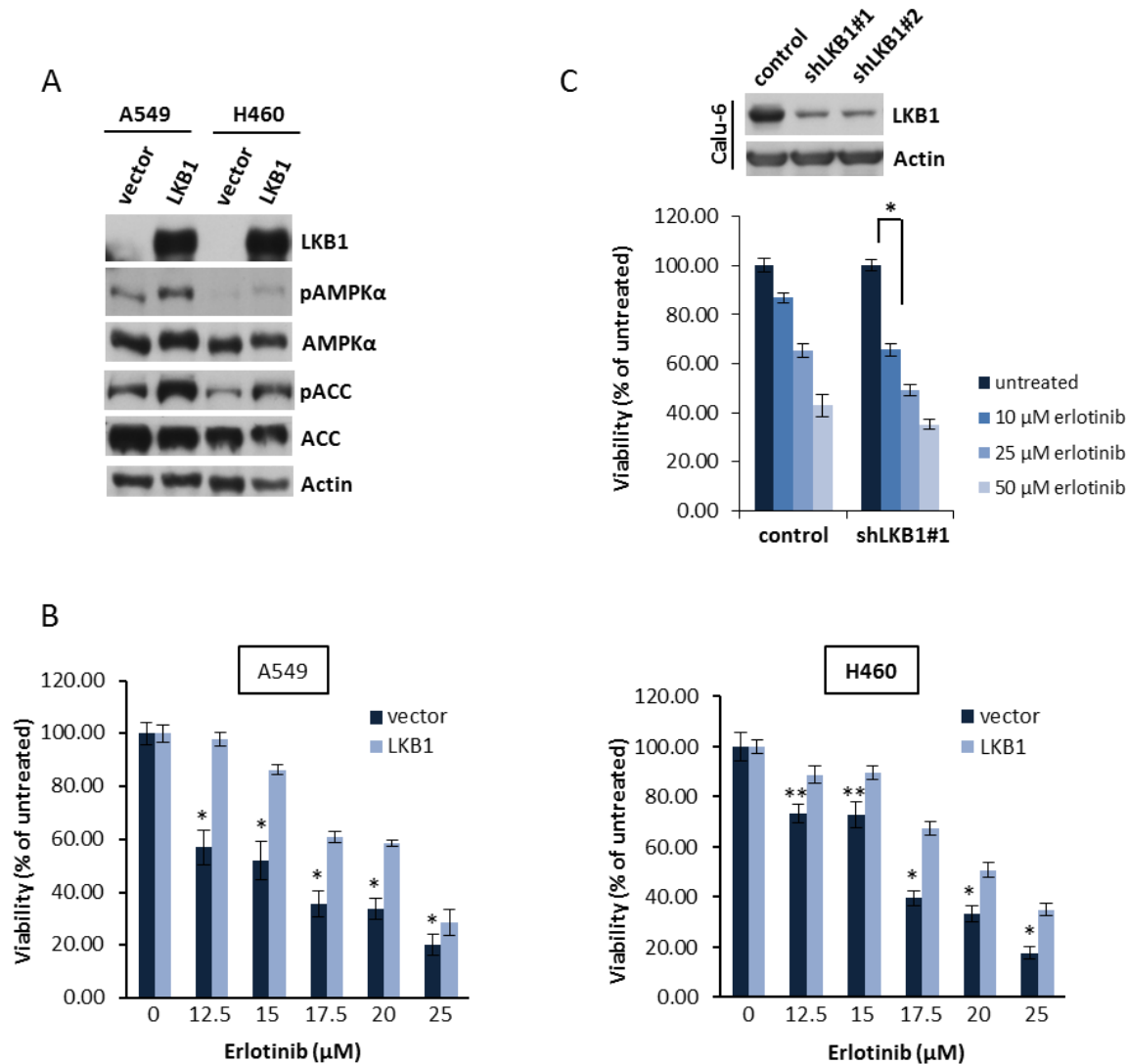


Figure 2. Erlotinib induces enhanced growth inhibition in LKB1 mutant cells and LKB1 knockdown cells. A, levels of LKB1 expression and LKB1-related downstream proteins in A549 and H460 stably expressing empty pBABE vector (vector) and wild-type LKB1 (LKB1). Western blots of whole-cell lysates from A549 and H460 stable vector and LKB1-expressing cells after puromycin selection. LKB1 was stably expressed in both cell lines, as detected using Western blot analysis. Wild-type LKB1-overexpressing cells induced phosphorylation of AMPKα and targeted the phosphorylation of acetyl-CoA carboxylase, whereas empty vector did not. β-actin was used as a loading comparison. Vector, control pBABE vector; LKB1, wild-type LKB1 expressing vector; ACC, acetyl-CoA carboxylase. B, cell proliferation was significantly reduced by erlotinib treatment in A549 and H460 cells expressing the empty vector. Cells were seeded in 96-well plates. After 24 h, the cells were treated with indicated concentrations of erlotinib for 48 h. Cell viability was determined by MTT assay. *, $p < 0.0001$, **, $p < 0.005$, untreated/erlotinib in vector cells. C, LKB1 stable knockdown Calu-6 cells were more sensitive to erlotinib treatment. Western blots of whole-cell lysates from LKB1 stable knockdown and non-target shRNA control Calu-6 cells after puromycin selection. Calu-6 cells were infected with

lentiviral LKB1 short-hairpin RNA (shRNA) constructs using two different alternative splicing sequences for silenced LKB1 gene. Upper panel: LKB1 expressions were depleted in both LKB1 stable knockdown cells, as detected using Western blot analysis. β -actin was used as a loading comparison. control, pLKO.1 control vector; shLKB1#1 and shLKB1#2, LKB1 shRNA vector. Lower panel: Cell proliferation was significantly reduced by erlotinib treatment in LKB1 stable knockdown cells. Cells were seeded in 96-well plates. After 24 h, the cells were treated with indicated concentrations of erlotinib for 48 h. Cell viability was determined by MTT assay. *; $p < 0.0001$, untreated/erlotinib in shLKB1 cells.

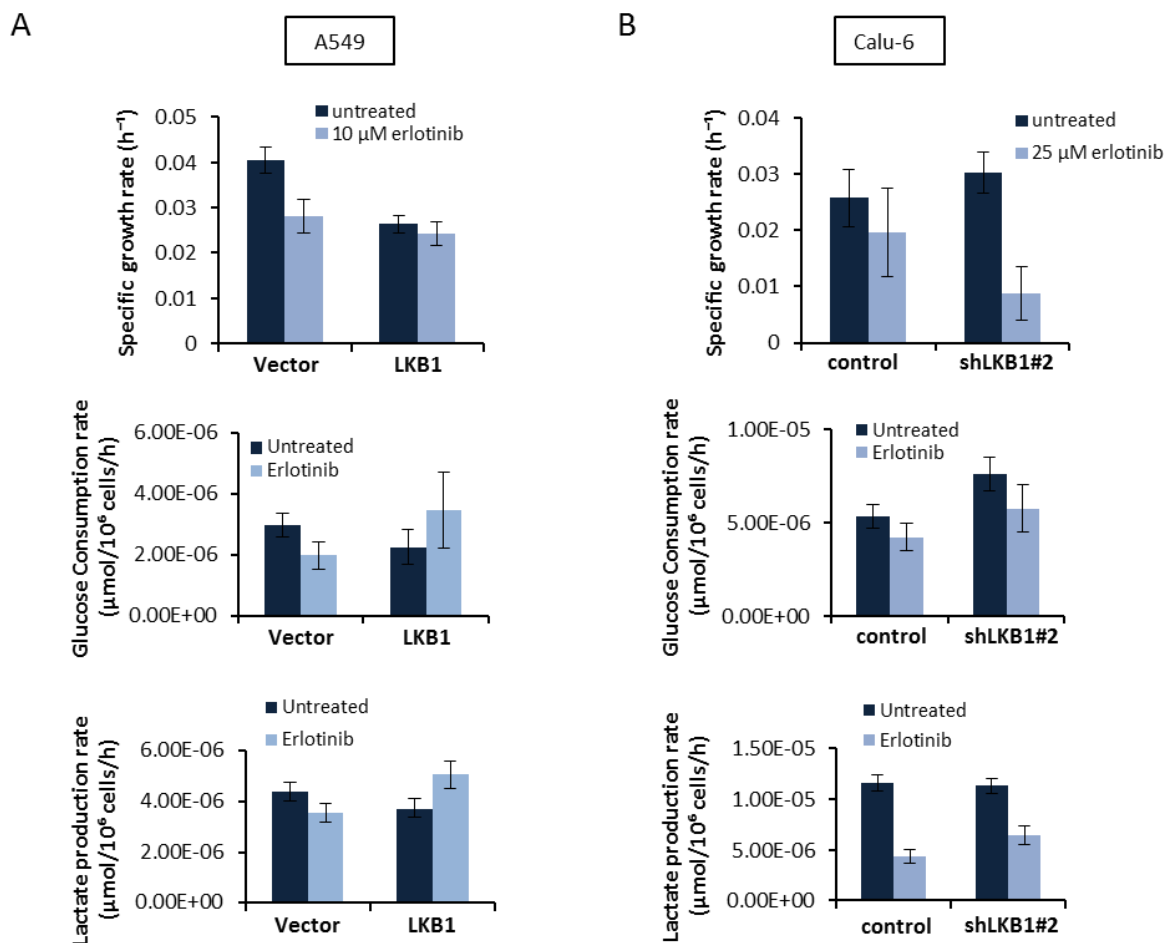


Figure 3. Erlotinib reduces growth rate and aerobic glycolysis in LKB1-deficient cells. Cells were seeded into 96-well plates. Growth was measured by CyQuant assay while glucose and lactate concentrations were measured by a YSI biochemical analyzer. A, A549 cells stably expressing empty pBABE vector (vector) and wild-type LKB1 (LKB1) were treated with erlotinib for 3days. B, shLKB1 Calu-6 cells (shLKB1#2, the depleted LKB1 expression showed at upper panel in Figure 2) and control were treated with erlotinib for 3days. Vector, control vector; LKB1, wild-type LKB1-expressing vector; control, pLKO.1 control vector; shLKB1#2, LKB1 shRNA vector.

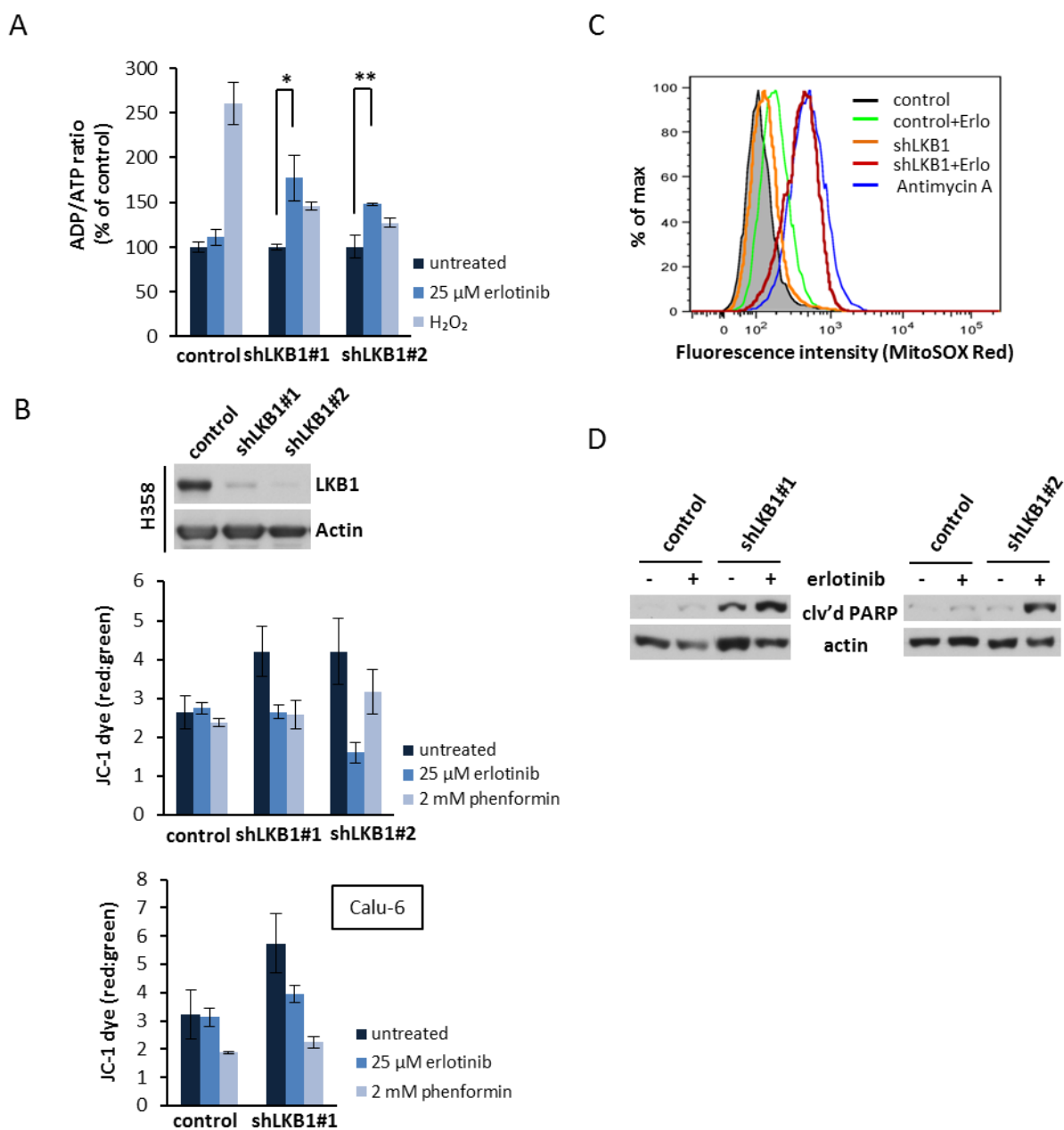


Figure 4. Erlotinib induces mitochondrial defects and enhanced apoptosis in LKB1 stable knockdown NSCLC cells. A, erlotinib increased ADP/ATP ratio in shLKB1 Calu-6 cells. Cells were treated with 25 μ M erlotinib for 24 h or 1 mM H₂O₂ for 1 h prior to measurement of ADP/ATP ratio. ADP/ATP levels were represented as relative luminescence values for comparisons between cells treated with erlotinib and untreated. Erlotinib significantly enhanced ADP/ATP ratio in shLKB1 Calu-6 cells. The data in all bar graphs are mean $\pm$ SEM. All P values are from Student's t-test. *, p<0.05, **, p<0.005. B, erlotinib disrupted mitochondrial integrity as a result of changes in the mitochondrial membrane potential in shLKB1 cells. H358 cells were infected with lentiviral LKB1 shRNA constructs using two different alternative splicing

sequences for silenced LKB1 gene. LKB1 were depleted in both LKB1 stable knockdown cells, as detected using Western blot analysis. β -actin was used as a loading comparison. control, pLKO.1 control vector; shLKB1, LKB1 shRNA vector. Cells were treated with 25 μ M erlotinib for 24 h. After treatment, cells were incubated with JC-1 solution at 37 °C for 1 h in the dark. Cells were washed with PBS and then the fluorescent intensities were measured at Ex/Em = 490/525 nm and 490/590 nm with a microplate reader. C, erlotinib elevated mitochondrial ROS in shLKB1Calu-6 cells. Cells were treated with 25 μ M erlotinib for 24 h or 20 μ M antimycin for 40 min prior to staining with MitoSOX Red and analyzed by flow cytometry. D, erlotinib highly induced apoptosis in shLKB1 cells. Control and shLKB1 Calu-6 cells were treated with 25 μ M erlotinib for 24 h and cell lysates were then subjected to Western blot analysis using antibody to cleaved PARP. β -actin was used as a loading comparison.

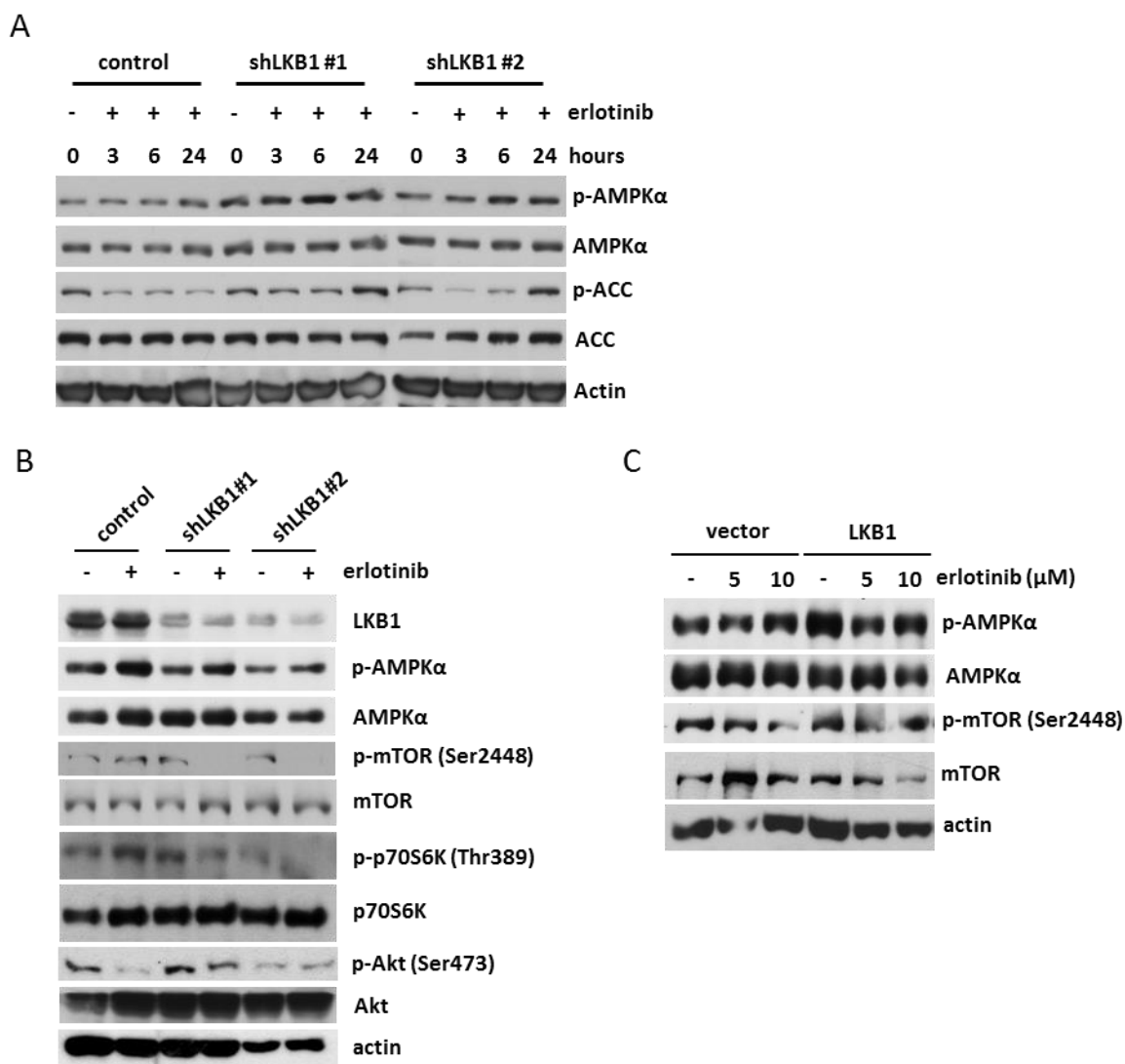


Figure 1. Erlotinib-induced activation of AMPK α selectively inhibits mTOR signaling pathway in LKB1-deficient cells. A, erlotinib activated AMPK α and inhibits ACC in shLKB1 Calu-6 cells. Following treatment with 10 μ M erlotinib for the indicated time, cell lysates were then analyzed by Western blotting. After 6h, erlotinib induced phosphorylation of AMPK α and then targeted the phosphorylation of ACC in shLKB1 Calu-6 cells, whereas control did not. B, erlotinib blocked mTOR signaling pathway by mediation of activated AMPK α in shLKB1 Calu-6 cells. After 24 h of 10 μ M erlotinib treatment, cell lysates were then subjected to Western blot analysis using specific antibodies for the indicated proteins. Blockade of mTOR activity following erlotinib treatment resulted in decrease in phosphorylation of p70S6K in shLKB1 Calu-6 cells. Erlotinib reduced phosphorylation of Akt in both cells. C, A549 cells expressing LKB1 and empty vector were treated with 5 or 10 μ M erlotinib for 24 h and then subjected to Western blot analysis using specific antibodies for indicated proteins. Phosphorylation of AMPK α increased by 10 μ M erlotinib in empty vector and resulted decrease in phosphorylation of mTOR. β -actin was used as a loading comparison. The blots are representative of three independent experiments.

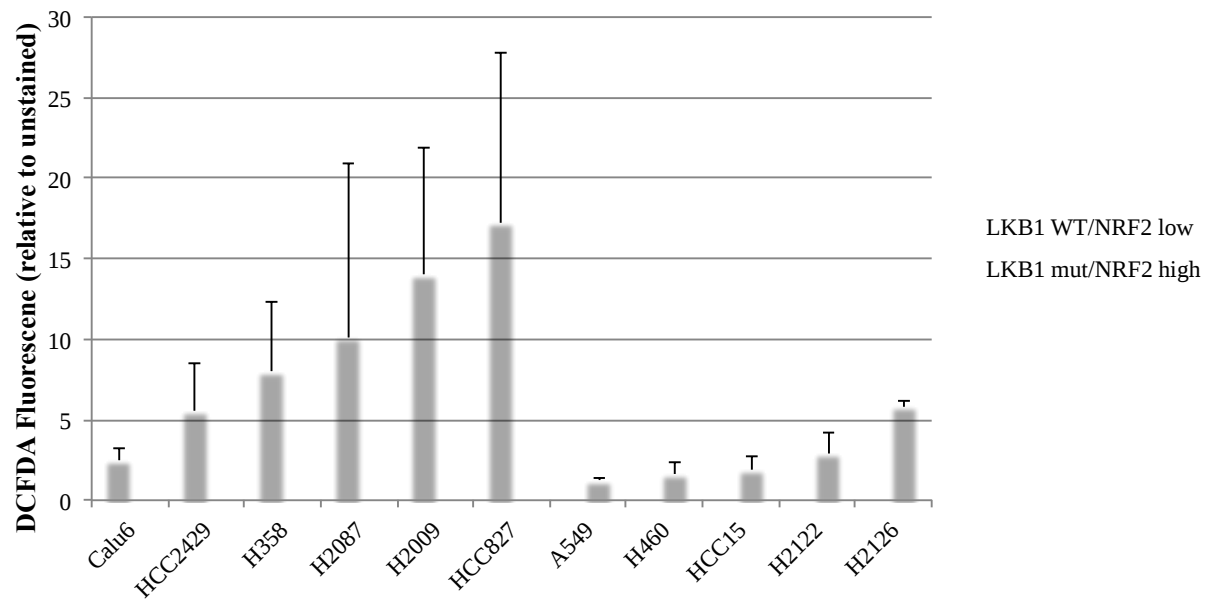


Figure 6. Endogenous ROS production among NSCLC cell lines. The activation of NRF2, as determined by gene expression profiling, was associated with significantly lower levels of endogenous ROS in cell lines. A549 and H460 have known KEAP1 mutations.