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APPLICATION NOTE 45

Metabolic Isotopic Analysis Reveals Mitochondrial Loss of Function in pRb-Deficient Cells *in vivo*

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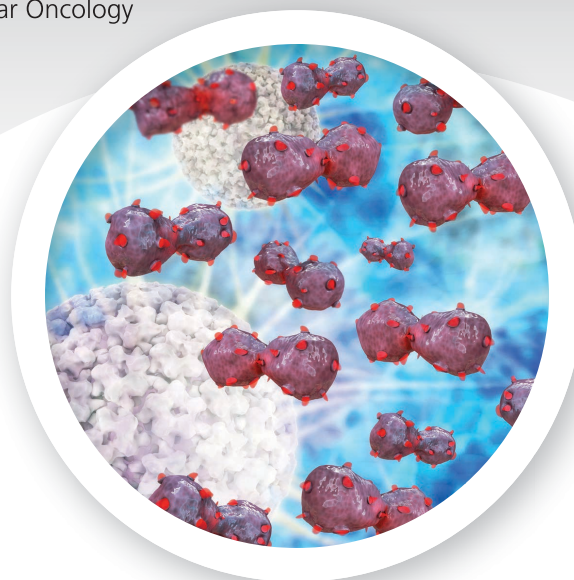
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Metabolic isotopic analysis (MIA) utilizes stable isotope-enriched substrates and mass spectrometry to study metabolism in living cells and tissue. Because isotope-labeled substrates make the determination of metabolic pathways and flux in living cells or tissue possible, MIA has become an integrated platform in oncology research.

This application note presents an example of MIA for studying metabolism in mice and cultured human cells used in oncology research by summarizing research conducted by Nicolay, et al. Previously, these researchers used MIA to study the inactivation of the retinoblastoma protein (pRb) tumor suppressor in the fly model. In the presented work, Nicolay and coworkers found that acute loss of pRB in adult mouse tissues led to rapid changes in glucose oxidation (Nicolay, et al., 2015). This subsequent study shows that MIA of glucose-derived intermediates in the TCA cycle for mitochondria in pRb^{-/-} tissue are less functional than the pRb^{+/+} tissue. These effects are mimicked in human cell culture models of pRb loss.

Methods and Results

An *in vivo* isotopic analysis of ¹³C₆-glucose (CIL Catalog No. CLM-1396) in both the colon and the lung using protocols similar to previously published work was performed (Fan, et al., 2011; Lane, et al., 2011; Yuneva, et al., 2012; Sellers, et al., 2015). Briefly, mice were given a single bolus of ¹³C₆-glucose and were sacrificed after 20 minutes for tissue isolation. An initial timecourse analysis found that 20 minutes after injection the levels of ¹³C₆-glucose had peaked within the blood, and ¹³C enrichments were sustained in downstream intermediates of glycolysis and the TCA cycle within both the lung and colon (Figure 1A-D). To determine that the glucose clearance would be similar in both Rb^{+/+} and RbKO mice, glucose tolerance tests (GTT) (of the same concentrated bolus of ¹³C₆-glucose) showed no differences between genotypes 96 hrs



after Cre induction (Figure 1E). These control experiments demonstrated that qualitative differences in glucose-derived metabolites could be determined using this methodology.

In support of the GTT results, analysis of ¹³C₆-glucose uptake from the serum revealed no differences between the Rb^{+/+} and RbKO colon or lung tissues (Figure 2A). In contrast, loss of Rb1 induced a significant enrichment (two-fold) of M+2 citrate in both tissues (Figure 1F). No difference was seen in the amount of M+3 pyruvate or M+3 lactate produced in either tissue (Figure 1G, H). Interestingly, no increase in glycolysis was detected following Rb1 loss (as indicated by pyruvate and lactate production (Figure 2B,C)). This curious result suggests that RbKO tissues had increased entry of glucose into the TCA. In agreement with this, a ratiometric that qualitatively measures pyruvate dehydrogenase (PDH) activity (M+2 citrate/M+3 pyruvate) was significantly elevated upon loss of Rb1 (Figure 1I). Furthermore, a significant enrichment of M+2 acetyl-CoA in both RbKO tissues was observed, as well as a significant increase in total acetyl-CoA in the RbKO lung (Figure 1J-K). Using this technique we could not qualitatively measure the TCA cycle activity directly. Nevertheless, these results show that the loss of pRb *in vivo* did not produce

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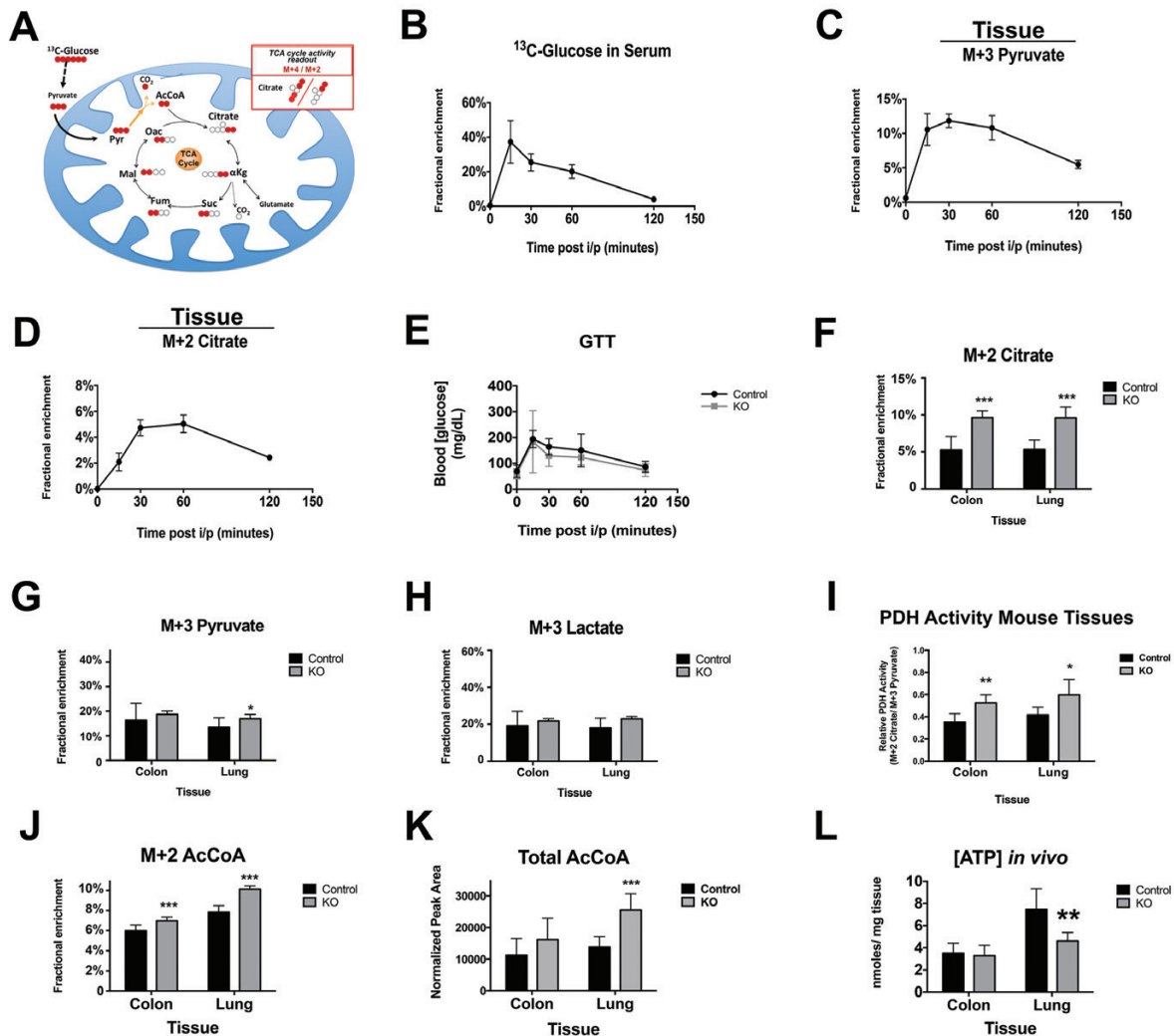


Figure 1. *In vivo* $^{13}\text{C}_6$ -glucose analysis of the effects of the loss of Rb on TCA cycle entry. (A) Cartoon of $^{13}\text{C}_6$ -glucose fate mapping the TCA cycle through pyruvate oxidation. Red circles are ^{13}C and open circles are ^{12}C . Pyruvate is completely comprised of ^{13}C and is labeled M+3. Abbreviations used are: AcCoA – Acetyl-CoA; alphaKG – alpha-ketoglutarate; CO_2 – carbon dioxide; Suc – Succinate; Mal – Malate; Fum – Fumarate; Oac – Oxaloacetate; Pyr – Pyruvate. (B-D) Timecourse analysis of the kinetics of the $^{13}\text{C}_6$ -glucose in the blood and tissues of control mice. Shown is the fractional enrichment of $^{13}\text{C}_6$ -glucose in the blood or tissues at the indicated timepoints post (i/p) injection of the $^{13}\text{C}_6$ -glucose, $n = 9-24$ mice/timepoint. (B) Kinetics of the clearance of $^{13}\text{C}_6$ -glucose from the blood. (C-D) Kinetics of the oxidation of $^{13}\text{C}_6$ -glucose into downstream glycolytic (M+3 pyruvate) and TCA cycle (M+2 citrate) intermediates in adult mouse tissues. Data trend is representative of colon and lung tissues; shown is data of colon samples. (E) Glucose tolerance test (GTT) shows similar glucose absorption between genotypes in mice after 96 hrs following Rb loss, $n = 4$ mice/genotype. (F-H) Rb loss elevates $^{13}\text{C}_6$ -glucose-derived citrate in both the colon and lung, but does not affect $^{13}\text{C}_6$ -glucose-derived pyruvate or lactate, $n = 8$ mice. (I) Ratio of PDH activity (M+2 citrate/M+3 pyruvate). Rb loss elevates PDH activity in both the colon and the lung. (J-K) Loss of Rb increases enrichment of glucose-derived acetyl-CoA and increases total pools of acetyl-CoA. (L) Loss of Rb impacts ATP levels *in vivo*. Error bars are the 95% confidence intervals. Statistical significance shown as P-values as follows: * < 0.05 , ** < 0.02 , *** < 0.001 . Statistical differences are between effects from $\text{Rb}^{-/-}$ compared to control cells.

a glycolytic phenotype, and, despite increased entry of glucose into the TCA cycle, ATP output decreased under comparable energetic conditions.

To support these *in vivo* results, a similar analysis was performed using the retinal pigment epithelium (RPE) cell lines. Strikingly, RbKO RPE cells showed a significant increase in the enrichment for M+2 citrate (Figure 3A). This effect again correlated with an elevated ratio of PDH activity (Figure 3B). These results show that Rb/RB1 ablation similarly affects pyruvate oxidation in the TCA cycle in mice and humans; furthermore, this change is irrespective of effects of Rb/Rb1 loss on cell proliferation. Unlike the *in vivo* analysis, a 24 hr pulse of $^{13}\text{C}_6$ -glucose in the RPE cells produced a fully labeled fraction of ^{13}C -citrate. From this we estimated the TCA cycle activity as a ratio between the M+4 citrate/M+2 citrate. M+4 citrate represents the second pass of ^{13}C -enriched citrate through the TCA cycle (Figure 1A). Thus, a larger ratio would reflect an accelerated TCA cycle. The loss of Rb1 severely decreased this ratio (Figure 3C), confirming that the TCA cycle was indeed diminished in these cells. As glutamine is also a large source of carbon oxidation for the TCA cycle *in vitro*, we performed similar fate mapping of $^{13}\text{C}_5$ -glutamine-derived TCA intermediates, and we found that oxidation of $^{13}\text{C}_5$ -glutamine was also diminished in RbKO RPE cells (Figure 3D).

Given the reduced mitochondrial capacity associated with Rb1 mutation, we tested whether these changes could create a cellular vulnerability. Therefore, we cultured control RPE and RbKO RPE cells for 72 hrs in DMEMPhysio in which the glucose concentration was reduced from 5 mM to 1 mM. Impressively, this nutrient shift, by itself, led to a 50% reduction in growth in RbKO RPE cells when compared to control cells (Figure 3E). Additional treatment with two different oxidative phosphorylation inhibitors (rotenone and phenformin, both CI inhibitors) further reduced the viability of RbKO RPE cells compared to similarly treated control cells (Figure 3F). These results strongly support the conclusion that loss of Rb/Rb1 impairs oxidative phosphorylation capacity and show that the mitochondrial changes have physiological consequences.

For methodology and further study details please see PubMed PMID: 26314710.

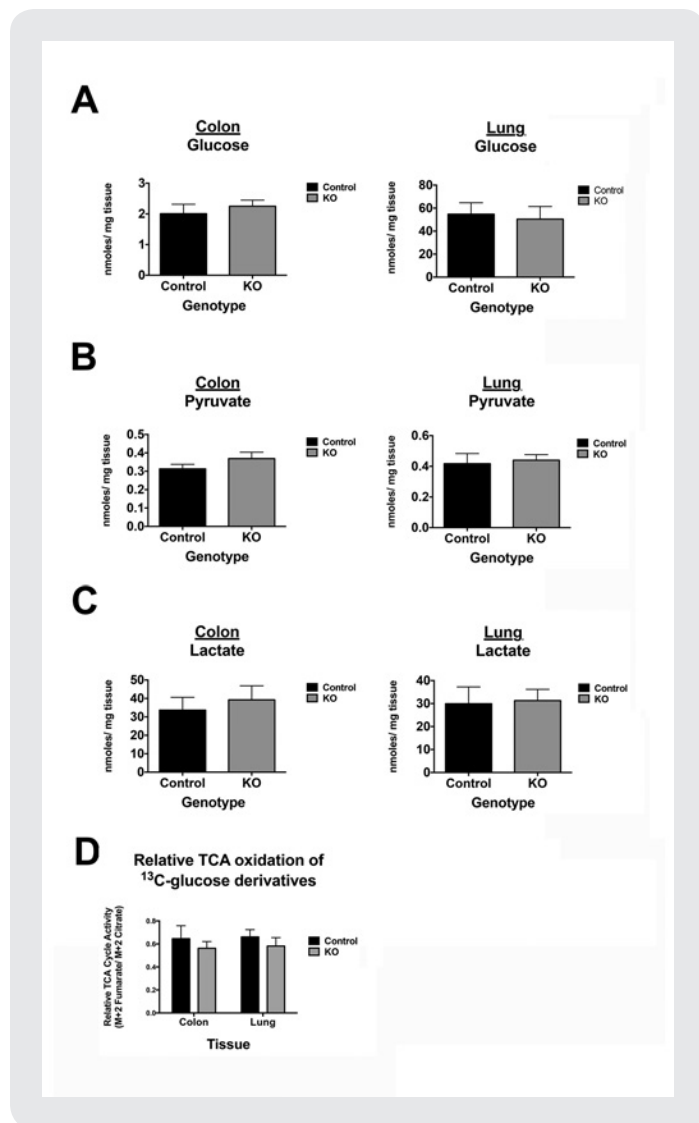


Figure 2. Loss of pRb does not influence glycolytic metabolite levels. (A-C) Loss of Rb1 does not affect steady state levels of $^{13}\text{C}_6$ -glucose in the tissues of mice following $^{13}\text{C}_6$ -glucose bolus. Activity of glycolysis as shown by readout of steady state levels of pyruvate and lactate is unchanged in mouse RbKO tissues, $n = 8$ mice/genotype. (D) Loss of Rb1 in the colon and lung shows slight decreases in TCA cycle activity as a readout of M+2 fumarate/M+2 citrate, $n = 8-12$ mice/genotype. Error bars represent the 95% confidence intervals.

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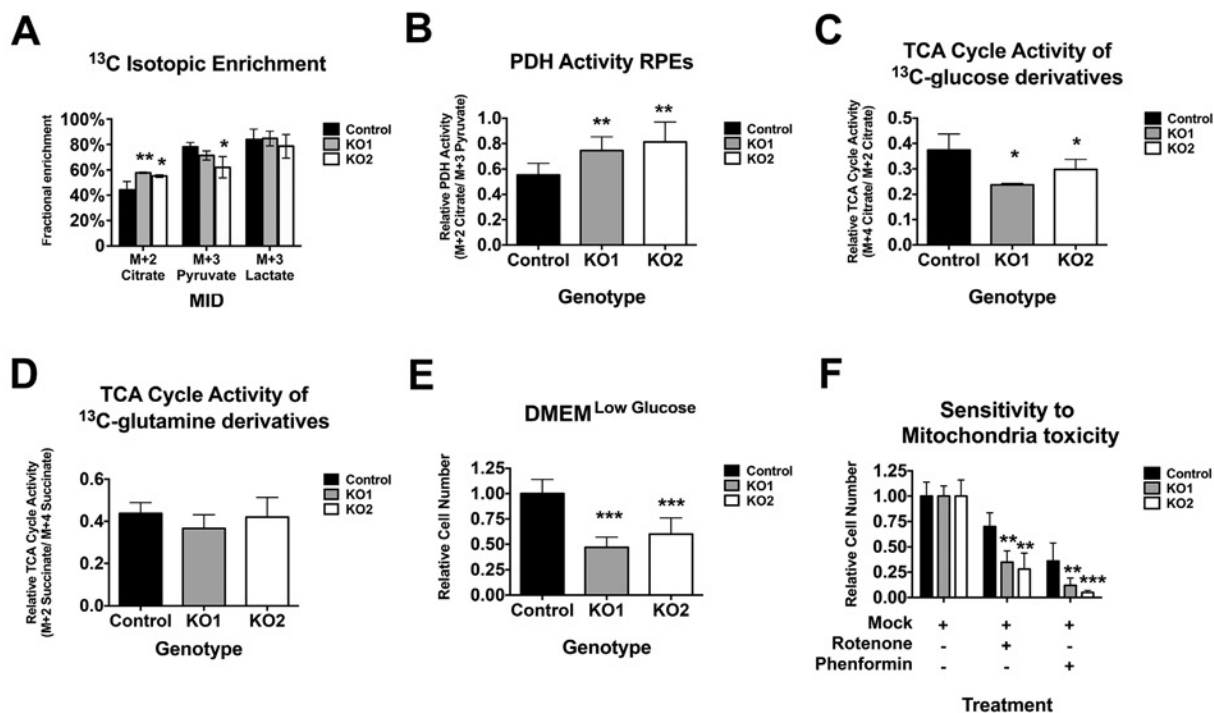


Figure 3. RbKO RPE cells have decreased TCA cycle activity and enhanced sensitivity to mitochondrial stress. (A) Rb loss elevates ^{13}C -glucose-derived citrate in RPE cells, but has little to no effect on the rates of ^{13}C -glucose-derived pyruvate or lactate, $n = 6$. (B) Ratio of PDH activity (M+2 citrate/M+3 pyruvate). Rb loss elevates PDH activity in RPE cells. (C-D) RbKO RPE cells show reduced TCA cycle oxidation of glucose (C) or glutamine (D). (E) RbKO RPE cells are growth impaired over 72 hrs when cultured in low glucose conditions and (F) show significantly enhanced sensitivity to rotenone (500 μM) and phenformin (10 μM), $n = 12$ samples per genotype, repeated twice. Error bars are the 95% confidence intervals. Statistical significance shown as P-values as follows, * < 0.05 , ** < 0.02 , *** < 0.001 . Statistical differences are between RbKO and RbWt, except in 3F: mock compared to drug treatment.

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