Mitochondrial electron transport chain (ETC) dysfunction elevates the NADH/NAD\(^+\) ratio to cause metabolic derangements. Here we describe a protocol to measure the NADH/NAD\(^+\) ratio and analyze the rewiring of glucose metabolism using [4-\(^2\)H]-glucose, [3-\(^2\)H]-glucose, and [U-\(^{13}\)C]-glucose in ETC-inhibited human cancer cells. We also describe a protocol to analyze the NADH/NAD\(^+\) ratio-sensitive metabolites in mouse plasma and mouse liver following phenformin treatment. These protocols comprehensively analyze the metabolic derangements resulting from increased NADH/NAD\(^+\) ratio in \textit{in vitro} and \textit{in vivo} models.
Protocol

Protocols for analyzing metabolic derangements caused by increased NADH/NAD\(^+\) ratio in cell lines and in mice

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SUMMARY

Mitochondrial electron transport chain (ETC) dysfunction elevates the NADH/NAD\(^+\) ratio to cause metabolic derangements. Here we describe a protocol to measure the NADH/NAD\(^+\) ratio and analyze the rewiring of glucose metabolism using [4-\(^2\)H]-glucose, [3-\(^2\)H]-glucose, and [U-\(^13\)C]-glucose in ETC-inhibited human cancer cells. We also describe a protocol to analyze the NADH/NAD\(^+\) ratio-sensitive metabolites in mouse plasma and mouse liver following phenformin treatment. These protocols comprehensively analyze the metabolic derangements resulting from increased NADH/NAD\(^+\) ratio in in vitro and in vivo models. For complete details on the use and execution of this profile, please refer to Liu et al. (2021).

BEFORE YOU BEGIN

The cellular NADH/NAD\(^+\) redox balance is fundamental to metabolism. NADH is generated by many metabolic pathways such as glycolysis, fatty acid oxidation, amino acid degradation, citric acid cycle, serine synthesis, and one carbon metabolism (Ducker and Rabinowitz, 2017; Hosios and Vander Heiden, 2018; Katsyuba et al., 2020; Lunt and Vander Heiden, 2011; Xiao and Loscalzo, 2020). ETC oxidizes NADH to regenerate NAD\(^+\) and maintain normal NADH/NAD\(^+\) ratio. ETC dysfunction or hypoxia increases the NADH/NAD\(^+\) ratio to inhibit NADH-generating reactions and cause metabolic derangements. Elevated NADH/NAD\(^+\) ratio inhibits cell proliferation in vitro, limits tumor growth in vivo, and underlies mitochondrial disease pathogenesis (Liu et al., 2021; McElroy et al., 2020). Thus, measurement of the elevated NADH/NAD\(^+\) ratio and the related metabolic derangements is necessary to understand the metabolic consequences under ETC dysfunction.

In cultured cells, the NADH/NAD\(^+\) ratio can be directly measured by measuring NADH and NAD\(^+\) though either liquid chromatography–mass spectrometry (LC-MS) or a commercial kit. Glycolysis compensates for ATP production and is essential for cell survival under ETC impairment. The glycolysis and its side pathways generate NADH at the steps catalyzed by GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) and PHGDH (phosphoglycerate dehydrogenase), and regenerate NAD\(^+\) by two side pathways catalyzed by cGPD (cytoplasmic Gro3P dehydrogenase) and LDH (lactate dehydrogenase) (Figure 1A). Glycolysis thus serves as a model pathway for analyzing cellular metabolic rewiring caused by the elevated NADH/NAD\(^+\) ratio. Here we describe a protocol to systematically analyze glucose metabolism under ETC inhibition. The deuterium of [4-\(^2\)H]-glucose can be transferred to NADH in the glycolysis pathway and is used to trace electron transfer of glycolytic NADH (Figure 1B); the deuterium of [3-\(^2\)H]-glucose can be transferred to NADPH in the oxidative
Figure 1. Schematic of glucose metabolism and deuterium glucose tracing

(A) Schematic of glucose metabolism. NADH/NAD\(^+\)-dependent reactions catalyzed by cGPD, GAPDH, PHGDH and LDH are highlighted. cGPD: cytosolic glycerol-3-phosphate dehydrogenase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; PHGDH: phosphoglycerate dehydrogenase. LDH: lactate dehydrogenase.

(B) Schematic of [4-\(^2\)H]-glucose tracing of NADH. The \(^2\)H can be transferred to NADH at the step catalyzed by GAPDH and then to Gro3P catalyzed by cGPD. DHAP: dihydroxyacetone phosphate; G3P: glyceraldehyde-3-phosphate; Gro3P: glycerol-3-phosphate.

(C) Schematic of [3-\(^2\)H]-glucose tracing of NADPH. The \(^2\)H can be transferred to NADPH in the oxidative pentose phosphate pathway. G6P: glucose-6-phosphate; 6PG: 6-phosphogluconate; RSP: ribose-5-phosphate.

pentose phosphate pathway (PPP) and is used to trace electron transfer of oxidative PPP (Figure 1C) (Lewis et al., 2014; Liu et al., 2021). We also use \([U-^{13}C]\)-glucose to trace glucose carbon.

In mouse tissue and plasma, total cellular measurements of NAD\(^+\), NADH, the NADH/NAD\(^+\) ratio are unchanged with known NADH/NAD\(^+\) ratio modulators, such as treatment with metformin/phenformin and expression of LbNOX (Goodman et al., 2020; Madiraju et al., 2014). The altered NADH/NAD\(^+\) ratio in tissues can be indicated by measuring the NADH/NAD\(^+\) ratio-sensitive metabolites, such as \(\alpha\)-hydroxybutyrate, alanine, aspartate and the lactate/pyruvate ratio (Goodman et al., 2020; Liu et al., 2021; Madiraju et al., 2014). We describe a protocol to measure the NADH/NAD\(^+\) ratio-sensitive metabolites and the altered glucose metabolism in mouse liver and plasma after phenformin treatment.

Preparations of cell line experiments

© Timing: around 1–2 h

1. On the day of glucose tracing and metabolite extraction from cell lines, the following needs to be prepared:

   a. Prepare the glucose tracing medium.
      i. \([U-^{13}C]\)-glucose tracing medium: glucose-free DMEM supplemented with 10% dialyzed FBS, 10 mM \([U-^{13}C]\)-glucose.
      ii. \([4-^{2}H]\)-glucose tracing medium: glucose-free DMEM supplemented with 10% dialyzed FBS, 10 mM \([4-^{2}H]\)-glucose.
      iii. \([3-^{2}H]\)-glucose tracing medium: glucose-free DMEM supplemented with 10% dialyzed FBS, 10 mM \([3-^{2}H]\)-glucose.
   b. Prepare ETC inhibitor stocks: 10 mM piericidin A and 10 mM antimycin A (dissolved in DMSO), and DMSO as control.
   c. Prepare the stock of NAD\(^+\) regeneration supplements: 1 M pyruvate and 1 M \(\alpha\)-ketobutyrate (dissolved in water and filtered through 0.22 \(\mu\)m filter).
   d. Prepare dry ice, cell lifters, labeled tubes and a tube rotator.
   e. Prepare extraction buffer (freshly made):
      i. \(-80^\circ\)C pre-chilled 80% methanol for \([U-^{13}C]\)-glucose tracing experiments;
ii. 15% ammonium bicarbonate and ice-cold acetonitrile/methanol/water (4/4/2) containing 0.1 M formic acid for [4-2H]-glucose and [3-2H]-glucose tracing experiments.

f. Prepare 0.1 M KOH and Bradford assay kit.

**Preparations of mouse experiments**

© Timing: around 3–4 h

2. On the day of mouse phenformin oral gavage, the following needs to be prepared:
   a. Prepare water for vehicle treatment.
   b. Prepare phenformin: dissolve phenformin in water at 10 mg/mL and store at –20°C.
   c. Separate mice into two groups: the vehicle group and the phenformin group.
   d. Prepare weighing scale, 1 mL syringes and gavage needles.
   e. Weigh the mice. Calculate the amount of vehicle (water) or phenformin at 100 mg/kg phenformin for a mouse.

3. On the day of mouse blood collection, tissue collection and plasma metabolite extraction, the following needs to be prepared:
   a. Prepare 25 gauge needles, ice-cold microtainer blood collection tubes for mouse blood collection.
   b. Prepare labeled 2 mL eppendorf tubes and liquid nitrogen for tissue collection.
   c. Prepare ice-cold acetonitrile/methanol/water (4/4/2) for plasma metabolite extraction.
   d. Prepare a vortex with the microtube holder and put it in the 4°C cold room.
   e. Cool down the centrifuge to 4°C.
   f. Prepare a SpeedVac vacuum concentrator.

4. On the day of mouse tissue metabolite extraction, the following needs to be prepared:
   a. Prepare the tissue homogenizing tube with 12 zirconia grinding beads.
   b. Prepare liquid nitrogen, ice-cold acetonitrile/methanol/water (4/4/2).
   c. Cool down the tissue homogenizer and put it in the 4°C cold room.
   d. Prepare a vortex with the microtube holder and put it in the 4°C cold room.
   e. Prepare a SpeedVac vacuum concentrator.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| Dulbecco’s modified Eagle’s medium (DMEM) | Gibco | Cat# C1196500BT |
| Fetal bovine serum (FBS) | Gibco-Biologicals | Cat# 900-108 |
| Dulbecco’s modified Eagle’s medium (DMEM)-glucose free | Gibco | Cat# C11966-025 |
| Dialyzed FBS | Gibco | Cat# 26400-044 |
| Penicillin and streptomycin (P/S) | Gibco | Cat# 151400122 |
| Piericidin A | Cayman Chemical | cat# 15379 |
| Antimycin A | Sigma-Aldrich | cat# A8674 |
| Phenformin (hydrochloride) | Sigma-Aldrich | cat# P7045 |
| Sodium pyruvate | Sigma-Aldrich | cat# P5280 |
| 2-Ketobutyric acid sodium salt hydrate (AKB) | Sigma-Aldrich | cat# 464422 |
| [U-13C]D-glucose | Sigma-Aldrich | cat# 389374 |
| [4-2H]D-glucose | Cambridge Isotope laboratories | cat# DLM-9294-PK |
| [3-2H]D-glucose | Cambridge Isotope laboratories | cat# DLM-9294-PK |
| Methanol for HPLC | Sigma-Aldrich | cat# 34860 |
| Acetonitrile for HPLC | Sigma-Aldrich | cat# 34851 |

(Continued on next page)
### MATERIALS AND EQUIPMENT

#### Table of metabolite standards

| Name                                           | Abbreviation          | Source           | Identifier | Cas number  |
|------------------------------------------------|-----------------------|------------------|------------|-------------|
| Nicotinamide adenine dinucleotide, reduced form | NADH                  | Sigma            | N8129      | 606-68-8    |
| Nicotinamide adenine dinucleotide              | NAD                   | Sigma            | N1511      | 53-84-9     |
| Nicotinamide adenine dinucleotide phosphate, reduced form | NADPH                | Sigma            | V900362    | 2646-71-1   |
| D-Glyceraldehyde 3-phosphate                    | G3P                   | Sigma            | 39705      | 591-57-1    |
| Dihydroxyacetone phosphate                      | DHAP                  | Sigma            | D7137      | 102783-56-2 |
| Glycerol 3-phosphate                            | Gro3P                 | Sigma            | G7886      | 29849-82-9  |
| D-Fructose 6-phosphate                          | F6P                   | Sigma            | F1502      | 103213-47-4 |
| D-Fructose 1,6-bisphosphate                     | F1,6BP                | Sigma            | F6803      | 38099-82-0  |
| D-Glycerate 3-phosphate                         | 3PG                   | Sigma            | P8877      | 80731-10-8  |
| D-Ribose 5-phosphate                            | R5P                   | Cayman           | 21344      | 17187-72-3  |
| D-Sedoheptulose 7-phosphate                     | S7P                   | Sigma            | 220116     | 5094-24-6   |
| alpha-hydroxybutyrate                           | α-HB                  | Sigma            | 9256       | 56-84-8     |
| Aspartate                                       | Asp                   | Sigma            | A7627      | 56-41-7     |
| Alanine                                         | Ala                   | Sigma            | 10127647001 | 3671-99-6   |
| D-Glucose 6-phosphate                           | G6P                   | Sigma            | 103302-15-4 |
| Erythrose 4-phosphate                           | E4P                   | Santa Cruz       | 214801     | Biotechnology |
Preparation of buffers

### 15% Ammonium bicarbonate

| Reagent           | Final concentration | Amount |
|-------------------|---------------------|--------|
| Ammonium bicarbonate | 15 % (w/v)         | 0.15 g |
| Ultrapure water   | n/a                | n/a    |
| Total             | n/a                | 1 mL   |

Note: Store at 4°C for years.

### 80% Methanol

| Reagent     | Final concentration | Amount |
|-------------|---------------------|--------|
| Methanol    | 80 %                | 40 mL  |
| Ultrapure water | 20 %              | 10 mL  |
| Total       | n/a                | 50 mL  |

Note: The solution is prepared just before use.

### Acetonitrile/methanol/water (4/4/2)

| Reagent           | Final concentration | Amount |
|-------------------|---------------------|--------|
| Acetonitrile      | 40 %                | 20 mL  |
| Methanol          | 40 %                | 20 mL  |
| Ultrapure water   | 20 %                | 10 mL  |
| Total             | n/a                | 50 mL  |

Note: The solution is prepared just before use.

### Acetonitrile/methanol/water (4/4/2) + 0.1 M formic acid

| Reagent             | Final concentration | Amount |
|---------------------|---------------------|--------|
| Acetonitrile        | 40 %                | 20 mL  |
| Methanol            | 40 %                | 20 mL  |
| Formic acid         | 0.1 M               | 192.5 μL|
| Ultrapure water     | 20 %                | 9.8 mL |
| Total               | n/a                 | 50 mL  |

Note: The solution is prepared just before use.

### LC mobile phase A

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| Ultrapure water  | 95 %                | 475 mL |
| Ammonium acetate | 0.01 M              | 0.385 g|
| Acetonitrile     | 5 %                 | 25 mL  |
| Total            | n/a                 | 500 mL |

Note: The solution is prepared just before use.
Note: The solution is prepared just before use.

**STEP-BY-STEP METHOD DETAILS**

**Isotope labeled glucose tracing under ETC inhibition in cultured cells**

**Timing:** 2–3 days

This step analyzes the rewiring of glucose metabolism under ETC inhibition in 143B cells including glycolysis, pentose phosphate pathway and glycerol-3-phosphate synthesis. We use [U-13C]-glucose to trace glucose carbon, [4-2H]-glucose to trace electron transfer of glycolytic NADH, and [3-2H]-glucose to trace electron transfer of oxidative PPP. The overall workflow is illustrated in Figure 2.

1. Cell maintenance and cell culture
   a. Culture 143B cells in DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 2 mM L-glutamine.
   b. Seed 3 million 143B cells onto a 6-cm dish and culture for 16 h, three repeats for one treatment.
   c. Aspirate medium and add fresh medium with DMSO or ETC inhibitors (piericidin A: 10 \( \mu \)M; antimycin A, 10 \( \mu \)M) in the presence or absence of NAD+ regeneration supplementations (3 mM pyruvate or 3 mM \( \alpha \)-ketobutyrate). Incubate for 2 h.

   Note: Pretreatment of cells with ETC inhibitors increases the NADH/NAD+ ratio to enable the detection of metabolic rewiring.

2. Incubate cells with isotope labeled glucose or normal glucose and add the extraction buffer.
   a. For deuterium-glucose tracing.
      i. Quickly wash cells with PBS twice.
      ii. Add 4 mL deuterium-glucose (10 mM) tracing medium supplemented with the same inhibitors as pre-treatment and incubate for the indicated time (12 h for [4-2H]-glucose, Figure 2A; 4 h for [3-2H]-glucose, Figure 2B). 3 repeats of normal glucose (10 mM) medium are used as control medium.

   Note: Normal glucose medium is used to correct and calculate the natural abundance of the isotopes in metabolic data processing. Inhibitor treatment in normal glucose medium is not required.

   iii. Quickly wash cells with PBS twice.
   iv. Aspirate PBS and add 1 mL ice-cold acetonitrile/methanol/water (4/4/2) containing 0.1 M formic acid to the dishes on ice.

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**LC mobile phase B**

| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| Ultrapure water  | 5 %                 | 25 mL  |
| Ammonium acetate | 0.01 M              | 0.385 g|
| Acetonitrile     | 95 %                | 475 mL |
| Total            | n/a                 | 500 mL |
Note: This extraction buffer decreases the interconversion between NAD(P)H and NAD(P)⁺⁺ (Lu et al., 2018).

v. Incubate cells on ice for 2 min.
vi. Add 87 μL 15% ammonium bicarbonate to neutralize samples. Mix immediately after addition.

b. For [U-13C]-glucose tracing.
i. Quickly wash cells with PBS twice.
ii. Incubate cells with the [U-13C]-glucose (10mM) tracing medium supplemented with the same inhibitors as pre-treatment (Figure 2C). Start a 5 min timer.
iii. After incubation for 4 min and 20 s, quickly aspirate medium and wash cells with PBS twice.
iv. Aspirate PBS and add 1 mL –80°C pre-chilled 80% methanol when the timer reaches 5 min.
v. Put the dish on dry ice immediately.

Note: [U-13C]-glucose quickly saturates the 13C-labeling of glycolysis intermediates within minutes. 5-min tracing has reached the steady state labeling of glycolysis intermediates, but the interchange of glycolysis intermediates (F6P and G3P) with non-oxidative PPP intermediates (R/X5P, S7P and E4P) are not saturated, allowing the detection of the altered labeling pattern of non-oxidative PPP intermediates under ETC inhibition (see results in Figure 7F).
3. Metabolite extraction
   a. Scrape cells on ice and collect the cell lysate in a tube.
   b. Vortex the cell lysate at 4°C for 1 min.
   c. Centrifuge the cell lysate at 20,000 g for 10 min at 4°C.
   d. Carefully transfer the supernatants to a new tube and keep the pellets.
   e. Dry the supernatants by a SpeedVac vacuum concentrator at 4°C.
   f. Store dried samples at −80°C.

   **CRITICAL:** Because of the short tracing time, we strictly control the total time from step ii-iv as 5 min (Figure 2C) and recommend handling the samples one by one.

   g. Dissolve the remaining cell pellets in step d in 600 μL 0.1 M KOH by shaking on a tube rotator at 4°C for 10–16 h.
   h. Measure protein concentration by the Bradford assay kit.

**Extraction of the NADH/NAD⁺ ratio-sensitive metabolites in mouse plasma and liver following phenformin treatment**

**© Timing:** 8 days for step 4

**© Timing:** 1–2 days for steps 5–7

Phenformin treatment increases hepatic NADH/NAD⁺ ratio and alters ratio-sensitive metabolism in mice. This step performs oral gavage of phenformin, followed by collection of plasma and liver samples, and metabolite extraction from collected samples. The overall workflow is illustrated in Figure 3.

**Note:** Any animal procedures described in this protocol should be cleared with local ethics and animal handling regulations of your institution.

4. Mouse maintenance and oral gavage administration
   a. Maintain C57BL/6J mice at 6–8 weeks old under a 12-h light/dark cycle and on a standard chow diet at the specific pathogen-free (SPF) facility.
   b. Gently restrain the mouse by grasping the loose skin of the neck and back to immobilize the head.

   **Note:** Try not to cause any animal vocalization or other signs of distress.

   c. Maintain the mouse in a vertical position and pass the gavage needle into the esophagus.

   **Note:** If resistance is encountered, alter the needle position.

   d. Inject the compound after the needle is passed to the stomach.

   **Note:** If the mouse struggles vigorously when compound administration begins, stop and withdraw the needle immediately. If compounds have been injected into the lungs, the mouse should be euthanized.

   e. Oral gavage mice daily with vehicle (water) or phenformin consecutively for 8 days before metabolic analysis.

5. Mouse blood collection and liver dissection
a. Restrain the mouse with the left hand by grasping the loose skin over the shoulders and behind the ears (Figure 4A).

b. Puncture the vein with a 25 gauge needle slightly behind the mandible, but in front of the ear canal (Figures 4B–4D).

**Note:** Use a swift motion to puncture and only the tip of the needle should enter the vessel to a shallow depth of 1–2 mm.

c. Collect about 350 μL blood into the blood collection tube held by another person (Figure 4E).

**Note:** Blood will flow immediately after puncture. The person holding the blood collection tube should be prepared right under the puncturing site.

△ **CRITICAL:** Do not squeeze the vessel during blood collection to avoid rupture of red blood cells.

d. Put the blood sample on ice.
e. Sacrifice the mice immediately after blood collection.

f. Dissect out the liver and immediately put it into the 2 mL Eppendorf tube and freeze in liquid nitrogen within seconds.

**Note:** If collecting multiple tissues (e.g., the liver and the brain) from the same mouse, we recommend collaboration by two people to shorten the processing time after mouse death.

g. Store frozen tissues at −80°C.

**Note:** Tissues can be stored at −80°C for weeks.

h. Extract plasma metabolites immediately after blood collection.

6. Metabolite extraction from mouse plasma

a. Centrifuge blood samples at 1,500 g for 10 min at 4°C.

**Note:** After centrifugation, if red blood cells are intact during blood collection, the upper plasma fraction is nearly colorless (Figure 4F). If the red blood cells are ruptured during blood collection, the upper plasma fraction is pink or red. The intracellular metabolites of ruptured red blood cells may enter the plasma and alter plasma metabolite concentration.

b. During centrifugation, prepare a new Eppendorf tube with 1 mL extraction buffer (acetonitrile/methanol/water (4/4/2)) on ice for each sample.

c. After centrifugation, carefully transfer 50 μL upper plasma fraction into the extraction buffer (Figure 4G).

d. Mix the extraction buffer and the plasma sample immediately by up and down shaking.

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**Figure 4. Blood collection and extraction**

(A) Depicting the mouse restraining gesture.

(B–D) To determine the accurate puncture site, draw a straight line from the bottom of the mandible to the caudal portion of the ear (B) and from the top of the eye to the point of the shoulder (C). Puncture the vein where these two lines cross (D).

(E) Blood collection after the puncture.

(F) Separation of plasma and red blood cells after centrifugation. The upper plasma layer is colorless, which suggests no occurrence of hemolysis.

(G) White flocculent precipitation after mixing plasma with the extraction buffer.

(H) The vortex with a microtube holder.
e. Vortex all the samples on a microtube holder (Figure 4H) for 1 min at 4°C.
f. Centrifuge samples at 20,000 g for 10 min at 4°C.
g. Transfer the supernatants to a new tube.

Note: Be careful not to perturb the pellets.

h. Dry the supernatants by a SpeedVac vacuum concentrator at 4°C.
i. Store dried samples at –80°C before mass-spec analysis.

△ CRITICAL: To avoid metabolites degradation, analyze the samples within 24 h.

7. Metabolite extraction from mouse liver
   a. Put –80°C frozen tissues into liquid nitrogen.
   b. Grind the tissue in a mixer mill MM400 (Figure 5A) at liquid nitrogen temperature.
      i. Prechill the rack in a liquid nitrogen bath and put the tissue containing tubes into the rack.
      ii. Run at 30 frequency/s for 30 s.
      iii. Put the tissue containing tubes into liquid nitrogen before weighing.
   
   Note: This dry cryogenic grinding can also be done by liquid nitrogen grinding in a mortar manually.
   c. Weigh approximate 30 mg tissues into the pre-cooled homogenizing tube with 12 zirconia grinding beads (Figure 5B). Seal the tube and put it into liquid nitrogen.
   d. After weighing all the tissue samples, put the tubes on ice and add approximately 600 μL ice-cold acetonitrile/methanol/water (4/4/2) (20 μL/mg).
   e. Homogenize tissue for 1 min at 6.5 m/s at 4°C in the tissue homogenizer (Figure 5C) and put on ice for 2 min. Repeat homogenization and cooling.

   Note: It is critical to maintain low sample temperature during homogenization. We perform tissue homogenization in the 4°C cold room.
   f. Centrifuge samples at 20,000 g for 1 min at 4°C and transfer the supernatants into new Eppendorf tubes.
   g. Centrifuge samples at 20,000 g for 10 min at 4°C and transfer the supernatants to new Eppendorf tubes.
   h. Dry the supernatants by a Speedvac vacuum concentrator at 4°C.
   i. Store dried samples at –80°C before mass-spec analysis.

   △ CRITICAL: To avoid metabolites degradation, analyze the samples within 24 h.

Liquid chromatography–mass spectrometry (LC-MS) analysis of metabolites

© Timing: 2 h for step 8

© Timing: 1–2 days for step 9

© Timing: 1 day for step 10

This step performs metabolite analysis of the cell line samples produced from steps 1–3 and mouse samples produced from steps 4–7. The cell line samples contain both isotope labeled and unlabeled metabolites, while mouse samples only contain unlabeled metabolites. The procedures for analyzing labeled and unlabeled metabolites are similar. We thus describe them together and also add notes in specific steps.
8. Preparation of stock solution of metabolite standards.

△ CRITICAL: The solutions of NAD, NADH, NADPH need to be prepared freshly on the day of LC-MS experiment. Other standard solution aliquots can be stored at −80°C for 4 weeks.

a. Prepare 1 mg/mL stock of metabolite standards (see table of metabolite standards) with ultra-pure water.

Note: If the compound is not fully dissolved, sonicate at 25°C for 20 min.

b. Prepare the 10 μg/mL mixed standard solution.
   i. Take 10 μL from each 1 mg/mL stock solution of metabolite standard.
   ii. Add to a new 1.5 mL tube.
   iii. Add 50% MeOH to make the final volume of 1 mL.

c. Split the 1 mL 10 μg/mL mixed standard solution into 10 aliquots of 100 μL and store at −80°C.

d. On the day of experiment, take one aliquot of mixed standard solution and run parallelly with biological samples.

9. LC-MS analysis of metabolites

a. Put dried samples stored at −80°C on ice.
b. Immediately add 100 μL 50% methanol to the samples and vortex 10 s to resuspend samples.
c. Centrifuge at 20,000 g at 4°C for 10 min.
d. Filter the supernatants through 0.45 μm filters.

Note: Filtration can remove the remaining insoluble particles to extend the column lifetime.

e. Transfer filtered supernatants to HPLC glass vials with micro-inserts and screw the cap.
f. Analyze the biological samples parallelly with the 10 μg/mL mixed standard solution using a high-resolution LC-MS system, e.g., Vanquish UHPLC - Q Exactive HF-X Hybrid Quadrupole-Orbitrap MS (Thermo Scientific).

Note: Other high resolution mass spectrometers, such as QTOF, can also be used for this application with only slight modification of MS parameters according to the specific instrumental setup. In case of unit resolution mass spectrometers, such as QQQ, multiple reaction monitoring (MRM) mode is recommended for higher specificity. For unlabeled metabolites, the MRM parameters need to be optimized using metabolite standards. For isotope-labeled
metabolites, few metabolite standards are commercially available; therefore, both the position of labeling and the fragmentation pattern of the metabolites need to be known to calculate the MRM product ion.

g. The following Liquid chromatography conditions were applied:
   Column: SeQuant ZIC-HILIC column, 3.5 µm, 100 Å, 100x2.1 mm;
   Mobile phase A: 10 mM ammonium acetate in ACN/water 5/95 (v/v);
   Mobile phase B: 10 mM ammonium acetate in ACN/water 95/5 (v/v);
   Flow rate: 0.5 mL/min;
   Column temperature: 40°C;
   Gradient: 0–5 min, 99% B; 5–20 min, 99%–20% B; 20–21 min, 20%–99% B; 21–25 min, 99% B.

h. The following MS parameters were applied for Q Exactive HF-X system:
   ESI source setting (positive mode): Spray voltage 3.5 kV, auxiliary gas heater temperature: 380°C, capillary temperature: 320°C, sheath gas flow rate: 35 units, auxiliary gas flow: 10 units.
   ESI source setting (negative mode): Spray voltage: 2.5 kV, auxiliary gas heater temperature: 380°C, capillary temperature: 320°C, sheath gas flow rate: 30 units, auxiliary gas flow: 10 units.

i. Scan parameters: MS1 full scan, m/z range: 66.7 to 1,000, resolution 60,000, AGC target 3e6, and maximum injection time 200 ms.

10. LC-MS data processing

The following data processing was performed by the Thermo Xcalibur Qual Browser software (4.2).

Note: If another mass spec instrument is used, use the corresponding vendor software to retrieve the ion chromatogram, retention time and peak height of the target metabolite.

a. Use m/z for unlabeled (M+0) metabolites (see table of retention time and m/z of targeted metabolites) to calculate their M+1, M+2, M+3... isotopologues using the following equation:

\[ m/z_{M+n} = m/z_{M+0} + n \times \text{Diff} \]

where \( m/z_{M+n} \) is the m/z of \( M+n \) isotopologue, \( n \) is the number of labeled atoms, \( m/z_{M+0} \) is the m/z of unlabeled metabolite, and Diff is the mass difference between \(^2\text{H}\) and \(^1\text{H}\), or \(^{13}\text{C}\) and \(^{12}\text{C}\) (see the following table).

| Isotope | Mass | Diff |
|---------|------|------|
| \(^1\text{H}\) | 1.0078 | 1.0063 |
| \(^2\text{H}\) | 2.0141 |      |
| \(^{12}\text{C}\) | 12   |      |
| \(^{13}\text{C}\) | 13.0034 |      |

For example, the m/z of NADH M+0 is 666.1321, then the m/z of \(^2\text{H}\)-labeled NADH M+1 is 666.1321+1* 1.0063=667.1384.

Note: NAD, NADP, NADPH are labeled by \(^2\text{H}\) while other glycolysis and TCA cycle metabolites are labeled by \(^{13}\text{C}\). For unlabeled metabolites such as alanine and aspartate, use m/z of M+0 in the following steps.

b. Open the metabolite data by the software. Retrieve the ion chromatograms for the target metabolite using its [M+H]+ or [M-H]− accurate m/z (table of retention time and m/z of targeted metabolites) with the mass tolerance of 10 ppm. Figure 6A shows the retrieve setting for NADH isotopologues (M+0 to M+4).
c. Identify the retention time of the target metabolite in the metabolite standard sample. Figure 6B shows the retention time of NADH isotopologues, e.g., 12.40 for NADH (M+0).

d. Use the above retention time (retention time window: 0.1 min) as the reference to identify the metabolites and record the peak heights, which represents metabolite concentration. As shown in Figure 6C, the NADH isotopologues in the normal glucose sample consist of M+0 (peak height: 2037327), M+1 (peak height: 537089) and M+2 (peak height: 172573). The NADH isotopologues in the [4-2H]-glucose sample consist of M+0 (peak height: 1229633), M+1 (peak height: 1192633), M+2 (peak height: 531912) and M+3 (peak height: 209730) (Figure 6D).

△ CRITICAL: Carefully check the retention times of all the metabolites. Only integrate isotopologues with retention time window less than 0.1 min.

e. Normalize the peak heights to protein concentration.

f. Calculate the mass isotopomer distribution of each metabolite after natural abundance correction using an open source IsoCor software (http://metasys.insa-toulouse.fr/software/isocor/) (Millard et al., 2012).

Table of retention time and m/z of targeted metabolites

| Compound | Formula  | Mode | Adduct | m/z (M+0) | RT (min) |
|----------|----------|------|--------|-----------|---------|
| NADH     | C21H29N7O14P2 | ESI+ | [M+H]+ | 666.1321 | 12.40   |
| NAD      | C21H27N7O14P2 | ESI+ | [M+H]+ | 664.1164 | 13.33   |
| NADPH    | C21H30N7O17P3 | ESI+ | [M+H]+ | 746.0984 | 14.04   |
| G3P      | C3H7O6P     | ESI- | [M-H]- | 168.9907 | 11.59   |
| DHAP     | C3H7O6P     | ESI- | [M-H]- | 168.9907 | 11.64   |
| Gro3P    | C3H9O6P     | ESI- | [M-H]- | 171.0064 | 11.58   |
| G6P      | C6H13O9P    | ESI- | [M-H]- | 259.0224 | 12.06   |
| F6P      | C6H13O9P    | ESI- | [M-H]- | 259.0224 | 11.81   |
| F1,6BP   | C6H14O12P2  | ESI- | [M-H]- | 338.9888 | 12.62   |
| 3PG      | C3H7O7P     | ESI- | [M-H]- | 184.9857 | 12.05   |
| R5P      | C5H11O8P    | ESI- | [M-H]- | 229.0119 | 11.76   |
| S7P      | C7H15O10P   | ESI- | [M-H]- | 289.0330 | 11.84   |
| E4P      | C4H9O7P     | ESI- | [M-H]- | 199.0013 | 12.06   |
| a-HB     | C4H8O3      | ESI- | [M-H]- | 103.0400 | 6.88    |
| Asp      | C4H7NO4     | ESI- | [M-H]- | 132.0302 | 10.95   |
| Ala      | C3H7NO2     | ESI- | [M-H]- | 88.0404  | 10.44   |

EXPECTED OUTCOMES

Rewiring of glucose metabolism under ETC inhibition in 143B cell lines

ETC inhibitor treatment increases the NADH/NAD+ ratio (Figure 7A).

[4-2H]-glucose tracing shows that ETC inhibition increases electron transfer from glucose to NADH at the step catalyzed by GAPDH; electrons are further transferred from NADH to Gro3P catalyzed by cGPD (Figure 7B). These results support that Gro3P synthesis regenerates NAD+ under ETC inhibition.

[3-2H]-glucose tracing shows that ETC inhibition decreases electron transfer from glucose to NADPH (Figure 7C), suggesting ETC inhibition suppresses oxidative pentose phosphate pathway.

[U,13C]-glucose tracing shows that ETC inhibition causes the accumulation of F6P, F1,6BP and G3P but decreases 3-PG (Figure 7D), indicating glycolysis is impeded at the NADH-producing step.
catalyzed by GAPDH. Glycolysis blockade forces glucose carbon into Gro3P synthesis (Figure 7E) and non-oxidative PPP intermediates R/X5P and S7P (Figure 7F). The increased labeling of M+3 R/X5P can only derive from the reverse flux from glycolysis intermediates to non-oxidative PPP (Figure 7G). Supplementation of pyruvate or AKB normalized the NADH/NAD⁺ ratio (Birsoy et al., 2015; Sullivan et al., 2015) and repressed these metabolic changes (Figures 7D–7F). The rewiring of glucose metabolism by the elevated NADH/NAD⁺ ratio is summarized in Figure 7H.
Measurement of the NADH/NAD+ ratio-sensitive metabolites in mouse plasma and liver after phenformin treatment

Previous (Goodman et al., 2020) and our (Liu et al., 2021) studies have shown that elevated NADH/NAD+ ratio increases α-hydroxybutyrate (αHB) and alanine levels, as well as decreases plasma...
aspartate levels in mouse plasma and liver. In mouse plasma, phenformin treatment increases αHB and alanine levels and decreases aspartate level (Figure 8A). Similarly, phenformin treatment increases αHB and alanine levels in mouse liver (Figure 8B). Phenformin treatment also rewires liver glucose metabolism to inhibit glycolysis at the NADH-producing step catalyzed by GAPDH (increased F6P and G6P levels and decreased 3PG level) and promotes the conversion of DHAP into Gro3P and the accumulation of non-oxidative PPP intermediates S7P and E4P (Figure 8B).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are expressed as mean ± SD for cell line experiments. Data are expressed as mean ± SEM for mouse experiments. All reported sample sizes (n) represent a biologically independent experiment, defined as follows: for 143B cell line data, this represents repetitive cells seeded on the same days within an experimental group. For mouse experiments, this represents blood or tissue from a distinct mouse. The results of all experiments were validated by independent repetitions.

Statistical significance was determined by two-tailed unpaired Student’s t-test. p values are denoted in figures as: not significant [ns], *p < 0.05, **p < 0.01, ***p < 0.001.

LIMITATIONS

Variation of mouse metabolite data is common. This relates to mouse status, varied mouse stress response during oral gavage and blood collection. Processing multiple samples at a time also complicates the process and increases the data variation.

TROUBLESHOOTING

Problem 1

[U-13C]-glucose tracing results vary greatly between biological repeats (steps 1 and 2).
Potential solution
Keep cell culture conditions exactly the same, e.g., use cells of the same passage, use dialyzed FBS aliquoted from the same bottle (step 1).

Because the tracing time is only 5 min for 143B cells, variation of tracing time will cause result fluctuation. We recommend collaboration between two people to keep accurate tracing time for each sample (step 2).

Problem 2
Weak metabolic changes in phenformin-treated mice (step 4).

Potential solution
Phenformin sensitivity varies among mice. Over-dosage of phenformin treatment causes mouse lethality. αHB is the most sensitive metabolite to altered NADH/NAD+ ratio (Goodman et al., 2020). We recommend testing several phenformin dosages and examining plasma αHB level after 8 days of phenformin treatment. Choose the dosage that is tolerated by mice and also increases the plasma αHB level.

Problem 3
Metabolite degradation in mouse tissue samples (steps 5–7).

Potential solution
The sample collection and processing time is critical for preventing metabolite degradation. Prepare and label all the tubes and materials before getting started. We strongly recommend collaboration between two people to collect blood and tissues. The blood should be put on ice immediately after collection, and dissected liver should freeze in liquid nitrogen within seconds.

Problem 4
Slow and insufficient amount of blood collection (step 5).

Potential solution
The accurate puncturing site and swift lancing motion are critical for the smooth blood flow. After puncturing, a large amount of blood should flow immediately. If blood flows drop by drop, try puncturing a second time instead of squeezing the vessel.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hui Jiang (jianghui@nibs.ac.cn).

Materials availability
This study did not generate new materials.

Data and code availability
This study did not generate unique datasets or code.

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AUTHOR CONTRIBUTIONS
All experiments were performed by S.L. and Y.M.; S.L., Y.M., and H.J. wrote the manuscript.
DECLARATION OF INTERESTS
The authors declare no competing interests.

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