Analyzing the metabolism of metastases in mice

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i Abstract

Metastasis formation is the leading cause of death in cancer patients. It has recently emerged that cancer cells adapt their metabolism to successfully transition through the metastatic cascade. Consequently, measuring and analyzing the *in vivo* metabolism of metastases has the potential to reveal novel treatment strategies to prevent metastasis formation. Here, we describe

two different metastasis mouse models and how their metabolism can be analyzed with metabolomics and ^{13}C tracer analysis.

1. Introduction

Metabolic alterations in cancer enable malignant transformation, uncontrolled cell proliferation [1,2] and promote tumor progression towards metastasis outgrowth [3–5]. The combination of cell intrinsic factors, such as genetic landscape and cell origin, and cell extrinsic factors, such as local nutrient concentrations and cell–cell interactions, regulate this reprogrammed tumor metabolism [4,6–9]. Understanding the interplay between these cell intrinsic and extrinsic factors in shaping *in vivo* metastasis progression is highly relevant to develop new therapeutic strategies against metastasis formation. To analyze the metabolism during metastasis formation requires specific methods such as *in vivo* metabolomics and ^{13}C tracer analysis.

Infusing tumor-bearing mice and cancer patients with isotope-labeled nutrients (*in vivo* ^{13}C tracer analysis) is emerging as a powerful technique for analyzing tumor metabolism [10,11]. Examination of label-enriched tumor metabolites after intravenous infusion with nutrients labeled with stable isotopes, such as ^{13}C, ^{2}H or ^{15}N, can provide the first direct assessment of the activity of metabolic pathways in tissues [11,12]. Using this cutting-edge technique, several studies have shown inter- and intratumor heterogeneity of nutrient preferences and pathway activity of intact tumors in mice and humans (**Table 1**). Based on such *in vivo* data, it emerges that cancer progression toward metastases formation is inherently linked to changes in the microenvironment [4,9,13–15]. However, the complete metabolic rewiring in secondary tumors still remains poorly studied, even though metabolic changes have an essential role in the metastatic outgrowth [4]. Therefore, it is highly relevant to measure and analyze the *in vivo* metabolism of cancer cells during the metastatic progression.

Here, we describe a protocol for determining the metabolism of mouse primary tumors (breast cancer) and metastases (lung) using stable isotope-labelled nutrients. In particular, we provide a protocol for the establishment of two different lung metastasis mouse models, catheterization, tracer administration by infusion, harvest of (cancer) tissue from mice for subsequent mass spectrometry (MS) analysis, and MS data interpretation.

2. Materials

Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations. Use a lab coat, gloves, and if required safety glasses at all times. Work, if necessary, under the chemical fume hood. Familiarize yourself with chemical/biological safety and ethical regulations before conducting the experiment.

2.1 Establishing a lung metastasis mouse model

2.1.1 Cell preparation

1. Culture medium (see **Note 1**) containing 10% Fetal Bovine Serum and 50 U/mL Penicillin/streptomycin.

2. Cultured 4T1, B16F10 or EMT6.5 cells.

3. Trypsin-EDTA (0.25%).

4. Dulbecco's Phosphate-Buffered Saline (DPBS).

5. 1.5 ml tube.

6. Ice.

7. Dry ice.

8. 37°C water bath.

9. Cell culture flask.

10. 70% ethanol.

11. Falcon tube.

2.1.2 Intravenous injection of cancer cells

1. Balb/c mice 6 weeks old or C57BL/6 mice 8 weeks old (see **Note 2**).

2. Infrared lamp (see **Note 3**).

3. Insulin syringe with needle 29G, 0.5ml, 12mm length.

4. Single cell solution on ice prepared in Subheading 2.1.1.

5. Clean mouse cage.

6. Mouse tail vein restrainer.

2.1.3 Injection of cancer cells in the mammary fat pad

1. Mouse shaver.

2. Hair removal cream.

3. Cotton swab.

4. Insulin syringe with needle 29G, 0.5 ml, 12mm length.

5. Single cell solution on ice prepared in Subheading 3.1.1.

2.2 Mouse surgery and ^{13}C glucose infusion

This technique can be performed as described by Broekaert and Fendt [10].

2.3 Collection of frozen tissue samples

1. Liquid Nitrogen.

2. Dry ice.

3. Ice.

4. Labelled polyzip bags.

5. Biosqueezer (e.g. Lab Services ref.1210).

6. 0.9% blood bank saline.

7. 1 ml Single-use tuberculin syringe with ml graduation, Luer tip.

8. Microvette for capillary blood collection.

2.4 Metabolite extraction

Work under the chemical fume hood when preparing extraction solutions.

1. Labelled 2 ml Eppendorf tubes (3 tubes per sample).

2. Grinding balls, stainless steel, 5mm.

3. Grinding balls, stainless steel, 3mm.

4. Analytical balance (range: 0.0001-50 g).

5. Forceps.

6. Liquid nitrogen.

7. Dry ice.

8. Ice.

9. MS-grade chloroform.

10. Chromasolv grade methanol.

11. Cryomill.

12. Milli-Q water.

13. 400x Norvaline:Glutarate internal standard stock solution for analysis of polar metabolites: Dissolve 1 mg each of both norvaline and glutarate in 1 ml Milli-Q water (see **Note 4**). Aliquots can be stored for up to one year at -20 °C.

14. Extraction solution 1 (water + Norvaline:Glutarate + methanol): Prepare per sample 0.75 µl of 400x Norvaline:Glutarate stock (i.e., 0.75 µg of each norvaline and glutarate per sample) in 300µl Milli-Q water. Add 500 µl methanol per sample and mix (final ratio water:methanol = 3:5). Store extraction solution 1 at -20°C until extraction (see **Note 4, 5** and **6**).

15. 100x internal standard stock solution for analysis of fatty acids: Dissolve 1 mg of heptadecanoic acid (C_{17}) in 1 ml chloroform (see **Note 4** and **5**). The C_{17} internal standard stock can be stored at -80 °C for 3-4 weeks.

16. Extraction solution 2: Prepare per sample 5 µl of 100x C_{17} stock (i.e., 5 µg C_{17} per sample) in 500 µl chloroform. Store this solution at -80°C until extraction.

17. Chemical fume hood.

18. Refrigerated acid resistant vacuum centrifuge

2.5 Derivatization of Polar Metabolites for Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Work under the chemical fume hood when preparing derivatization solutions

1. Methoxyamine derivatization solution: 20 mg/ml solution of O-Methoxyamine-HCl in anhydrous pyridine, counting for at least 20 µl of solution per sample. Weigh 20 mg methoxyamine in a safe lock Eppendorf tube. Wash a needle with pyridine using a syringe and discard the waste appropriately. Aliquot more than 1 ml of pyridine in a safe-lock Eppendorf tube using the washed needle. Add 1 ml of pyridine to the methoxyamine. Vortex until methoxyamine is entirely dissolved (see **Note 7**).
2. Glass GC-MS vials with insert and magnetic caps with septum.
3. Crimper.
4. *N*-(tert-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide with 1% tert-Butyldimethylchlorosilane (TBDMS) derivatization agent (see **Note 8**).
5. Heating Block.
6. Safe-Lock Eppendorf tubes.
7. Analytical scale (range: 0.0001-50 g).
8. Syringe with needle.
9. Chemical fume hood.
10. Refrigerated acid resistant vacuum centrifuge

2.6 Derivatization of Nonpolar Metabolites (Fatty acids) for GC-MS Analysis

Work under the chemical fume hood when preparing derivatization solutions

1. 2% sulfuric acid in MS-grade methanol (see **Note 9**).
2. Hexane (≥97.0%, GC grade).

3. Saturated NaCl: Dissolve sodium chloride in autoclaved Milli-Q water until solution is saturated. From then on, work only with the saturated (top) fraction.

4. Glass GC-MS vials with insert and nonmagnetic caps with septum.

5. Crimper.

6. Heating block.

7. Safe-Lock Eppendorf tubes.

8. Chemical fume hood.

9. Refrigerated acid resistant vacuum centrifuge

2.7 Preparation of Polar Metabolite Samples for LC-MS Analysis

1. 60% acetonitrile (LC-MS grade): prepare under the chemical fume hood.

2. Plastic vials with insert and plastic caps with septum.

3. Safe-Lock Eppendorf tubes.

4. Chemical fume hood.

5. Refrigerated acid resistant vacuum centrifuge

2.8 Protein Quantification

1. BCA protein assay kit.

2. 0.2 M NaOH: Weigh 0.8 g NaOH and transfer to a glass recipient. Add autoclaved Milli-Q water to a volume of 100 ml and mix (see **Note 10**).

3. Autoclaved Phosphate-Buffered Saline (PBS).

4. Heating Block.

5. Multichannel pipet.

6. Pipetting reservoir.

7. 96-well plate.

8. 37 °C incubator.

2.9 Collection of lungs for Haemotoxylin and Eosin (H&E) staining

1. Formalin solution, neutral buffered, 10% (see **Note 11**).

2. 27 G needle.

3. 1ml syringe without needle.

4. Mouse dissection tools: needle holder, scissors, standard forceps.

5. Closed container.

6. PBS.

7. Histology embedding cassettes.

3. Methods

Perform all procedures at room temperature unless otherwise specified.

3.1 Establishing a lung metastasis mouse model

3.1.1 Cell preparation

1. Thaw one vial of 4T1, B16F10 or EMT6.5 cells by removing it rapidly from liquid nitrogen and immediately placing it into a 37°C water bath (see **Note 12**).

2. Wash the vial with 70% ethanol before opening. Re-suspend the contents of the vial in a falcon tube containing 10 times the volume in the vial of the appropriate cell culture medium (RPMI for 4T1 and B16F10 cells, or MEM alpha for EMT6.5 cells). Spin down at 300 x g for 5 min. Aspirate the cell culture medium, and re-suspend the cell pellet in the desired volume of the appropriate cell culture medium. Transfer the cell suspension to a cell culture flask.

3. When the cell culture flask reaches 80-90% confluency, “split” the cells in an appropriate ratio depending on growth rates. 4T1, B16F10 and EMT6.5 cells are fast growing cells and can be split using trypsin-EDTA (0.25%) in a 1/10 ratio to reach confluency again after 3 days.

Expand the cells up to the desired amount (see **Note 13**). For intravenous injection, 100,000 cells per mouse will be needed. For injection in the mammary fat pad, 1 million cells per mouse will be needed (see **Note 14**).

4. When the desired amount of cells has been reached, dissociate the cells with trypsin-EDTA (0.25%) and wash 3 times with PBS to remove all Fetal Bovine Serum and penicillin/streptomycin. To wash the cells, spin down at 300 x g for 5min. Count the cells and re-suspend in the desired amount of PBS (see **Note 15**).

5. For intravenous injection, re-suspend 100,000 cells in 100 µl. For injection in the mammary fat pad, re-suspend 1 million cells in 50 µl (see **Note 16**). Transfer the cell suspension into an Eppendorf tube and put on ice.

3.1.2 Intravenous injection of cancer cells (lung metastasis w/o primary tumor)

Before you start, take a clean cage and direct an infrared (IR) lamp towards the cage to be able to dilate the blood vessels in the tail of the mice (Fig. 1a) (see **Note 3**). Put a tail vein restrainer in front of you. Transfer the first 5 mice to the clean cage with direct IR light.

1. Re-suspend the cells in the Eppendorf tube by pipetting up and down (see **Note 17**). Fill the insulin syringe (29G) with 100 µl cell suspension and remove all bubbles from the syringe (see **Note 18**).

2. Move the IR lamp further away from the clean cage, but keep the cage warm (see **Note 19**).

3. Take the first mouse and put it in a tail vein restrainer (Fig. 1b) (see **Note 20**). Hold the tail in your left hand (when right handed), identify the lateral tail vein and slightly bend the tail in between the index finger and thumb (Fig. 1c). To define the lateral tail vein you can place the mouse in front of you. The vein facing upwards is the dorsal tail vein, the vein facing downwards is the ventral artery (Fig. 1d red line) and the veins left and right from the dorsal tail vein are the lateral tail veins (Fig. 1d green lines). Insert the needle almost parallel to the

tail, with the needle opening upwards, and inject the cell suspension in one of the two lateral tail veins (Fig. 1d, e). The vein should change color when injecting correctly (see **Note 21**).

4. Put the mouse slowly back in its non-heated cage.

3.1.3 Injection of cancer cells in the mammary fat pad (spontaneous metastasis from primary tumor)

1. Shave the mice 2 days before injection around the nipple (Fig. 2) (see **Note 22**). To remove every single hair, you can evenly spread a drop of hair removal cream on the nipple area using a cotton swab. Wait approximately 2 min so the skin can absorb the cream and remove the cream using wet tissues.

2. Re-suspend the cells in the Eppendorf tube by pipetting up and down (see **Note 17**). Fill the insulin syringe (29G) with 50 µl cell suspension and remove the bubbles.

3. Inject the cells subcutaneously under the nipple, by inserting the needle about 10 mm distally from the nipple horizontally under the skin. Move the needle horizontally and subcutaneously towards the nipple, and inject when the needle tip is right under the nipple, with the needle opening upwards (see **Note 23**) (Fig. 3a, b). A small bubble under the nipple indicates a good injection (Fig. 3c) (see **Note 24**).

3.2 Mouse surgery and infusion of labelled metabolites

The duration of the model and moment of infusion differs for each mouse model, based on the humane endpoints to sacrifice the mice defined by the ethics regulations where the experiment is conducted (see **Note 25**) (Table 2).

Mouse surgery needs to be performed approximately 1 week before infusion.

3.3 Collection of snap-frozen tissue samples

1. Sacrifice the mouse with an overdose of Dolethal (140 mg/kg, 2.8 µl per gram of animal weight of a 50 mg/ml solution) (see **Note 27** and **40**).

2. Open the mouse and collect the blood by heart puncture. Put the blood on ice in a microvette for capillary blood collection, and spin down right after for 10 min at 10,000 x g. Transfer the plasma to an Eppendorf tube and store at -80 °C.

3. Collect the organs of interest as fast as possible. Wash the tissue in ice cold blood bank saline, remove the saline with a sterile compress, put the tissue in a labeled polyzip bag, squeeze it with the pre-cooled biosqueezer, and put it into liquid nitrogen (see **Note 28**). Store the tissues at -80 °C.

4. When collecting tumor tissue, it is important to separate tumor and healthy tissue immediately during collection, as frozen tissue is not malleable enough to allow for a correct separation of tumor tissue. However, keep in mind to limit the collection time to prevent any potential tissue degradation (see **Note 29**).

3.4 Metabolite extraction

Work under the chemical fume hood when handling the extraction solutions.

1. Weigh a piece of tissue (approximately 10 mg) while keeping it as cold as possible (see **Note 30**).

2. For grinding the tissue, add one 5 mm pre-cooled grinding ball and one 3 mm pre-cooled grinding ball to every tube with tissue. Cool down the Cryomill machine by opening the connection to the liquid nitrogen tank and grind the tissue for 30-40 s (frequency: 25 Hz). Place the sample tubes on dry ice immediately after grinding (see **Note 28**). Grounded tissue can be stored at -80 °C (see **Note 31**).

3. Transfer the tubes to a mixture of dry ice and ice. Add to each tube 800 µl of ice cold extraction solution 1, and 500 µl of ice cold extraction solution 2. Vortex the samples for 10

min at 4 °C, and then centrifuge them for 10 min at 4°C and maximum speed. The contents of the tube will now have separated into 3 layers (Fig. 4).

4. Pipet the polar top phase (consisting of extraction solution 1) into a new Eppendorf tube, and place the tube on dry ice. This layer will contain polar metabolites such as amino acids and organic acids. The middle layer consists of protein, DNA, and RNA. With a new pipet, cross through the middle layer while releasing some air, so that proteins, DNA, and RNA do not enter the pipet tip. Then, collect the nonpolar bottom phase (consisting of extraction solution 2), transfer into a new Eppendorf tube (trying not to disturb the middle layer while removing the pipet tip), and place the tube on dry ice (see **Note 32**). This lower phase will contain fatty acids and other nonpolar metabolites (Fig. 4). Bring also the tube with the remaining protein/DNA/RNA into dry ice.

5. Dry down the collected samples in a vacuum centrifuge. The samples containing polar metabolites should be dried at 4 °C for 8 h, while the protein and fatty acid containing samples can be dried down at 20 °C for 1 h in an acid resistant vacuum centrifuge. When dry, store the samples at -80 °C.

6. Perform this extraction process one additional time in triplicate in identical Eppendorf tubes but without any sample. These samples are “mock” extractions and will serve to assess the presence of impurities at a later point.

3.5 Derivatization of Polar metabolites for GC-MS Analysis

Work under the chemical fume hood when handling the derivatization solutions.

1. Add 20 µl of 20 mg/ml O-Methoxyamine-HCl dissolved in pyridine to each sample, vortex briefly, and incubate for 90 min in a heating block at 37 °C. Subsequently, centrifuge the samples for 3-5 min at maximum speed, transfer 7.5 µl of the supernatant into a glass GC-MS vial with insert, and seal with a magnetic cap using a crimper (see **Note 33**).

2. Fill a glass vial with insert with *N*-(tert-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide (TBDMS, 15 µl/sample + 25 µl extra per vial) and seal with a nonmagnetic cap (see **Note 34**). Fill a 2 ml glass vial without insert with pyridine, and seal with a nonmagnetic cap. Program the GC-MS autosampler to add 15 µl TBDMS to each sample, incubate for 1h at 60 °C, and re-suspend the sample prior to injection. Program also the autosampler to wash the injection needle with pyridine in between loading different samples (see **Note 35** and **36**).

3. Measure the samples using a GC-MS instrument. In our case, as described before [13,16], samples are measured on an Agilent 7890A GC system coupled to an Agilent 5975C Inert MS system. In brief, 1 µL of sample is injected into a DB35MS column in splitless mode, using an inlet temperature of 270 °C, and with helium at a flow rate of 1 ml/min as the carrier gas. Upon injection, the GC oven is first held at 100 °C for 1 min, then ramped up to 105 °C with a gradient of 2.5 °C/min, after that ramped up to 240 °C with a gradient of 3.5 °C/min, and finally ramped up to 320 °C with a gradient of 22 °C/min, followed by a 4 min post-run at 320 °C. The MS system is operated under electron impact ionization at 70 eV, and a mass range of 100–650 amu is scanned.

3.6 Derivatization of Non-polar Metabolites (Fatty Acids) for GC-MS Analysis

Work under the chemical fume hood when handling the derivatization solutions.

1. Add 500 µl of 2% sulfuric acid in methanol to each sample and incubate for 3 h at 60 °C or overnight at 50 °C (see **Note 28** and **33**).

2. Add 600 µl hexane and 100 µl saturated NaCl to each sample, vortex 10 min, and centrifuge 5 min at maximum speed. Transfer the upper hexane phase to a new Eppendorf tube, and dry the samples for 10 min at 20 °C in an acid resistant vacuum centrifuge (see **Note 37**).

3. Re-suspend in a defined amount of hexane, depending on the amount and type of tissue (e.g., a sample containing 5 mg normal liver was re-suspended in 440 µl hexane), vortex 10 min, transfer to a glass vial with insert, and seal with a nonmagnetic cap using a crimper.

4. Measure the samples using a GC-MS instrument. In our case, the approach is analogous to the one described above for polar metabolites, but using a higher helium carrier-gas flow rate (1.3 mL/min) and a different temperature gradient: upon injection of non-polar metabolite samples, the GC oven is first held at 140 °C for 2 min, then ramped up to 185 °C with a gradient of 1 °C/min, and after that ramped up to 300 °C with a gradient of 20 °C/min, followed by a 2 min post-run at 300 °C.The MS instrument is operated in dual (positive + negative) polarity, with the following ion source parameters: gas (N_2) temperature = 270 °C; gas flow = 10 L/min; nebulizer pressure = 35 psi; sheath gas (N_2) temperature = 300 °C; sheath gas flow = 12 L/min; capillary voltage = 3500/-3000 V (positive/negative polarity); nozzle voltage = 500 V. Ion detection is performed in multiple reaction monitoring (MRM) mode, with a total cycle time of 1500 ms.

3.7 Protein Quantification

1. Re-suspend the dried protein pellet in 200 µl 0.2 M NaOH and incubate for 20 min in a heating block 95 °C (see **Note 28**).

2. For the quantification continue as described in the manual of the BCA protein assay kit.

3. Use the protein amount to normalize the measured metabolite levels to the starting material used.

3.8 Collection of lungs for H&E staining

1. Sacrifice the mouse with an overdose of Dolethal (140mg/kg, 2.8 µl per gram of animal weight of a 50 mg/ml solution) (see **Note 27** and **38**).

2. Sanitize the mouse by spraying 70% ethanol on the fur along the abdominal region, so that any loose/dry hairs will not enter the region (Fig. 5a).

3. Carefully open the mouse thorax with a midline incision, cut away the diaphragm and cut open the lateral chest walls without touching the lungs (Fig. 5b) (see **Note 38**).

4. Open the skin at the tracheal area and remove all the glands in that area until you can easily see and reach the trachea. Remove the connective tissue around the trachea using scissors. (Fig. 5c, d) (see **Note 39**).

5. Take a 1 ml syringe with 27G needle and fill with 1 ml 10% neutral buffered formalin solution. Clamp the cranial part of the trachea using a needle holder (Fig. 5e). Hold the trachea with the needle holder while you insert slowly the needle in the caudal part of the trachea parallel to the trachea with the needle opening towards you. Inject 1 ml 10% neutral buffered formalin solution (Fig. 5f) (see **Note 11**).

6. Collect the lung, wash for 1 min in PBS and store for 24 h at 4 °C in a closed container filled with 10% neutral buffered formalin solution. Ensure that the fixative is at least 10x the volume of tissue (Fig. 5g, h) (see **Note 40**).

7. After 24 hours, wash the samples with 70% ethanol and proceed with embedding the lung in paraffin using an automated machine routinely used for tissue embedding in paraffin wax. When embedded, samples can be stored at room temperature [17,18].

3.9 Analysis of MS data – technical considerations.

1. Mass spectrometry is a highly specific and sensitive technique allowing the simultaneous detection of hundreds of metabolites. However, the complexity of biological samples can lead to difficulties when interpreting MS data. One important parameter for the interpretation of MS data is the retention time, i.e., the time at which the metabolites of interest are eluted from the chromatographic column and detected. The same metabolite can elute at different times and

positions depending on many technical differences (type of column, elution gradients, buffers, etc.), so it is best to, prior to analysis of biological samples, establish an internal library of retention times. To do this, standards for each metabolite of interest, must be processed individually as described above.

The necessary amount to be used for this purpose can be different from metabolite to metabolite, but a good starting point is 1-10 μg total amount. It is also important to perform calibration curves with increasing amounts of the metabolite of interest (for example 0, 1, 5, 10 and 15 μg). This way, comparing between each chromatogram, the peak arising from the metabolite of interest should increase with increasing starting amounts, making peak assignment easier. Moreover, the sensitivity and the linear range of concentration for each metabolite can be determined. The sensitivity (limit of detection) is defined as the lowest amount of metabolite that can be detected, and must be determined for each metabolite considering the slope of the calibration curve or a signal-to-noise ratio above 3 [19]. Once a retention time library has been established, it is possible to mix all metabolites of interest (provided that they do not co-elute) in a single set of samples and perform standard curves for all metabolites in one go. Due to factors such as detector- and derivatization-efficiency, it is possible that the calibration curves from a specific run might not apply to a future run and, therefore, a new calibration curve should be performed each time.

2. The total amount of a metabolite present in the sample is directly proportional to its peak area. Thus, if information about the absolute amount of a metabolite (i.e. a metabolite concentration) in biological samples is necessary, it is important to create a standard curve. It is important to extract the samples used to generate the standard curve in the same way as the biological sample. Consequently, the peak area of the standard curve samples also need to be normalized to the internal standard (see Subheading 3.10.1). Due to the complexity of biological

samples there is the possibility that metabolites will interact with each other, leading to shifts in retention times and/or peak intensity. This is known as the matrix effect.

To correct for this effect, perform an additional 3 extractions for each of your biological samples using a similar amount of starting material to that of the biological samples, and pool them together (to prevent any possible metabolite differences between extraction). Afterwards, split the samples into 3 aliquots and perform standard addition of 0x, 2x and 5x of the initial quantity of the metabolite of interest, as determined by the standard curves mentioned in Subheading 3.9, **step 1**.

If there are no matrix effects, this new calibration curve, performed in the presence of the biological matrix, should have the same slope as that of the pure metabolite, any deviation will be due to the matrix effect and the new standard curve should be used to quantify the metabolite. Since the matrix effect is dependent on the metabolites present in the samples and these can vary wildly from tissue to tissue, it is recommended to perform matrix correction for each of the different biological origins of the samples. For example, a matrix effect present in a liver sample might not be present in a kidney sample, the same is true for liquid samples: a urine sample and plasma sample can possess different matrix effects that should be corrected separately.

3.10 Interpretation of MS-derived biological data.

3.10.1– Calculation of total metabolite levels.

1. For each sample and metabolite of interest, identify all possible mass isotopologues potentially contributing to that metabolite's total signal. We will designate these as $M + i$, where M is the mass of the unlabeled metabolite, and i ($i = 0,1, \ldots, N$) denotes a specific isotopologue, based on the number of tracer atoms incorporated into the metabolite

backbone (with N being the maximum number of tracer atoms that the metabolite can incorporate). For instance, if a ^{13}C tracer (such as $^{13}C_6$-glucose) is fed to the animal, there are 4 possible mass isotopologues potentially arising for pyruvate ($C_3H_4O_3$), namely those due to the incorporation of 0, 1, 2 or 3 ^{13}C atoms into its carbon backbone (i.e. $M+0$, $M+1$, $M+2$ and $M+3$, respectively).

2. Determine the ion counts measured for each of those isotopologues (IC_{M+i}), by appropriately integrating the area under the corresponding spectral peaks using any commercially available peak integration software.
3. Add up the ion counts for all isotopologues corresponding to every given metabolite, to obtain total ion counts for each metabolite, $TC = \sum_{i=0}^{N} IC_{M+i}$. Furthermore, since some metabolites may be present as impurities arising from the extraction buffers or the tubes where the derivatization was performed, "mock" samples, as defined in Subheading 3.4, **step 6** can be used identify these impurities. Since these "mock" samples did not have any contact with any biological material, any metabolite present in these samples is considered an impurity. Consequently, metabolite abundances below or at the level of the mock should not be used for biological interpretation.
4. Standardize the total ion counts for each metabolite relative to those of the appropriate internal standard (e.g., norvaline, glutarate or heptadecanoic acid for amino acids, organic acids and fatty acids, respectively) in the same sample. This removes the influence of any potential loss of material during the extraction process (see **Note 41**).
5. Using the previously mentioned calibration curves (standardized in an analogous manner), transform these standardized ion counts into absolute levels (concentrations, or amounts). This step can be skipped if all that is sought from the experiment is a comparison between the relative levels of the same metabolites among different samples (e.g. when comparing pyruvate levels between primary tumor and metastasis tissues).

6. Finally, normalize the above levels (absolute or relative) by the starting amount of biological sample (i.e. fluid volume, tissue weight, or cell number). These values can then be compared between different groups.

3.10.2 – Analysis of fractional nutrient contributions and pathway activities.

1. For each sample and metabolite of interest, determine a (measured) fractional mass isotopologue distribution, or mass distribution vector (MDV), $Y = \{Y_{M+0}, Y_{M+1}, \dots, Y_{M+N}\}$, based on the isotopologue counts IC_{M+i} and total ion counts TC defined in Subheading 3.10.1, according to

$$Y_{M+i} = \frac{IC_{M+i}}{TC} \quad (i = 0,1, \dots, N) \qquad (1)$$

2. For appropriate interpretation, the measured MDV must first be corrected to account for the natural abundance of heavier isotopes. This is because, for every metabolite, and independently of the presence of tracer atoms, there is a probability for each of the atoms in it to naturally appear in the form of a heavier isotope. Thus, these natural abundances must be corrected to avoid overestimating the label incorporation into the metabolites of interest. The theory underlying natural abundance correction and its practical implementation have been widely covered in the literature [20,21]. Readers are thus encouraged to use one of the several programmatic tools already developed and tested to perform these corrections (see for instance https://pypi.org/project/IsoCor). For the purpose of this manuscript, it is sufficient to state that the latter all involve solving a linear system of equations of the form

$$Y = \bar{\bar{L}} \cdot X \qquad (2)$$

Where Y is the measured MDV, $X = \{X_{M+0}, X_{M+1}, \dots, X_{M+N}\}$ is the actual (natural abundance-corrected) MDV, and $\bar{\bar{L}}$ is a correction matrix, whose components depend both on the full

atomic composition of the metabolite of interest upon measurement (including all atoms, not only those susceptible of tracer incorporation) and on the nature and number of tracer atoms that can be incorporated by the latter. Consequently, it is important to keep in mind that, if there is a chemical derivatization process (such as the aforementioned TBDMS derivatization), the atomic compositions of the whole derivatives (and not just those of the bare metabolites) must be taken into consideration when correcting for natural abundance. Conversely, the number of potentially labeled atoms considered for the correction will stem from the bare metabolite backbone, since the chemical derivatization process will not add labeled atoms to the metabolite. For instance, in the case of the TBDMS derivative of pyruvate, the formula to be used for natural abundance correction is $C_6H_{12}O_3NSi$ (corresponding to the full derivative ion), and not $C_3H_4O_3$ (corresponding to pyruvate only). However, the correction must consider that only up to 3 carbon atoms (and not 6) may be labeled. The same principle applies whenever any atoms are added to/removed from the metabolite as a result of an ionization process (e.g. H^+ removal upon negative electrospray ionization in LC-MS measurements).

3. After correcting for natural abundance, it is possible to calculate the fractional contribution (FC) of the tracer to each metabolite of interest, by using the equation

$$FC = \frac{\sum_{i=0}^{N} i \cdot X_{M+i}}{N} \qquad (3)$$

Where N is the number of atoms in the metabolite susceptible of tracer incorporation, and the X_{M+i} are the corrected fractional abundances of each of its different isotopologues. In isotope-labeling experiments with uniformly-labeled tracers, FC represents the fraction of a given metabolite pool that originates from those labeled tracers, and thus can be used to infer the relative contributions of labeled and unlabeled nutrients towards the production of a given metabolite [12]. For a faithful comparison between FCs for different samples, the FC of each metabolite of interest must be standardized towards the actual FC of the original

tracer in the same sample. For example, if $^{13}C_6$-glucose is infused, the FC of plasma glucose in each sample should be used to standardize the FC values for all other metabolites in that sample. This standardization corrects for any possible dilutions of the initial tracer in the bloodstream, which will significantly alter the distribution of label across the metabolic network. Following the example of $^{13}C_6$-glucose and pyruvate, let the FC values determined for each metabolite be 0.90 and 0.72, respectively. This would indicate that 0.72/0.9 = 80% of the measured pyruvate originated from glucose.

As an exception to this, it is possible to directly compare the FC of the same metabolite in two different tissues of the same mouse (for example, hepatic and renal pyruvate FC) as the enrichment of the tracer in the blood stream of the mouse is the same measure for both tissues.

4. In addition to obtaining information about differential nutrient contributions (by comparing the FCs for given metabolites between samples), it is also possible to infer conclusions about differential pathway activities by comparing the underlying MDVs. Indeed, since each isotopologue can only arise from a subset of specific metabolic pathways, comparing the relative abundances of specific isotopologues of a given metabolite can provide information on the relative activities of the metabolic pathways converging into that metabolite. For instance, when analyzing citrate labelling from a uniformly labeled $^{13}C_6$-glucose tracer, multiple isotopologues can be detected, including $M+2$ (if doubly-labeled acetyl-CoA is incorporated into citrate via citrate synthase), $M+3$ (if uniformly labeled pyruvate is incorporated into oxaloacetate via pyruvate carboxylase, and from there further into citrate), or $M+5$ (if both pathways are active). Thus, comparing the fractional abundances of the citrate $M+2$, $M+3$ and $M+5$ isotopologues will provide information about the relative activities of those pathways.

It is important to keep in mind that, unlike FC, individual isotopologues should never be corrected for the enrichment of the initial tracer in the blood stream.

4. Notes

1. Culture media needs to be adapted to the individual cell lines, e.g. RPMI 1640 Medium for 4T1 and B16F10 cells and MEM Alpha Medium for EMT6.5 cells.
2. Mouse strains need to be syngeneic with the injected cell line, e.g. murine 4T1 or EMT6.5 cell lines can be injected in Balb/c mice without any rejection response. The B16F10 cell line can be injected in C57BL/6 mice without any rejection response.
3. Alternative: heating chamber. You can heat the tail by putting it in warm water or in a heating chamber.
4. Use glass bottles and glass pipets or “inert plastics” if possible. Normal plastic will get dissolved.
5. Work under the chemical fume hood.
6. When performing LC-MS measurements without infusion of labeled metabolites, add ^{13}C yeast internal standard solution to extraction solution 1, instead of Norvaline:Glutarate for normalization[10].
7. Work under the chemical fume hood. Seal both the methoxyamine and pyridine bottles with Parafilm and store in a desiccator, since humidification impairs the derivatization efficiency.
8. Store bottle at -20°C, warm up to room temperature and dry immediately before use, since humidification impairs the derivatization efficiency.
9. Sulfuric acid needs to be of 99.999% purity, since water will inhibit the derivatization reaction. Add sulfuric acid dropwise to the methanol. Work under the chemical fume hood, wear safety glasses, use clean glassware and clean glass pipets. Buffer can be

stored up to 14 days at room temperature. If you would like to reuse the glass pipet for H_2SO_4, rinse the glass pipet three times with water and three times with isopropanol.

10. Work with safety glasses.
11. Work in a chemical fume hood or beneath a strong exhaust to not breathe the formalin damps.
12. Transport the vials in dry ice. Care should be taken on thawing, as liquid nitrogen may cause vials to explode. Liquid nitrogen is hazardous. Use gloves and protective face equipment during handling. DMSO can penetrate skin and carry dissolved products across the skin barrier. Handle DMSO with caution.
13. Keep the cells at least one week and maximum 4 weeks in culture before using them for the mouse model.
14. Culture more cells than needed in case the cells did not grow as fast as expected. It is better to have more flasks that are less confluent than flasks that are more than 90% confluent. Never use cells that were grown too confluent.
15. If the effect of culture medium is not important in your tumor model, the survival of cells is better when re-suspended and injected in culture medium without Fetal Bovine Serum and penicillin/streptomycin instead of PBS.
16. Always take into account the dead volume of your syringes. Make the cell suspension for 5 extra mice.
17. The best results are obtained by re-suspending the cells every time before filling a syringe.
18. Injecting bubbles can cause air embolisms. To easily remove all bubbles, take more than 100 µl, tap the syringe to relocate the bubbles to the needle tip and push out the air and extra cell suspension.

19. The mice should not suffer because of the heat. Make sure the cage is not too hot by holding your hand in the cage for a couple of seconds.
20. Put the mouse very tight with its nose positioned in a way it can breathe.
21. Start injecting distally so you can move proximally when correct insertion in the vein fails. When you feel pressure and the injection site thickens you are not injecting in the vein. Do not waste any cell suspension and try again more proximally. When the injection goes smoothly, you are in the vein. Inject slowly but smoothly. Inject the cell suspension every time in the same way to increase the reproducibility of your results.
22. Wounds that can have been caused by shaving will be healed at the time of injection.
23. Inject every mouse in the same way to have reproducible results.
24. The organ where the metastases will manifest first is cell type specific. For example, 4T1 and EMT6.5 metastatic cancer cells seed first into the lungs.
25. Humane endpoints need to be in accordance with the local regulations. Here, we determined them as follows: The size of the primary tumor cannot exceed 2 cm^3, loss of ability to ambulate, labored respiration, surgical infection or weight loss over 10% of initial body weight.
26. Upon detection of one of these symptoms the animal was euthanized: loss of ability to ambulate, labored respiration, surgical infection or weight loss over 10 % of initial body weight.
27. Each method of anaesthesia or euthanasia induces specific changes in the metabolome, which need to be considered when interpreting results. The procedure used for anaesthesia, euthanasia and tissue collection should be fully documented in all publications [22–24].
28. Work with safety glasses.

29. When collecting tumors or metastases, limit the amount of normal adjacent tissue that is collected along. It can be useful to split the tumor section into two parts lengthwise, and use one of the halves for histological determination of actual tumor area, and the other for metabolic extractions. Performing this extra analysis allows researchers to confirm the purity of the collected tumor tissue. However, this determination cannot be used to correct the metabolite abundances detected. As an example, if a specific collected tumor piece contains 90% tumor and 10% non-transformed tissue, this does not mean that, for each metabolite, 90% of its total amount arose from the tumor tissue.
30. Label the Eppendorf tube on the cap and on the side to avoid losing the label. Keep the tissues in plastic bags in a box with liquid nitrogen. Pre-cool the Eppendorf tube in liquid nitrogen, place the empty Eppendorf tube on the balance and tare the balance. Quickly break off a piece of tissue with a liquid nitrogen cooled tweezer. Place the tissue into the Eppendorf tube as fast as possible, write down the weight, and transfer the closed Eppendorf tube with the tissue into a tube holder floating in liquid nitrogen.
31. Wear face protection when using the Cryomill, as tubes could break if liquid nitrogen enters the tube. In a case where liquid nitrogen has entered a tube, open the tube as fast as possible and let the liquid nitrogen evaporate. Regrind the tissue if it is not completely ground after one round in the Cryomill. Beads can stay in the tube for the whole extraction process.
32. Work under a fume hood. Release air when going through the middle layer (protein phase) by depressing the pipet by 80% and press the rest out as bubbles while crossing the middle layer. Do not take the complete liquid from each phase to not disturb the middle (protein containing) layer, as the loss of some volume from each phase can later be corrected by the internal standards.

33. Work under the chemical fume hood. Samples must be completely dry before addition of the derivatization solution. If the samples were stored at -80 °C, re-dry them for 10 min in an acid resistant vacuum centrifuge at 20 °C.
34. While adding 20 µl O-Methoxyamine–HCl dissolved in pyridine solution to your sample, wash the walls of the Eppendorf tubes by pipetting up and down.
35. Work under the chemical fume hood. The 25 µl extra volume of TBDMS is needed to correct for evaporation of TBDMS.
36. The derivatization of polar metabolites for GC-MS analysis can also be performed by hand. Add 20 µl 20 mg/ml methoxyamine dissolved in pyridine to each sample, vortex briefly and incubate for 90 min in a heating block at 37 °C. Subsequently, add 40 µl TBDMS, vortex briefly and incubate for 60 min in a heating block at 60 °C. Centrifuge the samples for 2 min at maximum speed, transfer the supernatant to a glass GC-MS vial with insert and seal with a non-magnetic cap using a crimper.
37. Work under the chemical fume hood. Make sure to only collect the upper phase. Any addition of the lower phase will destroy the GC-MS column. Bubbles detected when flicking the vials are indicative of liquid from the lower phase in your sample. When using hexane, prepare an aliquot in a cleaned glass bottle, the aliquot should not be older than 2 weeks.
38. Wait until the mouse does not respond anymore when you pinch the toe.
39. Be careful to not rupture the trachea.
40. For the collection of lungs for H&E staining you will have to fix the complete lung. If you want to collect lung tissue for histology and metabolite analysis you will need 2 mice.

41. If no infusion or injection with stable isotopic tracers are performed, also stable and heavy labelled metabolites can be used as internal standard to calculate metabolite concentrations.

5. References

1. Sullivan LB, Gui DY, Vander Heiden MG (2016) Altered metabolite levels in cancer: implications for tumour biology and cancer therapy. *Nat Rev Cancer* **16**: 680–693.
2. Lorendeau D, Christen S, Rinaldi G, Fendt S-M (2015) Metabolic control of signalling pathways and metabolic auto-regulation. *Biol Cell* **107**: 251–272.
3. Lunt SY, Fendt S-M (2018) Metabolism – A cornerstone of cancer initiation, progression, immune evasion and treatment response. *Curr Opin Syst Biol* **8**: 67–72.
4. Elia I, Doglioni G, Fendt S-M (2018) Metabolic Hallmarks of Metastasis Formation. *Trends Cell Biol* **28**: 673–684.
5. Doglioni G, Parik S, Fendt S-M (2019) Interactions in the (Pre)metastatic Niche Support Metastasis Formation . *Front Oncol* **9**: 219.
6. Vander Heiden MG, DeBerardinis RJ (2017) Understanding the Intersections between Metabolism and Cancer Biology. *Cell* **168**: 657–669.
7. Rinaldi G, Rossi M, Fendt S-M (2018) Metabolic interactions in cancer: cellular metabolism at the interface between the microenvironment, the cancer cell phenotype and the epigenetic landscape. *Wiley Interdiscip Rev Syst Biol Med* **10**: e1397.
8. Elia I, Schmieder R, Christen S, Fendt S-M (2015) Organ-Specific Cancer Metabolism and Its Potential for Therapy. In, *Handbook of experimental pharmacology* pp 321–353.

9. Elia I, Fendt S-M (2016) In vivo cancer metabolism is defined by the nutrient microenvironment. *Transl Cancer Res* **5**: S1284–S1287.

10. Broekaert D, Fendt S-M (2019) Measuring In Vivo Tissue Metabolism Using 13C Glucose Infusions in Mice. In, *Methods in molecular biology (Clifton, N.J.)* pp 67–82.

11. Faubert B, DeBerardinis RJ (2017) Analyzing Tumor Metabolism In Vivo. *Annu Rev Cancer Biol* **1**: 99–117.

12. Buescher JM, Antoniewicz MR, Boros LG, Burgess SC, Brunengraber H, Clish CB, DeBerardinis RJ, Feron O, Frezza C, Ghesquiere B, et al. (2015) A roadmap for interpreting 13 C metabolite labeling patterns from cells. *Curr Opin Biotechnol* **34**: 189–201.

13. Christen S, Lorendeau D, Schmieder R, Broekaert D, Metzger K, Veys K, Elia I, Buescher JM, Orth MF, Davidson SM, et al. (2016) Breast Cancer-Derived Lung Metastases Show Increased Pyruvate Carboxylase-Dependent Anaplerosis. *Cell Rep* **17**: 837–848.

14. Elia I, Broekaert D, Christen S, Boon R, Radaelli E, Orth MF, Verfaillie C, Grünewald TGP, Fendt S-M (2017) Proline metabolism supports metastasis formation and could be inhibited to selectively target metastasizing cancer cells. *Nat Commun* **8**: 15267.

15. Elia I, Rossi M, Stegen S, Broekaert D, Doglioni G, van Gorsel M, Boon R, Escalona-Noguero C, Torrekens S, Verfaillie C, et al. (2019) Breast cancer cells rely on environmental pyruvate to shape the metastatic niche. *Nature* **568**: 117–121.

16. Lorendeau D, Rinaldi G, Boon R, Spincemaille P, Metzger K, Jäger C, Christen S, Dong X, Kuenen S, Voordeckers K, et al. (2017) Dual loss of succinate dehydrogenase (SDH) and complex I activity is necessary to recapitulate the metabolic phenotype of SDH mutant tumors. *Metab Eng* **43**: 187–197.

17. Feldman AT, Wolfe D (2014) Tissue Processing and Hematoxylin and Eosin Staining. In pp 31–43. Humana Press, New York, NY.

18. Hewitson TD, Wigg B, Becker GJ (2010) Tissue Preparation for Histochemistry: Fixation, Embedding, and Antigen Retrieval for Light Microscopy. In pp 3–18. Humana Press, Totowa, NJ.

19. Shrivastava A, Gupta V (2011) Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles Young Sci* **2**: 21.

20. Fernandez CA, Rosiers C Des, Previs SF, David F, Brunengraber H (1996) Correction of13C Mass Isotopomer Distributions for Natural Stable Isotope Abundance. *J Mass Spectrom* **31**: 255–262.

21. Millard P, Letisse F, Sokol S, Portais J-C (2012) IsoCor: correcting MS data in isotope labeling experiments. *Bioinformatics* **28**: 1294–1296.

22. Pierozan P, Jernerén F, Ransome Y, Karlsson O (2017) The Choice of Euthanasia Method Affects Metabolic Serum Biomarkers. *Basic Clin Pharmacol Toxicol* **121**: 113–118.

23. Overmyer KA, Thonusin C, Qi NR, Burant CF, Evans CR (2015) Impact of Anesthesia and Euthanasia on Metabolomics of Mammalian Tissues: Studies in a C57BL/6J Mouse Model. *PLoS One* **10**: e0117232.

24. Stephen P.J. Brooks BJL and CGB (1999) *The Influence of Euthanasia Methods on Rat Liver Metabolism*.

25. Hui S, Ghergurovich JM, Morscher RJ, Jang C, Teng X, Lu W, Esparza LA, Reya T, Le Zhan L, Yanxiang Guo J, et al. (2017) Glucose feeds the TCA cycle via circulating lactate. *Nature* **551**: 115–118.

26. Davidson SM, Papagiannakopoulos T, Olenchock BA, Heyman JE, Keibler MA,

Luengo A, Bauer MR, Jha AK, O'Brien JP, Pierce KA, et al. (2016) Environment Impacts the Metabolic Dependencies of Ras-Driven Non-Small Cell Lung Cancer. *Cell Metab* **23**: 517–528.

27. Faubert B, Li KY, Cai L, Hensley CT, Kim J, Zacharias LG, Yang C, Do QN, Doucette S, Burguete D, et al. (2017) Lactate Metabolism in Human Lung Tumors. *Cell* **171**: 358-371.e9.

28. Courtney KD, Bezwada D, Mashimo T, Pichumani K, Vemireddy V, Funk AM, Wimberly J, McNeil SS, Kapur P, Lotan Y, et al. (2018) Isotope Tracing of Human Clear Cell Renal Cell Carcinomas Demonstrates Suppressed Glucose Oxidation In Vivo. *Cell Metab* **28**: 793-800.e2.

29. Fan TWM, Lane AN, Higashi RM, Farag MA, Gao H, Bousamra M, Miller DM (2009) Altered regulation of metabolic pathways in human lung cancer discerned by 13Cstable isotope-resolved metabolomics (SIRM). *Mol Cancer* **8**: 1–19.

30. Sellers K, Fox MP, Bousamra M, Slone SP, Higashi RM, Miller DM, Wang Y, Yan J, Yuneva MO, Deshpande R, et al. (2015) Pyruvate carboxylase is critical for non–small-cell lung cancer proliferation. *J Clin Invest* **125**: 687–698.

31. Hensley CT, Faubert B, Yuan Q, Lev-Cohain N, Jin E, Kim J, Jiang L, Ko B, Skelton R, Loudat L, et al. (2016) Metabolic Heterogeneity in Human Lung Tumors. *Cell* **164**: 681–694.

32. Tardito S, Oudin A, Ahmed SU, Fack F, Keunen O, Zheng L, Miletic H, Sakariassen PØ, Weinstock A, Wagner A, et al. (2015) Glutamine synthetase activity fuels nucleotide biosynthesis and supports growth of glutamine-restricted glioblastoma. *Nat Cell Biol* **17**: 1556–1568.

33. Marin-Valencia I, Yang C, Mashimo T, Cho S, Baek H, Yang X-L, Rajagopalan KN,

Maddie M, Vemireddy V, Zhao Z, et al. (2012) Analysis of Tumor Metabolism Reveals Mitochondrial Glucose Oxidation in Genetically Diverse Human Glioblastomas in the Mouse Brain In Vivo. *Cell Metab* **15**: 827–837.

34. Maher EA, Marin-Valencia I, Bachoo RM, Mashimo T, Raisanen J, Hatanpaa KJ, Jindal A, Jeffrey FM, Choi C, Madden C, et al. (2012) Metabolism of [U-13C]glucose in human brain tumors in vivo. *NMR Biomed* **25**: 1234–1244.

35. Yuneva MO, Fan TWM, Allen TD, Higashi RM, Ferraris D V., Tsukamoto T, Matés JM, Alonso FJ, Wang C, Seo Y, et al. (2012) The metabolic profile of tumors depends on both the responsible genetic lesion and tissue type. *Cell Metab* **15**: 157–170.

36. Kucejova B, Duarte J, Satapati S, Fu X, Ilkayeva O, Newgard CB, Brugarolas J, Burgess SC (2016) Hepatic mTORC1 Opposes Impaired Insulin Action to Control Mitochondrial Metabolism in Obesity. *Cell Rep* **16**: 508–519.

37. Satapati S, Kucejova B, Duarte JAG, Fletcher JA, Reynolds L, Sunny NE, He T, Nair LA, Livingston KA, Livingston K, et al. (2015) Mitochondrial metabolism mediates oxidative stress and inflammation in fatty liver. *J Clin Invest* **125**: 4447–4462.

38. Satapati S, Sunny NE, Kucejova B, Fu X, He TT, Méndez-Lucas A, Shelton JM, Perales JC, Browning JD, Burgess SC (2012) Elevated TCA cycle function in the pathology of diet-induced hepatic insulin resistance and fatty liver. *J Lipid Res* **53**: 1080–1092.

39. Mashimo T, Pichumani K, Vemireddy V, Hatanpaa KJ, Singh DK, Sirasanagandla S, Nannepaga S, Piccirillo SG, Kovacs Z, Foong C, et al. (2014) Acetate is a bioenergetic substrate for human glioblastoma and brain metastases. *Cell* **159**: 1603–1614.

40. Kim C-W, Addy C, Kusunoki J, Anderson NN, Deja S, Fu X, Burgess SC, Li C, Ruddy M, Chakravarthy M, et al. (2017) Acetyl CoA Carboxylase Inhibition Reduces Hepatic

Steatosis but Elevates Plasma Triglycerides in Mice and Humans: A Bedside to Bench Investigation. *Cell Metab* **26**: 394-406.e6.

41. Kennedy KM, Scarbrough PM, Ribeiro A, Richardson R, Yuan H, Sonveaux P, Landon CD, Chi J-T, Pizzo S, Schroeder T, et al. (2013) Catabolism of Exogenous Lactate Reveals It as a Legitimate Metabolic Substrate in Breast Cancer. *PLoS One* **8**: e75154.

42. Rauckhorst AJ, Gray LR, Sheldon RD, Fu X, Pewa AD, Feddersen CR, Dupuy AJ, Gibson-Corley KN, Cox JE, Burgess SC, et al. (2017) The mitochondrial pyruvate carrier mediates high fat diet-induced increases in hepatic TCA cycle capacity. *Mol Metab* **6**: 1468–1479.

43. Vatner DF, Majumdar SK, Kumashiro N, Petersen MC, Rahimi Y, Gattu AK, Bears M, Camporez J-PG, Cline GW, Jurczak MJ, et al. (2015) Insulin-independent regulation of hepatic triglyceride synthesis by fatty acids. *Proc Natl Acad Sci U S A* **112**: 1143–1148.

44. DeLany JP, Windhauser MM, Champagne CM, Bray GA (2000) Differential oxidation of individual dietary fatty acids in humans. *Am J Clin Nutr* **72**: 905–911.

45. Sidossis LS, Coggan AR, Gastaldelli A, Wolfe RR (1995) Pathway of free fatty acid oxidation in human subjects. Implications for tracer studies. *J Clin Invest* **95**: 278–284.

46. Blaak EE, Wagenmakers AJM (2002) The fate of [U-(13)C]palmitate extracted by skeletal muscle in subjects with type 2 diabetes and control subjects. *Diabetes* **51**: 784–789.

47. Gallego S, Hermansson M, Liebisch G, Hodson L, Ejsing C, Gallego SF, Hermansson M, Liebisch G, Hodson L, Ejsing CS (2018) Total Fatty Acid Analysis of Human Blood Samples in One Minute by High-Resolution Mass Spectrometry. *Biomolecules* **9**: 7.

48. Ducker GS, Chen L, Morscher RJ, Ghergurovich JM, Esposito M, Teng X, Kang Y, Rabinowitz JD (2016) Reversal of Cytosolic One-Carbon Flux Compensates for Loss of the Mitochondrial Folate Pathway. *Cell Metab* **23**: 1140–1153.

49. Neinast MD, Jang C, Hui S, Murashige DS, Chu Q, Morscher RJ, Li X, Zhan L, White E, Anthony TG, et al. (2018) Quantitative Analysis of the Whole-Body Metabolic Fate of Branched-Chain Amino Acids. *Cell Metab*.

50. Strong JM, Anderson LW, Monks A, Chisena CA, Cysyk RL (1983) A 13C tracer method for quantitating de novo pyrimidine biosynthesis in vitro and in vivo. *Anal Biochem* **132**: 243–253.

51. Busch R, Kim Y-K, Neese RA, Schade-Serin V, Collins M, Awada M, Gardner JL, Beysen C, Marino ME, Misell LM, et al. (2006) Measurement of protein turnover rates by heavy water labeling of nonessential amino acids. *Biochim Biophys Acta - Gen Subj* **1760**: 730–744.

52. Pinnick KE, Gunn PJ, Hodson L (2019) Measuring Human Lipid Metabolism Using Deuterium Labeling: In Vivo and In Vitro Protocols. In pp 83–96. Humana Press, New York, NY.

Figures

Figure 1.

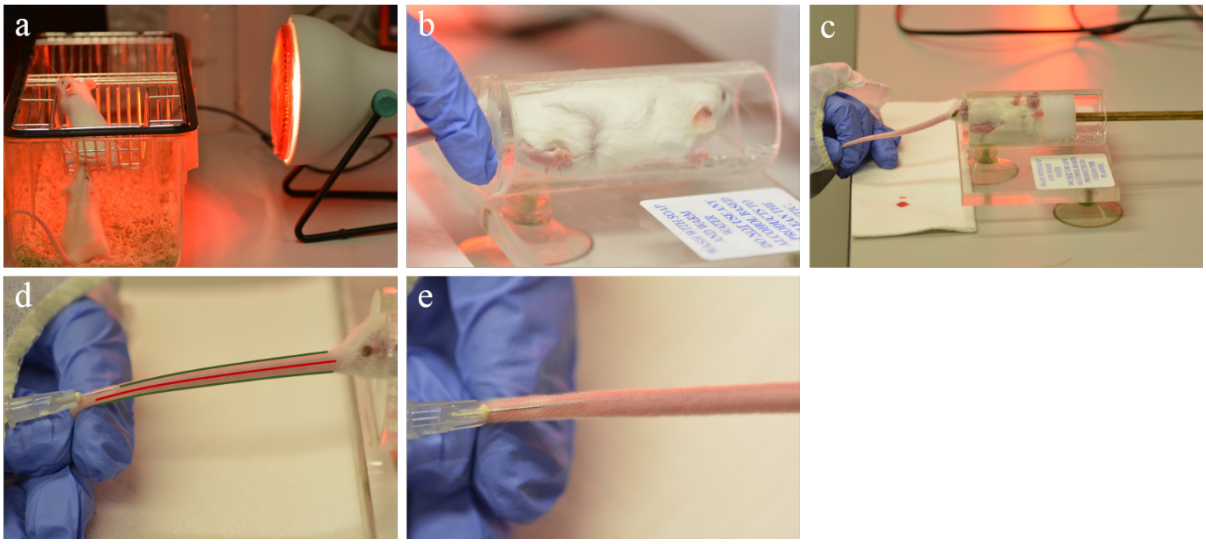

Fig. 1 Generation of lung metastasis mouse model without primary tumor by intravenous injection of cancer cells in the mouse tail vein. (**a**) Infrared lamp is directed towards the mice in the cage. (**b**) Position the mouse in a tail vein restrainer. (**c**) The tail is bend between the index finger and thumb. (**d**) Identify the lateral tail veins (indicated by the green lines) left and right from the ventral artery (indicated by the red line). (**e**) Insert the needle almost parallel to the tail.

Figure 2.

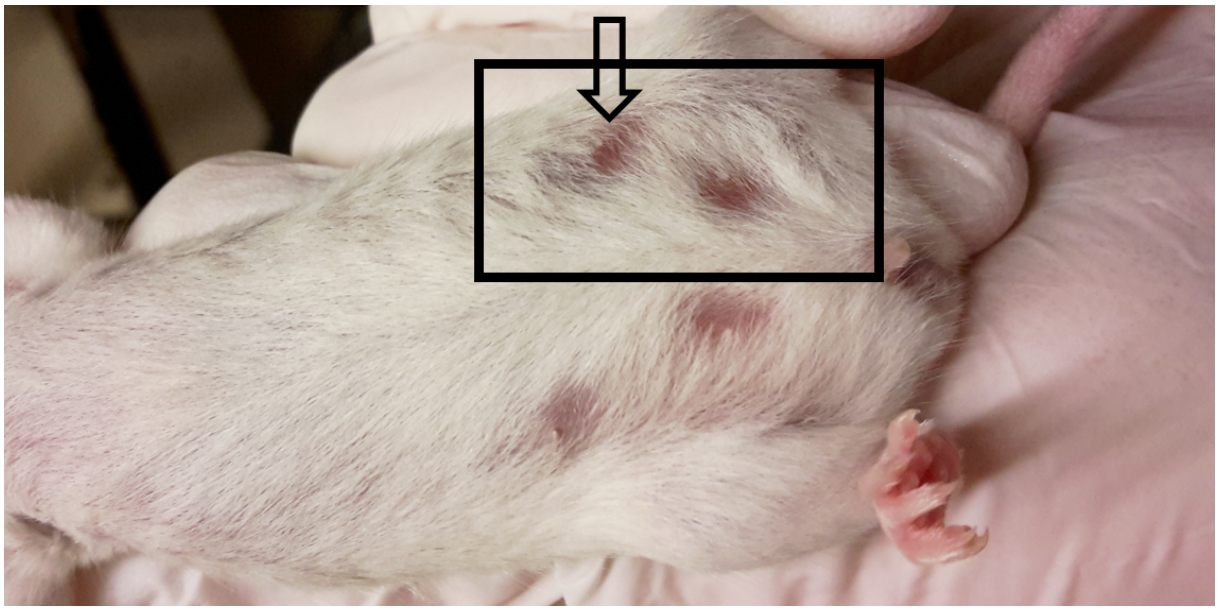

Fig. 2 Area around the mouse nipple that needs to be shaved. Cells will be injected in the mammary fat pad under the nipple indicated by the arrow.

Figure 3.

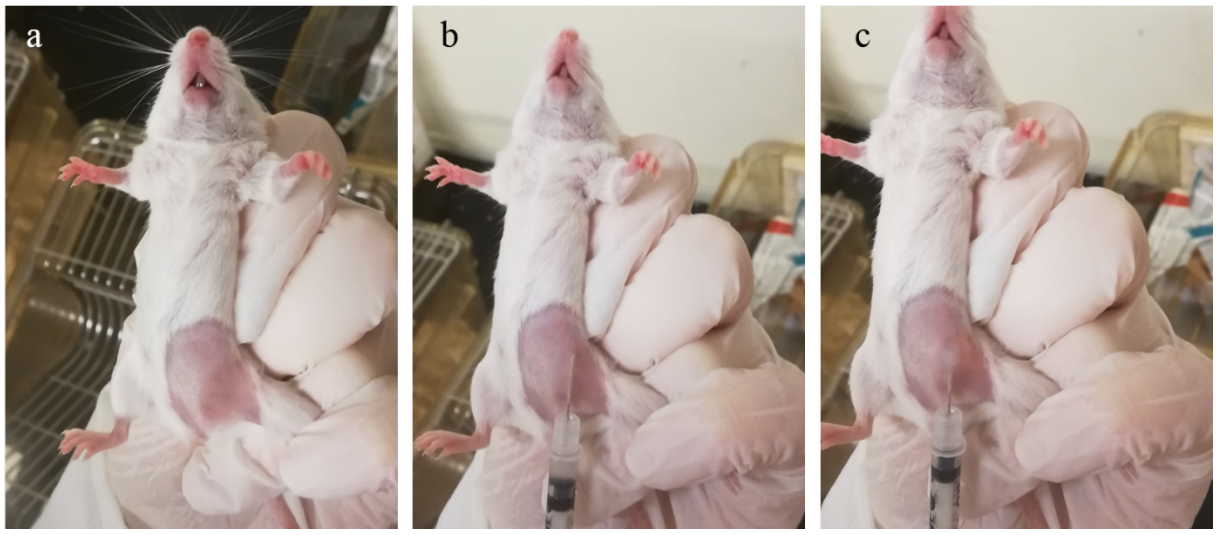

Fig. 3 Generation of spontaneous metastasis mouse model (with primary tumor) through injection of cancer cells in the mammary fat pad. (**a**) Pick up the mouse with your left hand and locate the nipple. (**b**) The needle was inserted about 10 mm distally from the nipple horizontally under the skin and moved to the nipple. (**c**) Inject when the needle tip is right under the nipple. A small bubble under the nipple is an indication of a good injection.

Figure 4

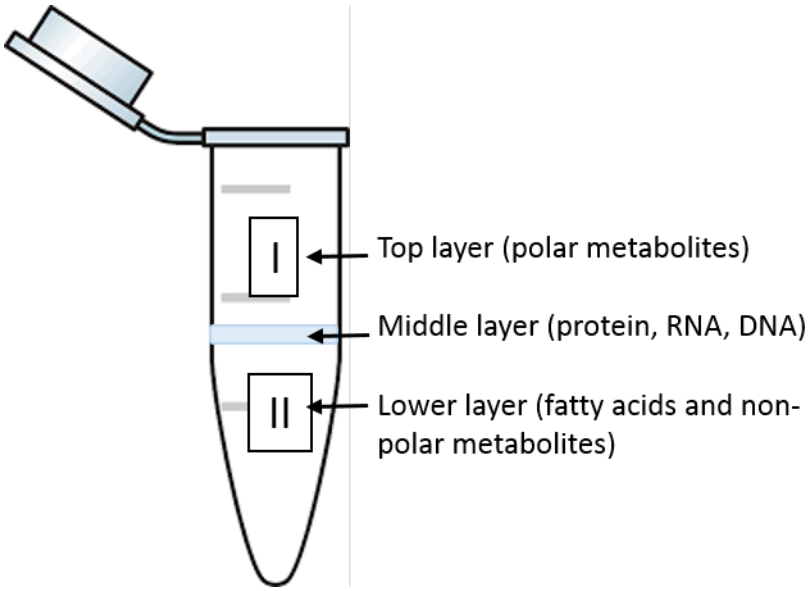

Fig. 4 Eppendorf tube with the three layers after metabolite extraction. The top layer consists of extraction solution 1 containing polar metabolites. The middle layer consists of protein, DNA and RNA. The lower layer consists of extraction solution 2 containing fatty acids and nonpolar metabolites.

Figure 5

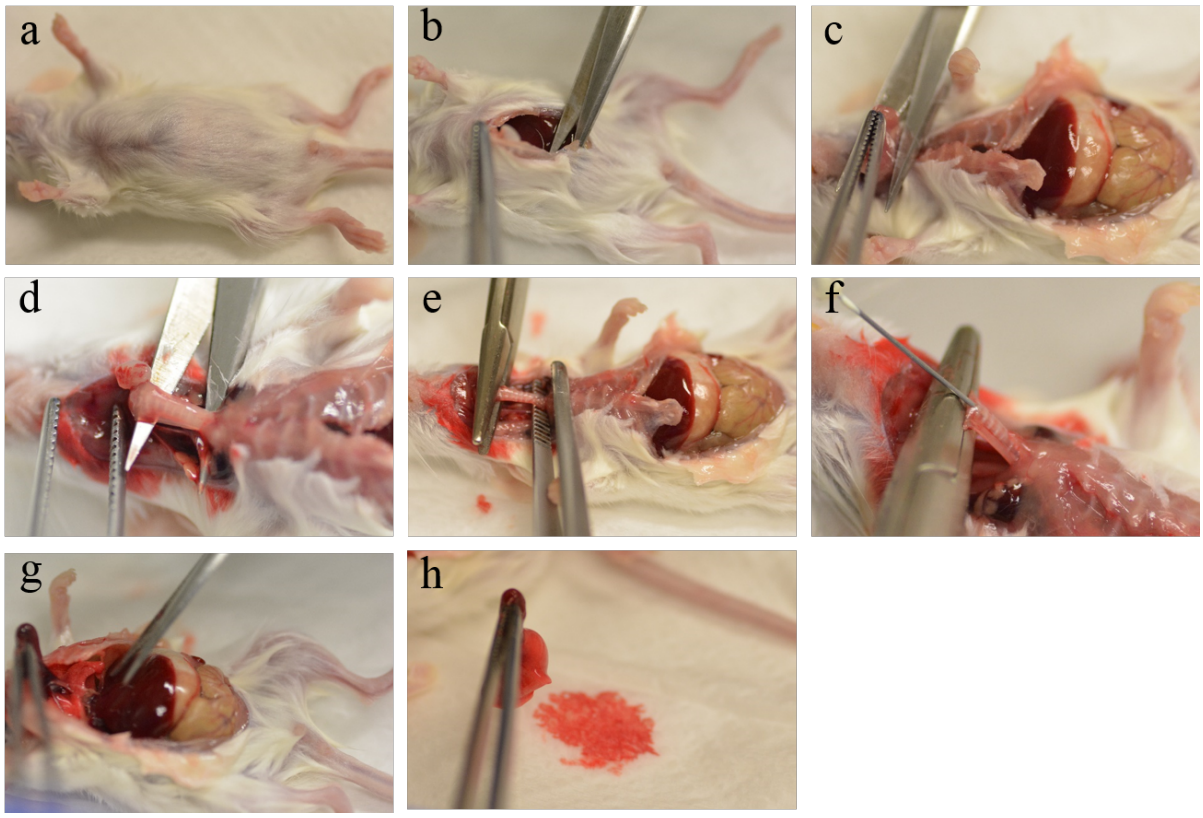

Fig. 5 Collection of lungs for H&E staining. (**a**) The mouse has been sanitized using 70% ethanol. (**b**) Open the mouse thorax with a midline incision. (**c**) Open the skin at the tracheal area and remove all the glands. (**d**) All glands and connective tissue have been removed around the trachea. (**e**) Clamp the cranial part of the trachea using a needle holder. (**f**) Insert the needle in the caudal part of the trachea parallel to the trachea. (**g**) Collect the lung. (**h**) Wash the lung in PBS, remove the blood and store in 10% neutral buffered formalin solution.

Tables

Table 1. Isotopic tracers used for measuring metabolic pathways in rodents and humans

Rodent	Human
$^{13}C_6$-glucose	
Healthy C57BL/6 mice [25] Normal tissues from mouse lung [26] [27]	Clear cell renal cell carcinoma [28]
Subcutaneous xenografts non-small cell lung carcinoma [27]	Lung cancer [27] [29] [30] [31]
Primary orthotopic human glioblastoma xenografts in mice [32] [33]	Glioblastoma [32][34]
Lung cancer in genetically engineered mice [25] [26] [30] [35]	Adjacent lung tissue [27] [30] [31]

Pancreatic cancer in genetically engineered mice [25]	
Primary breast cancers and the corresponding lung metastases [13] [14]	
1,6-$^{13}C_2$-glucose	
Primary orthotopic human glioblastoma xenografts in mice [33]	
1,2-$^{13}C_2$-glucose	
Lung cancer in genetically engineered mice [26]	
3,4-$^{13}C_2$-glucose	
Genetically engineered mice and rats [36] [37] [38]	
$^{13}C_2$-acetate, 1-$^{13}C_1$-acetate	
Glioblastoma and brain metastases in mice [39]	Clear cell renal cell carcinoma [28]
	Fatty acid synthesis in healthy subjects [40]
$^{13}C_3$-lactate, 2-$^{13}C_1$-lactate	
Lung adenocarcinoma (A549 and HCC827) in Ncr nude mice [31] Subcutaneous xenografts non-small-cell lung carcinoma and normal lung in mice [27]	Non-small-cell lung cancer and adjacent lung tissue [27]
Lung cancer in genetically engineered mice [25]	
Pancreatic cancer in genetically engineered mice [25]	
R3230Ac mammary carcinoma in rats [41]	
Genetically engineered mice (hepatocyte-specific) [42]	
Healthy C57BL/6 mice [25]	
$^{13}C_3$-pyruvate, 1-$^{13}C_1$-pyruvate	
Subcutaneous xenografts non-small-cell lung carcinoma and normal lung in mice [27]	
Lung cancer in genetically engineered mice [26]	
Genetically engineered mice (hepatocyte-specific) [42]	
$^{13}C_3$-alanine	
Subcutaneous xenografts non-small-cell lung carcinoma and normal lung in mice [27]	
$^{13}C_5$-glutamine	
Primary orthotopic human glioblastoma xenografts [32] [33]	
Lung cancer in genetically engineered mice [35] [25]	
Healthy and pancreatic cancer in genetically engineered mice [25]	
3,4-$^{13}C_2$-acetoacetate	
Genetically engineered mice and rats [36] [37] [38]	
$^{13}C_4$-sodium β-hydroxybutyrate	
Genetically engineered mice and rats [36] [37] [38]	
$^{13}C_3$-propionate	
Genetically engineered mice and rats [36] [37] [38]	
^{13}C-labeled fatty acids	
Healthy and high fat-fed insulin-resistant Sprague–Dawley rats [43]	Healthy [44] [45] and type 2 diabetes subjects [46]
	Normoinsulinemic and hyperinsulinemic subjects [47]
2, 3-$^{2}H_2$-serine	
HCT-116 colon cancer xenografts in CD1/nude mice [48]	
^{13}C-branched-chain amino acids	
Healthy C57BL/6 and insulin-resistant mice [49]	
^{13}C-bicarbonate/$^{13}CO_2$	
Lymphocytic leukemia in CDF1 mice [50]	
$^{2}H_2O$	
Healthy Sprague–Dawley rats and C57Bl/6J male mice [51]	Healthy volunteers [51] [52]

Table 2

Duration of described mouse models to generate lung metastases.

Mouse strain	Cell line	Type of injection	Number of cells injected	Injection volume	Model duration
Balb/c	4T1	Mammary fat pad	1 million	50 µl	~3 weeks
Balb/c	4T1	IV	100,000	100 µl	~2 weeks
Balb/c	EMT6.5	Mammary fat pad	1 million	50 µl	~3.5 weeks
Balb/c	EMT6.5	IV	100,000	100 µl	~2.5 weeks
C57BL/6J	B16F10	IV	150,000	100 µl	~3 weeks

Mouse surgery and infusions can be performed as described before [10] (see **Note 26**).