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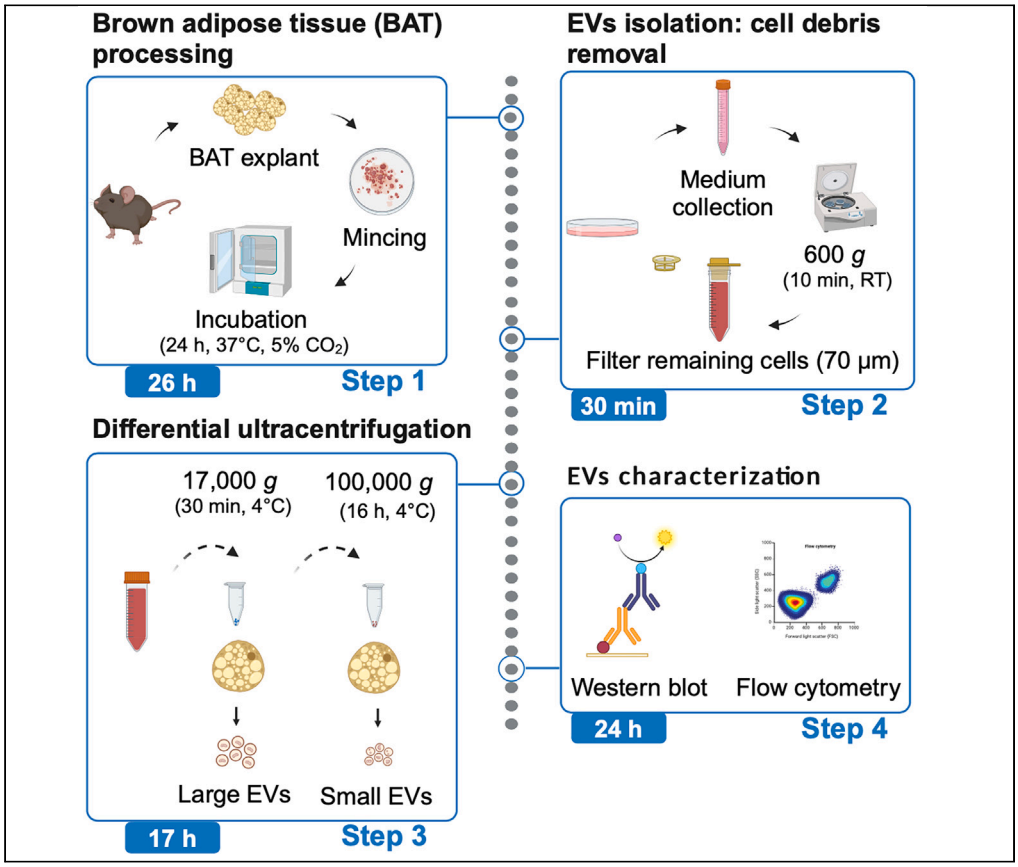
Protocol for detecting mitochondria extracellular vesicles of brown adipose tissue in mice



Brown adipose tissue (BAT) is mitochondria rich, enabling high oxidative metabolism for non-shivering thermogenesis. The release of large/small extracellular vesicles (EVs) containing mitochondria or mitochondrial fragments, termed mito-EVs, may support mitochondrial quality control or intercellular communication. We present a protocol to isolate and characterize mito-EVs. We detail steps for BAT processing, cell debris removal, differential centrifugation (dC), and mito-EV analysis by flow cytometry and immunoblotting assays.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Isolation of mito-EVs from murine brown adipose tissue (BAT) cultures

Differential ultracentrifugation-based separation of large/small mito-EVs

mito-EV analysis by flow cytometry and immunoblotting

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Protocol for detecting mitochondria extracellular vesicles of brown adipose tissue in mice

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SUMMARY

Brown adipose tissue (BAT) is mitochondria rich, enabling high oxidative metabolism for non-shivering thermogenesis. The release of large/small extracellular vesicles (EVs) containing mitochondria or mitochondrial fragments, termed mito-EVs, may support mitochondrial quality control or intercellular communication. We present a protocol to isolate and characterize mito-EVs. We detail steps for BAT processing, cell debris removal, differential centrifugation (dC), and mito-EV analysis by flow cytometry and immunoblotting assays.

For complete details on the use and execution of this protocol, please refer to Rosina et al.[1]

BEFORE YOU BEGIN

Extracellular vesicles (EVs) are membranous particles enclosed by lipid bilayers, ranging from nano- to micro-sized dimensions, devoid of a functional nucleus, and believed to be discharged by diverse cellular types. In the absence of distinct markers delineating their subcellular origin, EVs are commonly classified based on physical attributes such as size or density. EVs smaller than 200 nm are typically termed small EVs, while those exceeding 200 nm are referred to as large EVs.[2] Several studies have demonstrated that EVs can transport mitochondria-derived vesicles (MDVs) or whole mitochondria. Mitochondria encapsulated in vesicles within the cell are clearly visible through transmission electron microscopy analysis.[1] EVs containing either whole mitochondria or mitochondrial fragments have been termed mito-EVs and are captured by recipient cells, which function as an effective means for intercellular mitochondria transfer. In physiological contexts, mito-EVs are crucial in modulating energy metabolism, apoptosis, and immune responses, contributing to cellular homeostasis and inter-organ communication.[1,3,4] Our recent research has shown that during thermogenic stress, brown adipocytes release mito-EVs carrying dysfunctional mitochondrial parts to assure mitochondria quality. In particular, we reported confocal fluorescence microscopy micrographs of MTG+ mito-EVs released by brown adipose tissue (BAT) and taken-up by bone marrow-derived macrophages: BAT-resident macrophages engulf these mito-EVs, thus upholding BAT functional integrity.[1]

The process of mito-EV isolation and characterization is essential for studying their critical functions and understanding their impact on cellular and systemic metabolism. The protocol below details the techniques and methodologies for the efficient isolation and detection of mito-EVs, highlighting their significance in the broader context of cellular homeostasis and energy balance.

Note: This protocol describes the steps for isolating large and small EVs from *ex vivo* BAT cultures of healthy mice and characterizing their mitochondrial protein cargo. Given the

physiological relevance of mito-EVs, which extends to different organs and is associated with various diseases, this protocol can also be applied to *ex vivo* cultures of other tissues from either wild type or mutant mice, as well as to cell cultures.

Note: The small mito-EV fraction predominantly comprises mitochondrial fragments, whereas the large EV fraction may encompass vesicles containing larger mitochondrial components. It should be remarked that besides mito-EVs another significant mechanism of mitochondrial ejection involves the release of free (also termed naked) mitochondria, which are not encapsulated within another membrane layer.[5,6] These naked mitochondria might be pelleted together with the large EV fraction. Hence, through this protocol it is not possible to discriminate whether naked mitochondria can be isolated together with vesicles.

Institutional permissions

All experiments involving mice in this protocol have been approved by University of Rome Tor Vergata Animal Care and Italian Ministry of Health Committee (protocol 378/2017-PR).

Preparation of reagents and tools

Timing: 2–3 h

1. Prepare the tools and reagents for mouse dissection and tissue processing.
   a. Autoclave all surgical instruments (tweezers and scissors).

   ⚠ CRITICAL: Use sterile materials suitable for cell culture at all steps of the protocol.

   b. Prepare the working area by sterilizing with 70% ethanol and placing a Bunsen burner adjacent to the dissecting station.

   Note: The Bunsen burner provides a sterile working environment by creating an updraft that minimizes the risk of airborne contaminants settling on the dissected tissue or media.

   c. Prepare two sterile 50 mL centrifuge tubes: one filled with 70% ethanol and one filled with Pen/Strep in PBS (1% final concentration).

   Note: These will be employed to clean and sterilize the tweezers during the process of BAT cleaning from additional fur.

2. Prepare 12 mL culture medium per dissected BAT by adding Pen/Strep (1% final concentration) and glutamine (1% final concentration) into DMEM (4.5 g/L glucose and 110 mg/L sodium pyruvate).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-ACO2 (1:1,000) antibody	Abcam	ab129105
Anti-CD63 (1:1,000) antibody	System Biosciences	EXOAB-CD63A-1
Anti-CD81 (1:1,000) antibody	System Biosciences	EXOAB-CD81A-1
Anti-tubulin (1:1,000) antibody	Abcam	Ab4074
Total OXPHOS rodent WB antibody cocktail (1:1,000)	Abcam	ab110413
Goat anti-rabbit IgG (H + L)-HRP conjugated (1:2,000) antibody	Bio-Rad	1706515
Goat anti-mouse IgG (H + L)-HRP conjugated (1:2,000) antibody	Bio-Rad	1706516

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
β-2-mercaptoethanol	Sigma-Aldrich	444203
Acetic acid	Sigma-Aldrich	695092
DMEM 4.5 g/L glucose	Gibco	21969-035
Glutamine	Euroclone	ECB3000D
Glycerol	Sigma-Aldrich	G5516
KCl	Sigma-Aldrich	P9541
Methanol	VWR	1.06009.2511
NaCl	Sigma-Aldrich	S9888
PageRuler	Thermo Scientific	26617
PBS (sterile)	Euroclone	ECV4004L
Penicillin/streptomycin (Pen/Strep)	Euroclone	ECB3001D
Phosphatase inhibitor	Sigma-Aldrich	P0044
Ponceau S	Sigma-Aldrich	141194
Protease inhibitor	Sigma-Aldrich	P8340
SDS	Sigma-Aldrich	S-L3771
Skim milk powder	Sigma-Aldrich	70166
Sodium deoxycholate	Sigma-Aldrich	30970
Sodium fluoride	Sigma-Aldrich	201154
Sodium orthovanadate	Sigma-Aldrich	567540
Tris	Sigma-Aldrich	T1502
TritonX-100	Sigma-Aldrich	T8787
Tween 20	VWR	0777
UltraPure glycine	VWR	0167
Critical commercial assays		
Clarity and Clarity Max ECL	Bio-Rad	102031794
Experimental models: Organisms/strains		
3-months-old C57BL/6J male mice	Charles River Laboratories	
Other		
0.22 μm filter	ClearLine	257195
60 mm cell culture dish	Corning	430167
4%–15% Mini-PROTEAN TGX precast protein gels	Bio-Rad	4561083
ChemiDoc MP imaging system	Bio-Rad	12003154
CytoFLEX	Beckman Coulter	A00-1-1102
Infinite 200 PRO	Tecan	30050303 01
Rotor SW28	Beckman Coulter	
Rotor SW40	Beckman Coulter	
Surgical scissors	Fine Science Tools GmbH	14002-12
Tweezers	Outils Rubis SA	OUTI00-NANO
Ultracentrifuge	Beckman Coulter	L7-65
Ultracentrifuge tubes	Beckman Coulter	3444059

MATERIALS AND EQUIPMENT

Tris-HCl, pH 6.8

Reagent	Final concentration	Amount (weight or volume)
Tris base	125 mM	0.6 g
ddH_2O	N/A	to 50 mL
Total	**N/A**	**50 mL**

Adjust to pH 6.8.

Tris-NaCl, pH 8.0		
Reagent	Final concentration	Amount (weight or volume)
Tris base	50 mM	0.60 g
NaCl	150 mM	0.88 g
ddH_2O	N/A	to 70 mL
Total	**N/A**	**70 mL**
Adjust to pH 8.0 with HCl.		

RIPA Lysis Buffer		
Reagent	Final concentration	Amount (weight or volume)
Tris-NaCl, pH 8.0	N/A	70 mL
NP-40	1%	1 mL
Sodium deoxycholate	0.5%	0.5 g
SDS	0.1%	0.1 g
Sodium orthovanadate	1 mM	0.018 g
Sodium fluoride	1 mM	0.004 g
ddH_2O	N/A	to 100 mL
Total	**N/A**	**100 mL**
RIPA lysis buffer can be aliquoted and stored at −20°C (up to 1 year).		

10× TGS Electrophoresis Buffer		
Reagent	Final concentration	Amount (weight or volume)
Tris base	25 mM	3.03 g
Glycine	192 mM	14.4 g
SDS	0.1%	1 g
ddH_2O	N/A	to 1 L
Total	**N/A**	**1 L**
10× TGS electrophoresis buffer can be stored at room temperature (up to 3 months). Prepare the 1× TGS electrophoresis buffer right before use by diluting the 10× TGS with ddH_2O.		

1× Transfer Buffer		
Reagent	Final concentration	Amount (weight or volume)
Tris Base	25 mM	3.03 g
Glycine	192 mM	14.4 g
Methanol	20%	200 mL
ddH_2O	N/A	to 1 L
Total	**N/A**	**1 L**
1× Transfer buffer can be stored at 4°C (up to 2 weeks).		

1× SDS PAGE Loading Buffer		
Reagent	Final concentration	Amount (weight or volume)
Tris-HCl, pH 6.8	125 mM	60 mL
SDS	4%	4 g
Glycerol	20%	20 mL
β-2-mercaptoethanol	10%	10 mL
Bromophenol blue	0.004%	0.004 g
ddH_2O	N/A	to 100 mL
Total	**N/A**	**100 mL**
1× SDS PAGE loading buffer can be aliquoted and stored at −20°C for up to 1 year.		

10× Tris Buffer Saline (TBS)		
Reagent	Final concentration	Amount (weight or volume)
Tris base	24.7 mM	3 g
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
ddH_2O	N/A	to 1 L
Total	**N/A**	**1 L**
Adjust the pH to 7.4 with HCl. 10× TBS can be stored at room temperature (up to 1 month).		

1× Tris Buffer Saline (TBS)		
Reagent	Final concentration	Amount (weight or volume)
10× TBS	1×	100 mL
ddH_2O	N/A	to 1 L
Total	**N/A**	**1 L**
1 x TBS can be stored at room temperature (up to 2 weeks).		

1× Tween-Tris Buffer Saline (TTBS)		
Reagent	Final concentration	Amount (weight or volume)
10× TBS	1×	100 mL
Tween-20	0.1%	1 mL
ddH_2O	N/A	to 1 L
Total	**N/A**	**1 L**
1× TTBS can be stored at room temperature (up to 2 weeks).		

1× Ponceau		
Reagent	Final concentration	Amount (weight or volume)
Ponceau S	0.1%	1 g
Acetic Acid	0.5%	50 mL
ddH_2O	N/A	to 1 L
Total	**N/A**	**1 L**
1× Ponceau solution may be reused once nitrocellulose membrane staining is ended.		

STEP-BY-STEP METHOD DETAILS

Dissection of BAT

Timing: 26 h

During this step, the interscapular BAT is dissected out of the mouse and cut into small pieces (1–2 mm^3) within the DMEM medium prepared according to the instructions given in step 1d. In this way, the vesicles will be released into the medium and can be collected in the following steps.

1. Sacrifice the mouse by the approved means and spray it with 70% ethanol to limit bacterial growth and fluttering of hair in the dorsal and ventral external surfaces.

⚠ CRITICAL: Ensure adequate wetting to minimize fur-related contamination during dissection.

⚠ CRITICAL: Avoid placing the fingers directly over the BAT to prevent any damage to the tissue.

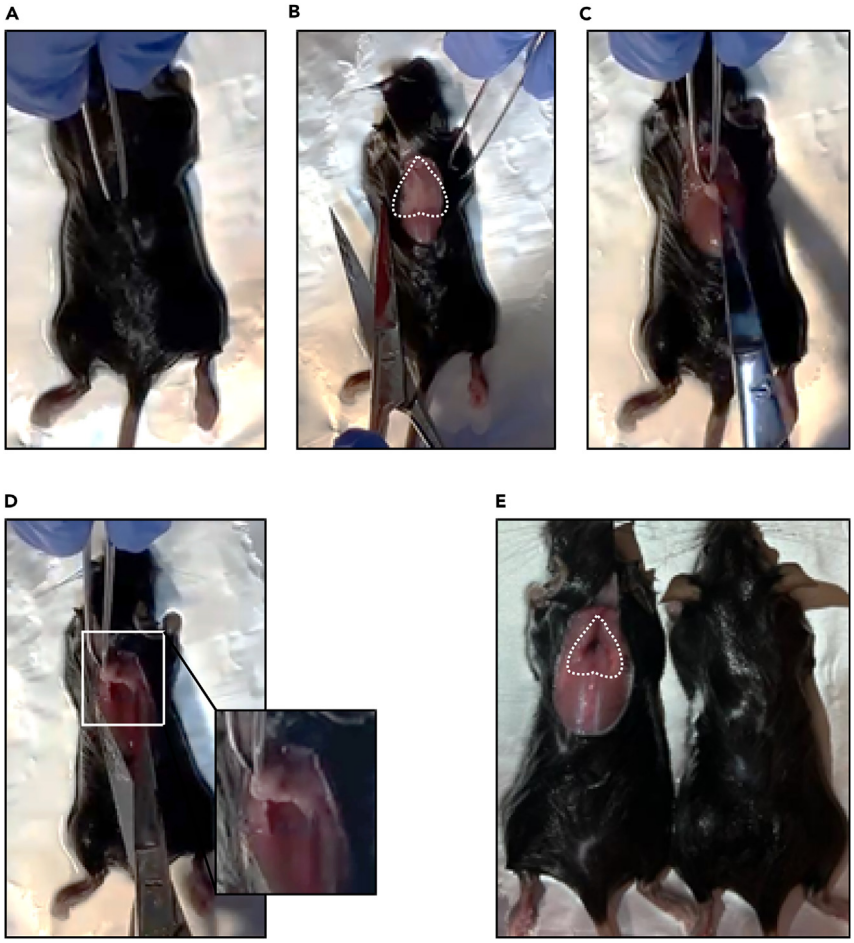

Figure 1. Dissection of interscapular BAT from a C57BL/6 mouse

(A) Place the mouse in a prone position with its stomach facing downward. Elevate the dorsal skin at the nape of the neck using forceps. Create a small incision in the skin.

(B) Insert the scissors into the initial incision and execute a vertical incision through the skin, originating from the nape of the neck and descending along the spine to the mid-back. Gently peel back the skin using forceps to expose the BAT depot.

(C) Use the forceps to lift the BAT and perform a horizontal incision along the lower border of the BAT and create two vertical incisions along the lateral borders of the depot, following the natural tissue borders.

(D) Employ forceps to delicately lift the depot, unveiling the butterfly-shaped interscapular brown adipose tissue nestled within the white adipose tissue (WAT).

(E) Mouse prior to and after BAT dissection. Note the typical butterfly-shape.

2. Tare the scale to a 60 mm sterile dish that will be used to weight the dissected BAT.
3. Dissect the interscapular BAT (Figure 1), place it in a sterile 60 mm dish and weight it.

Note: All the details for the dissection are reported in Figure 1. Follow them thoroughly for a clean dissection procedure.

a. Position the mouse on its stomach, ensuring the abdomen faces downwards.
b. Using forceps, lift the dorsal skin at the back of the neck and make a small cut (Figure 1A).
c. Insert the scissors into the initial cut and make a vertical incision along the spine, starting from the back of the neck and descending to the middle of the back.
d. Carefully peel back the skin with forceps to reveal the BAT depot (Figure 1B).
e. With the forceps, raise the BAT and make a horizontal cut along its lower edge.

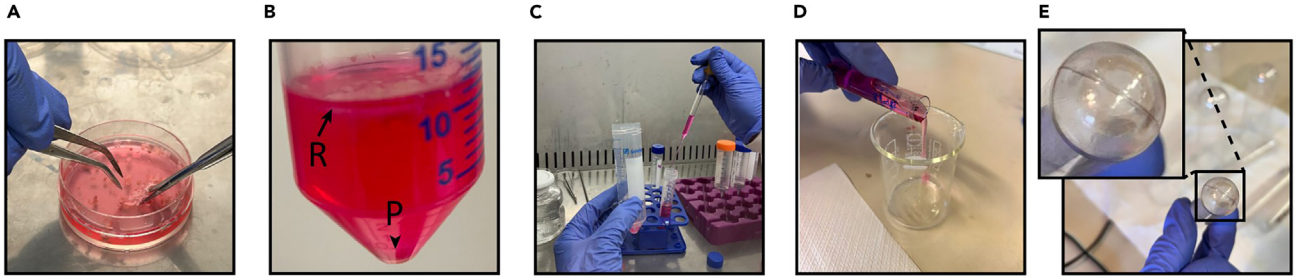

Figure 2. Representative images showing the steps of mito-EV purification from *ex vivo* culture

(A) Mincing of the tissue.

(B) Appearance of the sample medium following the first centrifugation. P (arrowhead) is indicative of the pellet and R (arrow) of the lipidaceous debris ring.

(C) Collection of the interphase, carefully avoiding disturbance of the lipidaceous debris ring and debris pellet at the bottom of the centrifuge tube.

(D) After ultracentrifugation (100,000 *g*, 16 h), remove the medium.

(E) After drying the excess liquid on absorbent paper, visualize the pellet containing isolated large and small mito-EVs.

f. Then, make two vertical cuts along the sides of the depot, following natural tissue boundaries (Figure 1C).
g. Use forceps to gently lift the depot, exposing the butterfly-shaped interscapular BAT within the white adipose tissue (WAT) (Figure 1D).

Note: Images of the mouse before and after BAT dissection reveal the characteristic butterfly shape (Figure 1E).

h. Sterilize the surgical instruments in the previously prepared 50 mL centrifuge tube with 70% ethanol.
i. Rinse them in the previously prepared 50 mL centrifuge tube with Pen/Strep in PBS (1% final concentration).

4. Cleaning the BAT.
   a. Pipette 10 mL of cold PBS containing Pen/Strep (1% final concentration) in a 60 mm dish.
   b. Pick up the dissected BAT and rinse it as quickly as possible in a sterile environment.

Note: Using scissors, remove any connective tissue or WAT residues associated with the BAT.

⚠ CRITICAL: Remove any contamination deriving from fur. This means taking precautions to prevent any fur from coming into contact with the tissue samples during the dissection process. This is particularly important because fur can carry bacteria, debris, or other contaminants that could compromise the integrity of the tissue samples or lead to inaccurate results in subsequent analyses. During this process, clean the surgical instruments in the previously prepared 50 mL centrifuge tube with 70% ethanol and rinse them in the previously prepared 50 mL centrifuge tube with Pen/Strep in PBS (final concentration: 1%).

⚠ CRITICAL: From this step onwards, work under a laminar flow cabinet.

5. Transfer the tissue to a new 60 mm dish with fresh medium (10 mL per BAT).

⚠ CRITICAL: Adjust the medium volume based on BAT weight (i.e., 10 mL per BAT weighting 0.12 g).

6. Mince BAT into small pieces (1–2 mm^3) using sterile scissors (Figure 2A).

⚠ CRITICAL: Consistency in the number of incisions during the mincing process is imperative for maintaining precision and uniformity. A typical number of incisions could be 100.

7. Incubate at 37°C, 5% CO_2 in a humidified atmosphere for 24 h.

EV isolation: Cell debris removal

Timing: 30 min

This step aims at eliminating pieces of tissues and cell debris from the medium.

8. The following day, place a 70 μm cell strainer into a 50 mL centrifuge tube.
9. Wash the strainer with 5 mL of medium.
10. Aspirate with a serological pipette the content of the 60 mm dish and release it onto the cell strainer.

⚠ CRITICAL: Make sure to pick up all the medium, pieces of tissues will be aspirated in the serological pipette.

11. Collect the flow-through in the 50 mL tube and centrifuge at 600 *g* for 10 min to remove large cell debris.
12. After centrifugation, a lipidaceous debris ring will form at the top, while other particulate matter will pellet at the bottom of the centrifuge tube (Figure 2B).
13. Retrieve the supernatant into a fresh 50 mL centrifuge tube, thereby avoiding to collect the lipidaceous ring at the top (Figure 2B, arrow R) and the pellet (Figure 2B, arrowhead P) on the bottom.

⚠ CRITICAL: It is suggested to carefully traverse the lipidaceous debris ring using a glass Pasteur pipette and aspirate only the intermediate phase containing the medium (Figure 2C).

EV isolation: Large and small EVs

Timing: 17 h

This step allows for the isolation of small and large EV-containing fractions. With a single centrifugation step at 17,000 *g*, it is possible to obtain a fraction of large EVs. A successive ultracentrifugation step at 100,000 *g* allows for collection of small EVs. When performing only a single ultracentrifugation at 100,000 *g*, it is possible to collect both small and large EVs.

For the isolation of large mito-EVs, centrifuge the collected medium at 17,000 *g* for 30 min at 4°C. The pellet contains the large EVs. Keep the supernatant for the following step.

⚠ CRITICAL: Large and small EVs should be resuspended in the same volume in order to visualize differences based on their protein content. As specified in point 16, the resuspension depends on the downstream application.

14. For the isolation of small mito-EVs, ultracentrifuge the supernatant of step 14 at 100,000 *g* for 16 h at 4°C (use Rotor SW28 or SW40, according to sample volume).

⚠ CRITICAL: Before ultracentrifugation, ensure uniform weight distribution in the ultracentrifuge tubes by filling them to two-thirds of their volume. Adjust the volume of all samples on a balance using 0.22 μm filtered and sterile PBS, ensuring the same weight of all tubes.

15. Before collecting the pellets, carefully decant the supernatant (Figure 2D) and remove it by inverting the tube onto absorbent paper (Figure 2E).
16. The small mito-EVs are localized within the pellet (Figure 2E).
17. Carefully resuspend the pellets at the bottom of the tube with approximately 100 repetitions and avoiding foaming. Resuspend the pellets of the small and large EVs in the filtered PBS according to the downstream analysis:
    a. For flow cytometry analysis, resuspend the mito-EVs in 100 μL of 0.22 μm filtered PBS for each BAT.
    b. For immunoblotting analysis, resuspend the mito-EVs in 100 μL of RIPA buffer supplemented with fresh protease and phosphatase inhibitor cocktail, according to the manufacturer's instructions.

⚠ CRITICAL: During the resuspension of EVs for the experiments of interest, ensure that a consistent number of resuspension cycles is maintained.

EV analysis: Immunoblotting

Timing: 24 h

This step enables the visualization of proteins present in the isolated EVs fractions.

18. Mix the EVs samples resuspended in RIPA with an equal volume of sample buffer.
19. Seal the tubes by caps and heat them at 95°C for 5 min.

⚠ CRITICAL: Samples can be immediately used or be stored at −20°C.

20. Select the precast gel percentage based on the molecular weight of the target proteins. In this protocol, 10% Mini-PROTEAN precast protein gels are employed. Choose the right percentage (as indicated in the key Resources Table) based on your proteins of interest.
21. Assemble the gels in the running cassette.
22. Fill the running cassette with 1× TGS Electrophoresis Buffer.
23. Load equal volumes of each sample alongside a pre-stained protein ladder.
24. Run the SDS-PAGE at 20 V/gel to separate proteins by molecular weight.

⚠ CRITICAL: The running time depends on the equipment and percentage of the gel.

25. Transfer the proteins from the gel to a nitrocellulose membrane using a wet transfer system for either 30 or 60 min at 100 mA.

⚠ CRITICAL: The pore dimension of the nitrocellulose membrane and the transfer time depend on the percentage of the gel.

26. After transfer, incubate the membrane with the 1× Ponceau solution until the protein bands are visibly sharp on the membrane.
27. Remove the 1× Ponceau.
28. Photograph the membrane to confirm equal loading of the proteins.
29. Rinse the membrane in 1× TTBS until the Ponceau stain is completely removed.
30. Block the membrane in 1× TTBS containing 5% milk powder for 1 h at 20°C–22°C with gentle agitation.
31. Incubate the membrane with primary antibodies, diluted as recommended by the manufacturer, 15 h at 4°C with gentle agitation.
32. Wash the membrane three times with 1× TTBS for 5 min at 20°C–22°C with gentle agitation.

33. Incubate the membrane with an anti-rabbit or an anti-mouse HRP-conjugated secondary antibody diluted 1:2000 in milk powder (3% final concentration) in 1× TTBS for 1 h at 20°C–22°C with gentle agitation.
34. Wash the membrane three times with 1× TTBS for 5 min at 20°C–22°C with gentle agitation.
35. Wash the membrane with 1× TBS and keep it in 1× TBS until ready for imaging.
36. Prepare the membrane for chemiluminescence reaction by incubating with the preferred Clarity and Clarity Max ECL detection reagents for the time given by the manufacturer instructions in the dark.
37. Visualize the protein bands using a gel imaging system.

EV analysis: Flow cytometry

Timing: 3 h

The flow cytometry analysis described in this step enables the quantification of EV numbers in each fraction and the assessment of fractions positive or negative for specific fluorescence signals. In our study, we focus on EVs positive for MitoTracker Green (MTG), which is indicative of the presence of mitochondrial components within the EVs (mito-EVs). Single-vesicle flow cytometry analysis is conducted using a CytoFLEX S instrument. The latter, thanks to the lateral diffusion of the violet and blue lasers, allows to increase the range of particles that can be detected and studied.

38. Execute CytoFLEX Daily Quality Control (QC) procedures prior to each analytical session, to validate the optical alignment of the lasers and the integrity of the fluidic system.

Note: To avoid any instances of swarm detection, use distilled water to cleanse the fluidics system before each analysis, ensuring that the background noise level drops below 150 events per second.

⚠ CRITICAL: Rigorous sample preparation is crucial for vesicle analysis in flow cytometry. The buffer used for resuspending and labeling samples should be filtered through 0.22 μm filters to eliminate potential background debris that could interfere with the analysis of the target populations.

⚠ CRITICAL: Caution must be taken to avoid vigorous resuspension of samples immediately before analysis to prevent the formation of air bubbles. Such bubbles could disrupt sample acquisition by refracting light, thereby registering as false events.

39. For mito-EVs staining, prepare a fresh solution of PBS + MTG (0.5 μM final concentration).
40. Filter the PBS + MTG solution to limit the fluorescence background noise as much as possible.

⚠ CRITICAL: It is essential to filter PBS through a 0.22 μm filter immediately before preparing the solution. This precautionary measure is implemented to prevent the introduction of contaminants that may affect the quantification of events during flow cytometry analysis.

41. Add 50 μL of PBS + MTG solution to each sample and incubate at 37°C, 5% CO_2 for 30 min.
42. Add an equal volume of sterile and filtered PBS (1:1 v/v) to stop the labeling reaction after the incubation.
43. Run PBS, PBS + MTG and unlabeled samples through the flow cytometer.

⚠ CRITICAL: It is suggested to conduct readings of PBS with and without the probe, as well as EVs samples, at a "slow" reading speed.

Note: The gating process involves manually drawing regions using the dedicated tool within the CytExpert software. The first step involves selecting the population on VioletSSC-A/FSC-A

dot plots. Next, it is necessary to examine the autofluorescence of the MTG probe by opening the VioletSSC-A/FITC-A dot plots. This step is crucial for excluding autofluorescence originating from PBS + MTG in the dot plot where sample fluorescence is analyzed. This procedure is conducted for both PBS and PBS + MTG samples.

44. Select the EV population on VioletSSC-A/FSC-A graphs.
45. Open VioletSSC-A/FITC-A dot plots.
46. Draw the gating region as the region with minimal or absent event occurrence (Figure 4A), then proceed with sample analysis.

Note: Only events within the MTG gate are considered positive for analysis, effectively filtering out any non-vesicle-specific signals during acquisition. As illustrated in Figure 4A, the green area exclusively encompasses MTG-positive events associated with mitochondrial presence in the samples, while excluding non-specific signals from PBS, the probe, or unlabeled samples.

47. Set a fixed number of events within the gate (e.g., 100,000 events) for data acquisition.
48. Run all samples one after the other on the flow cytometer.
49. Plot histograms of the event counts for the FITC-A channel to improve the graphical visualization of the samples. These histograms represent the number of events for each FITC intensity.

Note: As shown in Figure 4B, shifting of the histogram peak towards the right is indicative of increased fluorescence in the observed sample (i.e., a major content of mitochondrial proteins within each EV event).

50. At the end of reading all the samples, Triton X-100 is added to the sample to a final concentration of 0.5%.
    a. Incubate for 5 min at room temperature.
    b. Run the sample on the flow cytometer.
    c. Note the reduction of the MTG fluorescence upon Triton X-100 incubation.

Note: To make sure that the mitochondrial signal is enclosed in the EVs, it is necessary to treat the vesicles with a detergent. This procedure is helpful to lyse the membrane enclosing the vesicles, resulting in the leakage of the fluorescence MTG signal.

⚠ CRITICAL: After each analysis, utilize ddH_2O to clean the fluidic system thoroughly.

EXPECTED OUTCOMES

The protocol designed to isolate and detect both large and small EVs followed by immunoblotting and flow cytometry analysis is anticipated to yield crucial insights into the molecular composition of these distinct EV populations and holds promise for advancing our understanding of EV biology and their clinical relevance.

Immunoblotting allows the identification and quantification of specific proteins within the EVs, providing preliminary information about their cargo and potential biomarkers. Among the cargos, mitochondria or mitochondrial-fragments can be transported by EVs thereby being highly relevant for various physiological processes, including cellular homeostasis, tissue repair, neuroprotection, immune regulation, and disease pathogenesis.[7–9] Delivery of functional mitochondria through EVs can serve as a mechanism to rescue cells with dysfunctional or damaged mitochondria. By providing functional mitochondria or mitochondrial components, EVs can restore cellular bioenergetics, maintain oxidative phosphorylation, and prevent cell death, thereby promoting cell survival in conditions of mitochondrial stress or dysfunctional mitochondria.[10] On a greater scale, this

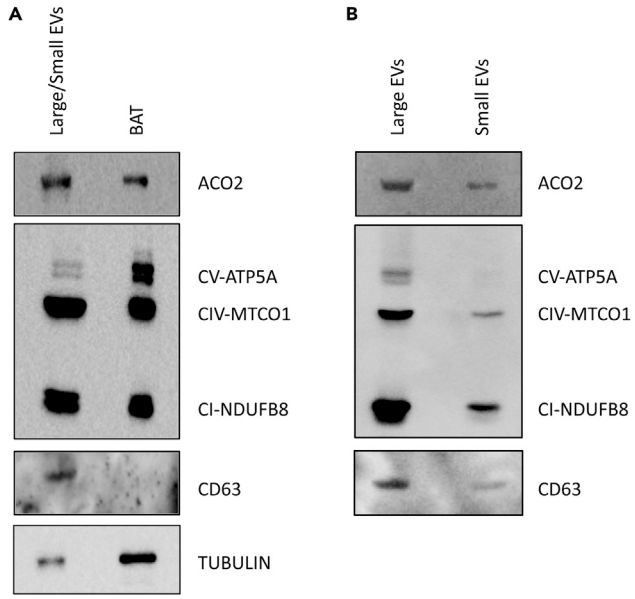

Figure 3. Immunoblotting analysis of the isolated EV fractions

(A) Immunoblotting analysis of BAT-derived mito-EVs and BAT lysate. Samples are analyzed for mitochondrial markers using anti-ACO2 and the total OXPHOS Rodent WB Antibody Cocktail. Anti-CD63 is a vesicle marker. Anti-Tubulin highlights the content of a typical cytoplasmatic protein, which is depleted in vesicles. Samples are normalized by volume.

(B) Immunoblotting analysis of large and small mito-EVs. Samples are analyzed for mitochondrial (ACO2, ATP5A, MTCO1, NDUFB8) and vesicle (CD63) markers. Samples are normalized by volume.

process could be functional for tissue repair and regeneration by providing functional mitochondria in situations of tissue injury or damage, to support cellular metabolism, and enhance ATP production. On the other hand, the release of EVs containing damaged mitochondria can serve as a pro-survival signal under certain circumstances. In situations where the damage to mitochondria is limited and the cell is still able to maintain overall cellular homeostasis, the release of EVs containing damaged mitochondria could be a mechanism to remove these dysfunctional organelles from the cell without triggering cell death.[11] Yet, this release may signal to neighboring cells or the immune system the presence of cellular stress, prompting adaptive responses that enhance cell survival and tissue resilience.[12] This is the case of thermally stressed brown adipocytes that release EVs containing oxidatively damaged mitochondrial components as a protective measure to prevent failure of the thermogenic program. The removal of these vesicles by the phagocytic activity of BAT-resident macrophages plays a crucial role in maintaining BAT physiology. Besides BAT and thermogenesis, the release of damaged mitochondria is crucial for the progression of other diseases. Among others, cancer cells exhibit altered mitochondrial metabolism, and the transfer of dysfunctional mitochondria or mitochondrial components from cancer cells to surrounding stromal cells or immune cells through EVs can promote tumor growth, metastasis, and immune evasion, contributing to cancer progression and aggressiveness.[13]

By performing immunoblotting analyses, it is possible to identify the presence of mitochondrial markers in the EVs collected from the BAT secretome (Figure 3A). In Figure 3A, the amount of ACO2 and some of the OXPHOS components in the total fraction of EVs (small and large) is shown. BAT tissue lysate, which is very rich in mitochondria, has been loaded for reference. The CD63 protein, a member of the tetraspanin family that is present only on the surface of EVs, has been used as EV marker. Tubulin is a cytoskeletal protein that forms microtubules and is generally abundant in the cellular cytoplasm has been used as a cellular marker, depleted in the EV fraction. In Figure 3B we compare small and large EVs originating from the same BAT secretome. The media have been

initially centrifuged at 17,000 g for 30 min to obtain a sample of large mitochondrial fragments and whole mitochondria. Subsequently, by means of ultracentrifugation at 100,000 g for 17 h, we obtain a fraction containing small EVs. By loading the same volume of the samples, we are able to compare the relative abundance of the mitochondrial component in the two different fractions (Figure 3B). As illustrated in Figure 3B, BAT releases mitochondrial proteins encapsulated within both large and small EVs into the culture medium. However, large EVs contain a higher yield of mitochondrial proteins with respect to small EVs.

The second technique mentioned in this protocol, i.e., flow cytometry analysis, offers quantitative data on the EV number and presence of markers inside and/or on the membrane of EVs, allowing for the characterization of their heterogeneity and potential functional differences. This approach could reveal differences in protein expression profiles between large and small EVs, shedding light on their roles in intercellular communication, disease pathology, and potential therapeutic applications.

EVs resuspended in PBS are labeled with MTG, which binds cysteine residues of the mitochondrial membrane, allowing detection of mitochondrial components in the EVs. However, this labeling does not allow to assess mitochondrial activity, hence it is not possible to evaluate whether the isolated mitochondria and/or mitochondrial fragments are functional. To tackle this issue, functional mitochondria could be visualized by MitoTracker Red staining (not illustrated in this protocol). Indeed, it should be noted that MitoTracker Green binds to mitochondrial proteins regardless of mitochondrial membrane potential, staining all mitochondria based on their mass, not activity. In contrast, MitoTracker Red accumulates specifically in mitochondria with an active membrane potential, marking only functional mitochondria. This makes MitoTracker Red suitable for assessing mitochondrial function, while MitoTracker Green cannot differentiate between functional and dysfunctional mitochondria. In Figure 4A, filtered PBS with and without MTG as well as unlabeled samples are read by flow cytometry in order to obtain a gating strategy that allows to identify positive events without overlapping with background noise. The dot plots in Figure 4A demonstrate that both large and small EVs exhibit positive MTG labeling, indicating the presence of mitochondrial proteins in both fractions. However, as reported in the histograms, the MTG signal was higher in the large EV samples with respect to the small EV samples, which is consistent with an higher content of mitochondrial proteins as also demonstrated by Western blot analysis (Figure 3B).

Overall, the integration of immunoblotting and flow cytometry analysis within this protocol holds promise for advancing our understanding of the biology of EVs and their clinical relevance.

Besides the use described in this protocol, the flow cytometry analysis could be also employed to detect other cargos by using specific dyes (i.e., Bodipy for lipids, Calcein-AM for viability, SytoRNA for RNAs) or specific antibodies to determine the surface marker expression on EVs.

LIMITATIONS

Several limitations or challenges, potentially affecting the reliability of our protocol are mentioned in the following paragraph. As previously mentioned, naked mitochondria can be present in the large EV fraction. Our methodology does not allow to discriminate between these conditions, for this purpose an analysis under a transmission electron microscope can be applied, if needed.

TROUBLESHOOTING

Problem 1

Environmental factors such as temperature fluctuations, humidity levels, and air quality in the laboratory environment can impact the stability and integrity of EVs during isolation. Contamination from airborne particles or microbial contaminants can also affect the validity of the results. Furthermore, the handling and processing of tissue samples prior to centrifugation can influence the quality and

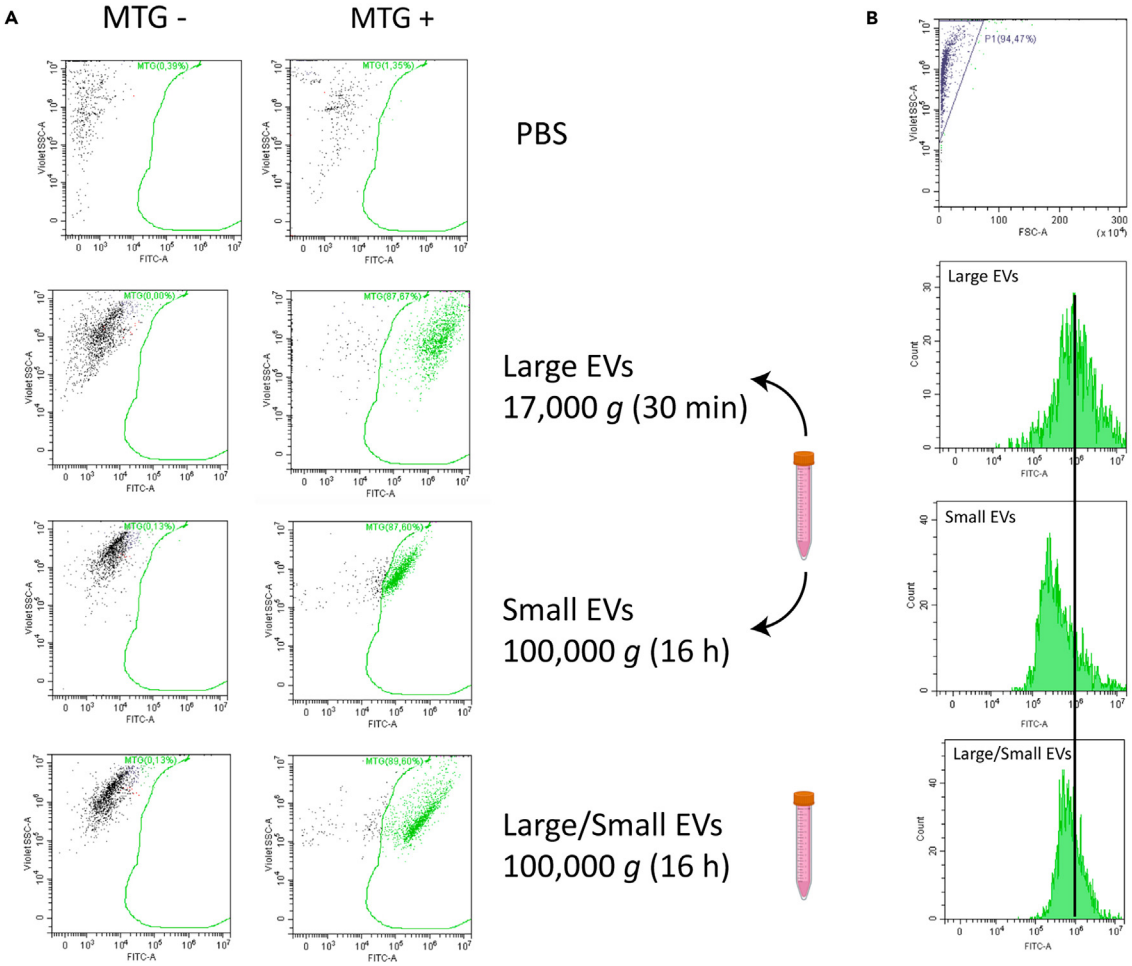

Figure 4. Flow cytometry analysis of MTG-stained EV populations

(A) PBS filtered through a 0.22 μm is used to resuspend the vesicle samples, which are then analyzed by flow cytometry, in the presence and absence of the MTG probe, to circumvent false positives. The populations of large and small mito-EVs are analyzed separately. The ultracentrifuged sample reveals the concurrent presence of both whole mitochondria and mitochondrial fragments contained in EV fractions.

(B) Histograms of the event counts for the FITC-A channel. After having select the EVs population on VioletSSC-A/FSC-A graphs (top), the counts for each FITC-A intensity can be plotted. The movement of the histogram peak towards the right suggests heightened fluorescence in the observed sample, indicating a significant presence of mitochondrial proteins within each EV event.

yield of isolated EVs. Factors such as tissue storage conditions and sample homogenization methods can affect EV stability and recovery during isolation (related to steps 1–7).

Potential solution

Try to work in a controlled laboratory environment with stable temperature and humidity. Adhere to strict sterile techniques throughout the isolation process to minimize the risk of microbial contamination. Use certified sterile equipment, work in laminar flow hoods or biosafety cabinets, and regularly clean and disinfect laboratory surfaces and equipment. Standardize sample collection, storage, and processing protocols to maintain sample integrity and minimize protease activity. Implement quality control measures throughout the experimental procedure and use complementary techniques to validate the results, such as electron microscopy or HPLC purification of EVs.

Problem 2

The media in which BAT is minced and cultured, and the collected EVs could be contaminated (related to preparation of reagents and tools, step 2).

Potential solution

After sacrifice, spray the mouse's fur with ethanol. Immediately after explanting BAT from the mouse, immerse the tissue in a Pen/Strep solution. Work as sterilely as possible by performing the mouse dissection steps next to a lit Bunsen burner.

Problem 3

During centrifugation, EVs may adhere to tubes or other surfaces, leading to their loss from the isolated fraction. Additionally, inefficient recovery of EVs from the pellet or supernatant can result in decreased yield and sensitivity of the isolation protocol (related to step 17).

Potential solution

In order to tackle this issue, it is important to optimize the EV recovery steps. For example, resuspending EVs not only on the bottom but also on the side of the tubes. Moreover, it is recommended to carefully pipette avoiding harsh or excessive manipulation. This can help preserving EV integrity and enhancing recovery efficiency.

Problem 4

Overstaining/understanding with the MTG probe. There has to be a balance between vesicle number and probe concentration (related to step 41).

Potential solution

Estimate the number of vesicles with the flow cytometer (1st run). The number of events should not be more than 50,000. Adjust the concentration of MTG accordingly.

Problem 5

The efficiency of EV isolation using differential centrifugation (dC) can vary depending on factors such as centrifugation speed, duration, and temperature, as well as the type and condition of the tissue sample. Inconsistent isolation conditions may introduce variability in EV yield and composition among experimental replicates (related to steps 14–17).

Potential solution

For EV isolation with dC, as reported by the MISEV guidelines,[2] it is necessary to standardize all the variables and keep them consistent throughout the experiments. Furthermore, when isolating EVs with dC, it is necessary to report all details of the method for reproducibility, such as the tube type, adaptor (if relevant), time, temperature, and brake.

RESOURCE AVAILABILITY

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Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

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AUTHOR CONTRIBUTIONS

F.T. and C.D.B. performed the experiments and wrote the paper, which was edited by all the authors. K.A. and D.L.-B. contributed essential reagents and designed and supervised the experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Rosina, M., Ceci, V., Turchi, R., Chuan, L., Borcherding, N., Sciarretta, F., Sánchez-Díaz, M., Tortolici, F., Karlinsey, K., Chiurchiù, V., et al. (2022). Ejection of damaged mitochondria and their removal by macrophages ensure efficient thermogenesis in brown adipose tissue. Cell Metab. *34*, 533–548.e12. https://doi.org/10.1016/j.cmet.2022.02.016.

2. Thery, C., Witwer, K.W., Aikawa, E., Alcaraz, M.J., Anderson, J.D., Andriantsitohaina, R., Antoniou, A., Arab, T., Archer, F., Atkin-Smith, G.K., et al. (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J. Extracell. Vesicles *7*, 1535750. https://doi.org/10.1080/20013078.2018.1535750.

3. Zhou, X., Liu, S., Lu, Y., Wan, M., Cheng, J., and Liu, J. (2023). MitoEVs: A new player in multiple disease pathology and treatment. J. Extracell. Vesicles *12*, 12320. https://doi.org/10.1002/jev2.12320.

4. Brestoff, J.R., Wilen, C.B., Moley, J.R., Li, Y., Zou, W., Malvin, N.P., Rowen, M.N., Saunders, B.T., Ma, H., Mack, M.R., et al. (2021). Intercellular Mitochondria Transfer to Macrophages Regulates White Adipose Tissue Homeostasis and Is Impaired in Obesity. Cell Metab. *33*, 270–282.e8. https://doi.org/10.1016/j.cmet.2020.11.008.

5. Borcherding, N., Jia, W., Giwa, R., Field, R.L., Moley, J.R., Kopecky, B.J., Chan, M.M., Yang, B.Q., Sabio, J.M., Walker, E.C., et al. (2022). Dietary lipids inhibit mitochondria transfer to macrophages to divert adipocyte-derived mitochondria into the blood. Cell Metab. *34*, 1499–1513.e8. https://doi.org/10.1016/j.cmet.2022.08.010.

6. Boudreau, L.H., Duchez, A.C., Cloutier, N., Soulet, D., Martin, N., Bollinger, J., Paré, A., Rousseau, M., Naika, G.S., Lévesque, T., et al. (2014). Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. Blood *124*, 2173–2183. https://doi.org/10.1182/blood-2014-05-573543.

7. Clement, E., Lazar, I., Attané, C., Carrié, L., Dauvillier, S., Ducoux-Petit, M., Esteve, D., Menneteau, T., Moutahir, M., Le Gonidec, S., et al. (2020). Adipocyte extracellular vesicles carry enzymes and fatty acids that stimulate mitochondrial metabolism and remodeling in tumor cells. EMBO J. *39*, e102525. https://doi.org/10.15252/embj.2019102525.

8. D'Acunzo, P., Pérez-González, R., Kim, Y., Hargash, T., Miller, C., Alldred, M.J., Erdjument-Bromage, H., Penikalapati, S.C., Pawlik, M., Saito, M., et al. (2021). Mitovesicles are a novel population of extracellular vesicles of mitochondrial origin altered in Down syndrome. Sci. Adv. *7*, eabe5085. https://doi.org/10.1126/sciadv.abe5085.

9. Todkar, K., Chikhi, L., Desjardins, V., El-Mortada, F., Pépin, G., and Germain, M. (2021). Selective packaging of mitochondrial proteins into extracellular vesicles prevents the release of mitochondrial DAMPs. Nat. Commun. *12*, 1971. https://doi.org/10.1038/s41467-021-21984-w.

10. Liu, D., Gao, Y., Liu, J., Huang, Y., Yin, J., Feng, Y., Shi, L., Meloni, B.P., Zhang, C., Zheng, M., and Gao, J. (2021). Intercellular mitochondrial transfer as a means of tissue revitalization. Signal Transduct. Target. Ther. *6*, 65. https://doi.org/10.1038/s41392-020-00440-z.

11. Stephens, O.R., Grant, D., Frimel, M., Wanner, N., Yin, M., Willard, B., Erzurum, S.C., and Asosingh, K. (2020). Characterization and origins of cell-free mitochondria in healthy murine and human blood. Mitochondrion *54*, 102–112. https://doi.org/10.1016/j.mito.2020.08.002.

12. Jiang, D., Gao, F., Zhang, Y., Wong, D.S.H., Li, Q., Tse, H.-f., Xu, G., Yu, Z., and Lian, Q. (2016). Mitochondrial transfer of mesenchymal stem cells effectively protects corneal epithelial cells from mitochondrial damage. Cell Death Dis. *7*, e2467. https://doi.org/10.1038/cddis.2016.358.

13. Yu, D., Li, Y., Wang, M., Gu, J., Xu, W., Cai, H., Fang, X., and Zhang, X. (2022). Exosomes as a new frontier of cancer liquid biopsy. Mol. Cancer *21*, 56. https://doi.org/10.1186/s12943-022-01509-9.