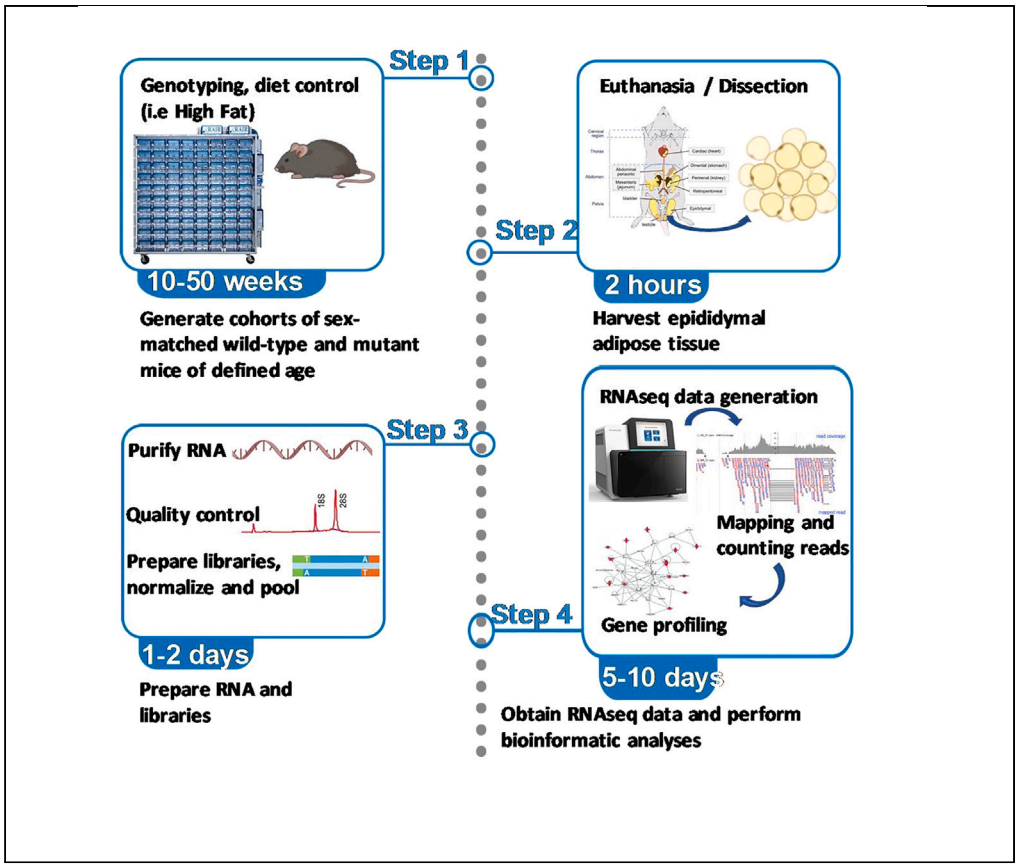
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Measuring the transcriptome-wide effects of aging on murine adipocytes using RNAseq



Adipose tissue plays a central role in age-related diseases. While RNAseq protocols exist for many tissues, few data have been generated with this technology to explore gene expression in adipocytes, particularly during aging. Here, we present a protocol to analyze the transcriptional changes that occur in adipose tissue during normal and accelerated aging in mouse models. We describe steps for genotyping, diet control, euthanasia, and dissection. We then detail RNA purification and genome-wide data generation and analysis.

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

Aurore De Cauwer, Angélique Pichot, Anne Molitor, Tristan Stemmelen, Raphael Carapito, Seiamak Bahram, Philippe Georgel

adecauwer@unistra.fr (A.D.C.)
pgeorgel@unistra.fr (P.G.)

Highlights

RNAseq-based transcriptomic analysis of murine adipose tissue

Differential analysis the transcriptome of adipocytes during aging

Mandatory quality control steps to ensure successful final analysis

De Cauwer et al., STAR Protocols 4, 102397 September 15, 2023
https://doi.org/10.1016/j.xpro.2023.102397

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Measuring the transcriptome-wide effects of aging on murine adipocytes using RNAseq

Aurore De Cauwer,[1,3,*] Angélique Pichot,[1] Anne Molitor,[1] Tristan Stemmelen,[1,2] Raphael Carapito,[1,2] Seiamak Bahram,[1,2] and Philippe Georgel[1,4,*]

[1]Laboratoire d'ImmunoRhumatologie Moléculaire, Institut National de la Santé et de la Recherche Médicale (INSERM) UMR_S 1109, Institut Thématique Interdisciplinaire (ITI) de Médecine de Précision de Strasbourg, Transplantex NG, Faculté de Médecine, Fédération Hospitalo-Universitaire OMICARE, Fédération de Médecine Translationnelle de Strasbourg (FMTS), Université de Strasbourg, 67085 Strasbourg, France

[2]Laboratoire d'Immunologie, Plateau Technique de Biologie, Pôle de Biologie, Nouvel Hôpital Civil, 67000 Strasbourg, France

[3]Technical contact

[4]Lead contact

*Correspondence: adecauwer@unistra.fr (A.D.C.), pgeorgel@unistra.fr (P.G.)
https://doi.org/10.1016/j.xpro.2023.102397

SUMMARY

Adipose tissue plays a central role in age-related diseases. While RNAseq protocols exist for many tissues, few data have been generated with this technology to explore gene expression in adipocytes, particularly during aging. Here, we present a protocol to analyze the transcriptional changes that occur in adipose tissue during normal and accelerated aging in mouse models. We describe steps for genotyping, diet control, euthanasia, and dissection. We then detail RNA purification and genome-wide data generation and analysis.

For complete details on the use and execution of this protocol, please refer to De Cauwer et al. (2022) *iScience*. Sep 16;25(10):105149.

BEFORE YOU BEGIN

The protocol below describes the specific steps for isolating epididymal white adipose tissue from mice and analyzing transcriptional modification of adipocytes that occur during aging.

Adipocytes are known to be fragile cells,[1] exhibiting important sex- and age-related physiological differences. Furthermore, tissue abundance and distribution also differs between males and females[2] and appears to exhibit some variation depending on the genetic background. It is therefore essential to generate cohorts of male (or female) mice that are similar in age to obtain exploitable data. If mutant animals (such as Dicer1 $^{d/d}$ in our case, which exhibit early aging) have to be investigated, wild-type controls must share the same background (here, C57Bl6/J).

The high lipid content of adipocytes renders RNA isolation challenging. Therefore, it is important to harvest enough tissue (at least 300 mg) and perform appropriate quality control check before starting a whole genome transcriptomic analysis by RNAseq.

Maintaining an RNAse-free environment at the bench and regarding, tools, plastics and solutions is critical to obtain high quality material for subsequent RNAseq analysis.

Institutional permissions

Experiments on live vertebrates must be performed in accordance with relevant institutional and national guidelines and regulations. Therefore, a statement identifying the committee approving the

experiments and confirming that all experiments conform to the relevant regulatory standards must be included.

In addition, researchers performing some of the experiments described in this protocol will need to acquire permissions from their relevant institutions.

Animal care and epididymal white adipose tissue collection

Timing: weeks

1. Animal housing and husbandry.
2. Euthanasia and dissection.

Total mRNA isolation and quality controls

Timing: 2 h

3. Total RNA preparation.
4. RNA cleanup and DNase I treatment.
5. RNA quality control.

Library construction and RNA sequencing

Timing: 2–3 days

6. Library construction.
   a. First-strand cDNA synthesis.
   b. PCR1 - Addition of Illumina adapters and indexes.
   c. Purification of the RNA-Seq library using AMPure XP beads.
   d. Depletion of ribosomal cDNA with ZapR v2 and R-probes v2.
   e. PCR2 - final RNA-Seq library amplification.
   f. Purification of final RNA-Seq library using AMPure beads.
   g. Validation using the Agilent 2100 bioanalyzer.
7. NGS sequencing of libraries.
   a. Planning the run.
   b. Pooling the libraries.
   c. Libraries and PhiX denaturation.
   d. Preparation of the reagents, loading and running of the samples.

Analysis of RNAseq data

Timing: 5 days

8. Quality controls of RNAseq data and alignment on the mouse genome.
9. RNAseq data exploitation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Custom diet for Rat & Mice, 61% of the energy from fat (lard)	SAFE custom Diets	SAFE® U8954 version 205
Chloroform	Sigma Aldrich	32211-1L-M

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Isopropanol	VWR	20838.294
Absolute ethanol ≥99.8%	VWR	20821.365
RNA 6000 Pico Labchip Kit	Agilent	5067-1513
RNeasy Mini Kit	Qiagen	74104
RNase-Free DNase Set	Qiagen	79254
SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian	Takara	634413
QUBIT dsDNA HS ASSAY KIT	Thermo Fisher	Q32854
High Sensitivity DNA Labchip Kit	Agilent	5067-4626
NextSeq PhiX Control Kit	Illumina	FC-110-3002
NextSeq™ 500/550 High Output Kit v2.5 (150 Cycles)	Illumina	20024907
NaOH 10 N	VWR	E584-100ML
Tris-HCl, 1 M Solution (pH 7.0)	Fisher scientific	BP1756-100
OMNI International Tissue MASTER 125	VWR	10046-866
Fresco™ 21 Centrifuge	Fisher scientific	
VWR Analog Vortex Mixer	VWR	10153-838
Nanodrop 2000c	Thermo Fisher	ND-2000C
2100 Bioanalyzer Instrument	Agilent	G2939BA
Applied Biosystems™ Veriti™ Thermal Cycler, 96-Well	Fisher scientific	4375305
Invitrogen™ Qubit™ 3 Fluorometer	Fisher scientific	Q33216
NextSeq 550 System	Illumina	
NUCLEASE-FREE WATER 500 ML	Thermo Fisher	AM9930
AMPure XP beads	Beckman coulter	A63881
TRIzol® Reagent	Thermo Fisher	15596018
IScript™ cDNA Synthesis Kit	BIO-RAD	1708891
Agilent RNA 6000 Pico kit	Agilent	5067-1513
SMARTer Stranded total RNA-seq ki v2 – Pico input mammalian	Takara	634417
Experimental models: Organisms/strains		
Dicer1$^{gt(\beta\text{-}geo)han}$ mouse strain. Males aged from 10 to 80 weeks-old.	Otsuka et al. [3]	N/A
Software and algorithms		
FastQC	Babraham Institute	
STAR	Dobin et al.[4]	RRID:SCR_004463 http://code.google.com/p/rna-star/
Bowtie2	Langmead and Salzberg[5]	
HTseq	Anders et al.[6]	
DESeq2	Love et al.[7]	
Ingenuity Pathway Analysis IPA	Ingenuity	RRID:SCR_008653
ImmQuant	Frishberg et al.[8]	http://csgi.tau.ac.il/ImmQuant/
Galaxy pipeline browser	Galaxy Community. Nucleic Acids Res. 2022 Jul 5;50(W1):W345-W351.	https://galaxyproject.org/use/
Other		
Databases to study the genetics of aging	Human Ageing Genomic Resources	https://genomics.senescence.info/

STEP-BY-STEP METHOD DETAILS

Animal care and epididymal white adipose tissue collection

Timing: weeks

This section provides the requirements that are necessary to adequately prepare mouse cohorts and isolate the adipose tissue.

1. Animal housing and husbandry.
   a. Keep all animals under specific pathogen-free conditions.

   Note: Only males were used in the experiments described in this protocol, though some funding agencies (e.g NIH) require strong justification if the study is performed in only one sex.

   b. House mice in groups (2–7 animals) containing both wild-type and mutants to minimize potential microbiota-specific effect.
   c. Keep animals under stabilized temperature (20°C–22°C) with a 12 h dark-light cycle and fed water/food ad libitum.

   Note: Cages contain wood-based bedding composed of a mixture of spruce flakes and aspen chips and are enriched with cotton tubes for nests and red polycarbonate tunnels.

   d. Assign one experimenter to perform handling and experiments.

   Note: The experimenter must be aware of mice genotype at all time.

2. Euthanasia and dissection.
   a. Euthanized mice by cervical dislocation after halothane (isoflurane 4%) anesthesia.
   b. Dissect the peritoneal cavity
   c. Collect and weight epididymal fat depots (see Figure 1) immediately
   d. Place tissues in a 1.5 mL Eppendorf tube snap-frozen in liquid nitrogen.

   Note: Samples are stored at −80°C until further use.

Total mRNA isolation and quality controls

Timing: 3 h

This section describes the steps enabling the preparation and assessment of high quality total RNA from adipose tissue.

3. Total RNA preparation.
   a. Mince adipose tissue (300 mg) on a Petri dish sitting on dry ice, using a scalpel blade and forceps renewed with each sample. Place pieces in a 2 mL polypropylene tube maintained in ice and add 1 mL cold Trizol (TRI Reagent T9424-200ML). Homogenize with a tissue homogenizer (OMNI International Tissue MASTER 125).
   b. Transfer homogenates in 1.5 mL Eppendorf tube for a first centrifugation (10 min, 4°C, 12,000 g). Transfer the liquid phase into a new working 1.5 mL tube by,

   Note: Pipette carefully to avoid the floating fat layer.

   c. Add chloroform (0.2 mL), vigorously mix the samples and incubate for 2 min on ice.
   d. Centrifugation (30 min, 4°C, 12,000 g).
   e. Collect the upper aqueous phase and transfer it in RNA low binding, RNAse free, 1.5 mL Eppendorf tube.
   f. Precipitate RNA by adding isopropanol (0.5 mL) to the collected aqueous phase.
   g. Incubate the samples for 10 min on ice and centrifuge (10 min, 4°C, 12,000 g).
   h. Wash the pellet two times with 70% ethanol before centrifugation (5 min, 4°C, 7,500 g).
   i. Air-dry the pellets and solubilize in nuclease-free water (30 μL).

   Note: the solution is stored at −80°C until further use.

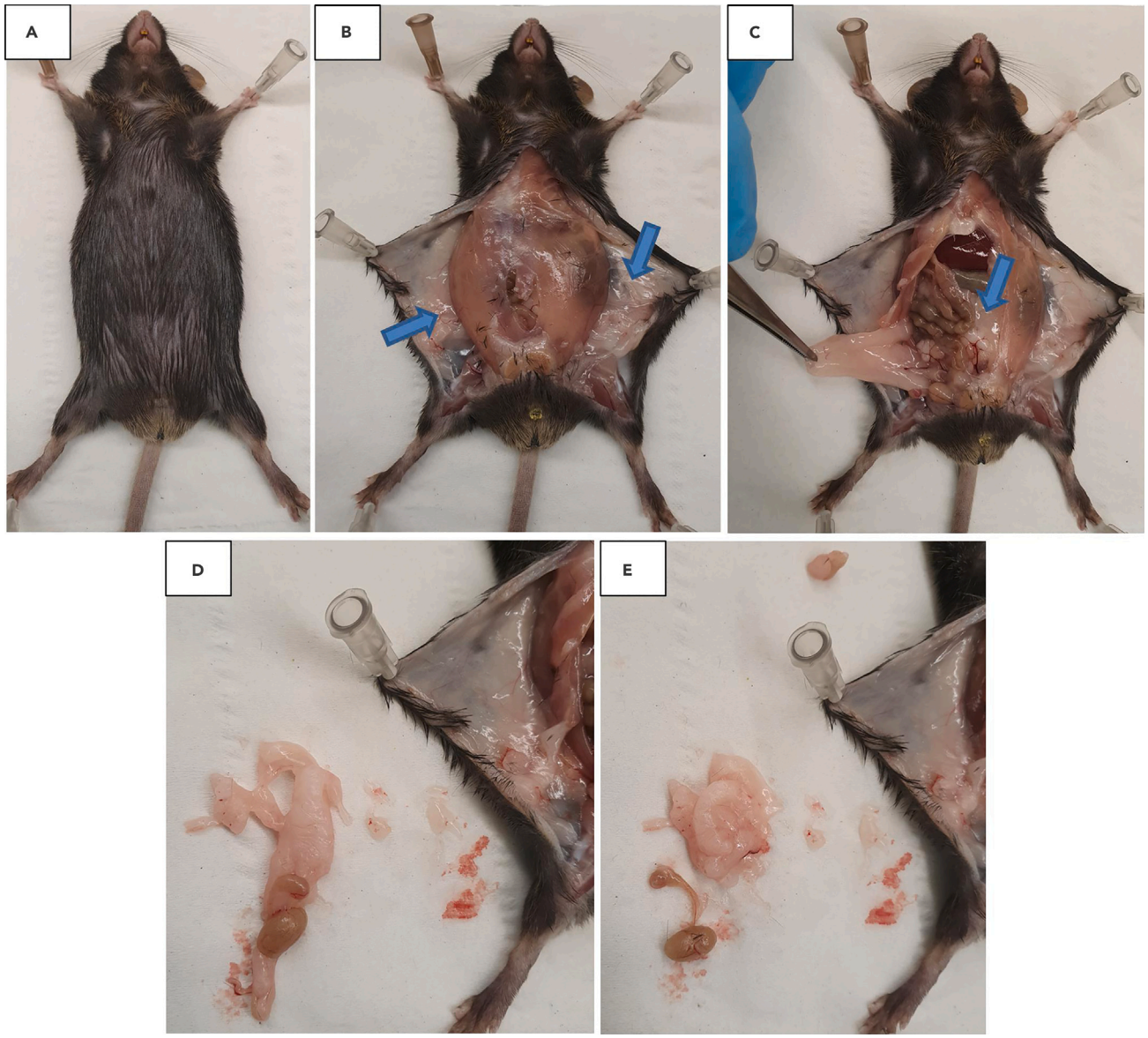


Figure 1. Dissection of the epididymal white adipose tissue

(A) The sedated (with isoflurane exposure) animal is euthanized and prepared for dissection. The fur is sprayed with 70% ethanol and the tools are cleaned with disinfectant solution and rinsed with ethanol.

(B) Make a midline ventral incision (5–6 cm) through the skin with scissors, from the base of the sternum to the base of the tail. Use forceps to peel back the skin from the peritoneal cavity and secure the outstretched skin with dissection pins. The sub-cutaneous fat depot (inguinal WAT) is visible (arrows) and can be harvested if necessary, after removal of the inguinal lymph node.

(C) After the opening of the peritoneal cavity, the adipose tissue surrounding the reproductive system is visible. Other adipose masses are mesenteric (associated with the intestines, arrow) and perirenal (sticked to kidneys, not visible here). Hang on the epididymal adipose tissue with forceps and cut at the base, where the prostate and seminal vesicles adhere. If those are damaged, rinse the tissue with cold PBS.

(D) A testis, the epididymis and the connecting the duct are associated with the adipose mass.

(E) Carefully dissociate the testis and epididymis, rinse the adipose tissue with PBS, dry on a clean tissue, weigh on a weighing balance and immediately freeze in liquid nitrogen.

⚠ CRITICAL: In step 3b, the first centrifugation is essential to remove the lipids that will aggregate into a monolayer of fat. In step 3c, 30 min centrifugation must be respected, since a shorter centrifugation time leads to poor phase separation. In step 3d, it is important that purified total RNA is solubilized in nuclease-free water, not in TE or other buffers

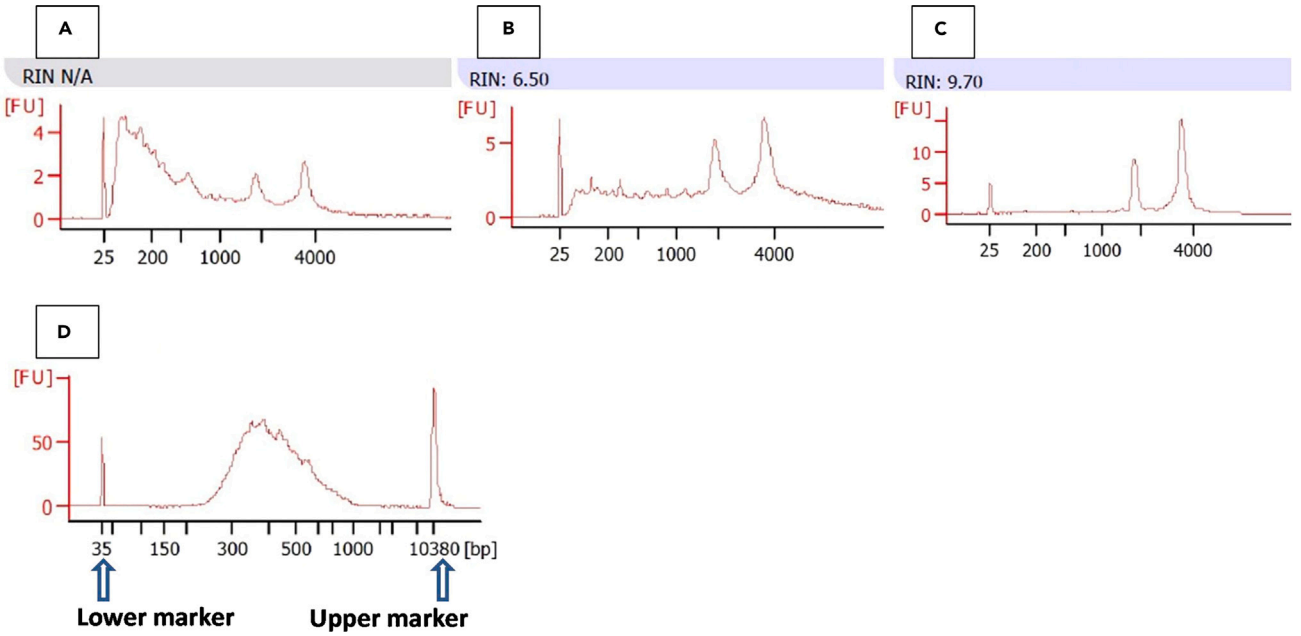


Figure 2. Quality controls of purified RNAs and libraries

(A–C) Bioanalyzer profiles and highly degraded (A), partially degraded (B) and high quality (C) RNA samples extracted from adipose tissue. Only high quality RNA is retained for further library production.

(D) Expected bioanalyzer profile of a high quality library.

containing EDTA. Chelation of divalent cations by EDTA will interfere with RNA fragmentation and the efficiency of reverse transcription.

4. RNA cleanup and DNase I treatment.

   See also https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/rna-purification/total-rna/rneasy-kits?catno=74104.

   a. Adjust the sample to a volume of 100 µL with RNAse-free water. Add 350 µL Buffer RLT, and mix well.
   b. Add 250 µL ethanol (96%–100%) to the diluted RNA, and mix well by pipetting. Do not centrifuge. Proceed immediately to next step.
   c. Transfer the sample (700 µL) to an RNeasy Mini spin column placed in a 2 mL collection tube. Close the lid gently, and centrifuge for 15 s at 12000 × *g*. Discard the flow-through.
   d. Reuse the collection.
   e. After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.
   f. Add 350 µL Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 12000 × *g*. Discard the flow-through.
   g. Reuse the collection tube.
   h. In a new 1.5 mL tube, add 10 µL DNase I stock solution to 70 µL Buffer RDD. Mix by gently inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.
   i. Add the DNase I incubation mix (80 µL) directly to the RNeasy spin column membrane, and place on the benchtop (20°C–30°C) for 15 min.
   j. Add 350 µL Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 12000 × *g*. Discard the flow-through.
   k. Add 500 µL Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at 12000 × *g* to wash the spin column membrane. Discard the flow-through.
   l. Reuse the collection tube.

m. Add 500 µL Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at 12000 × *g*.
n. Place the RNeasy spin column in a new 2 mL collection tube, and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at 20000 × *g* for 1 min.
o. Place the RNeasy spin column in a new 1.5 mL collection tube (supplied). Add 30–50 µL RNAse-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 × *g* (≥10,000 rpm) to elute the RNA.
p. The solution is stored at −80°C until further use.

5. RNA quality control.
   a. Measure the RNA concentrations of samples by spectrophotometry (Nanodrop) using nuclease-free water as blank.

Note: Sample purity is determined using the absorption spectrum and the ratio 260/280 nm absorbance. Samples with a ratio < 1.9 and/or absorption in short wavelengths (230 nm) are considered contaminated and RNA extraction must be performed again, starting at the chloroform (3c) step.

   b. Determine the total RNA integrity with the Agilent total RNA Pico Kit on a 2100 Bioanalyzer instrument (Agilent Technologies, Paolo Alto, USA).

⚠ CRITICAL: Only high quality RNA (RIN>8) is used for RNA sequencing. A Qubit kit can also be used for RNA quantification and quality control. Examples of degraded, partially degraded and high quality RNA extracted from adipose tissue are shown in Figures 2A–2C.

See also the manufacturer's instructions at: https://www.agilent.com/en/product/automated-electrophoresis/bioanalyzer-systems/bioanalyzer-instrument/2100-bioanalyzer-instrument-228250 https://www.agilent.com/cs/library/usermanuals/public/2100_Bioanalyzer_Expert_USR.pdf https://www.agilent.com/en/product/automated-electrophoresis/bioanalyzer-systems/bioanalyzer-rna-kits-reagents.

Library construction and RNA sequencing

Timing: 1.5 days (for step 6)

Timing: Preparation: 1 h; Run: 16 h (for step 7)

Manufacturer's protocols can be found at: https://www.takarabio.com/learning-centers/next-generation-sequencing/technical-notes/rna-seq/stranded-libraries-from-picogram-input-total-rna-(v2). Working in RNase-free conditions is again of crucial importance for these steps.

In this section, the step-by-step procedure to elaborate libraries from total RNA is detailed, followed by the evaluation of their quality.

6. Library construction.
   Library construction is performed with the SMARTer® Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian" (TaKaRa Bio USA, Inc., Mountain View, CA, USA) with a final multiplexing of 11 libraries. This number depends on the total number of samples in the RNAseq experimental design.
   a. First-strand cDNA synthesis.
      i. Mix the following components on ice:
         1–8 µL RNA.
         1 µL SMART Pico Oligos Mix v2.
         4 µL 5× First-Strand Buffer.
         0–7 µL Nuclease-Free Water.
         13 µL Total volume per reaction.

ii. Incubate the tubes 4 min at 94°C in a preheated, hot-lid thermal cycler, then immediately place the samples on an ice-cold PCR chiller rack for 2 min.

⚠ CRITICAL: The samples should be taken out of the thermal cycler immediately after the time indicated to avoid over-fragmentation. Make sure to wait by the thermal cycler when the incubation time is over and immediately chill the samples.

The next reaction steps are critical for first-strand synthesis and should not be delayed after step ii. Prepare the First-Strand Master Mix, while your tubes are incubating (step ii), or have it almost ready before starting step ii.

iii. Prepare enough First-Strand Master Mix for all reactions, plus 10%, by combining the following reagents on ice, in the order shown:

4.5 µL SMART TSO Mix v2.
0.5 µL RNase Inhibitor.
2 µL SMARTScribe Reverse Transcriptase.
7 µL Total volume per reaction.

⚠ CRITICAL: The SMART TSO Mix v2 is very viscous. Make sure to homogenize the First-Strand Master Mix very well by pipetting up and down 10 times with a pipette set at a volume larger than the final master mix volume.

iv. Add 7 µL of the First-Strand Master Mix to each reaction tube from step ii. Mix the contents of the tubes by vortexing for 2 s, then spin the tubes briefly to collect the contents at the bottom.

⚠ CRITICAL: The First-Strand Master Mix is very viscous—make sure to homogenize the content of the tubes very well.

v. Incubate the tubes in a preheated hot-lid thermal cycler with the following program:

42°C 90 min.
70°C 10 min.
4°C forever.

vi. Leave the samples in the thermal cycler at 4°C until the next step.

Pause point: Samples can be left overnight in the thermal cycler at 4°C. If not processed the next day, freeze the cDNA at –20°C for up to 2 weeks.

b. PCR1 - Addition of Illumina adapters and indexes.

i. Prepare a PCR1 Master Mix for all reactions. Combine the following reagents in the order shown, then mix well and spin the tube briefly in a microcentrifuge:

2 µL Nuclease-Free Water.
25 µL SeqAmp CB PCR Buffer (2×).
1 µL SeqAmp DNA Polymerase.
28 µL Total volume per reaction.

ii. Add 28 µL of PCR Master Mix to each sample from step a.vi.

iii. Add 1 µL of each 5′ and 3′ PCR Primer HT to each sample. Mix by gentle vortexing or tapping of the tubes, then spin down briefly. Make sure that each tube has a unique combination of 5′/3′ indexes.

iv. Place the tubes in the preheated hot-lid thermal cycler. Perform PCR using the following program:

94°C 1 min.
5 cycles:
98°C 15 s.

55°C 15 s.
68°C 30 s.
68°C 2 min.
4°C forever.

Pause point: Samples can be left for up to 1 hr in the thermal cycler at 4°C. If not processed within the next hour, freeze the PCR products at –20°C for up to 2 weeks.

c. Purification of the RNA-Seq library using AMPure XP beads.
   i. Allow AMPure beads to come to room temperature before use (~30 min). Add 40 µL of AMPure beads to each sample.

⚠ CRITICAL: Mix by pipetting the entire volume up and down at least 10 times. The beads are viscous; pipette the entire volume up and then out slowly.

   ii. Incubate at room temperature for 8 min to allow the DNA to bind to the beads.
   iii. Briefly spin the sample tubes to collect the liquid at the bottom. Place the sample tubes on the magnetic separation device for 5 min or longer, until the solution is completely clear.
   iv. While the tubes are sitting on the magnetic separation device, pipette out the supernatant and discard.
   v. Keeping the tubes on the magnetic separation device, add 200 µL of freshly made 80% ethanol to each sample - without disturbing the beads - to wash away contaminants. Wait for 30 s and carefully pipette out and discard the supernatant. cDNA will remain bound to the beads during the washing process.
   vi. Repeat Step v once.
   vii. Briefly spin the tubes (~2,000 g) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic separation device for 30 s, then carefully remove any remaining ethanol with a pipette, without disturbing the beads.
   viii. Let the open sample tubes rest at room temperature for 3–5 min until the pellets appear dry.

⚠ CRITICAL: The dried bead should not look glossy. Cracking might indicates over-drying which can result in lower library yield.

   ix. Once the beads are dry, add 52 µL of Nuclease-Free Water to cover the beads. Remove the tubes from the magnetic separation device and mix thoroughly by pipetting up and down until all the beads have been washed off the sides of the tubes.
   x. Incubate at room temperature for 5 min to rehydrate.
   xi. Briefly spin the sample tubes. Place the sample tubes on the magnetic separation device for 1 min or longer, until the solution is completely clear.
   xii. Pipette 50 µL of supernatant from each sample into respective wells of a new 8-well strip.
   xiii. Add 40 µL of AMPure beads to each sample and mix well.

⚠ CRITICAL: Mix by vortexing for 5 s (recommended) or by pipetting the entire volume up and down at least 10 times. The beads are viscous; pipette the entire volume up and then out slowly.

   xiv. Incubate at room temperature for 8 min to allow the DNA to bind to the beads. During the incubation time, proceed immediately to the next step (d).

d. Depletion of ribosomal cDNA with ZapR v2 and R-probes v2.
   i. Thaw R-Probes v2 and ZapR Buffer at room temperature. Place R-Probes v2 on ice as soon as it is thawed, but keep ZapR Buffer at room temperature.

⚠ CRITICAL: ZapR v2 should be kept on ice at all times and returned to the freezer immediately after use.

ii. Preheat the thermal cycler in anticipation of Step d.v.
iii. Upon completion of the 8-min incubation in Step c.xiv, briefly spin the sample tubes to collect the liquid at the bottom. Place the sample tubes on the magnetic separation device for 5 min or longer, until the solution is completely clear.

Note: It is acceptable—and in some cases necessary—to leave the tubes on the magnetic separation device for more than 5 min.

iv. During the 5-min incubation time in Step d.iii, pipette into a pre-chilled PCR tube a sufficient volume of R-Probes v2 for the number of reactions to be performed - 1.5 µL per reaction, (see Step d.xii) plus 10% to account for pipetting errors. Keep the PCR tube containing R-Probes v2 on ice and immediately return the remaining unused R-Probes v2 to a −70°C freezer.
v. Incubate the PCR tube containing R-Probes v2 at 72°C in a preheated hot-lid thermal cycler using the following program:
    72°C 2 min.
    4°C forever.
vi. Leave the R-Probes v2 tube in the thermal cycler at 4°C for at least 2 min, but for no more than 10–15 min, before using it in the next step (Step d.xii).
vii. Once the 5-min incubation on the magnetic separation device is complete (Step d.iii) and the samples are clear, pipette out the supernatant and discard, while keeping the tubes sitting on the magnetic separation device.
viii. Keeping the tubes on the magnetic separation device, add 200 µL of freshly made 80% ethanol to each sample - without disturbing the beads - to wash away contaminants. Wait for 30 s and carefully pipette out and discard the supernatant. cDNA will remain bound to the beads during the washing process.
ix. Repeat Step viii once.
x. Briefly spin the tubes (2,000 g) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic separation device for 30 s, then carefully remove any remaining ethanol with a pipette, without disturbing the beads.
xi. Let the open sample tubes rest at room temperature until the pellets appear dry.

Note: The beads will dry more quickly than in Step c.viii. 1–2 min may be sufficient, but the beads can be left to dry for up to 5 min during preparation of the ZapR Master Mix in Step d.xii.

xii. While the beads are drying, prepare the ZapR Master Mix. Prepare enough Master Mix for all reactions, plus 10%, by combining the following reagents at room temperature in the order shown. Make sure to add the preheated and chilled R-Probes v2 from Step d.vi last. Return ZapR v2 to a −20°C freezer immediately after use. Mix the components well by vortexing briefly, and spin the tubes briefly in a microcentrifuge.
    16.8 µL Nuclease-Free Water.
    2.2 µL 10× ZapR Buffer.
    1.5 µL ZapR v2.
    1.5 µL R-Probes v2.
    22 µL Total volume per reaction.
xiii. To each tube of dried AMPure beads from Step d.xi, add 22 µL of the ZapR Master Mix. Remove the tubes from the magnetic separation device and mix thoroughly to resuspend the beads.
xiv. Incubate at room temperature for 5 min to rehydrate.

xv. Briefly spin the sample tubes to collect the liquid at the bottom. Place the sample tubes on the magnetic separation device for 1 min or longer, until the solution is completely clear.

xvi. Pipet out 20 μL of supernatant, being careful not to disturb the beads, into a new PCR strip.

xvii. Incubate the tubes in a preheated hot-lid thermal cycler using the following program:

37°C 60 min.
72°C 10 min.
4°C forever

Note: Samples can be left in the thermal cycler at 4°C for up to 1 h. However, we recommend proceeding immediately to section e.

e. PCR2 - final RNA-Seq library amplification

i. Prepare a PCR2 Master Mix for all reactions (plus 10%). Combine the following reagents in the order shown, then mix well and spin the tubes briefly in a microcentrifuge:

26 μL Nuclease-Free Water.
50 μL SeqAmp CB PCR Buffer.
2 μL PCR2 Primers v2.
2 μL SeqAmp DNA Polymerase.
80 μL Total volume per reaction

⚠ CRITICAL: DO NOT reduce the reaction volume. The 100 μL final volume is important for yield. If your thermal cycler cannot accommodate 100 μL sample volumes, it is important to equally divide each sample into two tubes (containing ~50 μL each) after the PCR Master Mix has been added, mixed, and spun down (prior to Step iii).

ii. Add 80 μL of PCR2 Master Mix to each tube from Step d.xvii. Mix by tapping gently, then spin down. The total is 100 μL.

iii. Place the tubes in a preheated hot-lid thermal cycler. Perform PCR using the following program:

94°C 1 min.
9–16 cycles:
98°C 15 s.
55°C 15 s.
68°C 30 s.
4°C forever.

Note: The actual number of cycles varies depending on the starting material. We do not recommend performing more than 16 cycles, as it may lead to background amplification. We recommend that you perform a pilot experiment with a small number of samples to determine the optimal number of cycles for your input material. In our case, we used 10 ng of input RNA and performed 14 PCR cycles.

f. Purification of final RNA-Seq library using AMPure beads.

i. Allow AMPure beads to come to room temperature before use (~30 min). Add 100 μL of AMPure beads to each sample.

⚠ CRITICAL: Mix by vortexing for 5 s or by pipetting the entire volume up and down at least 10 times. The beads are viscous; pipette the entire volume up, and then out slowly.

ii. Incubate at room temperature for 8 min to let the cDNA to bind to the beads.

iii. Briefly spin the sample tubes to collect the liquid at the bottom. Place the sample tubes on the magnetic separation device for 5–10 min or longer, until the solution is completely clear.
iv. While the tubes are sitting on the magnetic separation device, pipette out the supernatant and discard.
v. Keep the tubes on the magnetic separation device. Without disturbing the beads, add 200 μL of freshly made 80% ethanol to each sample to wash away contaminants. Wait for 30 s and carefully pipette out the supernatant. cDNA will remain bound to the beads during the washing process.
vi. Repeat Step v once.
vii. Briefly spin the tubes (2,000 g) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic separation device for 30 s, then carefully remove any remaining ethanol with a pipette, without disturbing the beads.
viii. Let the sample tubes rest open at room temperature for 5–7 min until the pellets appear dry.

Note: You may see a tiny crack in each pellet. Do not overdry.

ix. Once the beads are dry, add 20 μL of Tris Buffer to cover the beads. Remove the tubes from the magnetic separation device and mix thoroughly by pipetting up and down several times until all the beads have been washed off the sides of the tubes.

Note: Consider eluting in 12 μL instead of 20 μL if anticipated yield is low.

x. Incubate at room temperature for 5 min to rehydrate.
xi. Briefly spin the sample tubes. Place the sample tubes on the magnetic separation device for 2 min or longer, until the solution is completely clear.
xii. Transfer the supernatants to nonsticky tubes. Proceed to validation immediately or store at −20°C.

g. **Validation using the Agilent 2100 bioanalyzer.** See also the manufacturer's instructions at : https://www.agilent.com/en/product/automated-electrophoresis/bioanalyzer-systems/bioanalyzer-instrument/2100-bioanalyzer-instrument-228250
https://www.agilent.com/cs/library/usermanuals/public/2100_Bioanalyzer_Expert_USR.pdf
https://www.agilent.com/en/product/automated-electrophoresis/bioanalyzer-systems/bioanalyzer-rna-kits-reagents
https://www.agilent.com/en/product/automated-electrophoresis/bioanalyzer-systems/bioanalyzer-dna-kits-reagents
i. Quantify libraries with Qubit dsDNA HS kit.
ii. Evaluate library size distribution by running samples on the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) or an equivalent microfluidic device/kit. Dilute libraries to about 1.5 ng/μL prior to loading the chip. Successful cDNA synthesis and amplification should produce a distinct curve spanning 200–1,000 bp, with a local maximum at ~300–400 bp. A small amount of products ~150–200 bp in size will not interfere with sequencing. However, consider repeating the final clean-up (Section f) if an excessive amount of products <200 bp in size is present. A representative example of an expected bioanalyzer profile is shown in Figure 2D. In our hands, dimers were never observed when using the Agilent kit.

7. **NGS sequencing of libraries.** Manufacturer's instructions can be found at: https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/nextseq-series-kits-v2-5.html
In this section, the generation of high throughput sequence reads is described. The library pool is sequenced on a NextSeq 500 (Illumina Inc., San Diego, CA, USA).
a. Planning the run.

Prepare the planned run using the software provided with the sequencer and applying the following features: Paired-end 2*76 bp; Dual-index (in our case, we use the indexes corresponding to those described in the Illumina TruSeq HT kit).

b. Pooling the libraries.
In our experiment, 11 libraries are pooled in equimolar quantities, at a 4 nM concentration. This depends on the number of reads desired. A good quality RNAseq also depends on the number of reads related to the genome size.

c. Libraries and PhiX denaturation.
   i. Prepare a fresh NaOH 0.2 N dilution by mixing:
      980 µL nuclease-free water.
      20 µL concentrated (10 N) NaOH.
      Vortex. This solution can be used for 12 h.
   ii. Allow HT1 (Next Seq® Accessory Box v2) to defreeze at room temperature and store at +4°C until further use.
   iii. Prepare a fresh solution of 200 mM Tris-HCl, pH 7 by mixing:
      8 µL nuclease-free water.
      2 µL of stock (1M) Tris-HCl, pH 7.
   iv. Denaturation. Add 5 µL NaOH 0.2 N to 5 µL of the pool of libraries at 4 nM. Vortex briefly and centrifuge at 500 g for 1 min. Incubate 5 min at room temperature.
   v. Neutralize by addition of 5 µL Tris-HCL 200 mM, pH 7. Briefly vortex and centrifuge.
   vi. Add 985 µL of pre-chilled HT1 to denaturated libraries. Final concentration is now 20 pM. Briefly vortex and centrifuge. Place the tube on ice.
   vii. Dilute the libraries to 1.4 pM with HT1 by mixing:
      91 µL of denaturated libraries.
      1209 µL of pre-chilled HT1.
      Mix by inverting the tube and centrifuge.
   viii. Add the sequencing internal control (PhiX DNA) by mixing:
      26 µL of denaturated PhiX DNA at 1.4 pM.
      1274 µL of denaturated and diluted libraries at 1.4 pM.

d. Preparation of the reagents, loading and running of the samples.
   i. Defreeze a reagents cartridge by placing it in a container with water at room temperature for 2 h. **Do not submerge the cartridge.** Check that all the compartments (specifically #29–32) are thawed and dry the cartridge. Mix the reagents by inverting the cartridge and gently tap it on the bench to remove air bubbles.
   ii. Take a new flow cell (stored in the fridge) and keep it at room temperature in its original packaging.
   iii. Clean the cap of the compartment #10 labeled "Load library here" with Kimtech wipes and pierce it with a clean 1 mL pipette tip.
   iv. Transfer the libraries (1300 µL) into compartment #10 without touching the aluminum cap.
   v. Initiate the sequencer using appropriate user name and password and insert the flow cell. Press "Load".
   vi. Insert the buffer cartridge and close the door. Press "Next".
   vii. Insert the reagents cartridge. Close the door and press "Load" then "Next".
   viii. Select an analysis name and press "Next". Confirm the parameters, press "Next". The system autocheck is engaged. Once finished, select "Start".

Assessment of RNAseq data quality and bioinformatic analyses

Timing: 5 days

In this section, RNAseq data are processed through various bioinformatics software to assess the quality of the reads, identify statistically-significant differentially expressed genes and analyze the pathways, as well as the cellular and molecular mechanisms in which they are involved.

8. Quality controls of RNAseq data and alignment on the mouse genome

For every sample, perform a quality control test on RNAseq raw data with the NGS Core Tools FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Map sequence reads using STAR[4] and remap unmapped reads with Bowtie2[9] using the very sensitive local option. The total mapped reads are finally available in a BAM (Binary Alignment Map) format for raw read counts extraction. Generate read counts with the htseq-count tool of the Python package HTSeq[6] with default parameters to generate an abundance matrix. Only genes with a total read count across selected samples superior to 10 are kept. At last, perform differential analyses using the DESEQ2[7] package of the Bioconductor framework. Select up- and down-regulated genes based on the adjusted p-value and the fold-change information. Finally, map the Ensembl gene names to the HGNC gene names with biomart R function.[10]

Note: Since many readers of this protocol might not have a training in advanced bioinformatics, it is also worth mentioning that all these bioinformatic tools (e.g. FastQC, STAR, Bowtie2, HTSeq, DESEQ2 etc.) can be performed using the open-source web-based Galaxy platform.[11] Galaxy has all these tools built-in and using them requires no prior training in coding languages, which makes these tools incredibly accessible to all bench scientists.

9. RNAseq data interpretation.

Analyze gene expression using dedicated R scripts to build volcano plots. Built heatmaps using the online application heatmapper (http://www.heatmapper.ca/). Identify pathways with IPA (Ingenuity Pathway Analysis, Qiagen). Perform Gene set enrichment using the web-based GEne SeT AnaLysis Toolkit (http://www.webgestalt.org/). Predict the proportion of immune cell populations that infiltrate the adipose tissue from bulk RNAseq data with the ImmQuant deconvolution software[8]

EXPECTED OUTCOMES

Yield of RNA extraction: We usually obtain 4.86 +/− 2.6 μg of RNA / 100 μg of adipose tissue.

QUANTIFICATION AND STATISTICAL ANALYSIS

In order to check if the samples of each group segregate together, perform a Multi-Dimensional Scaling (MDS) analysis and plot the corresponding graph with R package ggplot2 (https://ggplot2.tidyverse.org/).

LIMITATIONS

Like any tissue, the adipose tissue is not a homogeneous assembly of a single cell type. It contains infiltrating immune cells, the number of which increases with age, as well as stem cells. Therefore, the transcriptomic analysis described here, although describing the metabolic changes of adipocytes, also reflects changes in abundance and activity of additional cell types present in minority. To evaluate potential changes in immune cells infiltration, we use a deconvolution software of bulk RNAseq data (ImmQuant[8]), as mentioned in the step-by-step protocol. We also would like to stress that accelerated aging seen in Dicer 1 mutant is accompanied with reduced epididymal fat depots and an almost complete disappearance of the sub-cutaneous white adipose tissue. This represents an additional difficulty for investigators aiming at analyzing ageing-associated perturbations of adipose tissues.

TROUBLESHOOTING

Problem 1

During the library preparation (step 6), dimer formation between primers or adapters (which could be visualized during the bioanalyzer quality control, see Figure 2D for an example of an expected profile) might negatively impact PCR steps.

Potential solution

To maximize the removal of these dimers, step 6f is reiterated with fresh AMPure XP beads in the same conditions.

Problem 2

The final amount of libraries (determined in step 6g) is insufficient.

Potential solution

A re-amplification is performed, starting from step 6e, with a reduced number of cycles (usually 6 to 8) and the same pair of indices.

Problem 3

The Multi-Dimensional Scaling analysis described in step 9 (which can be replaced by a Principal Component Analysis) of the various samples shows that the biological replicates are scattered, indicating low repeatability.

Potential solution

RNA must be prepared from additional adipose tissue harvested from more control and experimental animals. The adipose tissue exhibits considerable metabolic and transcriptomic variation, depending on sex, location, diet and age.

Problem 4

Only subtle, non-significant changes in gene expression (upon analysis described in step 9) can be observed between controls and experimental samples.

Potential solution

Bulk RNAseq obtained from whole tissue could possibly mask important transcriptomic changes occurring in minor cell populations, such as preadipocytes. In this case, single cell RNAseq (not described in the present protocol) can solve the issue.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Philippe Georgel (pgeorgel@unistra.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new code. Raw RNAseq data can be obtained from the lead contact.

ACKNOWLEDGMENTS

Work in S.B. laboratory is supported by the Strasbourg's Interdisciplinary Thematic Institute (ITI) for Precision Medicine, TRANSPLANTEX NG, as part of the ITI 2021-2028 program of the University of Strasbourg, CNRS and INSERM, funded by IdEx Unistra (ANR-10-IDEX-0002) and SFRI-STRAT'US (ANR-20-SFRI-0012), the INSERM UMR_S 1109, the University of Strasbourg (IDEX UNISTRA), the European regional development fund (European Union) INTERREG V program (project PERSONALIS), and the MSD-Avenir grant AUTOGEN. Technical help from Cécile Macquin and the Genomax sequencing facility personnel is acknowledged.

AUTHOR CONTRIBUTIONS

A.D.C. and A.P. performed experiments. A.M. and R.C. supervised RNAseq experiments. T.S. performed RNAseq bioinformatics analyses. S.B. obtained funding. P.G. supervised the work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Dufau, J., Shen, J.X., Couchet, M., De Castro Barbosa, T., Mejhert, N., Massier, L., Griseti, E., Mouisel, E., Amri, E.Z., Lauschke, V.M., et al. (2021). In vitro and ex vivo models of adipocytes. Am. J. Physiol. Cell Physiol. *320*, C822–C841. https://doi.org/10.1152/ajpcell.00519.2020.

2. White, U.A., and Tchoukalova, Y.D. (2014). Sex dimorphism and depot differences in adipose tissue function. Biochim. Biophys. Acta *1842*, 377–392. https://doi.org/10.1016/j.bbadis.2013.05.006.

3. Otsuka, M., Jing, Q., Georgel, P., New, L., Chen, J., Mols, J., Kang, Y.J., Jiang, Z., Du, X., Cook, R., Das, S.C., Pattnaik, A.K., Beutler, B., and Han, J. (2007). Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. Immunity *27*, 123–134. https://doi.org/10.1016/j.immuni.2007.05.014.

4. Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics *29*, 15–21. https://doi.org/10.1093/bioinformatics/bts635.

5. Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat Methods. *9*, 357–359. https://doi.org/10.1038/nmeth.1923.

6. Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq–a Python framework to work with high-throughput sequencing data. Bioinformatics *31*, 166–169. https://doi.org/10.1093/bioinformatics/btu638.

7. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550. https://doi.org/10.1186/s13059-014-0550-8.

8. Frishberg, A., Brodt, A., Steuerman, Y., and Gat-Viks, I. (2016). ImmQuant: a user-friendly tool for inferring immune cell-type composition from gene-expression data. Bioinformatics *32*, 3842–3843. https://doi.org/10.1093/bioinformatics/btw535.

9. Langmead, B., Wilks, C., Antonescu, V., and Charles, R. (2019). Scaling read aligners to hundreds of threads on general-purpose processors. Bioinformatics *35*, 421–432. https://doi.org/10.1093/bioinformatics/bty648.

10. Durinck, S., Moreau, Y., Kasprzyk, A., Davis, S., De Moor, B., Brazma, A., and Huber, W. (2005). BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. Bioinformatics *21*, 3439–3440. https://doi.org/10.1093/bioinformatics/bti525.

11. Galaxy Community (2022). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update. Nucleic Acids Res. *50*, W345–W351. https://doi.org/10.1093/nar/gkac247.