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Supplementary Information for

# Obesity-associated exosomal miRNAs modulate glucose and lipid metabolism in mice

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## Supplemental materials and methods

**Animal studies.** All *in vivo* studies were performed at the School of Medicine Animal Facilities (University of Barcelona). Procedures were conducted in accordance with principles of laboratory animal care (European and local government guidelines) and approved by the Animal Research Committee of the University of Barcelona (register number:404/13). C57BL/6J male mice were used throughout the study. Mice were fed either standard chow or a high-fat diet (HFD, 45% calories from fat, *Open Source Diets*) for the periods indicated below. Glucose tolerance was determined by intraperitoneal glucose tolerance test (IpGTT) after 6h fasting by injecting a dose of 2g/Kg body weight. Tail blood glucose was measured at 0, 15, 30, 60 and 120min by using reactive strips in an *Accutrend Glucometer (Roche) (1)*. Insulin sensitivity was determined by insulin tolerance test (ITT) after 6h fasting by injecting different insulin doses as follows: 0.75U/Kg (HFD-model), 0.35U/Kg (EXO-model) and 0.5U/Kg (siPPARA and MIMIC-models). Tail blood glucose was measured at 0, 15, 30 and 60min as described for the IpGTT. Circulating TG levels were measured in tail blood from 6h fasted mice by using reactive strips in an *Accutrend GCT Glucometer (Roche).* Circulating FFA levels were determined in 5μl plasma from 6h fasted mice by using NEFA-HR(2) Kit (*Wako*) by following the instructions of the manufacturer. Plasma was obtained from 10μl of tail blood centrifuged at 1,500xg for 20 min (4ºC). Hepatic FFA content was measured in 10mg of homogenized liver by using Free Fatty Acid Quantitation Kit (*Sigma-Aldrich*) by following the instructions of the manufacturer. Hepatic TG content was measured by a colorimetric method in liver extracts. Briefly, 100mg tissue was powdered in liquid $N_2$ and digested for 1h at 70ºC in 100 µl KOH 3M prepared in ethanol 65%. Samples were then incubated o/n at 37ºC in a water bath and neutralized by addition of Tris-HCl 2M to achieve a final concentration of 50mM. An aliquot of each sample was then measured using a Triglyceride quantification kit (*Spinreact*). At the sacrifice, 1ml of blood was obtained by cardiac puncture with 6% EDTA and used for exosome isolation and miRNA analysis. Selected tissues (liver, the gastrocnemius-soleus muscle complex and the epididymal and subcutaneous adipose tissues) were dissected, weighed and flash-frozen in liquid nitrogen for posterior RNA analysis where appropriate.

**Experimental animal models.** HFD: 8-week old mice were maintained in either standard chow or a HFD for 15 weeks. EXO: 8-week old mice were injected through the tail vein with a 100μl PBS suspension containing 5μg exosomes isolated from plasma of either control or HFD mice. Injections took place biweekly during 4 weeks for a total of 8 injections. Half of the mice in each

of the groups were administered HFD during the next 4 weeks while maintaining the exosome injections. MIMIC: 11-week old mice were injected through the tail vein with a 100μl PBS suspension containing 25μg exosomes isolated from plasma of lean mice and transfected with either a negative control (*cel-miR-39-3p*) or with a cocktail of artificial miRNA mimics (*miR-192*, *miR-122*, *miR-27a-3p* and *miR-27b-3p*). Injections took place biweekly during 4 weeks for a total of 8 injections. A new cohort of mimic-injected mice was simultaneously administered with lipolysis inhibitor Acipimox dissolved in tap water (100mg/Kg/day, *Sigma-Aldrich*) or PPARα agonist Fenofibrate dissolved in 0.5% carboxymethylcellulose (50mg/Kg/day, both from *Sigma-Aldrich*) by oral gavage daily from Monday to Friday. A group of mimic-treated mice were treated with the equivalent volume of 0.5% carboxymethylcellulose as a control, but as the results were indistinguishable from the mimic group, these data are not presented. siPPARA: 11-week old mice were injected through the tail vein with a 100μl PBS suspension containing 25μg exosomes isolated from plasma of lean mice and transfected with a non-targeting siRNA or with a siRNA targeting *Ppara*. Two injections were performed during a week. A new cohort of both control and treated siPPARA mice was simultaneously administered with Acipimox (100mg/Kg/day) dissolved in drinking water. A third cohort was injected and sacrificed 48h afterwards. Biodistribution studies: 13-week old mice were injected once through the tail vein with a 200μl PBS suspension containing 50μg exosomes isolated from plasma of lean mice and transfected with a non-mammalian miRNA (*cel-miR-39-3p*) or PBS and sacrificed 4h afterwards for Real time RT-PCR analysis. A second cohort of mice was injected intravenously with either 100μg PKH67-labelled exosomes or PBS as a negative control 6h prior to sacrifice for immunohistochemistry analysis in eWAT and liver. Adipocytes and hepatocytes were isolated as described (2, 3) and used to study mimic accumulation and *Ppara* expression by Real time RT-PCR.

**Exosome isolation, quantification and fluorescent labeling.** Exosomes were isolated from 500μl mouse plasma by sequential centrifugation (4). Briefly, plasma was diluted with an equal volume of PBS and samples were sequentially centrifuged at 2,000xg for 30min (4ºC), 10,000xg for 45min (4ºC), filtered through a 0.22μm syringe filter and ultracentrifuged o/n at 120,000xg (4ºC) in a S110AT rotor in a Sorvall MX 150 ultracentrifuge (*Thermo Scientific Inc*). Pellets were resuspended in PBS and ultracentrifuged again at 120,000xg for 3h (4ºC). The final pellets were resuspended in 100μl PBS. Total exosome protein was quantified by Bradford Assay (*Sigma*) and equal volumes (9μl) were resolved by 9% SDS-PAGE and transferred to a PVDF membrane

(*Millipore*). Anti-CD63 (H-193) (sc-15363, *Santa Cruz Biotechnology*) was diluted 1/250 in TBS (20mM Tris, 150mM NaCl, pH7.5) supplemented with 5% bovine serum albumin (BSA) and visualized by blotting with HRP-conjugated secondary anti-rabbit antibody (*GE Healthcare Bio-Sciences* NA934V). Chemiluminiscence was detected by using the ECL Plus Reagents (*GE Healthcare Bio-Sciences*), in a LAS4000 Lumi-Imager (*Fuji Photo Film Inc.*). Vesicle morphology was analyzed after negative staining by using transmission electron microscopy. Briefly, 30µl of exosome samples diluted 1/10 to 1/20 with PBS were allowed to dry on top of Formvar carbon-coated grids for 25min and contrasted with 2% uranyl acetate for 2min. Preparations were observed in a JEOL 1010 100kV Electron Microscope. Diameter size and concentration of vesicles was determined using NanoSight LM10 equipment (*Malvern*) using different dilutions (1/10 to 1/50) and the following parameters: camera at 30 frames per second (FPS), camera level at 16, temperature between 21–25 °C and video recording time 60 s. Nanosight NTA Software analyzed raw data videos by triplicate. Complementary analysis by measuring the esterase activity known to be within exosomes were also performed in 10µl of exosomes in suspension by using Exocet Exosome Quantification Assay Kit (*SBI System Biosciences*) by following the instructions of the manufacturer. Exosomes were fluorescently labeled with the PKH67 Fluorescent Cell Linker Kit (*Sigma-Aldrich*). Briefly, aliquots up to 25µl exosomes were diluted with Diluent C and mixed with 4µl PKH67 dye. Incubation was stopped by adding exosome-free fetal bovine serum (FBS) and ultracentrifuging at 120,000xg for 3h (4ºC). Pellets were resuspended in exosome-free FBS and ultracentrifuged again at 120,000xg for 3h (4ºC). The final pellets were resuspended in 100µl PBS.

**Exosome transfections.** All the exosomes used for the different transfections were isolated from plasma of control mice and mixed in a pool to ensure consistency. For fluorescent miRNA mimic transfection, 50µg exosomes isolated from control mice were transfected with 370pmol fluorescent miRNA mimic by using Exo-Fect™ Exosome Transfection Reagent (*SBI System Biosciences*) and following the instructions of the manufacturer. For MIMIC mice model, 50µg exosomes isolated from control mice were transfected with 250pmol negative control (*cel-miR-39-3p*) or with a cocktail of 250pmol artificial miRNA mimics (*miR-192*, *miR-122*, *miR-27a-3p* and *miR-27b-3p*) by using Exo-Fect™ Exosome Transfection Reagent. Each transfection was enough for 2 injections. For siPPARA mice model, 50µg exosomes isolated from control mice were transfected with 380pmol siRNA targeting *Ppara* or 380pmol non-targeting siRNA by using Exo-Fect™ Exosome Transfection Reagent. Each transfection was enough for 2 injections. See Table S5 for sequences and references.

***In vitro* capture of labelled exosomes.** 3T3-L1 cells (ATCC, CL-173) were maintained in Dulbecco's modified Eagle's Medium (DMEM) (*Sigma-Aldrich*) supplemented with 10% FBS and antibiotics. Cells were trypsinized and distributed into $5x10^4$cells/well. Cells were incubated for 24h with 7μg/ml PKH67-labelled exosomes or PBS as a negative control. 3T3-L1 cells also were incubated for 24h with 7μg/ml exosomes transfected with fluorescent miRNA mimic, or were transfected with 15pmol fluorescent miRNA mimic naked in DMEM supplemented with 10% FBS ultracentrifuged for exosome depletion. Cells were fixed in 4% PFA for 10min, washed with PBS and nuclei were stained with 1:300 Hoestch in PBS for 3 min. The preparations were mounted and coverslipped using FA mounting fluid (*Dako*). Images were collected using TCS SPE confocal microscope (*Leica*) and processed using Adobe Photoshop 7.0 (*Adobe Systems Inc*.) and ImageJ FIJI (5).

**Cell culture and mimic/siRNA transfection.** 3T3-L1 cells were transfected with 8pmol negative control or with the 4 selected obesity-associated miRNA mimics (2pmol for each miRNA) individually or in combination by using Metafectene Pro (*Biontex*) at a 1:3 weight ratio with the siRNA/miRNA (6). In the case of siRNA transfection, cells were transfected with 60nM siPPARA or a scrambled control siRNA (*Applied Biosystems*). After 48h, cells were collected for RNA extraction and analysis as described below.

**RNA isolation and Real time RT-PCR.** For exosomal miRNA analysis, total RNA was extracted from 10μl isolated exosomes with miRNeasy Mini Kit (*Qiagen*). An equal volume of each RNA sample was retrotranscribed by using mircury LNA™ Universal RT microRNA PCR (*Exiqon*). A miRNA profiling of 378 miRNAs was performed by Real time RT-PCR using predesigned panels with LNA specific primers (*Exiqon*) in a 7900HT Fast Thermocycler (*Applied Biosystems*). Differential expression was determined with GenEx Software (*Exiqon*) by normalizing to the mean Ct of the whole plate. In the case of tissue and cell samples, total RNA was extracted by using the miRNAeasy kit (*Qiagen*). For mRNA expression 500ng were analyzed by RT-PCR using the High-Capacity Reverse Transcription Kit (*Applied Biosystems*) and house-made primers Results were normalized by housekeeping *actb*, and *hprt*. For miRNA expression in tissues, 5ng total RNA were retrotranscribed with mircury LNA™ Universal RT kit and analyzed by Real time PCR using commercial SYBRGreen primers (Exiqon). Data were normalized to *RNU5G* values. Results are presented as fold change values calculated as $2^{-ddCt}$, after further normalizing the dCt of the case samples with the mean dCt of the control samples (7). Heat

maps of gene expression in liver and eWAT representing log2 of fold change were created by using Plotly Software (https://plot.ly). See Table S6 for primer sequences and references.

**Western blotting.** Total protein was extracted from eWAT samples using RIPA lysis buffer. Appropriate protease and phosphatase inhibitors were added fresh to the lysis buffer. Equal amounts (35µg) quantified by Bradford Assay (*Sigma*) were resolved by 9% SDS-PAGE and transferred to a PVDF membrane (*Millipore*). Mitoprofiler antibody (*MitoSciences-Abcam* ab110413) was diluted 1/1000 in TBS-BSA and visualized by blotting with HRP-conjugated secondary anti-mouse antibody (*GE Healthcare Bio-Sciences* NA931V). Anti-actin (*Sigma* 20-33) was diluted 1/1000 in TBS-BSA and visualized by blotting with HRP-conjugated secondary anti-rabbit antibody. Chemiluminiscence was detected using the ECL Plus Reagents (*GE Healthcare Bio-Sciences*), in a LAS4000 Lumi-Imager (*Fuji Photo Film Inc.*).

**Immunohistochemistry.** Tissues were fixed in 4% PFA o/n at 4ºC. Liver was transferred to 30% sucrose in PBS for 24h at 4ºC and embedded in OCT. 10µm sections were obtained with a CM1860 cryostat (*Leica*) and applied to poly-lysine coated slides. eWAT was embedded in paraffin and 5µm sections were obtained with a RM2135 microtome (*Leica*) and applied to poly-lysine coated slides. H&E and Oil Red staining were performed by following the protocols at IHCWorld (http://www.ihcworld.com). Images were collected using a BX41TF Microscope (*Olympus*) and processed using Adobe Photoshop 7.0 (*Adobe Systems Inc*., San José, CA) and ImageJ FIJI (5). Macrophage infiltration of eWAT was determined with anti-F4/80 antibody (*Abcam*) staining of paraffin sections following the instructions of the manufacturer. Briefly, dewaxed sections were incubated with 20 µg/ml Proteinase K solution in TE buffer for 3 min at RT and rinsed with PBS. Sections were then incubated o/n at 4ºC in a wet chamber with 1:100 anti-F4/80 in antibody diluent (*Dako*) followed by 1:250 donkey anti-rat-Cy3 secondary antibody for 2h at RT in the dark (*Jackson Immunoresearch*). To study biodistribution of PKH67-labelled exosomes, liver was fixed in 4% PFA o/n at 4ºC, transferred to 30% sucrose in PBS for 24h at 4ºC and embedded in OCT. 10 µm sections were obtained with a CM1860 cryostat (*Leica*) and applied to poly-lysine coated slides. Slides were then washed with PBS and nuclei were stained with 1:300 Hoestch in PBS for 3min, and the preparations were mounted and coverslipped using FA mounting fluid (*Dako*). In the case of eWAT, tissue was finely minced with scissors, fixed in 4% PFA for 10min and then washed with PBS and nuclei were stained with 1:300 Hoestch in PBS for 3min. Preparations were mounted and coverslipped using FA mounting fluid (*Dako*).

Images were collected using TCS SPE confocal microscope (*Leica*) and processed using Adobe Photoshop 7.0 (*Adobe Systems Inc*., San José, CA) and ImageJ FIJI (5).

**Statistical analyses.** Differences between groups were determined by either t-test analysis when only two groups were compared or by One-way ANOVA with t-test analysis for the pair wise comparison of 3 or more groups with different number of values. Asterisks indicate significance with respect to control group, unless otherwise specified. Correlation analyses were performed by Pearson regression.

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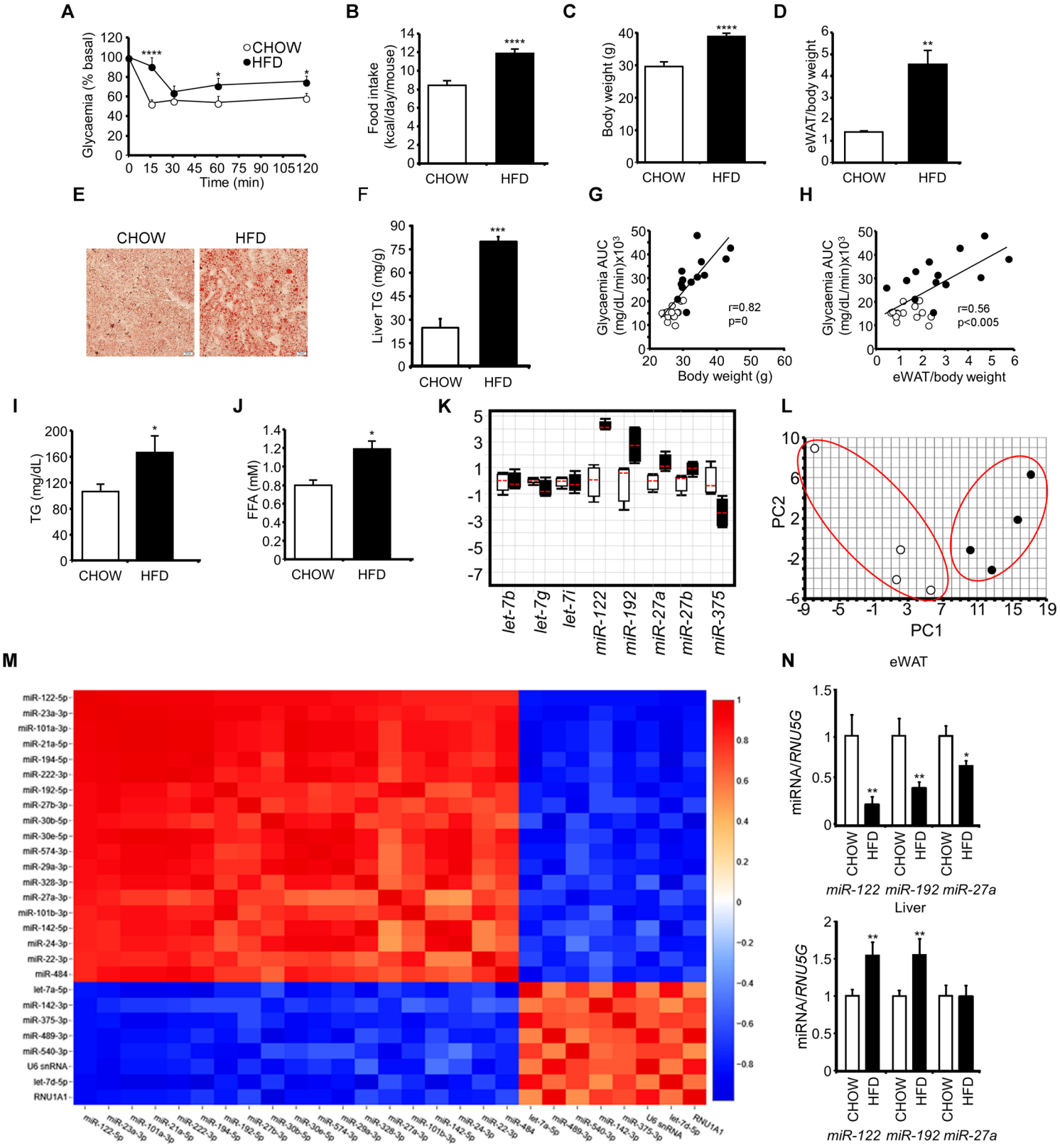


**Fig. S1. Diet-induced central obesity changes the profile of circulating exosomal miRNAs.** (A-D) ITT (0.75U/Kg) (A), food intake (B), body weight (C) and ratio between the weight of epididymal fat and body weight (D) in C57B6J mice after 15 weeks of HFD feeding. (E-F) Representative Oil Red staining of liver sections (E) and TG quantification in the liver of chow-fed and HFD mice (F). (G-H) Correlation between the glycaemia AUC obtained from the IpGTT and either body weight (G) or percentage of eWAT (H). (I-J) Plasma TG (I) and FFA (J) concentrations from chow-fed and HFD mice. (K-M) Real time RT-PCR profiling of the miRNA content of lean and obese plasma exosomes. Box plots of selected invariant, decreased and increased miRNAs (K), principal component analysis (L), and heat map showing Pearson correlation coefficients between differentially expressed miRNAs across all samples (M). (N) Obesity-associated miRNA expression in eWAT and liver tissues of the mice described in (A-D). Data are presented as mean ± SEM. n=10 per group (A,B); n=8 per group (C); n=5 per group (D); n=2 per group (E); n=4 per group (F, J-M); n=14 per group (G,H); n=5 per group (I); at least n=4 per group (N). *p<0.05, **p<0.01, ***p<0.005, ****p<0.001, Student's t-test.

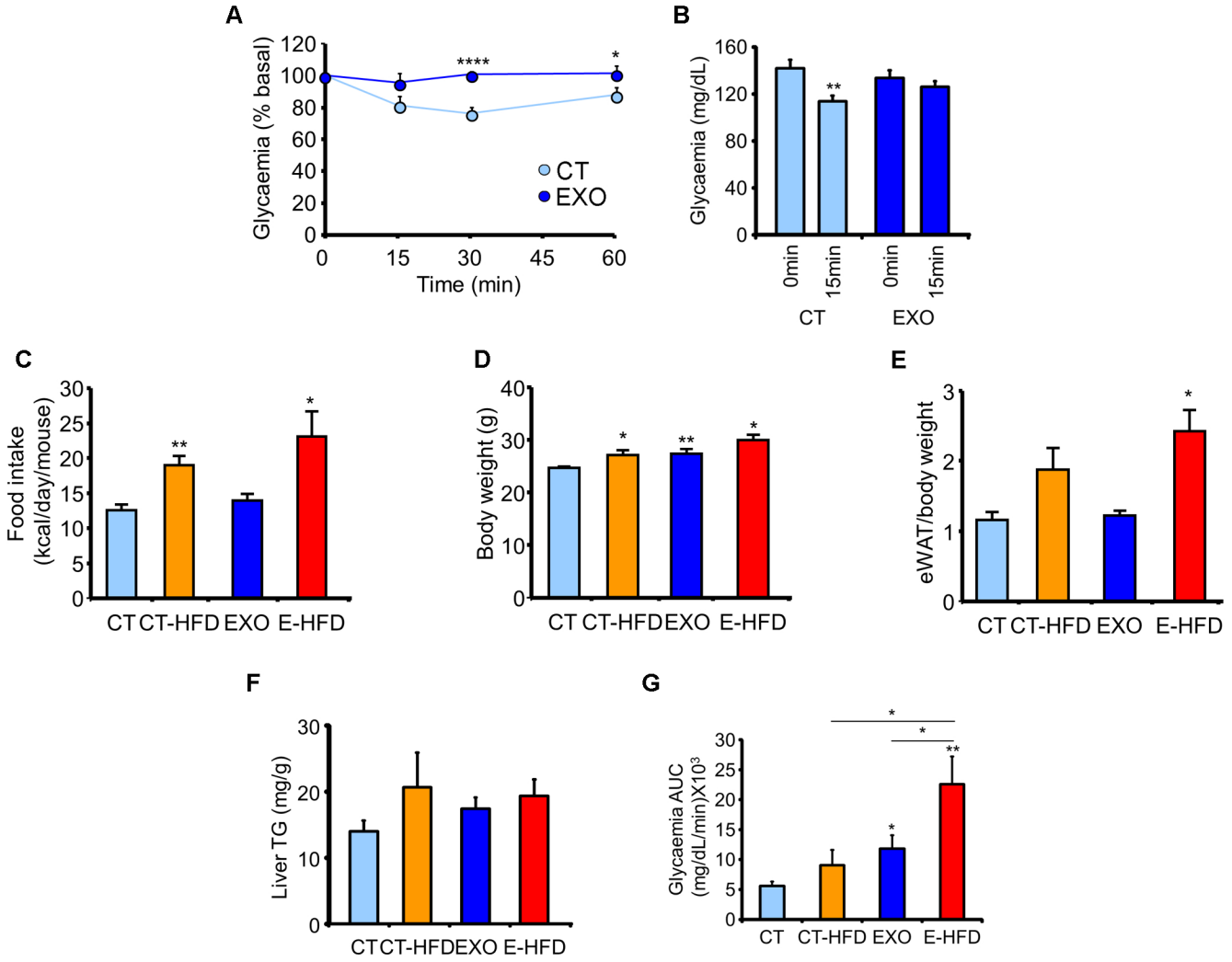


**Fig. S2. Exosomes from obese mice induce glucose intolerance in lean mice.** (A-B) ITT (0.175U/Kg) of chow-fed mice after 4 weeks of biweekly systemic injections of obese exosomes (A) and glucose values at T0 and T15 obtained from the ITT. (C-G) Food intake (C), body weight (D), ratio between the weight of epididymal fat and body weight (E), liver TG content (F), and glycaemia AUC from IpGTT (G), in C57B6J mice after 8 weeks of biweekly systemic injections of obese exosomes, with HFD feeding during the last 4 weeks. Data are presented as mean ± SEM. n=10 per group (A-B); n=5 per group, except n=4 CT-HFD (C-D, G); n=3 CT and EXO, n=4 CT-HFD and n=5 E-HFD (E); n=3 per group (F). *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 with respect to CT group unless otherwise indicated, Student's t-test.

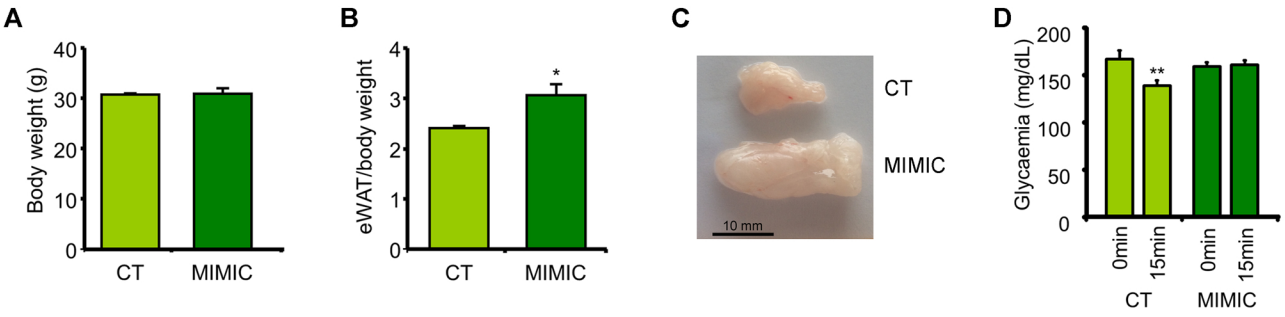


**Fig. S3. Exosomes transfected with obesity-associated miRNAs induce glucose intolerance dissociated from obesity.** (A-B) Body weight (A) and ratio between the weight of epididymal fat and body weight (B) from chow-fed mice after 4 weeks of injections of exosomes loaded with mimics of 4 miRNAs enriched in obese exosomes. (C) Representative image of the eWAT of mimic-treated mice. (D) Glucose values at T0 and T15 obtained from the ITT (0.175U/Kg) shown in Fig. 3D. Data are presented as mean ± SEM. n=5 per group (A-D). *p<0.05, **p<0.01, Student's t-test.

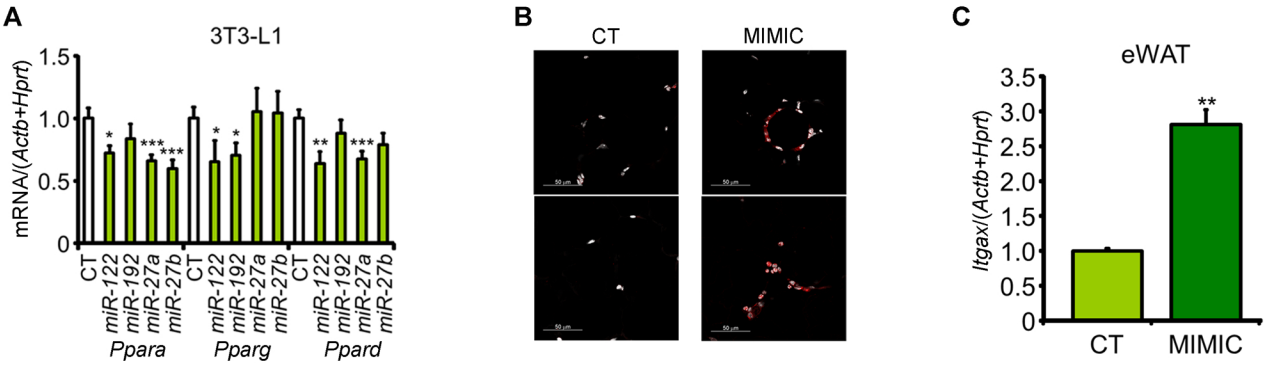


**Fig. S4. Mimic treatment induces eWAT inflammation and hepatic steatosis.** (A) mRNA expression level of *Ppar* family members in 3T3-L1 cells after transfection with individual selected obesity-associated miRNA mimics. (B-C) Representative micrograph showing staining of F4/80 in adipose tissue sections (B) and mRNA expression level of *Itgax* in eWAT (C) from chow-fed mice after 4 weeks of injections of exosomes loaded with mimics of 4 miRNAs enriched in obese exosomes. Data are presented as mean ± SEM. At least n=6 per condition (A); n=4 per group (B-C). *p<0.05, **p<0.01, ***p<0.005, Student's t-test.

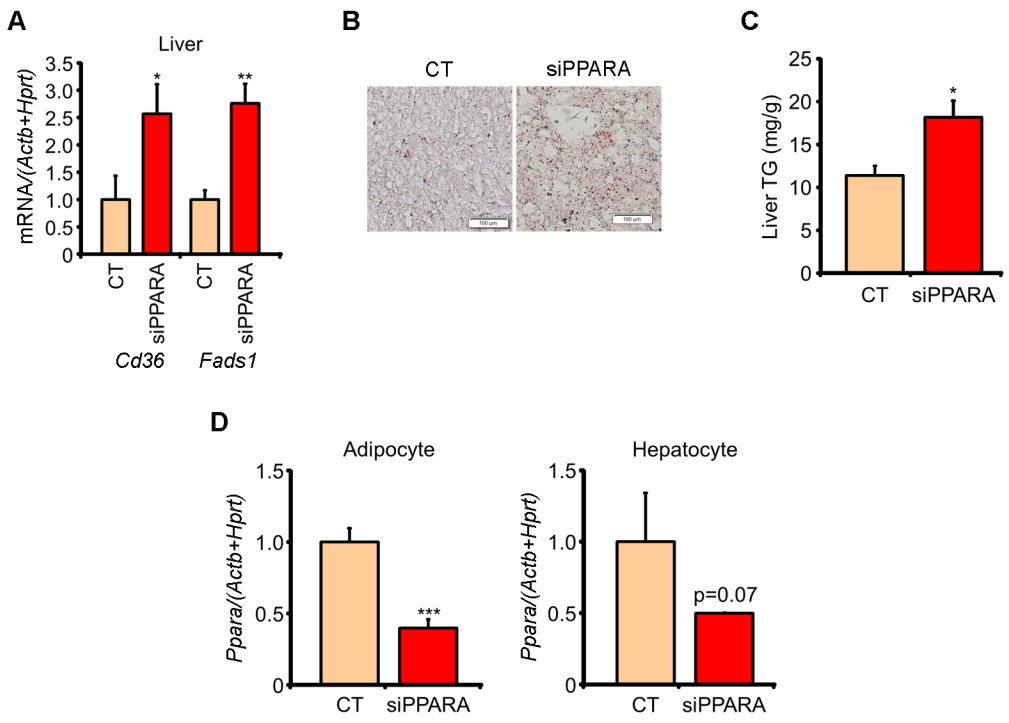


**Fig. S5. Treatment with siPPARA-transfected exosomes recapitulates the central obesity phenotype of mimic-treated mice.** (A) Hepatic mRNA expression of genes involved in lipogenesis in chow-fed mice after 2 injections of lean exosomes loaded with siPPARA siRNA. (B-C) Representative Oil Red staining of liver sections (B) and TG quantification in the liver of mice described in (A). (D) *Ppara* mRNA expression in visceral adipocytes and hepatocytes isolated from chow-fed mice after 1 injection of exosomes loaded with siPPARA siRNA. Data are presented as mean ± SEM. n=4 per group (A-C); n=3 per group (D). *p<0.05, **p<0.01, ***p<0.005, Student's t-test.

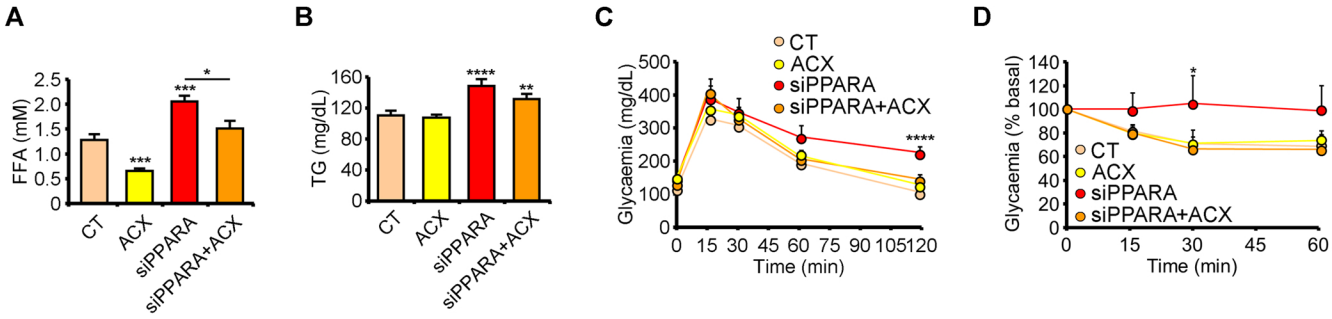


**Fig. S6. Decreasing FFA plasma levels partially reverts the pathologic phenotype.** (A-B) Plasma FFA (A) and TG (B) concentrations from chow-fed mice after 2 injections of exosomes loaded with siPPARA siRNA and simultaneously treated with ACX dissolved in drinking water. (C-D) IpGTT (C) and ITT (0.5U/Kg) (D) in the mice described in (A-B). Data are presented as mean ± SEM. n=14 CT, n=5 ACX, n=9 siPPARA and n=5 siPPARA+ACX (A-B); n=5 per group (C); n=10 CT, n=5 ACX, n=5 siPPARA and n=5 siPPARA+ACX (D). *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 with respect to CT group unless otherwise indicated, Student's t-test.

**Table S1. Experimental groups.** Parameters related to glucose tolerance and body composition for the different mouse models analyzed. Boldface indicates at least p<0.05 as compared with the respective control groups. ND: Not determined. Data are presented as mean ± SEM.

| | | **Fasting glycaemia (mg/dL)** | **Glycaemia AUC x10$^3$ (mg/dL/min)** | **Body weight (g)** | **Liver weight (g)** | **eWAT weight (g)** | **scWAT weight (g)** | **eWAT/scWAT ratio** | **Muscle (g)** gastroc+soleus |
|---|---|---|---|---|---|---|---|---|---|
| **EXP** | **CHOW** | 61.6 ± 4.3 | 18.2 ± 1.2 | 29.4 ± 1.5 | 1.50 ± 0.10 | 0.60 ± 0.10 | 0.42 ± 0.05 | 1.36 ± 0.10 | 0.30 ± 0.02 |
| **HFD** | **HFD** | **122.6 ± 14.3** | **27.9 ± 2.6** | **38.7 ± 1.0** | 1.70 ± 0.10 | **1.83 ± 0.10** | **1.99 ± 0.14** | **0.94 ± 0.10** | 0.30 ± 0.02 |
| **EXP** | **CT** | 136.8 ± 5.5 | 5.6 ± 0.7 | 24.6 ± 0.2 | 1.20 ± 0.10 | 0.28 ± 0.03 | 0.27 ± 0.01 | 1.0 ± 0.06 | 0.21 ± 0.01 |
| **EXO-HFD** | **CT-HFD** | **158.5 ± 14.3** | **9.0 ± 2.6** | **27.0 ± 0.9** | 1.18 ± 0.10 | **0.50 ± 0.06** | 0.33 ± 0.03 | **1.5 ± 0.06** | 0.23 ± 0.01 |
| | **EXO** | **110.8 ± 8.3** | **11.8 ± 2.3** | **27.4 ± 0.8** | 1.16 ± 0.20 | 0.32 ± 0.03 | 0.32 ± 0.01 | 1.0 ± 0.10 | 0.23 ± 0.01 |
| | **EXO-HFD** | **160.2 ± 11.5** | **22.6 ± 4.6** | **29.9 ± 1.1** | 1.14 ± 0.04 | **0.71 ± 0.08** | **0.47± 0.05** | **1.5 ± 0.12** | 0.26 ± 0.02 |
| **EXP** | **CT** | 163.2 ± 12.5 | 9.6 ± 1.1 | 30.7 ± 0.3 | 1.33 ± 0.08 | 0.69 ± 0.02 | 0.46 ± 0.02 | 1.56 ± 0.07 | 0.30 ± 0.02 |
| **MIMIC** | **MIMIC** | 173.4 ± 12.1 | **16.1 ± 2.0** | 30.9 ± 1.1 | 1.26 ± 0.07 | **0.89 ± 0.07** | 0.57 ± 0.06 | 1.66 ± 0.13 | 0.27 ± 0.01 |
| **EXP** | **CT** | 159.4 ± 8.0 | 3.1 ± 0.5 | 29.7 ±1.2 | 1.33 ± 0.07 | 0.47 ± 0.05 | 0.27 ± 0.02 | 1.74 ± 0.11 | 0.33 ±0.02 |
| **MIMIC2** | **MIMIC** | 150.8 ± 6.6 | **9.6 ± 0.6** | 29.6 ±0.7 | 1.40 ± 0.03 | **0.71 ± 0.08** | **0.38 ± 0.03** | 1.88 ± 0.14 | 0.30 ±0.01 |
| | **MIMIC+ACX** | 151.0 ± 3.6 | 3.4 ± 0.5 | 28.1 ±0.4 | 1.30 ± 0.03 | 0.55 ± 0.02 | **0.34 ± 0.01** | 1.63 ± 0.05 | 0.31 ±0.01 |
| | **MIMIC+FF** | 153.6 ± 9.6 | 2.1 ± 1.1 | 28.3 ±0.4 | 1.34 ± 0.05 | 0.47 ± 0.05 | 0.30 ± 0.04 | 1.62 ± 0.14 | 0.30 ±0.01 |
| **EXP** | **CT** | 116.0 ± 8.6 | 10.7 ± 2.1 | 28.1 ± 0.4 | 1.45 ± 0.07 | 0.23 ± 0.02 | 0.21 ± 0.01 | 1.2 ± 0.07 | 0.26 ± 0.01 |
| **siPPARA** | **ACX** | **151.0 ± 7.3** | 9.7 ± 1.2 | 27.8 ± 0.7 | 1.48 ± 0.09 | **0.42 ± 0.06** | ND | ND | ND |
| | **siPPARA** | 146.0 ± 6.7 | **16.3 ± 2.4** | 28.4 ± 0.5 | 1.66 ± 0.10 | **0.36 ± 0.03** | 0.24 ± 0.03 | 1.61 ± 0.13 | 0.28 ± 0.02 |
| | **siPPARA+ACX** | 132.0 ± 12.6 | 12.3 ± 1.1 | 28.3 ± 0.4 | **1.73 ± 0.10** | **0.39 ± 0.05** | 0.27 ± 0.04 | 1.49 ± 0.07 | 0.30 ± 0.05 |

**Table S2. Exosomal miRNA profiling.** Results of the real time RT-PCR miRNA profiling of exosomes isolated from chow-fed control and HFD mice plasma (n=4 samples/group).

| miRNA | Fold change CHOW/HFD | Difference (A-B log scale) | P-Value |
|---|---|---|---|
| ***miR-122-5p*** | -19.14532 | -4.25892 | 0.00047329 |
| ***miR-194-5p*** | -7.59782 | -2.92559 | 0.00353313 |
| ***miR-192-5p*** | -6.97126 | -2.80142 | 0.03284819 |
| ***miR-22-3p*** | -5.51714 | -2.46392 | 0.00472715 |
| ***miR-101b-3p*** | -5.31998 | -2.41142 | 0.01194403 |
| ***miR-484*** | -3.78796 | -1.92142 | 0.04629613 |
| ***miR-30e-5p*** | -3.42575 | -1.77642 | 0.04009519 |
| ***miR-101a-3p*** | -3.2635 | -1.70642 | 0.01308352 |
| ***miR-21a-5p*** | -3.23535 | -1.69392 | 0.02688733 |
| ***miR-27a-3p*** | -2.59174 | -1.37392 | 0.02321927 |
| ***miR-222-3p*** | -2.39732 | -1.26142 | 0.00280695 |
| ***miR-378a-3p*** | -1.99504 | -0.99642 | 0.02138253 |
| ***miR-532-5p*** | -1.86144 | -0.89642 | 0.02692559 |
| ***miR-328-3p*** | -1.82629 | -0.86892 | 0.03104197 |
| ***miR-142-3p*** | 1.71247 | 0.77608 | 0.03527462 |
| ***miR-214-3p*** | 2.00961 | 1.00691 | 0.03947225 |
| ***let-7a-5p*** | 2.68716 | 1.42608 | 0.01508044 |
| ***miR-103-3p*** | 3.23645 | 1.69441 | 0.0214863 |
| ***miR-297a-5p*** | 3.82665 | 1.93608 | 0.03226725 |
| ***let-7d-5p*** | 4.21173 | 2.07441 | 0.02347649 |
| ***miR-26b-5p*** | 4.75215 | 2.24858 | 0.04133171 |
| ***miR-375-3p*** | 5.20025 | 2.37858 | 0.02239404 |
| ***miR-540-3p*** | 7.89121 | 2.98025 | 0.01431059 |
| ***U6 snRNA*** | 12.96078 | 3.69608 | 0.04875204 |
| ***RNU1A1*** | 5.05805 | 2.33858 | 0.05220366 |
| ***miR-202-3p*** | 10.99351 | 3.45858 | 0.05538575 |
| ***miR-27b-3p*** | -1.99159 | -0.99392 | 0.05604487 |
| ***miR-574-3p*** | -3.84083 | -1.94142 | 0.05806141 |
| ***miR-18a-3p*** | 2.22594 | 1.15441 | 0.06938457 |
| ***rno-miR-146b-5p*** | 2.71367 | 1.44025 | 0.07255721 |
| ***miR-125a-5p*** | 3.47878 | 1.79858 | 0.07568019 |
| ***miR-29a-3p*** | -2.69712 | -1.43142 | 0.0915877 |
| ***miR-146a-5p*** | -1.8679 | -0.90142 | 0.09457178 |
| ***miR-187-3p*** | 5.23037 | 2.38691 | 0.09787929 |
| ***miR-29b-3p*** | -2.12346 | -1.08642 | 0.09891505 |
| ***miR-30b-5p*** | -1.3603 | -0.44392 | 0.09911454 |
| ***miR-489-3p*** | 2.87006 | 1.52108 | 0.10622981 |
| ***rno-miR-214-3p*** | 2.2714 | 1.18358 | 0.1132423 |
| ***miR-23a-3p*** | -1.90715 | -0.93142 | 0.12299883 |
| ***miR-200b-3p*** | 2.32583 | 1.21775 | 0.13073063 |
| ***miR-148a-3p*** | -2.71588 | -1.44142 | 0.13089863 |
| ***miR-24-3p*** | -1.81998 | -0.86392 | 0.13127296 |
| ***let-7g-5p*** | 1.50116 | 0.58608 | 0.1339603 |
| ***miR-15a-5p*** | -2.45193 | -1.29392 | 0.13509952 |
| ***miR-142-5p*** | -1.65166 | -0.72392 | 0.13765375 |
| ***miR-107-3p*** | 1.97851 | 0.98441 | 0.13793932 |
| ***miR-140-5p*** | 1.8707 | 0.90358 | 0.13958098 |

| | | | |
|---|---|---|---|
| ***miR-376c-3p*** | 2.55103 | 1.35108 | 0.14941205 |
| ***miR-23b-3p*** | -1.76408 | -0.81892 | 0.14984889 |
| ***miR-19a-3p*** | -1.58988 | -0.66892 | 0.15586609 |
| ***miR-106b-5p*** | -1.67762 | -0.74642 | 0.18458018 |
| ***miR-185-5p*** | 1.7596 | 0.81525 | 0.21608726 |
| ***rno-miR-208a-3p*** | 2.3071 | 1.20608 | 0.21751361 |
| ***miR-320-3p*** | -2.05113 | -1.03642 | 0.22594986 |
| ***miR-191-5p*** | -1.51458 | -0.59892 | 0.24812553 |
| ***miR-144-3p*** | -1.79181 | -0.84142 | 0.278872 |
| ***miR-193b-3p*** | 2.11196 | 1.07858 | 0.28628917 |
| ***miR-322-5p*** | -1.65453 | -0.72642 | 0.34209411 |
| ***miR-125b-5p*** | 1.25578 | 0.32858 | 0.34269414 |
| ***miR-25-3p*** | -1.67472 | -0.74392 | 0.3522066 |
| ***miR-758-3p*** | 2.00497 | 1.00358 | 0.3547427 |
| ***miR-151-3p*** | 1.79553 | 0.84441 | 0.35839148 |
| ***miR-145a-5p*** | -1.37451 | -0.45892 | 0.36404507 |
| ***miR-29c-3p*** | -1.69516 | -0.76142 | 0.36902513 |
| ***miR-18a-5p*** | 1.5029 | 0.58775 | 0.37683357 |
| ***miR-365-3p*** | -1.73078 | -0.79142 | 0.38376366 |
| ***rno-miR-143-3p*** | 1.2471 | 0.31858 | 0.39545821 |
| ***miR-221-3p*** | -1.67472 | -0.74392 | 0.42023894 |
| ***miR-130a-3p*** | -1.76408 | -0.81892 | 0.42625515 |
| ***miR-30a-5p*** | -1.59725 | -0.67559 | 0.43380765 |
| ***miR-199a-3p*** | 1.12201 | 0.16608 | 0.44655439 |
| ***miR-20a-5p*** | -1.51721 | -0.60142 | 0.45028195 |
| ***miR-350-3p*** | 1.50899 | 0.59358 | 0.45165893 |
| ***miR-30c-5p*** | -1.12227 | -0.16642 | 0.46202313 |
| ***miR-451a*** | -1.35794 | -0.44142 | 0.47235919 |
| ***miR-3107-5p*** | -1.43288 | -0.51892 | 0.47427613 |
| ***miR-1a-3p*** | 1.45254 | 0.53858 | 0.49636082 |
| ***miR-423-5p*** | -1.44953 | -0.53559 | 0.51552718 |
| ***miR-149-5p*** | 1.48564 | 0.57108 | 0.51608069 |
| ***miR-223-3p*** | -1.18421 | -0.24392 | 0.53643725 |
| ***miR-148b-3p*** | -1.41806 | -0.50392 | 0.56598704 |
| ***miR-205-5p*** | -1.26043 | -0.33392 | 0.58835016 |
| ***miR-19b-3p*** | -1.68637 | -0.75392 | 0.59233825 |
| ***miR-139-5p*** | -1.21961 | -0.28642 | 0.60974373 |
| ***miR-16-5p*** | -1.20909 | -0.27392 | 0.61293538 |
| ***miR-361-5p*** | -1.14189 | -0.19142 | 0.62891038 |
| ***miR-425-5p*** | 1.27331 | 0.34858 | 0.6325603 |
| ***miR-150-5p*** | -1.19038 | -0.25142 | 0.65225899 |
| ***miR-195a-5p*** | 1.10082 | 0.13858 | 0.65445048 |
| ***miR-143-3p*** | -1.11452 | -0.15642 | 0.6787174 |
| ***miR-126a-3p*** | -1.12812 | -0.17392 | 0.67885501 |
| ***miR-423-3p*** | 1.16763 | 0.22358 | 0.70258654 |
| ***miR-34a-5p*** | 1.26232 | 0.33608 | 0.7040076 |
| ***rno-miR-223-3p*** | -1.10299 | -0.14142 | 0.71452209 |
| ***miR-93-5p*** | -1.07097 | -0.09892 | 0.74565538 |
| ***miR-99a-5p*** | 1.15956 | 0.21358 | 0.74896529 |
| ***let-7i-5p*** | 1.09892 | 0.13608 | 0.74990102 |
| ***miR-210-3p*** | 1.11619 | 0.15858 | 0.76257545 |
| ***miR-10b-5p*** | 1.12071 | 0.16441 | 0.79625444 |
| ***miR-15b-5p*** | 1.06887 | 0.09608 | 0.80492377 |
| ***miR-301a-3p*** | 1.13635 | 0.18441 | 0.80622759 |
| ***miR-676-3p*** | -1.10938 | -0.14975 | 0.81499887 |
| ***miR-342-3p*** | -1.09727 | -0.13392 | 0.83841979 |

| | | | |
|---|---|---|---|
| ***miR-30d-5p*** | -1.09727 | -0.13392 | 0.84095652 |
| ***let-7c-5p*** | 1.06517 | 0.09108 | 0.85913002 |
| ***miR-26a-5p*** | 1.09322 | 0.12858 | 0.8906536 |
| ***miR-152-3p*** | -1.04833 | -0.06809 | 0.92117586 |
| ***miR-31-5p*** | -1.02557 | -0.03642 | 0.96562229 |
| ***miR-132-3p*** | -1.01613 | -0.02309 | 0.97352208 |
| ***miR-215-5p*** | -1.01145 | -0.01642 | 0.97519844 |
| ***miR-203-3p*** | -1.0062 | -0.00892 | 0.98026715 |
| ***miR-133b-3p*** | -1.0097 | -0.01392 | 0.98405818 |
| ***miR-133a-3p*** | -1.0132 | -0.01892 | 0.98480783 |
| ***let-7b-5p*** | 1.00597 | 0.00858 | 0.98714822 |
| ***miR-339-5p*** | -1.00388 | -0.00559 | > 0.99 |

**Table S3. Candidate pathways and networks.** Main candidate canonical pathways regulated by the 14 miRNAs up-regulated and 10 miRNAs down-regulated in obese mice exosomes and representative molecules identified.

| Up-regulated miRNAs Pathways | p-value | Representative Molecules |
|---|---|---|
| **Activation of BH3 only proteins** | 1.07 E-05 | *Akt1, Akt3, E2f1, Tfdp2, Ywhag, Ywhaz* |
| **Circadian clock** | 2.62 E-05 | *Ppara, Hif1a, Nr3c1, Sirt1, Cry2, Ccrn4l, Serpine1, Skp1a,* |
| **Transcriptional regulation by TP53** | 2.59 E-04 | *Socs2, Ccne2, Ccng1, Rictor, Csnk2a1, Jun, Ddit4, Pten, Tnrc6b, Cdk9, Trp53inp1, Cdk13, Pmaip1, Tnfrsf10b, E2f1, Bax, Prdm1, Cdc25c, Cdkn1b* |
| **Generic transcription pathway** | 4.08 E-04 | *Ppara, Pparg, Ppargc1b, Pten, Socs2, Ccne2, Rictor, Bbc3, Hdac4, Tfdp2, Arid1a, Ddit4, Nr3c1, Trp53inp1, Max, Tcf7, Polr2l, Smarcd1, Setd1b, Runx1* |
| **Nuclear receptor transcription** | 4.69 E-04 | *Ppara, Pparg, Nr2c1, Pgr* |

| Down-regulated miRNAs Pathways | p-value | Representative Molecules |
|---|---|---|
| **RUNX3 regulates YAP1-mediated transcription** | 3.3 E-04 | *Yap1, Wwtr1* |
| **Neurexins and neuroligins** | 0.006 | *Grm5, Dlg3, Sharpin, Nrxn1, Lin7a* |
| **Regulation of cortical dendrite branching** | 0.009 | *Robo2* |
| **Transcriptional activation of mitochondrial biogenesis** | 0.013 | *Gabpb2, Tgs1, Nrf1* |
| **Activation of RAC1** | 0.017 | *Sos1, Pak2* |

**Table S4. Candidate pathways and networks.** Main candidate canonical pathways, biological networks and genes targeted in eWAT and liver. Target sites for the selected miRNAs in mRNAs of the *Ppar* family members, as described in the scientific literature.

| **Ingenuity Canonical Pathways** | **p-value** | **Molecules** |
|---|---|---|
| **PXR/RXR Activation** | 3.38 E-06 | *Scd, Cpt1a, Prkar2a, Foxo1, Foxo3* |
| **TCA Cycle II** | 5.37 E-04 | *Sdha, Cs, Idh3g, Aco2, Idh3a, Fh, Ogdh* |
| **PPAR Signaling** | 4.37 E-03 | *Ppard, Med1, Sos2, Ep300, Fos, Il1rn, Insr* |
| **IGF-1 Signaling** | 3-24 E-03 | *Igfbp2, Fos, Prkci, Igf1, Foxo1, Foxo3, Fgfbp1* |

| **Top Networks** | **p-value** |
|---|---|
| **Lipid Metabolism** | 2.08E-02- 1.85E-03 |
| **Molecular Transport** | 2.08E-02- 1.85E-03 |
| **Small Molecule Biochemistry** | 2.08E-02- 1.85E-03 |

| **Gene** | ***miR-122-5p*** | ***miR-192-5p*** | ***miR-27a/b-3p*** | **Refs** |
|---|---|---|---|---|
| *Ppara* | | | 5'TAGACAAAGACAGG ATGAGCCCT3' | (1) |
| *Ppard* | 5'AGCCUACUCACAAC ACUCC3' | | | (2) |
| *Pparg* | | 5'CGGAGACAGACATG AGTCTT3' | 5'CAGGAAAGTCCCAC CCGCTGACAA3' 5'TAAGAAATTTACTGT GAA3' | (1,3) |

**References**


1. Sun L, Trajkovski M (2014) *MiR-27* orchestrates the transcriptional regulation of brown adipogenesis. Metabolism 63(2):272–282.
2. Gatfield D *et al.* (2009) Integration of microRNA *miR-122* in hepatic circadian gene expression. Genes Dev 23(11):1313–1326.
3. Wang J *et al.* (2012) Cardiomyocyte overexpression of *miR-27b* induces cardiac hypertrophy and dysfunction in mice. Cell Res 22(3):516–527.

**Table S5. Mimics and siRNAs.** References and sequences of the miRNA mimics (*Exiqon*) and siRNA duplexes (*Applied Biosystems*) used throughout the study.

| miRNA mimic | Ref. (*Exiqon*) | Batch number |
|---|---|---|
| **miR-122-5p LNA mimic** | 470430-001 | 623673 |
| **miR-192-5p LNA mimic** | 471355-001 | 623676 |
| **miR-27a-3p LNA mimic** | 471338-001 | 623675 |
| **miR-27b-3p LNA mimic** | 470553-001 | 623674 |
| **cel-miR-39-3p LNA mimic** | 479902-001 | 623677 |

| siRNA | Ref. (*ABI*) | Sense strand | Antisense strand |
|---|---|---|---|
| **siC** | AM463-6 | UAAGGCUAUGAAGAGAUACUU | AAGUAUCUCUUCAUAGCCUUA |
| **siPPARA** | S72003 | GGAAAGUCCCUUAUCUGAA | UUCAGAUAAGGGACUUUCC |

**Table S6. Real time RT-PCR primers.** Sequences of the SYBRGreen primers used throughout the study are shown. When commercial primers were used, the specific reference (*Exiqon*) is presented.

| Gene | Forward | Reverse |
|---|---|---|
| ***Actb*** | TCAGCAAGCAGGAGTACGATG | AACGCAGCTCAGTAACAGTCC |
| ***Ccl2*** | CTGGAGCATCCACGTGTTGG | TGGTGAATGAGTAGCAGCAGG |
| ***Cd36*** | TTGTGGCCTTGCACTCTCTC | AACCATCCACCAGTTGCTCC |
| ***Cpt1a*** | ACACCATCCACGCCATACTG | GCAGAGCAGAGGGGAATTGT |
| ***Fads1*** | CGGGTCATCAGCCACTACG | GACCCTTGTTGATGTGGAATGC |
| ***Fgf21*** | AATCCTGGGTGTCAAAGCCTC | AGGCCTCAGGATCAAAGTGAG |
| ***Hprt*** | TGCCGAGGATTTGGAAAAAGTG | TGGCCTCCCATCTCCTTCAT |
| ***Lipe*** | CTTCCAGTTCACACCTGCCA | CGTTGCGTTTGTAGTGCTCC |
| ***Plin2*** | GTCCCTCAGCTCTCCTGTTA | CCACTCTCATCACCACGCT |
| ***Pnpla2*** | AACGCCACTCACATCTACGG | AATGTTGGCACCTGCTTCAC |
| ***Ppara*** | TTGTGGCTGGTCAAGTTCGG | GCTCTCTGTGTCCACCATGT |
| ***Ppard*** | CTTCCACTACGGGGTCCAC | TCGAGCTTCATGCGGATTGT |
| ***Pparg2*** | CTGCCTATGAGCACTTCACAA | ATGCGAGTGGTCTTCCATCA |
| ***Tnf*** | GATCGGTCCCCAAAGGGATG | GGCTACAGGCTTGTCACTCG |

| miRNA | Ref. (*Exiqon*) |
|---|---|
| ***cel-miR-39-3p*** | 203952 |
| ***miR-192-5p*** | 204099 |
| ***miR-122-5p*** | 205664 |
| ***miR-27a-3p*** | 206038 |
| ***RNU5G*** | 203908 |