Maternal obesity increases hypothalamic miR-505-5p expression in mouse offspring leading to altered fatty acid sensing and increased intake of high-fat food

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The authors have declared that no conflict of interest exists

Abstract

In utero exposure to maternal obesity programs increased obesity risk. Animal models show that programmed offspring obesity is preceded by hyperphagia, but the mechanisms that mediate these changes are unknown. Using a mouse model of maternal obesity, we observed increased intake of a high-fat diet in offspring of obese mothers that precedes the development of obesity. Through small RNA sequencing, we identified programmed overexpression of hypothalamic miR-505-5p that is established in the fetus, lasts to adulthood and is maintained in hypothalamic neural progenitor cells cultured *in vitro*. Metabolic hormones and long-chain fatty acids associated with obesity increase miR-505-5p expression in hypothalamic neurons *in vitro*. We demonstrate that targets of miR-505-5p are enriched in fatty acid metabolism pathways and over-expression of miR-505-5p decreased neuronal fatty acid metabolism *in vitro*. miR-505-5p targets are associated with increased BMI in human genetic studies. Intra-cerebroventricular injection of miR-505-5p in wild-type mice increased high-fat diet intake, mimicking the phenotype observed in offspring exposed to maternal obesity. Conversely, maternal exercise intervention in an obese mouse pregnancy rescued the programmed increase of hypothalamic miR-505-5p in offspring of obese dams and reduced high-fat diet intake to control offspring levels. This study identifies a novel mechanism by which maternal obesity programs obesity in offspring via increased intake of high-fat foods.

45 **1. Introduction**

46 The rapid global rise in obesity represents a significant health challenge. A genetic influence
47 on body weight is undisputed, but the recent rapid increase in obesity prevalence suggests a
48 robust interplay between genetic factors and the environment (1). It is now well established
49 that an individual's long-term health can be influenced by *in utero* and early post-natal
50 environmental conditions, giving rise to the concept of "developmental programming" (2).
51 Numerous studies in humans and animal models have highlighted the association between
52 maternal obesity and several long-term adverse health outcomes in the offspring, including
53 the risk of obesity and type 2 diabetes (3). Growing evidence from animal models shows
54 that exposure to maternal obesity can disrupt the hypothalamic circuits responsible for
55 maintaining energy homeostasis, which may contribute to increased offspring susceptibility
56 to obesity later in life (4).
57
58 The hypothalamus is the crucial region of the brain responsible for maintaining energy
59 homeostasis in the body and contains specialised neurons that sense circulating nutrients
60 and hormones. Exposure to over-nutrition during critical windows of development has been
61 shown to program increased food intake in animal models (4), including increased
62 preference for high- fat foods in rats and non-human primates (5-7). Studies in mouse
63 models report that maternal obesity alters hypothalamic neuronal projections (8-10), but
64 aside from this little is known about the molecular mechanisms by which maternal
65 overnutrition programs permanent changes in feeding behaviour in offspring.
66
67 The stable nature of metabolic phenotypes in offspring exposed to maternal obesity during
68 development suggests lasting changes to gene profiles across the lifespan. There is
69 accumulating evidence that miRNAs, which are small non-coding RNAs that regulate gene
70 function through degradation of mRNAs and/or inhibition of protein translation (11), may
71 reflect an important mechanism by which the maternal environment can alter long-term
72 phenotypes in the offspring (3, 12). We have reported previously that programmed cell-
73 autonomous up-regulation of miR-126 in adipose tissue of offspring of obese mouse dams is
74 responsible for the downregulation of the genes *Irs1* and *Lnpk*, contributing to insulin
75 resistance and endoplasmic reticulum stress, respectively, in these animals (13, 14). Few
76 studies to date have explored the role of miRNAs in regulating hypothalamic energy-sensing

77 (15-19), or examined the potential contribution of miRNAs to programming of long-term
78 hypothalamic function by the perinatal environment (20).
79
80 A growing body of research suggests that neurons within the hypothalamus can sense and
81 respond to changes in fatty acid (FA) levels in the circulation (21-24), as reported previously
82 for glucose-sensing neurons (25). Circulating FAs, particularly long-chain fatty acids, may act
83 as cellular messengers, which inform sub-populations of neurons in the hypothalamus about
84 whole body nutrient status, allowing the regulation of food intake, hepatic glucose
85 production and insulin secretion in order to maintain energy homeostasis (22, 26, 27).
86 Studies in rodents and humans have shown that oleic and palmitic acid reduce the
87 expression of neuropeptide Y (NPY) and increase expression of pro-opiomelanocortin
88 (POMC)(28-30), and are associated with a reduction in food intake. Furthermore, both oleic
89 (31) and octanoic acid (32) can reduce food intake and increase energy expenditure via
90 regulation of POMC neuron excitability, confirming that several FAs can act centrally to
91 regulate food intake. Notably, some studies have suggested that neuronal responses to FAs
92 are blunted in diet- induced obese animals (33, 34), and this blunting of neuronal FA sensing
93 may contribute to unbalanced energy homeostasis. Whether the nutritional environment in
94 early life impacts long-term neuronal FA sensing- as has been reported for glucose sensing
95 (35, 36)- remains unclear. However, one study has suggested that maternal HFD
96 consumption during pregnancy alters the sensitivity of offspring ventromedial hypothalamic
97 neurons to oleic acid (35).
98
99 Therefore, this study aimed to explore the contribution of hypothalamic miRNAs in
100 mediating the effects of maternal obesity on offspring obesity risk. We profiled miRNA
101 expression in the offspring hypothalamus to identify miRNAs that could mediate increased
102 high-fat diet intake and adopted a combination of *in vitro* and *in vivo* approaches to
103 demonstrate the causal effects of altered levels of a specific miRNA on hypothalamic
104 neuronal function and feeding behaviour.
105

106 ## 2. Methods

107 ### 2.1. Mouse model

108 We utilised a mouse model of maternal diet-induced obesity published previously by our
109 group (37). Briefly, female C57BL/6J mice were randomly assigned prior to mating to either
110 a control chow diet (RM1; Special Dietary Services, Witham UK) or a highly palatable
111 obesogenic high-fat diet (45% total calories from fat, Special Dietary Services, Witham UK)
112 supplemented with condensed milk (Nestle, UK) fortified with mineral mix AIN93G (Special
113 Dietary Services, Witham UK). After approximately three weeks on the respective diets,
114 females were mated with chow-fed males for their first pregnancy. Dams remained on their
115 respective diets throughout pregnancy and lactation and the first litter was culled after
116 weaning. After first pregnancy, proven breeders from the obese group when they had
117 achieved a body composition of around 30–35% fat and body weight exceeding 35g were
118 mated for second pregnancy along with age matched lean dams. A subset of dams was
119 culled on embryonic day 13 (E13) to collect fetal hypothalamus and remaining pregnancies
120 were allowed to deliver naturally. On post-natal day 2 litters were reduced to 6 pups to
121 ensure standardized milk supply (38). One male and female mouse per litter was included in
122 each separate experiment, therefore the statistical number relates to the dam. For diet-
123 induced obesity experiments in adult male mice in Figure 2k, mice were fed *ad-libitum* chow
124 diet (DS-105, Safe diets) or HFD (60% total calories from fat, D12492, Research diets) for 21
125 weeks.
126

127 ### 2.2. Food intake and body composition analyses in offspring

128 At weaning, male and female mice from control or obese mothers were randomly assigned
129 to either a control chow (as above) or obesogenic diet (as above), creating 4 experimental
130 offspring groups: offspring born to control mothers weaned onto a chow diet (Off-C Chow),
131 offspring born to control mothers weaned onto a HFD (Off-C HFD), offspring born to obese
132 mothers weaned onto a chow diet (Off-Ob Chow), offspring born to obese mothers weaned
133 onto a HFD (Off-Ob HFD) (illustrated in Figure 1A). *Ad libitum* food intake in the home cage
134 was measured twice a week from the age of 3 weeks to 8 weeks. Animals were weighed
135 weekly, and body composition determined in fed animals using Time-Domain Nuclear
136 Magnetic Resonance imaging (TD-NMR; Minispec Plus, Bruker, Billerica, MA, USA).

137 For two-bottle choice tests for sucrose mice were single housed at six weeks of age. At eight
138 weeks of age, baseline measurements of body weight, chow intake and water intake were
139 measured daily for three days. There were no differences in baseline measurements. A
140 sucrose preference test was conducted across a range of sucrose solutions -0.5%, 1%, 2%,
141 4%, 8%, 16% and 32% [w/v] in water. Mice were habituated to the set-up for three days,
142 with one bottle containing 0.5% sucrose ([w/v] in drinking water) and the other water,
143 during which body weight, chow intake, and the weight of the water and sucrose bottles
144 were measured daily at 1300. Bottles were weighed before they were placed in the top of
145 the cages. After 24 hours, the bottles were carefully removed and weighed again. The side
146 order of the bottles was switched daily to avoid side preference.
147

148 **2.1. Exercise intervention in dams**

149 From one week prior to mating for the second pregnancy, a group of obese dams
150 underwent an exercise intervention protocol that we have published previously (39). Briefly,
151 dams were trained to run 20 minutes daily on a treadmill, starting at 5 m/min on the first
152 day and with daily incremental increases in speed until they were running at a final speed of
153 12.5 m/min on day 5. Dams were then rested for 2 days during the weekend when they
154 were time-mated and day 1 of pregnancy was defined by the appearance of a copulatory
155 plug. Exercise was then maintained at the final running speed and following a routine of 5
156 days of daily exercise (weekdays) followed by 2 days of rest (weekends), until day 17. The
157 duration of running at the top speed of 12.5 m/min was reduced in the final 3 days to adapt
158 to the physical constraints of pregnancy. At weaning, half of the offspring were weaned
159 onto an obesogenic HFD (as above- to assess the impact of the intervention on feeding
160 behaviour) and half were weaned onto a chow diet (to examine hypothalamic gene
161 expression independent of the effects of an HFD).
162

163 **2.2. Small RNA sequencing in the offspring hypothalamus**

164 Small RNA sequencing was conducted in 8-week-old male offspring of control and obese
165 mothers fed a chow diet (Off-C Chow and Off-Ob Chow groups), to avoid confounding
166 factors of HFD consumption on hypothalamic gene expression. After culling by cervical
167 dislocation, brains were immediately frozen and stored at -80°C until sectioning at 12 µm on

168 a cryostat (Leica Biosystems, Wetzlar, DE). PVH and ARC tissue was collected from brain
169 sections using laser capture microdissection with a PALM Robot Microbean (Carl-Zeiss,
170 Gottingen, DE), and RNA extracted as described below. Library preparation and small RNA
171 sequencing of the PVH and ARC samples was performed as described previously (40) using a
172 TruSeq® Small RNA Library Preparation kit (Illumina, Cambridge, UK). Libraries were
173 sequenced using a HiSeq 4,000 platform. Raw reads were mapped to the mouse genome
174 through bowtie v1.2.3. Reads per Kilobase of transcript per Million mapped reads were
175 produced from RNA Sequencing raw output and statistically analysed through likelihood
176 ratio test using edgeR version 3.36.0. A P value cut off of <0.05 was used to determine
177 significantly regulated miRNAs. Sequencing data has been deposited on the GEO data
178 repository.
179

180 **2.3. Cell culture and *in vitro* treatments**

181 For neural progenitor cell culture, fetal hypothalamus (embryonic day 13) was rapidly
182 dissected in ice cold buffer and neural progenitor cells were harvested by centrifugation
183 (according to (41)) and cultured in Neurocult™ proliferation media (Stem Cell Technologies,
184 Cambridge UK) containing recombinant human EGF according to manufacturer's
185 instructions. Cells were cultured for 8 days *in vitro* before extraction of RNA as described
186 below. mHypoE-N46 cells (generously donated by Dr. Clemence Blouet) were cultured in
187 growth medium (High glucose Dulbecco's modified Eagle Medium supplemented with 5%
188 fetal bovine serum, 1% Penicillin-Streptomycin, 2% L-glutamine) and treated for 24h with
189 glucose (20 mM), oleic acid (250 μM), linoleic acid (250 μM), palmitic acid (250 μM) or
190 stearic acid (250 μM) (all from Sigma- Aldrich, Saint Louis, Missouri, USA). Mouse brain
191 astrocyte cell line (C8-D1A, CRL-2541, ATCC ®, Manassas, VA USA) were cultured in ATCC-
192 formulated Dulbecco's Modified Eagle's Medium, (Catalog No. 30-2002) supplemented with
193 10% fetal bovine serum and treated for 24h with glucose (20 mM), oleic acid (250 μM),
194 palmitic acid (250 μM) or stearic acid (250 μM). After respective treatments, RNA was
195 extracted as described below. To confirm regulation by miR-505-5p of predicted miR
196 targets, mHypoE-N46 cells were transfected with a miR-505-5p mimic (miRCURY LNA miRNA
197 mimic, Qiagen) or negative control, using Lipofectamine-3000 transfection kit (Invitrogen,

198 Waltham, Massachusetts, USA). After 48h of transfection, RNA was extracted for gene
199 expression analyses.
200

201 **2.4. Pulsed stable isotope labelling by amino acids in cell culture (pSILAC)**

202 pSILAC proteomic methodology was performed as described previously (13). Briefly, GT1-7
203 cells were cultured in regular DMEM media containing light lysine (Lys 0) in two cell culture
204 dishes for 24h and then transfected with miR-505-5p mimic or negative control. After 8h,
205 normal growth medium from both cell populations was replaced with SILAC media
206 containing either heavy lysine [L-lysine-$^{13}C_6$-$^{15}N_2$ (Lys8)] or medium-heavy lysine [L-Lysine-
207 $^{2}H_4$ (Lys4)] for the miR-505-5p mimic cells and miRNA negative control cells, respectively.
208 After 24h, protein extracts from both cell populations were combined and subjected to SDS-
209 PAGE separation, digested with trypsin and submitted to high performance liquid
210 chromatography tandem mass spectrometry (HPLC/MS/MS). Raw signal data were
211 processed in Maxquant 1.5.2.8 using a Uniprot Mus musculus database (downloaded on 29
212 Jan 2016, 24,802 sequences). Results were merged with predicted targets for miR-505-5p
213 obtained from Target Scan (http://www.targetscan.org/vert_72/). After bioinformatic
214 analysis, differentially expressed transcripts were validated by qPCR analysis. Pathway
215 enrichment analysis was performed in Ingenuity® Pathway Analysis (Qiagen). pSILAC raw
216 data is available in Supplementary data file 2.
217

218 **2.5. RNA extraction and gene expression analysis**

219 All RNA extraction from tissue and cultured cells was performed using the miRNeasy Micro
220 Kit (Qiagen) to isolate total RNA including small RNAs. cDNA was reverse transcribed using a
221 RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) or miRCURY LNA RT Kit (Qiagen
222 Sciences) for analysis of mRNA or miRNA respectively. Gene expression was analysed using a
223 StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA) and either Power SYBR
224 Green PCR Master Mix or Taqman Universal PCR Master Mix (both Thermo Fisher Scientific,
225 USA). Relative quantification of mRNA and miRNA was calculated by the $2^{-\Delta\Delta Ct}$ method. Data
226 were normalized to the geometric average of housekeeping genes *Snord68* and *Unisp6*
227 when miRNA was analysed or to *Sdha* when target genes were analysed (the expression of
228 housekeepers did not change between groups) and values were reported as fold changes

229 compared to values obtained from the control group (set at 1.0). Primers for SYBR Green-
230 based PCR of mRNA were obtained from Sigma Aldrich, miRCURY LNA assays for qPCR of
231 miRNA were purchased from Qiagen, and TaqMan probes were obtained from
232 ThermoFisher Scientific. All primer sequences and assay IDs are listed in Supplementary
233 table 1.
234

235 **2.6. Fatty acid uptake**

236 mHypoE-N46 cells were cultured in a 24 well plate and transfected with miR-505-5p mimic
237 or negative control as described above. After 48h, cells were treated with 1 µM [1-^{14}C]-oleic
238 acid (PerkinElmer) diluted in high glucose DMEM containing 0.05% BSA for 1h. Cells used for
239 protein quantification were incubated in uptake medium not containing the radionuclide.
240 After the incubation, media from wells were removed and transferred to 4 ml of scintillation
241 fluid. Cells were carefully washed three times in 1 ml ice cold 0.1% fatty acid free BSA in
242 PBS. After washing, cells were scraped into 200 µl RIPA buffer, transferred to
243 microcentrifuge tubes and centrifuged at 16,000 g for 10 min. 100 µl of lysate was added to
244 4 ml of scintillation fluid and a beta scintillation counter used to measure concentration of
245 radionuclide. The fatty acid uptake rate was calculated using the concentration of
246 radionuclide, incubation time and total protein amount (and expressed as pmol/min/mg).
247

248 **2.7. Fatty acid metabolism**

249 A fatty acid oxidation protocol was adapted from Pantaleao *et al.* (40). Briefly, mHypoE-N46
250 cells were cultured in a 24 well cell culture dish and transfected with miR-505-5p mimic or
251 negative control in triplicate. After 48h of transfection, cells were washed with warm PBS
252 and incubated in 500 µl of High-glucose DMEM containing 12.5 mM HEPES, 0.3% fatty acid-
253 free BSA, 1 mM L-carnitine, 100 µM oleic acid and 0.4 µCi/ml [1-^{14}C]-oleic acid. 200 µl of
254 concentrated hydrochloric acid was injected into the wells, and the acid solution was left to
255 incubate for an extra hour. The acid solution was then centrifuged at maximum speed (≥
256 14,000 × g) for 10 min at 4 °C and 500 µl supernatant transferred to a scintillation vial for
257 radioactivity measurement of acid soluble metabolites from fatty acid metabolism (Acetyl-
258 CoA, acyl-carnitines etc).
259

260 **2.8. Characterisation of miR-505-5p targets in human datasets**

261 miR-505-5p fatty acid metabolism related target genes were mapped to orthologous genes
262 in the human genome using ENSEMBL and integrated with common variant genome-wide
263 association study (GWAS) data for body mass index (BMI), from the GIANT BMI study (42).
264 Each of the miR-505-5p target genes was annotated based on proximity to genome-wide
265 significant signals ($p<5x10^{-8}$), in 1Mb windows; 500kb up- or downstream of the gene's start
266 or end site. For genes with proximal GWAS signals, we calculated genomic windows of high
267 linkage disequilibrium (LD; $R^2>0.8$) for each given signal and mapped these to the locations
268 of known enhancers for the target genes, using the activity-by-contact (ABC) enhancer maps
269 (43). We also performed a gene-level Multi-marker Analysis of GenoMic Annotation
270 (MAGMA) analysis (44), which collapses all observed genomic variants within a given gene
271 and calculates aggregate gene-level associations to the phenotypic trait. Genes exhibiting an
272 FDR-corrected MAGMA p-value <0.05 were considered significant. Finally, for genes in loci
273 containing at least a suggestive level of GWAS significance ($p<5x10^{-5}$), we performed
274 summary-data-based Mendelian randomization (SMR) and heterogeneity in dependent
275 instruments (HEIDI) tests(v1.02; (45)) using blood expression quantitative trait loci (eQTL)
276 data from the eQTLGen study (46). For the eQTL analyses, we considered gene expression to
277 be associated with BMI if the FDR-corrected p-value for the SMR-test was p<0.05 and the p-
278 value for the HEIDI test was >0.001.
279

280 **2.9. Intra-cerebroventricular (ICV) injection of miR-505-5p mimic**

281 Adult C57Bl/6J male mice were implanted with an intra-cerebroventricular (ICV) canula
282 (Plastics One, Inc.) in the lateral ventricle (coordinates from Bregma: anteroposterior -0.4;
283 mid-lateral -1.0 and dorsoventral -2.3 mm). One week prior to the ICV surgery, mice were
284 exposed to a high fat diet (60% fat) for familiarization and for basal food intake
285 measurement. After 5 days of recovery from surgery, mice received a 0.5µM dose of a miR-
286 505-5p mimic (Qiagen) or negative control combined with HiPerfect transfection reagent
287 (Qiagen) into the lateral ventricle. The ICV treatment was repeated once every 24h for 3
288 days through the cannula and *ad lib* food intake was measured once every 24h (as shown in
289 Figure 5a).
290

291 **2.10. Statistics**

292 Data were analysed using Prism 8 (Graphpad, La Jolla, USA) by different tests according to
293 the numbers of groups and time points: unpaired or paired t-test (as specified) for
294 experiments with two treatment groups, one-way ANOVA for experiments with 3 or more
295 groups at one time point, mixed effects analysis for experiments with 2 or more groups over
296 time. Tukey or Sidaks multiple comparisons test were performed after mixed effects analysis
297 (as specified in figure legends). Outliers were detected by Grubb's method. For pSILAC,
298 statistical analysis of normalized labelled: unlabelled protein ratios was performed using
299 multiple linear models with Bayesian correction using limma package (v.2.16.0) for R
300 (v.4.1.2). All data are shown as mean ± SEM. In each case n refers to the number of
301 different litters represented.
302

303 **2.11. Study approval**

304 The animal studies performed in this manuscript were licensed by the UK Home Office
305 under project license P5FDF0206 and approved by the University of Cambridge Animal
306 Welfare and Ethical Review Body and by the Ethics Committee of Animal Welfare from
307 Instituto de Ciencias Biomedicas- Universidade de Sao Paulo (protocol number 72/2017).
308
309

310 **3. Results**

311

312 **3.1. Male offspring born to obese mothers show increased intake of a high-fat diet and**

313 **subsequent increased adiposity**

314 Maternal diet-induced obesity during pregnancy has been suggested to program increased
315 food intake and subsequently adiposity in the offspring. We recorded food intake, body
316 weight and adiposity of male offspring born to obese and control mothers, who were
317 weaned onto either a normal chow or an obesogenic high fat diet (HFD) from weaning until
318 8 weeks of age (Figure 1a). Male offspring of obese mothers (Off-Ob chow) did not consume
319 more calories during *ad lib* feeding over this period when weaned onto a chow diet
320 compared to the offspring of lean mothers fed a chow diet (Off-C chow) (Figure 1b).
321 However, when weaned onto an HFD, offspring born to obese mothers (Off-Ob HFD)
322 showed significantly higher daily caloric intake from 6 weeks of age compared to offspring
323 of lean mothers weaned onto the same HFD (Off-C HFD) (Figure 1c). In a two- bottle choice
324 test for sucrose performed in male mice, all mice preferred sucrose solution to water (i.e., a
325 preference > 50%) and both sucrose preference and absolute intake of sucrose increased
326 with increasing sucrose concentration (Figure 1d and 1e). There was no significant effect of
327 perinatal exposure to maternal obesity on absolute sucrose preference (Figure 1d) or
328 sucrose intake (Figure 1e) in offspring.

329

330 There was no effect of perinatal exposure to maternal obesity on offspring body weight over
331 the period studied (i.e. up to 8 weeks of age), regardless of whether offspring were weaned
332 onto a chow diet (Figure 1f) or a HFD (Figure 1h). Longitudinal adiposity measurements
333 showed increased adiposity in all animals weaned onto a HFD compared to chow fed

334 animals; but this increase in adiposity was greater in offspring of obese mothers (Figure 1i).
335 We have previously shown that energy expenditure is unchanged in male mice exposed to
336 maternal obesity at 8 weeks of age (14) so the increased intake of a HFD is the likely cause
337 of increased adiposity.
338

339 **3.2. Maternal obesity programs increased expression of miR-505-5p in offspring**
340 **hypothalamus from fetal to adult life**

341 In order to investigate the effect of exposure to maternal obesity on miRNA expression
342 profile in the offspring hypothalamus, we analysed the miRNA profile of two key nuclei, the
343 paraventricular nucleus (PVH) and arcuate nucleus (ARC) of the hypothalamus, in young
344 adult chow-fed male mice born to obese mothers or lean control mothers. Small RNA
345 sequencing analysis highlighted 4 miRNA that were dysregulated in the PVH (Suppl. Table 2)
346 and 14 in the ARC (Suppl. Table 3) of offspring exposed to maternal obesity. The miRNA
347 miR-505-5p, which is conserved between mice and humans (Figure 2a) showed a striking
348 increased expression in both the ARC (Figure 2b) and PVH (Figure 2c) of offspring born to
349 obese mothers in comparison to offspring of control mothers. The results of the small RNA
350 sequencing analysis were validated by qPCR for miR-505-5p in a separate cohort of young
351 adult male mice (Fig 2d). In control animals, miR-505-5p was expressed at a higher level in
352 the hypothalamus than two other regions of the brain we examined (cortex: 1.00± 0.06,
353 hippocampus: 1.64 ± 0.14, hypothalamus: 2.66± 0.19 (A.U. normalised to cortex)). In
354 contrast to findings in the hypothalamus, exposure to maternal obesity did not result in
355 altered expression of miR-505-5p in either the cortex (OffC: 1.00 ± 0.06, OffOb: 1.01 ± 0.10
356 (A.U. normalised to OffC cortex)) or hippocampus (OffC: 1.64 ± 0.14, OffOb: 1.41 ± 0.36
357 (A.U. normalised to OffC cortex)), confirming that exposure to maternal obesity increases
358 expression of miR-505-5p specifically in the hypothalamus.
359

360 In order to ascertain how early in life the over-expression of miR-505-5p is established, we
361 analysed miR-505-5p expression in fetal hypothalamic samples and found a significant
362 upregulation of the miRNA in E13 fetuses exposed to maternal obesity (Figure 2e). To

363 determine if this overexpression is maintained outside the intrauterine environment, which
364 would suggest a cell-autonomous programmed effect, we analysed hypothalamic NPC from
365 E13 offspring and found overexpression of miR-505-5p in NPC after 8 days grown *in vitro*
366 (Figure 2f). miR-505-5p sits in the gene *Atp11c*, a type IV P-type ATPase. The expression of
367 *Atp11c* was also increased (though to a lesser extent than the miRNA) in the hypothalamus
368 of fetuses exposed to maternal obesity, showing that miR-505-5p is at least in part
369 regulated along with the host gene (Figure 2g). These data show that miR-505-5p
370 overexpression is permanently programmed in the hypothalamus following perinatal
371 exposure to maternal obesity and is a potential candidate to mediate the long-term effects
372 of maternal overnutrition on offspring hypothalamic function.
373

374 **3.3. Insulin and specific fatty acids increase the expression of miR-505-5p in a**
375 **hypothalamic neuronal cell line *in vitro***

376 We next sought to uncover which factors related to maternal obesity could be responsible
377 for the increase in miR-505-5p in hypothalamus. We have previously reported an increase in
378 circulating levels of insulin and leptin in the serum of diet-induced obese dams in our mouse
379 model (37). Application of insulin, but not leptin, to a hypothalamic neuronal cell line
380 (mHypoE-N46) caused an increase in expression of miR-505-5p (Fig 2h). We have previously
381 conducted mass spectrometry analysis of the HFD pellet to establish the primary FA
382 components of the obese dam diet, and found the FAs in greatest abundance were palmitic,
383 stearic, oleic and linoleic acid (47). We therefore sought to identify whether any of these
384 FAs can alter the expression of miR-505-5p in mHypoE-N46 cells. The expression of miR-505-
385 5p was increased in neurons after oleic and stearic acid treatment (Figure 2i). Neither
386 palmitic nor linoleic acid treatment caused overexpression of miR-505-5p in neurons (Figure
387 2i). As the obesogenic diet fed to obese dams is supplemented with condensed milk which is
388 high in sucrose, we also tested to see whether glucose could regulate expression of miR-
389 505-5p *in vitro*. Glucose did not alter the expression of miR-505-5p in hypothalamic cells *in*
390 *vitro* (Figure 2i). As astrocytes are also reported to be involved in hypothalamic FA sensing,
391 we also tested whether miR-505-5p was expressed in C8-D1A cells and if so whether the
392 expression was regulated by components of the obesogenic diet used in our model.
393 Although miR-505-5p is expressed in astrocytes, it was not regulated by FAs in the same

394 manner we observed in neurons (Fig 2j). In fact, we observed a down-regulation of miR-505-
395 5p expression in response to palmitic acid in astrocytes (Fig 2j).
396
397 In addition to regulation by nutrients and hormones known to be altered in pregnancies
398 complicated by obesity, we also examined whether miR-505-5p levels are modulated *in vivo*
399 by long-term obesity. We used two separate obesity models to delineate the effects of
400 obesity *per se* from consumption of a high- calorie diet: diet- induced obese animals and
401 leptin deficient *Ob/Ob* animals (fed a chow diet). The expression of miR-505-5p in the
402 hypothalamus was increased in male mice with diet-induced obesity (Figure 2k) and there
403 was a trend towards increase in mice with genetic obesity (analysed by one way ANOVA).
404 This shows that hypothalamic miR-505-5p is regulated by current obesity in adult animals
405 (independent of an HFD), as well as by factors associated with maternal obesity.
406
407 **3.4. miR-505-5p targets identified by pSILAC are involved in FA uptake and metabolism**
408 miRNAs are known to regulate gene function through degradation of mRNAs and/or via
409 inhibition of protein translation. We performed a proteomic analysis to identify targets of
410 miR-505-5p by pSILAC following transfection of a miR-505-5p mimic in a hypothalamic cell
411 line, GT1-7 (Figure 3a). Cumulative fraction analysis showed that proteins encoded from
412 mRNAs containing binding sites for miR-505-5p according to TargetScan (v. 7.2) were
413 downregulated by the miR-505-5p mimic, showing that the pSILAC was effective in
414 identifying proteins regulated as a consequence of over-expression of miR-505-5p (Figure
415 3b). pSILAC data was overlaid with predicted targets containing the miR-505-5p seed
416 sequence identified by TargetScan and miRbase to identify potential direct targets (Figure
417 3c).
418
419 Ingenuity Pathway Analysis (IPA) revealed that miR-505-5p targets are enriched for
420 processes related to fatty acid metabolism, followed by oxidative stress and mitochondrial
421 processes (Figure 3d). After conducting a manual search in addition to the IPA, we identified
422 30 proteins related to FA metabolism pathways that were significantly dysregulated in the
423 pSILAC experiment. Twenty-one of these FA related proteins were downregulated after miR-
424 505-5p over-expression (Figure 3e), suggesting an overall reduction in FA metabolism

425 processes in the cells. We next investigated the functional consequences of over-expression
426 of miR-505-5p in a hypothalamic cell line on FA oxidation and FA uptake processes. We used
427 radio-labelled oleic acid to show that oleic acid metabolism into acid-soluble metabolites
428 (Figure 3f) and oleic acid uptake (Figure 3f) were decreased in hypothalamic neurons after
429 miR-505-5p over-expression. As FA uptake and metabolism to Acetyl-CoA and TCA cycle
430 intermediates are thought to be key steps through which neurons are able to sense
431 circulating FA levels, these results suggest that increased miR-505-5p *in vivo*- through an
432 impact on hypothalamic fatty acid uptake and oxidation- could alter the subsequent ability
433 of the neurons to sense circulating LCFA levels.
434

435 **3.5. miR-505-5p and its targets are associated with BMI in humans**

436 A recent study of 3000 adults has identified circulating miR-505-5p levels as being one of
437 only a few miRNA positively associated with BMI in humans (48). To further understand the
438 contribution of miR-505-5p targets to human metabolic health, we mined phenotypic and
439 genetic data from human population studies by assessing data from a common variant
440 GWAS of BMI (N=806,834)(42). Of the 30 human orthologues to the FA metabolism related
441 targets of miR-505-5p, 12 genes were proximal to common variant genome-wide significant
442 signals; when variants were analysed at the gene-level using MAGMA, 7 genes were
443 significantly associated with BMI (Suppl. Table 4). As most common variant GWAS signals
444 are intronic or intergenic, we overlayed these associations with other datasets to
445 understand whether the associated variants can be functionally linked to our target genes
446 (Figure 3g). Genomic variants within 6 of the BMI association signals are within regions of
447 known enhancers(43). We also observed colocalization between these signals and gene
448 expression levels for 3 target genes.
449

450 Across these analyses, our human genetic analyses had the greatest support for CPT1A,
451 HINT1, HSD17B12 and SLC27A4, which had the greatest number of variant to gene
452 predictors (Figure 3g). For CPT1A, the BMI GWAS colocalised with CPT1A eQTLs (FDR-
453 corrected P SMR=5.94×10^{-5} and P HEIDI=0.002, Suppl. Table 4), while signals proximal to
454 HINT1, HSD17B12 and SLC27A4 all overlapped with known enhancers for these genes.

455 Collectively, these observations suggest that several of the novel miR-505-5p target genes
456 we identified are functionally associated with increased BMI in humans.
457
458 **3.6. miR-505-5p overexpression *in vitro,* as well as *in utero* exposure to maternal**
459 **obesity, causes downregulation of genes related to FA sensing in hypothalamic cells.**

460 As described above, a manual search combined with the IPA identified 30 genes linked to FA
461 metabolism (Figure 3e). Among the genes related to FA metabolism that were
462 downregulated by miR-505-5p were genes related to fatty acid transport (*Slc25a10* and
463 *Slc27a4* (that encodes the protein FATP4)) and fatty acid oxidation (*Acot9*, *Agpat3*, *Cpt1a*
464 and *Soat1*). To confirm the downregulation of predicted miR-505-5p targets relevant to FA
465 metabolism, we measured expression of these genes following transfection of a miR-505-5p
466 mimic in the mHypoE-N46 hypothalamic cell line. miR-505-5p mimic treatment decreased
467 the mRNA expression of *Acot9*, *Agpat3*, *Cpt1a*, *Slc25a10, Slc27a4* and *Soat1* (Figure 4a),
468 compared to cells treated with a vehicle control.
469
470 Decreased mRNA expression of the genes *Acot9*, *Agpat3*, *Cpt1a*, *Slc25a10* and *Slc27a4* was
471 also observed in the fetal hypothalamus at E13 of offspring from obese mothers, compared
472 to the offspring of lean mothers (Figure 4b). This is consistent with our observed increase in
473 hypothalamic miR-505-5p levels at E13 (Figure 2e). These data show that both over
474 expression of miR-505-5p in a hypothalamic cell line mHypoE-N46 *in vitro*, and exposure to
475 maternal obesity *in vivo* - which we have shown programs increased miR505-5p in the
476 hypothalamus - downregulate the expression of genes related to FA metabolism.
477
478 **3.7. Intra-cerebroventricular injection of miR-505-5p increases high-fat- but not chow-**
479 **pellet intake**

480 Our results demonstrate that targets of miR-505-5p in the hypothalamus include genes
481 involved in neuronal FA sensing, and that manipulation of miR-505-5p levels reduces rates
482 of neuronal FA uptake and metabolism. Previous studies have suggested that neuronal FA
483 uptake and metabolism are key processes that allow neurons to sense circulating FA levels
484 and initiate appropriate adjustments to parameters including food intake (22). We therefore
485 hypothesised that over-expression of miR-505-5p would alter the ability of hypothalamic

486 neurons to sense circulating FA levels and result in a change in food intake. To verify
487 whether miR-505-5p has a direct effect *in vivo* in the brain in FA sensing - and therefore
488 food intake - we performed ICV administration of a miR-505-5p mimic or a negative control
489 and measured *ad lib* intake of high- fat diet or chow diet for 3 consecutive days following
490 surgery (Figure 5a). Increased miR-505-5p in the hypothalamus after ICV injection of the
491 miR-505-5p mimic was confirmed on post-mortem dissection (Figure 5b). Mice that received
492 the miR-505-5p mimic ICV had increased high-fat diet intake compared to mice that
493 received a negative control treatment (Figure 5c). The increase in food intake caused by ICV
494 miR-505-5p was exclusively seen when animals were given the high fat diet; ICV miR-505-5p
495 did not increase food intake when animals were fed a chow diet (Figure 5d).
496
497 **3.8. Maternal exercise intervention in an obese pregnancy rescues the programmed**
498 **over-expression of hypothalamic miR-505-5p and normalises high- fat diet intake in**
499 **the offspring**
500
501 We have previously shown that a moderate exercise intervention in obese dams during
502 pregnancy rescues maternal insulin sensitivity- independent of changes in adiposity (39)-
503 and is associated with a rescue of the cardiac dysfunction observed in offspring exposed to
504 maternal obesity (49) (Figure 5e). We therefore sought to investigate whether the
505 correction of maternal metabolic health was sufficient to rescue the programmed over-
506 expression of hypothalamic miR-505-5p offspring and changes in offspring feeding
507 behaviour. Offspring of obese exercised dams (Off-FEx) show a reduced expression of miR-
508 505-5p in the ARC compared to Off-Ob to a level comparable to that observed in controls
509 (Figure 5f). As we have shown that over-expression of miR-505-5p in the hypothalamus
510 causes increased intake of a high- fat diet, we would expect Off-FEx to show a reduced
511 intake of HFD compared to Off-Ob. Indeed, offspring of obese- exercised dams with
512 normalised expression of miR-505-5p show significantly reduced intake of a HFD from 7
513 weeks of age, despite their exposure to perinatal obesity (Figure 5g). At this age there is no
514 difference in body weight between the three groups of offspring (OffC 25.65g $\pm$ 0.67, OffOb
515 26.66g $\pm$ 0.32, OffFEx 25.67g $\pm$0.63). Therefore effects of the maternal exercise intervention
516 are not related to changes in offspring body weight.

517

518 **4. Discussion**

519

520 In the current study, we have used a rodent model to examine the effects of exposure to
521 maternal obesity on offspring food intake, small RNA sequencing in the hypothalamus to
522 identify programmed miRNAs and a series of *in vitro* and *in vivo* experiments- including
523 human genetic analysis- to establish how miRNA may causally influence feeding behaviour.
524 Our results showed that maternal obesity alters the miRNA profile in the ARC and PVH and
525 identified miR-505-5p as a miRNA that is programmed in the fetal hypothalamus with
526 changed expression maintained through to adulthood. We have shown that targets of miR-
527 505-5p are related to FA uptake and metabolism, that some of these targets are associated
528 with increased BMI in humans, and that manipulation of miR-505-5p levels in hypothalamic
529 neurons *in vitro* alters these processes. Importantly, we have shown that ICV injection of a
530 miR-505-5p mimic increases intake specifically of an HFD, and an exercise intervention in
531 obese dams rescues programmed miR-505-5p levels and reduces HFD intake. Altered
532 expression of hypothalamic miR-505-5p *in utero* is therefore a novel route by which
533 perinatal exposure to maternal obesity programs permanent changes to offspring feeding
534 behaviour, leading to obesity.

535

536 The increased intake of an HFD in the offspring of obese mothers precedes the increased
537 adiposity observed in these animals and given that we have previously reported no
538 differences in energy expenditure in mouse offspring exposed to maternal obesity, suggests
539 a causal relationship. Indeed, many studies have now linked changes in food intake to the
540 increased susceptibility to develop obesity in animals exposed to obesity in the perinatal
541 period, and suggested that altered hypothalamic development and/or function plays a key
542 role in this process (4). To our knowledge, we are the first to conduct small RNA sequencing
543 to identify the full spectrum of miRNAs that are dysregulated in the ARC and PVH following
544 exposure to maternal obesity. A role for hypothalamic miRNAs in maintaining energy
545 homeostasis is largely unexplored, but deletion of the miRNA processing enzyme DICER in
546 POMC neurons results in obesity (50), and hypothalamic miR-29a and miR-103 have been
547 shown to protect against obesity (18, 51). Although a few studies have demonstrated
548 altered hypothalamic miRNA expression in mice in response to nutritional status or leptin

549 administration (16, 52, 53), whether exposure to altered nutritional status *in utero*
550 programs hypothalamic miRNA profile was previously unknown. Our small RNA sequencing
551 analysis identified miR-505-5p as being highly up-regulated in both the ARC and PVH of male
552 adult offspring after exposure to maternal obesity when offspring themselves were fed a
553 chow diet and at a time when there was no difference in offspring body weight. We
554 subsequently demonstrated that increased expression of miR-505-5p is detectable as early
555 as E13 in the fetal hypothalamus. Notably, a recent study found miR-505-5p to be one of
556 four miRNAs associated with several markers of cardiometabolic phenotypes in humans
557 (48). The increased expression of miR-505-5p was maintained *in vitro* in hypothalamic
558 neural progenitor cells after one week, indicating this is a programmed effect in the cell,
559 rather than an immediate effect of the intra-uterine environment on gene expression.
560 Interestingly, the miR-505-5p host gene Atp11c, which encodes a Type IV P-type ATPase, a
561 flippase that is involved in the transport of phospholipids, is also increased in the
562 hypothalamus of fetuses of obese mothers. It is intriguing that this apparent nutrient-
563 sensitive miRNA sits within another gene linked to cellular energy levels and that this gene is
564 altered by exposure to maternal obesity. This study does not address the expression or
565 regulation of miR-505-5p in female offspring exposed to maternal obesity and this is an
566 important avenue for future work. Circulating levels of miR-505-5p have been reported to
567 be lower in human females (54), but the Framingham study that reported an association
568 between circulating miR-505-5p and cardiometabolic phenotypes (48) was conducted in a
569 mixed sex group, suggesting miR-505-5p may also play a role in metabolism in females.
570
571 The fact that hypothalamic miR-505-5p was up-regulated in both diet- induced obese
572 animals and leptin deficient *Ob/Ob* obese animals shows that consumption of a HFD is not
573 required for miR-505-5p over-expression. This suggests it is a factor related to the maternal
574 obesity *per se* (such as a circulating hormone) that programs over-expression, rather than a
575 metabolite of the HFD consumed by the dam that passes directly to the fetus. Furthermore,
576 this indicates that direct regulation of this miRNA by obesity in adulthood may further
577 compound the ability of an individual with obesity to regulate their food intake. We show
578 that miR-505-5p is increased in response to insulin, oleic and stearic acid in a hypothalamic
579 cell line *in vitro*. We have shown that fatty acids and insulin are increased in the serum of
580 dams fed an obesogenic diet near the end of pregnancy (14), and insulin is also high in

581 fetuses of a human obese pregnancy as well as in our mouse model. Regulation of miR-505-
582 5p by glucose (another metabolic signal known to be high in fetuses exposed to maternal
583 obesity) was ruled out by our *in vitro* data. Interestingly, the human Framingham study that
584 identified miR-505-5p as positively associated with cardiometabolic parameters found no
585 correlation with circulating glucose, and the strongest association was with circulating
586 triglycerides (48) supporting our *in vitro* data. The maternal exercise intervention normalises
587 dam insulin sensitivity, but not maternal body weight, adiposity or leptin levels (39), and this
588 was sufficient to rescue the programmed over-expression of hypothalamic miR-505-5p in
589 offspring exposed to maternal obesity and prevented programmed increased in intake of a
590 high fat diet. Our results therefore suggest that increased circulating free- fatty acids or
591 insulin levels may be key mechanisms by which maternal obesity programs changes to the
592 developing offspring hypothalamus.
593
594 As offspring remained in the cage with the dam (who was consuming the obesogenic diet
595 throughout both the gestation and weaning period), we cannot discount the possibility that
596 some of the effects we see on offspring phenotype are due to the juvenile offspring eating
597 the HFD pellets themselves in the latter part of the weaning period. However, it should be
598 noted that exercise intervention during the pregnancy period alone prevented the
599 programmed increase miR-505-5p expression as well as increased food intake. This
600 therefore suggests that targeting the pregnancy period alone is sufficient to prevent at least
601 some of the programmed phenotypes. This issue could be further explored in the future
602 with a cross-fostering experiment where offspring of obese dams could be raised by lean
603 dams consuming a chow diet.
604
605 Unlike the well characterised populations of hypothalamic glucose sensing neurons (25), the
606 presence, location and molecular signature of FA sensing neurons in the hypothalamus is
607 not well defined. FAs are able to cross the blood brain barrier (55, 56), and the presence of
608 FA transporters such as FATP1, FATP4 and FAT/CD36, as well as genes required for
609 metabolism of FA (27) support the theory that at least some hypothalamic neuronal
610 populations are FA-sensing. A recent study has shown that knockdown of Free Fatty Acid
611 Receptor-1 in POMC neurons promotes hyperphagia and body weight gain (57). Therefore,
612 it is very likely that FA sensing is a mechanism by which the hypothalamus responds to

613 nutrient status changes, particularly that of an energy surfeit (58). Current hypotheses
614 suggest neuronal cells use the rate of uptake and/or metabolism of FAs to sense circulating
615 levels, (although there may also be direct regulation of some neurons via
616 electrophysiological effects (31, 59) as is the case for glucose-sensing neurons). For this
617 reason, it is relevant that we have shown that miR-505-5p targets include both FA
618 transporters (FATP4) and key components of FA oxidation pathways (such as CPT1A, which
619 has recently been shown to be reduced in offspring in a different model of maternal HFD
620 consumption (60)), and that over-expression of miR-505-5p in a hypothalamic cell line
621 disrupted FA uptake and metabolism. Therefore, altered expression of miR-505-5p caused
622 by *in utero* exposure to high FA levels has the potential to permanently disrupt
623 hypothalamic FA sensing mechanisms. This is supported by the observation that CNS
624 administration of a miR-505-5p mimic results in increased intake of a high-fat diet (but not a
625 chow diet), and that reduced levels of miR-505-5p in offspring of obese exercised dams
626 results in reduced intake of a high-fat diet. Thus, our results support the hypothesis that if
627 neurons cannot sense circulating FA levels due to altered FA uptake and metabolism
628 pathways, animals cannot maintain energy homeostasis, resulting in increased consumption
629 of foods high in fat. This is also supported by a previous study showing that maternal HFD
630 consumption during pregnancy reduced the sensitivity of offspring ventromedial
631 hypothalamic neurons to oleic acid (35).
632
633 Although we have highlighted only six critical genes in our experiments, the pSILAC analysis
634 identified many other targets of miR-505-5p that may contribute to a nutrient sensing
635 phenotype in the offspring of obese mothers. Among these are the SRCAP chromatin
636 remodelling complex, which activates a transcriptional profile that shifts metabolism from
637 anaerobic glycolysis to oxidative metabolism (61). Interestingly, the pSILAC revealed that
638 many indirect targets of miR-505-5p (i.e. downregulated in pSILAC but do not contain miR-
639 505-5p seed sequence) are related to FA sensing, and many of these also showed genetic
640 associations with BMI in humans. Whilst we have focused on validating a few key direct
641 target genes in the current study, it is unlikely that miR-505-regulates a complex process
642 such as nutrient sensing via only one key target gene, and therefore it is likely that miR-505-
643 5p controls the expression of several master regulators (e.g. transcription factors or
644 chromatin remodelling complexes such as SRCAP) that modulate whole profiles of FA

645 sensing genes. Notably, many of the proteins we found to be altered following over-
646 expression of miR-505-5p are expressed in mitochondria, so future studies should aim to
647 examine the effects of maternal obesity on offspring mitochondrial dynamics.
648
649 In conclusion, we have identified miR-505-5p as a novel regulator of FA metabolism
650 pathways that is programmed in mice exposed to maternal obesity and could contribute to
651 the increased HFD intake and adiposity seen in the offspring of obese mothers. This is
652 consistent with previous animal studies showing a role for hypothalamic FA sensing in
653 modulating food intake, and is corroborated by our human genetic data analysis. This study
654 is one of the first reports defining a molecular route by which exposure to an altered
655 nutritional environment *in utero* is able to alter feeding behaviour. Further defining the
656 mechanisms underlying the programming of miR-505-5p by maternal obesity may hold
657 promise for developing novel interventional strategies to prevent the adverse impacts of
658 maternal obesity on offspring long-term health.

5. Authors contributions

LD and SEO conceived the project. ICF, LD, and SEO designed the experiments and interpreted the results. LD, ICF, LP, LWPW, DFT, JF, MC, and GB performed experiments. JD, AVP, LD and SEO supervised experiments. KAK, KKO and JRBP carried out the genetic analysis. LD wrote the manuscript. All authors reviewed and commented on the final version of the manuscript.

6. Acknowledgments

We would like to thank Tom Ashmore, Claire Custance and Laura Hunter for assistance with animal husbandry, the Genomics and Transcriptomics Core for assistance with RNA-sequencing, and Dr. Robin Antrobus for assistance with mass spectrometry. Illustrative figures were generated using BioRender. This research was conducted using the UK Biobank Resource under application 9905.

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846

847

848 Figure Legends

849

850 Fig 1. Offspring of obese mothers eat more of a high- fat pellet and develop increased
851 adiposity compared to offspring of lean mothers fed the same diet. a) Diagram depicting
852 mouse model of maternal obesity. At weaning offspring are weaned onto either a chow or
853 HFD, and food intake, body weight and adiposity are measured weekly. b) Normal chow
854 daily kilocalorie intake of male offspring born to control (Off-C; n=21) and obese mothers
855 (Off-Ob; n=15). c) High fat diet (HFD) pellet daily kilocalorie intake of male offspring born to
856 control (Off-C; n=22) and obese mother (Off-Ob; n=19). d) Sucrose preference of male

857 offspring during a two-bottle choice test (Off-C n=6, Off-Ob n=4). e) Intake (g) of sucrose
858 solution during two bottle choice test (Off-C n=6, Off-Ob n=4). f) Body weight and (g) fat
859 mass from 4 to 8 weeks of age in offspring weaned onto a chow diet (Off-C n=12, Off-Ob
860 n=21). h) Body weight and (i) fat mass from 4 to 8 weeks of age in offspring weaned onto a
861 high-fat diet (Off-C n=12 Off-Ob n=13). **P<0.01, ***P<0.001 Statistical significance was
862 determined using mixed effects analysis with Sidak's post-hoc test. The underlying data are
863 provided as a Source Data file
864
865 Figure 2. Hypothalamic miR-505-5p expression is increased in offspring of obese mothers
866 from fetal to adult life, and *in vitro* in neurons by exposure to metabolic hormones and fatty
867 acids. a) Sequence overlap of mature miR-505-5p in mice and humans. b & c) Volcano plot
868 of small RNA sequencing results showing significant overexpression of miR-505-5p (red dot
869 surrounded by blue diamond) in the ARC (b) and PVH (c) of 8-week-old offspring born to
870 obese mothers compared to offspring of lean mothers. d-f) qPCR analysis of miR-505-5p
871 expression in (d) ARC at 8 weeks of age; (e) in whole hypothalamus at E13; (f) in
872 hypothalamic neural progenitor cells extracted at E13 and cultivated as neurospheres for 8
873 days *in vitro*. g) *Atp11c* (miR-505-5p host gene) mRNA levels in E13 whole hypothalamus. h)
874 miR-505-5p relative expression of mHypoE-N46 cells exposed to insulin or leptin. i) miR-505-
875 5p relative expression of mHypoE-N46 cells exposed to oleic acid, palmitic acid, stearic acid,
876 linoleic acid and glucose for 24h. j) miR-505-5p relative expression of C8-D1A (astrocyte)
877 cells exposed to oleic acid, palmitic acid, stearic acid and glucose for 24h. k) miR-505-5p
878 relative expression in mediobasal hypothalamus (MBH) in lean, diet-induced obese or
879 genetically obese (Ob/Ob) male mice.* P<0.05, **P<0.01. Statistical significance was
880 determined with unpaired t-test. Outliers were excluded from (g) for obese group (outlier
881 excluded value 0.878) and j for oleic acid treatment (outlier excluded value 1.554). The
882 underlying data are provided as a Source Data file
883
884
885 Figure 3. miR-505-5p targets are involved in fatty acid metabolism and genetically
886 associated with BMI in humans. a) Diagram of pulsed SILAC experiment in GT1-7 cells to
887 identify targets of miR-505-5p b) Cumulative fraction analysis of lys8-labelled: lys4-labelled
888 proteins ratio according to occurrence and type of mRNA binding site for miR-505-5p, as

889 predicted by TargetScan (v. 7.2) c) Workflow used to identify direct targets of miR-505-5p.
890 d) Top regulated pathways among miR-505-50 direct targets as reported in Ingenuity
891 Pathway Analysis (IPA). e) miR-505-5p target proteins of interest identified through IPA and
892 manual search of pSILAC data. f) Radiolabelled oleic acid metabolism into acid-soluble
893 metabolites and oleic acid uptake in mHypoE-N46 following transfection with either a miR-
894 505-5p mimic or control for 48h. g) Heatmap showing the overlap between human
895 orthologs of identified miR-505-5p fatty acid metabolism related target genes in mice and
896 human genetic datasets. Target genes were annotated on the basis of (i) proximity to GWAS
897 signals, (ii) aggregate gene-level associations to the trait, (iii) colocalization between the
898 GWAS and eQTL data, and (iv) the presence of known enhancers within the GWAS signals.
899 Total number of the observed concordant predictors (out of 4) is displayed in the summary
900 panel. Expanded results can be found in Supplementary Table 4. *p<0.05, ** p<0.01, ***
901 p<0.001. Statistical significance was determined by unpaired T-test. The underlying data are
902 provided as a Source Data file
903
904 Figure 4. miR-505-5p target genes related to fatty acid sensing are downregulated after
905 treatment with miR-505-5p *in vitro* and in fetal hypothalamus of offspring from obese
906 mothers. a) Relative mRNA expression of genes related to lipid metabolism in mHypoE-N46
907 cells after treatment with a miR-505-5p mimic and (b) in the hypothalamus of fetuses from
908 control or obese pregnancy at E13 * P<0.05, ** P<0.01, *** P<0.001. Statistical significance
909 was determined by unpaired T-test. Outliers were excluded from (a) for miR-505-5p mimic
910 (Cpt1a outlier excluded value 1.19, Soat1 outlier excluded value 1.71). The underlying data
911 are provided as a Source Data file
912
913 Figure 5. Intra-cerebroventricular injection of miR-505-5p in adult mice increases intake of a
914 high-fat diet, whilst a maternal exercise intervention corrects offspring hypothalamic miR-
915 505-5p levels and reduces intake of a high-fat diet.
916 a) Diagram depicting surgical schedule. Mice were allowed to recover for 5 days post-
917 implanted of an ICV cannula into the lateral ventricle. Mice then received injection of either
918 a vehicle or miR-505-5p mimic into the ICV cannula and food intake was measured over 3
919 days. b) qPCR confirming over-expression of miR-505-5p in the hypothalamus after ICV
920 injection of miR-505-5p mimic. c) Chow diet cumulative food intake after 3 consecutive days

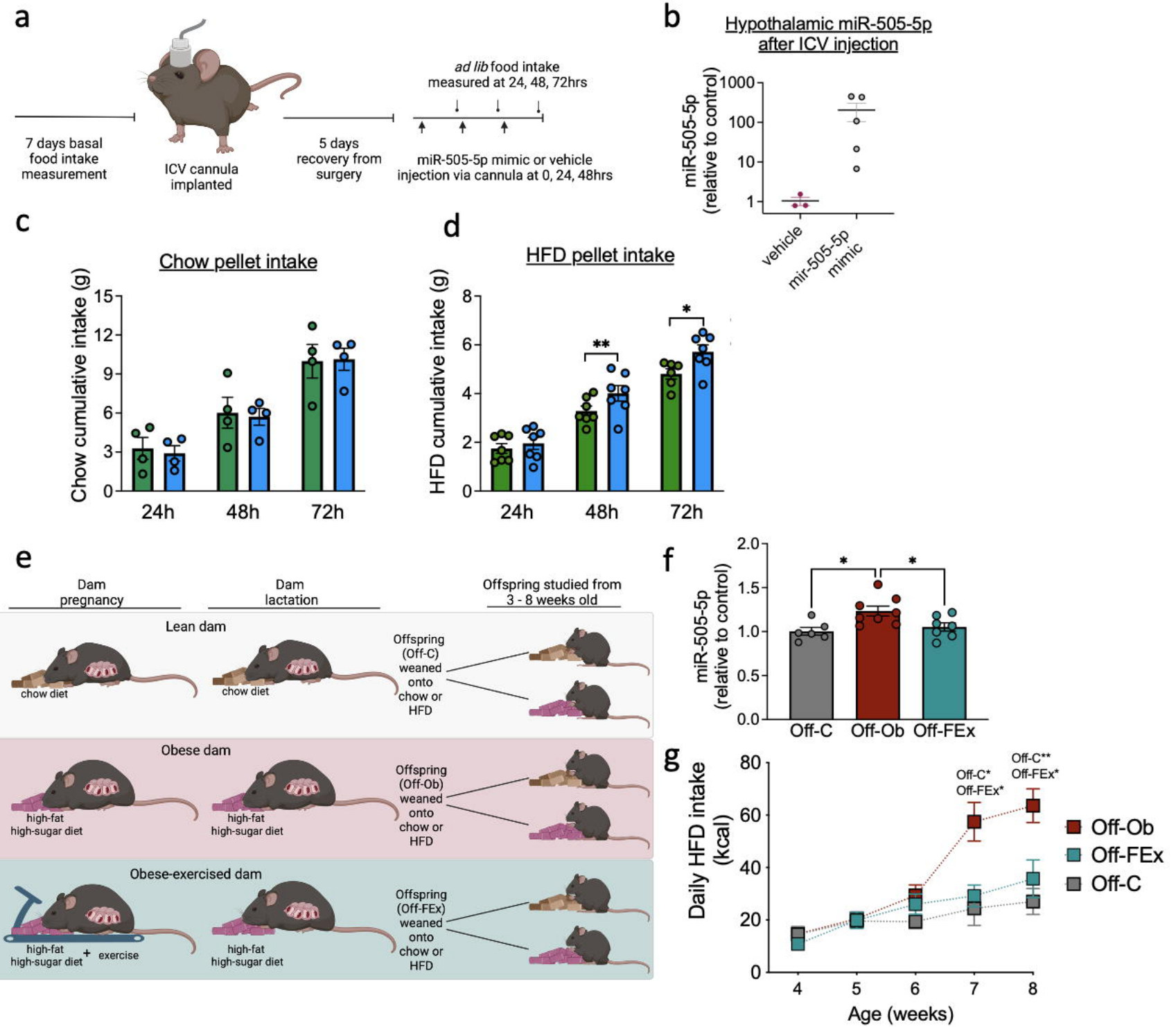
921 of treatment (24- 1 dose, 48- 2 doses and 72 hours- 3 doses- after first treatment). d) High-
922 fat diet cumulative food intake after 3 consecutive days of treatment (24- 1 dose, 48- 2
923 doses and 72 hours- 3 doses- after first treatment). e) Graphical depiction of maternal
924 obesity model with third group of dams that underwent moderate exercise intervention
925 during obese pregnancy. f) relative expression of miR-505-5p in the arcuate nucleus of 8
926 week old male offspring born to control, obese or obese- exercised dams. g) HFD pellet daily
927 kilocalorie intake of male offspring born to control, obese or obese- exercised dams *
928 $p<0.05$, ** $p<0.01$. Statistical significance was determined by unpaired T-test (c & d), one
929 way ANOVA (f), and mixed effects analysis with Sidak post-hoc test (g). The underlying data
930 are provided as a Source Data file.
931
932 Supplementary Material Figure legends
933
934 **Supplementary Table 1:**
935 A) Primer sequences of primers used in SYBR qPCR purchased from Sigma Aldrich B) Taqman
936 Assay IDs of probes used in Taqman qPCR purchased from Thermofisher Scientific C)
937 miRCURY LNA miRNA PCR assays purchased from Qiagen.
938
939 **Supplementary Table 2:**
940 Significantly regulated miRNAs detected in paraventricular nucleus of the hypothalamus of
941 offspring from obese mothers in miR-sequencing analysis.
942
943 **Supplementary Table 3:**
944 Significantly regulated miRNAs detected in arcuate nucleus of the hypothalamus of offspring
945 from obese mothers in miR-sequencing analysis.
946
947 **Supplementary Table 4:** Overlap of miR-505-5p targets with human genetic data on BMI
948 variation and other functional datasets. Gene; gene symbol for human orthologue of mir505
949 target, chr; chromosome where Gene is locates, Summary; summarised count of supporting
950 evidence across the four analyses, MAGMA P-value; p-value from the gene-level MAGMA
951 test, MAGMA FDR-corrected P-value; MAGMA P-value after FDR correction, GWAS Signal;
952 rsid for the GWAS signal proximal to Gene, position; chromosomal position of GWAS signal

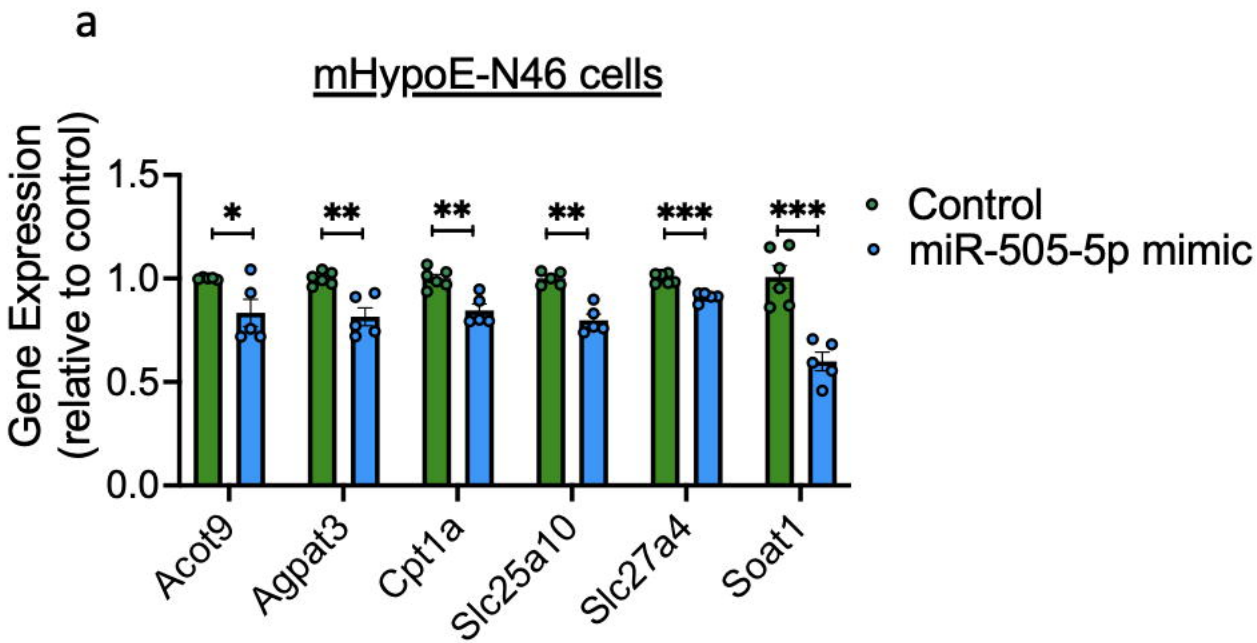
(GRCh 37), a1; effect allele, a0; alternate allele, freq1; observed frequency of a1, beta1; effect size estimate per copy of a1, se; standard error of beta1, p; p-value for the association, Is closest?; is Gene the closest gene to GWAS Signal, N ABC Enhancers; number of known ABC enhancers within the LD window (R-sq>0.8) of the GWAS Signal, P SMR; p-value for the SMR test, FDR-corrected P SMR; P SMR after FDR correction, P HEIDI; p-value for the Heidi test .

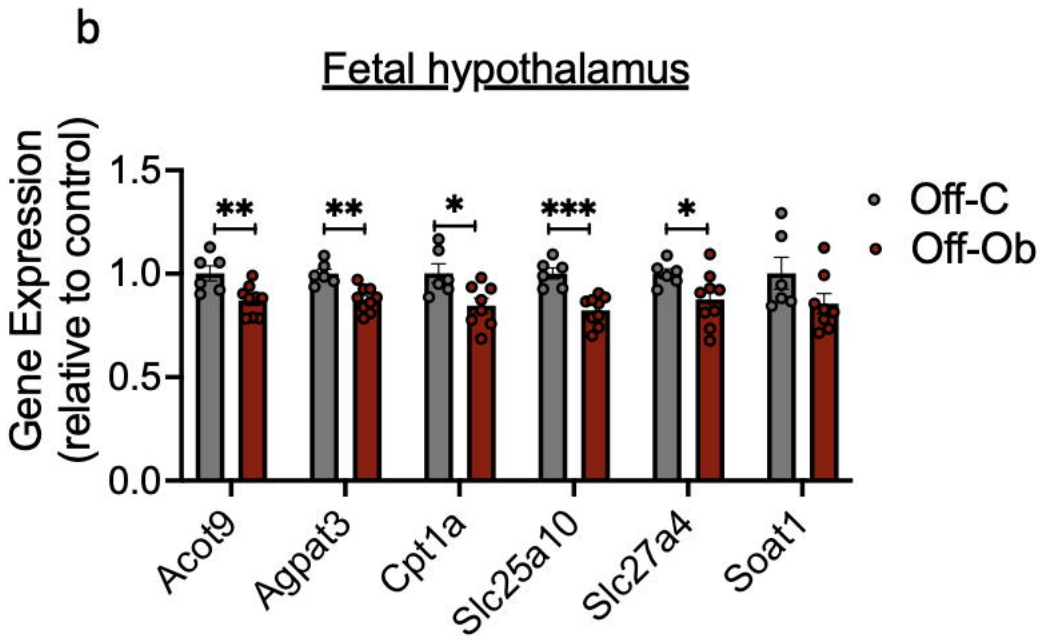
S1 Data: Original data for the graphs in Figure 1-5.
Each tab includes data for the noted graphs in Figure 1-5. plus a tab for data mentioned in the text.

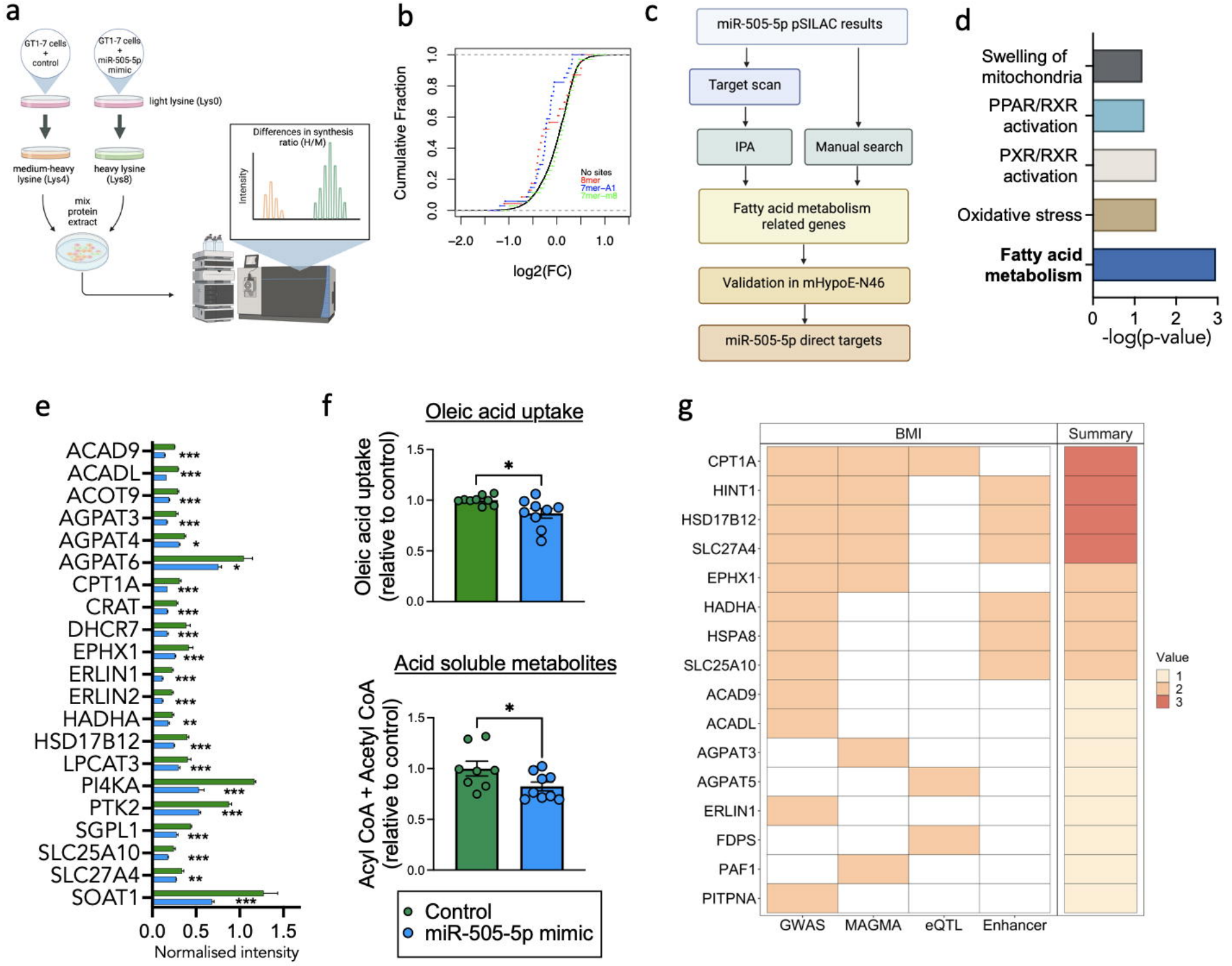
S2 Data: Raw data from p-SILAC experiment performed to identify changes in protein expression following miR-505-5p over-expression in GT1-7 cells.

966


a
7 days basal food intake measurement
ICV cannula implanted
5 days recovery from surgery
ad lib food intake measured at 24, 48, 72hrs
miR-505-5p mimic or vehicle injection via cannula at 0, 24, 48hrs
b
Hypothalamic miR-505-5p after ICV injection
miR-505-5p (relative to control)
1000
100
10
1
vehicle
mir-505-5p mimic
c
Chow pellet intake
Chow cumulative intake (g)
15
12
9
6
3
0
24h
48h
72h
d
HFD pellet intake
HFD cumulative intake (g)
8
6
4
2
0
**
*
24h
48h
72h
e
Dam pregnancy
Dam lactation
Offspring studied from 3 - 8 weeks old
Lean dam
chow diet
chow diet
Offspring (Off-C) weaned onto chow or HFD
Obese dam
high-fat high-sugar diet
high-fat high-sugar diet
Offspring (Off-Ob) weaned onto chow or HFD
Obese-exercised dam
high-fat high-sugar diet + exercise
high-fat high-sugar diet
Offspring (Off-FEx) weaned onto chow or HFD
f
miR-505-5p (relative to control)
2.0
1.5
1.0
0.5
0.0
*
*
Off-C
Off-Ob
Off-FEx
g
Daily HFD intake (kcal)
80
60
40
20
0
Off-C*
Off-FEx*
Off-C**
Off-FEx*
Off-Ob
Off-FEx
Off-C
4
5
6
7
8
Age (weeks)


a
mHypoE-N46 cells
Gene Expression
(relative to control)
1.5
1.0
0.5
0.0
*
**
**
**
***
***
Control
miR-505-5p mimic
Acot9
Agpat3
Cpt1a
Slc25a10
Slc27a4
Soat1

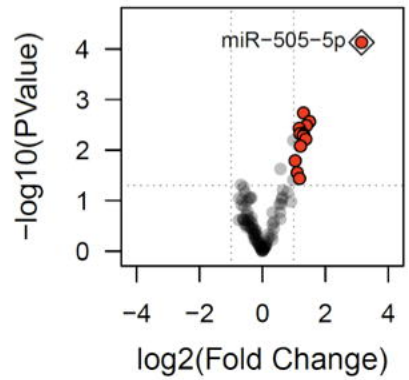

b
Fetal hypothalamus
Gene Expression
(relative to control)
1.5
1.0
0.5
0.0
**
**
*
***
*
Off-C
Off-Ob
Acot9
Agpat3
Cpt1a
Slc25a10
Slc27a4
Soat1


a
GT1-7 cells + control
GT1-7 cells + miR-505-5p mimic
light lysine (Lys0)
medium-heavy lysine (Lys4)
heavy lysine (Lys8)
mix protein extract
Differences in synthesis ratio (H/M)
Intensity
b
Cumulative Fraction
log2(FC)
No sites
8mer
7mer-A1
7mer-m8
c
miR-505-5p pSILAC results
Target scan
IPA
Manual search
Fatty acid metabolism related genes
Validation in mHypoE-N46
miR-505-5p direct targets
d
Swelling of mitochondria
PPAR/RXR activation
PXR/RXR activation
Oxidative stress
Fatty acid metabolism
-log(p-value)
e
ACAD9
ACADL
ACOT9
AGPAT3
AGPAT4
AGPAT6
CPT1A
CRAT
DHCR7
EPHX1
ERLIN1
ERLIN2
HADHA
HSD17B12
LPCAT3
PI4KA
PTK2
SGPL1
SLC25A10
SLC27A4
SOAT1
Normalised intensity
f
Oleic acid uptake
Oleic acid uptake (relative to control)
Acid soluble metabolites
Acyl CoA + Acetyl CoA (relative to control)
Control
miR-505-5p mimic
g
BMI
Summary
CPT1A
HINT1
HSD17B12
SLC27A4
EPHX1
HADHA
HSPA8
SLC25A10
ACAD9
ACADL
AGPAT3
AGPAT5
ERLIN1
FDPS
PAF1
PITPNA
GWAS
MAGMA
eQTL
Enhancer
Value

a

```
>mmu-miR-505-5p   GGGAGCCAGGAAGTATTGATGT   22
                  ||||||||||||||||||||||
>hsa-miR-505-5p   GGGAGCCAGGAAGTATTGATGT   22
```

b



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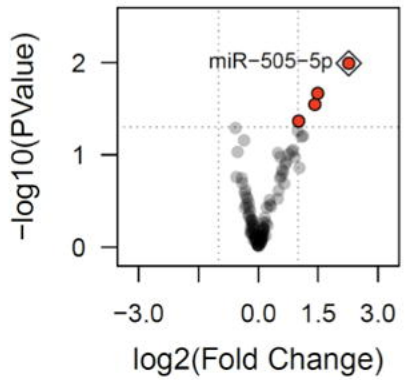

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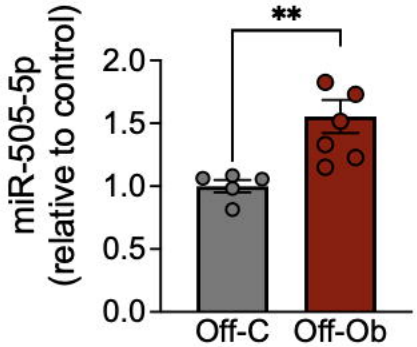

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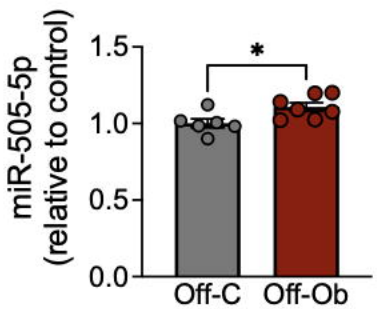

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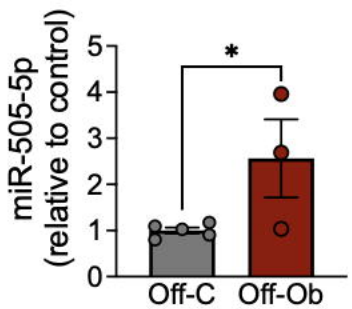

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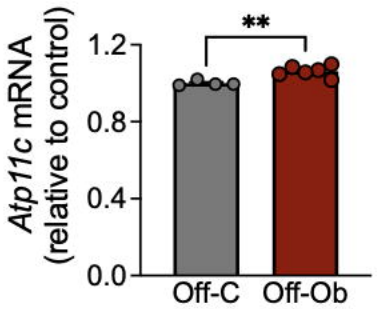

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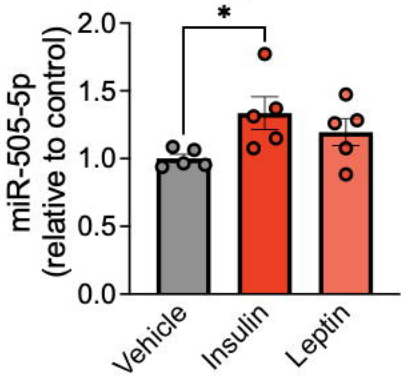

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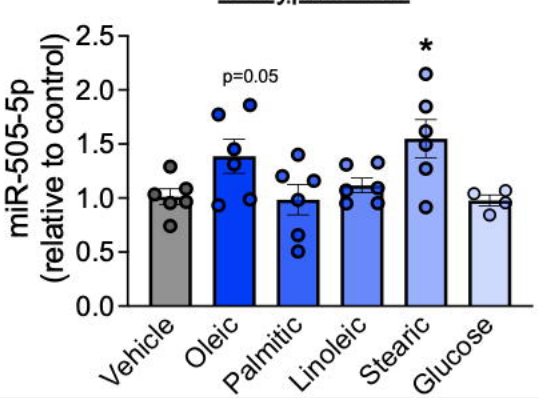

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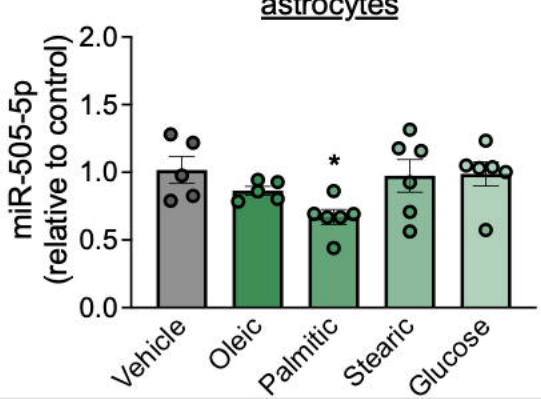

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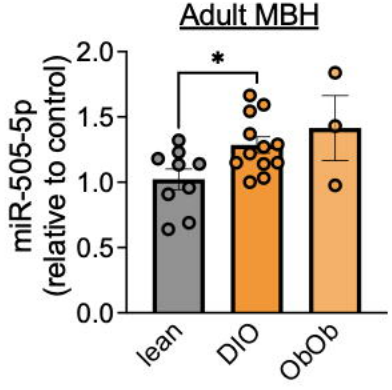

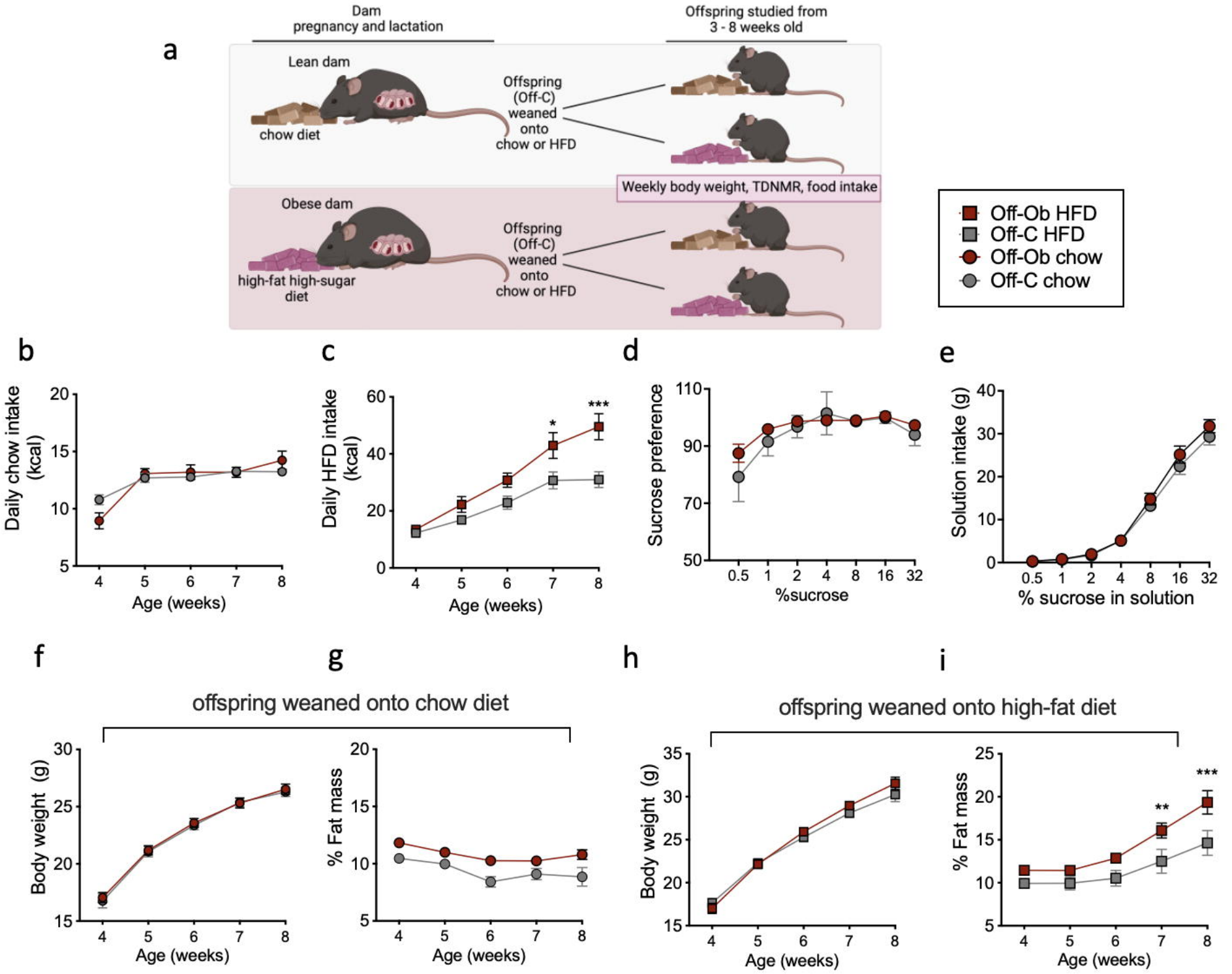
a
Dam
pregnancy and lactation
Offspring studied from
3 - 8 weeks old
Lean dam
chow diet
Offspring
(Off-C)
weaned
onto
chow or HFD
Weekly body weight, TDNMR, food intake
Obese dam
high-fat high-sugar
diet
Offspring
(Off-C)
weaned
onto
chow or HFD
Off-Ob HFD
Off-C HFD
Off-Ob chow
Off-C chow
b
Daily chow intake
(kcal)
Age (weeks)
c
Daily HFD intake
(kcal)
Age (weeks)
d
Sucrose preference
%sucrose
e
Solution intake (g)
% sucrose in solution
f
g
offspring weaned onto chow diet
Body weight (g)
% Fat mass
Age (weeks)
h
i
offspring weaned onto high-fat diet
Body weight (g)
% Fat mass
Age (weeks)