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High-fat diet disrupts metabolism in two generations of rats in a parent-of-origin specific manner

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Experimental and epidemiological evidence demonstrate that ancestral diet might contribute towards offspring health. This suggests that nutrition may be able to modify genetic or epigenetic information carried by germ cells (GCs). To examine if a parental high fat diet (HFD) influences metabolic health in two generations of offspring, GC-eGFP Sprague Dawley rats were weaned onto HFD (45% fat) or Control Diet (CD; 10% fat). At 19 weeks, founders (F0) were bred with controls, establishing the F1 generation. HFD resulted in 9.7% and 14.7% increased weight gain in male and female F0 respectively. F1 offspring of HFD mothers and F1 daughters of HFD-fed fathers had increased weight gain compared to controls. F1 rats were bred with controls at 19 weeks to generate F2 offspring. F2 male offspring derived from HFD-fed maternal grandfathers exhibited increased adiposity, plasma leptin and luteinising hormone to testosterone ratio. Despite transmission via the founding male germline, we did not find significant changes in the F0 intra-testicular GC transcriptome. Thus, HFD consumption by maternal grandfathers results in a disrupted metabolic and reproductive hormone phenotype in grandsons in the absence of detectable changes in the intra-testicular GC transcriptome.

31 **Introduction**

32 The prevalence of obesity has doubled since 1980 with an estimated $28billion annual
33 increase in associated medical costs in the USA[1–3]. Whilst lifestyle influences obesity risk,
34 other factors can contribute to weight accumulation and its effects on general health. Twin
35 studies suggest that 40-70% of bodyweight can be explained by inherited factors[4], however,
36 with a few exceptions, specific genes have remained elusive and genome wide association
37 studies have only accounted for 2-4% of the heritability of obesity.

38 Several human studies have identified relationships between parental weight and weight of
39 offspring[5], albeit this is confounded by parental and offspring environmental exposures.
40 Experimental evidence suggests that this might occur via non-genetic mechanisms. For
41 example, the diet of a male prior to conception can impact upon the metabolic and
42 reproductive health of his offspring[6–9], with some studies showing that such 'programmed'
43 effects are transmissible to further generations[10–12]. It has been proposed that such
44 environmental exposures might affect the germline epigenome; for example the miRNA
45 profile[6,13,14], chromatin dynamics/histone modifications[8,9] and DNA methylome[12] of
46 spermatozoa. However, the mechanisms linking these germline epigenetic modifications, the
47 stage of spermatogenesis affected and the phenotypic changes observed in offspring are
48 unknown.

49 The present study shows that grandparental exposure to a high-fat diet influences the
50 metabolism of two generations of rats in a grandparent-of-origin and sex-specific manner.
51 We demonstrate that the maternal grand-sire has the strongest effect on the metabolic
52 phenotype of his male grand-offspring. Using a rat model in which germ cells (GCs) express
53 eGFP[15], enabling the isolation of a pure intra-testicular germ cell population, we did not
54 identify any changes to the gene-coding or miRNA transcriptome of GCs from the testes of

55 HFD exposed adult males, implying that other (e.g. downstream) HFD-induced changes must
56 account for the intergenerational effects which we identify.

57 **Results**

58 **HFD induces adiposity but not altered glucose tolerance in exposed animals (F0)**

59 From weaning male and female rats were placed onto a control diet (CD) or onto a high fat
60 diet (HFD) for 16 weeks. Following 14 weeks on a HFD, F0 males and females were
61 respectively 9.3% and 14.7% heavier compared to animals fed the CD, with a significant
62 difference in male weight from 9 weeks of age and in female weight from 15 weeks (Figure
63 1A). In males, the adiposity index (the sum of fat pads divided by body weight) was
64 increased by 36% and leptin increased 3-fold, although this was not statistically significant
65 (Table 1). Insulin secretion in response to glucose tolerance testing (GTT) was increased 150%
66 in HFD fed males, without any changes in glucose concentration. There were no differences
67 between groups in plasma lipids or triglycerides, or in testosterone, luteinising hormone (LH)
68 or the LH:Testosterone ratio or in sperm count or testicular apoptosis (Table 1).

69 **Effects of parental (F0) HFD on F1 offspring**

70 At 19 weeks, adult males and females reared since weaning on CD or HFD were mated with
71 opposite sex CD-fed controls to generate F1 offspring. No differences were observed in litter
72 size, days taken to plug, gestation length, birthweight or proportion of males per litter in the
73 F1 animals born to parents who had consumed a HFD or a CD (Supplementary Table 1).
74 There were differences in the growth trajectories of the F1 offspring, with increased weight
75 gain in F1 offspring of mothers that had consumed the HFD and in female offspring of HFD-
76 fed males when compared to offspring of CD mothers (Figure 1B). Bodyweight at sacrifice
77 was increased for male (10%) and female (7%) F1 offspring born to HFD mothers (Table 2),
78 although there were no differences in adiposity, or in leptin, insulin and glucose levels (Table

79 2). Testosterone was elevated by ~70% in the sons of HFD-exposed mothers although there
80 was no difference in plasma LH or the LH:T ratio (Table 2).

81 **Grandparental diet affects F2 metabolic physiology in a parent-of-origin and sex-**
82 **specific manner**

83 To determine if grandparental exposure to HFD exerted effects on grandchildren (F2
84 offspring), representative F1 adult males and females derived from the relevant CD-exposed
85 or HFD-exposed F0 parents were mated with opposite sex CD-fed controls to generate F2
86 offspring. No differences were observed in litter size, percentage of males per litter or birth-
87 weights of F2 grand-offspring of CD or HFD fed rats (Supplementary Table 2). From 6
88 weeks of age, the F2 males whose maternal grandfather consumed a HFD were heavier than
89 the comparable F2 male offspring of rats fed CD (Figure 1C).

90 In adulthood, the adiposity index of the F2 male rats whose maternal grandfather consumed a
91 HFD was 31% greater than control males (Table 3). There was an associated 97% increase in
92 plasma leptin (Table 3) and evidence for decreased insulin sensitivity during the GTT, with
93 insulin area under the curve increased by ~70% following a glucose challenge, although this
94 was not statistically significant following Bonferroni adjustment (Table 3).

95 There was a trend for reduced plasma testosterone and increased LH levels in F2 males
96 whose maternal grandfather had consumed a HFD (Table 3), which resulted in a significant
97 three-fold increase in the LH:testosterone ratio in males of this group; this is indicative of
98 compensated Leydig cell failure. There was no observable influence of grandpaternal diet on
99 sperm count in F2 males (Table 3).

100 Female F2 animals derived from an HFD-fed grandparent showed no differences in body
101 weight, adiposity or size of organs when compared to controls (Table 3).

102 **High fat diet does not alter the transcriptome of F0 male intra-testicular GCs**

103 As F0 male exposure to HFD resulted in intergenerational effects in F2 male offspring, we
104 investigated if HFD exposure altered the gene expression profile of intra-testicular GCs from
105 F1 males. Purity of FACS sorted GCs was verified by qPCR for the GC specific protein *Vasa*
106 and expression of *Sox9* (Sertoli cell-specific) and *3βHSD* (Leydig cell-specific)
107 (Supplementary Figure 1). Total RNA was extracted from the GCs and underwent deep
108 sequencing; 5.0-9.0x10^7 reads per animal for RNA and 1.2-1.7x10^7 reads for small RNA
109 were uniquely aligned to the rat genome (rn5). The distribution of gene expression between
110 rats on HFD and CD, and between biological replicates, was highly consistent for protein
111 coding genes (Figure 2A) and miRNAs (Figure 3A) indicating little overall change in GC
112 transcription in this model. Clustering and principal components analysis showed strong
113 homogeneity between GCs isolated from HFD- and CD-exposed males (Figure 2B, 2C, 3B
114 and 3C). Three protein coding mRNAs and 1 microRNA were down-regulated with statistical
115 significance in GCs following HFD consumption; *collagen3a1*, *gelsolin* and *decorin* (Figure
116 2E) and miRNA rno-mir-10b (Figure 3E). Although each of these showed reduced expression
117 in qPCR validation, these failed to reach statistical significance (Figures 2F and 3F).

118 There were no differentially expressed piRNAs or repeat elements when comparing GCs
119 from CD- or HFD-exposed males (Figure S2D). Both piRNA and repeat elements showed
120 consistent distribution between treatment and biological replicates (Supplementary Figure
121 2A), and strong homogeneity was demonstrated by clustering analysis (Supplementary Figure
122 2B) and PCA (Supplementary Figure 2C).

123 Given recent evidence that dietary exposure can affect the abundance of specific tRNA
124 fragments in mature spermatozoa[13,16], the expression of tRNA derived species was examined.
125 Our library preparation selected for small RNA species of 22-30nt so we were only able to
126 estimate expression (Sharma *et al* found tRNA fragments to be 28-34nt), however we were

127 able to quantify 10 species of tRNA, none of which exhibited differential expression in the
128 germ cells extracted from the testes of CD or HFD exposed animals. This is consistent with
129 the findings of Sharma *et al.* who were unable to identify differential expression in testis
130 tissue[13].

131 **RNA-seq data from FACS sorted GCs was adequately powered to detect differences in**
132 **protein coding gene expression**

133 Given the few differentially expressed protein-coding genes and miRNAs in GCs of HFD-
134 exposed F0 males, we next determined if our study design was adequately powered. We
135 performed a *post hoc* power analysis in which data from the initial dataset were shuffled to
136 generate a simulated dataset, selected for genes with differential expression of |log2fold| ≤ 2.0
137 (see Methods). This simulated dataset was analysed using the same approach as described for
138 all other annotations. Our analysis revealed 2781 simulated genes with statistically significant
139 differences in expression in this data set, suggesting the study was adequately powered to
140 detect expression changes at a log2fold change ≤ 0.6 (Figure 2D and Supplementary Figure
141 4). A similar *post hoc power* analysis was conducted on the miRNA data (Figure 3D) which
142 identified 3 differentially expressed miRNAs, a proportion of differentially expressed
143 miRNAs similar to our original analysis (0.48% vs 0.35% respectively) (Supplementary
144 Figure 4).

145 **Discussion**

146 The aim of the present studies was to investigate if feeding rats a HFD results in metabolic or
147 reproductive changes in subsequent generations and, if so, whether this might be mediated
148 via altered gene expression in the GCs of the HFD-exposed parents (males in this case). Our
149 data show that parental HFD exposure for a 16-week period can alter the metabolic
150 phenotype of offspring and grand-offspring, with the most pronounced effects occurring
151 when the maternal grandfather was exposed to a HFD. These effects occurred despite only

152 modest changes in the adiposity of the HFD-exposed grandfathers. We did not detect any
153 significant HFD-mediated changes in the transcriptome of the GCs from the testes of F0 male
154 rats.

155 Consistent with previous studies, we found that exposure of the maternal grandfather to HFD
156 had the greatest impact on bodyweight, adiposity and insulin resistance in grandsons[6,11]. The
157 animal model data also agrees with epidemiological data from Överkalix, in Sweden, in
158 which the environment of men during puberty predicted cardiovascular disease in grandsons,
159 although in this instance via the paternal line[17,18].

160 F2 males whose maternal grandfather had been exposed to HFD exhibited compensated
161 Leydig cell failure, with an elevation in LH in the presence of normal testosterone levels.
162 Although the relevance of compensated hypogonadism remains unclear, it has been
163 associated with increased mortality[19] and cardiovascular disease[20,21]. In humans, obesity is
164 associated with hypogonadism[22], although the causality of this is unclear[23]. The etiology of
165 many cases of primary hypogonadism in men is unknown although there is some evidence
166 that an altered *in utero* environment can program this effect in adulthood[24].

167 Postnatal exposure of male rats to HFD resulted in metabolic changes in their grand-offspring,
168 and previous studies have demonstrated changes in sperm RNA profiles following
169 environmental exposures[6,14] ; however, we found no differences in miRNA expression in
170 intra-testicular GCs. This is in contrast to studies reporting alterations in miRNA in
171 spermatozoa[6,25], and suggests that HFD may affect the post-transcriptional stability of
172 miRNA within maturing spermatozoa in the epididymis rather than in altered expression in
173 the GC within the testis. Comparable studies in mice have shown that HFD exposure
174 significantly altered the whole testis transcriptome[6,14], with a proportion of transcripts
175 showing altered expression in epididymal sperm. One key difference to the data presented

176 here is that we excluded somatic cells from our analysis. Although this could suggest that
177 such differences are due to the changes to somatic cells as a result of the nutritional exposure,
178 Grandjean *et al*. went on to show that microinjection of miRNA mir-19b, which had
179 increased expression in the testis and epididymal sperm of HFD fed mice, into one-cell
180 embryos resulted in an altered metabolic phenotype mirroring that of offspring of HFD
181 exposed fathers[14]. We found remarkable homogeneity in the RNA and small RNA-seq data
182 from the intra-testicular germ cells of male rats exposed to CD or HFD, in a study which was
183 powered to identify any changes in expression of log2fold change $\geq$ 0.6. This suggests that
184 the changes identified may be species and/or experimental model specific. Unfortunately, due
185 to difficulties in obtaining sperm populations with sufficient purity and abundance to
186 interrogate miRNA profiles, we are unable to say if HFD-exposure induced changes in
187 miRNA expression in mature epididymal sperm in our study. As several groups have now
188 reported changes in expression of RNA species in epididymal spermatozoa following dietary
189 interventions, and in the case of Sharma *et al.* in the absence of alterations in expression in
190 testicular tissue[13], we suggest that sperm maturation may be the stage of germ cell
191 development most vulnerable to environmentally-induced perturbations.

192 Phenotypic changes may be transmissible across generations without changes in the GC
193 transcriptome, for example in a model of maternal undernutrition, in which F2 mice exhibited
194 an altered metabolic phenotype, the methylome of epididymal sperm was disrupted as a result
195 of *in utero* undernourishment[12] and in humans methylation of mature sperm DNA was altered
196 in obese individiuals[26]. Such changes in the GCs in our model could have occurred without
197 affecting transcription. Two further studies suggest that intergenerational effects of diet might
198 be mediated by alterations in chromatin structure in mature spermatozoa[8,9], which may occur
199 in the absence of altered expression of mRNA in GCs, especially given that during
200 spermiogenesis, the germline becomes largely transcriptionally inactive[27].

201 The time-point(s) of importance for an environmental exposure to affect sperm, and the
202 health of offspring and grand-offspring are unknown. Our results indicate that exposure to a
203 HFD from weaning through puberty to adulthood is sufficient to program an effect in
204 offspring, as has been shown in mouse models[11,28]. It is not clear how long such effects may
205 persist; in mice, 7 weeks exposure to exercise or CD following 8 weeks exposure to HFD
206 normalised insulin sensitivity in offspring, indicating some reversibility of the programmed
207 phenotype[29]. One might speculate that a short-term, reversible effect would be more likely to
208 arise from perturbations to sperm maturation than to spermatogonia, as has been suggested by
209 studies that found programmed phenotypes following relatively short paternal exposures, for
210 example as a consequence of exposure to 48h high sugar in *Drosophila*[8] and 24h fasting in
211 mice[30]. The timing of these acute exposures along with the results presented here, might point
212 towards perturbations in epididymal sperm maturation, rather than effects on spermatogenesis.
213 This is supported by evidence suggesting that tRNA/tRNA fragment accumulation in
214 maturing spermatozoa may play a role in influencing offspring phenotype[13,16]. Thus our data
215 add further weight to the argument that sperm maturation is most susceptible to
216 environmental influences. Alternatively, our data might suggest a non-GC mediated
217 transmission of the effects of HFD exposure, for example alterations in seminal fluid, which
218 is important for establishing normal conception and healthy development[31,32], and which is
219 altered by obesity[31,33].

220 Our study has a number of limitations. The HFD exposure in our study resulted in only
221 modest weight gain, although it clearly implicates dietary fat, or the physiological response to
222 it, as a factor resulting in changes in the health of offspring. The study utilised a semi inbred
223 rodent line, which may result in greater variability between animals than studies in inbred
224 mice. Furthermore, we cannot rule out genetic variation as a potential cause for the altered
225 phenotype. However, intergenerational programmed effects have been reported in both inbred

226 and outbred models[12,34], and outbred strains are a better model of the human population. We
227 have not further explored the epigenome in the purified GCs as, having found no changes to
228 transcription, the relevance of any differences would be difficult to interpret; thus, we cannot
229 discount possible epigenetic changes in regions in non-transcribed DNA in GCs. Finally,
230 since we purified germ cells from the testis, we cannot account for any stage-specific GC
231 effects, which could theoretically mask changes in gene expression; for example, if
232 expression of a gene was increased in early spermatogonia but reduced in spermatids.

233 In conclusion, we show that postnatal exposure of male rats to HFD results in impaired
234 metabolism in grandsons, a trait specifically transmitted down the maternal line. We did not
235 detect significant changes in the intra-testicular GC transcriptome as a result of exposure to
236 HFD that would explain the intergenerational effects. Further work is clearly necessary to
237 discover mechanisms, to determine the time points at which males are most susceptible to
238 HFD-induced changes, and establish if the effects are reversible. Given the rapid rise in the
239 prevalence of obesity our data highlight that the environment of our immediate ancestors
240 could play a role in this epidemic.

241 **Materials and methods**

242 **Study design and animal model**

243 Studies were performed according to the Animals (Scientific Procedures) Act 1986 following
244 specific approval from the UK Home Office (Project License 60/3962), following review by
245 the University of Edinburgh Animal Research Ethics Committee. Animals were maintained
246 under controlled lighting (lights on 0700-1900) and temperature (22°C). Sprague Dawley
247 GCS-eGFP rats[15] (a gift from R. Hammer, University of Texas Southwestern Medical Center,
248 USA) were mated in-house. Animals had access to water and diet *ad libitum* and were killed
249 by CO_2 inhalation followed by cervical dislocation.

250 30 male and 18 female 21-day-old founder (F0) rats were weaned onto either a high-fat, soya
251 free diet (45% fat from lard) (HFD), or a matched control diet (10% fat) (CD) (Research
252 Diets, NJ, USA) (for details see Supplementary Table 3) and weighed every 2 weeks. At 17
253 weeks, rats underwent metabolic testing. Two weeks later, 3 groups of virgin breeding pairs
254 of F0 animals were established (n=5-9), 1) mother CD/ father CD, 2) father HFD/ mother CD,
255 and 3) mother HFD/ father CD. Animals were used once for breeding. The days taken for a
256 plug to be observed and the length of gestation were recorded. Following delivery, F1 litters
257 were culled to eight pups (four males and four females where possible), and were weighed
258 and weaned onto CD on day 21.

259 At 19 weeks, a female and male rat from each F1 litter (n=4-7) were bred with a rat from the
260 F1 control group to generate 4-5 F2 litters for each experimental arm and 7 F2 control litters.
261 F2 litters were culled to 8 pups at birth and then culled to two animals per litter at 5-6 weeks
262 of age. A total of 48 F2 males and 48 F2 females were thus derived (from 25 litters). Animals
263 from the same treatment group were housed together with a maximum of 4 per cage and
264 maintained throughout on CD.

265 **Metabolic testing**

266 For F0 and F1 animals, one animal per litter underwent oral glucose tolerance testing (OGTT)
267 (F0 n=9, F1 n= 5-6). For F2 animals, two animals from each litter were tested (n= 8-14 from
268 4-7 litters). Following an overnight fast, at 09.00, blood was obtained by tail nicking into
269 EDTA coated micro tubes (Starstedt, Germany) and plasma separated. 2g/kg of 0.5g/ml
270 glucose solution (Sigma) was administered by oral gavage. Further blood was collected at 30
271 and 120 min.

272 For F0 animals, glucose was measured using a colorimetric kit (Cayman, USA) and for F1
273 and F2 with a kit (Alpha Laboratories Ltd., UK) adapted for use on a Cobas Fara centrifugal

274 analyser (Roche, UK). Insulin was measured using a Rat Insulin ELISA kit (Mercodia,
275 Sweden,). Fasting plasma triglyceride and cholesterol were determined using kits (Alpha
276 Laboratories Ltd., UK and Olympus Diagnostics Ltd, UK, respectively), adapted for use on a
277 Cobas Fara centrifugal analyser (Roche, UK). Terminal plasma leptin was measured using a
278 Rat Leptin ELISA (Crystal-Chem, IL, USA).

279 **Testosterone and Luteinising Hormone (LH)**

280 Plasma testosterone levels were measured at termination using an in-house
281 radioimmunoassay described previously[35]. Plasma Luteinising Hormone (LH) was
282 determined using an in house ELISA with the capture by a monoclonal anti-beta chain
283 antibody (from Dr. Jan F Roser, University of California, USA) and a signal biotinylated
284 anti-beta-chain monoclonal antibody (Medix, Finnland)[36].

285 **Epididymal sperm count**

286 Epididymides were dissected and the caput and cauda nicked once prior to placement into
287 5ml F12:Ham's (Life Technologies, UK) supplemented with 10% fetal calf serum (FCS) and
288 2% bovine serum albumin (BSA) (Sigma, UK). The epididymis was incubated for 1 hour at
289 37°C with inversion at 30 mins. Sperm in the medium were counted using a modified
290 Neubauer haemocytometer at x40 magnification; 3-4 fields were counted per animal.

291 **Organ wet weights**

292 Following sacrifice, pancreas, liver, testes, epididymal fat pads, and left retroperitoneal fat
293 pads were dissected and weighed. Anogenital distance was measured (using a 30cm rule for
294 F0 and F1 and digital callipers for F2) from the midpoint of the anus to the scroto-penile
295 junction. For F2 animals, the penis was dissected and measured using callipers. Body length
296 was measured using a 30cm rule from the tip of the nose to the end of the rump.

297 **TUNEL staining**

298 TUNEL staining used a modified protocol for the Promega DeadEnd kit (Promega). Testes
299 were fixed in Bouin's for 6 hours with bisection at 4 hours before paraffin embedding. 5µm
300 sections were dewaxed, rehydrated and washed in PBS, fixed for 15 min in 4%
301 paraformaldehyde in PBS, treated with 20µg/ml Proteinase K for 10mins prior to further
302 fixation in 4% paraformaldehyde. The remaining process was carried out as per
303 manufacturer's instructions. Slides were counterstained with DAPI prior to aqueous
304 mounting. Four 40x 10x10 tiled images were captured from each section using a Zeiss LSM
305 710 microscope. The number of positive pixels per tile was determined using ImageJ[37].

306 **RNA-seq**

307 Testes were decapsulated and minced in 5 ml ice cold Hanks Buffered Saline Solution with
308 0.1% Collagenase IV (Sigma, UK). The suspension was dissociated before incubation at
309 37°C with gentle rotation for 10 min, and passage through a 40µm cell strainer. Cells were
310 washed 3x by centrifugation at 500 *g* for 5 min and resuspended in 10ml 2% FCS (Invitrogen,
311 UK). Samples were kept on ice until FACS on a BD Aria II, gating for expression of eGFP.
312 $6x10^6$ sorted cells were centrifuged and RNA immediately extracted using the Qiagen
313 miRNAeasy mini kit (Qiagen, UK) according to manufacturer's instructions. Quality and
314 purity of total RNA was verified using spectrophotometry and the Agilent RNA 6000 nano
315 kit before library preparation (Illumina RNA library prep kit v2 and the Illumina Truseq
316 small RNA kits (Illumina, CA, USA)). The small RNA library preparation selected for RNA
317 species with length 22-30nt. Sequencing small RNA (smRNA) and RNA was performed on
318 the Illumina HiSeq2500 (Edinburgh Genomics, Edinburgh, UK). Intended library size for
319 total RNA was 37.5 million single end reads of 125bp and for small RNA was 20 million
320 single end reads of 50bp. Quality of the sequencing was verified using FastQC. Adaptor
321 contamination was removed from the smRNA-seq libraries using trimmomatic to a minimum

322 length of 25 nucleotides[38]. SmRNA-seq reads were aligned to the *Rattus norvegicus* genome
323 version rn5 using Butter with default parameters[39]. Differential expression was determined
324 using DESeq2[40]. RNA-seq libraries were aligned to Rn5 using Star v2.3.0[41] on a custom-built
325 splice junctions database based on Rn5 ensembl73 protein-coding annotations, with the
326 maximum proportion of mismatches over the read length of 0.05 and minimum mapping read
327 length of 125nts. As positive controls, liver and testis RNA-seq data from the Rat bodymap
328 were subject to the same bioinformatic pipeline[42] (Supplementary Figures 3A-D). All raw
329 RNA-seq data, and processed count tables are archived at the Gene Expression Omnibus with
330 accession number GSE80721. The code used to perform read quality control
331 (pipeline_readqc.py) and short read alignment (pipeline_mapping.py) can be found at
332 https://github.com/CGATOxford/CGATPipelines.

333 **Sample clustering and principal components analysis**

334 Variance stabilising transformed (VST) read counts for protein-coding genes, miRNAs,
335 repeats and piRNAs were calculated using DESeq2. Between-sample Pearson correlations (r)
336 were calculated using VST counts and used to hierarchically cluster samples using average
337 linkage clustering, with distances defined as 1 - | r |. Principal components analysis (PCA)
338 was performed on scaled and centred VST counts using the R *prcomp* function. Sample
339 clustering was visualised using the R gplots package function *heatmap.2*, and PCA results
340 were plotted using the R grammar of graphics package, *ggplot2*.

341 **Differential expression testing**

342 Uniquely aligned reads were counted over rn5 genomic annotations (ensemble v73 protein-
343 coding genes, miRBase miRNAs[43], piRNAQuest piRNAs[44] and repBase repeat classes[45]
344 using featureCounts[46]. Genomic annotations with a mean read count <1 across all samples
345 were excluded from analysis. This resulted in the differential expression testing of 18,025
346 protein-coding genes, 285 miRNAs, 7390 piRNA annotations and 522 repeat classes.

347 Statistical testing was carried out separately for protein-coding genes, miRNAs, piRNAs and
348 repeat elements. Differential expression testing was performed using a negative binomial
349 general linear model, regressing genomic annotation read counts on diet, adjusted for library
350 size, in the Bioconductor package DESeq2[40]. P-values were calculated based on the Bayesian
351 shrinkage moderated log2 fold changes by a Wald test with H_0: log2 fold change = 0, H_A:
352 log2 fold change $\neq 0$ and adjusted for multiple testing using the Benjamini & Hochberg
353 procedure[47].

354 **Power analysis**

355 Evaluation of statistical power was determined by simulation. For any given annotation, read
356 counts were randomly and iteratively shuffled to generate a simulated dataset of read counts,
357 preserving the experimental design, i.e. counts were shuffled between different genes, but not
358 different treatment groups. Shuffled annotations were retained with |log2 fold change| ranges
359 0.0-2.0 for protein-coding genes, and 0.0-0.5 for miRNA annotations. These ranges were
360 selected to reflect the observed fold changes in the experimental data. The counts tables
361 derived from the shuffled data sets were used as input into the same differential expression
362 testing procedure described above. The shuffled counts tables were generated using the
363 *counts2counts.py* Python script in the CGAT code collection[48], found at
364 https://github.com/CGATOxford/cgat. To generate Figures 2D and 3D, the relevant table of
365 simulated counts were spiked into the table of relevant annotation counts, and the differential
366 expression analysis was performed on the combined table. Statistical power was calculated in
367 bins of 0.1 as the proportion of statistically significantly differentially expressed annotations,
368 relative to all tested annotations of that class (i.e. either miRNAs or protein-coding genes).
369 The R code to generate the power curves and all other code to perform differential expression
370 testing can be found at https://github.com/MikeDMorgan/proj035, including the counts tables
371 on which the power analysis in Supplementary Figure 4 was performed.

372 **qPCR validation of sequencing**

373 cDNA was prepared using SuperScript VILO (Invitrogen) as per the manufacturer's
374 instructions. The most stably expressed genes from the RNA-seq were used to determine
375 reference genes using normfinder[49]. Expression was thus calculated relative to the mean
376 expression of *Ldha* and *Ropn1L*. qPCR was performed on the ABI Prism Sequence Detection
377 System (Applied Biosystems). Expression was determined using the primers and universal
378 probes (Roche, UK) in supplementary table 4. For miRNA, the ABI TaqMan miRNA assay
379 for rno-mir-10b was used according to manufacturer's instructions; snoRNA-U6 was used as
380 control. RNA from two independent cohorts of rats was used. All samples were analysed in
381 triplicate.

382 Validation of the purity of sorted GCs was achieved by qPCR as above but with expression
383 compared to RNA from adult rat testis (Ambion), using 18S as internal control.

384 **Statistics**

385 For experiments examining F0 founders, outcomes were analysed using linear mixed model
386 with diet and sex as fixed factors and cohort as a random factor. For analysis of F1 and F2
387 data, a mixed linear model was used with group and sex as fixed factors and litter number as
388 a random factor. Data are presented either as a % difference to the control group or, as mean
389 ± SEM with total animals as the denominator. For bodyweight, a mixed linear model was
390 used with group and sex as fixed factors, litter as a random factor and weight as a repeated
391 measure with an autoregressive covariance structure. Goodness of fit of these models was
392 checked using the maximum likelihood method and comparison of -2 log likelihood
393 information criteria for the lowest value. *Post hoc* Bonferroni analysis was conducted to
394 account for multiple comparisons. Where no differences were determined by Bonferroni
395 adjustment, but an interaction was identified, least significant difference analysis was

conducted as indicated in the results tables. Levels of significance were set at alpha=0.05, statistics were computed using SSPS version 19 (IBM, USA).

398 **References**

399 1. Ng, M. *et al.* Global, regional, and national prevalence of overweight and obesity in
400 children and adults during 1980-2013: a systematic analysis for the Global Burden of
401 Disease Study 2013. *Lancet* **384,** 766–781 (2014).
402 2. Wang, Y. C., McPherson, K., Marsh, T., Gortmaker, S. L. & Brown, M. Health and
403 economic burden of the projected obesity trends in the USA and the UK. *Lancet* **378,**
404 815–825 (2011).
405 3. WHO. WHO | Obesity and overweight. *WHO* (2014). Available at:
406 http://www.who.int/mediacentre/factsheets/fs311/en/. (Accessed: 21st May 2014)
407 4. Locke, A. E. *et al.* Genetic studies of body mass index yield new insights for obesity
408 biology. *Nature* **518,** 197–206 (2015).
409 5. Patro, B. *et al.* Maternal and Paternal Body Mass Index and Offspring Obesity: A
410 Systematic Review. *Ann. Nutr. Metab.* **63,** 32–41 (2013).
411 6. Fullston, T. *et al.* Paternal obesity initiates metabolic disturbances in two generations of
412 mice with incomplete penetrance to the F2 generation and alters the transcriptional
413 profile of testis and sperm microRNA content. *FASEB J.* **27,** 4226–42243 (2013).
414 7. Ng, S.-F. *et al.* Chronic high-fat diet in fathers programs β-cell dysfunction in female rat
415 offspring. *Nature* **467,** 963–966 (2010).
416 8. Öst, A. *et al.* Paternal Diet Defines Offspring Chromatin State and Intergenerational
417 Obesity. *Cell* **159,** 1352–1364 (2014).
418 9. Carone, B. R. *et al.* Paternally Induced Transgenerational Environmental Reprogramming
419 of Metabolic Gene Expression in Mammals. *Cell* **143,** 1084–1096 (2010).
420 10. King, V. *et al.* Maternal obesity has little effect on the immediate offspring but impacts
421 on the next generation. *Endocrinology* **154,** 2514–24 (2013).

422 11. Fullston, T. *et al.* Diet-induced paternal obesity in the absence of diabetes diminishes the
423 reproductive health of two subsequent generations of mice. *Hum. Reprod.* **27,** 1391–1400
424 (2012).
425 12. Radford, E. J. *et al.* In utero undernourishment perturbs the adult sperm methylome and
426 intergenerational metabolism. *Science* **345,** 1255903 (2014).
427 13. Sharma, U. *et al.* Biogenesis and function of tRNA fragments during sperm maturation
428 and fertilization in mammals. *Science* **351,** 391–396 (2016).
429 14. Grandjean, V. *et al.* RNA-mediated paternal heredity of diet-induced obesity and
430 metabolic disorders. *Sci. Rep.* **5,** 18193 (2015).
431 15. Cronkhite, J. T. *et al.* Male and female germline specific expression of an EGFP reporter
432 gene in a unique strain of transgenic rats. *Dev. Biol.* **284,** 171–183 (2005).
433 16. Chen, Q. *et al.* Sperm tsRNAs contribute to intergenerational inheritance of an acquired
434 metabolic disorder. *Science* **351,** 397–400 (2016).
435 17. Pembrey, M. E. *et al.* Sex-specific, male-line transgenerational responses in humans. *Eur.*
436 *J. Hum. Genet.* **14,** 159–166 (2006).
437 18. Kaati, G., Bygren, L. O., Pembrey, M. & Sjöström, M. Transgenerational response to
438 nutrition, early life circumstances and longevity. *Eur. J. Hum. Genet.* **15,** 784–790 (2007).
439 19. Agergaard Holmboe, S. *et al.* The association of reproductive hormone levels and all-
440 cause, cancer and cardiovascular disease mortality in men. *J. Clin. Endocrinol. Metab.*
441 jc20152460 (2015). doi:10.1210/jc.2015-2460
442 20. Hyde, Z. *et al.* Elevated LH predicts ischaemic heart disease events in older men: the
443 Health in Men Study. *Eur. J. Endocrinol. Eur. Fed. Endocr. Soc.* **164,** 569–577 (2011).
444 21. Giannetta, E., Gianfrilli, D., Barbagallo, F., Isidori, A. M. & Lenzi, A. Subclinical male
445 hypogonadism. *Best Pract. Res. Clin. Endocrinol. Metab.* **26,** 539–550 (2012).

446 22. Wu, F. C. W. *et al.* Hypothalamic-pituitary-testicular axis disruptions in older men are
447 differentially linked to age and modifiable risk factors: the European Male Aging Study.
448 *J. Clin. Endocrinol. Metab.* **93,** 2737–2745 (2008).
449 23. Corona, G., Vignozzi, L., Sforza, A., Mannucci, E. & Maggi, M. Obesity and late-onset
450 hypogonadism. *Mol. Cell. Endocrinol.* (2015). doi:10.1016/j.mce.2015.06.031
451 24. Kilcoyne, K. R. *et al.* Fetal programming of adult Leydig cell function by androgenic
452 effects on stem/progenitor cells. *Proc. Natl. Acad. Sci. U. S. A.* **111,** E1924–1932 (2014).
453 25. Gapp, K. *et al.* Implication of sperm RNAs in transgenerational inheritance of the effects
454 of early trauma in mice. *Nat. Neurosci.* (2014). doi:10.1038/nn.3695
455 26. Donkin, I. *et al.* Obesity and Bariatric Surgery Drive Epigenetic Variation of
456 Spermatozoa in Humans. *Cell Metab.* **23,** 369–378 (2016).
457 27. Rathke, C., Baarends, W. M., Awe, S. & Renkawitz-Pohl, R. Chromatin dynamics during
458 spermiogenesis. *Biochim. Biophys. Acta BBA - Gene Regul. Mech.* **1839,** 155–168 (2014).
459 28. Fullston, T. *et al.* Female offspring sired by diet induced obese male mice display
460 impaired blastocyst development with molecular alterations to their ovaries, oocytes and
461 cumulus cells. *J. Assist. Reprod. Genet.* (2015). doi:10.1007/s10815-015-0470-x
462 29. McPherson, N. O., Owens, J. A., Fullston, T. & Lane, M. Preconception diet or exercise
463 intervention in obese fathers normalizes sperm microRNA profile and metabolic
464 syndrome in female offspring. *Am. J. Physiol. - Endocrinol. Metab.* **308,** E805–E821
465 (2015).
466 30. Anderson, L. M. *et al.* Preconceptional fasting of fathers alters serum glucose in offspring
467 of mice. *Nutrition* **22,** 327–331 (2006).
468 31. Binder, N. K., Sheedy, J. R., Hannan, N. J. & Gardner, D. K. Male obesity is associated
469 with changed spermatozoa Cox4i1 mRNA level and altered seminal vesicle fluid
470 composition in a mouse model. *Mol. Hum. Reprod.* (2015). doi:10.1093/molehr/gav010

471 32. Bromfield, J. J. *et al.* Maternal tract factors contribute to paternal seminal fluid impact on
472 metabolic phenotype in offspring. *Proc. Natl. Acad. Sci.* 201305609 (2014).
473 doi:10.1073/pnas.1305609111
474 33. Leisegang, K., Bouic, P. J. D., Menkveld, R. & Henkel, R. R. Obesity is associated with
475 increased seminal insulin and leptin alongside reduced fertility parameters in a controlled
476 male cohort. *Reprod. Biol. Endocrinol. RBE* **12,** 34 (2014).
477 34. Anway, M. D., Memon, M. A., Uzumcu, M. & Skinner, M. K. Transgenerational Effect
478 of the Endocrine Disruptor Vinclozolin on Male Spermatogenesis. *J. Androl.* **27,** 868–
479 879 (2006).
480 35. Corker, C. S. & Davidson, D. W. A radioimmunoassay for testosterone in various
481 biological fluids without chromatography. *J. Steroid Biochem.* **9,** 373–374 (1978).
482 36. Tyndall, V. *et al.* Effect of androgen treatment during foetal and/or neonatal life on
483 ovarian function in prepubertal and adult rats. *Reproduction* **143,** 21–33 (2012).
484 37. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of
485 image analysis. *Nat. Methods* **9,** 671–675 (2012).
486 38. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina
487 sequence data. *Bioinforma. Oxf. Engl.* **30,** 2114–2120 (2014).
488 39. Axtell, M. J. Butter: High-precision genomic alignment of small RNA-seq data. *bioRxiv*
489 007427 (2014). doi:10.1101/007427
490 40. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion
491 for RNA-seq data with DESeq2. *Genome Biol.* **15,** 550 (2014).
492 41. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinforma. Oxf. Engl.* **29,**
493 15–21 (2013).
494 42. Yu, Y. *et al.* A rat RNA-Seq transcriptomic BodyMap across 11 organs and 4
495 developmental stages. *Nat. Commun.* **5,** 3230 (2014).

496 43. Kozomara, A. & Griffiths-Jones, S. miRBase: annotating high confidence microRNAs
497 using deep sequencing data. *Nucleic Acids Res.* **42,** D68–D73 (2014).
498 44. Sarkar, A., Maji, R. K., Saha, S. & Ghosh, Z. piRNAQuest: searching the piRNAome for
499 silencers. *BMC Genomics* **15,** 555 (2014).
500 45. Jurka, J. *et al.* Repbase Update, a database of eukaryotic repetitive elements. *Cytogenet.*
501 *Genome Res.* **110,** 462–467 (2005).
502 46. Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general-purpose program
503 for assigning sequence reads to genomic features. *Bioinformatics* btt656 (2013).
504 doi:10.1093/bioinformatics/btt656
505 47. Benjamini, Y. & Hochberg, Y. Controlling the False Discovery Rate: A Practical and
506 Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B Methodol.* **57,** 289–300
507 (1995).
508 48. Sims, D. *et al.* CGAT: computational genomics analysis toolkit. *Bioinformatics* **30,**
509 1290–1291 (2014).
510 49. Andersen, C. L., Jensen, J. L. & Ørntoft, T. F. Normalization of real-time quantitative
511 reverse transcription-PCR data: a model-based variance estimation approach to identify
512 genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.*
513 **64,** 5245–5250 (2004).
514

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526 **Conflict of interest**

527 The authors declare no conflicts of interest

528 **Author contributions**

529 TC, AD and RS designed the studies. TC conducted wet benchwork. TC, MM, AH designed
530 and conducted analysis of RNA-seq data. All authors contributed to manuscript preparation.

531

532 **Figure captions**

533 **FIGURE 1 Growth curves of rats in each generation**

534 **a**. Body weight of F0 rats fed a control diet (CD; black lines, closed symbols) or a high fat
535 (HFD; grey lines, open symbols) for 14 weeks. Data are shown for male (squares) and female
536 (circles) rats. Data were analysed by linear mixed model with diet and sex as fixed factors,
537 time as a repeated factor and cohort as a random factor with Bonferroni post hoc testing for
538 effect of diet within a sex. N= 22-35 males and 10-18 females from two cohorts. *p<0.05
539 HFD vs CD. **b**. Body weight of F1 rats according to diet of the F0 parents. Analysis was by
540 linear mixed model with group and sex as fixed factors and litter as a random factor. N= 13-
541 32 males from 5-9 litters and 10-21 females from 5-9 litters. *p<0.05 maternal high fat diet
542 vs. both parents on control diet. ^ p<0.05 maternal or paternal high fat diet vs. both parent
543 control diet. **c**. Bodyweight of F2 rats from birth to 18 weeks of age. Analysis was by linear
544 mixed model with sex and group as fixed factors, time as a repeated factor and litter as a
545 random factor and post hoc Bonferroni analysis. *p<0.05 maternal grandfather high fat diet
546 vs. control. Data are means ± SEM.

547 **FIGURE 2. Effect of 14-week exposure to a high fat diet (HFD) or a control diet (CD)**
548 **on the intra-testicular GC protein-coding transcriptome of F0 male rats.**

549 **a.** The distribution of variance stabilising transformed (VST) expression of annotated protein-
550 coding genes was unaffected by diet and was highly consistent across biological replicates. **b.**
551 Hierarchical clustering on the sample correlation matrix of gene expression indicated that the
552 HFD and CD samples are highly similar. **c.** Principal components analysis (PCA) was unable
553 to distinguish samples according to diet. **d.** Differential expression testing identified 3
554 protein-coding genes that were statistically significantly down-regulated in response to HFD
555 (see **e**). A *post hoc* power analysis by simulation showed that the few HFD-induced changes
556 were not due to a lack of statistical power given the same expression value and fold change

range (Orange points = HFD vs. CD comparison; black points = statistically significantly differentially expressed genes between HFD and CD, including the simulated genes; purple points = simulated genes comparison). **e.** The top 5 most differentially expressed genes in GCs of HFD-fed rats when compared with CD-fed rats. Mean expression indicates the expression level in GCs from CD animals, normalised for library size and averaged across 4 replicates; p-values were adjusted for multiple testing. **f.** RT-qPCR validation analysis for the differentially expressed genes in **E**. (Means ±SEM for n=11-12).

FIGURE 3. Effect of 14-week exposure to a high fat diet (HFD) or a control diet (CD) on regulatory miRNA expression in the intra-testicular GCs of male F0 rats. a. The distribution of expression of annotated *Rattus norvegicus* miRNAs within GCs was unaffected by paternal diet. Expression was more variable between replicates than for the protein-coding mRNAs (Figure 2), but showed that the majority of miRNAs were relatively lowly expressed (shown as Variance Stabilised expression). **b.** Hierarchical clustering of miRNA expression showed that the HFD and CD samples had high similarity. **c**. Principle components analysis (PCA) did not separate samples according to HFD or CD over the first two principal components, which together explained the majority of the variance in miRNA expression between samples. **d**. Differential expression testing of miRNA expression identified a single miRNA (rno-mir-10b) that was significantly down-regulated in the HFD group compared with CD. This small but statistically significant change was mirrored in a *post hoc* power analysis by simulation that demonstrated a similar proportion of differentially expressed spike-in miRNAs (0.35% vs 0.48%) (Orange points = HFD vs. CD comparison; black points = statistically significantly differentially expressed genes between HFD and CD (including simulated genes); purple points = simulated gene comparison). **e**. Top 3 most differentially expressed miRNAs in GCs of HFD-fed rats when compared with CD-fed rats. The miRNA rno-mir-10b is highly expressed and demonstrates a modest difference between

582 HFD and CD groups. P-values were adjusted for multiple testing. **f**. RT-qPCR validation

583 analysis for the down-regulation of rno-mir-10b. Values are Means ±SEM for n=8.

Tables

TABLE 1. Effect of feeding with a high fat (HFD) or control diet (CD) for 14 weeks on the metabolic and reproductive phenotype of F0 males.

Data are derived from post-mortem dissection at 19 weeks of age. Biochemical data derives from 09.00 fasting plasma obtained during glucose tolerance testing at 17 weeks of age. Data were analysed by linear mixed model with diet as fixed factor and cohort as a random factor with post hoc Bonferroni analysis. Values significantly different ($p<0.05$) from control (CD) are shown in bold.

	Control diet	High fat diet	
N (litters)	7-15(6)	7-15(6)	
	Mean±SEM	Mean±SEM	p
Body size			
Weight (g)	416±14	**454±14**	**0.003**
Body length (cm)	23.95±0.25	24.10±0.31	0.565
Organ weights			
Pancreas weight/bodyweight (mg/g)	1.9±0.09	1.8±0.08	0.416
Liver/bodyweight (mg/g)	38.3±1.2	**31.0±0.9**	**<0.001**
Adipose weights			
Retroperitoneal Fat/bodyweight (mg/g)	4.7±0.03	**6.9±0.04**	**<0.001**
Gonadal fat/bodyweight (mg/g)	6.3±0.3	**8.3±0.3**	**<0.001**
Adiposity Index (mg/g)	11.0±0.7	**15.2±0.7**	**<0.001**
Biochemistry			
Leptin (ng/ml)	1.67±1.28	5.25±1.21	0.054
Insulin AUC µg/l.min	96.4±11.1	**144.7±10.1**	**0.003**
Glucose AUC mM.min	647.1±22.7	672.2±17.6	0.4
Triglycerides mM	0.81±0.06	0.73±0.07	0.719
Cholesterol mM	1.53±0.07	1.58±0.10	0.414
Reproduction			
AGD (mm)	46.91±1.29	45.93±0.80	0.52
Testis weight (g)	1.79±0.06	1.73±0.05	0.334
Penis length (mm)	10.69±1.22	12.14±0.11	0.25
Sperm count (10^{-6})	19.10±2.60	17.50±1.44	0.598
Testosterone (ng/ml)	5.14±1.53	7.31±0.66	0.075
LH (ng/ml)	0.37±0.05	0.73±0.3	0.322
LH:T	0.08±0.01	0.09±0.03	0.236
% motile sperm	32.36±4.37	26.64±2.46	0.277
TUNEL (pixels)	1111±414	1451±263	0.504

TABLE 2. Phenotypic analysis of F1 offspring of F0 mothers and fathers that were fed a high fat (HFD) or control diet for 14 weeks.

Data are derived from post-mortem dissection at 19 weeks of age. Biochemical data derives from 09.00 fasting plasma obtained during glucose tolerance testing at 17 weeks of age. Data was analysed by linear mixed model with group and sex as fixed factors and litter as a random factor with post hoc Bonferroni analysis. Values significantly different ($p<0.05$) from control are shown in bold. * indicates different only with least significant difference analysis (not taking multiple testing into account). ^ indicates sex not used as a fixed factor as data only available for males.

High-fat diet disrupts metabolism in two generations of rats in a parent-of-origin specific manner without affecting the intra-testicular germ-cell transcriptome

Chambers TJG, Morgan MD, Heger AH, Sharpe RM, Drake AJ.

Supplementary information

Figure S1 Validation of the purity of FACS-sorted GCs.
Relative expression (RT-qPCR) of the cell specific genes Vasa (GCs), Cdkn1b and Sox9 (Sertoli cells) and 3βHSD (Leydig cells) compared to whole testis are shown (Means ± SEM, N=4).

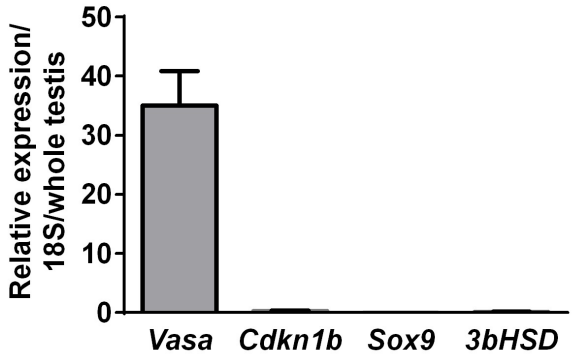


Figure S2 Effect of 14-week exposure to a high fat diet (HFD) or a control diet (CD) on the GC retrotransposon, repeat element and piRNA transcriptome of F0 male rats

A. The distribution of variance stabilising transformed (VST) expression values for annotated *Rattus norvegicus* piRNAs and expressed repeat elements was unaffected by diet and was highly consistent across biological replicates. The bimodal distribution of repeats expression was more similar to that of protein-coding genes than either miRNAs (Figure 3) or piRNAs. **B**. Hierarchical clustering of expression correlation of piRNAs (top) and repeat elements (bottom) between samples indicated that the HFD and CD samples were highly similar. **C**. Principal component analysis (PCA) across expressed piRNAs (top) and repeats (bottom) was unable to distinguish between the transcriptomes. **D**. Differential expression testing detected no differences in either the piRNA (top) or repeat element (bottom) expression.

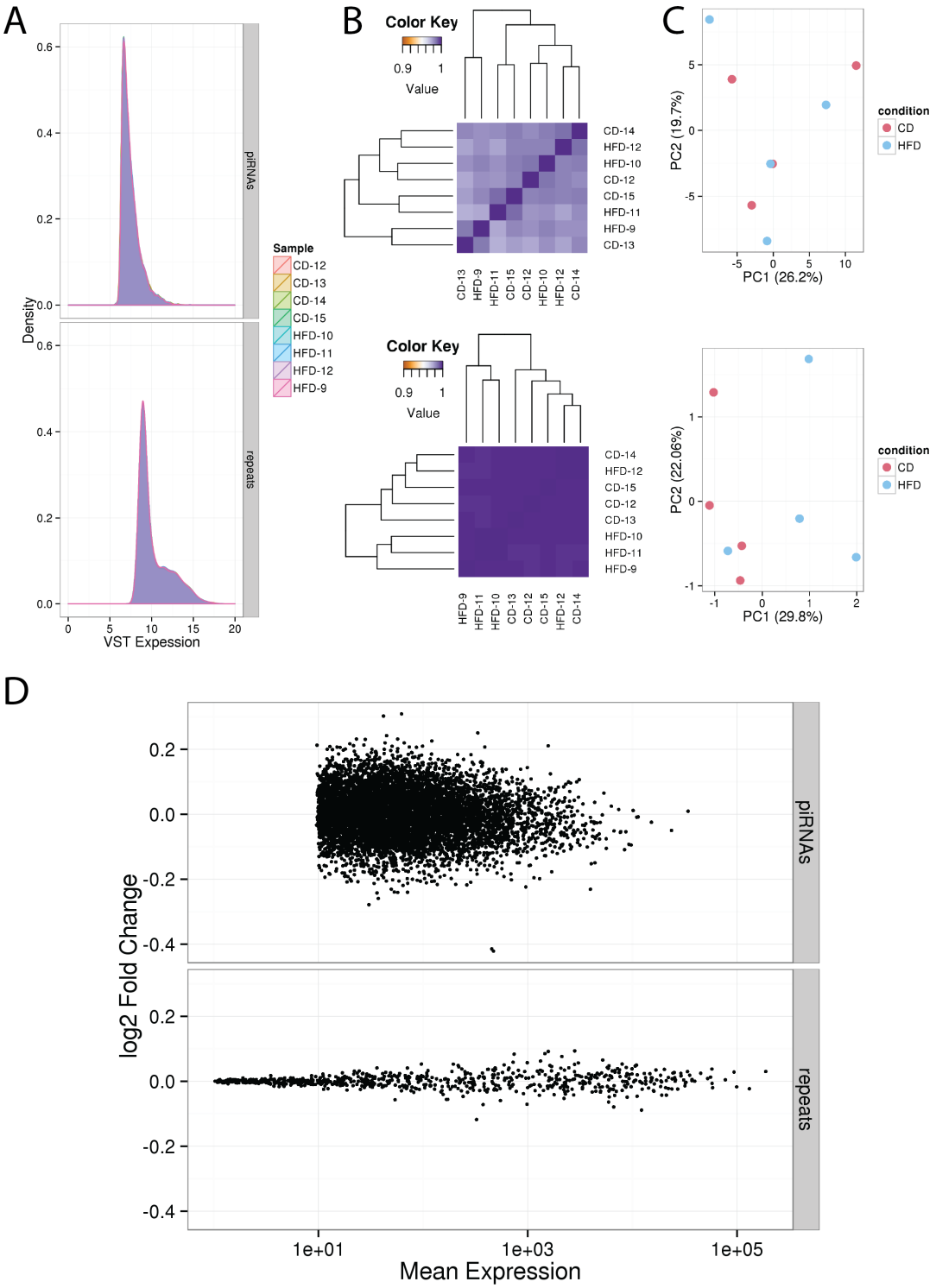

A
Density
VST Expression
piRNAs
repeats
Sample
CD-12
CD-13
CD-14
CD-15
HFD-10
HFD-11
HFD-12
HFD-9
B
Color Key
Value
C
PC1 (26.2%)
PC2 (19.7%)
PC1 (29.8%)
PC2 (22.06%)
condition
CD
HFD
D
log2 Fold Change
Mean Expression
piRNAs
repeats

Figure S3 Rat bodymap data demonstrate large tissue differences in mRNA expression between liver and testes

A. Distribution of expression values for annotated *Rattus norvegicus* protein-coding mRNAs displayed obvious differences between tissues. **B**. Hierarchical clustering of protein-coding gene expression separated samples by tissue type, in contrast to that found for comparison of CD- and HFD-fed rats (Figures 4B, 5B and supplementary figure 2B-3). **C**. Principal component analysis across expressed protein-coding genes differentiated between liver and testis samples, with 90% of the variation in expression explained by tissue differences. **D**. Differential expression testing detected large differences in gene expression between liver and testis tissue in the Rat Bodymap dataset that was not observed in the transcriptome components of the GCs of rats fed CD or HFD diets (red dots indicate significance with adjusted p-value ≤ 0.05).

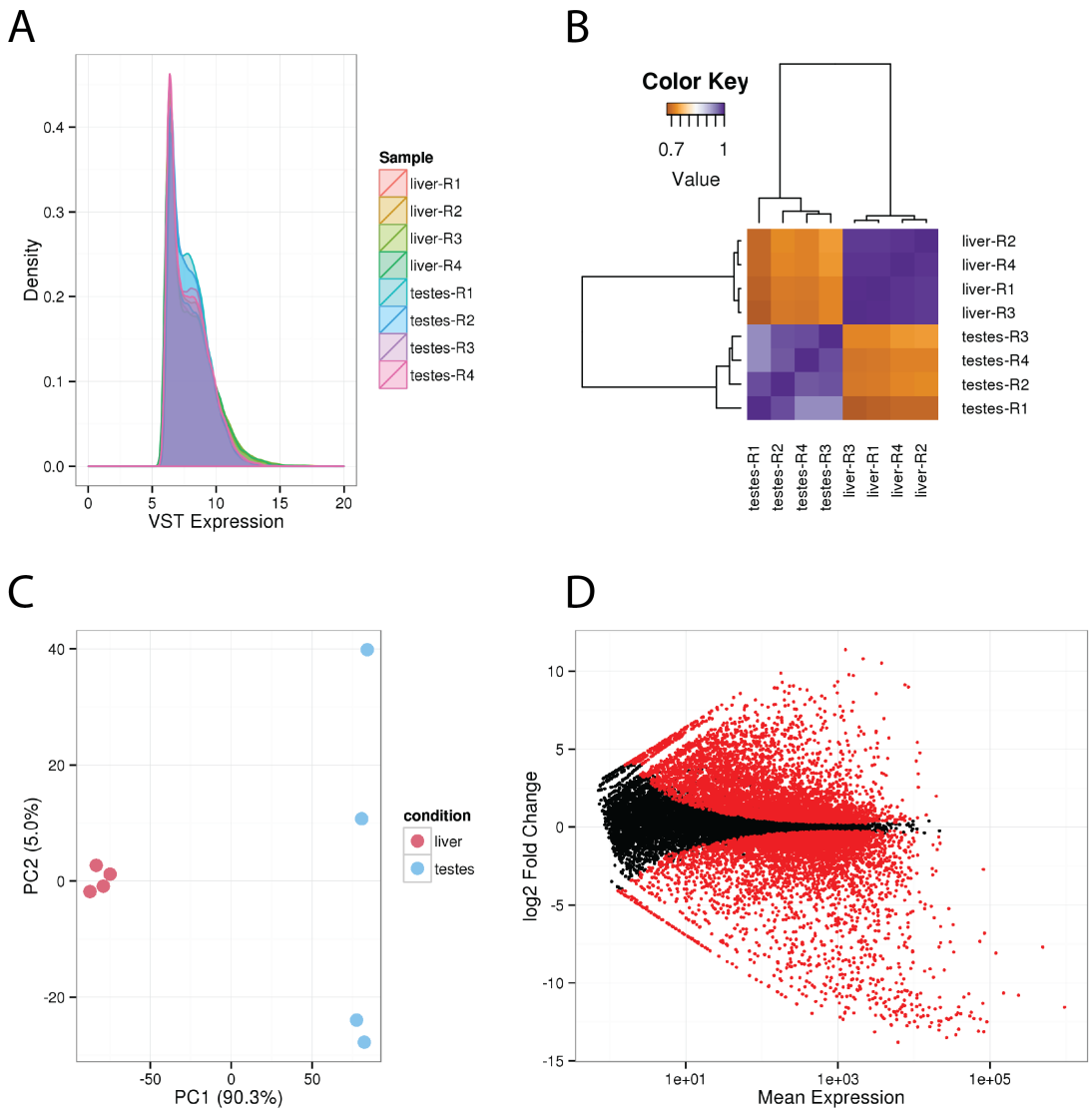


Figure S4 Comparison of RNAseq data from FACS sorted GCs in the present study with the top 50 up- and down-regulated genes identified from microarray analysis of whole testis extracts from mice following exposure to HFD from Fullston *et al.* (ref. 6). Spearman R correlation was used to compare the two data sets.

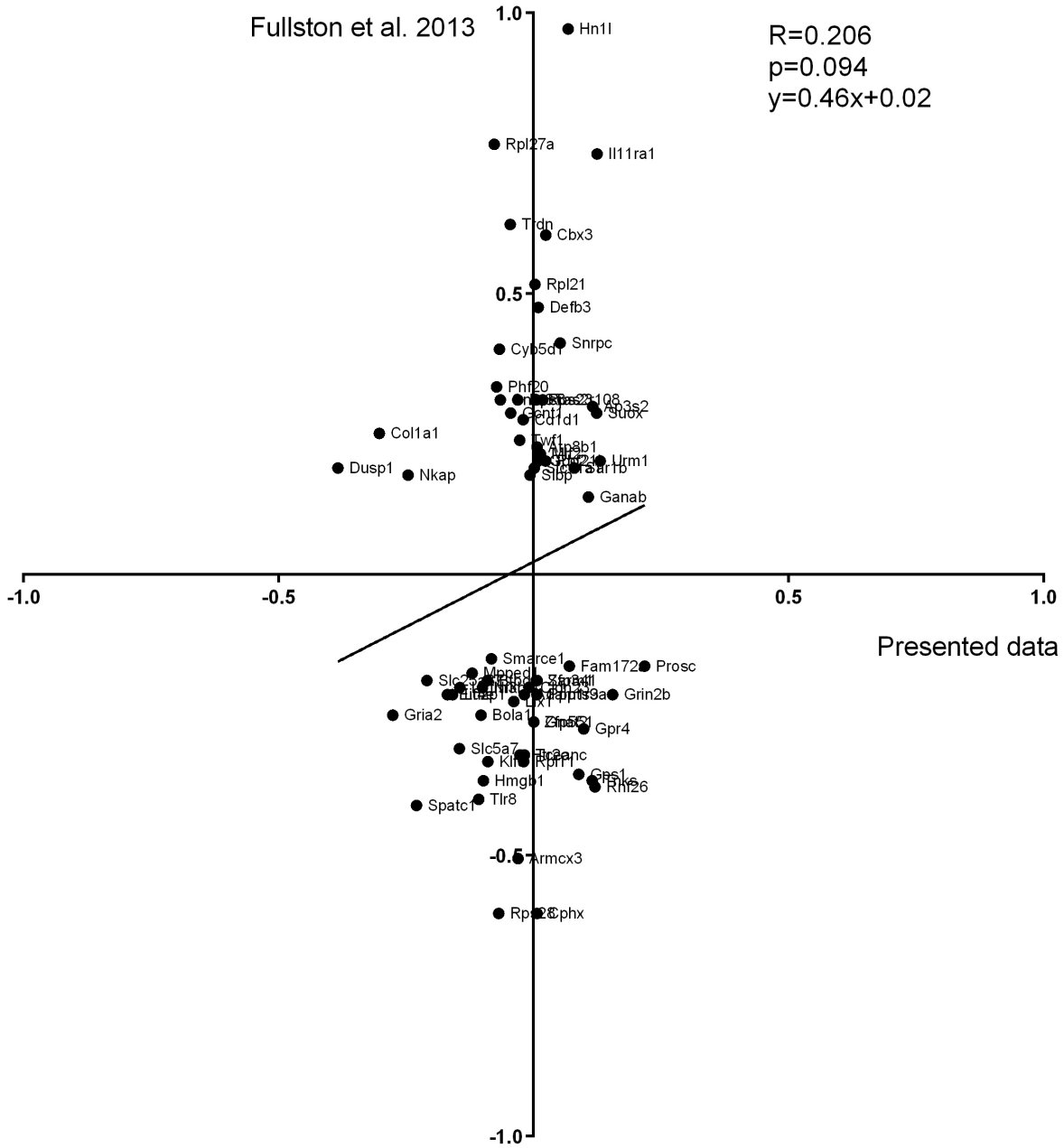
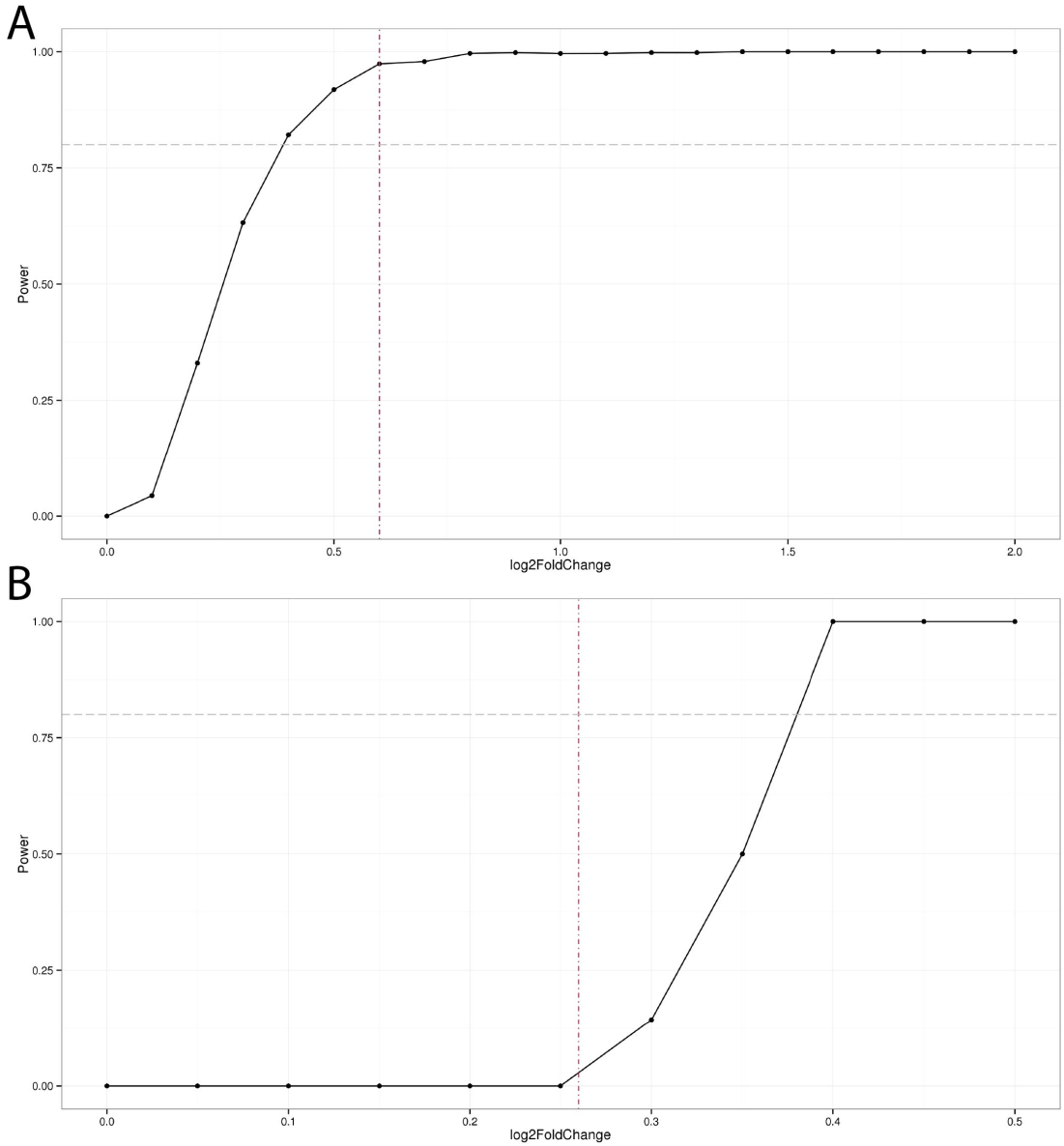


Figure S5 Power curves of spike-in experiments. **A**. Spike-in analysis of protein-coding genes demonstrated sufficient statistical power (80%) to detect estimated absolute log2 fold changes greater than ~|0.4| (horizontal grey line). The smallest observed estimate was ~|0.6|, shown by the vertical red line, indicating there was sufficient statistical power to detect a wide range of log fold changes. **B**. Spike-in analysis of miRNA data showed lower statistical power for the smallest observed difference, but there was sufficient power (grey line) at a similar level as for the protein-coding genes (~|0.4|). This suggests the sample size used in this study would have sufficient power to detect any observations above this threshold.



	HFD	CD
Research Diets ™ #	D06071701	D06072701
Cysteine (% w/w)	0.35	0.28
Casein (% w/w)	23.31	18.96
Corn-starch (% w/w)	8.48	29.86
Maltodextrin (% w/w)	11.65	3.32
Sucrose (% w/w)	20.14	33.17
Cellulose (% w/w)	5.83	4.74
Corn oil (% w/w)	2.91	2.37
Mineral mix (% w/w)	1.17	0.95
Vitamin mix (% w/w)	1.17	0.95
Lard (% w/w)	20.68	1.9
calories/100g	473	385
% of total energy from carbohydrate	35	70
% of total energy from protein	20	20
% of total energy from lipid	45	10

Table S1. Composition of control (CD) and high fat (HFD) - soya free diets, which were obtained from Research Diets; some of the carbohydrate was replaced by fat (lard) in the HFD.

	CD	Mother HFD	Father HFD	
litters	5	5	5	
	Mean±SEM	Mean±SEM	Mean±SEM	p
Litter size	10.20±1.39	12.60±0.87	12.40±0.75	0.236
Birthweight (g)	6.85±0.14	6.67±0.22	6.64±0.32	0.794
% males per litter	48.2±10.7	29.4±3.4	48.5±3.5	0.114
Days to plug	5.33±1.15	3.17±0.17	3.50±0.67	0.136
Gestation (days)	22.8±0.2	22.5±0.2	22.6±0.2	0.638

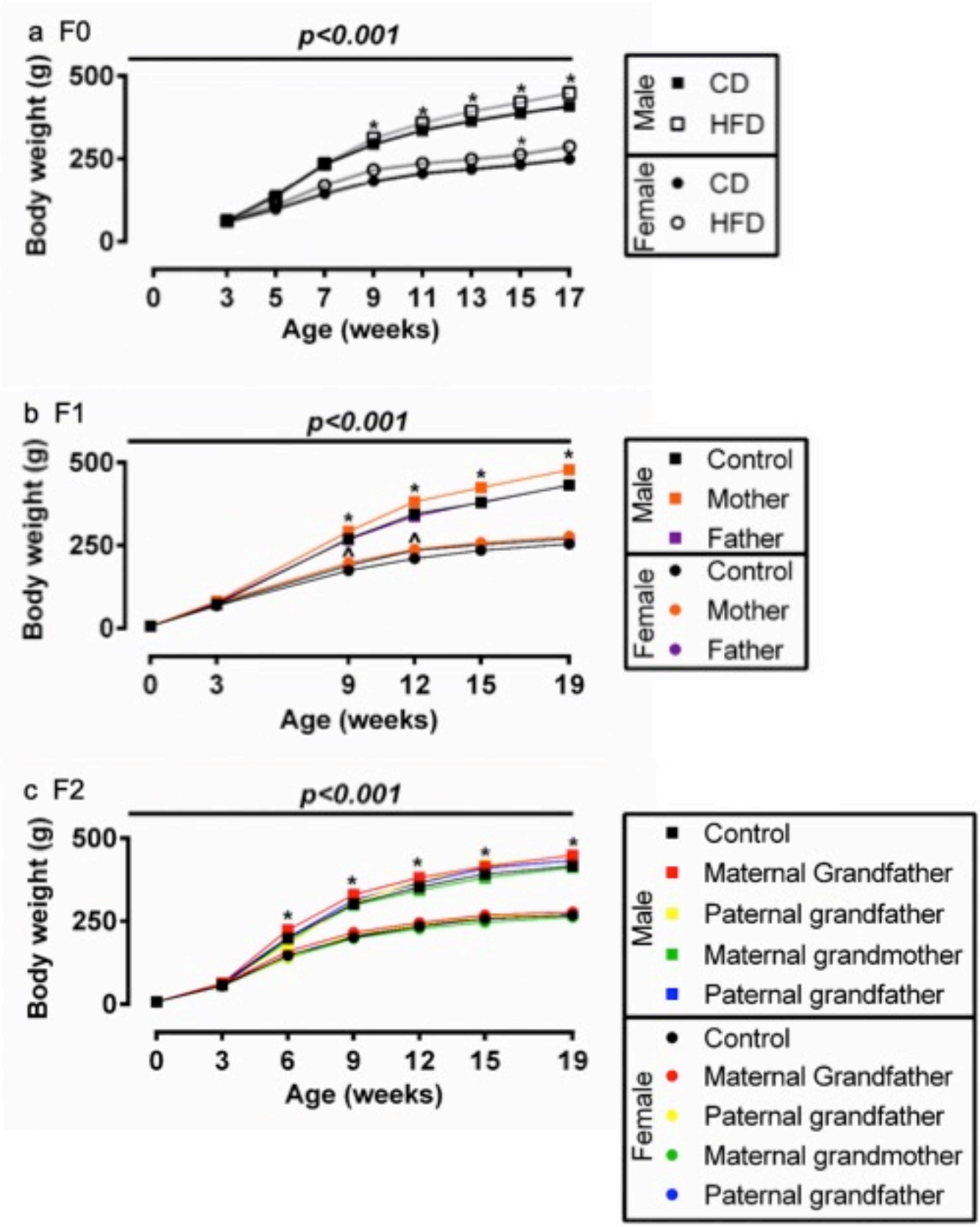
Table S2. Demographics of F1 litters born to F0 rats in which the mother or father had been exposed for 14 weeks to a control (CD) or high fat diet (HFD).

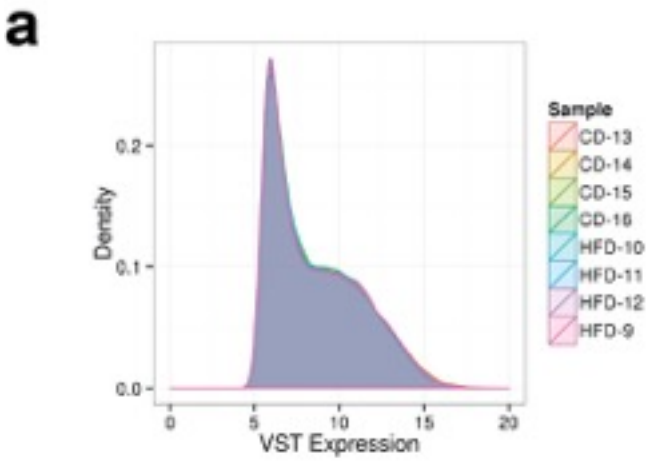
	Control	Maternal Grandfather	Maternal Grandmother	Paternal Grandfather	Paternal Grandmother	
Litters	7	4	4	5	5	
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	p
Birthweight (g)	6.93±0.24	6.86±0.42	6.89±0.31	7.05±0.32	6.85±0.19	0.986
% male pups	46.5±2.8	58.2±4.0	46.0±5.6	55.0±8.4	58.6±7.6	0.384
Litter size	12.57±0.48	12.5±1.66	12.25±1.49	11.8±0.58	11.2±2.13	0.934

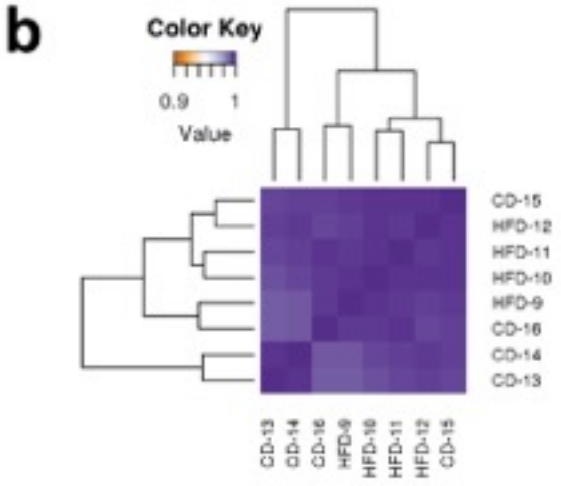
Table S3. Demographics of F2 litters according to the diet of the maternal or paternal grandparent. Top row indicates which grandparent consumed a high fat diet (HFD). Control indicates grandparents and parents consumed the CD. Animals consumed a CD unless otherwise stipulated.

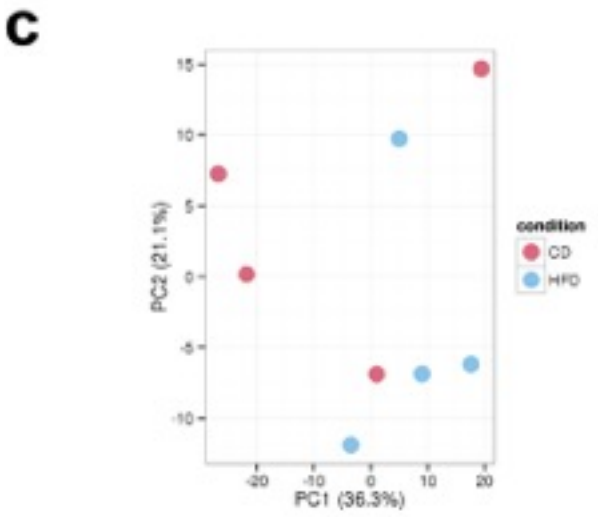
Primer	**F**	**R**	**Probe**
Col3a1	cctgcaggaaaggatgga	gaggtccaggcagtccac	80
Dcn	ctccgagtggtgcagtgtt	gcaatgttgtgtcaggtgga	115
Gsn	ctggccaagctctacaaggt	agccacgagggagactgac	16
Ldha	gatctcgcgcacgctact	cacaatcagctggtccttgag	129
Ropn1L	catcctcaagcagttcacca	tacgggaagtgggtctcct	121
vasa	cattcagaagaggtgggagaga	tgctggtttcctagaaccaaa	77
3bHSD	gaccagaaaccaaggaggaa	ctggcacgctctcctcag	105
Sox9	atcttcaaggcgctgcaa	cggtggaccctcagattg	63

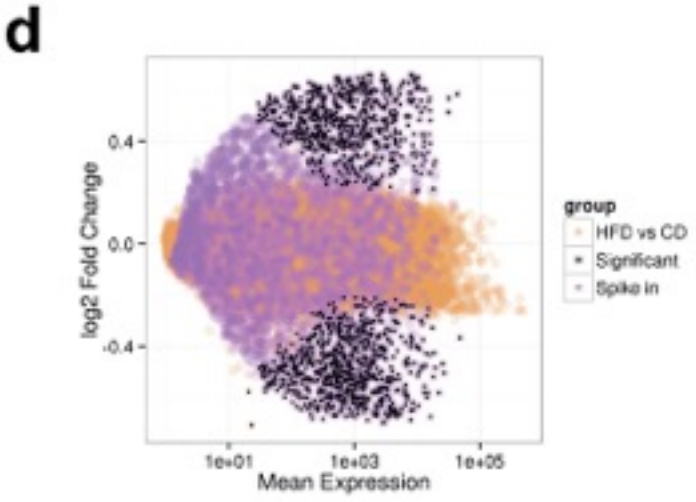
Table S4. Primers and probes used for qPCR


a F0
p<0.001
Body weight (g)
500
250
0
0 3 5 7 9 11 13 15 17
Age (weeks)
Male
CD
HFD
Female
CD
HFD
b F1
p<0.001
Body weight (g)
500
250
0
0 3 9 12 15 19
Age (weeks)
Male
Control
Mother
Father
Female
Control
Mother
Father
c F2
p<0.001
Body weight (g)
500
250
0
0 3 6 9 12 15 19
Age (weeks)
Male
Control
Maternal Grandfather
Paternal grandfather
Maternal grandmother
Paternal grandfather
Female
Control
Maternal Grandfather
Paternal grandfather
Maternal grandmother
Paternal grandfather



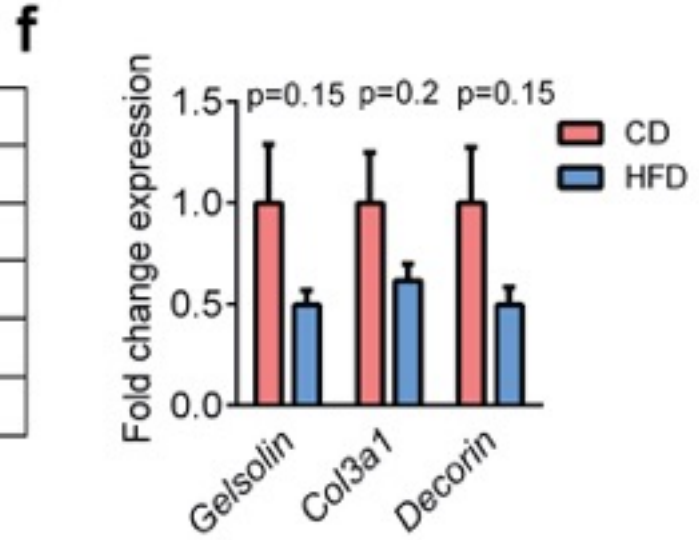




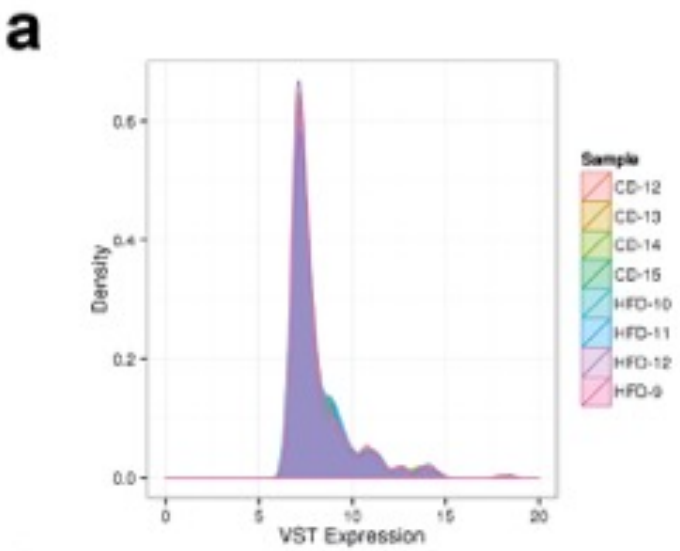


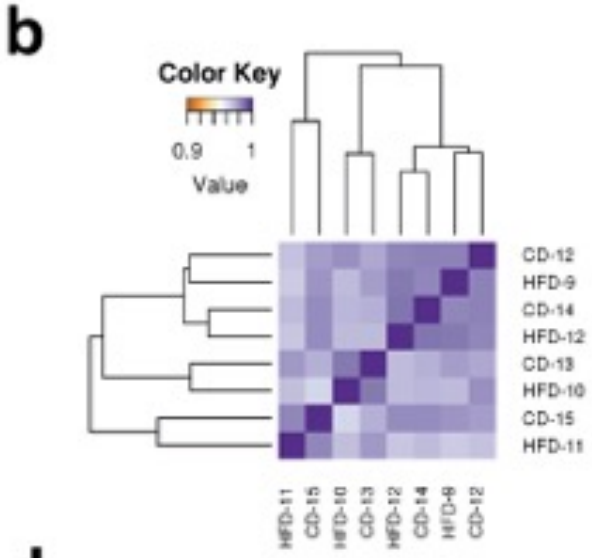
e

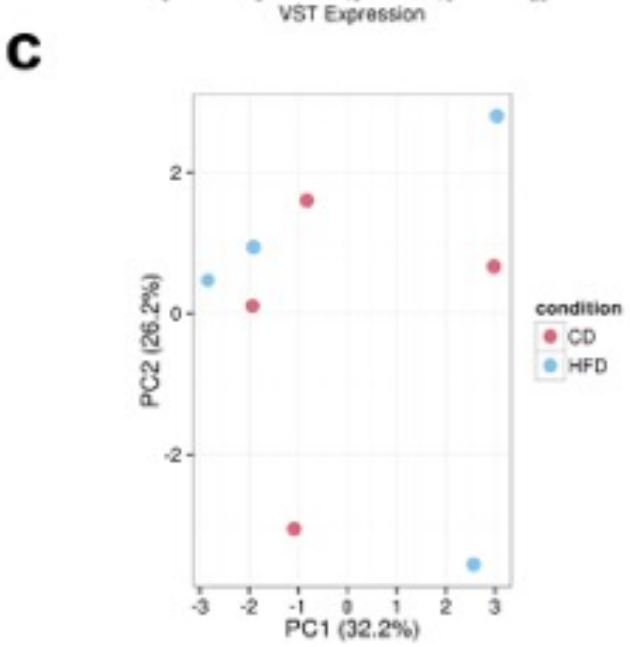
Gene	Mean Expression	log2 fold change	p-value
Col3a1	22.92	-0.71	**0.004**
Gsn	128.43	-0.59	**0.021**
Dcn	20.55	-0.58	**0.037**
Anxa1	11.99	-0.49	0.056
Lyc2	123.39	-0.57	0.056

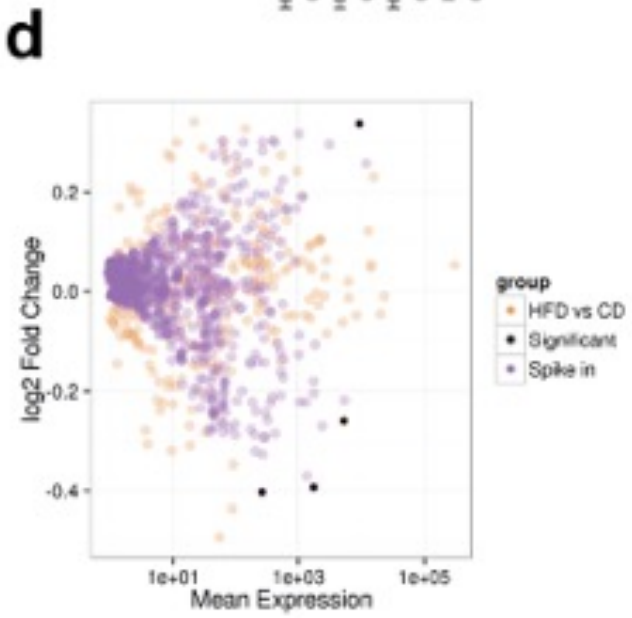


Sex	Male			Female			
Group	Control	Maternal HFD	Paternal HFD	Control	Maternal HFD	Paternal HFD	p
N (litters) unless stated below	19-32 (9)	13-19 (5)	10-17 (5)	10-17 (9)	14-20 (5)	13-21 (5)	
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	
Body size							
Weight (g)	434±4	**478±10**	431±8	260±5	278±4	270±4	**0.003**
Length (cm)	23.8±0.2	24.1±0.3	23.5±0.3	21.2±0.2	21.1±0.2	21.2±0.2	0.114
AGD (mm)	47.8±0.5	45.9±2.2	44.8±0.8	21.3±0.3	19.3±0.8	18.58±0.8	0.945
Adiposity							
Gonadal fat/bw (mg/g)	7.7±0.3	7.6±0.3	6.7±0.4	6.3±0.3	7.2±0.7	6.2±0.5	0.062
Retroperitoneal fat/bw (mg/g)	5.3±0.3	5.0±0.4	4.5±0.4	5.1±0.3	4.9±0.6	3.7±0.3	0.177
Adiposity index (mg/g)	12.9±0.6	12.6±0.7	11.2±0.7	11.4±0.5	12.1±1.2	10.0±0.6	0.062
Biochemistry							
Leptin (ng/ml)(n=5)	1.30±0.29	1.72±0.53	0.94±0.12				0.346^
Insulin AUC (µg/l.min) (n=5)	108.6±21.6	76.65±11.1	77.9±14.7	51.39±10.4	65.7±13.3	47.05±9.8	0.249
Glucose AUC (mM.min) (n=5)	984±32.4	931.2±36.5	951±39.3	931.2±36.5	938.4±24.0	904±73.3	0.846
Reproduction							
Penis length (mm)	12.1±0.08	11.8±0.18*	11.7±0.16*				**0.023^**
Gonad weight (g)	1.90±0.02	2.00±0.07	1.90±0.07	0.08±0.00	0.08±0.00	0.12±0.02	0.148
Testosterone (ng/ml) (n=5)	4.22±0.64	**7.38±0.90**	2.73±0.51				**0.002^**
LH (ng/ml) (n=5)	0.38±0.06	0.34±0.04	0.26±0.04				0.255^
LH:T ratio (n=5)	0.10±0.03	0.05±0.01	0.11±0.03				0.234^



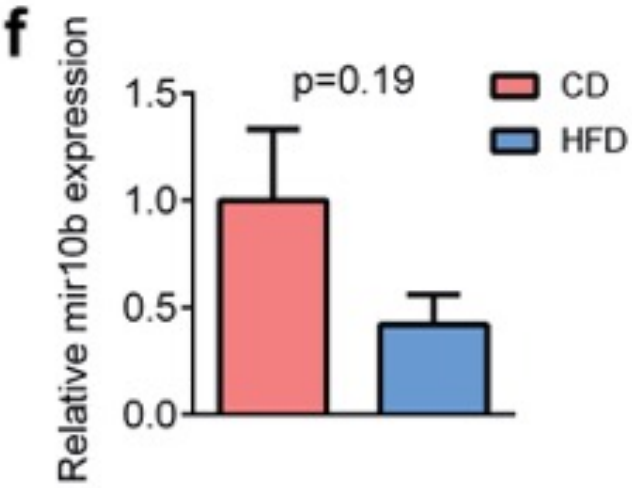






e

miRNA	Mean Expression	log2 Fold Change	p-value
rno-mir-10b	5258.49	-0.26	**2.2×10^{-7}**
rno-mir-871	520.04	0.27	0.08
rno-mir-103-2	532.91	0.17	0.60



	Male					Female					
	Control	Maternal grandfather	Maternal grandmother	Paternal grandfather	Paternal grandmother	Control	Maternal grandfather	Maternal grandmother	Paternal grandfather	Paternal grandmother	p
N (litters)	14 (7)	8 (4)	8 (4)	10 (5)	10 (5)	14 (7)	8 (4)	8 (4)	10 (5)	10 (5)	
Body size											
Weight (g)	416±5.2	448±13.4	446±8.8	412±11.4	433±9.5	268±7.6	277±5.4	269±7.5	262±5.9	271±5.3	0.124
Length (cm)	23.3±0.1	23.5±0.2	23.5±0.2	22.8±0.1	23.4±0.2	21.1±0.2	20.9±0.1	21.0±0.2	20.7±0.2	21.1±0.2	0.613
Organ weights											
Liver/bw (mg/g)	43.2±0.8	40.8±0.5	42.3±1.4	42.8±1.2	44.4±1.4	38.9±0.9	37.0±1.5	34.3±1.5	38.4±1.4	37.6±1.1	0.24
Pancreas/bw (mg/g)	2.6±0.1	2.4±0.2	2.1±0.1	2.3±0.1	2.2±0.1	3.5±0.1	3.5±0.2	3.8±0.4	4.0±0.2	3.0±0.2	**0.007**
Adiposity											
Retroperitoneal fat/bw (mg/g)	4.8±0.4	**6.6±0.5**	5.1±.4	6.3±0.5	6.1±0.4	4.1±0.5	3.6±0.2	3.9±0.7	4.9±0.3	5.2±0.3	**0.003**
Gonad fat/bw(mg/g)	6.6±0.3	8.4±0.6*	7.0±0.2	7.9±0.5	6.9±0.4	5.9±0.5	5.2±0.3	5.9±0.4	6.3±0.4	6.3±0.5	**0.017**
Adiposity index (mg/g)	18.0±1.0	**23.4±1.7**	19.0±0.7	22.0±1.3	19.9±1.0	16.0±1.3	14.0±0.6	15.7±1.3	17.4±0.9	17.8±1.2	**0.012**
Biochemistry											
Insulin AUC (µg/l.min)	90.2±10.7	148.7±18.2*	104.0±7.1	119.0±13.3	128.5±18.6	60.3±7.4	86.2±16.2	48.4±2.9	103.3±8.4	72.0±11.9	**0.016**
Glucose AUC (mM.min)	964±18.5	904±6.6	947±36.5	954±10.7	966±6.4	947±24.1	931±18.9	921±27.7	1146±13.4	978±9.1	0.15
Leptin (ng/ml)	2.5±0.3	**5.0±0.8**	3.3±0.2	3.6±0.5	2.9±0.4	1.8±0.2	1.7±0.2	1.9±0.2	1.6±0.1	1.9±0.2	**0.001**
Cholesterol (mM)	1.08±0.03	1.16±0.05	1.03±0.07	1.18±0.05	1.03±0.04	1.02±0.04	1.10±0.08	1.06±0.06	1.25±0.06	1.08±0.07	0.474
Triglycerides (mM)	1.08±0.05	1.3±0.189	1.54±0.13	1.16±0.10	1.27±0.12	0.82±0.05	0.78±0.04	1.21±0.12	0.83±0.07	1.14±0.13	0.106
Reproduction											
LH (ng/ml)	0.6±0.1	1.2±0.2	0.7±0.7	1.0±0.2	1.0±0.2						0.250^
Testosterone(ng/ml)	8.5±0.5	6.1±0.8	8.6±1.4	8.7±1.4	7.80±0.6						0.270^
LH:T ratio	0.07±0.01	**0.21±0.04**	0.09±0.02	0.13±0.04	0.12±0.02						**0.017^**
Penis Length (mm)	12.1±0.1	12.3±0.2	12.5±0.2	12.3±0.2	12.3±0.1						0.614^
Sperm count (10^6)	6.0±12	36.3±7.5	22.4±7.3	33.2±8.8	23.3±7.7						0.629^
AGD (mm)	48.6±0.7	49.5±0.9	49.9±1.1	47.8±0.8	50.9±0.6	21.3±0.5	23.1±0.2	21.7±0.4	22.4±0.4	22.7±0.4	0.121

TABLE 3. Phenotypic analysis of F2 offspring of grandparents (F0 mothers and fathers) that were fed a high fat (HFD) or control diet for 14 weeks.

Data are derived from post-mortem dissection at 19 weeks of age. Biochemical data derives from 09.00 fasting plasma obtained during glucose tolerance testing at 17 weeks of age. Data was analysed by linear mixed model with group and sex as fixed factors and litter as a random factor with post hoc Bonferroni analysis. Comparing groups within each sex, values significantly different ($p<0.05$) from control are shown in bold. * indicates different only with least significant difference analysis (not taking multiple testing into account). ^ indicates sex not used as a fixed factor as data only available for males.