# Supporting Information

# Maternal overnutrition programs hedonic and metabolic phenotypes across generations through sperm tsRNAs

## Authors

[1]Gitalee Sarker,[1]Wenfei Sun, [2]David Rosenkranz, [3]Pawel Pelczar, [4]Lennart Opitz, [1]Vissarion Efthymiou, [1]Christian Wolfrum, [1*]Daria Peleg-Raibstein

## Affiliations

[1]Laboratory of Translational Nutrition Biology, Department of Health Sciences and Technology, ETH Zurich, Schwerzenbach, 8603 Switzerland.

[2]Institute of Anthropology, Johannes Gutenberg University Mainz, 55099 Mainz, Germany.

[3]Center for Transgenic Models, University of Basel, Basel, Switzerland.

[4]Functional Genomics Center Zurich, ETH Zurich, Switzerland.

*Correspondence to: daria-peleg@ethz.ch

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## Methods

### Mice

Male and female C57BL/6N mice were purchased from Charles River (Charles River, Germany) at the age of 10 weeks and were housed in cages at temperature of 21 ± 1 °C and humidity of 55 ± 5%. All mice were given standard chow (Kliba 3430) and water ad libitum and were maintained on 12:12h reversed light–dark cycle with lights on at 7.00 pm. All animal experiments and procedures were approved by the Zurich Cantonal Veterinarian's Office, Switzerland.

### Feeding and breeding design

To generate F1 offspring, 40 female mice (F0) were randomly divided into two groups (20 in each group) to receive either a HFD (60% energy from fat, Diet Kliba 2127, energy content = 22 kj/g, protein (w/w): 26%, carbohydrates: 1%, fat: 38%) or a standard chow diet (Kliba 3430, energy content = 13 kj/g, protein (w/w): 21%, carbohydrates: 39.8%, fat: 5%) for a total duration of 9 weeks (3 weeks prior to conception, 3 weeks gestation and 3 weeks lactation). The shorter duration of HFD exposure led the mother not to develop any obesogenic phenotypes as well as any alteration in maternal behavior during gestation and lactation (1). The 20 male breeders (F0) kept on standard chow diet were randomly chosen and allocated as one male per 2 female breeders during the mating. The males were removed from the mating cage once the pregnancy was confirmed by vaginal copulation plug assessment to exclude paternal effect. Upon weaning at postnatal day 21, F1 offspring were housed as four littermates of same sex per cage and were provided with chow diet and water ad libitum throughout their life.

### Sperm collection, RNA extraction, small RNAs isolation and sperm RNAs microinjection

### Sperm isolation

Total sperm RNAs or separated RNA fractions (tsRNA, tRNA and T40 RNA) were adjusted to a concentration of 2ng/ul and was microinjected individually into the male pronuclei of fertilized mouse oocytes until 20-30% distension of the organelle was observed. Embryos that survived the microinjection were transferred on the same day into the oviducts of pseudopregnant females that had been mated with sterile vasectomized males (1) the day before embryo transfer. Pregnant females delivered and raised their pups until weaning. To collect purified motile sperm, we followed 'Swim-up' purification method as described elsewhere (2). Mature sperm was isolated from cauda epididymis of F1 male mice (N = Total RNA, 8 HFD and 8 CTR; sncRNAs, 12 HFD and 12 CTR). After careful removal of the both epididymis and vas deferens with the least amount of fat attached, the membrane of cauda epididymis was punched and squeezed to press out the sperm mass. The sperm mass was then collected in M2 medium (Sigma Aldrich, M7167) and incubated for 1h at 37◦C to capacitate the sperm, after which the upper 2/3$^{rd}$ of the fluid was collected to eliminate somatic cell contamination. Purity of the supernatant was confirmed by observing the live motile sperm under a light microscope. Finally, the sperm was pelleted by centrifugation at 10000 rpm at 4◦C for 10 minutes and stored at -80◦C.

### RNA extraction

Total RNA from sperm sample was extracted using the Trizol-chloroform method (Invitrogen, CA, USA) according to the manufacturer's instruction. The sperm pellet was suspended in Trizol reagent, treated with chloroform, homogenated, after which the upper aqueous solution containing RNA was carefully

collected. The RNA sample was then treated with DNAse (Biolabs INc, CA, USA) for 30 min at 37°C to exclude any DNA contamination. The final RNA pellet was suspended in 20ul RNAse free water and stored at -80◦C.

## Sperm small RNAs isolation

Isolated sperm total RNA was dissolved in denaturing RNA loading buffer and heated 65◦C for 5 min before loading into the gel. 10-15 ug of total RNA was loaded on a vertical 6% TBE gel with 19:1 acrylamide: bis-acrylamide, 8M urea, 1× TBE buffer and was run at 15 W to ensure optimal temperature (~60◦C) for sample denaturation. Prior to loading the samples, the gel was pre-run for 45 min. The approximate location of the desired RNA fractions was determined by the position of small RNA markers (NEB) which were visualized by illuminating the gel with long wave UV light on a fluorescent indicator plate. Each RNA fragment was excised together with a gel slice and placed separately in the RNA elution buffer (20 mM Tris-HCl (pH 7.5), 300 mM sodium acetate, 2 mM EDTA, 0.25% SDS). The elution was carried out overnight at room temperature. In the purification step, the eluted sample was collected in a fresh tube and the RNA was precipitated with 3 volumes of 96% ethanol. The pellet was then washed with 70% ethanol and dissolved directly in 30ul of RNA injection buffer (1mM Tris-Cl, pH7.5, 0.1mM EDTA).

## Zygotes and early embryo collection

C57BL/6J female mice aged 4 week underwent ovulation induction by intraperitoneal injection (i.p.) of 5 IU equine chorionic gonadotrophin (PMSG; Folligon–InterVet), followed by i.p. injection of 5 IU human chorionic gonadotropin (hCG; Pregnyl–Essex Chemie) 48 h later. For the recovery of zygotes, C57BL/6J females were mated with males of the same strain immediately after the administration of hCG. All zygotes were collected from oviducts 24 h after the hCG injection, and were then freed from any remaining cumulus cells by a 1–2 min treatment of 0.1% hyaluronidase (Sigma-Aldrich) dissolved in M2 medium. Mouse embryos were cultured in M16 (Sigma-Aldrich) medium at 37°C and 5% CO2. For micromanipulation, embryos were then transferred into M2 medium.

## Sperm RNAs microinjection and embryo transfer

All microinjections were performed using a microinjection system comprised of an inverted microscope equipped with Nomarski optics (Nikon), a set of micromanipulators (Narashige), and a FemtoJet microinjection unit (Eppendorf). Total sperm RNAs or separated RNA fractions (tsRNA, tRNA and T40 RNA) were adjusted to a concentration of 2ng/ul and was microinjected individually into the male pronuclei of fertilized mouse oocytes until 20-30% distension of the organelle was observed. Embryos that survived the microinjection were transferred on the same day into the oviducts of 8–16-wk-old pseudopregnant Crl:CD1 (ICR) females (0.5 d used after coitus) that had been mated with sterile genetically vasectomized males (1) the day before embryo transfer. Pregnant females were allowed to deliver and raise their pups until weaning age.

## Small RNA library preparation and sequencing

The small sperm RNA fraction with nucleotide length of 30-40nt was isolated for RNA sequencing. Small RNA libraries were constructed according to TruSeq Small RNA Sample Preparation Kit (Illumina), the small RNA libraries were followed by library quality validation for sequencing. Sequencing is performed by the Function Genomic Center Zurich.

## Small RNA-seq data processing and analysis

Initial data processing was conducted using a series of Perl scripts (in brackets) which are part of the NGS TOOLBOX (3). Adapter sequences were trimmed from raw sequence reads searching for TGGAATTCTC$(N)_x$-3' motifs (clip). Sequence reads <16 nt were removed (length-filter) and datasets were collapsed to non-identical sequences while keeping information on sequence read counts (collapse). Low-complexity sequences were removed (duster) and the remaining sequences were mapped to the mouse genome (GRCm38p6) with bowtie [v.1.2, (4)] using default settings. Mapped sequences were annotated with unitas [v.1.5.0, -skip_dust, (5)], which was also used to detect differentially expressed sRNA sequences between CTL (n=3) and HFD (n=3) with Bonferroni-Holm correction of alpha error.

## tsRNA target prediction

Based on their association with PIWI proteins (6, 7), we predicted tsRNA target transcripts applying targeting rules recently described for piRNAs (8) which are more strict compared to the commonly applied miRNA targeting rules. We run miRanda (9) with mouse cDNA data from Ensembl database (release 90) and subsequently considered alignments which are in compliance with the criteria defined by Zhang and colleagues using the Perl script miRanda_wrapper which is freely available at https://sourceforge.net/projects/piranha-targetprediction/. Having identified putative targets of the differentially expressed tsRNAs we assigned a score value to each target which describes the difference in the number of molecules (measured in reads per million) targeting the transcript in HFD and CTL condition. Positive score values imply a higher number of targeting tsRNA molecules in HFD compared to CTL, which should result in downregulation of the corresponding transcript in HFD. The opposite is true for negative score values. The absolute value of the score can be used as a proxy for the likelihood that expression of the corresponding target is affected by differentially expressed tsRNAs.

## Experimental design

All the experiments were conducted when the mice reached adulthood (PND 70-110). HFD preference test, sucrose preference test and alcohol preference test were conducted chronologically with the same set of animals from each experimental group with the wash time period of at least 7 days in between tests. For the amphetamine sensitivity test, a separate set of mice was selected from each group and sex. To assess the metabolic parameters, a separate set of behaviorally naïve mice from each group was allocated. For junk food choice test, a new set of behaviorally naïve mice from HFD and control tsRNA groups were selected. The metabolic experiments were conducted in following orders: CT scan, insulin sensitivity test, metabolic cage study, plasma insulin and lipid profiles measurement. For gene expression analysis by qRT-PCR, samples were collected from a separate set of behaviorally naïve mice from each group. The sample size for each experiment was included in the figure legends and also summarized in the supplementary tables **S2-S4**.

## Behavioral experiments

### HFD preference

During the test, mice were single housed to enable the measurement of food intake. Mice received free access to laboratory chow and HFD for 3 hours with ad libitum water. The test was conducted for 4 consecutive days. The weight of each type of food was measured before and after testing. Body weight

was recorded daily. The intake of chow and HFD was calculated as an amount of each food consumption in mg per gram body weight. The average consumption during the last 3 days was used to analyze the food preference.

### Sucrose preference

The preference test was carried out in the animal home cage. The mice were presented with 2 polypropylene tubes (15ml; Sarstedt, Germany). One tube contained plain drinking water and the second one contained sucrose solution. Prior to the test, mice were caged individually and had free access to ad libitum food. Mice were habituated to drink from both tubes containing water for 2 days. On day 3, one of the drinking tubes was filled with sucrose solution and the other one with water for a period of 9 days. Three different concentrations of sucrose (0.5%, 1% and 3%) were tested and gradually increased every 3rd day. The position of the bottles was changed daily to exclude positional effects. The intake of water and sucrose solution as well as body weight were measured daily. Sucrose and water consumption was calculated as total sucrose or water consumption in mg per gram body weight per day and averaged over the 3 days intake for each concentration.

### Alcohol preference

The same protocol was used as for the sucrose preference test. After the habituation period, mice were given a choice to drink from two tubes- one containing water and the other containing either 2%, 5% or 8% ethanol. The mice were exposed to each concentration of ethanol in ascending order for 3 days. The water and ethanol consumption as well as body weight were measured daily. The consumption was calculated as total alcohol consumed in mg per gram body weight and averaged over the 3 days intake for each concentration.

### Amphetamine sensitivity

The test was conducted in the open field paradigm. The test apparatus consisted of four square shaped arenas (40cm×40 cm×35cm), made from grey colored plastic laminated wood. A digital camera was mounted on top of the apparatus to capture images at a rate of 5Hz which were processed by the Ethovision software (Noldus, Wagening, Nenederlands). At the beginning, basal locomotor activity was recorded for 30 min. The mice were then briefly removed and injected with 0.9% NaCl solution (intraperitoneal; i.p) and tested for another 30 min. Following that, mice received an i.p injection of d-amphetamine sulfate (2.5mg/kg body weight dissolved in 0.9% NaCl; i.p). The locomotion was tested for 2h after the drug challenge. Data were indexed by the distance travelled in the entire arena and summed for each successive 10 min bins.

### Metabolic phenotype

### Body weight

Body weights of each offspring were measured weekly from post-natal week 3 up to post-natal week 11 with an electronic scale (Mettler PM 2000).

### Insulin tolerance test (ITT)

The mice were fasted for 6 hours before the experiment. During the test, an i.p injection of insulin (Actrapid; Novo Nordisk A/S) at a dose of 0.75 unit/kg body weight for chow treated males, 2 unit/kg body

weight for junk treated males, 0.65 unit/kg body weight for chow treated females and 0.85 unit/kg body weight for junk treated females was administered. Blood glucose was measured before (0 min) and 15, 30, 60, 90 and 120 min after the insulin challenge using Accu-Chek Aviva device (Roche).

## Fat distribution assay

The distribution of body fat was measured using a computed tomography (CT) X-ray scanner (La Theta LCT-100; Aloka Inc, Japan). The scanner consisted of a cylindrical view of 48 mm by 41 mm with a pixel resolution of 100 µm on 480 × 480 pixel images. The X-ray source was set at 50 kV with a constant 1mA current. Anesthetized mice were laid on supine position in on the transparent mouse holder with the caudal end held towards the CT and the rostral end towards the anesthetic tube where the gas anesthesia was maintained with 1.5% - 2.5% isoflurane. The scan area was set based on sagittal pre-scan and fixed anatomical landmarks. A whole body scan excluding the head and tail was performed at a rate of 4.5 s/image for 5 min. The images were analyzed using the La Theta 2.10 software.

## Metabolic cage study

TSE Phenomaster cages (TSE Systems GmbH, Bad Homburg, Germany) were used for the measurement of energy expenditure. Mice were single housed for 2 days prior to the experiment for acclimatization to the new cage environment. Mice were individually placed in the Phenomaster metabolic cage unit for a total of 3 days. The temperature was set to 23 °C and the reversed light/dark cycle 12:12 h (lights off at 07:00 and lights on at 19:00) was maintained throughout the experiment. Mice had food and water ad libitum. $O_2$ consumption and $CO_2$ production were calculated from the gas concentrations and the air flow through the individual cage measured in parallel. Heat production, locomotor activity and food intake were also monitored during this period. Data were analyzed with Phenomaster software. $O_2$ consumption, $CO_2$ production and heat production was normalized to lean body mass.

## Plasma parameters measurement

Plasma sample was collected from 6h fasted mice. Plasma insulin and leptin were measured using commercially available ELISA kits designed for mouse (Mouse Insulin ELISA Kit, Crystal Chem, USA; Murine Leptin ELISA Kit, Invitrogen). Plasma cholesterol (Roche, Switzerland), triacylglycerol (Hitachi, Switzerland) and non-esterified fatty acids (Wako, Germany) were analyzed colorimetric assay.

## Junk food choice test

In the junk food preference test, five weeks old HFD-tsRNA and CTR-tsRNA offspring were given free access to HFD and 1% sucrose solution together with regular chow and water for a duration of 12 weeks. On the starting day, the body weight of the mice was measured (week 0). The control groups received equivalent amount of chow and two bottles of water for the same duration. Body weight and consumption of junk foods, chow and water were measured every week. At the end of the experiment, distribution of fat, insulin sensitivity, energy expenditure and plasma parameters were evaluated.

## Dissection of brain regions

Adult F1 father and tsRNA offspring from both HFD and CTR groups were euthanized, the brains were rapidly isolated, and 1 mm thick coronal sections were made. The slices were placed on an ice-chilled dissection plate for the extraction of the brain regions using a 1 mm micropunch. All brain areas of interest

were dissected according to the Mouse Brain Atlas in Stereotaxic Coordinates (Paxinos and Franklin, 2008) which were as follows: dorsal striatum (bregma +1.34 to +0.14 mm), nucleus accumbens (bregma +1.60 to +0.98 mm) and ventral tegmental area (bregma −2.92 to −3.64 mm) and immediately frozen at −80°C.

## Gene expression analysis

Total RNA from brain tissue was extracted using the Trizol-chloroform method (Invitrogen, CA, USA) according to the manufacturer's instruction and treated with DNase (Biolabs INc, CA, USA) for 30 min at 37°C to exclude any DNA contamination. Following RNA extraction, 1µg of total RNA was converted to cDNA using High Capacity cDNA Reverse Transcription Kit [Applied Biosystems (ABI), Foster City, CA)]. The RT-qPCR reactions were performed using the SYBER Green master mix (ABI) in the presence of specific primer pairs on an Applied Biosystems ViiA$^{TM}$ 7 RT-qPCR system. Data for tissue samples were normalized to Endogenous control m36B4. RT-qPCR reactions were conducted as follows: after a pre-denaturation and polymerase-activation program (5min at 95◦C), 40 cycles each consisting of 95◦C for 3 sec and 60◦C for 30 sec were followed by a melting curve program (60 to 95◦C with heating rate of 0.1◦C/sec). The relative expression of mRNA was calculated using the 2- ΔΔCt method. All primers were purchased from Microsynth AG, Switzerland. The primers are listed in **Supplementary Table 1.**

## Supplementary Table 1

| Gene Name | Primer Forwar (5'-3') | Primer Reverse (5'-3') |
|---|---|---|
| ZCCHC11 | AACATGCACCACGCCATTTC | GTGGCACATCTGTCTGCTTG |
| CHRNA2 | TGCTGACTCTTCGGTGAAGG | TCCCCAGGAAGCAGACGATA |
| DHRS3 | TGCACGTCAAAAGCATCAGC | GGTTGGGAAACCTGACTCTCA |
| VAV3 | AAAAGATTTCTGACGGCGGC | TGCCCATAAATAACCAATCTTTCC |
| EEFA1 | ATATAAGTGCGGCAGTCGCC | CACAACACCTGCGTTCTGA |
| DAB2IP | CATGAACGCGCAGTTGTTAGA | CTGCCAGATCCTTTTCTGCTTG |
| GRIN3A | ACGTGTGGAAAAGAGCAGATG | CGTTGGTTGTCGTGACTCAG |
| m36B4 | GCCGTGATGCCCAGGGAAGA | CATCTGCTTGGAGCCCACGTT |

**Table S1:** Summary of the primers used for gene expression analysis in brain.

## Protein extraction and Western blot

dSTR (dorsal striatum), VTA (ventral tegmental area) and Nac (nucleus accumbens) tissues were homogenized in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 2mM EDTA, 1.0% Triton X100, 0.5% sodium deoxycholate) supplemented with protease (Complete, Roche) and phosphatase (Halt phosphatase inhibitor cocktail, Thermo Fisher) inhibitor cocktails. Lysates were cleared by centrifugation at 12,000g for 15 minutes at 40C. Protein concentration of the supernatants was determined by BCA Protein Assay (ThermoScientific). Western Blotting was carried out following standard procedures. Equal amount of proteins (60 µg) were separated on 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane via wet transfer in mini gel transfer chambers (Bio-Rad) and blotted for CHRNA2 (1:500, Sigma)

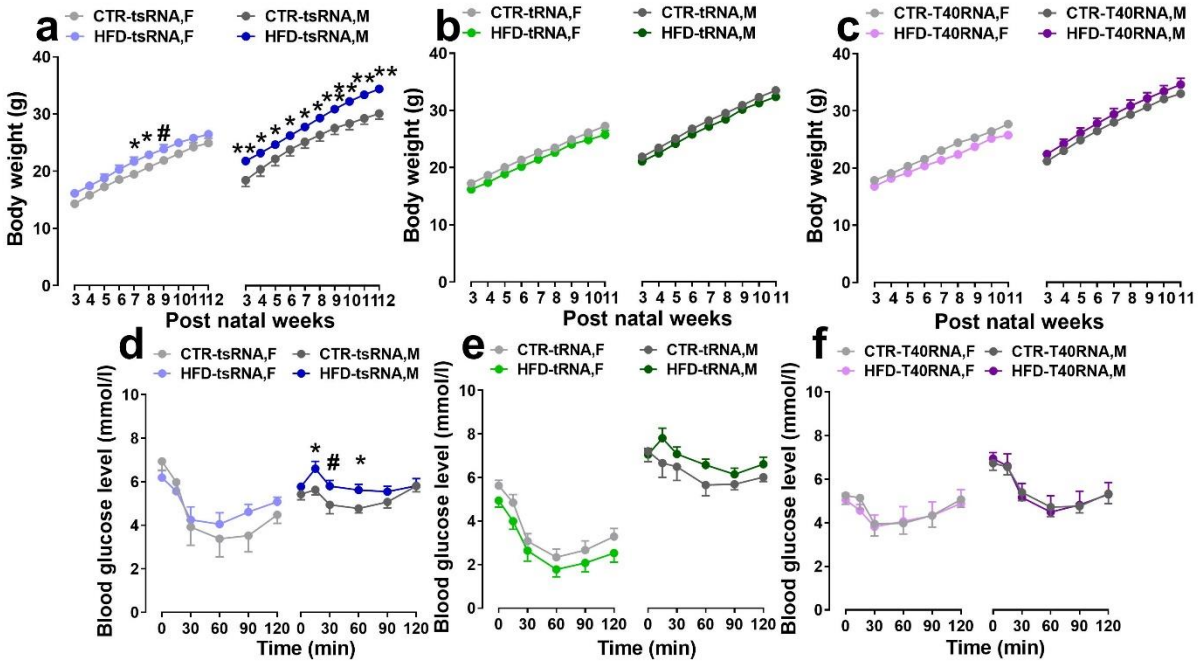
and GAPDH (1:1000 Cell Signaling). Signal of the HRP-conjugated secondary antibodies (Cell Signaling) was visualized by the LAS 4000 mini Image Quant system (GE Healthcare Life Sciences).

**Statistical analysis:**

Statistical analysis was performed using the StatView software (version 5.0). Analysis of variance (ANOVA) followed by *post-hoc* comparisons (Fisher's least significant difference) or factorial ANOVA was applied whenever appropriate. For the weekly body weight measurement, a 2 × 2 × 5 (group × sex × weeks) repeated measure ANOVA in the total RNA group, a 2 × 2 × 9 (group × sex × weeks) repeated measure ANOVA in the sncRNA groups and a 2 × 2 × 12 (group × sex × weeks) repeated measure ANOVA in the junk challenged tsRNA groups were employed. To analyze the preference for HFD an 2 x 2 x 2 (group x sex x food) ANOVA was used. For the sucrose and alcohol preference tests, a 2 × 2 × 2 x 3 (group x sex x preference substance x substance concentrations) repeated-measure ANOVA was used. The locomotion during the baseline and saline phases prior to the amphetamine challenge was subjected to a 2 x 2 x 3 (group x sex x 10-min bins) repeated-measure ANOVA. Amphetamine induced locomotor activity was subjected to a 2 × 2 × 12 (group x sex x 10-min bins) repeated-measure ANOVA. To analyze the insulin sensitivity, a 2 x 2 x 5 (group x sex x time) repeated measure ANOVA was conducted. In the junk food choice test, a 2 × 2 × 2 x 12 (group x sex x preference substance x weeks) repeated-measure ANOVA was used. Two-tailed Student t-test was used to analyze the differences in gene expression in different brain tissue. Statistical significance was set at $P < 0.05$. All data were presented as means ± standard error of mean (SEM). In the behavioral, metabolic and molecular tests, no difference was detected in the variance between the HFD and CTR-RNA injected groups ($F>1$).

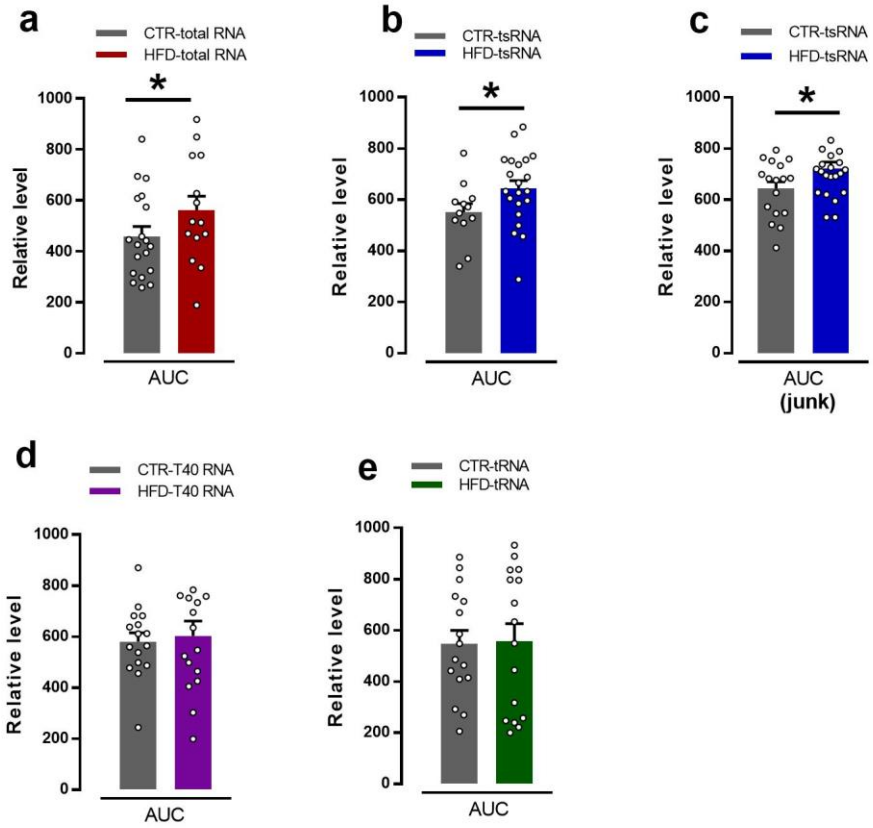
## Supplementary Figures

## Figure S1 metabolic sperm RNA fractions



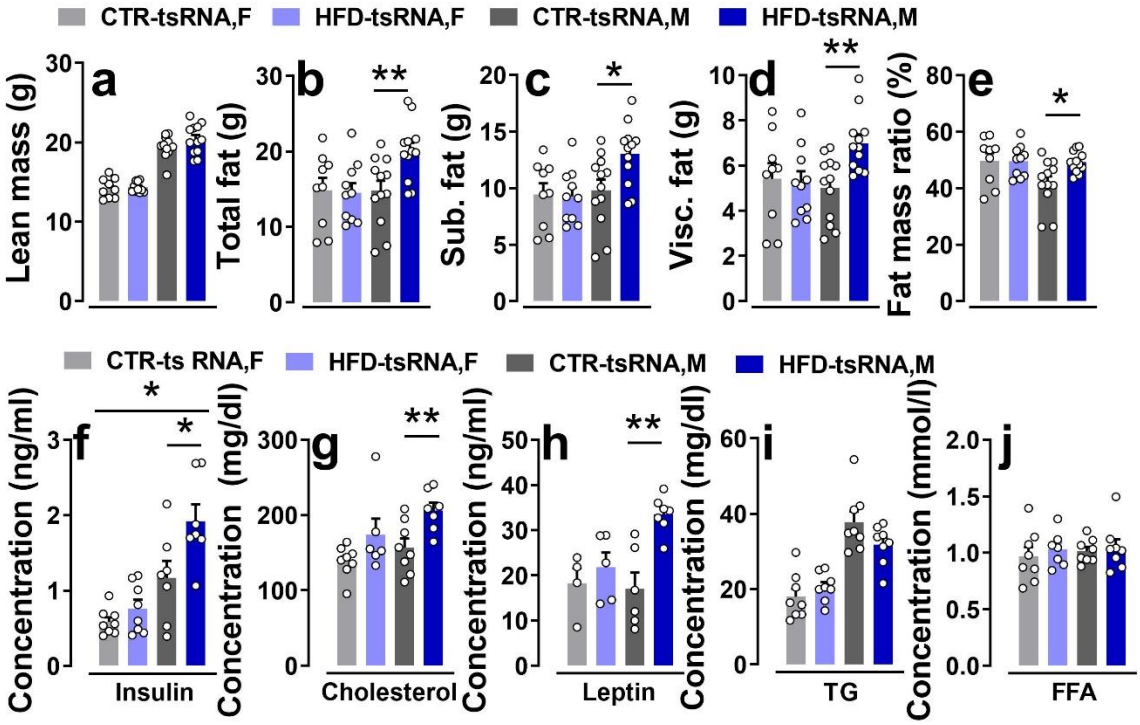

**Supplementary Figure 1. Altered metabolic phenotypes in sperm RNA fragments injected offspring.** Body weight: (a) HFD-tsRNA offspring gained more weight as compared to the CTR-tsRNA offspring. N (CTR-tsRNA) = 16 (8 M, 8 F); N (HFD-tsRNA) = 20 (10M, 10 F). (b) No difference was detected in body weight between the HFD-tRNA and CTR-tRNA offspring. N (CTR-tRNA) = 23 (12 M, 11 F); N (HFD-tRNA) = 20 (11 M, 9 F). (c) Offspring from HFD-T40RNA group did not show any difference in body weight compared to the CTR-T40RNA group. N (CTR-T40RNA) = 23 (15 M, 8 F); N (HFD-T40RNA) = 18 (10 M, 8 F). Insulin tolerance test: (d) HFD-tsRNA offspring showed significantly higher blood glucose level following an i.p insulin injection, indicating impaired insulin sensitivity in this group. A stronger impairment of insulin sensitivity was depicted in the male HFD-tsRNA offspring. N (CTR-tsRNA) = 14 (7 M, 7 F); N (HFD-tsRNA) = 19 (11 M, 8 F). (e) HFD-tRNA offspring did not show any difference in insulin sensitivity compared to the CTR-tRNA offspring. N = (8 M, 8 F) per group. (f) The insulin sensitivity of HFD-T40RNA offspring was comparable to that of CTR-T40RNA offspring. N = (8 M, 8 F) per group. Data are represented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. CTR = control, HFD = high fat diet, M = male, F = female.

## Figure S2



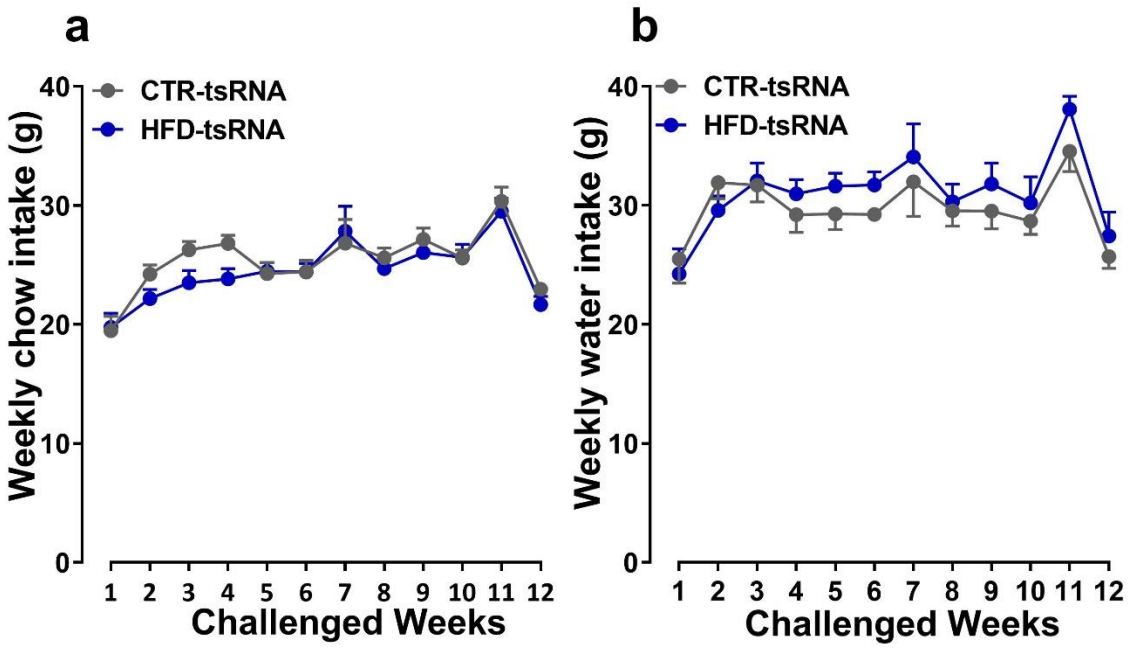

**Supplementary Figure 2**. **Area under curve (AUC) analysis of ITT data in different groups.** The bar graph shows the comparison of AUC between HFD and CTR groups for figure 3(b), 6 (d), 7(c), 6(f) and 6(e) respectively. Data are represented as mean ± SEM. * $P < 0.05$.

**Figure S3**



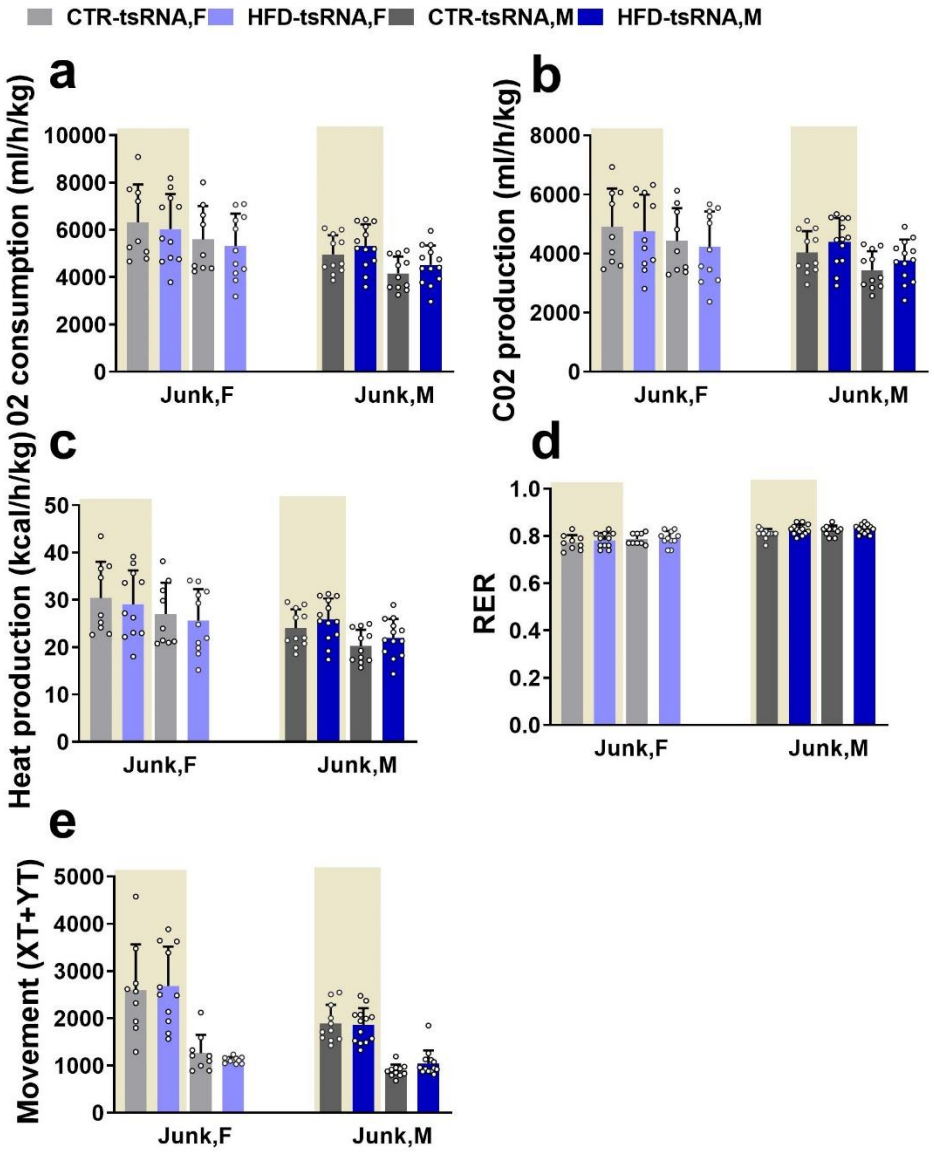

**Supplementary Figure 3. Metabolic parameters of tsRNA offspring in junk food choice test .** (a-e) Fat Distribution : Male HFD-tsRNA offspring developed a marked increase in total fat, subcutaneous fat, visceral fat as well as fat mass ratio compared to their CTR-littermates. No difference was observed in total lean mass between the groups. N (CTR-tsRNA) = 21 (12 M, 9 F); N (HFD-tsRNA) = 22 (12 M, 10 F). (f) Plasma Insulin: HFD-tsRNA group showed increased fasted plasma insulin compared to the CTR-tsRNA offspring. Male HFD-tsRNA had higher insulin level compared to others. N = (8M, 8F) per group. (h) Plasma leptin: HFD-tsRNA group showed higher fasted leptin level compared to CTR-tsRNA. Male HFD-tsRNA offspring had marked increase in leptin level compared to others. N (CTR-tsRNA) = 10 (6 M, 4 F); N (HFD-tsRNA) = 12 (7 M, 5 F). (g,i-j) Lipid profile: Male HFD-tsRNA offspring showed increased plasma cholesterol level compared to the others. N = (8M, 8F) per group. Data are represented as mean ± SEM. *P <0.05, **P < 0.01, ***P < 0.001. FFA = free fatty acid, TG = triglycerides, CTR = control, HFD = high fat diet, M = male, F = female.

**Figure S4**



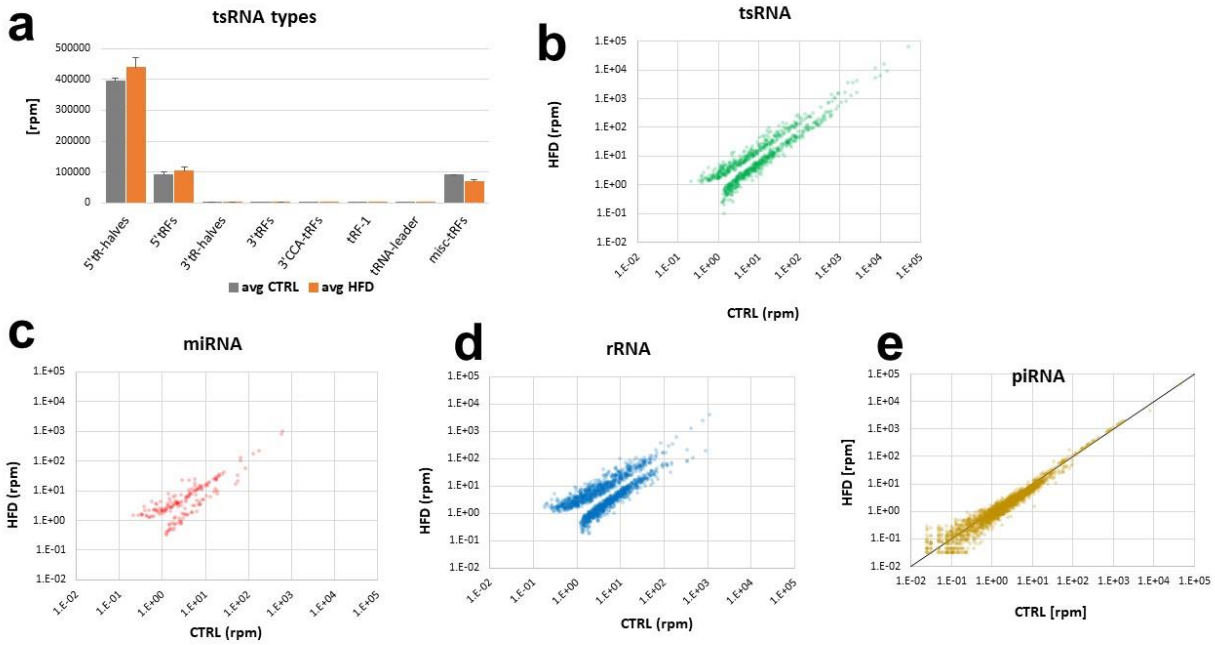

**Supplementary Figure 4:** Food and water intake in control groups during junk test: No difference in chow food intake was detected between HFD-tsRNA and CTR-tsRNA offspring (a). Both offspring group did not differ in their water consumption (b). N (CTR-tsRNA) = 20 (10 M, 8 F); N (HFD-tsRNA) = 23 (9 M, 8 F). Data are represented as mean ± SEM. CTR = control, HFD = high fat diet, M = male, F = female.

**Figure S5**



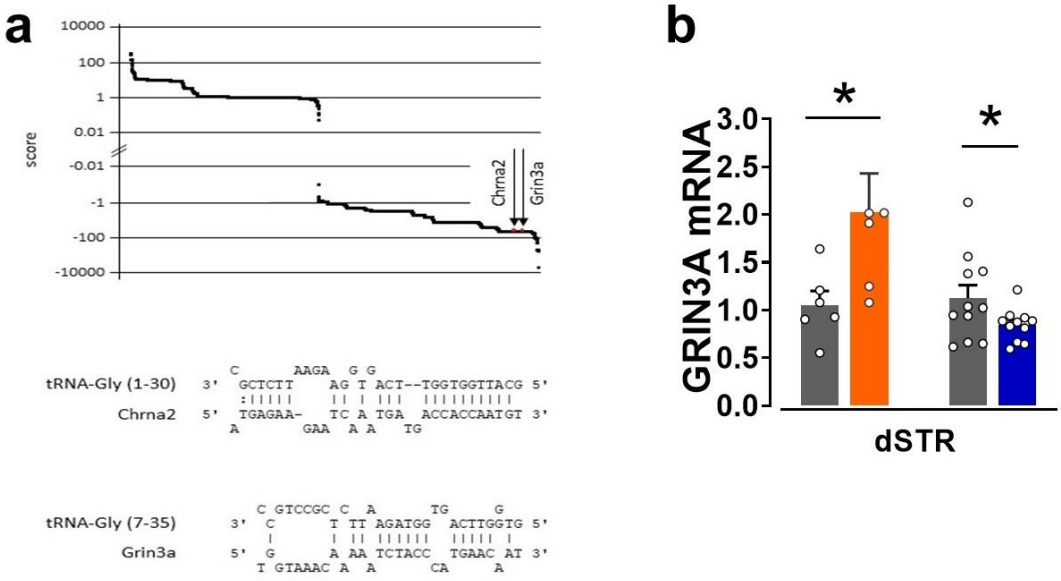

**Supplementary Figure 5. Energy expenditure analysis in the offspring tested for junk food choice.**
**(a)** There was no difference in $V0_2$ consumption between the HFD-tsRNA offspring and the CTR-tsRNA offspring. **(b)** No difference was observed in $VCO_2$ production between the groups . **(c)** Heat production was comparable between the groups. **(d)** No difference was detected in RER between HFD-tsRNA offspring and CTR-tsRNA offspring. **(e)** Male showed less activity than females. No difference in activity was detected between the groups. Data are represented as mean ± SEM. CTR = control, HFD = high fat diet, RER = respiratory exchange ratio, M = male, F = female.

## Figure S6




**Supplementary Figure 6. The expression profile of F1 sperm sncRNAs. (a)** The relative expression of different tRNA fragments in the HFD and CTR F1 sperm. **(b-e)** The scattered plots showing the relative expression of tsRNAs, miRNAs, rRNA, and piRNAs between HFD and CTR sperm. miRNA = microRNA; rRNA = ribosomal RNA; piRNAs = PIWI interacting RNAs; CTR = control, HFD = high fat diet.

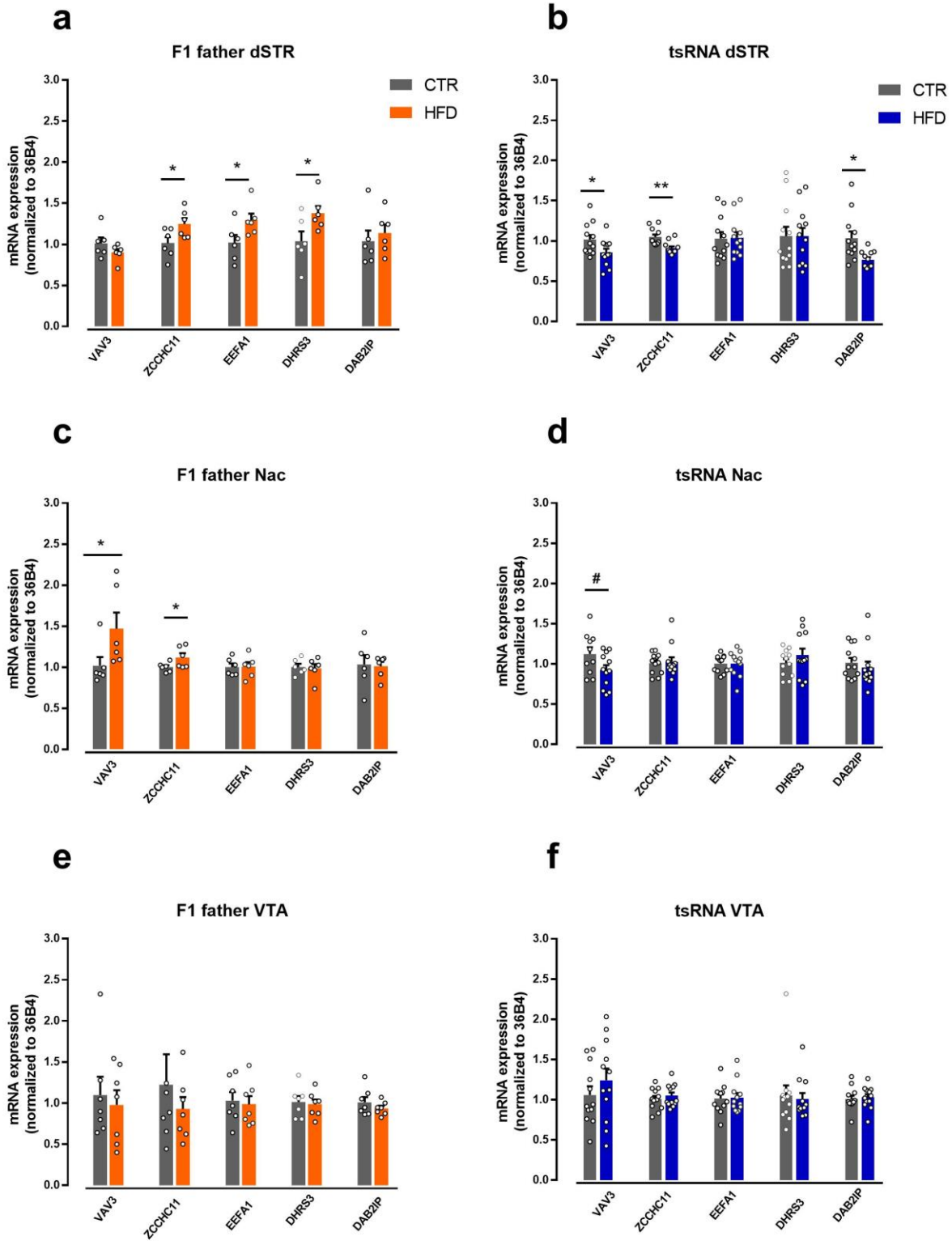
## Figure S7




**Supplementary Figure 7. Sperm tsRNAs profile in F1 fathers and differences in transcriptomes in brain of F1 father and tsRNA offspring.** (a) Scores for 6017 predicted tsRNA target transcripts. Positive scores forecast higher expression in CTR sperm, negative scores forecast higher expression in HFD. Exemplary target sites for CHRNA2 and GRIN3A are shown. (b) Altered expression of GRIN3A in the dSTR of F1-HFD and HFD-tsRNA offspring compared to their CTR littermates. The statistical significance was calculated using two-tailed Student t-test. N (F1-CTR) = 6; N

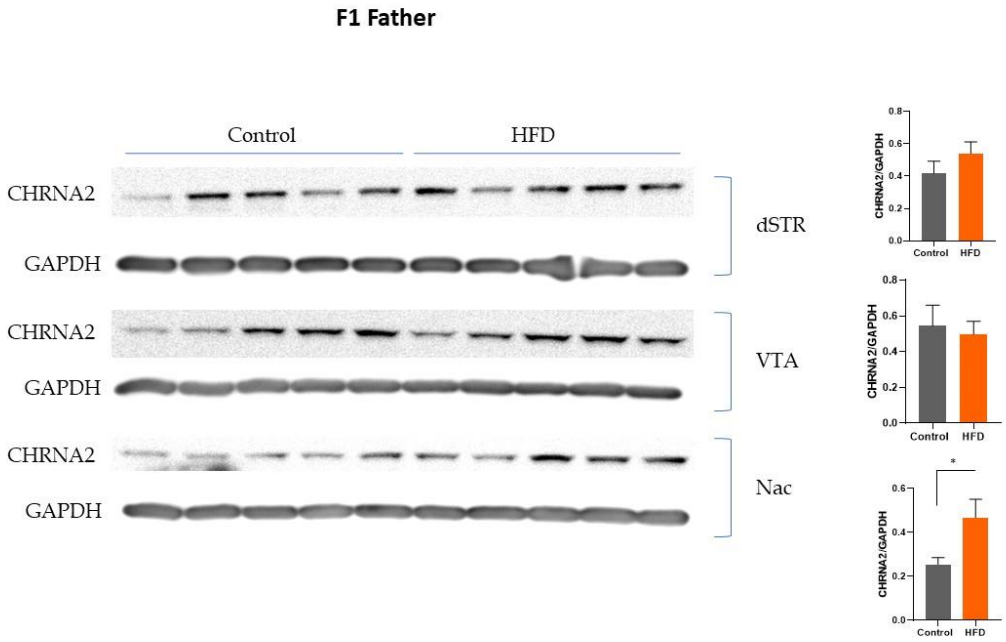
317 (F1-HFD) = 6; N (CTR-tsRNA) = 12; N (HFD-tsRNA) = 12. Data are represented as mean ± SEM. * P < 0.05. CTR = control, HFD = high fat diet, dSTR
318 = dorsal striatum, Nac = nucleus accumbens, VTA = ventral tegmental area.

**Figure S8**


a
F1 father dSTR
CTR
HFD
mRNA expression (normalized to 36B4)
VAV3
ZCCHC11
EEFA1
DHRS3
DAB2IP
b
tsRNA dSTR
c
F1 father Nac
d
tsRNA Nac
e
F1 father VTA
f
tsRNA VTA

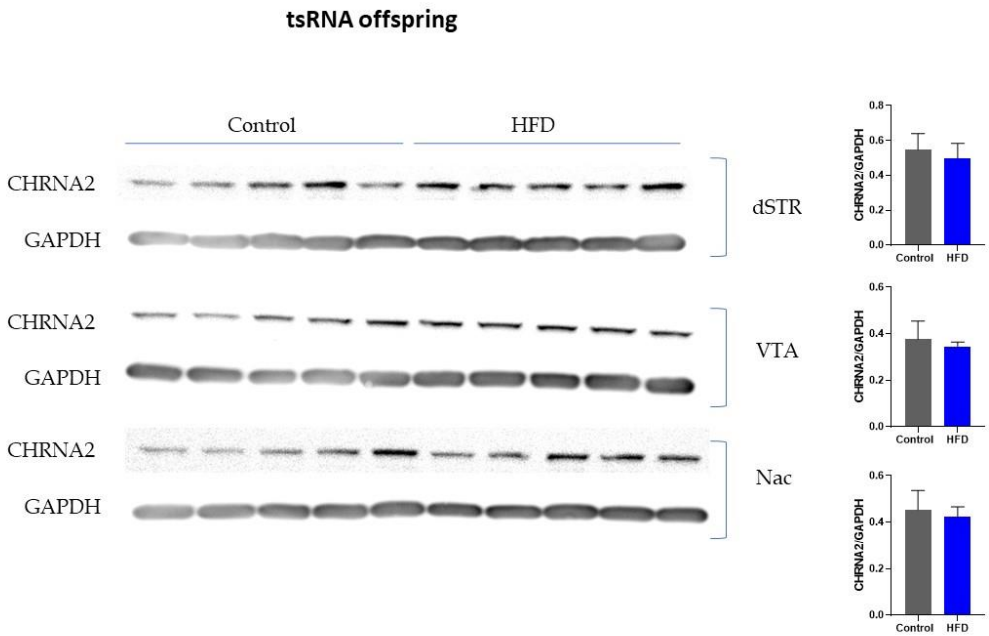
**Supplementary Figure 8. Expression of potential candidate genes in the dSTR, Nac and VTA.** The expression of VAV3, ZCCHC11, EEFA1, DHRS3 and DAB2IP in the dSTR, Nac and VTA of F1 and tsRNA offspring **(a-f)**. **(a)** The levels of ZCCHC11, EEFA1 and DHRS3 were increased in F1-HFD compared to F1-CTR males in the dSTR. **(b)** The levels of VAV3, ZCCHC11 and DAB2IP were reduced in tsRNA-HFD compared to tsRNA-CTR offspring in the dSTR. **(c)** The levels of VAV3 and ZCCHC11 were increased in F1-HFD compared to F1-CTR males in the Nac. **(d)** The expressions of VAV3 tended to be reduced in tsRNA-HFD compared to tsRNA-CTR offspring in the Nac. **(e-f)** The expression of VAV3, ZCCHC11, EEFA1, DHRS3 and DAB2IP remained unaltered in the VTA of F1-HFD and tsRNA-HFD offspring compared to the control groups. The statistical significance was calculated using two-tailed Student t-test. N (F1-CTR) = 6; N (F1-HFD) = 6; N (CTR-tsRNA) = 12; N (HFD-tsRNA) = 12. Data are represented as mean ± SEM. * P <0.05, ** P < 0.01. CTR = control, HFD = high fat diet, dSTR = dorsal striatum, Nac = nucleus accumbens, VTA = ventral tegmental area.

## Figure S9




**Supplementary Figure 9**. Western Blots (left) and quantifications (right) of CHRNA2 in dSTR, VTA and Nac of F1 fathers that were exposed to 9 weeks maternal HFD or chow diet. dSTR: dorsal striatum, VTA: ventral tegmental area, Nac: nucleus accumbens.Quantifications are presented as Mean +/- SEM. Student's t-test *=p<0.05

## Figure S10




**Supplementary Figure 10.** Western Blots (left) and quantifications (right) of CHRNA2 in dSTR, VTA and Nac in mice born after microinjection of sperm tsRNA from F1-HFD and F1-CTR male into naïve zygote. dSTR: dorsal striatum, VTA: ventral tegmental area, Nac: nucleus accumbens. Quantifications are presented as Mean +/- SEM. Student's t-test *=p<0.05.

## Supplementary Table 2

| Cohorts | Test | HFD-total RNA | CTR-total RNA |
|---|---|---|---|
| Cohort 1 | 1. HFD preference | 7 M, 9 F | 8 M, 11 F |
| | 2. Sucrose preference | | |
| | 3. Alcohol preference | | |
| Cohort 2 | Amphetamine sensitivity | 5 M, 5 F | 5 M, 5 F |
| Cohort 3 | 1. BW | 8 M, 10 F | 8 M, 11 F |
| | 2. CT scan | 6 M, 8 F | 7 M, 10 F |
| | 3. ITT | 8 M, 8 F | 8 M, 11 F |
| | 4. Plasma profile | 6 M, 6F | 6 M, 6 F |

**Table S2:** Summary of the number of the offspring in each experiment and the sequence of the tests conducted in the HFD-total RNA and control-total RNA offspring.

## Supplementary Table 3

| Cohorts | Test | HFD-tsRNA | CTR-tsRNA |
|---|---|---|---|
| Cohort 1 | 1. HFD preference | 10 M, 10 F | 10 M, 10 F |
| | 2. Sucrose preference | 10 M, 7 F | 10 M, 9 F |
| | 3. Alcohol preference | 9 M , 10 F | 7 M , 6 F |
| Cohort 2 | Amphetamine sensitivity | 5 M, 5 F | 5 M, 5 F |
| Cohort 3 | 1. BW | 10 M, 10 F | 8 M, 8 F |
| | 2. ITT | 11 M, 8 F | 7 M, 7 F |
| Cohort 4 (junk food choice test) | 1. BW | 13 M, 10 F | 12 M, 9 F |
| | 2. CT | 12 M, 10 F | 12 M, 9 F |
| | 3. ITT | 11 M, 11 F | 9 M, 8 F |
| | 4. Metabolic cage | 13 M, 11 F | 11 M, 9 F |
| | 5. Plasma Parameter | 8 M, 8 F | 8 M , 8 F |
| Cohort 5 | Gene expression analysis | 6 M, 6 F | 6 M, 6 F |

**Table S3:** Summary of the number of the offspring in each experiment and the sequence of the tests conducted in the HFD-tsRNA and control-tsRNA offspring.

## Supplementary Table 4

| Cohorts | Test | HFD-tRNA | CTR-tRNA |
|---|---|---|---|
| Cohort 1 | 1. HFD preference | 8 M, 8 F | 8 M, 8 F |
| | 2. Sucrose preference | | |
| | 3. Alcohol preference | | |
| Cohort 2 | Amphetamine sensitivity | 5 M, 5 F | 5 M, 5 F |
| Cohort 3 | 1. BW | 11 M, 9 F | 12 M, 11 F |
| | 2. ITT | 8 M, 8 F | 8 M, 8 F |
| Cohorts | Test | HFD-T40RNA | CTR-T40RNA |
| Cohort 1 | 1. HFD preference | 8 M, 8 F | 8 M, 8 F |
| | 2. Sucrose preference | | |
| | 3. Alcohol preference | | |
| Cohort 2 | Amphetamine sensitivity | 5 M, 5 F | 5 M, 5 F |
| Cohort 3 | 1. BW | 10 M, 8 F | 15 M, 8 F |
| | 2. ITT | 8 M, 8 F | 8 M, 8 F |

**Table S4:** Summary of the number of the offspring in each experiment and the sequence of the tests conducted in the HFD-tRNA and control-tRNA offspring; HFD-T40RNA and control-T40RNA offspring.

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