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Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice

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Small non-coding RNAs (sncRNAs) are potential vectors at the interface between genes and environment. We found that traumatic stress in early life altered mouse microRNA (miRNA) expression, and behavioral and metabolic responses in the progeny. Injection of sperm RNAs from traumatized males into fertilized wild-type oocytes reproduced the behavioral and metabolic alterations in the resulting offspring.

Although the genetic make-up of an individual contributes to disease risk and heritability[1], environmental factors, particularly adverse and traumatic experiences in early life, are also critical. How they mediate their influence is poorly understood, but likely involves non-genetic mechanisms. sncRNAs are potential mediators of gene-environment interactions that can relay signals from the environment to the genome and exert regulatory functions on gene activity[2]. They have been implicated in gene dysregulation in many diseases[2–4]. Recent studies in *C. elegans*[5,6] and mice[7,8] have suggested that sncRNAs can mediate non-Mendelian inheritance of traits or phenotypes acquired across life. sncRNAs are abundant in the mature sperm in mammals and may therefore convey transgenerational inheritance[9,10]. Whether sncRNAs in germ cells are influenced by environmental factors like early traumatic stress and contribute to associated pathological traits is unknown.

We investigated the involvement of sperm sncRNAs in the effect of traumatic stress in early life across generations, and began by examining the sncRNA content of adult mouse sperm in normal conditions. Deep sequencing of purified sperm RNA (**Supplementary Fig. 1**) identified several populations of short RNAs mapping to the mouse genome to a different degree and fidelity (**Supplementary Figs. 2a** and **3a**). Many had typical miRNA size (21–23 bp), and others aligned to Piwi-interacting RNA (piRNA) clusters and had typical piRNA size (26–31 bp) (**Supplementary Fig. 3b**,**c**). Some 15–44-bp reads mapped to ribosomal RNAs, to small cytoplasmic, nuclear or transfer RNAs, or to repeat regions such as retrotransposons (**Supplementary Fig. 2b**,**c**). A 16-bp read giving an unusually large peak mapped to a specific piRNA sequence (**Supplementary Fig. 4**). Reads of all sizes mapped uniquely to mitochondrial DNA sequences (**Supplementary Fig. 2d**), consistent with the presence of mitochondria in adult sperm[11].

We next examined the effect of exposure to traumatic stress in early life on sperm sncRNAs using a mouse model of unpredictable maternal separation combined with unpredictable maternal stress (MSUS) (**Supplementary Fig. 5**)[12–14]. In these mice, behavioral responses are affected by MSUS across generations. On the elevated plus maze, a test based on the natural avoidance of mice for open and unfamiliar

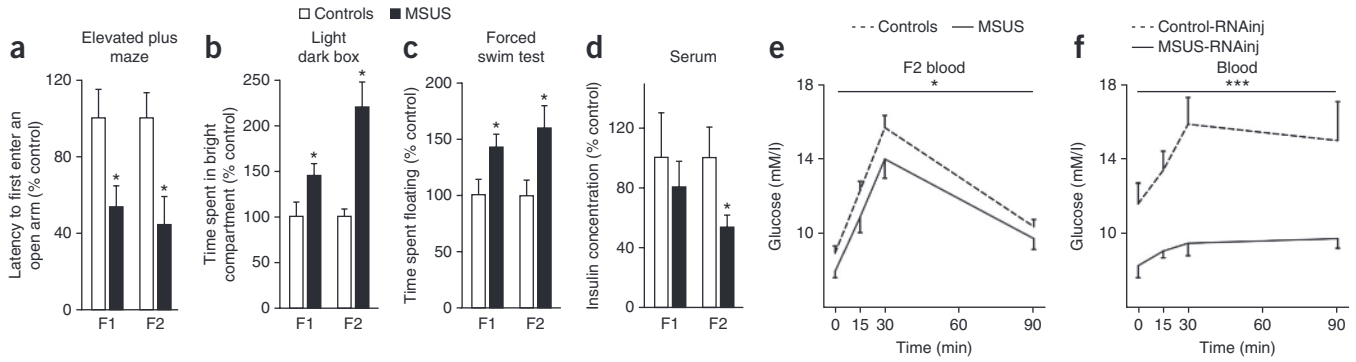

Figure 1 Behavioral and metabolic responses in MSUS males across generations and in mice derived from RNA-injected oocytes. (**a**) Latency to first enter an open arm on an elevated plus maze in F1 (control, $n = 8$; MSUS, $n = 18$; $t_{24} = 2.37$) and F2 (control, $n = 30$; MSUS, $n = 25$; $t_{41.98} = 3.74$) mice. (**b**) Time spent in the bright compartment of the light dark box in F1 (control, $n = 16$; MSUS, $n = 21$; $t_{35} = -2.14$) and F2 (control, $n = 33$; MSUS, $n = 36$; $t_{41.61} = -3$) mice. (**c**) Time spent floating on the forced swim test in F1 (control, $n = 14$; MSUS, $n = 16$; $t_{28} = -2.34$) and F2 (control, $n = 19$; MSUS, $n = 20$; $t_{37} = -2.36$) mice. Results were replicated in two independent experiments. (**d**) Insulin concentration in serum in F1 (control, $n = 5$; MSUS, $n = 9$; $t_{12} = 0.28$) and F2 (control, $n = 10$; MSUS, $n = 10$; $t_{18} = 2.1$) males. (**e**,**f**) Glucose level in blood in F2 control and MSUS (control, $n = 8$; MSUS, $n = 7$; $F_{1,13} = 5.64$; **e**) and control-RNAinj ($n = 8$) and MSUS-RNAinj ($n = 8$) ($F_{1,14} = 9.72$) males at baseline (time = 0) and 15, 30 and 90 min after stress initiation. Data represent mean ± s.e.m. $^{*}P < 0.05$, $^{***}P < 0.001$.

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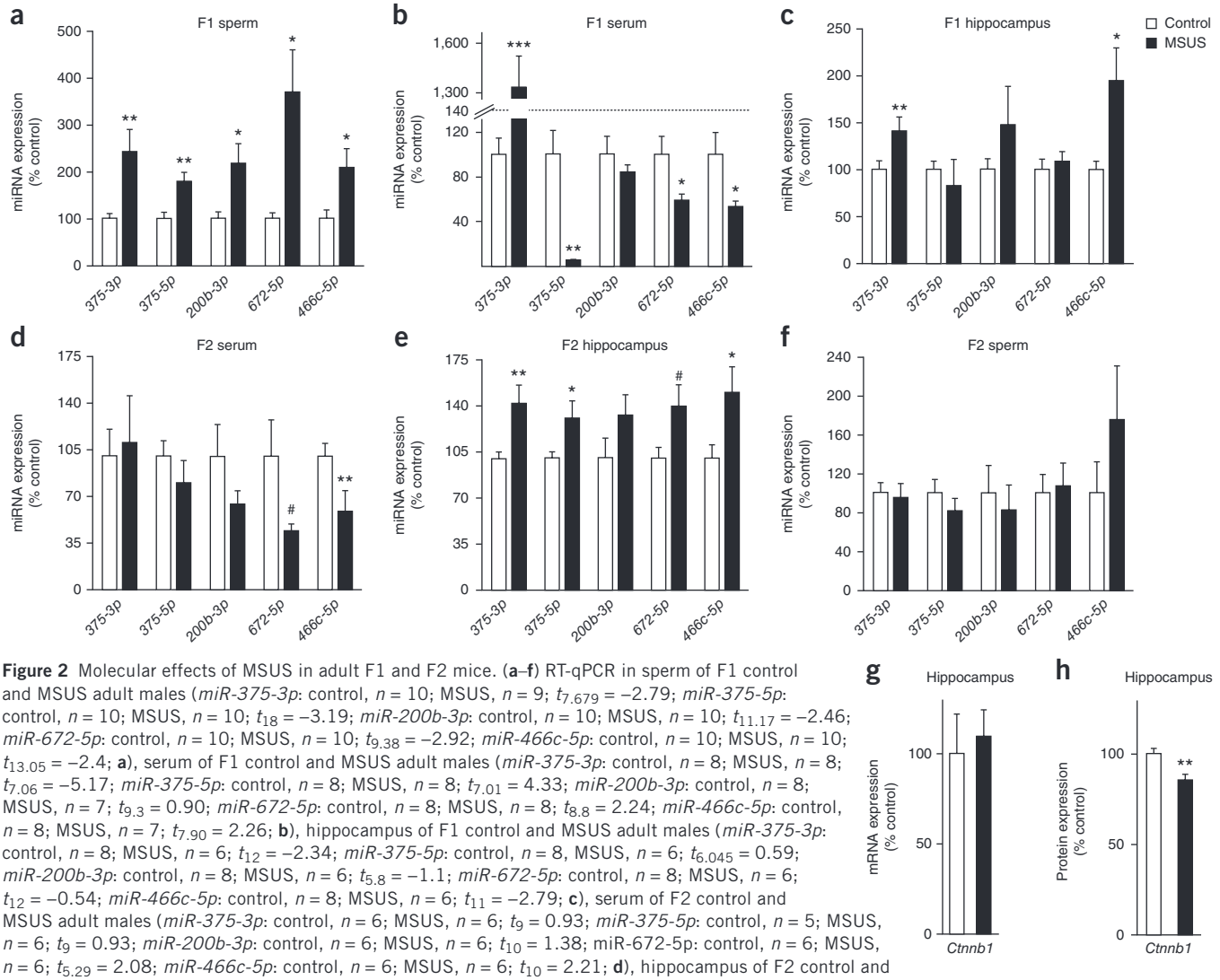

Figure 2 Molecular effects of MSUS in adult F1 and F2 mice. (**a**–**f**) RT-qPCR in sperm of F1 control and MSUS adult males (*miR-375-3p*: control, $n = 10$; MSUS, $n = 9$; $t_{7.679} = -2.79$; *miR-375-5p*: control, $n = 10$; MSUS, $n = 10$; $t_{18} = -3.19$; *miR-200b-3p*: control, $n = 10$; MSUS, $n = 10$; $t_{11.17} = -2.46$; *miR-672-5p*: control, $n = 10$; MSUS, $n = 10$; $t_{9.38} = -2.92$; *miR-466c-5p*: control, $n = 10$; MSUS, $n = 10$; $t_{13.05} = -2.4$; **a**), serum of F1 control and MSUS adult males (*miR-375-3p*: control, $n = 8$; MSUS, $n = 8$; $t_{7.06} = -5.17$; *miR-375-5p*: control, $n = 8$; MSUS, $n = 8$; $t_{7.01} = 4.33$; *miR-200b-3p*: control, $n = 8$; MSUS, $n = 7$; $t_{9.3} = 0.90$; *miR-672-5p*: control, $n = 8$; MSUS, $n = 8$; $t_{8.8} = 2.24$; *miR-466c-5p*: control, $n = 8$; MSUS, $n = 7$; $t_{7.90} = 2.26$; **b**), hippocampus of F1 control and MSUS adult males (*miR-375-3p*: control, $n = 8$; MSUS, $n = 6$; $t_{12} = -2.34$; *miR-375-5p*: control, $n = 8$, MSUS, $n = 6$; $t_{6.045} = 0.59$; *miR-200b-3p*: control, $n = 8$; MSUS, $n = 6$; $t_{5.8} = -1.1$; *miR-672-5p*: control, $n = 8$; MSUS, $n = 6$; $t_{12} = -0.54$; *miR-466c-5p*: control, $n = 8$; MSUS, $n = 6$; $t_{11} = -2.79$; **c**), serum of F2 control and MSUS adult males (*miR-375-3p*: control, $n = 6$; MSUS, $n = 6$; $t_9 = 0.93$; *miR-375-5p*: control, $n = 5$; MSUS, $n = 6$; $t_9 = 0.93$; *miR-200b-3p*: control, $n = 6$; MSUS, $n = 6$; $t_{10} = 1.38$; miR-672-5p: control, $n = 6$; MSUS, $n = 6$; $t_{5.29} = 2.08$; *miR-466c-5p*: control, $n = 6$; MSUS, $n = 6$; $t_{10} = 2.21$; **d**), hippocampus of F2 control and MSUS adult males (*miR-375-3p*: control, $n = 7$; MSUS, $n = 8$; $t_{8.62} = -2.74$; *miR-375-5p*: control, $n = 14$; MSUS, $n = 15$; $t_{17,89} = -2.14$; *miR-200b-3p*: control, $n = 8$; MSUS, $n = 8$; $t_{14} = -1.47$; *miR-672-5p*: control, $n = 7$; MSUS, $n = 8$; $t_{13} = -2.01$; *miR-466c-5p*: control, $n = 7$; MSUS, $n = 8$; $t_{13} = -2.15$; **e**), sperm of F2 control and MSUS adult males (*miR-375-3p*: control, $n = 8$; MSUS, $n = 8$; $t_{14} = 0.26$; *miR-375-5p*: control, $n = 8$; MSUS, $n = 8$; $t_{14} = 0.94$; *miR-200b-3p*: control, $n = 4$; MSUS, $n = 3$; $t_5 = 0.44$; *miR-672-5p*: control, $n = 4$; MSUS, $n = 4$; $t_6 = -0.24$; *miR-466c-5p*: control, $n = 4$; MSUS, $n = 4$; $t_6 = -1.16$; **f**). (**g**,**h**) Level of *Ctnnb1* mRNA (control, $n = 7$; MSUS, $n = 7$; $t_{12} = 0.4$; **g**) and (**h**) protein (control, $n = 7$; MSUS, $n = 6$; $t_{11} = 3.26$) in hippocampus of F2 control and MSUS males. Results replicated in an independent experiment using samples from a different batch of animals. Data represent mean ± s.e.m. $^{\#}P < 0.1$, $^{*}P \leq 0.05$, $^{**}P < 0.01$, $^{***}P \leq 0.001$.

space, MSUS males had shorter latency to first enter an open arm than controls (F1; **Fig. 1a**). This effect was not a result of altered locomotor activity (**Supplementary Fig. 6a**), suggesting reduced avoidance and fear. In a light-dark box, a task based on the aversion of rodents for brightly lit areas, MSUS males spent more time in the illuminated compartment than controls (F1; **Fig. 1b**), suggesting an altered response to aversive conditions. On a Porsolt forced swim test, a test of behavioral despair, MSUS males spent more time floating than controls (F1; **Fig. 1c**). Notably, these behavioral traits were transmitted to the F2 offspring. On the elevated plus maze, F2 MSUS mice had a shorter latency to first enter an open arm than F2 controls (**Fig. 1a**), but normal locomotor activity (**Supplementary Fig. 6b**). They spent more time in the bright compartment of the light-dark box and had altered behaviors on the forced swim test (**Fig. 1b**,**c**).

Given that early stress can be a strong metabolic dysregulator[15], we next examined glucose metabolism. Insulin levels in serum were normal in F1 MSUS mice, but were lower in F2 MSUS progeny than in controls (**Fig. 1d**). Blood glucose was also normal in F1 animals (**Supplementary Fig. 7a**), but was lower in F2 MSUS mice, both at baseline and following an acute restraint stress (**Fig. 1e**). Furthermore, F1 MSUS males had normal baseline glucose levels and clearance on a glucose tolerance test (GTT), but showed a larger decline in blood glucose on an insulin tolerance test (ITT) (**Supplementary Fig. 7b**,**c**). F2 MSUS animals had normal glucose at baseline, but lower glucose rise on GTT and normal glucose decrease on ITT (**Supplementary Fig. 8a**,**b**). These anomalies suggest insulin hypersensitivity. F2 MSUS animals (but not F1) also showed hypermetabolism, as their body weight was lower than that of controls despite higher caloric intake (**Supplementary Figs. 7d**,**e** and **8c**,**d**). The alterations were overall more marked in F2 mice, probably because the effects of stress are present starting at conception, whereas they only occurred after birth in F1 mice.

Because the traits induced by MSUS are transmitted to the progeny, we examined whether sperm sncRNAs are affected. Deep sequencing analyses revealed that several miRNAs were upregulated in F1 MSUS sperm (**Supplementary Fig. 2e**), and 73 potential miRNAs targets, implicated in DNA and RNA regulation, epigenetic regulation or RNA binding and processing, were identified (**Supplementary Table 1**). piRNAs were also affected, particularly cluster 110, which was downregulated in MSUS sperm (**Supplementary Fig. 9a**,**b**). These results indicate an effect of MSUS on several sncRNA populations. Validation by reverse transcription quantitative PCR (RT-qPCR) confirmed that miR-375-3p, miR-375-5p, miR-200b-3p, miR-672-5p and miR-466-5p were upregulated in F1 MSUS sperm (**Fig. 2a**). miRNAs were also altered in serum, and in the hippocampus and hypothalamus (not cortex), brain structures that are involved in stress response, in adult F1 MSUS animals (**Fig. 2b**,**c** and **Supplementary Fig. 10a**,**b**). Moreover, miRNAs were affected in the serum and hippocampus of adult F2 MSUS mice, but not in F2 sperm (**Fig. 2d**–**f**). Consistently, miRNAs were normal in F3 animals (**Supplementary Fig. 11**). Given that F3 MSUS mice have behavioral symptoms similar to those of F1 and F2 animals[13] despite there being normal sperm miRNA level in F2 males, it is possible that the changes in miRNAs that initially occur in sperm cells as a result of MSUS are transferred to other non-genomic or epigenetic marks, such as DNA methylation or histone post-translational modifications, for maintenance and further transmission[16,17].

miR-375 has been implicated in stress response and metabolic regulation[18,19]. We found that mimicking the effect of stress by injecting corticosterone *in vivo* increased miR-375 expression in the hippocampus (saline, $n = 14$; corticosterone, $n = 14$; $t_{26} = 2.27$). One of miR-375's predicted targets is catenin β1 (Ctnnb1), a protein that has been implicated in stress pathways[20]. Cultured cells transfected with a miR-375 mimic showed downregulation of Ctnnb1 (control, $n = 3$; transfected, $n = 3$; mRNA, $t_4 = 2.78$; protein, $t_4 = 5{,}14$), confirming that miR-375 targets Ctnnb1. Consistently, Ctnnb1 was decreased in F2 MSUS hippocampus (**Fig. 2g**,**h**), suggesting that miR-375 alteration has functional consequences on Ctnnb1 expression *in vivo*.

Finally, we tested the causal link between sperm RNAs and the effects of MSUS across generations by microinjecting RNAs purified from sperm from MSUS males (**Supplementary Fig. 1**) into wild-type fertilized mouse oocytes. On the elevated plus maze, MSUS-RNAinj mice ($n = 20$) had a lower latency to first enter an open arm than control-RNAinj ($n = 19$) mice ($t_{37} = 2.67$), but displayed normal locomotion (**Supplementary Fig. 6c**). In the light-dark box, they spent more time in the bright compartment (control-RNAinj, $n = 15$; MSUS-RNAinj, $n = 17$ males; $t_{30} = -3.77$), and, on the forced swim test (control-RNAinj, $n = 18$; MSUS-RNAinj, $n = 20$ males; $t_{37} = -2.19$), they floated longer than control-RNAinj mice. Furthermore, in MSUS-RNAinj animals, insulin (control-RNAinj, $n = 4$; MSUS-RNAinj, $n = 7$; $t_9 = 2.31$) and glucose levels at baseline and after acute stress (**Fig. 1f**) were decreased, body weight was lower and miR-375-5p was upregulated in the hippocampus (**Supplementary Fig. 12a**,**b**). These results indicate comparable behavioral, metabolic and molecular effects by either direct exposure to MSUS during early postnatal life or injection of sperm RNAs from MSUS males. Notably, the offspring of MSUS-RNAinj mice also showed depressive-like behaviors (**Supplementary Fig. 13**), indicating transmission of the effects of injected sperm RNAs.

These findings provide evidence for the idea that RNA-dependent processes contribute to the transmission of acquired traits in mammals. They underscore the importance of sncRNAs in germ cells and highlight their sensitivity to early traumatic stress. Our findings demonstrate the consequences of germ cell exposure to such traumatic experience in early life across generations. The identification of several miRNAs and putative targets as mediators of these effects provide molecular markers of traumatic stress for potential use for the diagnostic of stress predisposition and stress-induced disorders in humans.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Sequencing data were deposited to NCBI's Gene Expression Omnibus with the accession number GSE50132.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

K.G. carried out all of the RT-qPCR, behavioral tests, metabolic measurements, and sperm RNA preparation for sequencing libraries and for RNA injection into fertilized oocytes, and part of the sequencing analyses. A.J. performed western blots and cell culture experiments and assisted with metabolic measurements. J.B. carried out the MSUS procedures and produced MSUS mice. J.P. and P.S. performed most RNA sequencing analyses. P.P. carried out the RNA injection experiments. E.M. and L.F. helped design the RNA sequencing analysis. K.G. and I.M.M. designed the study, interpreted the results and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57Bl/6J mice were maintained under a reverse light-dark cycle in a temperature and humidity-controlled facility with food and water *ad libitum*. All experimental manipulations were performed during the animals' active cycle in accordance with guidelines and regulations of the cantonal veterinary office, Zurich.

Mice treatment. For MSUS, C57Bl/6J dams (2–3 months old) and litters were selected at random and subjected to daily 3-h proximal separation from postnatal days 1–14 as described previously[13]. Control animals were left undisturbed apart from a cage change once a week until weaning (postnatal day 21). Once weaned, pups were reared in social groups (4–5 mice per cage) composed of mice subjected to the same treatment, but from different dams to avoid litter effects. To obtain second and third generations, adult F1 males (>5 months old) were bred with primiparous C57Bl/6J females.

Preparation of sperm samples. Mature sperm cells extracted from cauda epididymis from males were separated from somatic cells by counterflow centrifugal elutriation using a Beckman JE-5.0 elutriation rotor in a Sanderson chamber and a Beckman Avanti J-26 XPI Elutriation Centrifuge. Briefly, cauda epididymis and epididymis were collected in culture dishes in PBS (130 mM NaCl, 10 mM Na_2PO_4, 1.7 mM KH_2PO_4, 2 mM KCl, pH 7.4) containing 0.5% (wt/vol) BSA, 0.5% (wt/vol) non-fat dry milk powder, 5 mM $CaCl_2$, 5 mM $MgCl_2$ filtered through a cellulose acetate membrane (Sartorius) and cut into small pieces to release sperm cells. The suspension was loaded into the elutriation chambers, which form part of the centrifuge rotor, using a rotor speed of 3,500 rpm and a pump rate of 7 ml min^{-1}. Mature sperm was eluted by increasing the pump rate to 31 ml min^{-1}. Purity of the elutriate was confirmed by inspecting the eluted sperm cells under a light microscope.

RNA sequencing. Total RNA was prepared from adult mouse sperm using a standard Trizol protocol. The quantity and quality of RNAs were determined by Agilent 2100 Bioanalyser (Agilent Technologies), Qubit fluorometer (Life Technologies) and mass spectrometry (**Supplementary Fig. 1**). Pure RNA preparations with no DNA or protein contamination were used for sequencing. Sequencing was done using an Illumina Genome Analyzer (Illumina) at Fasteris AG. Small RNA libraries were prepared according to a modified Illumina v1.5 protocol. Briefly, small RNAs of <50 nt were purified on an acrylamide gel. Universal miRNA cloning linker (New England BioLabs) instead of 3′ adaptors and then 5′ Illumina adaptors were single-stranded ligated with T4 truncated RNA and T4 ligase, respectively. The constructs were purified on an acrylamide gel to remove empty adaptors and then reverse transcribed and PCR amplified. The primers used for cDNA synthesis and PCR were designed to insert an index in the 3′ adaptor. This index enables assignation of a specific read to the corresponding library, among the multiplexed libraries of one sequencing lane. High-throughput sequencing was performed on a Genome Analyzer HiSeq 2000 for 50 cycles plus 7 cycles to read the indexes. After demultiplexing and adaptor removal, an average of 16,067,416 pass filter reads was obtained in the libraries. Sequencing results were validated by RT-qPCR on the same samples as those used for deep sequencing.

Mass spectrometry. Tryptic digestions were performed as previously described with slight modifications[21]. In brief, 1 µl of sperm RNA samples was made up to 100 µl with 25 mM ammonium bicarbonate, pH 8.0. Samples were reduced with 10 mM dithiothreitol (DTT) for 45 min at 56 °C and alkylated with 40 mM iodoacetamide for 30 min. Samples were digested overnight with trypsin (Promega) at 37 °C and desalted using C18 Ziptip before MS analysis using ESI-LTQ-Orbitrap Velos. MS and MS/MS data were searched using Mascot and searches of MS/MS spectra used a Swiss-Prot protein database.

miRNA targets prediction. The DIANA-microT CDS miRNA target prediction algorithm[22], which is based on potential binding site in the 3′ untranslated region of the mRNA and predicted stable thermodynamic binding, was used to predict target genes of miRNAs. Binding score threshold was set to 0.9 (1 = highest potential binding predicted, 0 = no binding predicted) and only the top 100 targets were considered for each miRNA, to only consider predictions with highest probability.

RNAs injection in fertilized oocytes. Fertilized oocytes were collected from B6D2F1 (Janvier) females superovulated by intraperitoneal injection of 5 IU pregnant mare serum gonadotropin followed by 5 IU human chorionic gonadotrophin 48 h later, then mated with B6D2F1 males. 1–2 pl of 0.5 ng μl^{-1} solution of total RNA isolated and pooled from sperm from five adult MSUS or control males (same samples used for sequencing) dissolved in 0.5 mM Tris-HCl, pH 8.0, 5 µM EDTA were microinjected into the male pronucleus of fertilized eggs using a standard microscope and DNA microinjection method[23].

Behavioral testing. The experimenter was blind to treatment, and behaviors were monitored by direct observation and videotracking (Viewpoint). All behavioral tests were conducted in adult male animals.

Elevated plus maze. The elevated plus maze consisted of a platform with two open (without walls) and two closed (with walls) arms (dark gray PVC, 30 cm × 5 cm) elevated 60 cm above the floor. All experiments were performed in red light (15W). Each mouse was placed on the central platform, facing a closed arm and observed for a 5-min period. The latency to enter an open arm, the time spent in each arm and the total distance moved were automatically recorded by a videotracking system. The number of rearing, protected (body in closed arm) and unprotected (body in open arm) stretch-attend postures in the center of the maze were manually recorded.

Light-dark box. Each mouse was placed in the lit compartment (white walls, 130 lx) of a plastic box (40 × 42 × 26 cm) split into two unequal compartments (2/3 lit, 1/3 dark compartment with black walls and covered by a black lid) by a divider with an opening (5 × 5 cm). The animal can move freely from the lit to the dark compartment during a 10-min session. The time spent in each compartment and the latency to enter the dark compartment were measured manually.

Forced swim test. Mice were placed in a small tank of water (18 cm high, 13 cm diameter, 18 ± 1 °C, filled up to 12 cm) for 5 min. Floating duration was scored manually.

Serum insulin and blood glucose analyses. For non-fasted baseline measurements of insulin, blood was collected, stored overnight at 4 °C, centrifuged for 10 min at 2,000 *g* at 4 °C, then serum was collected and stored at −80 °C until analyzed. Insulin was measured in serum using a mouse insulin ELISA (Alpco). The sensitivity of the assay was 0.06 ng ml^{-1}, and the intra-assay coefficient of variation was 3.7%. Glucose in non-fasted animals was measured in blood samples at baseline and after acute stress. Mice were restrained for 30 min (between 14:00 and 16:00) in a plastic tube and blood samples were collected from a tail nick 0, 15, 30 and 90 min after initiation of restraint. For the glucose tolerance test, mice were fasted for 6 h. Glucose was measured in blood samples at baseline, and 0, 15, 30 and 90 min after intraperitoneal injection of 2 mg per g of body weight glucose in 0.45% (wt/vol) saline (injection started at 2 p.m.). For the insulin tolerance test, mice were fasted for 6 h. Glucose was measured in blood samples at baseline, and 0, 15, 30, 90 and 120 min after intraperitoneal injection of 1 mU per g body weight insulin (NovoRapid Novo Nordisk A/S) in sterile 0.9% saline. If blood glucose fell below 1.7 mM ml^{-1}, animals were rescued with intraperitoneal injection of 2 mg per g glucose and were removed from the experiment. Glucose level was determined in fresh tail blood using an Accu-Chek Aviva device (Roche).

Caloric intake measurement. The amount of consumed food was measured for each mouse (4 months old) every 24 h. Caloric intake was calculated as the mean amount of food intake over 48 h in relation to mean body weight (caloric intake = mean food intake/mean body weight).

Cell culture. Mouse neuroblastoma (N2a) cells were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum. Approximately 300,000 cells from three different passage number stocks were simultaneously plated in 6-well culture plates. Cells were treated with miScript miRNA mimic (Qiagen) and a negative control siRNA with no known targets in mammalian genome (All Stars Negative siRNA, Qiagen) at 60 nM for 48 h. Transfections were carried out using lipid-based HiPerfect transfection reagent (Qiagen). Cells were

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harvested 48 h after transfection and total RNA was isolated using standardized Trizol protocol.

Western blotting. Western blotting was performed as previously described[24]. Briefly, 30–60 μg of protein were resolved on 10–12% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked (milk, 5% wt/vol) then incubated in primary and secondary antibodies. Band intensity was determined and quantified using an Odyssey IR scanner (Li-Cor Biosciences). β-actin (1:15,000, mouse monoclonal, Sigma #A5316) was used as internal control. We used antibody to Ctnnb1 (1:2,000, mouse monoclonal, BD Transduction Laboratories #610153) and goat antibody to mouse (IRDye 680 nm, 1:10,000, Li-Cor Biosciences). Samples from different groups were processed on the same blots. Data are expressed as percent relative to controls.

RT-qPCR. For miRNAs, DNaseI-treated RNA isolated from pure sperm cells or hippocampal samples (Trizol) was reverse-transcribed using the miScript reverse transcription kit (Qiagen). RT-qPCR was performed in a LightCycler 480 qPCR (Roche) using miScript probes (Qiagen) according to the manufacturer's recommendations. For normalization of Ct values for miRNAs, we used miR-101b for sperm, ribosomal Rrnu6 for hippocampus and miR-195 for serum. For mRNAs, RT-qPCR were performed using SYBR Green (Roche) on a Light-Cycler II 480 (Roche) according to the manufacturer's recommendations. Data for tissue samples were normalized to two endogenous controls (Tubd1 and Hprt), and data for cell samples were normalized to GAPDH. Cycling conditions: 5 min at 95 °C, 45 cycles with denaturation (10 s at 95 °C), annealing (10 s at 60 °C) and elongation (10 s at 72 °C). We used primers to Tubd1 (forward, TCTCTTGCTAA CTTGGTGGTCCTC; reverse, GCTGGGTCTTTAAATCCCTCTACG), Hprt (forward, GTTGGGCTTACCTCACTGCTTTC; reverse, CCTGGTTCATC ATCGCTAATCACG), Ctnnb1 (forward, ATGGAGCCGGACAGAAAAGC; reverse, CTTGCCACTCAGGGAAGGA) and GAPDH (forward, CCACTGGT GCTGCCAAGGCT; reverse, GGCAGGTTTCTCCAGGCGGC).

Deep sequencing data analyses. *Overall analysis of small RNAs libraries.* After adaptor trimming, sequence reads were sorted based on length (number of nucleotides) and only 15–44-bp reads were used for analysis. The number of reads of each size was counted and normalized to the total number of reads. The obtained counts were averaged across control libraries. Reads were aligned to the mouse genome (UCSC mm9, http://hgdownload.cse.ucsc.edu/goldenPath/mm9/bigZips/)[25], ncRNAs and repeat elements from rmsk (UCSC mm9) and mitochondrial DNA (UCSC mm9) using a BWA software[26] with mismatch tolerance of up to 2 bp for 15–17 bp inserts, and 3 and 4 bp for 18–38-bp and 37–44-bp inserts, respectively. Sequencing reads were aligned to different selected features (ncRNAs, mtDNA) separately to 'force' mapping (sequences of the mouse genome other than the respective feature were masked to prevent alignment to featured regions), and the different features matching each read were determined. The percentage of reads of a given size mapping once or multiple times (unique or multiple hits) to the mouse genome or to a given feature (100% represents all reads of a given size within a library) was determined and averaged across all control libraries. In **Supplementary Figure 2a**, un-mapped reads may result from PCR pre-sequencing amplification artifacts, incomplete trimming of adaptors or sequencing errors, or may reflect the presence of RNA splicing products. In **Supplementary Figure 2b**, some reads map to ribosomal RNAs (cleaved) with multiple hits, reflecting ribosomal RNAs cleavage and no functional ribosomes in sperm cells (transcriptionally quiescent)[27]. Reads mapping to mitochondrial DNA showing only unique hits allow unambiguous attribution to mitochondria DNA. For quantitative comparison, one control library showing a substantially lower number of total reads, possibly due to a bias in library preparation before sequencing, was excluded from the analyses.

Analysis of miRNA and piRNA sequences. Perfect matches to mature miRNA sequences downloaded from miRBase[28] were identified using custom Perl scripts. One sample was removed from the analysis due to much lower total read count. Read counts were identified for each miRNA and normalized using DESeq[29]. A Wilcox unpaired test on the normalized data was used to identify miRNAs showing a statistically significant difference after MSUS treatment. piRNAs were identified by determining sequences that aligned to annotated piRNA clusters[30] using Bowtie[31]. Alignments to piRNA cluster sequences were conducted as a custom-built 'genome' with parameters -k 1 -v 0 -best (to select only the best aligning read with 0 mismatch). After inspection to confirm enrichment of piRNA-like sequences (**Supplementary Fig. 3c**), all sequences with a length of 26–32 nucleotides and a T as the first nucleotide were selected from the libraries and used for alignment to piRNA clusters. DEseq was then used to normalize read counts for each cluster and the differential expression and statistical significance of the differential expression was calculated using a negative binomial test within the DESeq package.

Statistical analyses. No statistical methods were used to predetermine sample sizes but our sample sizes are similar to those reported in our previous publications on the MSUS model[12–14]. Two-tailed Student *t* tests were used to assess statistical significance for behavioral, RT-qPCR, insulin, bodyweight and caloric intake measurements. The remaining metabolic experiments were analyzed using repeated-measurements ANOVAs. All analyzed data matched the requirements for parametric statistical tests (normal distribution). If variance was not homogenous between groups (determined by Levene's test), adjusted *P* value, *t* value and degree of freedom were determined. miRNAs were analyzed using Mann Whitney *U* test and piRNAs were analyzed using negative binomial test with and without Bonferroni multiple test correction. Values over two s.d. away from the mean of each group were considered outliers and excluded from analysis. All statistics were computed with SPSS. All reported replicates were biological replicates, or pooled samples from biological replicates in the case of sequencing samples. Significance was set at $P < 0.05$ for all tests. Error bars represent s.e.m. in all figures.

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