Single paternal Dexamethasone challenge programs offspring metabolism and reveals circRNAs as novel candidates in RNA-mediated inheritance

Katharina Gapp^{#1,2,3,4}, Guillermo Parada^{1,2,5}, Fridolin Gross³, Alberto Corcoba², Evelyn Grau², Martin Hemberg^{1,2}, Johannes Bohacek^{3,4}, Eric A. Miska^{*1,2,5}

¹ Gurdon Institute, University of Cambridge, Tennis Court Rd, Cambridge, CB2 1QN, UK.

² Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, CB10 1SA, UK.

³ Institute for Neuroscience, Department of Health Science Technology, ETH Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland

⁴ Neuroscience Center Zurich, ETH Zurich and University of Zurich, Switzerland

⁵ Department of Genetics, University of Cambridge, Downing Street, Cambridge, CB2 3EH, UK.

* corresponding

co-corresponding author

1

2 **Summary**

3 Single traumatic events that elicit an exaggerated stress response can lead to the
4 development of neuropsychiatric conditions. Studies in mice suggests germline RNA as a
5 mediator of effects of chronic environmental exposures to the progeny. The effects of an
6 acute paternal stress exposure on the germline and their potential consequences on
7 offspring remain unknown. We find that acute administration of an agonist for the stress-
8 sensitive Glucocorticoid receptor, using the common corticosteroid Dexamethasone, affects
9 the RNA payload of post-meiotic transcriptionally silent, mature sperm as soon as 3 hours
10 post exposure. It further impacts early embryonic transcriptional trajectories, as determined
11 by single embryo sequencing, and metabolism in the offspring.
12 Importantly, we show persistent regulation of tRNA fragments in sperm and the descendant
13 2-cell- embryos, suggesting actual transmission from sperm to embryo. Lastly, we unravel
14 environmentally induced alterations in the previously underconsidered class of sperm
15 circRNAs, and their targets in the early embryo, highlighting this class as a novel candidate
16 in RNA-mediated inheritance.

17

18 **Introduction**

19 Acute stress elicits a complex but well-studied cascade of neuroendocrine responses
20 regulated by the hypothalamic pituitary adrenal axis. It involves the release of neuropeptides
21 in the brain that induce the secretion of corticosteroid hormones from the adrenals. These
22 hormones in turn activate mainly two types of nuclear glucocorticoid receptors (GR) (1)
23 expressed throughout the body, which then regulate gene expression and enable
24 physiological and behavioral adjustments (2). In vulnerable individuals, this response is
25 excessive and it can lead to long lasting maladaptations with consequences on psychological
26 and metabolic health (3).

27 It is also known that parental experiences can compromise the health of their progeny both in
28 humans (4–6) and in animal models (7, 8, 17–26, 9, 27, 10–16). Research on the

1 underlying mechanism of such transmission has found changes in germline epigenetic make-
2 up, in particular DNA methylation, histone post translational modifications (PTMs), histone
3 positioning and RNA (28). These epigenetic regulators are responsive to the environment
4 and have been implicated in a variety of environmentally induced diseases (29). Altered
5 modifications must circumvent epigenetic reprogramming events in zygote and, depending
6 on the timing of exposure, during germline development (28, 30). In the male germline, RNA
7 is excluded from reprogramming and therefore a promising candidate for transgenerational
8 information delivery (28, 31). Several studies carried out in *D. melanogaster* and *C. elegans*
9 reported on transgenerational inheritance of induced traits and provided firm evidence for the
10 involvement of small RNAs in the mechanism of transmission (32–34). In mammals, a causal
11 implication in the transmission of environmentally induced effects across generations has
12 also solely been demonstrated for sperm RNA (7, 24, 27, 35). Such RNA differs
13 substantially from somatic RNA since it mainly consists of small RNA, predominantly tRNA-
14 derived small fragments (tsRNAs), but also miRNAs, piRNAs and circRNAs, among others
15 (31)(28, 36). CircRNAs, comprise a very stable class of RNA that has recently been
16 observed to be present in high amounts in testis but also to some extent in sperm (37). Some
17 have been shown to act as miRNA sponges, thereby competing with mRNA targets, while
18 also regulating the expression of their host genes (37). Hence, circRNAs have a strong
19 potential for amplifying an inherited signal, which makes circRNAs exceptionally interesting
20 candidates for epigenetic germline inheritance. To date, the involvement of circRNAs in
21 soma-to-germline signalling has not yet been investigated.

22 tsRNAs and miRNAs are crucial regulators of early embryonic development and players in
23 non-genetic inheritance (7, 10, 24, 27, 35, 38, 39). They have been reported to be acquired
24 through exosomal uptake during epididymal transfer from caput to cauda epididymis (16,44).
25 This might explain their responsiveness to environmental perturbations, despite mature
26 sperm's presumably transcriptionally silent state caused by tightly packed chromatin. Sperm
27 RNA can indeed change in response to chronic stress or by chronic treatments that mimic
28 stress exposure, such as repeated injection of GR agonists (7, 23, 41, 42). Uptake of

1 epididysomal miRNA was sufficient to replicate a chronic stress induced effect on stress
2 response in offspring mice(43) . Surprisingly, acute stress has also recently been shown to
3 affect offspring weight and glucose metabolism in mice (44) and some of these effects were
4 germline dependent (45). Together these related lines of evidence led us to hypothesize that
5 acute GR activation has an intergenerational effect on offspring phenotype and that the
6 transmission potentially implicates changes in the germline. The male germline cells -
7 including mature sperm (46, 47) and their surrounding Sertoli cells (48) – as well es the
8 epididymal epithelial cells (49) express GRs that mediate the effects of glucocorticoids on
9 transcription. Dexamethasone (Dex) a specific GR agonist is known to directly activate GR
10 in the rat epididymis (50). It is unknown whether acute stress affects sperm RNA, and if so,
11 whether uptake via epididymosomes is involved in establishing germline changes that are
12 relevant for offspring phenotypic alterations.

13 Here we investigate the impact of acute GR agonist administration on the germline RNA
14 payload including circRNAs, at various time points post administration and interrogate the
15 fate of altered sperm RNA. We further test germ-line dependency of transmitted metabolic
16 effects and dissect the underlying molecular trajectories during early embryonic development
17 using single cell sequencing of in vitro fertilization (IVF) derived embryos. Identifying a
18 readout of transgenerational risk load at the level of the paternal sperm epigenome could
19 pave the way for future studies aiming at a prevention of the transmission of the effects of
20 acute GR activation to the offspring.

21

22

23 **Results**

24 *Effects of acute Dex injection on the germline small RNA payload*

25 Two reports have suggested that a single foot shock could elicit effects on offspring
26 phenotype (44, 45).To examine potential epigenetic mediators of such acute stressful
27 impacts we investigated sperm RNA of males 2 weeks after a single activation of the GR
28 (Figure 1A). This timeline was chosen to mimic the timing at which breeding occurred when

1 effects on offspring had been observed in a previous study (45). We injected the specific GR
2 agonist Dex once intraperitoneally into 8 adult males. This drug is in frequent clinical use,
3 now also as an apparently effective treatment for patients suffering from lower respiratory
4 tract infection as a consequence of Covid-19 virus (51–53). A sperm population was
5 harvested from each animal and RNA was extracted for ultra-deep small RNA sequencing,
6 resulting in 16 libraries representing one injected male each (8 vehicle and 8 Dex-injected).
7 Purity of the sperm samples was confirmed by inspecting RNA size profiles generated on the
8 bioanalyzer to be absent of ribosomal RNA peaks, that would indicate contamination by
9 somatic cells (Supplementary Fig. 1A). Reaching an average of 55.4 Million sequencing
10 reads while also using randomized adaptors for 3' ligation put us in a position to reduce PCR
11 biases (54) and accurately quantify less abundant miRNAs that are by far outnumbered in
12 sperm by other small RNAs e.g. tsRNAs(55). Our data showed an average of 60% mappable
13 reads across all libraries, including 34% of multimappers. We detected an expected
14 dominant prevalence of reads mapping to tsRNAs and abundant miRNAs in all samples
15 (Supplementary Fig. 2A). Differential gene expression analysis, using DEseq2 (56), revealed
16 that a single acute activation of GR receptors induced changes in tsRNAs and miRNAs
17 collected 14 days post injection (Figure 1B,C, FDR $q < 0.05$), as has been observed in
18 response to chronic environmental stress previously (7, 42). Interestingly, tsRNA-Gly-GCC, a
19 tsRNA previously associated with the effects of nutritional challenge (24), was among the
20 most strongly altered tsRNAs.

21
22 Some recent publications have suggested that sperm miRNAs and tsRNAs are acquired
23 during epididymal transit from caput to cauda (24, 40, 57). Further, it was shown that chronic
24 nutritional-challenge-induced change in sperm tsRNAs is acquired by uptake of distinct sets
25 of tsRNAs (24). To investigate whether the changes in small RNA after acute stress are due
26 to uptake during transit from caput to cauda epididymis we investigated the mature sperm
27 small RNA payload at two time points, 3 hours and 7 days following injection (Figure 2A,
28 Supplementary Fig. 1C). Cells collected from cauda 7 days after injection had already exited

1 testis, but have had time to pass through the entire epididymal tract before collection. Cells
2 collected 3 hours post injection will most likely not have passed through the corpus
3 epididymis, and already resided in cauda epididymis at the time of injection where sperm
4 resides up to 5 days (58, 59). Importantly, spontaneous ejaculation regularly voids cauda
5 epididymis of sperm, even in the absence of a mating partner(60), excluding the retention of
6 “old” mature sperm in cauda for prolonged periods of time. The cells collected 7 days after
7 exposure therefore represent a mixture of cells that might have already resided in the cauda
8 and those cells that indeed passed through the corpus epididymis, yet the spontaneous
9 ejaculation ensures that the sample predominantly contains the latter.

10 The collected samples were confirmed for their purity (Supplementary Fig. 1) and again
11 processed separately to represent sperm from one animal per library. The resulting libraries
12 were analysed jointly as to test for (1) effects of Dex injection independent of sampling time
13 post injection (2) effects of sampling time post injection independent of Dex treatment and (3)
14 effects depending on both Dex injection and the sampling time post injection (interaction).
15 We report an average of 64% mappable reads including 46% of multimappers and observe
16 that tsRNAs were significantly affected by sampling time post injection independent of
17 treatment. This demonstrates the fluctuation of tsRNAs over time in response to external
18 signals such as injections, or potentially due to uncontrollable external inputs from the animal
19 husbandry (Figure 2C). Interaction between treatment and time was statistically significant
20 for 27 tsRNA mapping loci including Gly-GCC-6-1, showing all upregulation after 7 days but
21 either no regulation (26 tsRNAs) or downregulation (Thr-TGT1-1) after 3 hours
22 (Supplementary Table2 sheet 3, $q < 0.05$). This finding is consistent with the dominating view
23 that tsRNAs are acquired during epididymal transit from caput to cauda epididymis. However,
24 most tsRNAs that showed a significant change in response to treatment not after 3 hours but
25 after 7 days (interaction between treatment and time post injection, Supplementary Table 2
26 sheet 3, $q < 0.05$) were not persistently altered in the dataset of 14 days after injection
27 (Supplementary Fig. 3). This indicates that on the one hand changes in sperm RNA are
28 dynamic and many do not persist for prolonged time. On the other hand, this suggests that

1 potentially relevant small RNA changes mostly require either sperm to reside in testis at
2 exposure time or rely on a prolonged residency in the exposed organism. Interestingly,
3 tsRNA-Leu-CAA and tsRNA-Arg-CCT, (Figure 2B) were persistently affected 3 hours and 7
4 days post exposure, that necessarily requires a mode of rapid acquisition of tsRNA-changes
5 in cauda epididymis. While the change in tsRNA-Leu-CAA was temporary and did not
6 persist, strikingly tsRNA-Arg-CCT-2 deregulation persisted until 14 days post injection
7 (Figure 1C). To additionally validate the Dex induced change of tsRNA-Arg-CCT-2
8 independent of epididymal transit from caput to cauda we replicated the effect observed in
9 mature sperm sampled from cauda epididymis 3 hours post injection using q-PCR (Figure
10 2D, Supplementary table 2). Additionally, we sampled caput sperm 3 hours post injection and
11 measured tsRNA-Arg-CCT-2 levels. Overall 2-way ANOVA revealed a significant interaction
12 between sperm sampling location (caput versus cauda) and treatment (vehicle versus Dex).
13 Post hoc tests confirm a significant increase in tsRNA-Arg-CCT-2 levels in response to Dex
14 in cauda but not in caput sperm and a significant increase in Arg-CCT-2 levels between
15 cauda and caput sperm independent of treatment.

16
17 The behaviour of miRNAs differed considerably from tsRNAs. As would be expected if
18 epididymal transit was required for miRNA changes to be implemented, we observe no group
19 effect of treatment on miRNAs (Supplementary Fig. 4A, Supplementary table 2) across 3
20 hours and 7 days post injection. Further, we detected no effect of time post injection on
21 sperm miRNA payload (Supplementary Fig. 4B, Supplementary table 2) confirming the
22 absence of an effect of injection on miRNAs per se. However, we neither detected an
23 interaction between Dex and time post injection (Supplementary Fig. 4C, Supplementary
24 table 2) in miRNAs 7 days and 3 hours post injection. Importantly, when inspecting those
25 miRNAs that were significantly altered 14 days after injection, no alterations were apparent 3
26 hours or 7 days post injection (Supplementary Fig. 4D), indicating that changes in miRNAs
27 occur more slowly or require sperm cells to reside in testis at the time of injection.

28

1 *Effects of acute GR activation on in vivo offspring metabolic phenotype*

2 Based on the two reports on effects of single foot shock on offspring weight and the impact of
3 a single GR activation on germline small RNA payload, we hypothesized that this acute
4 impact on the receptor is sufficient to elicit transgenerational effects. We thus injected Dex
5 once intraperitoneally, then harvested sperm 14 days post injection, and performed IVF using
6 naïve oocytes to generate offspring for phenotyping (Figure 3A). Dex treatment did not affect
7 sperm count, fertility rate or resulting litter-sizes (Supplementary Fig.10-12).

8 The weight and size of pups was measured every 2 to 4 weeks starting at weaning (3 weeks
9 of age) until adulthood (12 weeks of age) and body mass index (BMI) was calculated as a
10 ratio of weight and squared length. Overall ANOVA of the resulting offspring showed a
11 significant effect of treatment ((F1.71)= 76.55, $p<0.0001$), time post injection
12 ((F2.087,144.7)=41.99, $p<0.0001$) and sex (F (1, 71) = 76.55, $p<0.0001$) on BMI, and a
13 significant interaction between time and sex (F (3, 208) = 33.75, $p<0.0001$) and time and
14 treatment (F (3, 208) = 5.834, $p=0.0008$) (Figure 3B, Supplementary Fig. 5C, Supplementary
15 table 3). These results show that while males had generally higher BMI, both male and
16 female offspring of Dex injected fathers had a higher BMI.

17 To further explore potential causes and consequences of altered BMI, adult animals were
18 additionally tested for their glucose tolerance following glucose injection. Overall ANOVA
19 analysis of blood glucose levels revealed a significant effect of sex (F (1, 44) = 54.80,
20 $p<0.0001$) and time post injection (F (2.593, 114.1) = 196.6, $p<0.0001$) and significant
21 interactions between sex and time post injection (F (4, 176) = 6.115, $P=0.0001$), and sex and
22 treatment (F (1, 44) = 15.62, $P=0.0003$) (Supplementary Fig. 5C). Follow up repeated
23 measurements ANOVA analysis separated by sex showed a significant effect of treatment,
24 time and interaction in females (treatment: F (1, 22) = 12.35, $p=0.0020$; time: F (4, 88) =
25 110.1, $p<0.0001$; interaction: F (4, 88) = 2.835, $p=0.0291$) and significant effects of treatment
26 and time but no interaction in males (treatment: F (1, 22) = 6.019, $p=0.0225$; time: F (4, 88) =
27 96.36, $p<0.0001$; F (4, 88) = 0.5401, $p=0.7067$; Figure 3C). These data hence demonstrate a

1 sex-dependent effect of paternal Dex injection on glucose tolerance, with impaired tolerance
2 in females and decreased glucose levels in males in response to glucose challenge.
3 In addition, blood glucose levels were assessed during the insulin tolerance test. Overall
4 ANOVA analysis showed significant effects of sex ($F(1, 37) = 162.6, P < 0.0001$)
5 and time ($F(3.314, 122.6) = 23.85, P < 0.0001$) and revealed a significant interaction between
6 sex and time ($F(4, 148) = 12.49, P < 0.0001$), time and treatment ($F(4, 148) = 5.380,$
7 $P = 0.0005$) and time and treatment and sex ($F(4, 148) = 5.392, P = 0.0004$) (Supplementary
8 Fig. 5C). Follow-up repeated measurements ANOVA separated by sex showed a significant
9 effect of time ($F(2.982, 65.60) = 44.73, p < 0.0001$), yet no significant effect of treatment ($F(1,$
10 $22) = 0.3465, p = 0.5621$) nor an interaction between time and treatment ($F(4, 88) = 0.1373,$
11 $p = 0.9681$) in females (Figure 3D). In males we observe no effect of treatment $F(1, 15) =$
12 $1.467, p = 0.2446$ yet detected a significant effect of time post injection time: $F(2.914, 43.71)$
13 $= 4.538, p = 0.0079$, and a significant interaction between treatment and time post injection F
14 $(4, 60) = 7.003, p = 0.0001$, Figure 3D). These results indicate sex and time dependent
15 effects of paternal Dex on insulin tolerance. They further show no change in insulin tolerance
16 in female descendants of fathers injected with Dex, whereas impaired insulin tolerance in
17 male progeny.
18 Lastly, we explored a potential reflection of altered BMI in tissue composition by necropsy
19 and weighing the dissected organs and fat pads. Overall ANOVA of necropsy weights
20 revealed a significant effect of sex ($F(1, 140) = 28.27, P < 0.0001$), tissue ($F(4, 140) = 232.7,$
21 $P < 0.0001$) and a significant interaction between sex and tissue ($F(4, 140) = 3.379,$
22 $P = 0.0113$) yet no effect of treatment ($F(1, 140) = 0.2587, P = 0.6118$), or interaction between
23 treatment and sex ($F(1, 140) = 0.0004794, P = 0.9826$) or treatment and tissue ($F(4, 140) =$
24 $0.1635, P = 0.9565$) on tissue weight (Supplementary Fig. 5A,B,C). This confirms sex
25 dependency, yet no effect of paternal Dex injection on tissue weight in both sexes.
26
27 *Effects of acute Dex on offspring early embryonic small RNA*

1 The small quantity of paternal RNAs in the zygote relative to the large pool of maternal RNAs
2 poses serious obstacles to their accurate quantification (36). While initial reports on small
3 RNA transmission relied on comparative sequencing or microarrays analyses of unfertilized
4 oocytes and fertilized zygotes (61), today we are aware that such comparisons can be
5 deceiving, as they rely heavily on both assessment method (eg. microarray restricted to a
6 selective set versus unbiased genome-wide sequencing) and sequencing depth (54, 62). An
7 example are inconsistent results regarding miRNAs that are exclusively supplied from the
8 sperm, such as miR-34c, -99a, -214 (63, 64). Alternative approaches have used indirect
9 measures, e.g. assessing mRNA targets of paternally derived small RNAs (24, 64–66). We
10 attempted to directly examine the relative difference between the small RNA landscape in
11 early embryos resulting from IVF of naïve oocytes with sperm from either Dex or vehicle
12 injected males (Figure 4A). We used small-RNA sequencing to compare 2-cell embryos
13 derived from Dex treated or control fathers. We detected an average of 29 % mappable
14 reads including 21% multimappers. While we only detected subtle changes in miRNAs of
15 Dex exposed progeny (Supplementary Fig. 6), we observed downregulation of several
16 tsRNAs from 6 different genomic locations ($q < 0.1$) (Figure 4B). Strikingly, two of the
17 downregulated tsRNAs (Gly-GCC at several genomic loci and Gly-CCC) were consistently
18 downregulated in sperm 14 days post Dex injection. This likely indicates a direct delivery of
19 these tsRNAs in control conditions, yet absence or reduced delivery of this sperm RNA cargo
20 of Dex treated males to the oocytes they fertilize.

21

22

23 *Effects of acute Dex administration on offspring early embryonic transcriptome*

24 If sperm RNA was directly impacting the zygotic mRNA pool, or affecting early embryonic
25 gene expression we hypothesized this to be apparent in the 2 cell embryo's transcriptome
26 (Figure 5A). To examine the effect of paternal Dex on early embryonic RNA content we
27 subjected 2-cell embryos to the Smartseq single cell sequencing protocol (Supplementary
28 Fig. 7). After performing quality control and filtering the sequenced 2 cell embryo data on

1 criteria such as minimal read count/embryo (Supplementary Fig. 7A), we carried out
2 unsupervised clustering based on their gene expression profiles using SC3 (67). We
3 identified two distinct clusters (C1 and C2), which were composed by a balanced mixture of
4 treated and control cells. (Figure 5B). Since the resolution of single cell experiments allows
5 characterizing distinctive transcriptomic profiles within early cell division stages, we used
6 scmap (68), to project each 2 cell embryo gene expression profile onto a reference dataset
7 of single cells from 2 cell embryo states previously reported by Deng *et al.* (69) (Figure 5C.)
8 Most of the 2 cell embryos belonging to cluster C1 projected to the late 2 cell stage, whereas
9 embryos from C2 exclusively projected to cells from the mid 2 cell stage. This shows that the
10 two clusters identified through unsupervised clustering correspond to 2-cell embryos in the
11 mid and late 2-cell stage respectively.

12 Principal component analysis (PCA) revealed a prominent separation between C1 and C2
13 along the PC1 axis, suggesting a correlation between PC1 and developmental transitions
14 between mid and late single cell embryos (Supplementary Fig. 7B.) Interestingly, 2 cell
15 embryo offspring of males injected with Dex exhibited a significant shift of the C1 cluster
16 across PC1 (two-sided Wilcox test $p < 0.03$), while the C2 clusters did not show significant
17 differences across PC1 between treatment and control groups (Figure 5D.) These results
18 suggest that the effect of paternal Dex treatment on the transcriptome only becomes
19 apparent at the late 2 cell embryo stage. To further explore this hypothesis, we calculated the
20 silhouette coefficient (70) on PC1, as a measure of distance between C1 and C2 clusters, for
21 the control and treatment group. We observed a significant increase of PC1 silhouette
22 coefficient between treatment and controls for both C1 (one-sided Wilcoxon test p-value
23 < 0.005) and C2 (one-sided Wilcoxon test p-value $< 2 \times 10^{-5}$.) This confirms that Dex treatment
24 affects embryonic gene expression, promoting altered late 2 cell embryo stages since the
25 divergence from mid 2 cell embryos is significantly bigger in Dex offspring compared to
26 control offspring (Figure 5E.)

27 Accordingly, differential gene expression analysis using Monocle2 (71) focused on late 2-cell
28 embryos (cluster C1), revealed significant gene expression changes between offspring of

1 males injected with Dex and controls across 38 genes, some of which were already apparent
2 to a less significant extent during mid-2 cell embryos (cluster C1; e.g. Tc11; Supplementary
3 Fig. 8A,B.) In line with a potentially altered developmental trajectory becoming apparent in
4 cluster 1, the late 2-cell stage includes several affected genes that are involved in early
5 embryonic development. For example, Bcap31 (B-cell receptor-associated protein 31) is an
6 important element for endoplasmatic reticulum and Golgi apparatus function, and Bcap31
7 mutations lead to developmental diseases with metabolic disturbances (72). This is
8 reminiscent of the metabolic phenotype observed in the adult offspring of Dex injected
9 fathers. Hprt (Hypoxanthine-guanine phosphoribosyltransferase) is crucial for cell cycle
10 division, and Tc11 (T-cell leukemia/lymphoma) regulates cell proliferation (73, 74). Hence,
11 an upregulation of Hprt, and a concomitant down-regulation of Tc11 might indicate that cell
12 fate decisions later during development may be affected. Another differentially expressed
13 gene is Rbbp7 (RB binding protein 7), which is part of many histone deacetylase complexes
14 such as Nurd and PRC2/EED-EZH2, and thus plays an essential role in chromatin mediated
15 gene regulation (75). Interestingly, several forms of PRC mutations in humans lead to
16 different kinds of overgrowth phenotypes (76), an abnormality reminiscent of the increased
17 BMI observed in Dex-offspring (Figure 3B.)

18

19

20 *Effects of Dex administration on a novel candidate for sperm RNA mediated inheritance*

21 Despite the observed changes in sperm tsRNAs following acute Dex injection, we did not find
22 an obvious causal connection to the altered 2-cell embryonic transcripts. This prompted us to
23 investigate whether other germline changes might be more crucial for the offspring *in vivo*
24 alterations in our model. We previously showed that chronic stress exposure also led to
25 changes in sperm long RNAs that contributed functionally to the transmission of effects to the
26 offspring (79), yet the fact that sperm RNA is stable through transmission and that the minute
27 amounts of transmitted paternal RNA can elicit major changes in the embryo remains
28 puzzling. Therefore, we evaluated the impact of Dex injection on the highly stable class of

1 circRNAs in male sperm. CircRNAs were previously detected in swine(80) and human sperm
2 (81) and suggested to have functional implications in epigenetic regulation. They have been
3 attributed a critical role post transcriptional cessation in the male germline(82). Using
4 Circexplorer in combination with EdgeR, analysis of sperm long RNA sequencing of males
5 treated with Dex and controls revealed significant upregulation of two circRNAs (Figure 6A,
6 $q < 0.1$, Supplementary Fig. 1B.) while we also observed several significant changes in the
7 sperm long RNA protein coding transcripts following acute Dex treatment (Figure 6B,C,
8 Supplementary Fig. 1B, Supplementary Fig. 2B, Supplementary table 5). Both circRNAs are
9 hosted in genes relevant for immune function (Taspase 1: Tasp1 and DENN Domain
10 Containing 1B Dennd1b), yet the host genes did not show differential abundance of the protein
11 coding host- gene transcript (Supplementary table 5). CircAtlas(83) revealed several potential
12 miRNA sponge-targets to be captured by the altered circRNAs. Some of these miRNAs are
13 common sponge-targets of both circRNAs such as mir3110-5p, mir706, mir1955
14 (Supplementary Fig. 9). Diamine acetyltransferase 1 (Sat1), one of 3110-5p's high
15 confidence miRNA-targets, as predicted by TargetScan(84), is indeed significantly
16 upregulated in the embryos composing cluster 1 (later developmental stage). MiRNA-target-
17 upregulation is expected if mir3110-5p was downregulated through circRNA-mediated-
18 sponging and highlights a potential effective contribution of increased circRNA in sperm to
19 embryonic pathway regulation. This is the first report of a change induced by environmental
20 exposure in this compelling class of RNA in sperm.

21

22 Discussion

23 By generating offspring using assisted reproductive techniques (IVF), we circumvent
24 potential confounding variables such as transmission via RNA contained in seminal
25 exosomes (85) and affected maternal care by altered mating behavior (86) and prove
26 germline dependence (30). Consistent with the significant changes of miRNAs and tsRNAs in
27 the germline 2 weeks post GR activation, previous studies including our own have observed

1 regulation of mouse sperm small RNA in a variety of contexts (7, 23, 24, 27, 35, 38, 42, 87–
2 89).
3 Especially relevant specifically for our analysis, sperm RNA sequencing after drinking water
4 administration of corticosterone for 4 weeks followed by mating, led to strong downregulation
5 of tsRNA-GluCTC and tsRNA CysGCA, two of our top down-regulated tsRNAs, indicating
6 that these tsRNAs are responding similarly to acute and chronic insults. At the same time this
7 chronic manipulation elicits changes of several miRNAs, e.g. 34c and 471 (41), albeit in the
8 opposite direction of what we find in response to acute Dex treatment. These discrepancies
9 may arise either by the Dex induced short-term suppression of internal corticosteroid (90) or
10 due to adaptations in response to chronic administration.
11 While four (23, 41, 91, 92) out of five (93) previous studies did report phenotypic effects
12 following chronic paternal Dex exposure, only two assessed sperm small RNAs to associate
13 the alterations to the sperm RNA payload (91, 93) yielding conflicting outcomes.
14 These differences might be due to inconsistent life stages (adulthood versus gestational),
15 sperm collection (swim up, somatic lysis, or no purification) and/or dosage of exposure.
16 Depending on the dosage and timing, the complex autoregulation of the GR can lead to GR
17 downregulation after prolonged activation (94). Acute exposures have the advantage of
18 avoiding such long-term feedback regulation, and hence provide an elegant approach for
19 studying the signaling pathways leading to germline changes.
20 Mature sperm tsRNAs and miRNAs have been shown to be acquired during epididymal
21 transit (24, 40) and miRNAs are necessary for early embryonic development under certain
22 circumstances (57, 63). Furthermore, a recent publication suggests that chronic stress
23 induced sperm miRNAs are taken up primarily from epididymisomes originating from the
24 caput epididymis or the proximal epididymal tract (43). Chronic nutritional manipulation with
25 effects on offspring also report the necessity of epididymal transit to acquire tsRNA changes
26 in sperm(24). Harvesting mature sperm 3 hours after exposure yields a population enriched
27 for cells that had been exposed while already in the cauda epididymis, where spermatids
28 reside for roughly 5 days (59). These cells have not traveled through the epididymis nor have

1 they had a chance to potentially take up small RNA from caput-derived epididymisomes after
2 Dex administration. As expected, we detect no changes in miRNAs in these samples. We do
3 however detect changes in tsRNAs 3 hours post Dex, some of which even persist 14 days
4 post injection. These results for the first time show rapid acquisition of changes in vivo and
5 corroborate previous in vitro findings that show that incubation with epididymisomes can alter
6 sperm RNA payload(24). Our acute intervention for the first time assesses effects on
7 germline payload already after a short interval. Whereas chronic interventions - based on
8 their experimental design - do not assess changes in mature sperm soon after the first
9 intervention. Studies aiming at the elucidation of the origin of sperm RNA changes might
10 benefit from acute interventions to circumvent confounders such as dynamic exosomal RNA
11 supply as a result of cumulative interventive strain on animals.

12 An additional option for sperm RNA alterations in transcriptionally inert sperms was
13 suggested in a recent study that found mitochondrial tRNA cleavage in the T-loop in
14 response to a one-week high sugar diet in humans(95). In line with this observation, Dex
15 injection could trigger oxidative stress(96) that provokes such cleavage to increase tsRNA
16 levels(97). A role for oxidative stress in sperm RNA dynamics is further supported by a
17 recent study in boar sperm that found seasonal differences in sperm small and long RNA
18 associated with changed abundance of transcripts mapping to oxidative stress-, DNA
19 damage- and autophagy- related genes (98). Such potentially oxidative stress mediated
20 mechanism does not explain though a rapid decrease of tsRNAs 3 hours post Dex injection.

21 Importantly, we show alterations in sperm tsRNAs that persist in the oocyte concomitant with
22 changes in early embryonic gene expression and a metabolic phenotype in adulthood.
23 tsRNAs and tRNA-Gly derived fragments in particular are known to induce chromatin
24 structure mediated gene regulation and to regulate cell differentiation in various contexts (99,
25 100). Hence, we propose that the transmitted reduction in key tsRNAs such as Gly-GCC and
26 Gly-CCC explains in part the observed perturbations during late two cell embryo
27 developmental stage. This might reflect an accelerated developmental transcriptional

1 program in the preimplantation embryo of Dex injected males, ultimately resulting in aberrant
2 BMI and glucose metabolism later in life.

3 Additionally, we have discovered alterations in circRNA abundance in mature sperm that
4 might also impact the developmental program in the early embryo. CircRNAs have the
5 potential to be translated into proteins via back-splicing(101). Accordingly, they are crucial
6 contributors to spermiogenesis post transcriptional cessation, since they provide a stable
7 alternative to linear mRNA templates for protein translation(82). Transmitted sperm-circRNAs
8 could likewise contribute to translation post fertilization, yet the unconventional lattice-state of
9 ribosomes preventing normal rates of translation post transcription(102) accompanied by a
10 rapid increase in proteins of the ubiquitine/proteasome pathway(103) make this unlikely.

11 Never the less a study on human sperm detected abundant levels of circRNA with predicted
12 regulatory function of early developmental genes in sperm heads, suggesting transmission
13 and function post fertilization(81). By sponging miRNAs that regulate early embryonic
14 transcripts, circRNA could amplify minute signals from paternal environment, such as might
15 be the case for the gene transcript Sat1, that displays increased expression in Dex offspring
16 in 2-cell embryos from cluster 1.

17 Besides altered RNA identity, nucleic acid modifications especially of RNA but also DNA
18 methylation and chromatin accessibility might contribute further to the effects of Dex
19 injections on offspring metabolism. While detection of changes in each player should be
20 subject of further investigation and might reveal a glimpse of their potential implication, proof
21 of the individual relative causal contribution is extremely challenging since they likely require
22 tight interaction to unravel their orchestrated effects.

23 Finally, it might be useful to consider testing the translatability of our findings to humans.
24 Here we investigated the effects of a single Dex administration soon after the injection in
25 mice, mimicking a single GR activation such as elicited by treatment of an acute asthma
26 exacerbation (104). The recent report that Dex can reduce the number of deaths associated
27 with the Covid-19 pandemic (51), further prompts the re-evaluation of the impact of
28 prolonged Dex treatment on offspring phenotype. From a clinical perspective, additional

1 consideration is warranted for consequences on offspring health when extended time has
2 elapsed between treatment and time of conception. Such designs may pave the way for the
3 extrapolation of our findings.

4 We conclude that acute Dex treatment can induce germline epimodifications in the form of
5 small and long non-coding RNA, which likely are relevant in the transmission of the effects of
6 single traumatic events on offspring well-being. Our data suggest that sperm small RNAs are
7 not solely regulated through epididysomal uptake during transition from caput to cauda
8 epididymis. This expands the interpretation from chronic dietary and stress exposures(24,
9 43), where uptake of tsRNAs and miRNAs via epididymisomes has been suggested to lead
10 to differential sperm payload, yet required sperm to transit from caput to cauda to bring about
11 the changes. A persistent detection of significant fold changes of the exact same sperm
12 small RNA in the embryo suggests functional implication in the information transfer from
13 father to offspring. Together with potentially transmitted miRNA sponges in the form of
14 circRNAs, this likely contributes to a slight developmental acceleration of gene expression
15 programs in the early embryo and ultimately manifests in a metabolic phenotype. Future
16 studies may aim at testing the causal contribution of specific sperm RNAs to the transmission
17 of effects of acute impacts. Certainly, continuous methodological refinement will help dissect
18 the relative implication and the interplay of the distinct germline modifications such as DNA-
19 methylation, histone-PTMs and chromatin architecture in this highly complex process.

20

21

22 ***Conflict of interests***

23 The authors declare no competing interests. E.A.M. is a founder and Director of STORM
24 Therapeutics Ltd. STORM Therapeutics had no role in the design of the study and collection,
25 analysis, and interpretation of data as well as in writing the manuscript.

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Materials and Methods

Animals

C57Bl/6 mice were obtained from the Sanger Research support facility in-house-breeding colony. They were housed in a temperature and humidity-controlled facility in individually ventilated cages under a non-reversed light-dark cycle (Sanger Research support facility) or a reversed-light-dark cycle (ETH EPIC). Standard chow (LabDiet(r) 5021-3 supplied by IPS) and water were provided *ad libitum after weaning* unless stated otherwise (e.g. oocyte donors). Breeding colony was provided SAFE R03-10 breeding diet, supplied by SAFE diets. Experimental procedures were performed during the animals' inactive cycle at Sanger. Age and weight matched (margin of one week) males were used in each experimental group receiving Dex injections. Animals used for Dex injection followed by sperm sequencing were all sexually mature (14 and 7 days or 3 hours post treatment were 13, 11 and 9 weeks of age respectively) at the time of sperm collection.

C57Bl/6 males used for sperm sequencing 14 days post Dex injection and q-PCR experiments/validation 3 hours post Dex were obtained from the ETH's EPIC in house breeding colony in Zürich and were 14-18 weeks old. These mice were fed chow #3734 by Kliba/Granovit.

IVF oocyte donor females and embryo recipients were fed SAFE R03-10 breeding diet, supplied by SAFE diets until 10 days post embryo transfer. Until this time embryo recipients were housed in pairs after which they were split into single housing. IVF offspring was

1 weaned at PND21 and assigned to cages avoiding littermate cohousing. Offspring
2 phenotyping was carried out between 3.5 to 4 months and necropsy at 4.5 months of age in
3 balanced (offspring controls, offspring treatment) and age matched groups (all animals had
4 an age spread of 3 days). Animals were housed in groups of 4-5 mice/cage in the Sager
5 Institute barrier research support facility (all animals apart from animals for q-RT-PCR
6 experiments) and ETHZ's EPIC facility (animals for q-RT-PCR).
7 All experiments were approved by the UK home office (project license P176396F2) and
8 Cantonal commission for animal experimentation Zürich (project license ZH222/19).

9

10 *Dex treatment and sample collection*

11 Age matched males with an age spread of 1 week were randomly assigned to control and
12 treatment groups. Males were injected with either 2mg/kg of Dex in 10 % DMSO, 0.9% saline
13 or vehicle (10%DMSO in 0.9% saline). Males used for sperm collection did not undergo any
14 metabolic testing. They were sacrificed 2 weeks, 7 days and 3 hours after Dex or vehicle
15 treatment. Cauda epididymis and vas deference were dissected and placed in M2 medium.
16 After allowing sperm to diffuse into M2 medium, cells were pelleted by short centrifugation
17 and washed with PBS. For sperm RNA sequencing and q-PCR, mature sperm cells were
18 separated from potential somatic contamination by somatic lysis, followed by 2 washes with
19 PBS (105). Sperm counts and fertilization rate appeared unaffected post Dex injection
20 (Supplementary Fig. 10&11).

21

22 *Superovulation, in vitro fertilization and embryo culture*

23 12 randomly selected, C57BL/6 females were superovulated at 26-31 days of age with Card
24 Hyperova (Cosmo Bio, KYD-010-EX-X5), followed by 7.5 IU human chorionic gonadotrophin
25 (HCG) 48 hours later.
26 Cumulus-oocyte complexes (COCs) were released from the ampulla of the oviduct 16-17
27 hours after HCG administration, and preincubated in high calcium HTF with Glutathione
28 medium for 30-60 minutes (in CO2 incubator at 37 deg C, 5% CO2 in air) before

insemination. Frozen sperm used for insemination was pooled from 2 males that had been injected with Dex or vehicle 14 days prior to cryopreservation. Thawed sperm was preincubated for 30 minutes in TYH (with Methyl- β -cyclodextrin, Sigma C4555) medium at 37 deg C, 5% CO₂ in air, before being added to the COC complexes for fertilisation. 4 hours after insemination, the presumptive zygotes were washed through several drops of KSOM (Millipore, MR-121-D) and incubated overnight in KSOM.

For in vivo offspring, 14-20 x 2 cell embryos from overnight culture in 6 individual IVF dishes /group were implanted into 0.5 dpc pseudo-pregnant F1 females (6 females/group). Each dish contained oocytes from one female with the exception of 2 dishes (out of 6) in the Dex group that contained oocytes of the same female, since one female failed to super-ovulate. For molecular (single) embryo gene expression analysis at the two cell stage, 2-cell-embryos from overnight culture were frozen, and after thawing briefly cultured in preincubated KSOM until/during plating into 96 well plates. The females used to generate these embryos were superovulated with PMSG. The IVF Protocol is based on EMMA Harwell's protocol (adapted from Takeo & Nakagata 2011(106)), and the Sperm Freeze Protocol is based on Ostermeier G.C. et al (2008)(107). Resulting litter sizes did not differ between Vehicle and Dex injected offspring (Supplementary Fig. 12)

Sperm and embryo RNA extraction

Total RNA was prepared from adult mouse sperm using Trizol (Thermo Scientific 15596026) and Directzol (Zymo R2080). Total RNA was prepared from zygotes using the Trizol LS protocol. Quantity and purity of RNA were determined by Agilent 2100 Bioanalyser (Agilent Technologies) and Qubit fluorometer (Life Technologies). Absence of prominent ribosomal peaks indicated absence of somatic cell contamination.

Sperm RNA sequencing (RNAseq)

Sequencing was done using an Illumina Genome Analyzer HiSeq 2500 (Illumina) in Rapid

run mode for long 100bp and small 50 bp RNA sequencing runs respectively.

Libraries for long RNA sequencing were prepared using the TruSeq Stranded Total RNA kit according to the manufacturer's instructions with indices diluted at 1:3. 200 ng of total sperm RNA was subjected to removal of rRNA using Ribozero gold kit. Approximately 100ng of sperm RNA and total RNA of several 2-cell zygotes was subjected to TruSeq or Nextflex (sperm 14 days post injection) small RNA library preparation following the manufacturer's recommendations with the following modifications: adaptors were diluted 1:4 and PCR cycles were augmented to 18 and 22 (Nextflex) PCR cycles respectively. When library preparation of samples was split across days groups were balanced to circumvent batch effects.

Single embryo seq.

2 cell embryos were generated using the same conditions as indicated for in vivo offspring yet followed by embryo cryopreservation until processing for library preparation. They were thawed and those that appeared intact (34 controls and 37 Dex) pipetted into wells of 2 96 well culture plates containing lysis buffer and stored at -80°C before processing according to the Smartseq 2 protocol and manufacturer's recommendations (Nextera). Libraries contained a 1:19 Million dilution of External RNA Controls Consortium (ERCC) spike-ins (4456740 Ambion) and were amplified for 18 PCR cycles. Sequencing was performed on a HiSeq V4 under paired end 75bp mode.

Insulin and Glucose tolerance test

Animals were fasted 4 hours to establish a shared baseline glucose level. They received a single injection of insulin (insulin: 1mU/g body weight) (Actrapid Novo Nordisk), glucose (2mg/g body weight) or vehicle (saline) intraperitoneally. Blood samples were taken from lateral tail vein in adult animals to assess blood glucose level using an Accuheck aviva device.

Body mass index

1 Animal lengths were measured using a standard ruler and weighed for assessing body
2 weight. Body mass index was calculated using the following formula: weight (g)/(length
3 (cm)²).

4

5 *Necropsy*

6 Organs were dissected after sacrifice and weighed immediately on a scale using “g” as a unit
7 with an accuracy of 2 decimals (accurate down to 10 mg).

8

9 *q-RT-PCR*

10 5ng/sample RNA isolated from sperm was reverse transcribed (RT) using the miCURY LNA
11 RT kit (Qiagen #339340). Quantitative RT-PCR (qRT-PCR) was performed using SYBR
12 green based detection in a Biorad thermal cycler with MiRCURY LNA-based small RNA
13 probes designed against 5`end of tRNA ArgCCT-2
14 (5`GCCCCAGUGGCCUAAUGGAUAAGGCACUGGCC3`) with a polyA tail directed reverse
15 miRCURY primer (Qiagen # 339317). U6 was used as an internal control (Qiagen # 339306).

16

17 *Bioinformatic and statistical data analysis*

18

19 *Sperm RNA sequencing*

20 Each sequencing library represented sperm harvested from a single male. Sequencing
21 quality was assessed with FastQC (108) and MultiQC (109). Adapters were removed from
22 the 3` ends with cutadapt (110) (version 1.14) and resulting sequences with 14 nucleotides of
23 length or less were discarded. All other reads were aligned end to end (no soft clipping) to
24 the ENSEMBL *Mus musculus* genome (release 75) (111) with STAR (112). No mismatches
25 were allowed. Featurecounts was used to match the alignments against the miRbase(113)
26 annotation (version 21) and obtain a matrix of miRNA counts. We applied fractional counts
27 whenever alignment occurred at multiple genomic locations. Differential expression was
28 analyzed using DESeq2 (56). Quantification of tRNA fragments was performed as above, but

1 all CCA-3' trinucleotides were trimmed after adapter removal, sequences with 15
2 nucleotides or less were subsequently discarded and GtRNAdb (114) annotation
3 (GRCm38/mm10) was used to obtain the count matrix.
4 For the data set collected 14 days after Dex injection, library preparation included the
5 insertion of 2 random tetranucleotides between read and adapters. By including only unique
6 sequences in the analysis we removed duplicates due to PCR amplification.
7 Long RNAseq libraries were pre-processed with trimmomatic (115) to remove adapters. Reads were
8 aligned to the genome using STAR (112) and quantified using featurecounts (116). Circular RNAs
9 were quantified using Circexplorer2 (117) based on junction reads as detected by STAR. Differential
10 expression analysis was performed on the combined set of counts for circular and non-circular RNAs
11 using edgeR (118). Robust estimation of dispersion was used to avoid spurious significance due to
12 outliers.

13

14 *2-cell single embryo sequencing analysis*

15 Reads from 2 cell embryos were mapped to the mouse reference genome (mm10) and
16 ERCC spike-ins using STAR (112). Resultant alignments were processed to quantify the
17 expression of annotated genes by GENCODE (vM11) and ERCC spike-ins using
18 featureCounts (116). To filter low-quality sequenced embryos we only considered those
19 which had a total read count of at least 0.5 million reads with less than 15% and 10% their
20 read counts mapping to mitochondrial genes and ERCC spike-ins respectively. After these
21 filters were applied a total of 56 embryos (29 controls and 27 treated) remained. We
22 clustered their gene expression profiles using SC3 (67) obtaining two main clusters (C1 and
23 C2). Using scmap (68), we projected the gene expression profiles for the two cell embryos
24 onto an index containing expression profiles from zygotic, early/mid/late 2 cell embryos and
25 4 cell embryo cells reported by Deng *et al.* (69). We performed PCA analyses using scater
26 (119) (runPCA function) and we calculated the PC1 silhouette coefficient using in-house R
27 scripts. To perform differential gene expression analyses we normalized the read counts of
28 each embryo as FPKM and we used Census (71) algorithm to convert these values into

relative transcripts counts. We computed the obtained 'Census counts' using Monocle (v 2.99.2), assuming a negative binomial distribution and a lower detection limit of 0.5. We performed differential gene expression analyses between the total treated and control embryos, and also between the treated and control embryos inside of C1 and C2 clusters.

Remaining statistical analyses.

Sample size for in vivo offspring phenotyping was estimated based on previous work on similar models (44, 45). 3-Way repeated measures ANOVA was used to assess statistical significance for BMI, GTT and ITT measurements. Necropsy data were analysed using 3-way ANOVA followed by multiple t-tests and corrected for multiple comparisons using the Benjamini-Hochberg method. Normality was assessed with the Kolmogorov Smirnov test and met in all necropsy data. Homogeneity of variances was assessed and met in all necropsy data unless gonadal WAT. All t-tests did not assume homogeneity of variances (applied Welch's correction). All statistics of behavioural, metabolic tests and q-RT-PCR were computed with Prism. Outliers were removed from q-PCR results using Prism's inbuilt ROUT method and are depicted in the supplementary table 3 containing raw data with a star. Q-RT-PCR results were analysed with a 2-Way Anova followed by posthoc tests to compare individual groups applying the Bonferroni correction for multiple comparisons. All reported replicates were biological replicates. Significance was set at $p < 0.05$ for all tests.

Availability of Data.

The datasets supporting the conclusions of this article are included within the article (supplementary tables). All sequencing data were deposited to Gene Omnibus (accession number: xxx) and ENA (accession number: [xxxx](#)). All code is available on Github (<https://github.com/ETHZ-INS/Sperm-RNA-Dex>)

Figure legends

Figure 1 Effects of Dex on small RNA payload of sperm cells residing in testis at time of administration. (A) Experimental design depicting a time window of two weeks between injection of Dex and sperm collection for molecular analysis. (B) Volcano plot depicting fold changes and significance level of miRNAs and tsRNAs (C) in mature sperm 14 days post injection of Dex (n=8) versus vehicle (n=8) as assessed by small RNA sequencing.

Figure 2 Effect of Dex on sperm cells at different time points post Dex administration. (A) Experimental design showing the location of sperm at time of injection and timing of sperm harvest. MA (log-intensity ratios (M-values) versus log-intensity averages (A-values) plots depicting (B) effect of Dex (log2 fold changes control versus dexamethasone), (C) of time post injection (log2 fold changes 7 days versus 3 hours) (7 days Dex n =4 and controls n =4, 3 hours Dex n =3 and controls n =4). TsRNAs are indicated by sequence identity for display only, each dot represents one small RNA. MA plot depicts log2 fold changes on the y axis and the expression level on the x axis (the higher the expression the further to the right). Statistically significantly changed small RNAs are highlighted in red. (D) Relative expression of ArgCCT-2 as obtained by q-RT-PCR (cauda: Dex n=4, controls n=4, caput: Dex n=4, controls n=5; interaction $F(1,13)=6.34$, $p=0.0257$, treatment $F(1,13)=5.97$, $p=0.0040$, site of collection ($F(1,13)=12.15$, $p=0.0296$; cauda control versus cauda Dex $t(13)=3.42$, $p=0.0274$, cauda Dex versus caput Dex $t(13)=4.137$, $p=0.007$). Whiskers display minimum and maximum.

Figure 3 Effect of Dex on metabolic phenotype in the offspring (A) Experimental design depicting timeline between injection, sperm harvest, in vitro fertilization and phenotyping. (B) Impact of Dex on male and female adult offspring (B) Body mass index (males vehicle offspring n=21, Dex offspring n=22, females vehicle offspring n=17, Dex offspring n=17) (C) glucose tolerance (males vehicle offspring n=12, Dex offspring n=12, females vehicle offspring n=12, Dex offspring n=12) and (D) insulin tolerance (males vehicle offspring n=9, Dex offspring n=8, females vehicle offspring n=12, Dex offspring n=12). Error bars display

1 SEM. Detailed statistical results are depicted in Supplementary Fig. 5, raw data are provided
2 in supplementary table 3).

3

4 **Figure 4 Effect of paternal Dex injection on embryonic offspring small RNA (A)**

5 Experimental design depicting timeline between injection, sperm harvest, in vitro fertilization
6 and small RNA sequencing at 2-cell stage. (B) Volcano plot showing effect of paternal Dex
7 on small RNA tsRNAs (vehicle embryonic offspring n=5, Dex embryonic offspring n=4).
8 TsRNAs are grouped by sequence identity for display only.

9

10

11 **Figure 5 Effect of paternal Dex injection on embryonic offspring long RNA transcriptome (A)**

12 Experimental design depicting timeline between injection, sperm harvest, in vitro fertilization
13 and Smartseq2 sequencing at 2-cell stage. (B) Consensus matrix representing the similarity
14 between cells as reported by SC3. Similarity 0 indicates that a given pair of embryos were
15 never assigned to the same cluster, whereas similarity 1 means that a pair of embryos were
16 always assigned to the same cluster. (C) Sankey diagram showing projection of the
17 obtained clusters (C1 and C2) into clusters reported by Deng *et al.* for single cells obtained
18 from 2 cell embryos. (D) Principal component analysis of two-cell embryos gene expression.
19 The top panel indicates the density of 2 cell embryos along PC1 grouped by condition;
20 control (red) and Dex treatment (blue). The other two bottom panels show the distribution of
21 2 cell embryos across PC1 and PC2 for control (red) and treated (blue) groups. The cluster
22 membership of each embryo is denoted by the point shapes (C1 cycles; C2 triangles) and
23 centroids of each cluster is indicated with a black dot overlaid with an x. Wilcox tests were
24 performed to assess differences on PC1 values of C1 and C2 clusters between the treated
25 and control groups. NS denotes non-significant change for C2 cluster, while * indicates a
26 significant difference for C1 cluster (p-value < 0.05). (E) Silhouette coefficient comparison
27 between treatment and control, statistical significance was assessed with Wilcox test (** p-
28 value < 0.01; *** p-value < 0.005) (F) Selection of differentially expressed genes as

1 determined by Monocle within C1 corresponding to late 2-cell embryo stage (***) adjusted p-
2 value < 0.005).

3 **Figure 6** Effects of Dex on long RNA payload of sperm cells residing in testis at time of
4 administration. (A) Experimental design depicting a time window of two weeks between
5 injection of Dex and sperm collection for molecular analysis.
6 (B) Volcano plot depicting fold changes and significance level of long RNA in mature sperm
7 14 days post injection of Dex (n=4) versus vehicle (n=4) as assessed by small RNA
8 sequencing. (C) Heatmap showing significantly differentially expressed long RNA transcripts
9 of the same experiment (multiple comparison corrected, $q < 0.05$).

10

11

12 **Supplementary Material**

13 This article contains supplementary Figures and tables. Supplementary figures are compiled
14 in one word document.

15

16 **Supplementary table legend**

17

18 *Supplementary Table 1*

19 Sheet 1: List of normalized miRNA read counts of sperm harvested 14 days post Dex and
20 vehicle injection.

21 Sheet 2: Deseq2 results of a comparison between miRNA from sperm harvested 14 days
22 post Dex and vehicle injection.

23 Sheet 3: List of normalized tsRNA read counts of sperm harvested 14 days post Dex and
24 vehicle injection.

25 Sheet 4: Deseq2 results of a comparison between tsRNAs from sperm harvested 14 days
26 post Dex and vehicle injection.

27

28 *Supplementary table 2*

- 1 Sheet 1: Deseq2 results of the comparison of tsRNAs in sperm harvested 3 hours and 7
- 2 days post Dex with tsRNAs in sperm harvested 3 hours and 7 days post vehicle injection.
- 3 Sheet 2: Deseq2 results of the comparison between tsRNAs from sperm harvested post Dex
- 4 and vehicle injection at 3 hours with tsRNAs in sperm harvested 7 days post Dex and vehicle
- 5 injection.
- 6 Sheet 3: Deseq2 results of the interaction between treatment and time for tsRNAs from
- 7 sperm harvested 3 hours and 7 days post Dex and vehicle injection.
- 8 Sheet 4: Deseq2 results of the comparison of miRNAs in sperm harvested 3 hours and 7
- 9 days post Dex with miRNAs in sperm harvested 3 hours and 7 days post vehicle injection.
- 10 Sheet 5: Deseq2 results of the comparison between miRNAs from sperm harvested post Dex
- 11 and vehicle injection at 3 hours with miRNAs in sperm harvested 7 days post Dex and
- 12 vehicle injection.
- 13 Sheet 6: Deseq2 results of the interaction between treatment and time for miRNAs from
- 14 sperm harvested 3 hours and 7 days post Dex and vehicle injection.
- 15 Sheet 7: Raw values of qRT-PCR analysis for tsRNA-Arg-CCT-2 in caput and cauda sperm
- 16 sampled 3 hours post Dex and vehicle injection.

17

18

19 *Supplementary table 3*

- 20 Raw data for BMI (sheet 1), GTT (sheet 2) , ITT (sheet 3) and necropsy weights (sheet 4) of
- 21 adult offspring animals resulting from IVF of wildtype oocytes and sperm harvested 14 days
- 22 post Dex and vehicle injection.

23

24 *Supplementary table 4*

- 25 Sheet 1: Monocle output list on significantly differentially regulated genes between 2-cell
- 26 embryos resulting from IVF of wildtype oocytes and sperm harvested 14 days post Dex and
- 27 vehicle injection.

28

1 *Supplementary table 5*

2 Sheet 1: List of normalized long RNA seq counts of sperm harvested 14 days post Dex and
3 vehicle injection.

4 Sheet 2: EdgeR results of a comparison between long RNA reads from sperm harvested 14
5 days post Dex and vehicle injection.

6

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20 arising from this submission.

21 Author's contributions: KG performed animal exposures, collected samples and prepared
22 sequencing libraries. EG performed IVF and embryo culture. GP analysed single cell
23 sequencing data. MH supervised the single-embryo sequencing analysis. AC and FG
24 analyzed bulk sequencing data. KG, JB and EAM designed the study, interpreted the results
25 and wrote the manuscript with input from the other authors.

26

27

28 **References**

- 1 1. E. R. de Kloet, H. Karst, M. Joëls, Corticosteroid hormones in the central stress response:
2 Quick-and-slow. *Front. Neuroendocrinol.* **29**, 268–272 (2008).
- 3 2. E. R. de Kloet, M. Joëls, F. Holsboer, Stress and the brain: from adaptation to disease. *Nat.*
4 *Rev. Neurosci.* **6**, 463–475 (2005).
- 5 3. N. P. Daskalakis, M. S. Oitzl, H. Schachinger, D. L. Champagne, E. R. de Kloet, Testing the
6 cumulative stress and mismatch hypotheses of psychopathology in a rat model of early-life
7 adversity. *Physiol Behav.* **106**, 707–721 (2012).
- 8 4. M. E. Bowers, R. Yehuda, Intergenerational transmission of stress in humans.
9 *Neuropsychopharmacology.* **41** (2016), pp. 232–244.
- 10 5. B. T. Heijmans, E. W. Tobin, A. D. Stein, H. Putter, G. J. Blauw, E. S. Susser, P. E. Slagboom,
11 L. H. Lumey, Persistent epigenetic differences associated with prenatal exposure to famine in
12 humans. *Proc Natl Acad Sci U S A.* **105**, 17046–17049 (2008).
- 13 6. M. E. Pembrey, L. O. Bygren, G. Kaati, S. Edvinsson, K. Northstone, M. Sjöström, J. Golding,
14 T. Alspac, S. Team, Sex-specific, male-line transgenerational responses in humans. *Eur. J.*
15 *Hum. Genet.* **14**, 159–166 (2006).
- 16 7. K. Gapp, A. Jawaid, P. Sarkies, J. Bohacek, P. Pelczar, J. Prados, L. Farinelli, E. Miska, I. M.
17 Mansuy, Implication of sperm RNAs in transgenerational inheritance of the effects of early
18 trauma in mice. *Nat. Neurosci.* **17**, 667–669 (2014).
- 19 8. C. P. Morgan, T. L. Bale, Early Prenatal Stress Epigenetically Programs Dysmasculinization in
20 Second-Generation Offspring via the Paternal Lineage. *J. Neurosci.* **31**, 11748–11755 (2011).
- 21 9. D. M. Dietz, Q. Laplant, E. L. Watts, G. E. Hodes, S. J. Russo, J. Feng, R. S. Oosting, V.
22 Vialou, E. J. Nestler, Paternal transmission of stress-induced pathologies. *Biol. Psychiatry.* **70**,
23 408–414 (2011).
- 24 10. A. B. Rodgers, C. P. Morgan, N. A. Leu, T. L. Bale, Transgenerational epigenetic programming
25 via sperm microRNA recapitulates effects of paternal stress. *Proc. Natl. Acad. Sci.* **112**,
26 201508347 (2015).
- 27 11. T. B. Franklin, H. Russig, I. C. Weiss, J. Gräff, N. Linder, A. Michalon, S. Vizi, I. M. Mansuy,
28 Epigenetic Transmission of the Impact of Early Stress Across Generations. *Biol. Psychiatry.*
29 **68**, 408–415 (2010).
- 30 12. T. B. Franklin, N. Linder, H. Russig, B. Thöny, I. M. Mansuy, Influence of early stress on social
31 abilities and serotonergic functions across generations in mice. *PLoS One.* **6**, e21842 (2011).

- 1 13. I. C. Weiss, T. B. Franklin, S. Vizi, I. M. Mansuy, *Front. Behav. Neurosci.*, in press,
2 doi:10.3389/fnbeh.2011.00003.
- 3 14. T. L. Roth, F. D. Lubin, A. J. Funk, J. D. Sweatt, Lasting Epigenetic Influence of Early-Life
4 Adversity on the BDNF Gene. *Biol. Psychiatry*. **65**, 760–769 (2009).
- 5 15. F. M. Vassoler, S. L. White, H. D. Schmidt, G. Sadri-Vakili, R. C. Pierce, Epigenetic inheritance
6 of a cocaine-resistance phenotype. *Nat Neurosci*. **16**, 42–47 (2013).
- 7 16. D. C. Benyshek, C. S. Johnston, J. F. Martin, Glucose metabolism is altered in the adequately-
8 nourished grand-offspring (F3 generation) of rats malnourished during gestation and perinatal
9 life. *Diabetologia*. **49**, 1117–1119 (2006).
- 10 17. T. Pentinat, M. Ramon-Krauel, J. Cebria, R. Diaz, J. C. Jimenez-Chillaron, Transgenerational
11 inheritance of glucose intolerance in a mouse model of neonatal overnutrition. *Endocrinology*.
12 **151**, 5617–5623 (2010).
- 13 18. D. Martínez, T. Pentinat, S. Ribó, C. Daviaud, V. W. Bloks, J. Cebrià, N. Villalmanzo, S. G.
14 Kalko, M. Ramón-Krauel, R. Díaz, T. Plösch, J. Tost, J. C. Jiménez-Chillaron, In Utero
15 Undernutrition in Male Mice Programs Liver Lipid Metabolism in the Second-Generation
16 Offspring Involving Altered Lxra DNA Methylation. *Cell Metab.*, 1–11 (2014).
- 17 19. J. C. Jimenez-Chillaron, E. Isganaitis, M. Charalambous, S. Gesta, T. Pentinat-Pelegrin, R. R.
18 Faucette, J. P. Otis, A. Chow, R. Diaz, A. Ferguson-Smith, M. E. Patti, Intergenerational
19 transmission of glucose intolerance and obesity by in utero undernutrition in mice. *Diabetes*.
20 **58**, 460–468 (2009).
- 21 20. T. Fullston, E. M. C. O. Teague, N. O. Palmer, M. J. Deblasio, M. Mitchell, M. Corbett, C. G.
22 Print, J. A. Owens, M. Lane, Paternal obesity initiates metabolic disturbances in two
23 generations of mice with incomplete penetrance to the F2 generation and alters the
24 transcriptional profile of testis and sperm microRNA content. *FASEB J*. **27**, 4226–4243 (2013).
- 25 21. K. Shankar, P. Kang, A. Harrell, Y. Zhong, J. C. Marecki, M. J. J. Ronis, T. M. Badger,
26 Maternal overweight programs insulin and adiponectin signaling in the offspring.
27 *Endocrinology*. **151**, 2577–2589 (2010).
- 28 22. A. Marco, T. Kisiouk, T. Tabachnik, N. Meiri, A. Weller, Overweight and CpG methylation of
29 the Pomc promoter in offspring of high-fat-diet-fed dams are not “reprogrammed” by regular
30 chow diet in rats. *FASEB J*. **28**, 4148–4157 (2014).
- 31 23. L. Wu, Y. Lu, Y. Jiao, B. Liu, S. Li, Y. Li, F. Xing, D. Chen, X. Liu, J. Zhao, X. Xiong, Y. Gu, J.

- 1 Lu, X. Chen, X. Li, Paternal psychological stress reprograms hepatic gluconeogenesis in
2 offspring. *Cell Metab.* **23**, 735–743 (2016).
- 3 24. U. Sharma, C. C. Conine, J. M. Shea, A. Boskovic, A. G. Derr, X. Y. Bing, C. Belleannee, A.
4 Kucukural, R. W. Serra, F. Sun, L. Song, B. R. Carone, E. P. Ricci, X. Z. Li, L. Fauquier, M. J.
5 Moore, R. Sullivan, C. C. Mello, M. Garber, O. J. Rando, Biogenesis and function of tRNA
6 fragments during sperm maturation and fertilization in mammals. *Science (80-.).* **351**, 391–396
7 (2015).
- 8 25. B. R. Carone, L. Fauquier, N. Habib, J. M. Shea, C. E. Hart, R. Li, C. Bock, C. Li, H. Gu, P. D.
9 Zamore, A. Meissner, Z. Weng, H. a Hofmann, N. Friedman, O. J. Rando, Paternally induced
10 transgenerational environmental reprogramming of metabolic gene expression in mammals.
11 *Cell.* **143**, 1084–1096 (2010).
- 12 26. Y. Y. Zhang, X. Zhang, J. Shi, F. Tuorto, X. Li, Y. Y. Liu, R. Liebers, L. Zhang, Y. Qu, J. Qian,
13 M. Pahima, Y. Y. Liu, M. Yan, Z. Cao, X. Lei, Y. Cao, H. Peng, S. Liu, Y. Wang, H. Zheng, R.
14 Woolsey, D. Quilici, Q. Zhai, L. Li, T. Zhou, W. Yan, F. Lyko, Y. Y. Zhang, Q. Zhou, E. Duan,
15 Q. Chen, Dnmt2 mediates intergenerational transmission of paternally acquired metabolic
16 disorders through sperm small non-coding RNAs. *Nat. Cell Biol.* **20** (2018),
17 doi:10.1038/s41556-018-0087-2.
- 18 27. Q. Chen, M. Yan, Z. Cao, X. Li, Y. Zhang, J. Shi, G. H. Feng, H. Peng, X. Zhang, Y. Zhang, J.
19 Qian, E. Duan, Q. Zhai, Q. Zhou, Sperm tsRNAs contribute to intergenerational inheritance of
20 an acquired metabolic disorder. *Science (80-.).* **351**, 397–400 (2016).
- 21 28. K. Gapp, J. Bohacek, Epigenetic germline inheritance in mammals: looking to the past to
22 understand the future. *Genes, Brain Behav.* **17** (2017), doi:10.1111/gbb.12407.
- 23 29. R. L. Jirtle, M. K. Skinner, Environmental epigenomics and disease susceptibility. *Nat. Rev.*
24 *Genet.* **8**, 253–262 (2007).
- 25 30. J. Bohacek, I. M. Mansuy, A guide to designing germline-dependent epigenetic inheritance
26 experiments in mammals. *Nat. Methods.* **14**, 243–249 (2017).
- 27 31. J. Bohacek, M. Rassoulzadegan, Sperm RNA: Quo vadis? *Semin. Cell Dev. Biol.* (2019),
28 doi:10.1016/J.SEMCDB.2019.07.005.
- 29 32. T. Grentzinger, C. Armenise, C. Brun, B. Mugat, V. Serrano, A. Pelisson, S. Chambeyron,
30 piRNA-mediated transgenerational inheritance of an acquired trait. *Genome Res.* **22**, 1877–
31 1888 (2012).

- 1 33. A. Ashe, A. Sapetschnig, E. M. Weick, J. Mitchell, M. P. Bagijn, A. C. Cording, A. L. Doebley,
2 L. D. Goldstein, N. J. Lehrbach, J. Le Pen, G. Pintacuda, A. Sakaguchi, P. Sarkies, S. Ahmed,
3 E. A. Miska, piRNAs can trigger a multigenerational epigenetic memory in the germline of *C.*
4 *elegans*. *Cell*. **150**, 88–99 (2012).
- 5 34. M. Shirayama, M. Seth, H. C. Lee, W. Gu, T. Ishidate, D. Conte Jr., C. C. Mello, piRNAs initiate
6 an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell*. **150**, 65–77 (2012).
- 7 35. V. Grandjean, S. Fourre, D. A. F. De Abreu, M.-A. A. Derieppe, J.-J. J. Remy, M.
8 Rassoulzadegan, S. Fourré, D. A. F. De Abreu, M.-A. A. Derieppe, J.-J. J. Remy, M.
9 Rassoulzadegan, RNA-mediated paternal heredity of diet-induced obesity and metabolic
10 disorders. *Sci Rep*. **5**, 18193 (2015).
- 11 36. Q. Chen, W. Yan, E. Duan, Epigenetic inheritance of acquired traits through sperm RNAs and
12 sperm RNA modifications. *Nat. Rev. Genet.* **17**, 733–743 (2016).
- 13 37. S. P. Barrett, J. Salzman, Circular RNAs: analysis, expression and potential functions.
14 *Development*. **143**, 1838–1847 (2016).
- 15 38. E. Benito, C. Kerimoglu, B. Ramachandran, T. Pena-Centeno, G. Jain, R. M. Stilling, M. R.
16 Islam, V. Capece, Q. Zhou, D. Edbauer, C. Dean, A. Fischer, RNA-Dependent
17 Intergenerational Inheritance of Enhanced Synaptic Plasticity after Environmental Enrichment.
18 *Cell Rep*. **23**, 546–554 (2018).
- 19 39. S. Tyebji, A. J. Hannan, C. J. Tonkin, Pathogenic Infection in Male Mice Changes Sperm Small
20 RNA Profiles and Transgenerationally Alters Offspring Behavior. *Cell Rep*. **31**, 107573 (2020).
- 21 40. U. Sharma, F. Sun, C. C. Conine, B. Reichholf, S. Kukreja, V. A. Herzog, S. L. Ameres, O. J.
22 Rando, Small RNAs Are Trafficked from the Epididymis to Developing Mammalian Sperm.
23 *Dev. Cell*. **46**, 481–494.e6 (2018).
- 24 41. A. K. Short, K. A. Fennell, V. M. Perreau, A. Fox, M. K. O'Bryan, J. H. Kim, T. W. Bredy, T. Y.
25 Pang, A. J. Hannan, Elevated paternal glucocorticoid exposure alters the small noncoding RNA
26 profile in sperm and modifies anxiety and depressive phenotypes in the offspring. *Transl.*
27 *Psychiatry*. **6**, e837 (2016).
- 28 42. A. B. Rodgers, C. P. Morgan, S. L. Bronson, S. Revello, T. L. Bale, Paternal Stress Exposure
29 Alters Sperm MicroRNA Content and Reprograms Offspring HPA Stress Axis Regulation. *J.*
30 *Neurosci*. **33**, 9003–9012 (2013).
- 31 43. J. C. Chan, C. P. Morgan, N. Adrian Leu, A. Shetty, Y. M. Cisse, B. M. Nugent, K. E. Morrison,

- 1 E. Jašarević, W. Huang, N. Kanyuch, A. B. Rodgers, N. V Bhanu, D. S. Berger, B. A. Garcia,
2 S. Ament, M. Kane, C. Neill Epperson, T. L. Bale, Reproductive tract extracellular vesicles are
3 sufficient to transmit intergenerational stress and program neurodevelopment. *Nat. Commun.*
4 **11**, 1499 (2020).
- 5 44. C. Hoyer, H. Richter, C. Brandwein, M. A. Riva, P. Gass, Preconceptional paternal exposure to
6 a single traumatic event affects postnatal growth of female but not male offspring. **24** (2013),
7 doi:10.1097/WNR.0000000000000007.
- 8 45. J. Bohacek, S. von Werdt, I. M. Mansuy, Probing the germline-dependence of epigenetic
9 inheritance using artificial insemination in mice. *Environ. Epigenetics*. **2**, dvv015 (2016).
- 10 46. S. Haeussler, R. Claus, Expression of the glucocorticoid receptor and 11 β -hydroxysteroid
11 dehydrogenase 2 in pig testes cells along fetal development. *Reprod. Fertil. Dev.* **19**, 664
12 (2007).
- 13 47. S. H. Kaufmann, W. W. Wright, S. Okret, A. C. Wikström, J. A. Gustafsson, N. L. Shaper, J. H.
14 Shaper, Evidence that rodent epididymal sperm contain the Mr approximately 94,000
15 glucocorticoid receptor but lack the Mr approximately 90,000 heat shock protein.
16 *Endocrinology*. **130**, 3074–3084 (1992).
- 17 48. R. Hazra, D. Upton, M. Jimenez, R. Desai, D. J. Handelsman, C. M. Allan, In Vivo Actions of
18 the Sertoli Cell Glucocorticoid Receptor. *Endocrinology*. **155**, 1120–1130 (2014).
- 19 49. E. J. R. Silva, D. B. C. Queiróz, L. Honda, M. C. W. Avellar, Glucocorticoid receptor in the rat
20 epididymis: Expression, cellular distribution and regulation by steroid hormones. *Mol. Cell.*
21 *Endocrinol.* **325**, 64–77 (2010).
- 22 50. E. J. R. Silva, V. Vendramini, A. Restelli, R. P. Bertolla, W. G. Kempinas, M. C. W. Avellar,
23 Impact of adrenalectomy and dexamethasone treatment on testicular morphology and sperm
24 parameters in rats: insights into the adrenal control of male reproduction. *Andrology*. **2**, 835–
25 846 (2014).
- 26 51. Biggest COVID-19 trial tests repurposed drugs first. *Nat. Biotechnol.* **38**, 510 (2020).
- 27 52. EU Clinical Trials Register, Randomised Evaluation of COVID-19 Therapy (RECOVERY),
28 (available at <https://www.clinicaltrialsregister.eu/ctr-search/trial/2020-001113-21/GB>).
- 29 53. P. Horby, W. S. Lim, J. Emberson, M. Mafham, J. Bell, L. Linsell, N. Staplin, C. Brightling, A.
30 Ustianowski, E. Elmahi, B. Prudon, C. Green, T. Felton, D. Chadwick, K. Rege, C. Fegan, L. C.
31 Chappell, S. N. Faust, T. Jaki, K. Jeffery, A. Montgomery, K. Rowan, E. Juszczak, J. K. Baillie,

- 1 R. Haynes, M. J. Landray, R. C. Group, *medRxiv*, in press, doi:10.1101/2020.06.22.20137273.
- 2 54. C. Dard-Dascot, D. Naquin, Y. D'Aubenton-Carafa, K. Alix, C. Thermes, E. van Dijk,
3 Systematic comparison of small RNA library preparation protocols for next-generation
4 sequencing. *BMC Genomics*. **19**, 118 (2018).
- 5 55. H. Peng, J. Shi, Y. Zhang, H. Zhang, S. Liao, W. Li, L. Lei, C. Han, L. Ning, Y. Cao, Q. Zhou,
6 Q. Chen, E. Duan, A novel class of tRNA-derived small RNAs extremely enriched in mature
7 mouse sperm. *Cell Res*. **22**, 1609–1612 (2012).
- 8 56. M. I. Love, W. Huber, S. Anders, Moderated estimation of fold change and dispersion for RNA-
9 seq data with DESeq2. *Genome Biol*. **15**, 550 (2014).
- 10 57. C. C. Conine, F. Sun, L. Song, J. A. Rivera-Pérez, O. J. Rando, Small RNAs Gained during
11 Epididymal Transit of Sperm Are Essential for Embryonic Development in Mice. *Dev. Cell*. **46**,
12 470--480.e3 (2018).
- 13 58. J. Dadoune, M. Alfonsi, Autoradiographic investigation of sperm transit through the male
14 mouse genital tract after tritiated thymidine incorporation. *Reprod. Nutr. Dev*. **24**, 927–935
15 (1984).
- 16 59. M. L. Meistrich, Alteration of epididymal sperm transport and maturation in mice by oestrogen
17 and testosterone. *Nature* (1975).
- 18 60. M. H. R. Huber, F. H. Bronson, Social modulation of spontaneous ejaculation in the mouse.
19 *Behav. Neural Biol*. **29**, 390–393 (1980).
- 20 61. G. C. Ostermeier, D. Miller, J. D. Huntriss, M. P. Diamond, S. A. Krawetz, Reproductive
21 biology: Delivering spermatozoan RNA to the oocyte. *Nature*. **429**, 154 (2004).
- 22 62. A. Yeri, A. Courtright, K. Danielson, E. Hutchins, E. Alsop, E. Carlson, M. Hsieh, O. Ziegler, A.
23 Das, R. V Shah, J. Rozowsky, S. Das, K. Van Keuren-Jensen, Evaluation of commercially
24 available small RNAseq library preparation kits using low input RNA. *BMC Genomics*. **19**, 331
25 (2018).
- 26 63. W.-M. Liu, R. T. K. Pang, P. C. N. Chiu, B. P. C. Wong, K. Lao, K.-F. Lee, W. S. B. Yeung,
27 Sperm-borne microRNA-34c is required for the first cleavage division in mouse. *Proc. Natl.*
28 *Acad. Sci. U. S. A*. **109**, 490–494 (2012).
- 29 64. M. Amanai, M. Brahmajosyula, A. C. F. Perry, A restricted role for sperm-borne microRNAs in
30 mammalian fertilization. *Biol. Reprod*. **75**, 877–884 (2006).
- 31 65. F. Tang, M. Kaneda, D. O'Carroll, P. Hajkova, S. C. Barton, Y. A. Sun, C. Lee, A. Tarakhovsky,

- 1 K. Lao, M. A. Surani, Maternal microRNAs are essential for mouse zygotic development.
2 *Genes Dev.* **21**, 644–648 (2007).
- 3 66. S. A. Krawetz, A. Kruger, C. Lalancette, R. Tagett, E. Anton, S. Draghici, M. P. Diamond, A
4 survey of small RNAs in human sperm. *Hum Reprod.* **26**, 3401–3412 (2011).
- 5 67. V. Y. Kiselev, K. Kirschner, M. T. Schaub, T. Andrews, A. Yiu, T. Chandra, K. N. Natarajan, W.
6 Reik, M. Barahona, A. R. Green, M. Hemberg, SC3: consensus clustering of single-cell RNA-
7 seq data. *Nat. Methods.* **14**, 483–486 (2017).
- 8 68. V. Y. Kiselev, A. Yiu, M. Hemberg, scmap: projection of single-cell RNA-seq data across data
9 sets. *Nat. Methods.* **15**, 359–362 (2018).
- 10 69. Q. Deng, D. Ramsköld, B. Reinius, R. Sandberg, Single-Cell RNA-Seq Reveals Dynamic,
11 Random Monoallelic Gene Expression in Mammalian Cells. *Science (80-.).* **343**, 193–196
12 (2014).
- 13 70. P. J. Rousseeuw, Silhouettes: A graphical aid to the interpretation and validation of cluster
14 analysis. *J. Comput. Appl. Math.* **20**, 53–65 (1987).
- 15 71. X. Qiu, A. Hill, J. Packer, D. Lin, Y.-A. Ma, C. Trapnell, Single-cell mRNA quantification and
16 differential analysis with Census. *Nat. Methods.* **14**, 309–315 (2017).
- 17 72. P. Cacciagli, J. Suter-Sardo, A. Borges-Correia, J.-C. Roux, I. Dorboz, J.-P. Desvignes, C.
18 Badens, M. Delepine, M. Lathrop, P. Cau, N. Lévy, N. Girard, P. Sarda, O. Boespflug-Tanguy,
19 L. Villard, Mutations in BCAP31 Cause a Severe X-Linked Phenotype with Deafness, Dystonia,
20 and Central Hypomyelination and Disorganize the Golgi Apparatus. *Am. J. Hum. Genet.* **93**,
21 579–586 (2013).
- 22 73. T. H. Kang, Y. Park, J. S. Bader, T. Friedmann, The Housekeeping Gene Hypoxanthine
23 Guanine Phosphoribosyltransferase (HPRT) Regulates Multiple Developmental and Metabolic
24 Pathways of Murine Embryonic Stem Cell Neuronal Differentiation. *PLoS One.* **8**, e74967
25 (2013).
- 26 74. T. Miyazaki, S. Miyazaki, M. Ashida, T. Tanaka, F. Tashiro, J. Miyazaki, Functional Analysis of
27 Tc1 Using Tc1-Deficient Mouse Embryonic Stem Cells. *PLoS One.* **8** (2013),
28 doi:10.1371/JOURNAL.PONE.0071645.
- 29 75. N. Yu, P. Zhang, L. Wang, X. He, S. Yang, H. Lu, RBBP7 is a prognostic biomarker in patients
30 with esophageal squamous cell carcinoma. *Oncol. Lett.* **16**, 7204–7211 (2018).
- 31 76. O. Deevy, A. P. Bracken, PRC2 functions in development and congenital disorders.

- 1 *Development*. **146** (2019), doi:10.1242/DEV.181354.
- 2 77. T. S. Macfarlan, W. D. Gifford, S. Driscoll, K. Lettieri, H. M. Rowe, D. Bonanomi, A. Firth, O.
- 3 Singer, D. Trono, S. L. Pfaff, Embryonic stem cell potency fluctuates with endogenous
- 4 retrovirus activity. *Nature*. **487**, 57–63 (2012).
- 5 78. A. E. Peaston, A. V Evsikov, J. H. Graber, W. N. de Vries, A. E. Holbrook, D. Solter, B. B.
- 6 Knowles, Retrotransposons Regulate Host Genes in Mouse Oocytes and Preimplantation
- 7 Embryos. *Dev. Cell*. **7**, 597–606 (2004).
- 8 79. K. Gapp, G. van Steenwyk, P. L. Germain, W. Matsushima, K. L. M. Rudolph, F. Manuella, M.
- 9 Roszkowski, G. Vernaz, T. Ghosh, P. Pelczar, I. M. Mansuy, E. A. Miska, Alterations in sperm
- 10 long RNA contribute to the epigenetic inheritance of the effects of postnatal trauma. *Mol.*
- 11 *Psychiatry*, 1 (2018).
- 12 80. M. Gòdia, A. Castelló, M. Rocco, B. Cabrera, J. E. Rodríguez-Gil, S. Balasch, C. Lewis, A.
- 13 Sánchez, A. Clop, Identification of circular RNAs in porcine sperm and evaluation of their
- 14 relation to sperm motility. *Sci. Rep.* **10**, 1–11 (2020).
- 15 81. T. Chioccarelli, F. Manfredola, B. Ferraro, C. Sellitto, G. Cobellis, M. Migliaccio, S. Fasano, R.
- 16 Pierantoni, R. Chianese, Expression patterns of circular RNAs in high quality and poor quality
- 17 human spermatozoa. *Front. Endocrinol. (Lausanne)*. **10**, 1–14 (2019).
- 18 82. C. Tang, Y. Xie, T. Yu, N. Liu, Z. Wang, R. J. Woolsey, Y. Tang, X. Zhang, W. Qin, Y. Zhang,
- 19 G. Song, W. Zheng, J. Wang, W. Chen, X. Wei, Z. Xie, R. Klukovich, H. Zheng, D. R. Quilici,
- 20 W. Yan, m6A-dependent biogenesis of circular RNAs in male germ cells. *Cell Res.* **30**, 211–
- 21 228 (2020).
- 22 83. W. Wu, P. Ji, F. Zhao, CircAtlas: an integrated resource of one million highly accurate circular
- 23 RNAs from 1070 vertebrate transcriptomes. *Genome Biol.* **21**, 101 (2020).
- 24 84. V. Agarwal, G. W. Bell, J.-W. Nam, D. P. Bartel, Predicting effective microRNA target sites in
- 25 mammalian mRNAs. *Elife*. **4** (2015), doi:10.7554/eLife.05005.
- 26 85. L. Vojtech, S. Woo, S. Hughes, C. Levy, L. Ballweber, R. P. Sauteraud, J. Strobl, K.
- 27 Westerberg, R. Gottardo, M. Tewari, F. Hladik, Exosomes in human semen carry a distinctive
- 28 repertoire of small non-coding RNAs with potential regulatory functions. *Nucleic Acids Res.* **42**,
- 29 7290–7304 (2014).
- 30 86. R. Mashoodh, B. Franks, J. P. Curley, F. A. Champagne, Paternal social enrichment effects on
- 31 maternal behavior and offspring growth. *Proc Natl Acad Sci U S A.* **109 Suppl**, 17232–17238

- 1 (2012).
- 2 87. A. K. Short, S. Yeshurun, R. Powell, V. M. Perreau, A. Fox, J. H. Kim, T. Y. Pang, A. J.
3 Hannan, Exercise alters mouse sperm small noncoding RNAs and induces a transgenerational
4 modification of male offspring conditioned fear and anxiety. *Transl. Psychiatry*. **7**, e1114
5 (2017).
- 6 88. S. Yeshurun, A. J. Hannan, Transgenerational epigenetic influences of paternal environmental
7 exposures on brain function and predisposition to psychiatric disorders. *Mol. Psychiatry* (2018),
8 pp. 1–13.
- 9 89. G. R. Rompala, A. Mounier, C. M. Wolfe, Q. Lin, I. Lefterov, G. E. Homanics, Heavy Chronic
10 Intermittent Ethanol Exposure Alters Small Noncoding RNAs in Mouse Sperm and
11 Epididymosomes. *Front. Genet.* **9**, 32 (2018).
- 12 90. N. Barden, Regulation of corticosteroid receptor gene expression in depression and
13 antidepressant action. *J. Psychiatry Neurosci.* **24**, 25–39 (1999).
- 14 91. J. Cartier, T. Smith, J. P. Thomson, C. M. Rose, B. Khulan, A. Heger, R. R. Meehan, A. J.
15 Drake, Investigation into the role of the germline epigenome in the transmission of
16 glucocorticoid-programmed effects across generations. *Genome Biol.* **19**, 50 (2018).
- 17 92. S. Petropoulos, S. G. Matthews, M. Szyf, Adult Glucocorticoid Exposure Leads to
18 Transcriptional and DNA Methylation Changes in Nuclear Steroid Receptors in the
19 Hippocampus and Kidney of Mouse Male Offspring1. *Biol. Reprod.* **90** (2014),
20 doi:10.1095/biolreprod.113.115899.
- 21 93. C. Bönisch, M. Irmeler, L. Brachthäuser, F. Neff, M. T. Bamberger, S. Marschall, M. Hrabě de
22 Angelis, J. Beckers, Dexamethasone treatment alters insulin, leptin, and adiponectin levels in
23 male mice as observed in DIO but does not lead to alterations of metabolic phenotypes in the
24 offspring. *Mamm. Genome.* **27**, 17–28 (2016).
- 25 94. J. K. Gjerstad, S. L. Lightman, F. Spiga, Role of glucocorticoid negative feedback in the
26 regulation of HPA axis pulsatility. *Stress.* **21**, 403–416 (2018).
- 27 95. D. Nätt, U. Kugelberg, E. Casas, E. Nedstrand, S. Zalavary, P. Henriksson, C. Nijm, J.
28 Jäderquist, J. Sandborg, E. Flincke, R. Ramesh, L. Örkenby, F. Appelkvist, T. Lingg, N. Guzzi,
29 C. Bellodi, M. Löf, T. Vavouri, A. Öst, Human sperm displays rapid responses to diet. *PLoS*
30 *Biol.* **17**, 1–25 (2019).
- 31 96. S. Bera, S. Greiner, A. Choudhury, A. Dispenzieri, D. R. Spitz, S. J. Russell, A. Goel,

- 1 Dexamethasone-induced oxidative stress enhances myeloma cell radiosensitization while
2 sparing normal bone marrow hematopoiesis. *Neoplasia*. **12**, 980–992 (2010).
- 3 97. D. M. Thompson, C. Lu, P. J. Green, R. Parker, tRNA cleavage is a conserved response to
4 oxidative stress in eukaryotes. *Rna*. **14**, 2095–2103 (2008).
- 5 98. M. Gòdia, M. Estill, A. Castelló, S. Balasch, J. E. Rodríguez-Gil, S. A. Krawetz, A. Sánchez, A.
6 Clop, A RNA-seq analysis to describe the boar sperm transcriptome and its seasonal changes.
7 *Front. Genet.* **10**, 1–14 (2019).
- 8 99. N. Guzzi, C. Bellodi, RNA Biology Novel insights into the emerging roles of tRNA-derived
9 fragments in mammalian development (2020), doi:10.1080/15476286.2020.1732694.
- 10 100. Q. Li, B. Hu, G. Hu, C. Chen, X. Niu, J. Liu, S. Zhou, C. Zhang, Y. Wang, Z.-F. Deng, tRNA-
11 Derived Small Non-Coding RNAs in Response to Ischemia Inhibit Angiogenesis. *Sci. Rep.* **6**,
12 20850 (2016).
- 13 101. I. Legnini, G. Di Timoteo, F. Rossi, M. Morlando, F. Briganti, O. Sthandier, A. Fatica, T. Santini,
14 A. Andronache, M. Wade, P. Laneve, N. Rajewsky, I. Bozzoni, Circ-ZNF609 Is a Circular RNA
15 that Can Be Translated and Functions in Myogenesis. *Mol. Cell.* **66**, 22-37.e9 (2017).
- 16 102. S. Israel, M. Ernst, O. E. Psathaki, H. C. A. Drexler, E. Casser, Y. Suzuki, W. Makalowski, M.
17 Boiani, G. Fuellen, L. Taher, An integrated genome-wide multi-omics analysis of gene
18 expression dynamics in the preimplantation mouse embryo. *Sci. Rep.* **9**, 1–15 (2019).
- 19 103. S. Wang, Z. Kou, Z. Jing, Y. Zhang, X. Guo, M. Dong, I. Wilmut, S. Gao, Proteome of mouse
20 oocytes at different developmental stages. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 17639–17644
21 (2010).
- 22 104. K. P. Cross, R. I. Paul, R. D. Goldman, Single-dose dexamethasone for mild-to-moderate
23 asthma exacerbations: effective, easy, and acceptable. *Can. Fam. Physician.* **57**, 1134–1136
24 (2011).
- 25 105. U. Brykczynska, M. Hisano, S. Erkek, L. Ramos, E. J. Oakeley, T. C. Roloff, C. Beisel, D.
26 Schubeler, M. B. Stadler, A. H. F. M. Peters, Repressive and active histone methylation mark
27 distinct promoters in human and mouse spermatozoa. *Nat Struct Mol Biol.* **17**, 679–687 (2010).
- 28 106. N. Nakagata, (Humana Press, 2011; http://link.springer.com/10.1007/978-1-60761-974-1_4),
29 pp. 57–73.
- 30 107. G. C. Ostermeier, M. V Wiles, J. S. Farley, R. A. Taft, Conserving, Distributing and Managing
31 Genetically Modified Mouse Lines by Sperm Cryopreservation. *PLoS One.* **3**, e2792 (2008).

- 1 108. S. Andrews, FastQC: a quality control tool for high throughput sequence data. (2010).
- 2 109. P. Ewels, M. Magnusson, S. Lundin, M. Käller, MultiQC: summarize analysis results for
3 multiple tools and samples in a single report. *Bioinformatics*. **32**, 3047–3048 (2016).
- 4 110. M. Martin, Cutadapt removes adapter sequences from high-throughput sequencing reads.
5 *EMBnet.journal*. **17**, 10 (2011).
- 6 111. P. Flicek, M. R. Amode, D. Barrell, K. Beal, K. Billis, S. Brent, D. Carvalho-Silva, P. Clapham,
7 G. Coates, S. Fitzgerald, L. Gil, C. G. Girón, L. Gordon, T. Hourlier, S. Hunt, N. Johnson, T.
8 Juettemann, A. K. Kähäri, S. Keenan, E. Kulesha, F. J. Martin, T. Maurel, W. M. McLaren, D.
9 N. Murphy, R. Nag, B. Overduin, M. Pignatelli, B. Pritchard, E. Pritchard, H. S. Riat, M. Ruffier,
10 D. Sheppard, K. Taylor, A. Thormann, S. J. Trevanion, A. Vullo, S. P. Wilder, M. Wilson, A.
11 Zadissa, B. L. Aken, E. Birney, F. Cunningham, J. Harrow, J. Herrero, T. J. P. Hubbard, R.
12 Kinsella, M. Muffato, A. Parker, G. Spudich, A. Yates, D. R. Zerbino, S. M. J. Searle, Ensembl
13 2014. *Nucleic Acids Res.* **42**, D749–55 (2014).
- 14 112. A. Dobin, C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, T.
15 R. Gingeras, STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. **29**, 15–21 (2013).
- 16 113. A. Kozomara, S. Griffiths-Jones, miRBase: integrating microRNA annotation and deep-
17 sequencing data. *Nucleic Acids Res.* **39**, D152–D157 (2011).
- 18 114. P. P. Chan, T. M. Lowe, GtRNAdb 2.0: an expanded database of transfer RNA genes identified
19 in complete and draft genomes. *Nucleic Acids Res.* **44**, D184–9 (2016).
- 20 115. A. M. Bolger, M. Lohse, B. Usadel, Trimmomatic: a flexible trimmer for Illumina sequence data.
21 *Bioinformatics*. **30**, 2114–2120 (2014).
- 22 116. Y. Liao, G. K. Smyth, W. Shi, featureCounts: an efficient general purpose program for
23 assigning sequence reads to genomic features. *Bioinformatics*. **30**, 923–930 (2014).
- 24 117. X.-O. Zhang, R. Dong, Y. Zhang, J.-L. Zhang, Z. Luo, J. Zhang, L.-L. Chen, L. Yang, Diverse
25 alternative back-splicing and alternative splicing landscape of circular RNAs. *Genome Res.* **26**,
26 1277–1287 (2016).
- 27 118. S. Anders, D. J. McCarthy, Y. Chen, M. Okoniewski, G. K. Smyth, W. Huber, M. D. Robinson,
28 Count-based differential expression analysis of RNA sequencing data using R and
29 Bioconductor. *Nat. Protoc.* **8**, 1765–1786 (2013).
- 30 119. D. J. McCarthy, K. R. Campbell, A. T. L. Lun, Q. F. Wills, Scater: pre-processing, quality
31 control, normalization and visualization of single-cell RNA-seq data in R. *Bioinformatics*,

1 btw777 (2017).

2

3

Gapp et al. Figure 1

A

B

C

Gapp et al. Figure 2

A

B

C

Glucose Tolerance Test

D

Insulin Tolerance Test

A

B

Gapp et al. Figure 6

A

B

C