Paternal exposure to DDT programs breast cancer development in daughters via sperm miRNA in a mouse model

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Abstract

Background: DNA sequence accounts for the majority of disease heritability, including cancer. However, it is becoming clear that environmentally-induced epigenetic inheritance can also occur. Epidemiological studies have shown that maternal exposure to the pesticide DDT in pregnancy is associated with increased breast cancer risk in women. Yet, the effects of paternal exposure to this pesticide on the progeny's breast cancer development has not been investigated.

Methods: Male mice (*c57bl/6*) were exposed to DDT or to a control-vehicle (CO) solution and used for sperm collection or mating with unexposed females to produce the DDT or CO daughters. In another experiment, normal mouse embryos (zygote stage) were injected with miRNA-10b and implanted into surrogate mothers to produce miR-10b offspring. DDT daughters or miRNA-10b females were used to study breast cancer development and metabolic parameters. Paternal sperm was used for RNA-seq analysis and miRNA expression levels.

Results: Pre-conception paternal DDT exposure altered the sperm small non-coding RNA load, with a notable increase in miRNA-10b levels. DDT offspring weighed less at birth and at weaning, but became overweight and showed metabolic dysfunction in adulthood compared to CO. DDT daughters also showed increased mammary tumorigenesis, developing more aggressive tumors that grew faster than in CO. This tumor phenotype was linked to suppression of the AMPK energy sensing pathway and mTOR activation in mammary tissues. Remarkably, embryonic injection of miRNA-10b recapitulated the mammary gland and tumor phenotypes observed in DDT daughters.

Conclusions: To our knowledge, this is the first report of an association between paternal DDT exposure and breast cancer in offspring. Paternal DDT-induced programming of breast cancer

48 development in daughters is mechanistically linked to sperm miRNA-10b. The impact of DDT
49 and other endocrine disrupting chemicals on sperm and programming of breast and other
50 cancers in offspring needs to be evaluated in humans.
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71 **Introduction**

72 Dichlorodiphenyltrichloroethane (DDT), one of the first synthetic organochlorine pesticides, was
73 widely used in the United States (U.S.) from the 1940s to the 1970s and up to the 1980s in
74 Europe (Knower KC 2014; SE 2013). While the use of DDT has been restricted in the U.S. and
75 Europe for decades, developing countries with endemic malaria and other vector-borne diseases
76 continue to use it (Cohn BA 2015; Kabasenche and Skinner 2014; Knower KC 2014). This
77 pesticide has a prolonged half-life and is lipophilic, bio-accumulating in the food chain, and can
78 still be detected in fatty tissues and circulation of animals and humans (Knower KC 2014). In the
79 U.S., the highest concentrations of DDT are detected in minority populations and recent
80 immigrants (Daniels et al. 2018; Ruiz et al. 2018). Like other organochlorines, DDT is an
81 endocrine disruptor (Knower KC 2014; Kuiper et al. 1998) and it has been linked to metabolic
82 syndrome (La Merrill M 2014), obesity (Skinner et al. 2013) and cancer (Cohn et al. 2007; Cohn
83 BA 2015).

84 DNA sequence is responsible for the majority of disease heritability, including cancer. It is
85 becoming clear, however, that epigenetic inheritance can also occur. At conception, parents
86 not only contribute their genome but also transmit a molecular memory of past environmental
87 experiences to their progeny (YW Chen Q, Duan W 2016; OJ 2012). This can result in
88 offspring's predisposition to chronic diseases later in life (MK 2007). This environmentally-
89 induced risk of diseases, including breast cancer, has been shown to be transmitted to the
90 offspring via epigenetic mechanisms through both the female and male germ-lines (de Assis S
91 2012; Gapp K 2014; MK 2007). Although most of the evidence for this mode of disease
92 inheritance comes from maternal exposures in pregnancy, we (da Cruz et al. 2018; da Cruz et al.
93 2019; Fontelles et al. 2016a; Fontelles et al. 2016b) and others (Caronne BR 2010; Guido et al.

94 2016; Ng SF 2010) have shown that paternal exposures in the pre-conception window are also
95 important in determining disease outcomes in the offspring.
96 Several recent published reports demonstrated that the small RNA load in paternal sperm can
97 transmit environmentally-induced phenotypes to the offspring (YM Chen Q, Cao Z, Li X, Zhang
98 Y, Shi J, Feng GH, Peng H, Zhang X, Zhang Y, Qian J, Duan E, Zhai Q, Zhou Q 2016; YW
99 Chen Q, Duan W 2016; Gapp K 2014; Sharma U 2016). Some of those studies implicate specific
100 classes of small RNA and both miRNAs and tRFs obtained from sperm can recapitulate the
101 effect of specific paternal exposures in offspring when injected into normal embryos following in
102 vitro fertilization (YM Chen Q, Cao Z, Li X, Zhang Y, Shi J, Feng GH, Peng H, Zhang X, Zhang
103 Y, Qian J, Duan E, Zhai Q, Zhou Q 2016; Gapp K 2014; Rodgers AB 2015; Sharma U 2016).

104 Because the mammary gland bud first emerges during fetal development, it has been proposed
105 that some breast cancers may originate *in utero* (Trichopoulos 1990) and multiple reports on
106 maternal exposure in pregnancy have offered support to this hypothesis (Cohn BA 2015; de
107 Assis S 2006; de Assis S 2012; de Oliveira Andrade et al. 2014). In line with that, a recent
108 epidemiological analysis using the Child Health and Development Studies cohort (CHDS)
109 showed that daughters of women exposed to high levels of DDT in pregnancy have increased
110 rates of breast cancer (Cohn BA 2015) and are more likely to be diagnosed with advanced stage
111 and HER-2 positive tumors. Other studies in the same cohort found that women exposed to this
112 pesticide before age 14 have increased breast cancer risk, with the highest risk if the exposure
113 occurred in early infancy (Cohn et al. 2007; Cohn et al. 2019).

114 While there is a clear link between maternal exposure to DDT and breast cancer in the next
115 generation, no studies to date have investigated the contribution of pre-conception paternal
116 exposure to this or other pesticides to offspring's breast cancer development. Here, we tested the

117 hypothesis that paternal exposure to DDT can alter the sperm non-coding RNAs and program
118 breast cancer development in daughters, using a mouse model of this disease. We also examined
119 whether paternal DDT-induced programming of breast cancer development in daughters is
120 mechanistically linked to sperm miRNAs.
121

122 **Materials and Methods**

123 ***DDT exposures and breeding:*** Animals were purchased from Taconic Biosciences. The *c57bl/6*
124 strain of mice was used in all experiments. Adult male mice (8 weeks of age) were either treated
125 with a DDT solution (1.7 mg/kg) or vehicle-control solution (vegetable oil) via oral gavage for
126 two weeks. The DDT solution used mimics the formulation of DDT before its ban in the U.S.:
127 77.2% p,p'-DDT and 22.8% o,p'-DDT as described before (La Merrill M 2014). Animals body
128 weight was monitored weekly. At the end of the exposure period, DDT-exposed and control
129 male mice were mated to unexposed females to generate the female offspring (**Fig. S1**). Female
130 mice were kept on a standard chow diet during the breeding period, for the extent of pregnancy
131 (21 days) and after giving birth. The birth weight of pups and number of pups per litter was
132 determined. To avoid litter-effect, pups were cross-fostered one day after birth. Pups from 2-3
133 dams were pooled and housed in a litter of 8-10 pups per nursing dam. All pups were weaned on
134 postnatal day (PND) 21. Offspring's body weight was recorded weekly. The female offspring of
135 control or DDT-exposed fathers were used to study body weight, metabolic function, and
136 mammary tumorigenesis and for mammary gland collection as described in the following
137 sections. The number of contributing fathers and female offspring used in each experiment is
138 listed in **Table S1**. All animal procedures were approved by the Georgetown University Animal

139 Care and Use Committee, and the experiments were performed following the National Institutes
140 of Health guidelines for the proper and humane use of animals in biomedical research.

141 ***DDT and Phenobarbital (PB) treatment:*** PB treatment is known to promote hepatic enzymes
142 activation, accelerating DDT metabolism and excretion in humans (Davies et al. 1969; Kwalick
143 1971) and animals (Alary et al. 1971; Lambert and Brodeur 1975, 1976). A sub-set of control-
144 vehicle or DDT exposed males were concomitantly treated with phenobarbital (20 mg/kg of
145 body weight, i.p.) or saline and used for sperm harvesting.

146 ***Mature spermatozoa collection and purification:*** Control and DDT-exposed males as well as
147 those treated with PB were euthanized and caudal epididymis dissected for sperm collection. The
148 epididymis was collected, punctured, and transferred to tissue culture dish containing M2 media
149 (M2 Medium-with HEPES, without penicillin and streptomycin, liquid, sterile-filtered, suitable
150 for mouse embryo, SIGMA, product #M7167) where it was incubated for 1 hour at 37°C. Sperm
151 samples were isolated and purified from somatic cells. Briefly, the samples were washed with
152 PBS, and then incubated with SCLB (somatic cell lysis buffer, 0.1% SDS, 0.5% TX-100 in
153 Diethylpyrocarbonate water) for 1 hour. SCLB was rinsed off with 2 washes of PBS and the
154 somatic cell-free purified spermatozoa sample pelleted and used for RNA extraction.

155 ***Measurement of DDT metabolites:*** Liver tissues from control, DDT and DDT-PB treated males
156 (n=3 pooled samples/group), collected within 24 h after last dose, were used to determine the
157 levels of DDT main metabolites. Measurements were performed commercially by Pacific Rim
158 Labs, a fully accredited laboratory providing analysis of DDT and other persistent organic
159 pollutants. Briefly, samples were fortified with each of the six 13C-DDT isotopes and then
160 extracted with hexane. The extract was columned on Florisil and analyzed by GC-MS/MS. The
161 instrument was calibrated with a five-point calibration covering the range of 0.2-400 ng/g or mL.

162 ***Body weight monitoring:*** DDT female offspring's body weight was measured at birth (n=8-17
163 litters/group) and at weaning and weekly after that (n=25/group). Birth weights were analyzed by
164 t-test and longitudinal body weight analyzed using two-way ANOVA (group, time), followed by
165 post-hoc analyses.

166 ***Small RNA-Seq Analysis:*** Total RNA was isolated from paternal sperm (n=4/group) using
167 Qiagen's miRNeasy extraction kit, according to the manufacturer's instructions. Small non-
168 coding RNA transcript libraries were constructed according to Illumina TrueSeq Small RNA
169 Pre-Kit. Indexed, paired-end sequencing libraries were prepared from quality total RNA (RIN ≥
170 8). For each library, 10 million reads (raw data) were generated by Illumina Hi-Seq 4000. The
171 raw reads were subject to adapter trimming and low quality filtering using Trimmomatic
172 program. The high quality clean reads were aligned on the mouse genome. Non-coding RNA
173 tags were mapped to the mouse genome (GRCm38/mm10 reference genome) in order to analyze
174 their expression and distribution on the genome. Small RNA tags were annotated with miRNA,
175 t-RNA and pi-RNA to miRBase, Ref-seq, GenBank and Rfam database using blastn with
176 standard parameters. To analyze the differential expression of miRNA between control and DDT
177 groups, miRNAs were normalized to TPM (Transcripts Per Kilobase Million). miRNAs with a P
178 value less than 0.05 were considered significant, with an appropriate correction for multiple
179 testing (Clarke et al. 2008).

180 ***Quantitative real time PCR validation (qRT-PCR):*** cDNA was synthesized from total RNA
181 samples using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems).
182 PCR products were amplified from cDNA using TaqMan® Fast Advanced Master Mix and
183 sequence-specific primers from TaqMan Assays (Applied Biosystems). Fold change was
184 calculated from Ct values and the expression levels of specific miRNAs or genes were

185 determined by normalizing these values with the fold change values for appropriate endogenous
186 controls (miR-26a or *GAPDH*). Fold differences between the groups were analyzed using t-test
187 or two-way ANOVA (group, treatment), followed by post-hoc analyses.

188 ***Carcinogen-induced mammary tumorigenesis:*** Mammary tumors were induced in control and
189 DDT female offspring (n=25/group) by administration of MPA (medroxyprogesterone acetate,
190 15 mg, s.c.) at 6 weeks of age, followed by three weekly doses of 1mg of 7,12-
191 dimethylbenz[a]anthracene (DMBA) (Sigma, St. Louis, MO) dissolved in peanut oil by oral
192 gavage. This established model of breast cancer has been used by us and others (Apostoli et al.
193 2014; Fontelles et al. 2016a) . Mice were examined for mammary tumors by palpation once per
194 week, starting on week two after the last dose of DMBA and continue for a total of 20 weeks.
195 Tumor growth was measured using a caliper and the width and height of each tumor were
196 recorded. Other end-points for data analysis were latency to tumor appearance, the number of
197 animals with tumors (tumor incidence), and the number of tumors per animal (tumor
198 multiplicity). During follow-up, those animals in which tumor burden approximated 10% of total
199 body weight were euthanized, as required by our institution. Histopathology of tumors was
200 determined by our pathologist. All tumors included in our analyses were classified as mammary
201 carcinomas. Differences in tumor latency and multiplicity were analyzed by t-test (or
202 corresponding non-parametric test). Kaplan-Meier survival curves were used to compare
203 differences in tumor incidence, followed by the log-rank test. Tumor growth was analyzed using
204 two-way ANOVA (group, time), followed by post-hoc analyses.

205 ***Embryonic miRNA microinjections.*** To induce super-ovulation, female mice (*c57bl/6*) received
206 intra-peritoneal injections of 5 IU of pregnant mare's serum gonadotropin (PMSG). About 48
207 hours after the PMSG injection, mice were injected with 5 IU of human chorionic gonadotropin

208 (hCG). Super-ovulated females were mated with *c57bl/6* male mice. About 16 hours later,
209 fertilized eggs were harvested. Zygotes were injected with either synthetic miRNA-10b
210 (*mir*Vana®, ThermoFisher) diluted in TE solution (10mM Tris-HCl, pH7.4, 0.25 EDTA) or TE
211 solution only (to control for microjection). Each zygote received ~10 pL of TE or miRNA
212 preparation at 2ng/µL. Microinjections were confirmed by observing the swelling of the male
213 pro-nucleus. Injected zygotes were transferred into recipient female mice to produce offspring as
214 described before (Rodgers AB 2015). Briefly, recipient females were mated with vasectomized
215 males overnight and identified by copulation plug before the zygote transfer. Each recipient
216 female was implanted with 25-30 zygotes. Dams were housed in groups of two with free access
217 to food and water. Female offspring were used to study tumor growth, metabolic function and for
218 mammary tissue collection. Mature sperm cells contain approximately 20 femtograms of total
219 RNA, of which about 20% are small non-coding RNAs and 0.4% are miRNAs (Kawano M
220 2012). Thus, the amount of miRNA injected into zygotes did not exceed physiological levels.
221 Embryonic injections were performed at the Johns Hopkins Transgenic Core Facility. Resulting
222 offspring were subsequently transferred to our institution for follow-up studies.
223 ***Syngeneic orthotopic tumor implantation:*** Mouse EO771 breast cancer cells were cultured in
224 complete medium [Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10%
225 (v/v) FBS and 1% L-glutamine, at 37°C in 5% CO2 (v/v)] to 80% confluence, trypsinized,
226 counted, checked for viability and re-suspended in PBS for injection into both 4th inguinal
227 mammary glands. A total of $1x10^6$cells were injected into each of the 4th inguinal mammary
228 gland fat pads of miRNA-10b females (n=6-8/group). Tumor growth was monitored for at least
229 six weeks.

230 ***Immunohistochemistry analysis.*** Cell proliferation (Ki-67) was evaluated by
231 immunohistochemistry in mammary tumors (n=5-7/group). Briefly, tissues were fixed in 10%
232 buffered formalin, embedded in paraffin, and sectioned (5 µm). Sections were deparaffinized
233 with xylene and rehydrated through a graded alcohol series. Antigen retrieval was performed by
234 immersing the tissue sections at 98°C for 40 minutes in 1X Diva Decloaker (Biocare). Tissue
235 sections were treated with 3% hydrogen peroxide and 10% normal goat serum for 10 minutes,
236 and were incubated with the primary antibody (**Table S2**), overnight at 4°C. After several
237 washes, sections were treated to the appropriate HRP labeled polymer for 30 min and DAB
238 chromagen (Dako) for 5 minutes. Slides were counterstained with Hematoxylin (Fisher, Harris
239 Modified Hematoxylin), blued in 1% ammonium hydroxide, dehydrated, and mounted with
240 Acrymount. The sections were photographed using an Olympus IX-71 Inverted Epifluorescence
241 microscope at 40x magnification. Proliferation index (Ki-67 staining) was determined by
242 immunoRatio, a plugin Image J software (National Institute of Health, Bethesda, MD, USA) to
243 quantify hematoxylin and DAB-stained cells. Results were analyzed by t-test (or corresponding
244 non-parametric test).
245 ***Analysis of cell apoptosis:*** Cell apoptosis analysis was conducted in mammary tumors (n=14-
246 17/group) by morphological detection. Tissues were fixed in neutral buffered 10% formalin and
247 stained with hematoxylin and eosin (H&E). Cells presenting loss of adhesion between adjacent
248 cells, cytoplasmic condensation and formation of apoptotic bodies were considered apoptotic as
249 described before (Elmore et al. 2016). The sections were photographed using a Olympus IX-71
250 Epifluorescence microscope at 40x magnification. Twenty areas were photographed randomly
251 and the number of apoptotic bodies counted. The apoptotic index was determined as the

252 percentage of apoptotic bodies per total number of cells. Images were evaluated with Image J
253 software (NIH, USA). Results were analyzed by t-test (or corresponding non-parametric test).
254 ***Mammary gland development:*** Inguinal mammary glands (4th pair, n=5-16/group) collected on
255 post-natal day 50 (PND 50) were stretched onto a slide, placed in a fixative solution and stained
256 with a carmine aluminum solution (Sigma Chemical Co.) as previously described (de Assis et al.
257 2010). Whole mounts were examined under the microscope (AmScope) and ductal elongation
258 and number of TEBs (undifferentiated structure considered to be the targets of malignant
259 transformation), as previously described (de Assis et al. 2010). In addition, whole-mount slides
260 were digitized (Olympus SZX12 Stereomicroscope) and analyzed using the ImageJ software
261 (National Institute of Health, Bethesda, MD, USA): The area and integrated density of a
262 manually drawn perimeter around the mammary epithelial tree of each whole-mount were
263 measured. The average area as well integrated density measurements are reported for each group.
264 Differences were statistically tested using t test (or corresponding non-parametric test).

265 ***Analysis of protein expression:*** Protein levels were assessed by western blot on PND50
266 mammary tissues (n=6-7 group) and tumors (n=6/group). Total protein was extracted
267 from mammary tissues using RIPA buffer with Halt™ Protease Inhibitor Cocktail (Thermo
268 Fisher). Ten micrograms of protein extracts were resolved on a 4-12% denaturing
269 polyacrylamide gel (SDS-PAGE). Proteins were transferred using the iBlot® 7-Minute Blotting
270 System (Invitrogen, USA) and blocked with 5% non-fat dry milk for 1h at room
271 temperature. Membranes were incubated with the specific primary antibodies (for antibody
272 specifications and dilutions see **Table S2**) at 4°C overnight. After several washes, the
273 membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody
274 at room temperature for 1h. Membranes were developed using the Chemiluminescent HRP

275 antibody detection reagent (Denville Scientific Inc., USA), and exposed to blot imaging systems
276 (Amersham^{TM} Imager 600, GE Healthcare Life Sciences). Optical density of the bands was
277 quantified using Quantity-one software (BIO-RAD, USA). To control for equal protein loading,
278 expression of the proteins of interest was normalized to the β-actin or β-tubulin signal.
279 ***Metabolic Parameters:*** Glucose tolerance test (GTT) was performed after overnight fasting in
280 mice. Glucose (0.75 U/kg body weight) was administered intraperitoneally and tail blood
281 samples were collected at 0, 15, 30 and 60 minutes after injection (n=8/group). Circulating
282 insulin levels (n=6-7/group) were measured using an ELISA kit (Ultra Mouse insulin EIA Kit,
283 Crystal Chem). Differences in GTT were analyzed using two-way ANOVA (group, time),
284 followed by post-hoc analyses. Insulin levels were statistically tested using t test (or
285 corresponding non-parametric test).

286

287 **Results**

288 ***DDT offspring have altered body weight gain and metabolic function***

289 Male mice were either exposed to a vehicle-control or an environmentally relevant dose of DDT
290 (as described in the Material and Methods section) and mated with unexposed females to
291 generate the offspring (**Fig. S1**). The phenotypes in offspring from control and DDT-exposed
292 male mice were then examined. No differences in gender distribution or litter size were
293 observed between offspring born from DDT and control males (**Table S3**). However, paternal
294 DDT exposure was associated with a significant decrease in offspring's birthweight and weaning
295 weights ($P<0.05$, $P<0.01$ **Fig. 1a-b**). After weaning, DDT daughters underwent a catch-up
296 growth period and by 6 weeks of age no differences in body weight were observed between the
297 groups. In fact, DDT females became significantly heavier than controls by 28-weeks of age

298 (P<0.05, P<0.01, **Fig. 1c**). They also showed metabolic dysfunction with higher circulating
299 levels of insulin and impaired glucose tolerance compared to controls in adulthood (**Fig**. **2a-b**,
300 P<0.05).

301 ***DDT daughters have accelerated breast cancer growth***

302 Maternal DDT exposure has been linked to a near 4-fold increase in breast cancer in daughters
303 (Cohn BA 2015). Low birthweight, a phenotype observed in offspring DDT-exposed fathers,
304 has also been linked with higher risk for breast cancer in humans (Mellemkjaer L 2003;
305 Sanderson et al. 1996) and animal models (da Cruz et al. 2018; Fernandez-Twinn DS 2010).
306 Thus, we studied mammary cancer development in control and DDT daughters using the well-
307 established (Apostoli et al. 2014; Fontelles et al. 2016a) DMBA-induced mouse model of breast
308 cancer. Tumor growth was significantly increased in DDT daughters compared to controls
309 (*P*<0.001, **Fig. 3a**), with tumors weighing significantly more at the end of the monitoring period
310 (*P*<0.01, **Fig. 3b**). The incidence of mammary tumors in the DDT offspring was higher and
311 tumor latency slightly shorter than in controls but results did not reach statistical significance
312 (**Fig. 3c-d**).

313 Given the increased mammary tumor growth in the DDT daughters, we assessed the cell
314 proliferation and apoptosis rates in their tumors. We found a slight increase in cell proliferation
315 rates (*P*<0.1, **Fig. S2**) as well as a significant decrease in apoptosis rates in tumors of DDT
316 daughters compared to controls (*P*<0.05, **Fig. S2**).

317 ***DDT daughters' mammary gland morphological development is mostly normal***

318 Because DDT daughters showed accelerated breast cancer growth, we next analyzed the effects
319 paternal DDT on offspring's mammary morphological development in post-natal day (PND)50
320 inguinal mammary glands. We assessed number of terminal end buds (TEB), ductal elongation

321 and epithelial area/density. No major morphological changes were detected, except for a slight
322 increase in mammary ductal elongation in DDT daughters compared to controls (**Fig. S3**).

323 ***DDT daughters' mammary tissues have rewired nutrient sensing mechanisms***

324 We previously showed that low birth weight is linked to accelerated tumor growth, a phenotype
325 associated with suppression of the nutrient/energy sensing AMPK pathway (**Fig. 4b**) in
326 mammary tissues and tumors (da Cruz et al. 2018). In support of that, we found that both AMPK
327 expression and phosphorylation levels were decreased in DDT daughters' mammary tissues. We
328 also detected an increase in expression and activity of mTOR and its down-stream target, 4E-
329 BP1 (**Fig. 4b, Fig. S4a-f**). Similar patterns of mTOR and 4E-BP1 expression and activity were
330 also found in tumors of DDT daughters, although results were not statistically significant (**Fig.
331 4c, Fig. S4g-l**).

332 ***Pre-conception paternal DDT exposure alters the sperm non-coding RNA content***

333 To determine the impact of DDT exposure on paternal sperm, we performed RNA-seq analysis
334 to assess the small RNA patterns in mature cauda sperm of control and DDT-exposed males. The
335 distribution of the major small RNA species detected is in agreement with previous reports (YM
336 Chen Q, Cao Z, Li X, Zhang Y, Shi J, Feng GH, Peng H, Zhang X, Zhang Y, Qian J, Duan E,
337 Zhai Q, Zhou Q 2016; Sharma U 2016), with tRNA-derived fragments (tRFs) being the most
338 abundant sperm small RNA sub-type, followed by miRNAs and piRNAs in both groups (**Fig.
339 5a**). Interestingly, sperm of DDT-exposed males showed higher abundance of miRNAs (6.7%)
340 compare to controls (1.4%) (**Fig. 5a-b**). A total of 16 miRNAs were differentially expressed
341 (nine down- and seven up-regulated) in sperm of DDT-exposed mice compared to controls (**Fig.
342 5c**). Of the seven miRNAs upregulated in sperm of DDT-exposed males, miRNA-10b had the
343 highest surge, representing close to 60% of the overall miRNA increase (**Fig. 5d**).

344 To establish whether upregulation of miRNA-10b in sperm was, in fact, induced by DDT
345 exposure, we treated DDT-exposed mice with phenobarbital (PB), a drug known to promote
346 hepatic enzymes activation and accelerate DDT metabolism and excretion in humans (Davies et
347 al. 1969; Kwalick 1971) and animals (Alary et al. 1971; Lambert and Brodeur 1975, 1976).
348 Indeed, we observed an increase in hepatic phase I enzymes ($P<0.001$, **Fig.5e**) and a reduction in
349 DDT metabolites in males treated with both DDT and PB (**Table S4**). Further, compared to mice
350 exposed to DDT only, miRNA-10b was decreased in sperm of mice treated with both DDT and
351 PB with levels reversing to those observed in controls ($P<0.05$, **Fig. 5f**).

352 ***miRNA-10b embryonic injection replicates DDT daughters' phenotypes***

353 miRNA-10b along with other members of the miRNA-10 family play critical roles in embryonic
354 development and cell differentiation through the regulation of Homeobox (*Hox*) genes (Tehler et
355 al. 2011). These genes regulate organogenesis including development of the mammary gland
356 (Carroll and Capecchi 2015; Chen and Capecchi 1999; Garin et al. 2006).

357 Next, we examined whether miRNA-10b was responsible for the phenotypes observed in DDT
358 offspring using embryonic (zygote) microinjections. While no major changes in mammary gland
359 morphology were detected (**Fig. S3**), female mice derived from the miR-10b zygote injections
360 (miR-10b females from now on) showed mammary tissue suppression of AMPK and increased
361 expression and activity of mTOR and down-stream effector, 4E-BP1, compared to controls (**Fig.
362 6a**, **Fig. S5**), a phenotype also observed in mammary glands of DDT daughters. More
363 importantly, we also found increased orthotopic (EO771 cells) mammary tumor development in
364 miR-10b females, which showed both increased tumor growth and higher tumor weights
365 compared to controls ($P<0.05$, $P<0.01$, **Fig. 6b-c**). Survival after tumor implantation was also
366 worse in miRNA-10b females (**Fig. 6d**, $P<0.1$). Further, embryonic miR-10b injection

367 phenocopied the metabolic dysfunction of DDT daughters, with adult miR-10b females showing
368 hyperinsulinemia (P<0.01) compared to controls (**Fig. 6e**).
369
370 **Discussion**
371 Paternal environmental exposures have been shown to modulate offspring's health phenotypes
372 via sperm non-coding RNAs (YM Chen Q, Cao Z, Li X, Zhang Y, Shi J, Feng GH, Peng H,
373 Zhang X, Zhang Y, Qian J, Duan E, Zhai Q, Zhou Q 2016; Rodgers AB 2015; Sharma U 2016).
374 Here, we show that pre-conception paternal exposure to the pesticide DDT modulates the sperm
375 non-coding RNA load, particularly miRNAs, and programs female offspring's breast cancer
376 development in a pre-clinical animal model. We also found that miRNA-10b, the miRNA with
377 the highest increase in sperm of DDT-exposed fathers, can recapitulate phenotypes observed in
378 DDT daughters including alterations in the mammary tissue AMPK/mTOR axis and accelerated
379 breast tumor growth. These findings lend support to the notion that predisposition to diseases
380 such as cancer can be determined by factors other than genetic background and suggest that it
381 could be transmitted through sperm miRNAs. We have shown before that paternal obesity,
382 malnutrition or modulation of nutritional patterns can increase breast and other types of cancer in
383 the progeny (da Cruz et al. 2018; da Cruz et al. 2019; de Assis S 2012; Fontelles et al. 2016a).
384 This study further implies that environmentally-induced non-genetic or epigenetic predisposition
385 to cancer can occur and should be evaluated in human studies.
386 Previously published reports using the CHDS cohort showed that daughters of women exposed
387 to DDT in pregnancy have increased rates of breast cancer (Cohn BA 2015) and are more likely
388 to be diagnosed with advanced stage tumors. Likewise, we found that paternal DDT exposure in
389 the pre-conception window leads to increased breast cancer growth in daughters in a mouse

390 model. Our new observations and our previously published data (da Cruz et al. 2018; da Cruz et
391 al. 2019; Fontelles et al. 2016a; Fontelles et al. 2016b) indicate that paternal exposures are as
392 important as maternal ones in programming of breast and other cancers in offspring. This overlap
393 between the effects of maternal and paternal exposure on offspring's breast cancer phenotypes
394 suggest a common underlying mechanism that needs to be further investigated.

395 While no studies in humans have directly investigated the link between paternal DDT exposure
396 and breast cancer in their descendants, some evidence for a role of related paternal exposures
397 exists. For instance, an association between father's exposure to pesticides and a number of
398 childhood cancers in offspring has been reported (Bailey H 2014; Greenop KR 2013).
399 Epidemiologic studies show that paternal exposure to DDT and other pesticides is linked to low
400 birthweight (Robledo CA 2015) and our study supports those findings. Low birthweight is more
401 prevalent in minority populations (Lumpkins and Saint Onge 2017; Wang and Beydoun 2007)
402 which are more likely to be exposed to environmental toxicants such as DDT (CDC Upated
403 Tables-January 2019(2009); Daniels et al. 2018; Ruiz et al. 2018) and to develop more
404 aggressive breast tumors (Kohler et al. 2015).

405 It has been proposed that during the normal fertilization process, sperm small RNAs are
406 delivered to the oocyte and modulate the transcriptome during the first few cell divisions, setting
407 a signaling cascade that can impact embryonic development (Gapp and Miska 2016) and recent
408 reports support this view (YW Chen Q, Duan W 2016; Sharma U 2016). In line with that,
409 embryonic injection of miR-10b, the miRNA with the highest surge in sperm of DDT-exposed
410 males, replicates most of the major phenotypes observed in DDT daughters including higher
411 circulating levels of insulin, altered AMPK/mTOR signaling in mammary tissues and increased
412 tumor growth. While we have focused on miRNA-10b given its reported role in embryonic

413 development (Tehler et al. 2011), it is possible that the full phenotype observed in DDT
414 offspring reflects the interaction of that miRNA with other miRNAs and, likely, other non-
415 coding RNAs in sperm of DDT-exposed males. It is important to note, however, that other
416 paternal environmental exposures —including malnutrition and obesity— previously shown to
417 increase breast cancer development in daughters (da Cruz et al. 2018; Fontelles et al. 2016a;
418 Fontelles et al. 2016b) also lead to an increase in miRNA-10b levels in sperm (YM Chen Q, Cao
419 Z, Li X, Zhang Y, Shi J, Feng GH, Peng H, Zhang X, Zhang Y, Qian J, Duan E, Zhai Q, Zhou Q
420 2016; Cropley et al. 2016; da Cruz et al. 2018; Fontelles et al. 2016b), indicating that a common
421 mechanism underlying the effects of different paternal exposures on offspring phenotypes likely
422 exists and should be examined.

423 MicroRNA-10b along with other members of the miRNA-10 family play critical roles in
424 embryonic development and cell differentiation through the regulation of Homeobox (*Hox*)
425 genes (Tehler et al. 2011). These genes regulate organogenesis including development of the
426 mammary gland and other skin-derived tissues (Carroll and Capecchi 2015; Chen and Capecchi
427 1999; Garin et al. 2006). However, whether alterations in *Hox* genes in the embryonic mammary
428 gland play a mechanistic role in the increased breast cancer development in daughters of DDT-
429 exposed fathers needs to be examined in futures studies.

430 We previously showed that female offspring born small (due to paternal malnutrition) have re-
431 wiring of the nutrient sensing AMPK/mTOR pathway both in normal mammary tissues as well
432 as in tumors. Similarly, we found that DDT daughters, who are also born small, have alterations
433 in the mammary AMPK/mTOR axis, further suggesting a mutual mechanism between different
434 types of ancestral paternal exposures. Whether alterations in nutrient sensing mechanisms are
435 programmed by pre-conception exposure or are a consequence of the metabolic dysfunction

436 observed in DDT daughters remains to be determined. However, the same phenotype was
437 present in mammary glands of miRNA-10b females indicating that dysregulation of the
438 AMPK/mTOR axis is likely initiated through sperm miRNAs after fertilization but mechanisms
439 for how it impacts mammary development need to be further elucidated.

440 It also remains to be determined whether alterations in the AMPK/mTOR axis in mammary
441 tissues of DDT daughters and miR-10b females are functionally responsible for the observed
442 increase in tumor growth. In normal cells, proliferation is coupled to nutrient availability to
443 ensure proper cell growth and division (Viollet et al. 2009). The AMPK pathway functions as a
444 cellular energy sensor mechanism and provides a link between nutrient supplies and cell growth
445 and metabolism. This pathway controls mTOR activity and associated energy-consuming
446 biosynthetic activities such as protein synthesis (Shackelford and Shaw 2009). Not surprisingly,
447 suppression of AMPK and increased mTOR activity (due to loss of LKB1, the major AMPK
448 kinase) is responsible for the inheritable Peutz-Jeghers syndrome, in which patients have an 18-
449 fold increase in cancer risk, including breast cancer (Sebbagh et al. 2011). Alterations in
450 AMPK/mTOR axis have been shown to lead metabolic reprogramming in cancer cells (Faubert
451 et al. 2014; Mossmann et al. 2018; Saxton and Sabatini 2017) and our published studies confirm
452 those findings (da Cruz et al. 2018). Given its document role in cancer, the local mammary
453 changes in AMPK/mTOR signaling are likely mechanistic linked to accelerated tumor growth,
454 however, it will also be important to examined the role of systemic alterations in enhancing
455 breast cancer development in both DDT daughters and miRNA-10b females.

456 Although we clearly show that pre-conception paternal DDT exposure modulates the male
457 germline and programs female offspring's breast cancer development, our study has some
458 limitations. First, we only used one environmentally relevant dose of DDT in our investigations

459 and exposure was restricted to a short period of time in adulthood. In follow-up studies, it would
460 be important to examine whether other environmentally relevant doses of DDT would elicit the
461 same effects on the paternal germline and offspring's phenotypes. It would also be critical to
462 understand the impact of DDT exposure on sperm at different developmental windows and
463 possible differences stemming from longer exposure periods. Although embryonic injection of
464 miR-10b on its own replicated most of the effects of paternal DDT on offspring's mammary
465 cancer development, it will be important to test whether other miRNAs and small RNA subtypes
466 also play a role. Finally, due to age-specific requirements, we had to use different models of
467 breast cancer in studies involving DDT daughters (DMBA model) and miRNA-10b females
468 (orthotopic implantation). In both cases, we observed accelerated tumor growth, however, it is
469 likely that DMBA-induced primary tumors better mimic the three stages of cancer development
470 (initiation, promotion and progression) compared to orthotopic implantation models (Liu et al.
471 2015).

472 **Conclusions:** Our study shows that environmental toxicants such as DDT alter the male
473 germline non-coding RNAs and increase cancer susceptibility in the progeny. This could have
474 important implications for human health: According to published studies and the Center for
475 Disease Control and Prevention (CDC), minorities such as African Americans and Mexican-
476 Americans and recent immigrants have the highest circulating concentrations of this pesticide in
477 the U.S. (CDC 2009; Daniels et al. 2018; Ruiz et al. 2018). In addition, DDT is still used in
478 developing countries and Americans who could have been affected by paternal exposure to DDT
479 before its ban (Cohn BA 2015) are still alive today and reaching an age when the incidence of
480 breast and other cancers increases. While our study focused on DDT exposure, our findings are
481 likely to be relevant to other commonly used pesticides and flame retardants which, like DDT,

482 have anti-androgenic properties (Kharlyngdoh et al. 2018; Kortenkamp and Faust 2010; Stoker et
483 al. 2005).

484

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494 Assis).

495 The small RNA-seq (small RNA) data has been deposited in GEO (Gene Expression Omnibus)
496 database with accession code pending.

497

498

499 **Author contributions:** S.D.A. conceived the study, oversaw the research and wrote the
500 manuscript; R.S. supervised the animal work and tissue collection, performed molecular analysis
501 and wrote the manuscript; H.C and C.C.F performed animal work. A.N. and A.K. processed
502 tissues and/or helped perform molecular analysis; M.I.C. assisted with animal studies; L.J.
503 performed RNA-seq data.

504
505

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Figure Legends

Fig-1. Paternal DDT exposure programs offspring's body weight. Body weight (grams) at (a) birth (n=8-17 litter/group), at (b) weaning and in (c) adulthood [longitudinal body weight from 6 to 30 weeks of age (n=25/group)] in control (CO) and DDT offspring. All data are mean ± SEM. *p<0.05, **p<0.01 by t-test (birthweight and weaning) or repeated measures ANOVA (longitudinal body weight).

Fig-2. Paternal DDT exposure programs metabolic dysfunction in daughters. (a) Serum insulin levels (n=6-7/group) in adult control (CO) and DDT female offspring; (b) Glucose tolerance test (GTT) in adult control and DDT female offspring (n=8/group). All data are mean ± SEM. * p<0.05 by t-test (serum insulin levels) or repeated measures ANOVA (GTT).

Fig-3. Paternal DDT exposure programs breast cancer development in daughters. Carcinogen-induced mammary tumorigenesis in control (CO) and DDT female offspring: (a) Mammary tumor volume, (b) tumor weight (grams), (c) tumor incidence and (d) tumor latency (n=25/group). Tumor incidence is shown as percentage of animals with tumors. All other data are mean ± SEM. *p<0.05, **p<0.01 by t-test (tumor weight and tumor latency), repeated measures ANOVA (mammary tumor growth) or Kaplan-Meier analysis followed by log-rank test (tumor incidence).

Fig-4. Paternal DDT exposure programs the AMPK/mTOR signaling pathway in DDT daughters' mammary tissues. (a) Simplified schematic representation of the pathway; (b-c) Representative western blots showing protein expression (phosphorylated and total) levels of members of the AMPK/mTOR axis in (b) normal mammary tissues and in (c) tumors (n=6-7/group) of control (CO) and DDT female offspring. Protein expression levels were normalized by either β-actin or β-tubulin. Densitometry data is shown in Fig. S4.

Fig-5. DDT exposure reprograms the sperm non-coding RNA load. (a) Small RNA subtype distribution and (b) miRNA abundance in sperm of DDT-exposed and control (CO) males (n=4/group) assessed by RNA-seq; (c) Heat-map showing differentially expressed miRNAs in sperm from DDT-exposed and CO males; (d) miR-10b as a fraction of all upregulated miRNAs in sperm of DDT-exposed males; (e) Expression levels of Cyp2B10 assessed by q-PCR in liver tissues of CO and DDT-exposed male mice treated with phenobarbital (PB, 20mg/kg) or vehicle (n=4-5/group); (f) Expression levels of miRNA-10b, assessed by q-PCR, in sperm of control or DDT-exposed male mice treated with PB or vehicle (n=8-20/group). Expression levels of Cyp2B10 and miRNA-10b are relative to GAPDH and miR-26a, respectively. All data are mean ± SEM. *p<0.05, ***p<0.001, ****p<0.0001 by two-way ANOVA.

Fig-6. Embryonic (zygote stage) injection of miRNA-10b recapitulate the phenotypes observed in DDT daughters. (a) Representative western blots showing protein expression (phosphorylated and total) levels of members of the AMPK/mTOR axis in normal mammary tissues of control (CO) and miRNA-10b females (Protein expression levels were normalized by β-actin,

n=5/group. Densitometry data is shown in Fig. S5); (b-d) Orthotopic tumor growth (EO771 cells, n=6-8/group) in adult CO and miRNA-10b females [(b) tumor volume, (c) tumor weight (grams) and (d) survival]. (e) Serum insulin levels in adult control (CO) and miR-10b females (n=6/group). Survival is shown as percentage of animals still alive at a given time; All other data are mean ± SEM. *$p<0.05$, **$p<0.01$ by t-test (insulin levels, tumor weights, repeated measures ANOVA (mammary tumor volume) or Kaplan-Meier analysis followed by log-rank test (survival).

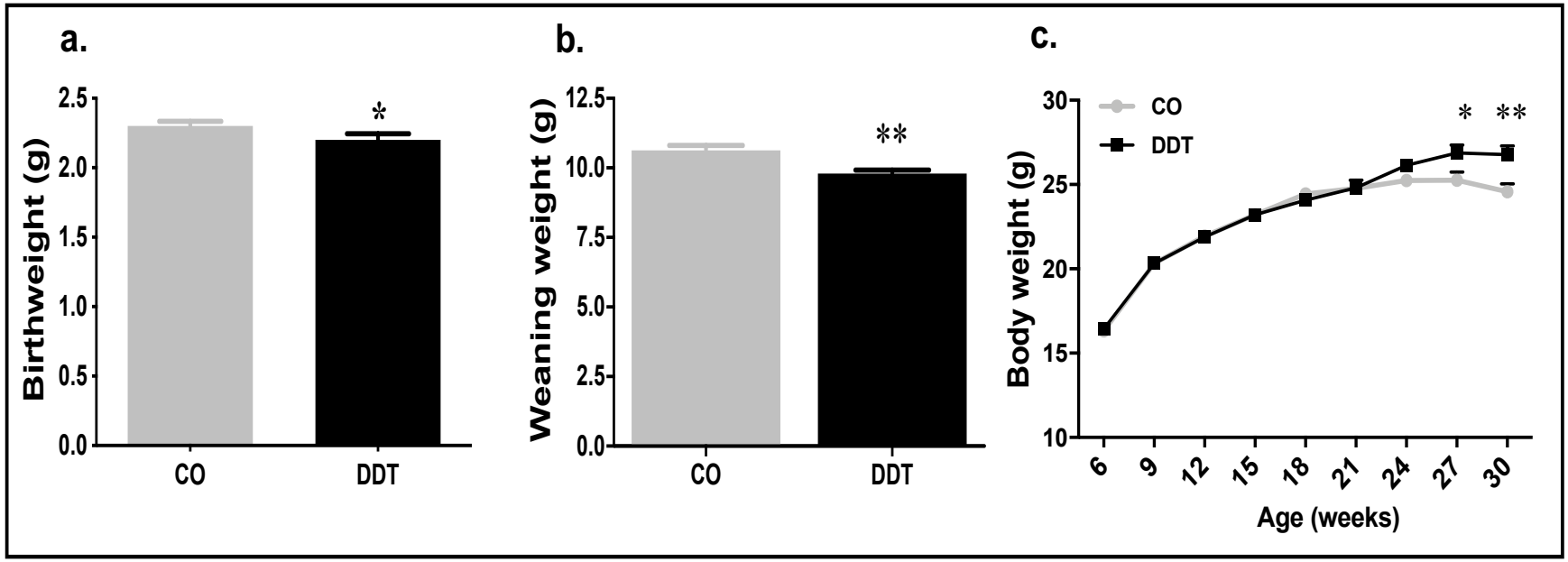
a.
Birthweight (g)
2.5
2.0
1.5
1.0
0.5
0.0
*
CO
DDT
b.
Weaning weight (g)
12.5
10.0
7.5
5.0
2.5
0.0
**
CO
DDT
c.
Body weight (g)
30
25
20
15
10
CO
DDT
*
**
6
9
12
15
18
21
24
27
30
Age (weeks)

Figure 1

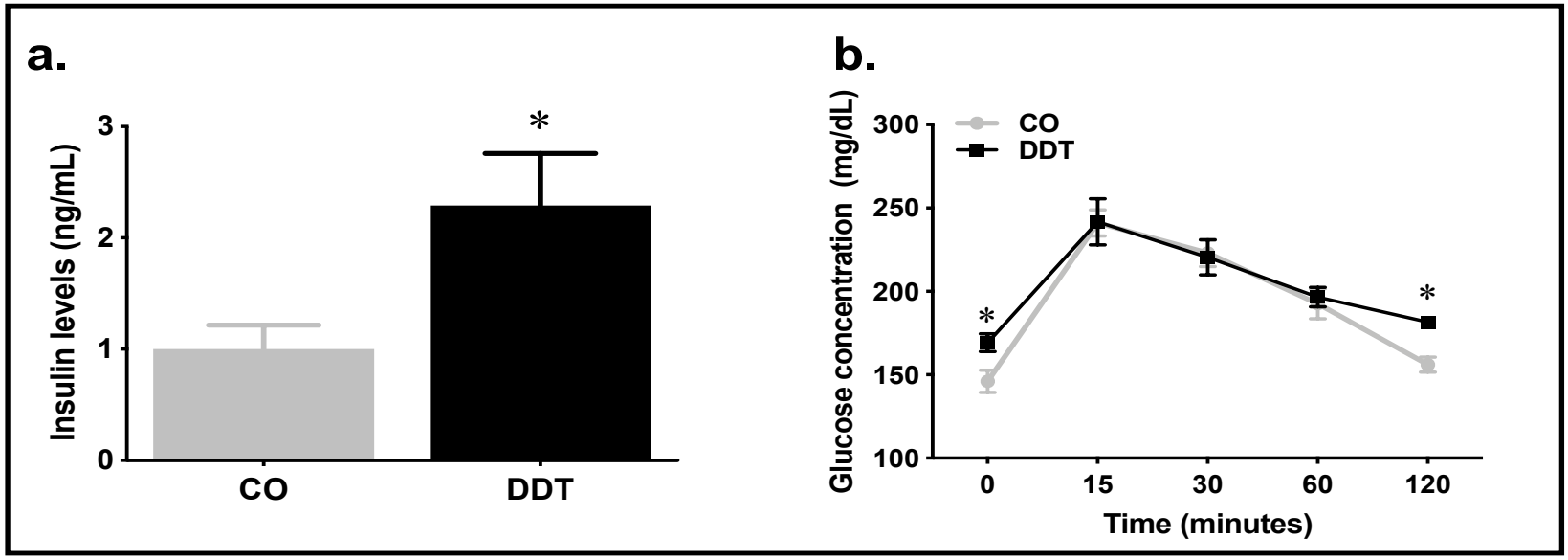
a.
Insulin levels (ng/mL)
3
2
1
0
*
CO
DDT
b.
Glucose concentration (mg/dL)
300
250
200
150
100
CO
DDT
*
*
0
15
30
60
120
Time (minutes)

Figure 2

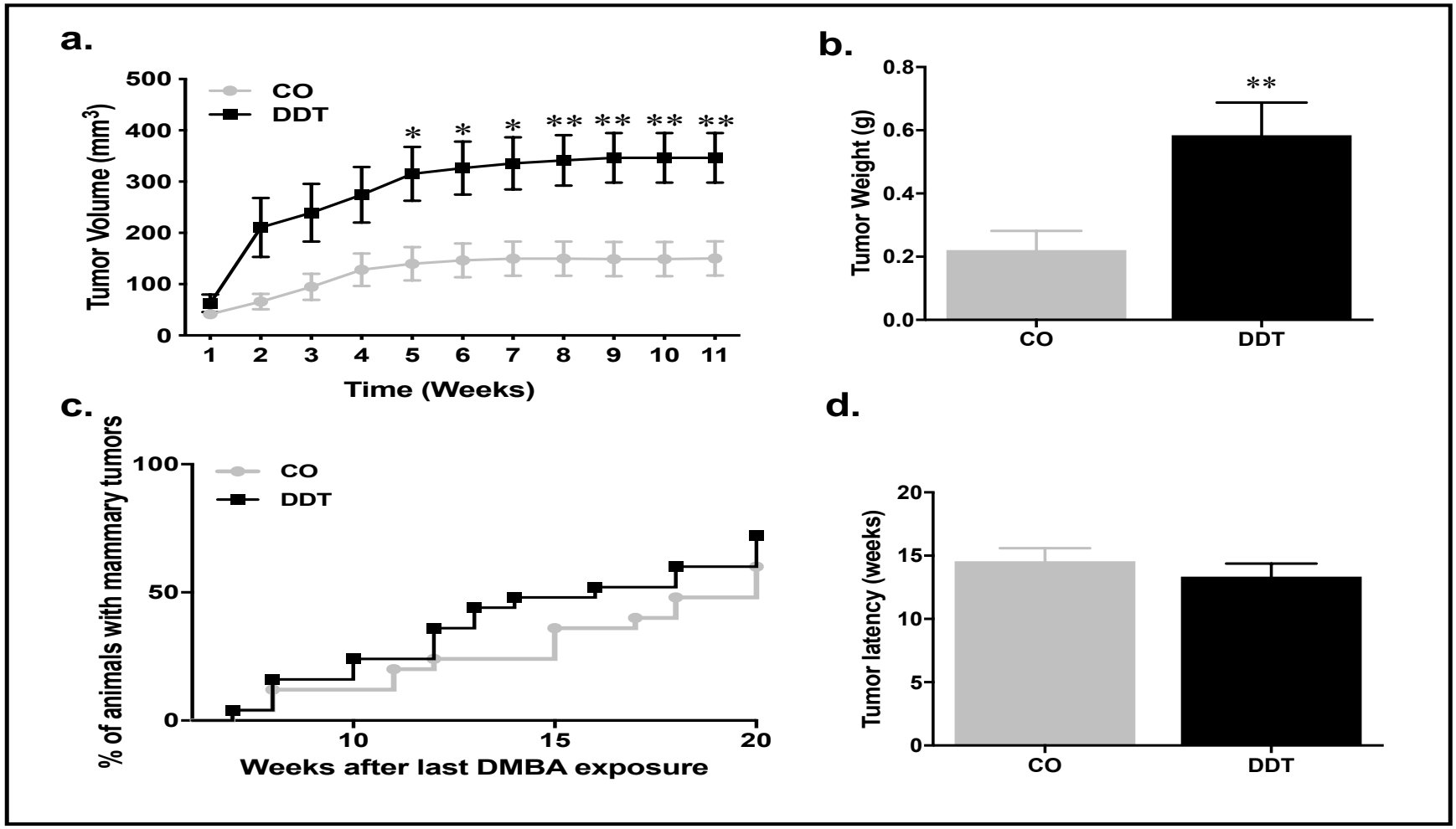
a.
Tumor Volume (mm3)
500
400
300
200
100
0
CO
DDT
*
*
*
**
**
**
**
1
2
3
4
5
6
7
8
9
10
11
Time (Weeks)
b.
Tumor Weight (g)
0.8
0.6
0.4
0.2
0.0
**
CO
DDT
c.
% of animals with mammary tumors
100
50
0
CO
DDT
10
15
20
Weeks after last DMBA exposure
d.
Tumor latency (weeks)
20
15
10
5
0
CO
DDT

Figure 3

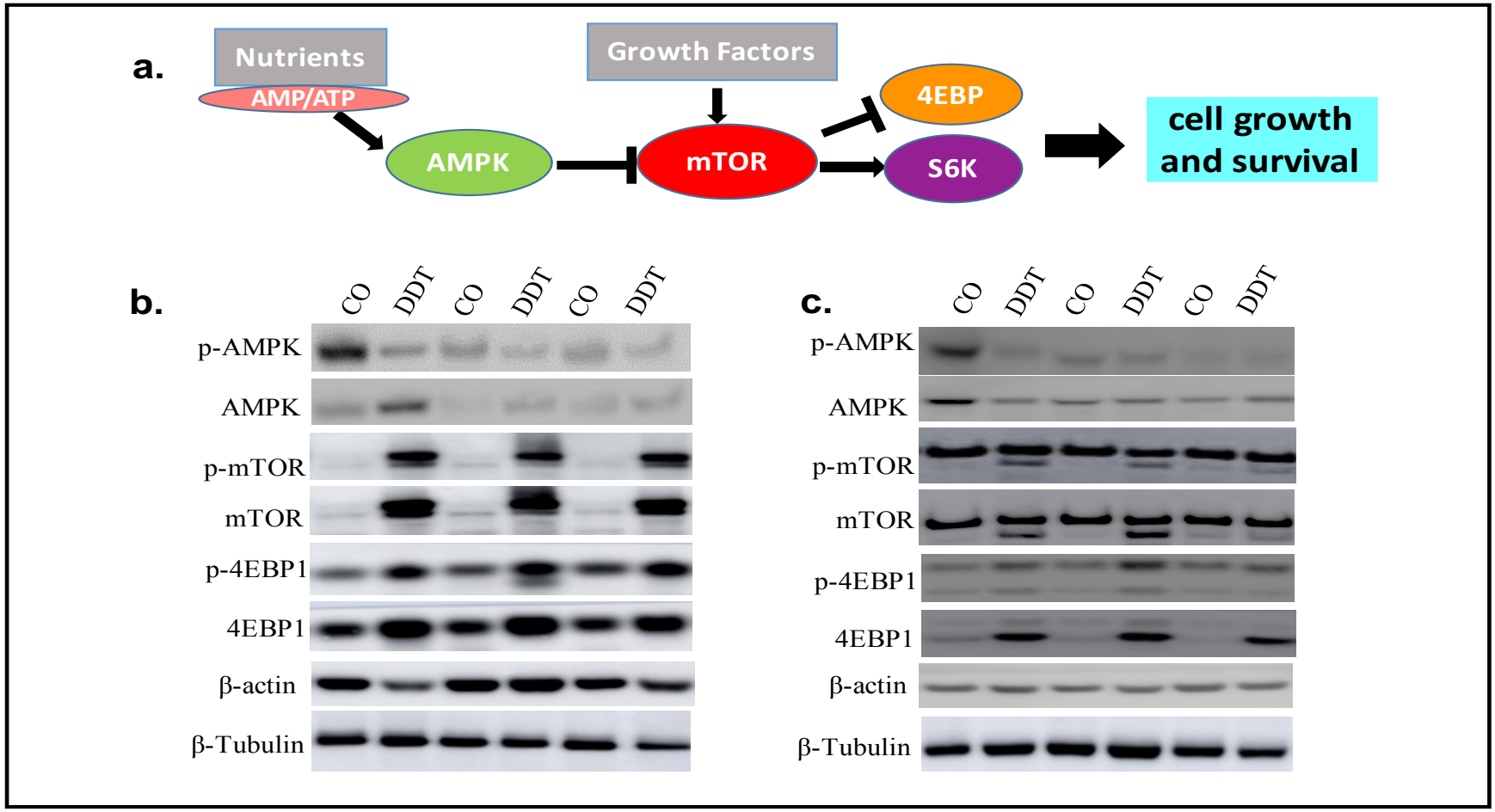
a.
Nutrients
AMP/ATP
Growth Factors
AMPK
mTOR
4EBP
S6K
cell growth and survival
b.
CO
DDT
CO
DDT
CO
DDT
p-AMPK
AMPK
p-mTOR
mTOR
p-4EBP1
4EBP1
β-actin
β-Tubulin
c.
CO
DDT
CO
DDT
CO
DDT
p-AMPK
AMPK
p-mTOR
mTOR
p-4EBP1
4EBP1
β-actin
β-Tubulin

Figure 4

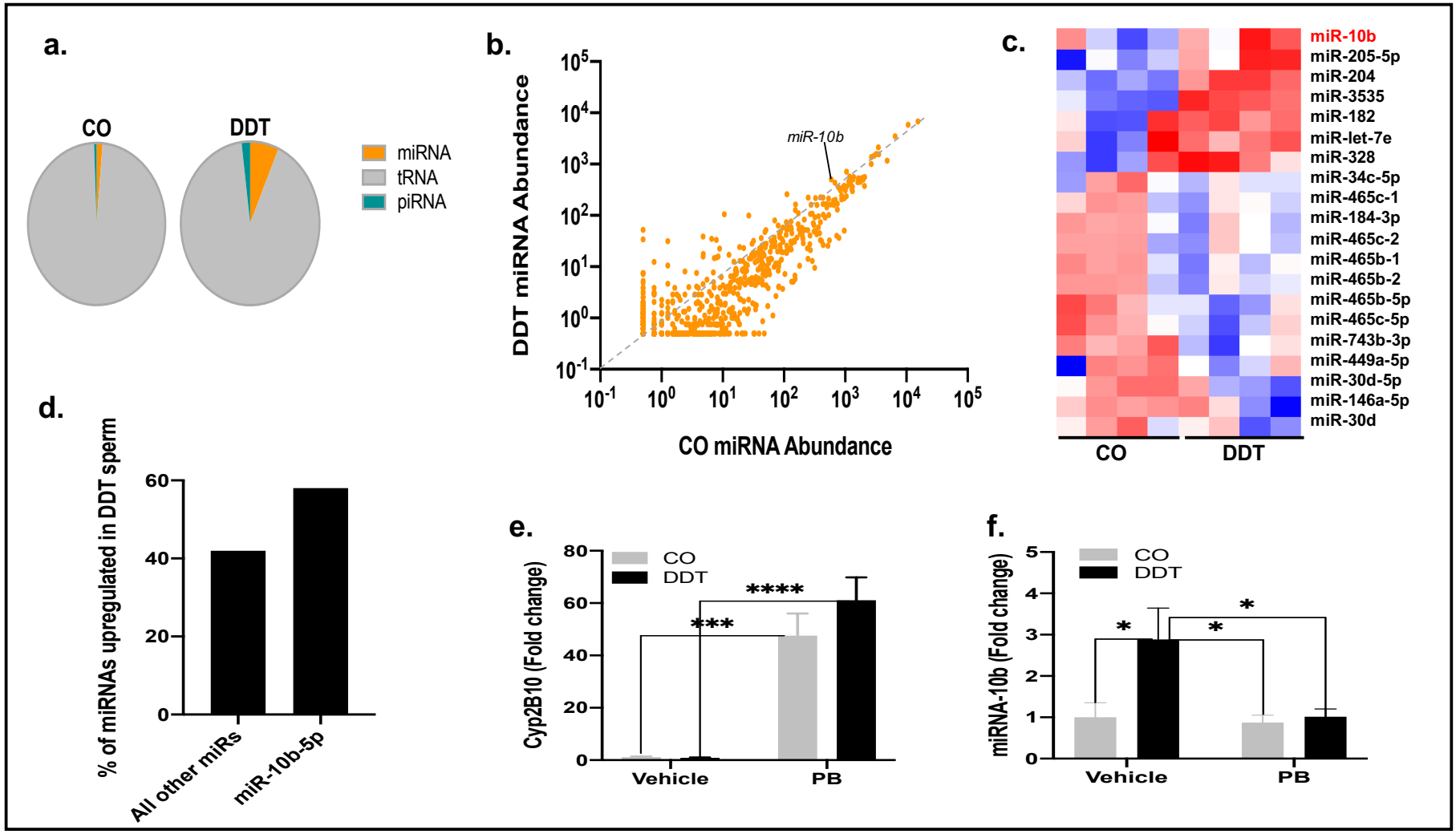
a.
CO
DDT
miRNA
tRNA
piRNA
b.
DDT miRNA Abundance
CO miRNA Abundance
miR-10b
c.
miR-10b
miR-205-5p
miR-204
miR-3535
miR-182
miR-let-7e
miR-328
miR-34c-5p
miR-465c-1
miR-184-3p
miR-465c-2
miR-465b-1
miR-465b-2
miR-465b-5p
miR-465c-5p
miR-743b-3p
miR-449a-5p
miR-30d-5p
miR-146a-5p
miR-30d
CO
DDT
d.
% of miRNAs upregulated in DDT sperm
All other miRs
miR-10b-5p
e.
Cyp2B10 (Fold change)
CO
DDT
Vehicle
PB
f.
miRNA-10b (Fold change)
CO
DDT
Vehicle
PB

Figure 5

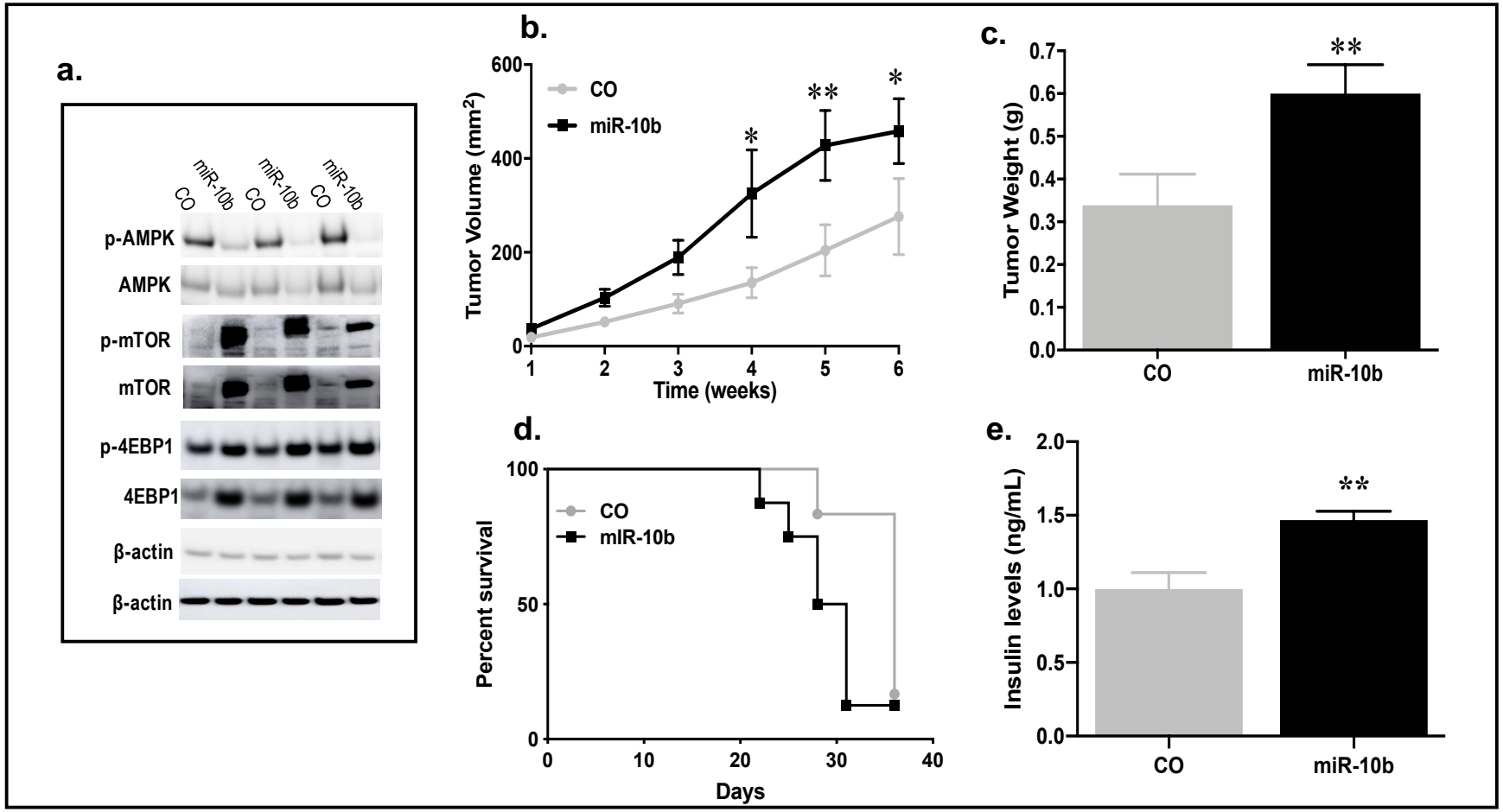
a.
CO
miR-10b
CO
miR-10b
CO
miR-10b
p-AMPK
AMPK
p-mTOR
mTOR
p-4EBP1
4EBP1
β-actin
β-actin
b.
CO
miR-10b
Tumor Volume (mm²)
Time (weeks)
c.
Tumor Weight (g)
CO
miR-10b
d.
CO
mIR-10b
Percent survival
Days
e.
Insulin levels (ng/mL)
CO
miR-10b

Figure 6

SUPPLEMENTARY INFORMATION

Supplementary Figures

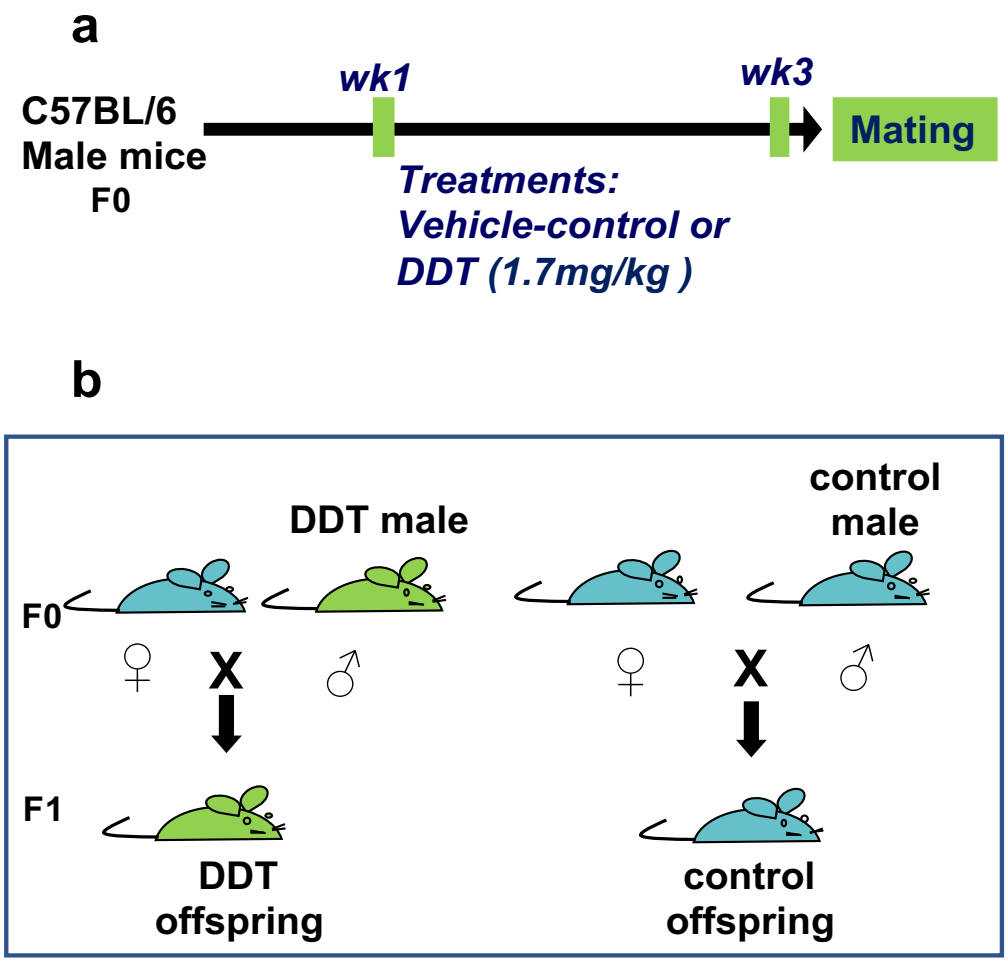

Figure S1

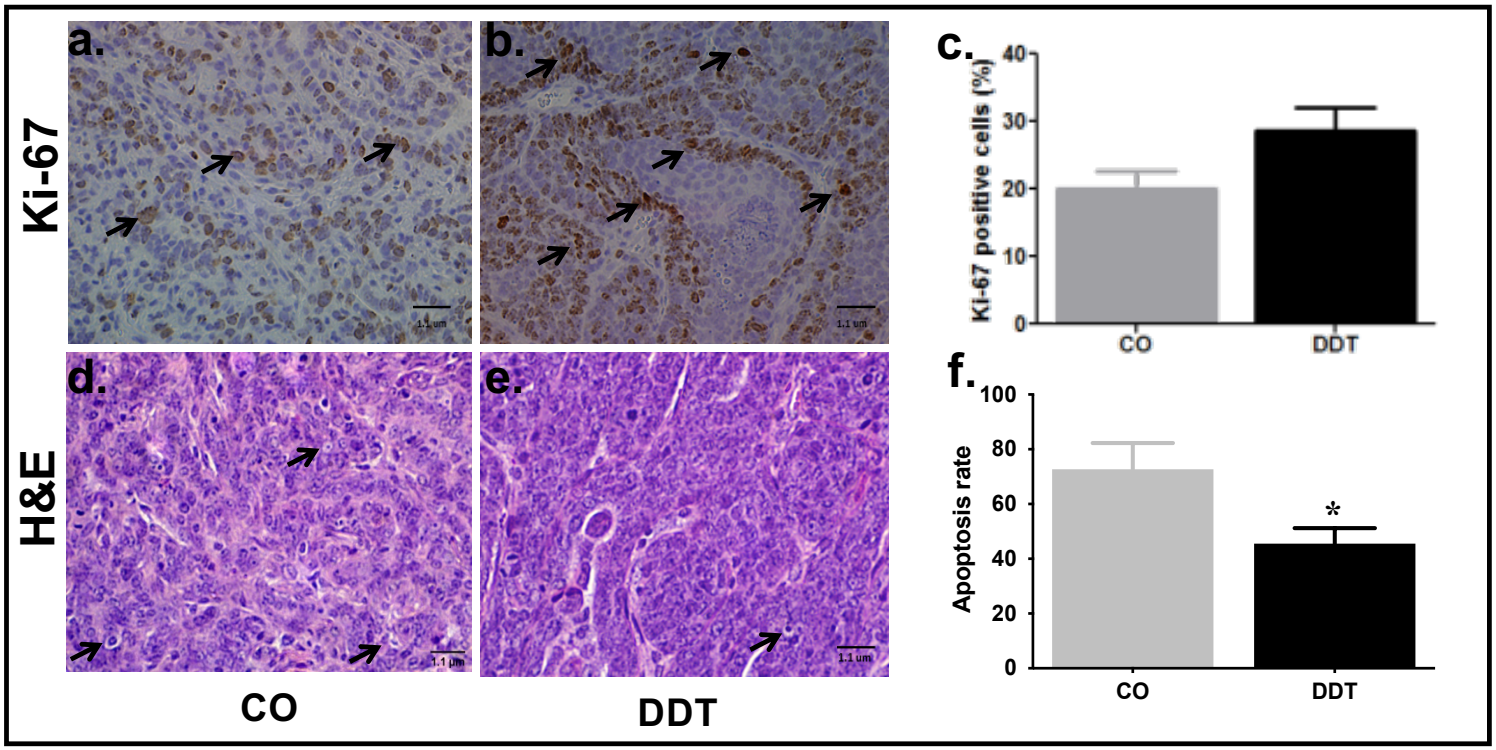
a.
b.
c.
d.
e.
f.
Ki-67
H&E
CO
DDT
Ki-67 positive cells (%)
0
10
20
30
40
CO
DDT
Apoptosis rate
0
20
40
60
80
100
*
CO
DDT

Figure S2

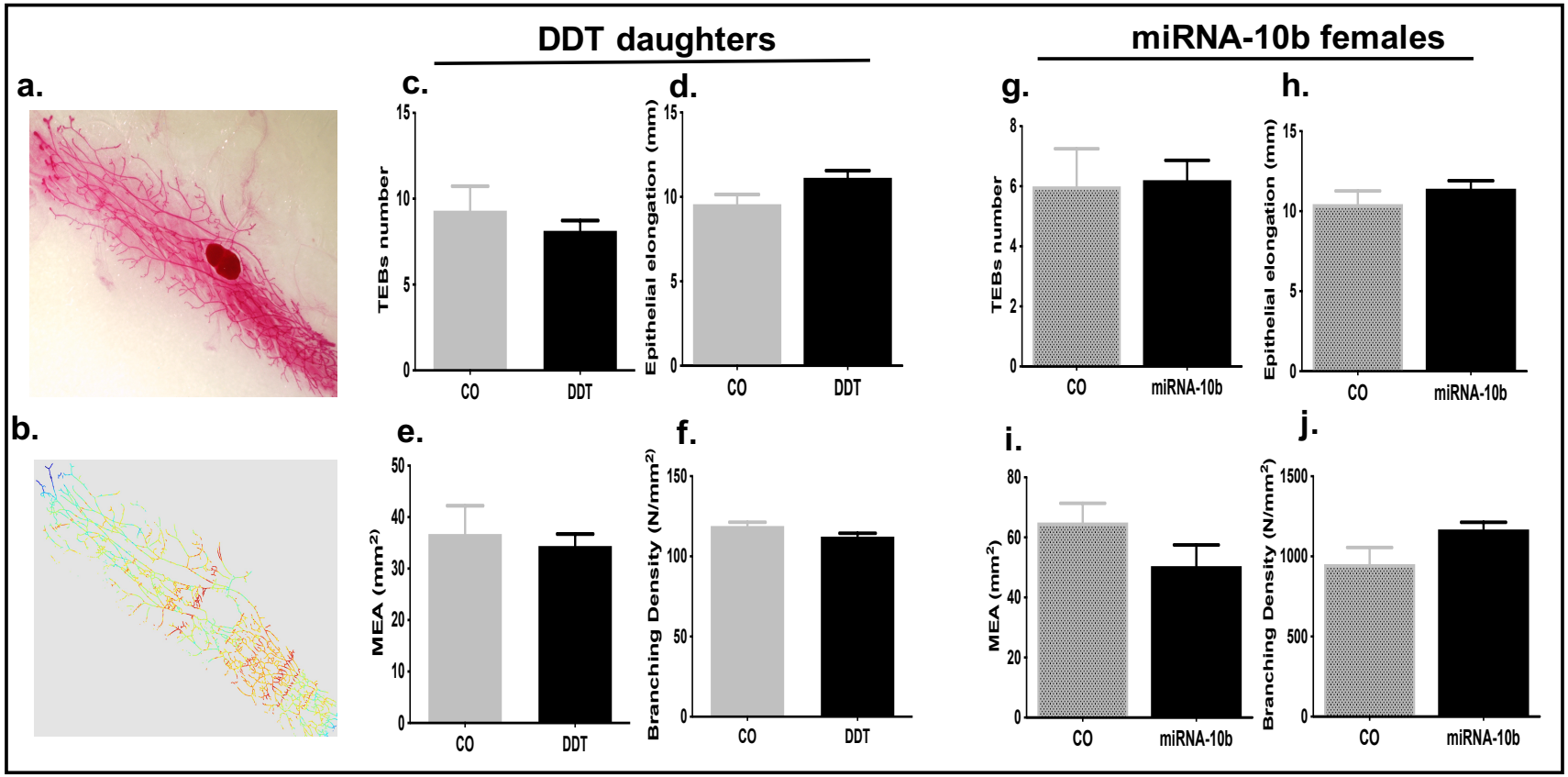
DDT daughters
miRNA-10b females
a.
b.
c.
d.
e.
f.
g.
h.
i.
j.
TEBs number
Epithelial elongation (mm)
MEA (mm²)
Branching Density (N/mm²)
CO
DDT
miRNA-10b

Figure S3

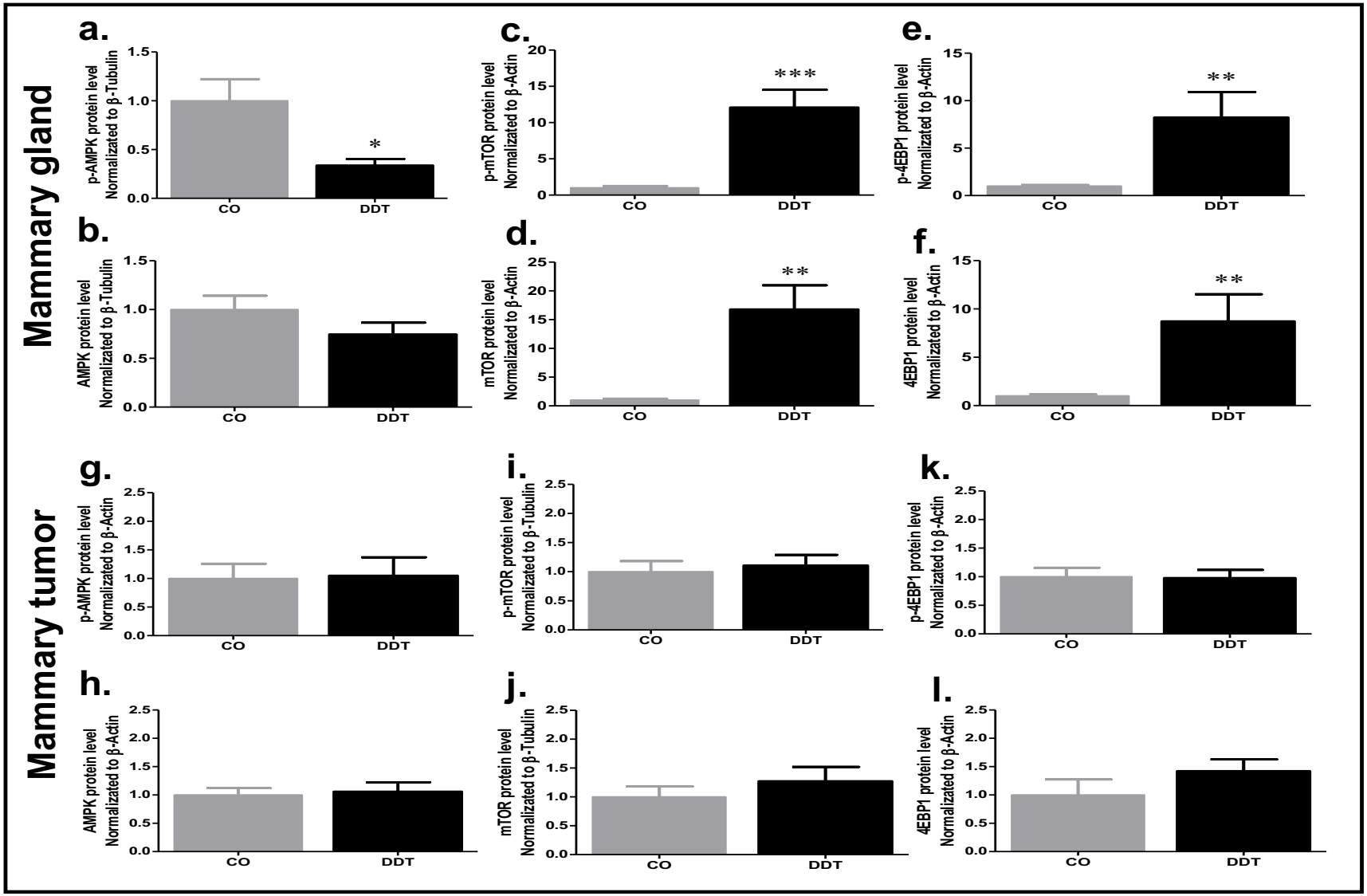
Mammary gland
Mammary tumor
a.
p-AMPK protein level
Normalizated to β-Tubulin
1.5
1.0
0.5
0.0
*
CO
DDT
b.
AMPK protein level
Normalizated to β-Tubulin
1.5
1.0
0.5
0.0
CO
DDT
c.
p-mTOR protein level
Normalizated to β-Actin
20
15
10
5
0
***
CO
DDT
d.
mTOR protein level
Normalizated to β-Actin
25
20
15
10
5
0
**
CO
DDT
e.
p-4EBP1 protein level
Normalizated to β-Actin
15
10
5
0
**
CO
DDT
f.
4EBP1 protein level
Normalizated to β-Actin
15
10
5
0
**
CO
DDT
g.
p-AMPK protein level
Normalizated to β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
CO
DDT
h.
AMPK protein level
Normalizated to β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
CO
DDT
i.
p-mTOR protein level
Normalizated to β-Tubulin
2.5
2.0
1.5
1.0
0.5
0.0
CO
DDT
j.
mTOR protein level
Normalizated to β-Tubulin
2.5
2.0
1.5
1.0
0.5
0.0
CO
DDT
k.
p-4EBP1 protein level
Normalizated to β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
CO
DDT
l.
4EBP1 protein level
Normalizated to β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
CO
DDT

Figure S4

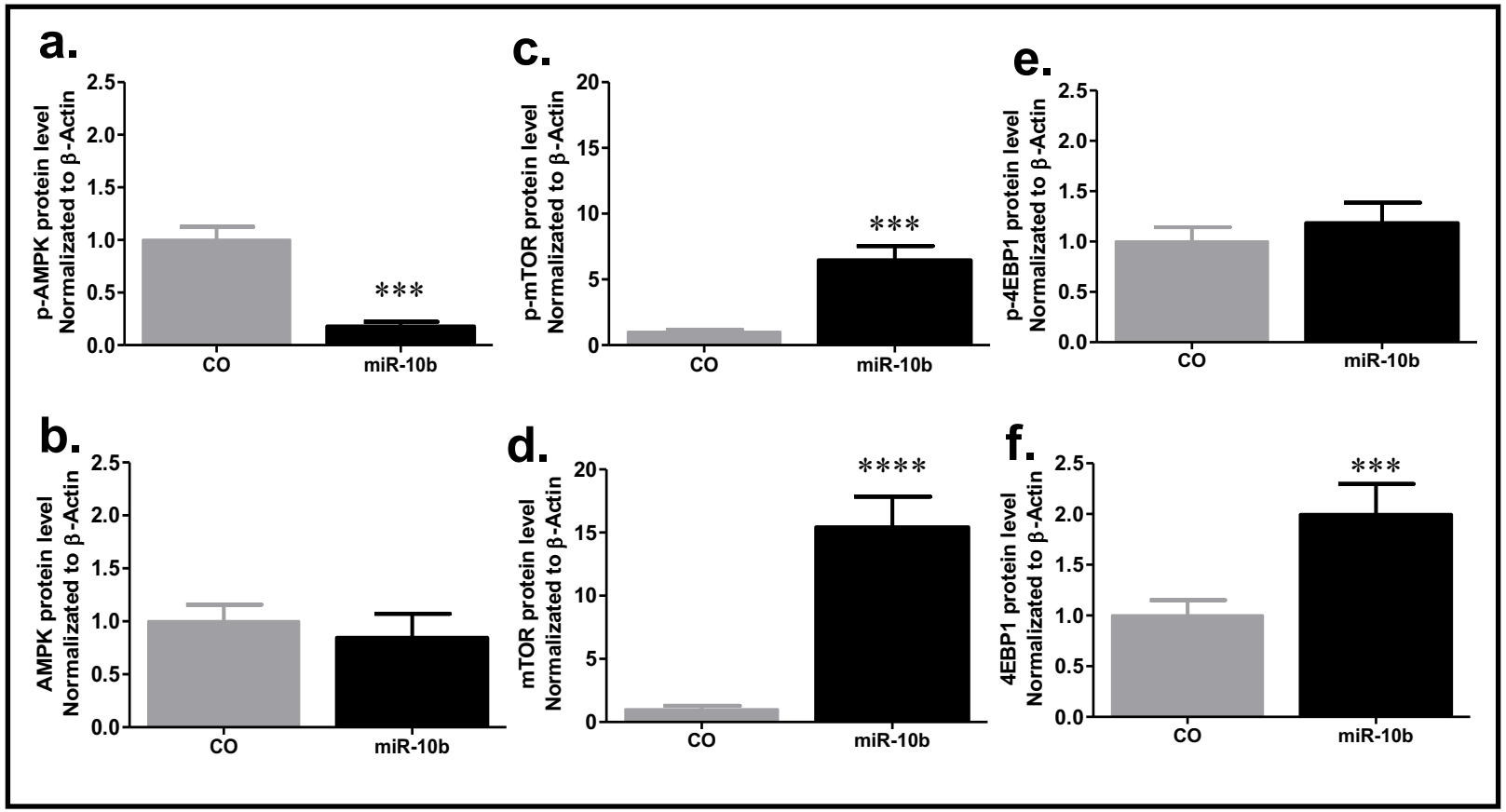
a.
p-AMPK protein level
Normalizated to β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
***
CO
miR-10b
c.
p-mTOR protein level
Normalizated to β-Actin
20
15
10
5
0
***
CO
miR-10b
e.
p-4EBP1 protein level
Normalizated to β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
CO
miR-10b
b.
AMPK protein level
Normalizated to β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
CO
miR-10b
d.
mTOR protein level
Normalizated to β-Actin
20
15
10
5
0
****
CO
miR-10b
f.
4EBP1 protein level
Normalizated to β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
***
CO
miR-10b

Figure S5

Supplementary Figure Legends

Fig-S1. Experimental design: C57BL/6 male mice were exposed to DDT (1.7mg/kg body weight) by oral gavage for two weeks. DDT or control-vehicle (CO)-exposed males were mated with unexposed female mice to generate the offspring. To avoid litter-effect, pups were cross-fostered one day after birth. Pups from 2-3 dams were pooled and housed in a litter of 8-10 pups per nursing dam. Pups were weaned from mothers at 21 days of age.

Fig-S2. Cell proliferation is increased and apoptosis rates decreased in mammary tumors of DDT female offspring. Representative images and quantification (percentage of the positively stained nuclear area) of proliferation staining (ki67; a-c, arrows, n=6/group) and apoptosis (d-f, morphological assessment, H&E sections, arrows, n=14-17/group) in control (CO) and DDT tumors. All images captured at 40x. Values are mean ± SEM. *P<0.05 by t-test.

Fig-S3. No major changes in mammary gland morphology on PND50 in DDT or miRNA-10b female offspring. (a,b) Representative photomicrography of mammary gland whole mount (6.25x) and digitized image of the whole mount. (c,g) Number of TEBs; (d,h) Epithelial elongation (distance between lymph node and the end of the mammary epithelial tree); (e,i) Mammary epithelial area measurement (MEA); (f,j) Branching density values; (n=5-16/group) in control (CO), DDT or miRNA-10b females. All data are mean ± SEM. PND, post-natal day.

Fig-S4. Paternal DDT exposure programs the AMPK/mTOR signaling pathway in DDT offspring's mammary tissues. (a-l) Quantification of protein expression levels of members of the AMPK/mTOR axis in (a-f) normal mammary tissues and in (g-l) tumors (n=6-7/group) of control (CO) and DDT offspring. Protein expression levels were normalized by either β-actin or β-tubulin. Optical density of individual bands was quantified using Quantity-one software. Values are mean ± SEM. *p<0.05, **p<0.01, ***p<0.01 by t-test.

Fig-S5. Embryonic injection of miR-10b recapitulate the DDT-induced programming of the AMPK/mTOR signaling pathway in mammary tissues. (a-f) Quantification of protein expression levels of members of the AMPK/mTOR axis in normal mammary tissues (n=5/group). Protein expression levels were normalized by β-actin. Optical density of individual bands was quantified using Quantity-one software. Values are mean ± SEM. ***p<0.01, ****p<0.01 by t-test.

Supplementary Tables

Table S1: Number of offspring and number of contributing fathers per experiment.

Samples	Assay	Group	Father	Female
Mammary gland	Terminal End Buds	**CO**	2	12
		DDT	3	16
	Western Blot	**CO**	2	6
		DDT	2	6-7
Mammary tumor	Apoptosis	**CO**	6	14
		DDT	9	17
	Ki67	**CO**	5	6
		DDT	5	6
	Tumorigenesis	**CO**	5	25
		DDT	8	25
	Western Blot	**CO**	3	6-7
		DDT	4	6
Plasma	ELISA	**CO**	1	6
		DDT	3	7

Table S2 - List of Antibodies

Antibody	Application	Dilution	Description	Supplier
AMPKα	Western Blot	1:1000	Rabbit polyclonal	Cell Signaling #2532
Anti-Ki67	IHC	1:7	Rabbit monoclonal	BIOCARE #CRM325
mTOR	Western Blot	1:1000	Rabbit monoclonal	Cell Signaling#2983
Phospho-AMPKα	Western Blot	1:1000	Mouse monoclonal	Cell Signaling #2535
Phospho-mTOR	Western Blot	1:1000	Rabbit monoclonal	Cell Signaling#2971
Phospho-4E-BP1	Western Blot	1:1000	Rabbit monoclonal	Cell Signaling#2855
4E-BP1	Western Blot	1:1000	Rabbit monoclonal	Cell Signaling#9452
β-Actin	Western Blot	1:1000	Rabbit monoclonal	Cell Signaling #8457
β-Tubulin	Western Blot	1:1000	Mouse monoclonal	Thermo Fisher Scientific #MA5-16308
Anti-mouse IgG, HRP	Western Blot	1:1000	Goat	Cell Signaling # 7076S
Anti-rabbit IgG, HRP	Western Blot	1:1000	Horse	Cell Signaling # 7074S

Table S3. Gender distribution (proportion) and number of pups per litter in control and DDT groups

Group	Female	Male	Number of pups per liter (mean±SEM)
CO	0.53	0.47	8.7±0.37
DDT	0.55	0.45	8.2±0.29

Table S4 – Levels of DDT metabolites in liver of mice treated with vehicle-control, DDT or DDT+ phenobarbital (**PB**)

DDT metabolite	**Group** (concentration, ng/g)		
	CO	**DDT**	**DDT- PB**
o,p' DDE	ND	ND	ND
p,p DDE	ND	0.6	ND
o,p DDD	ND	ND	ND
p,p DDD	ND	0.3	ND

Levels of DDT metabolites in liver (n=3 pooled samples/group) of control (CO), DDT (1.7/mg/kg) and DDT+phenobarbital (1.7mg+20mg/kg; DDT-PB) treated male mice measured by GC-MS/MS 24h after the last treatment. ND, non-detectable (readings below assay detection limits and not distinguishable from the blank control).