Paternal exposure to the pesticide DDT induces intergenerational programming of breast cancer predisposition via sperm miRNA

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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 and other pesticides 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 an increase in miRNAs and a specific surge 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 DNA sequence is responsible for the majority of disease heritability, including cancer. It is
73 becoming clear, however, that epigenetic inheritance can also occur. At conception, parents
74 not only contribute their genome but also transmit a molecular memory of past environmental
75 experiences to their progeny [1, 2]. This can result in offspring's predisposition to chronic
76 diseases later in life [3]. This environmentally-induced risk of diseases, including breast cancer,
77 has been shown to be transmitted to the offspring via epigenetic mechanisms through both the
78 female and male germ-lines [3-5]. Although most of the evidence for this mode of disease
79 inheritance comes from maternal exposures in pregnancy, we [6-9] and others [10-12] have
80 shown that paternal exposures in the pre-conception window are also important in determining
81 disease outcomes in the offspring.

82 Several recent published reports demonstrated that the small RNA load in paternal sperm can
83 transmit environmentally-induced phenotypes to the offspring [2, 5, 13, 14]. Some of those
84 studies implicate specific classes of small RNA and both miRNAs and tRFs obtained from sperm
85 can recapitulate the effect of specific paternal exposures in offspring when injected into normal
86 embryos following in vitro fertilization [5, 13-15].

87 Dichlorodiphenyltrichloroethane (DDT), one of the first synthetic organochlorine pesticides, was
88 widely used in the United States (U.S.) from the 1940s to the 1970s and up to the 1980s in
89 Europe [16, 17]. While the use of DDT has been restricted in the U.S. and Europe for decades,
90 developing countries with endemic malaria and other vector-borne diseases continue to use it
91 [17-19]. This pesticide has a prolonged half-life and is lipophilic, bio-accumulating in the food
92 chain, and is still detected in fatty tissues and circulation of animals and humans [17]. In the
93 U.S., the highest concentrations of DDT are detected in minority populations and recent

immigrants [20, 21]. Like other organochlorines, DDT is an endocrine disruptor [17, 22] and it has been linked to metabolic syndrome [23], obesity [24] and cancer [18, 25].

Because the mammary gland bud first emerges during fetal development, it has been proposed that some breast cancers may originate *in utero* [26] and multiple reports on maternal exposure in pregnancy have offered support to this hypothesis [4, 18, 27, 28]. In line with that, a recent epidemiological analysis using the Child Health and Development Studies cohort (CHDS) showed that daughters of women exposed to high levels of DDT in pregnancy have increased rates of breast cancer [18] and are more likely to be diagnosed with advanced stage and HER-2 positive tumors. Other studies in the same cohort found that women exposed to this pesticide before age 14 have increased breast cancer risk, with the highest risk if the exposure occurred in early infancy [25, 29].

While there is a clear link between maternal exposure to DDT and breast cancer in the next generation, no studies to date have investigated the contribution of pre-conception paternal exposure to this or other pesticides to offspring's breast cancer development. Here, we tested the hypothesis that paternal exposure to DDT can alter the sperm non-coding RNAs and program breast cancer development in daughters, using a mouse model of this disease. We also examined whether paternal DDT-induced programming of breast cancer development in daughters is mechanistically linked to sperm miRNAs.

Materials and Methods

DDT exposures and breeding: Animals were purchased from Taconic Biosciences. The *c57bl/6* strain of mice was used in all experiments. Adult male mice (8 weeks of age) were either treated with a DDT solution (1.7 mg/kg) or vehicle-control solution (vegetable oil) via oral gavage for

117 two weeks. The DDT solution used mimics the formulation of DDT before its ban in the U.S.:
118 77.2% p,p'-DDT and 22.8% o,p'-DDT as described before [23]. Animals body weight was
119 monitored weekly. At the end of the exposure period, DDT-exposed and control male mice were
120 mated to unexposed females to generate the female offspring (**Fig. S1**). Female mice were kept
121 on a standard chow diet during the breeding period, for the extent of pregnancy (21 days) and
122 after giving birth. The birth weight of pups and number of pups per litter was determined. To
123 avoid litter-effect, pups were cross-fostered one day after birth. Pups from 2-3 dams were pooled
124 and housed in a litter of 8-10 pups per nursing dam. All pups were weaned on postnatal day
125 (PND) 21. Offspring's body weight was recorded weekly. The female offspring of control or
126 DDT-exposed fathers were used to study body weight, metabolic function, and mammary
127 tumorigenesis and for mammary gland collection as described in the following sections. The
128 number of contributing fathers and female offspring used in each experiment is listed in **Table**
129 **S1**. All animal procedures were approved by the Georgetown University Animal Care and Use
130 Committee, and the experiments were performed following the National Institutes of Health
131 guidelines for the proper and humane use of animals in biomedical research.

132 ***DDT and Phenobarbital (PB) treatment:*** PB treatment is known to promote hepatic enzymes
133 activation, accelerating DDT metabolism and excretion in humans [30, 31] and animals [32-34].
134 A sub-set of control-vehicle or DDT exposed males were concomitantly treated with
135 phenobarbital (20 mg/kg of body weight, i.p.) or saline and used for sperm harvesting.

136 ***Mature spermatozoa collection and purification:*** Control and DDT-exposed males as well as
137 those treated with PB were euthanized and caudal epididymis dissected for sperm collection. The
138 epididymis was collected, punctured, and transferred to tissue culture dish containing M2 media
139 (M2 Medium-with HEPES, without penicillin and streptomycin, liquid, sterile-filtered, suitable

140 for mouse embryo, SIGMA, product #M7167) where it was incubated for 1 hour at 37°C. Sperm
141 samples were isolated and purified from somatic cells. Briefly, the samples were washed with
142 PBS, and then incubated with SCLB (somatic cell lysis buffer, 0.1% SDS, 0.5% TX-100 in
143 Diethylpyrocarbonate water) for 1 hour. SCLB was rinsed off with 2 washes of PBS and the
144 somatic cell-free purified spermatozoa sample pelleted and used for RNA extraction.

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

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

156 ***Small RNA-Seq Analysis:*** Total RNA was isolated from paternal sperm (n=4/group) using
157 Qiagen's miRNeasy extraction kit, according to the manufacturer's instructions. Small non-
158 coding RNA transcript libraries were constructed according to Illumina TrueSeq Small RNA
159 Pre-Kit. Indexed, paired-end sequencing libraries were prepared from quality total RNA (RIN ≥
160 8). For each library, 10 million reads (raw data) were generated by Illumina Hi-Seq 4000. The
161 raw reads were subject to adapter trimming and low quality filtering using Trimmomatic
162 program. The high quality clean reads were aligned on the mouse genome. Non-coding RNA

163 tags were mapped to the mouse genome (GRCm38/mm10 reference genome) in order to analyze
164 their expression and distribution on the genome. Small RNA tags were annotated with miRNA,
165 t-RNA and pi-RNA to miRBase, Ref-seq, GenBank and Rfam database using blastn with
166 standard parameters. To analyze the differential expression of miRNA between control and DDT
167 groups, miRNAs were normalized to TPM (Transcripts Per Kilobase Million). miRNAs with a P
168 value less than 0.05 were considered significant, with an appropriate correction for multiple
169 testing [35].

170 ***Quantitative real time PCR validation (qRT-PCR):*** cDNA was synthesized from total RNA
171 samples using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems).
172 PCR products were amplified from cDNA using TaqMan® Fast Advanced Master Mix and
173 sequence-specific primers from TaqMan Assays (Applied Biosystems). Fold change was
174 calculated from Ct values and the expression levels of specific miRNAs or genes were
175 determined by normalizing these values with the fold change values for appropriate endogenous
176 controls (miR-26a or *GAPDH*). Fold differences between the groups were analyzed using t-test
177 or two-way ANOVA (group, treatment), followed by post-hoc analyses.

178 ***Carcinogen-induced mammary tumorigenesis:*** Mammary tumors were induced in control and
179 DDT female offspring (n=25/group) by administration of MPA (medroxyprogesterone acetate,
180 15 mg, s.c.) at 6 weeks of age, followed by three weekly doses of 1mg of 7,12-
181 dimethylbenz[a]anthracene (DMBA) (Sigma, St. Louis, MO) dissolved in peanut oil by oral
182 gavage. This established model of breast cancer has been used by us and others [6, 36] . Mice
183 were examined for mammary tumors by palpation once per week, starting on week two after the
184 last dose of DMBA and continue for a total of 20 weeks. Tumor growth was measured using a
185 caliper and the width and height of each tumor were recorded. Other end-points for data analysis

186 were latency to tumor appearance, the number of animals with tumors (tumor incidence), and the
187 number of tumors per animal (tumor multiplicity). During follow-up, those animals in which
188 tumor burden approximated 10% of total body weight were euthanized, as required by our
189 institution. Histopathology of tumors was determined by our pathologist. All tumors included in
190 our analyses were classified as mammary carcinomas. Differences in tumor latency and
191 multiplicity were analyzed by t-test (or corresponding non-parametric test). Kaplan-Meier
192 survival curves were used to compare differences in tumor incidence, followed by the log-rank
193 test. Tumor growth was analyzed using two-way ANOVA (group, time), followed by post-hoc
194 analyses.

195 ***Embryonic miRNA microinjections.*** To induce super-ovulation, female mice (*c57bl/6*) received
196 intra-peritoneal injections of 5 IU of pregnant mare's serum gonadotropin (PMSG). About 48
197 hours after the PMSG injection, mice were injected with 5 IU of human chorionic gonadotropin
198 (hCG). Super-ovulated females were mated with *c57bl/6* male mice. About 16 hours later,
199 fertilized eggs were harvested. Zygotes were injected with either synthetic miRNA-10b
200 (*mir*Vana®, ThermoFisher) diluted in TE solution (10mM Tris-HCl, pH7.4, 0.25 EDTA) or TE
201 solution only (to control for microjection). Each zygote received ~10 pL of TE or miRNA
202 preparation at 2ng/µL. Microinjections were confirmed by observing the swelling of the male
203 pro-nucleus. Injected zygotes were transferred into recipient female mice to produce offspring as
204 described before [15]. Briefly, recipient females were mated with vasectomized males overnight
205 and identified by copulation plug before the zygote transfer. Each recipient female was
206 implanted with 25-30 zygotes. Dams were housed in groups of two with free access to food and
207 water. Female offspring were used to study tumor growth, metabolic function and for mammary
208 tissue collection. Mature sperm cells contain approximately 20 femtograms of total RNA, of

209 which about 20% are small non-coding RNAs and 0.4% are miRNAs [37]. Thus, the amount of
210 miRNA injected into zygotes did not exceed physiological levels. Embryonic injections were
211 performed at the Johns Hopkins Transgenic Core Facility. Resulting offspring were subsequently
212 transferred to our institution for follow-up studies.

213 ***Syngeneic orthotopic tumor implantation:*** Mouse EO771 breast cancer cells were cultured in
214 complete medium [Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10%
215 (v/v) FBS and 1% L-glutamine, at 37°C in 5% CO2 (v/v)] to 80% confluence, trypsinized,
216 counted, checked for viability and re-suspended in PBS for injection into both 4th inguinal
217 mammary glands. A total of $1x10^6$cells were injected into each of the 4th inguinal mammary
218 gland fat pads of miRNA-10b females (n=6-8/group). Tumor growth was monitored for at least
219 six weeks.

220 ***Immunohistochemistry analysis.*** Cell proliferation (Ki-67) was evaluated by
221 immunohistochemistry in mammary tumors (n=5-7/group). Briefly, tissues were fixed in 10%
222 buffered formalin, embedded in paraffin, and sectioned (5 μm). Sections were deparaffinized
223 with xylene and rehydrated through a graded alcohol series. Antigen retrieval was performed by
224 immersing the tissue sections at 98°C for 40 minutes in 1X Diva Decloaker (Biocare). Tissue
225 sections were treated with 3% hydrogen peroxide and 10% normal goat serum for 10 minutes,
226 and were incubated with the primary antibody (**Table S2**), overnight at 4°C. After several
227 washes, sections were treated to the appropriate HRP labeled polymer for 30 min and DAB
228 chromagen (Dako) for 5 minutes. Slides were counterstained with Hematoxylin (Fisher, Harris
229 Modified Hematoxylin), blued in 1% ammonium hydroxide, dehydrated, and mounted with
230 Acrymount. The sections were photographed using an Olympus IX-71 Inverted Epifluorescence
231 microscope at 40x magnification. Proliferation index (Ki-67 staining) was determined by

232 immunoRatio, a plugin Image J software (National Institute of Health, Bethesda, MD, USA) to
233 quantify hematoxylin and DAB-stained cells. Results were analyzed by t-test (or corresponding
234 non-parametric test).

235 ***Analysis of cell apoptosis:*** Cell apoptosis analysis was conducted in mammary tumors (n=14-
236 17/group) by morphological detection. Tissues were fixed in neutral buffered 10% formalin and
237 stained with hematoxylin and eosin (H&E). Cells presenting loss of adhesion between adjacent
238 cells, cytoplasmic condensation and formation of apoptotic bodies were considered apoptotic as
239 described before [38]. The sections were photographed using a Olympus IX-71 Epifluorescence
240 microscope at 40x magnification. Twenty areas were photographed randomly and the number of
241 apoptotic bodies counted. The apoptotic index was determined as the percentage of apoptotic
242 bodies per total number of cells. Images were evaluated with Image J software (NIH, USA).
243 Results were analyzed by t-test (or corresponding non-parametric test).

244 ***Mammary gland development:*** Inguinal mammary glands (4th pair, n=5-16/group) collected on
245 post-natal day 50 (PND 50) were stretched onto a slide, placed in a fixative solution and stained
246 with a carmine aluminum solution (Sigma Chemical Co.) as previously described [39]. Whole
247 mounts were examined under the microscope (AmScope) and ductal elongation and number of
248 TEBs (undifferentiated structure considered to be the targets of malignant transformation), as
249 previously described [39]. In addition, whole-mount slides were digitized (Olympus SZX12
250 Stereomicroscope) and analyzed using the ImageJ software (National Institute of Health,
251 Bethesda, MD, USA): The area and integrated density of a manually drawn perimeter around the
252 mammary epithelial tree of each whole-mount were measured. The average area as well
253 integrated density measurements are reported for each group. Differences were statistically tested
254 using t test (or corresponding non-parametric test).

255 ***Analysis of protein expression:*** Protein levels were assessed by western blot on PND50
256 mammary tissues (n=6-7 group) and tumors (n=6/group). Total protein was extracted
257 from mammary tissues using RIPA buffer with Halt™ Protease Inhibitor Cocktail (Thermo
258 Fisher). Ten micrograms of protein extracts were resolved on a 4-12% denaturing
259 polyacrylamide gel (SDS-PAGE). Proteins were transferred using the iBlot® 7-Minute Blotting
260 System (Invitrogen, USA) and blocked with 5% non-fat dry milk for 1h at room
261 temperature. Membranes were incubated with the specific primary antibodies (for antibody
262 specifications and dilutions see **Table S2**) at 4°C overnight. After several washes, the
263 membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody
264 at room temperature for 1h. Membranes were developed using the Chemiluminescent HRP
265 antibody detection reagent (Denville Scientific Inc., USA), and exposed to blot imaging systems
266 (Amersham™ Imager 600, GE Healthcare Life Sciences). Optical density of the bands was
267 quantified using Quantity-one software (BIO-RAD, USA). To control for equal protein loading,
268 expression of the proteins of interest was normalized to the β-actin or β-tubulin signal.
269 ***Metabolic Parameters:*** Glucose tolerance test (GTT) was performed after overnight fasting in
270 mice. Glucose (0.75 U/kg body weight) was administered intraperitoneally and tail blood
271 samples were collected at 0, 15, 30 and 60 minutes after injection (n=8/group). Circulating
272 insulin levels (n=6-7/group) were measured using an ELISA kit (Ultra Mouse insulin EIA Kit,
273 Crystal Chem). Differences in GTT were analyzed using two-way ANOVA (group, time),
274 followed by post-hoc analyses. Insulin levels were statistically tested using t test (or
275 corresponding non-parametric test).

276

277 **Results**

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

279 Male mice were either exposed to a vehicle-control or an environmentally relevant dose of DDT
280 (as described in the Material and Methods section) and mated with unexposed females to
281 generate the offspring (**Fig. S1**). The phenotypes in offspring from control and DDT-exposed
282 male mice were then examined. No differences in gender distribution or litter size were
283 observed between offspring born from DDT and control males (**Table S3**). However, paternal
284 DDT exposure was associated with a significant decrease in offspring's birtht and weaning
285 weights ($P<0.05$, $P<0.01$ **Fig. 1a-b**). After weaning, DDT daughters underwent a catch-up
286 growth period and by 6 weeks of age no differences in body weight were observed between the
287 groups. In fact, DDT females became significantly heavier than controls by 28-weeks of age
288 ($P<0.05$, $P<0.01$, **Fig. 1c**). They also showed metabolic dysfunction with higher circulating
289 levels of insulin and impaired glucose tolerance compared to controls in adulthood (**Fig**. **2a-b**,
290 $P<0.05$).

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

292 Maternal DDT exposure has been linked to a near 4-fold increase in breast cancer in daughters
293 [18]. Low birthweight, a phenotype observed in offspring DDT-exposed fathers, has also been
294 linked with higher risk for breast cancer in humans [40, 41] and animal models [8, 42]. Thus, we
295 studied mammary cancer development in control and DDT daughters using the well-established
296 [6, 36] DMBA-induced mouse model of breast cancer. Tumor growth was significantly increased
297 in DDT daughters compared to controls ($P<0.001$, **Fig. 3a**), with tumors in DDT offspring
298 weighing significantly more at the end of the monitoring period ($P<0.01$, **Fig. 3b**). The incidence
299 of mammary tumors in the DDT offspring was also higher and tumor latency slightly shorter
300 than in controls but results did not reach statistical significance (**Fig. 3c-d**).

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

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

306 Because DDT daughters showed accelerated breast cancer growth, we next analyzed the effects
307 paternal DDT on offspring's mammary morphological development in post-natal day (PND)50
308 inguinal mammary glands. We assessed number of terminal end buds (TEB), ductal elongation
309 and epithelial area/density. No major morphological changes were detected, except for a slight
310 increase in mammary ductal elongation in DDT daughters compared to controls (**Fig. S3**).

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

312 We previously showed that low birth weight is linked to suppression of the nutrient/energy
313 sensing AMPK pathway (**Fig. 4b**) in mammary tissues and tumors [8]. Indeed, we found that
314 both AMPK expression and phosphorylation levels were decreased in DDT daughters' mammary
315 tissues. Consistent with that, we also detected an increase in expression and activity of mTOR
316 and its down-stream target, 4E-BP1 (**Fig. 4b, Fig. S4a-f**). Similar patterns of mTOR and 4E-
317 BP1 expression and activity were also found in tumors of DDT daughters, although results were
318 not statistically significant (**Fig. 4c, Fig. S4g-l**).

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

320 To determine the impact of DDT exposure on paternal sperm, we performed RNA-seq analysis
321 to assess the small RNA patterns in mature cauda sperm of control and DDT-exposed males. The
322 distribution of the major small RNA species detected is in line with previous reports [13, 14],
323 with tRNA-derived fragments (tRFs) being the most abundant sperm small RNA sub-type,

324 followed by miRNAs and piRNAs in both groups (**Fig. 5a**). Interestingly, sperm of DDT-
325 exposed males showed higher abundance of miRNAs (6.7%) compare to controls (1.4%) (**Fig.**
326 **5a-b**). A total of 16 miRNAs were differentially expressed (nine down- and seven up-regulated)
327 in sperm of DDT-exposed mice compared to controls (**Fig. 5c**). Of the seven miRNAs
328 upregulated in sperm of DDT-exposed males, miRNA-10b had the highest surge, representing
329 close to 60% of the overall miRNA increase (**Fig. 5d**).
330 To establish whether upregulation of miRNA-10b in sperm was, in fact, induced by DDT
331 exposure, we treated DDT-exposed mice with phenobarbital (PB), a drug known to promote
332 hepatic enzymes activation and accelerate DDT metabolism and excretion in humans [30, 31]
333 and animals [32-34]. Indeed, we observed an increase in hepatic phase I enzymes ($P<0.001$,
334 **Fig.5e**) and a reduction in DDT metabolites in males treated with both DDT and PB (**Table S4**).
335 Compared to mice exposed to DDT only, miRNA-10b was significant decreased in sperm of
336 mice treated with both DDT and PB with levels reversing to those observed in controls ($P<0.05$,
337 **Fig. 5f**).
338 ***miRNA-10b embryonic injection replicates DDT daughters' phenotypes***
339 miRNA-10b along with other members of the miRNA-10 family play critical roles in embryonic
340 development and cell differentiation through the regulation of Homeobox (*Hox*) genes [43].
341 These genes regulate organogenesis including development of the mammary gland [44-46].
342 We next examined whether miRNA-10b was responsible for the phenotypes observed in DDT
343 offspring using embryonic (zygote) microinjections. While no major changes in mammary gland
344 morphology were detected (**Fig. S3**), female mice derived from the miR-10b zygote injections
345 (miR-10b females from now on) showed mammary tissue suppression of AMPK and increased
346 expression and activity of mTOR and down-stream effector, 4E-BP1, compared to controls (**Fig.**

347 **6a**, **Fig. S5**), a phenotype also observed in mammary glands of DDT daughters. More
348 importantly, we also found increased orthotopic (EO771 cells) mammary tumor development in
349 miR-10b females, which showed both increased tumor growth and higher tumor weights
350 compared to controls ($P<0.05$, $P<0.01$, **Fig. 6b-c**). Survival after tumor implantation was also
351 worse in miRNA-10b females (**Fig. 6d**, $P<0.1$). Further, embryonic miR-10b injection
352 phenocopied the metabolic dysfunction of DDT daughters, with adult miR-10b females showing
353 hyperinsulinemia ($P<0.01$) compared to controls (**Fig. 6e**).
354

355 **Discussion**

356 Paternal environmental exposures have been shown to modulate offspring's health phenotypes
357 via sperm non-coding RNAs [13-15]. Here, we show that pre-conception paternal exposure to
358 the pesticide DDT modulates the sperm non-coding RNA load, particularly miRNAs, and
359 programs female offspring's breast cancer development in a pre-clinical animal model. We also
360 found that miRNA-10b, the miRNA with the highest increase in sperm of DDT-exposed fathers,
361 can recapitulate phenotypes observed in DDT daughters including alterations in the mammary
362 tissue AMPK/mTOR axis and accelerated breast tumor growth. These findings are outlined in
363 the model presented in **Fig. 7**. Our results lend support to the notion that predisposition to
364 diseases such as cancer can be determined by factors other than genetic background and suggest
365 that it could be transmitted through sperm miRNAs. We showed before that paternal obesity,
366 malnutrition or specific nutrient intake can increase breast and other types of cancer in the
367 progeny [4, 6, 8, 9]. This study further implies that environmentally-induced non-genetic or
368 epigenetic predisposition to cancer can occur and should be evaluated in human studies.

369 Published reports using the CHDS cohort showed that daughters of women exposed to DDT in
370 pregnancy have increased rates of breast cancer [18] and are more likely to be diagnosed with
371 advanced stage tumors. Likewise, we found that paternal DDT exposure in the pre-conception
372 window leads to increased breast cancer growth in daughters in a mouse model. Our new
373 observations and previously published data [6-9] indicate that paternal exposures are as
374 important as maternal ones in programming breast and other cancers in offspring and ought to be
375 examined in epidemiologic studies. The overlap between the effects of maternal and paternal
376 exposure on offspring's breast cancer phenotypes in animal models suggest a common
377 underlying mechanism that also needs further investigation.

378 While no studies in humans have directly investigated the link between paternal DDT exposure
379 and breast cancer in their descendants, evidence for a role of related paternal exposures exists.
380 For instance, an association between father's exposure to pesticides and a number of childhood
381 cancers in offspring has been reported [47, 48]. Epidemiologic studies also show that paternal
382 exposure to DDT and other pesticides is linked to low birthweight [49] and our study supports
383 those findings. Low birthweight is more prevalent in minority populations [50, 51] which are
384 more likely to be exposed to environmental toxicants such as DDT [20, 21, 52] and to develop
385 more aggressive breast tumors [53].

386 Our study shows that DDT exposure alters the non-coding small RNA load in sperm. While the
387 proposed mechanisms by which sperm cells acquire their small RNA load in response to
388 environmental insults are still being worked out, studies have shown that extra cellular vesicles
389 produced by epididymal cells play a role [14, 54]. Further, it has been proposed that during the
390 normal fertilization process, sperm small RNAs are delivered to the oocyte and modulate the
391 transcriptome during the first few cell divisions, setting a signaling cascade that can impact

392 embryonic development [55] and recent reports support this view [2, 14]. In line with that,
393 embryonic injection of miR-10b, the miRNA with the highest surge in sperm of DDT-exposed
394 males, replicates most of the major phenotypes observed in DDT daughters including higher
395 circulating levels of insulin, altered AMPK/mTOR signaling in mammary tissues and increased
396 tumor growth.

397 Although we have focused on miRNA-10b given its DDT-induced surge in sperm and its role
398 during embryonic development [43], we acknowledge that the full phenotype observed in DDT
399 offspring may reflect the interaction of that miRNA not only with other miRNAs but also other
400 non-coding RNAs delivered by the sperm of DDT-exposed males during fertilization as reported
401 by others[13, 15]. It is important to note, however, that studies examining different paternal
402 environmental exposures —including obesity and malnutrition which also program breast cancer
403 risk in offspring— report a surge in levels in miRNA-10b and other members of this family in
404 sperm [7, 8, 13, 56], indicating that alterations in the miRNA-10 family could represent a
405 common mechanism underlying the effects of different paternal factors on offspring's
406 phenotypes.

407 MicroRNA-10b along with other members of the miRNA-10 family play critical roles in
408 embryonic development and cell differentiation through the regulation of Homeobox (*Hox*)
409 genes [43]. These genes regulate organogenesis including development of the mammary gland
410 and other skin-derived tissues [44-46]. However, whether alterations in *Hox* genes during
411 embryonic development of the mammary bud play a functional role in the increased breast
412 cancer development in daughters of DDT-exposed fathers will need to be examined in futures
413 studies.

414 We previously showed that low birth weight (induced by paternal malnutrition) leads to re-
415 wiring of the nutrient sensing AMPK/mTOR pathway both in normal mammary tissues as well
416 as in tumors. Similarly, we found that DDT daughters —who are also born small— have
417 alterations in the mammary AMPK/mTOR axis. Whether these molecular alterations in nutrient
418 sensors are directly by programmed by pre-conception paternal DDT exposure or are a
419 consequence of the metabolic dysfunction observed in DDT daughters remains to be determined.
420 However, miRNA-10b embryonic injection recapitulated the nutrient sensing machinery
421 phenotype in mammary tissues, indicating that dysregulation of the AMPK/mTOR axis is likely
422 initiated through sperm miRNAs after fertilization.

423 Given its documented role in cancer, the local mammary changes in AMPK/mTOR signaling are
424 likely mechanistic linked to accelerated tumor growth in both DDT daughters and miR-10b
425 females, although this remains to be studied experimentally. In normal cells, proliferation is
426 coupled to nutrient availability to ensure proper cell growth and division [57]. The AMPK
427 pathway functions as a cellular energy sensor mechanism and provides a link between nutrient
428 supplies and cell growth and metabolism. This pathway controls mTOR activity and associated
429 energy-consuming biosynthetic activities such as protein synthesis [58]. Not surprisingly,
430 suppression of AMPK and increased mTOR activity (due to loss of LKB1, the major AMPK
431 kinase) is responsible for the inheritable Peutz-Jeghers syndrome, in which patients have an 18-
432 fold increase in cancer risk, including breast cancer [59]. Alterations in AMPK/mTOR axis have
433 been shown to lead metabolic reprogramming in cancer cells [60-62]. Interestingly, we
434 previously reported that paternal nutrition can lead to metabolic reprogramming in mammary
435 tumors [8], suggesting that ancestral exposures can not only alter cancer predisposition but also
436 its biological phenotype.

437 In summary, our study shows that the environmental toxicant DDT alters the male germline non-
438 coding RNAs and increases cancer susceptibility in the progeny. While many questions remain,
439 our findings could have important implications for human health: According to published studies
440 and the Center for Disease Control and Prevention (CDC), minorities such as African Americans
441 and Mexican-Americans and recent immigrants have the highest circulating concentrations of
442 this pesticide in the U.S. [20, 21, 63]. In addition, DDT is still used in developing countries and
443 Americans who could have been affected by paternal exposure to DDT before its ban [18] are
444 still alive today and reaching an age when the incidence of breast and other cancers increases.
445 While our study focused on DDT exposure, our findings are likely relevant to other commonly
446 used pesticides and flame retardants which, like DDT, have anti-androgenic properties [64-66].

447

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457 Assis).
458 The small RNA-seq (small RNA) data has been deposited in GEO (Gene Expression Omnibus)
459 database with accession code pending.

460

461

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

467
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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).

Fig-7. Proposed mechanisms by which paternal DDT exposure programs offspring's breast cancer development. DDT exposure increase levels of miRNA-10b and other non-coding RNAs in sperm which are delivered to the oocyte during fertilization. This, in turn, leads to epigenetic programming of mammary bud in the developing fetus (perhaps through modulation of *Hox* genes). After birth, the ensuing mammary suppression of AMPK and sustained mTOR activity increase cell growth and survival allowing breast tumors to develop. M.G., mammary gland.

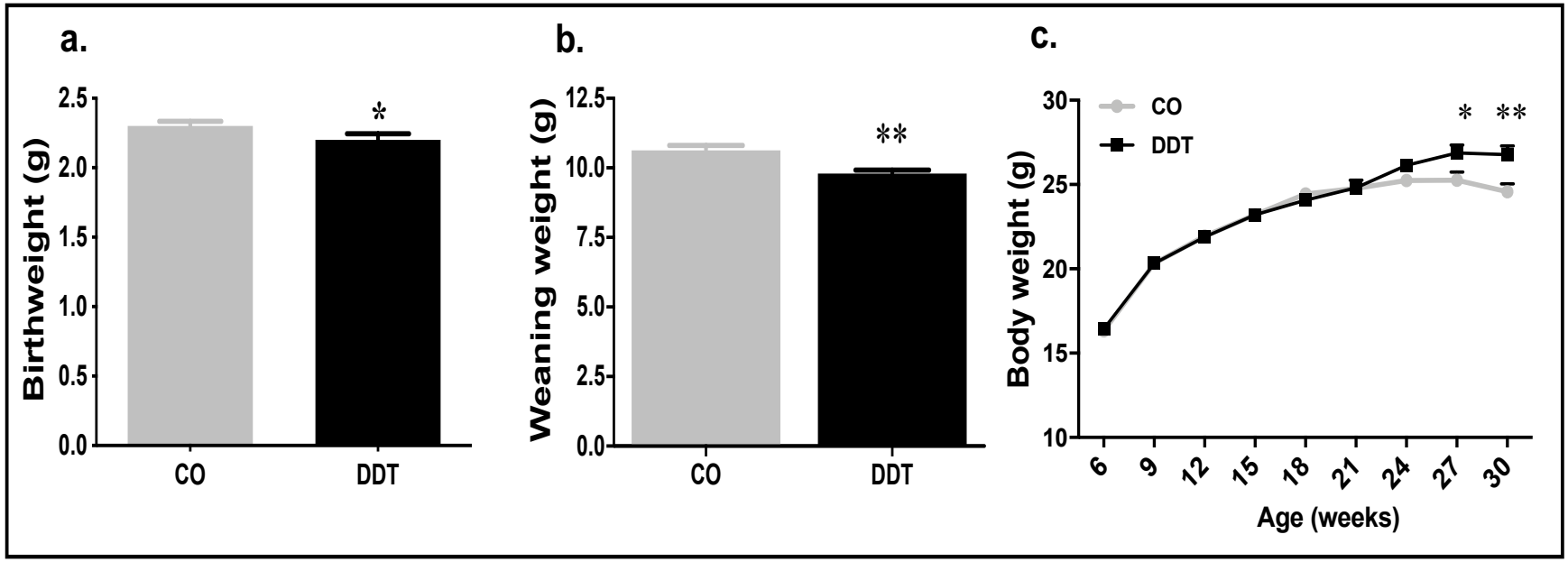
a.
Birthweight (g)
2.5
2.0
1.5
1.0
0.5
0.0
*
CO
DDT
b.
Weaning weight (g)
12.5
10.0
7.5
5.0
2.5
0.0
**
CO
DDT
c.
Body weight (g)
30
25
20
15
10
CO
DDT
*
**
6
9
12
15
18
21
24
27
30
Age (weeks)

Figure 1

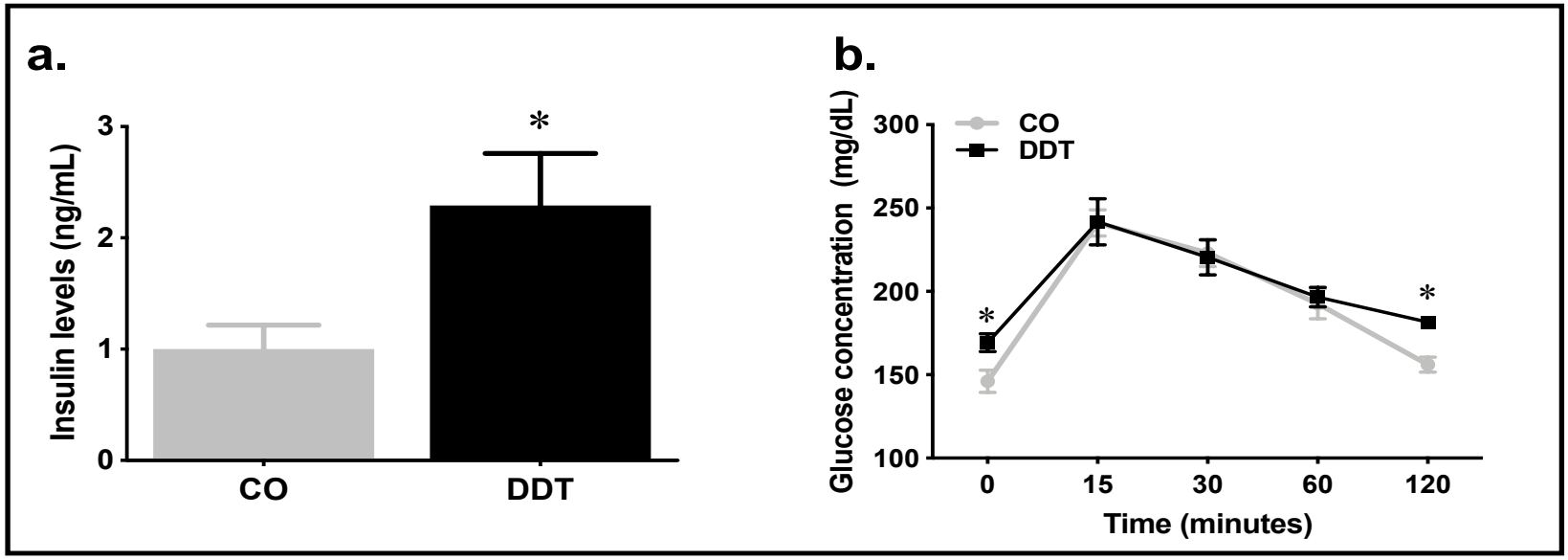
a.
Insulin levels (ng/mL)
3
2
1
0
*
CO
DDT
b.
Glucose concentration (mg/dL)
300
250
200
150
100
CO
DDT
*
*
0
15
30
60
120
Time (minutes)

Figure 2

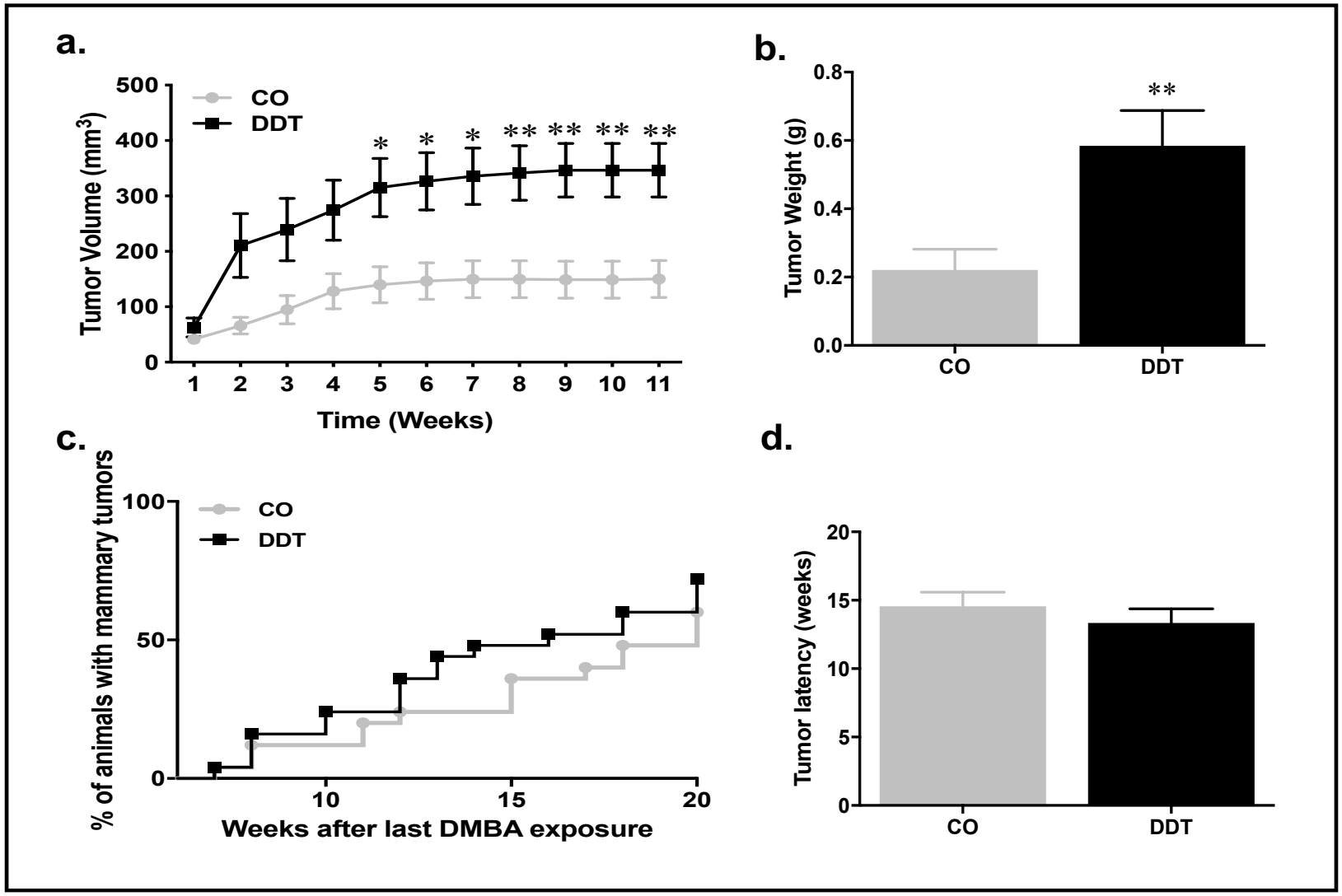
a.
CO
DDT
Tumor Volume (mm3)
500
400
300
200
100
0
1 2 3 4 5 6 7 8 9 10 11
Time (Weeks)
b.
Tumor Weight (g)
0.8
0.6
0.4
0.2
0.0
CO
DDT
c.
% of animals with mammary tumors
100
50
0
10 15 20
Weeks after last DMBA exposure
d.
Tumor latency (weeks)
20
15
10
5
0
CO
DDT

Figure 3

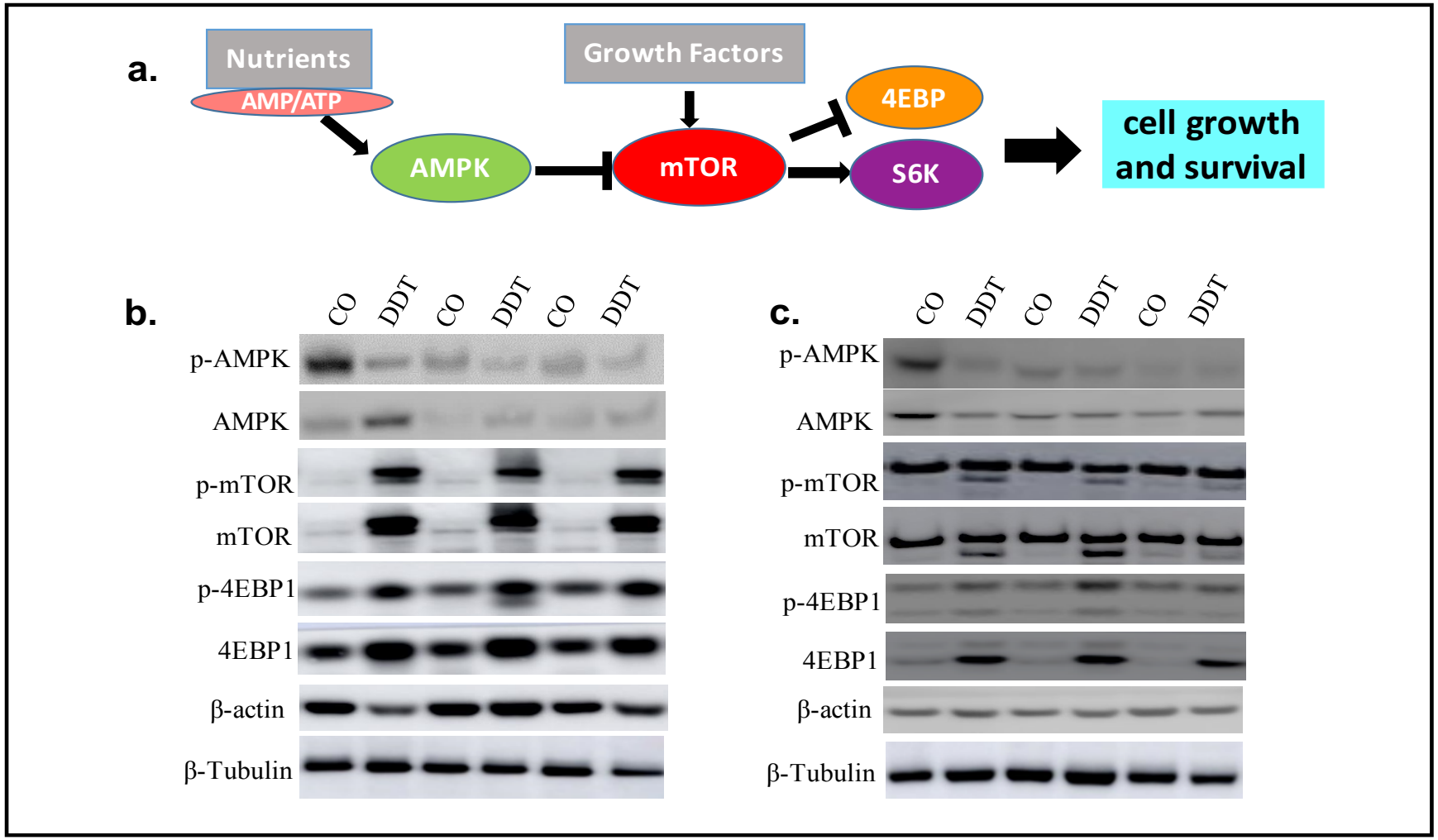
a.
Nutrients
AMP/ATP
Growth Factors
AMPK
mTOR
4EBP
S6K
cell growth and survival
b.
CO
DDT
CO
DDT
CO
DDT
p-AMPK
AMPK
p-mTOR
mTOR
p-4EBP1
4EBP1
β-actin
β-Tubulin
c.
CO
DDT
CO
DDT
CO
DDT
p-AMPK
AMPK
p-mTOR
mTOR
p-4EBP1
4EBP1
β-actin
β-Tubulin

Figure 4

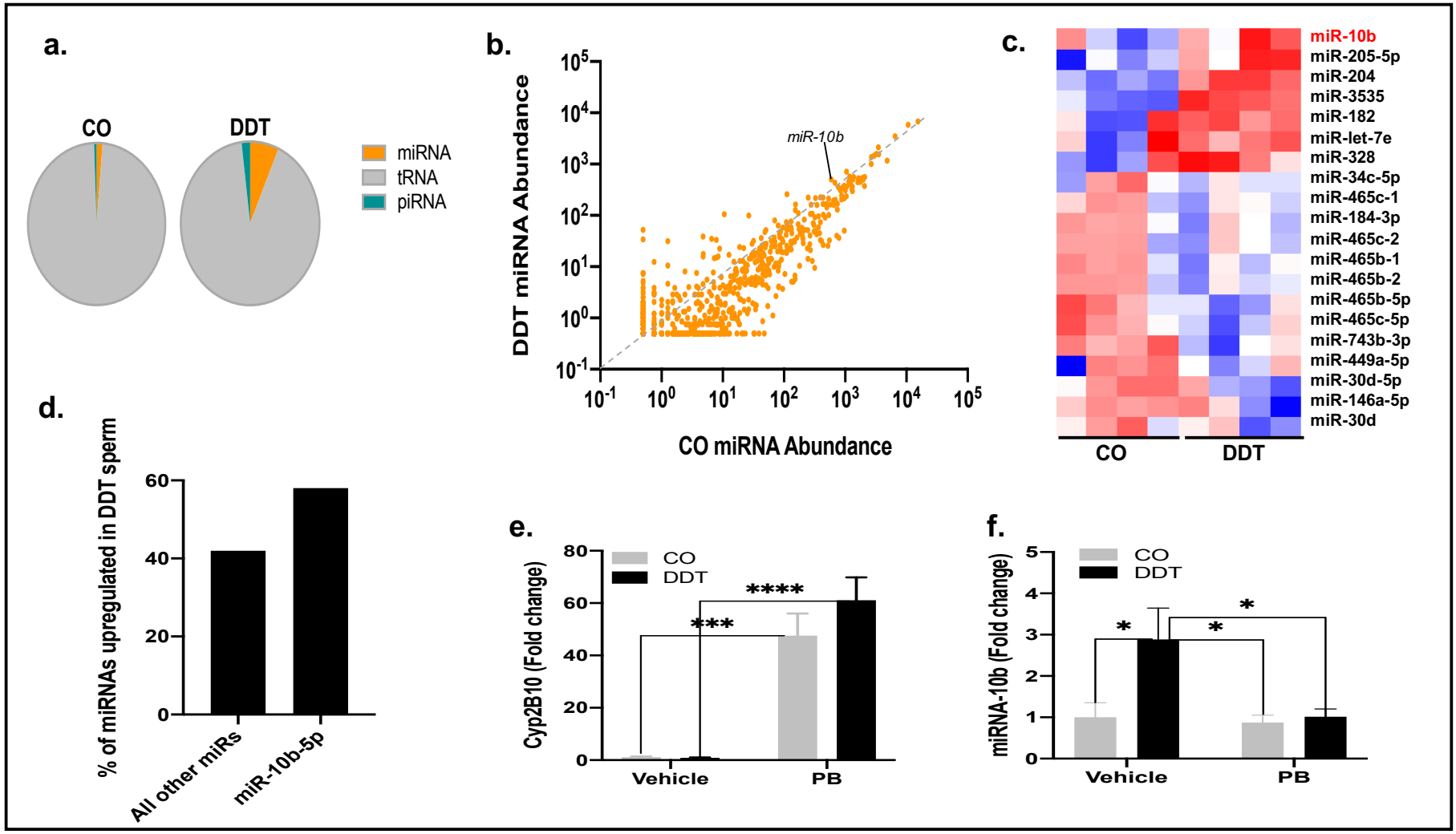
a.
CO
DDT
miRNA
tRNA
piRNA
b.
DDT miRNA Abundance
CO miRNA Abundance
miR-10b
c.
miR-10b
miR-205-5p
miR-204
miR-3535
miR-182
miR-let-7e
miR-328
miR-34c-5p
miR-465c-1
miR-184-3p
miR-465c-2
miR-465b-1
miR-465b-2
miR-465b-5p
miR-465c-5p
miR-743b-3p
miR-449a-5p
miR-30d-5p
miR-146a-5p
miR-30d
CO
DDT
d.
% of 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

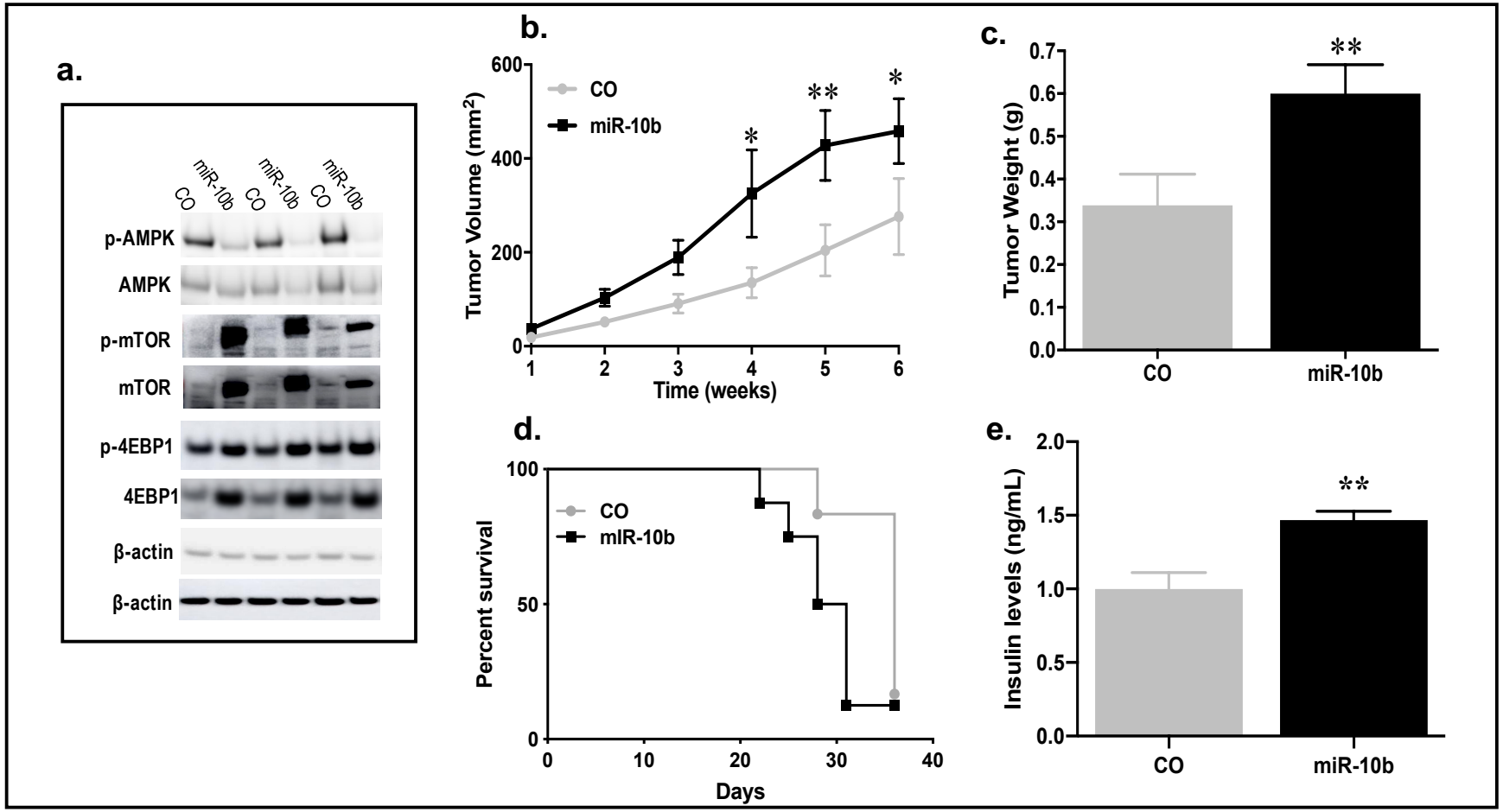
a.
CO
miR-10b
CO
miR-10b
CO
miR-10b
p-AMPK
AMPK
p-mTOR
mTOR
p-4EBP1
4EBP1
β-actin
β-actin
b.
CO
miR-10b
Tumor Volume (mm²)
Time (weeks)
c.
Tumor Weight (g)
CO
miR-10b
d.
CO
mIR-10b
Percent survival
Days
e.
Insulin levels (ng/mL)
CO
miR-10b

Figure 6

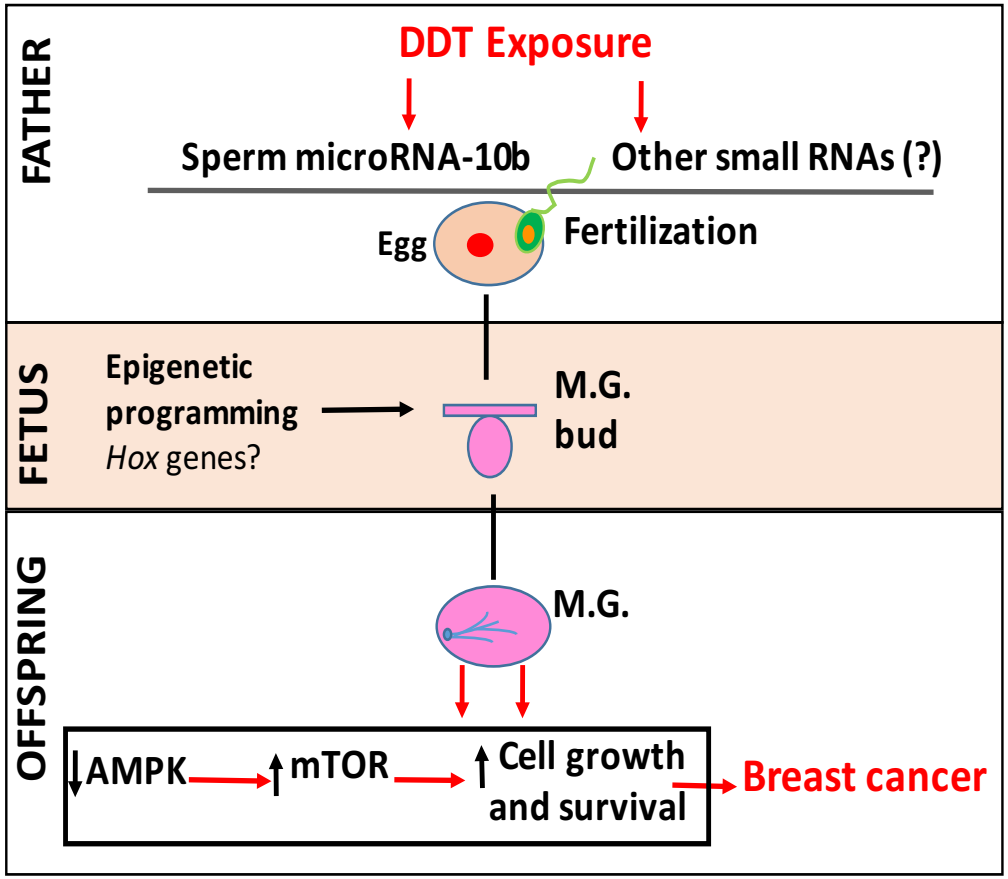
FATHER
DDT Exposure
Sperm microRNA-10b
Other small RNAs (?)
Egg
Fertilization
FETUS
Epigenetic
programming
Hox genes?
M.G.
bud
OFFSPRING
M.G.
AMPK
mTOR
Cell growth
and survival
Breast cancer

Figure 7

SUPPLEMENTARY INFORMATION

Supplementary Figures

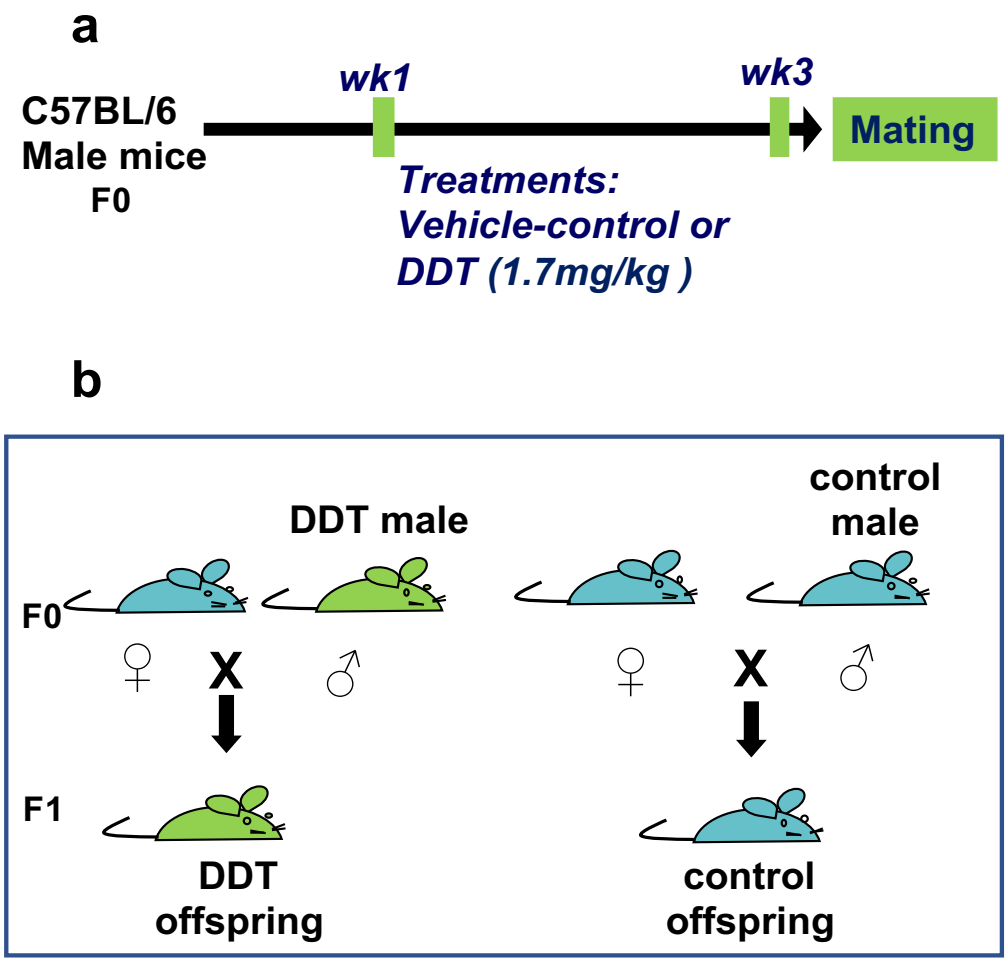

Figure S1

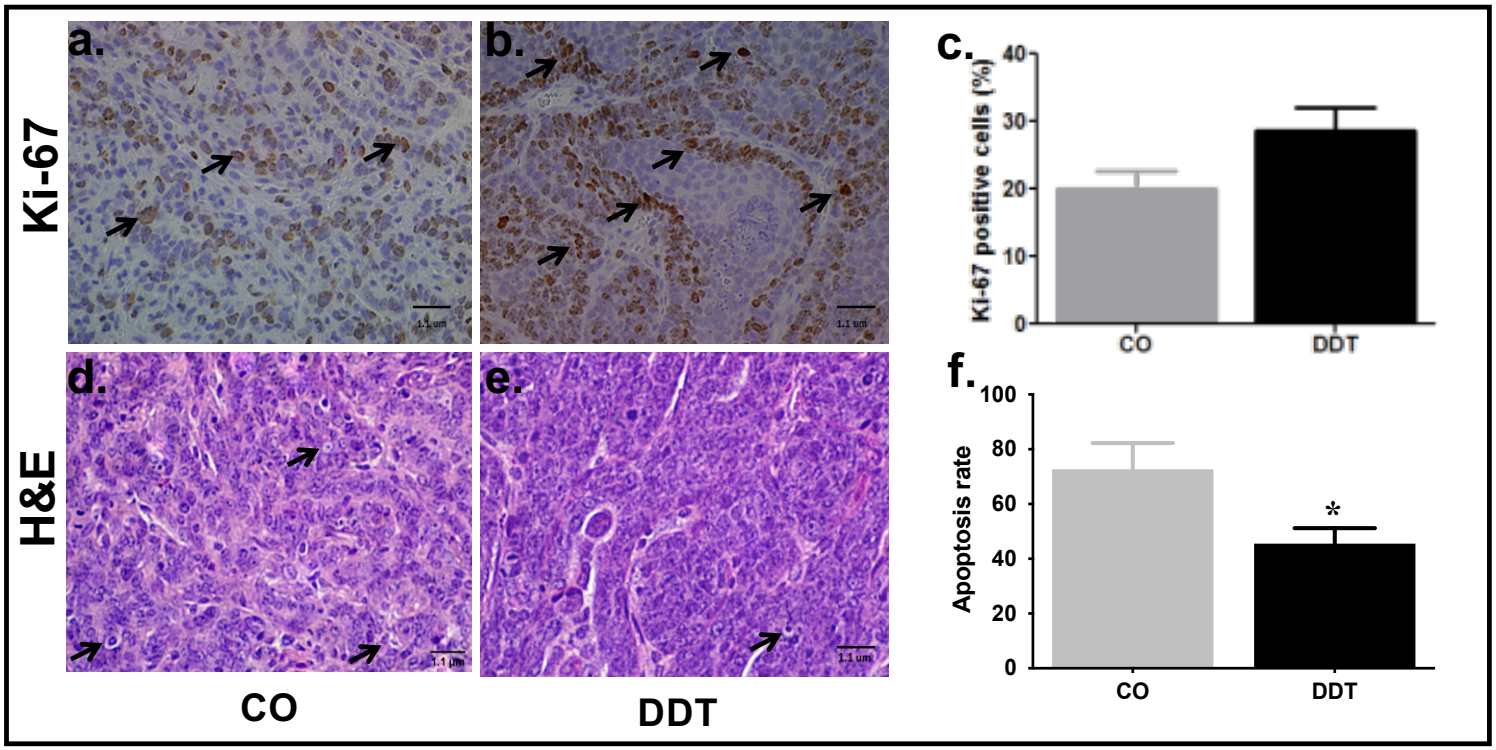
a.
b.
c.
d.
e.
f.
Ki-67
H&E
CO
DDT
Ki-67 positive cells (%)
0
10
20
30
40
CO
DDT
Apoptosis rate
0
20
40
60
80
100
*
CO
DDT

Figure S2

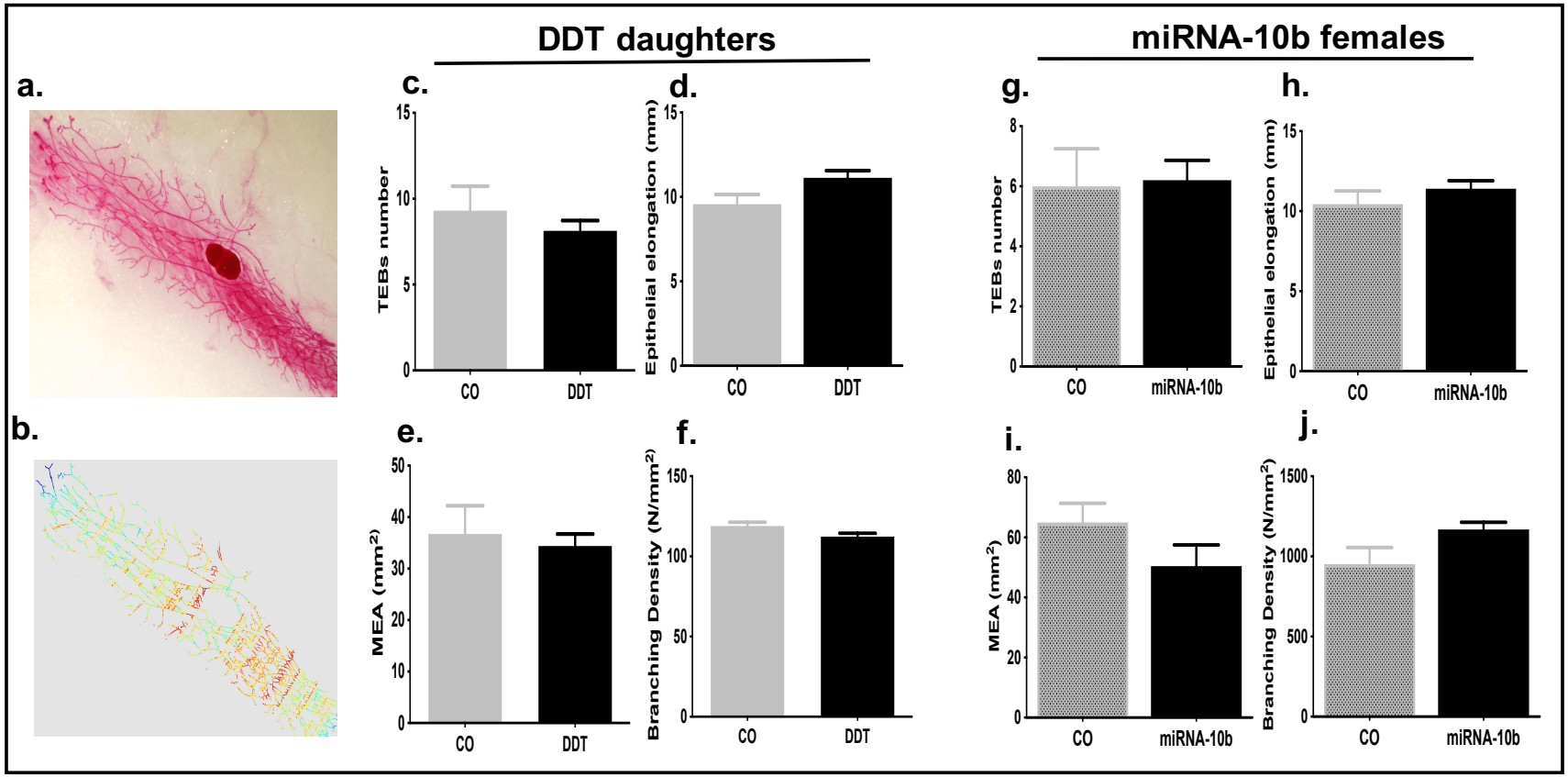
DDT daughters
miRNA-10b females
a.
b.
c.
d.
e.
f.
g.
h.
i.
j.
TEBs number
Epithelial elongation (mm)
MEA (mm²)
Branching Density (N/mm²)
CO
DDT
miRNA-10b

Figure S3

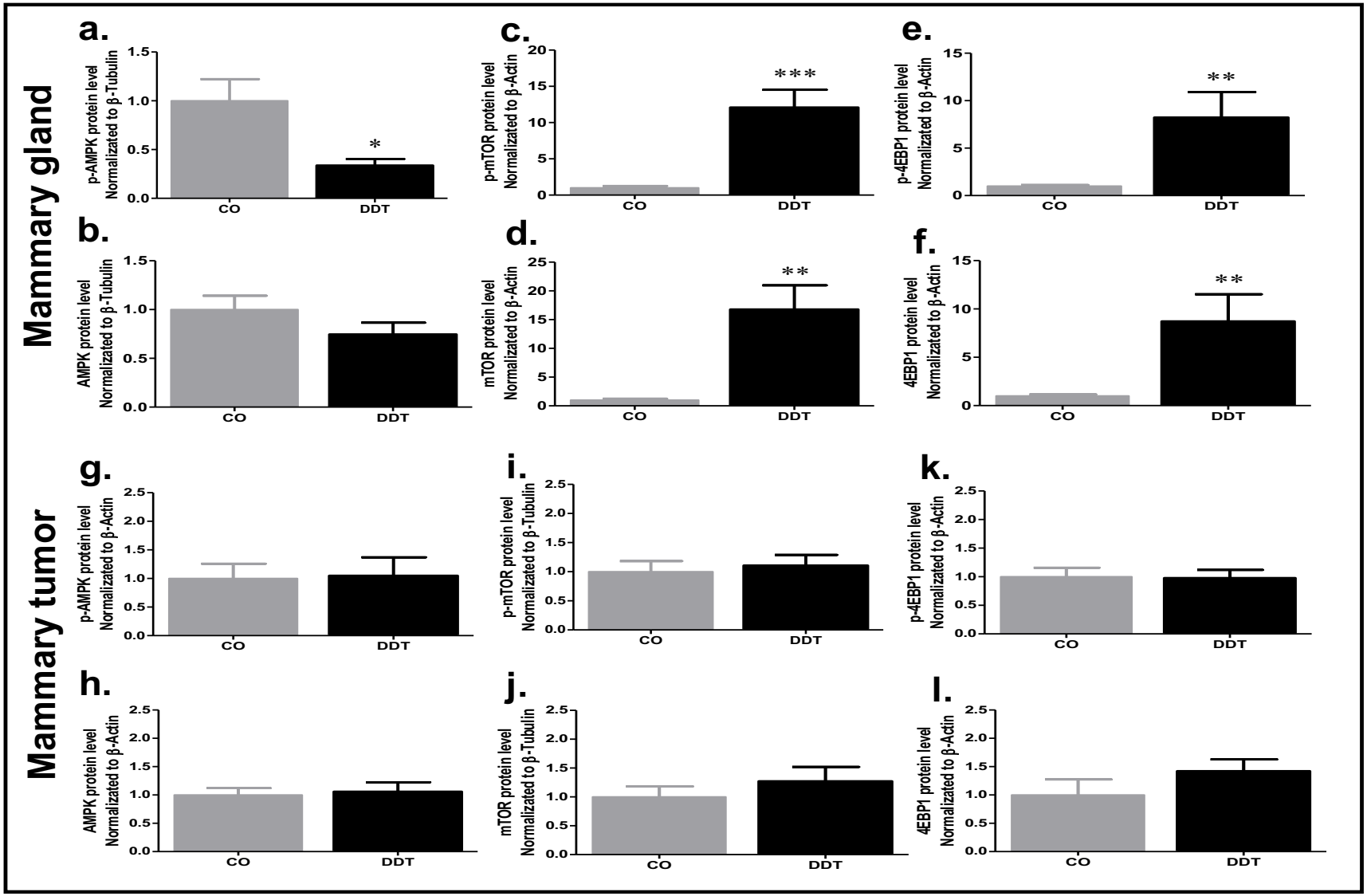
Mammary gland
Mammary tumor
a.
p-AMPK protein level
Normalizated to β-Tubulin
b.
AMPK protein level
Normalizated to β-Tubulin
c.
p-mTOR protein level
Normalizated to β-Actin
d.
mTOR protein level
Normalizated to β-Actin
e.
p-4EBP1 protein level
Normalizated to β-Actin
f.
4EBP1 protein level
Normalizated to β-Actin
g.
p-AMPK protein level
Normalizated to β-Actin
h.
AMPK protein level
Normalizated to β-Actin
i.
p-mTOR protein level
Normalizated to β-Tubulin
j.
mTOR protein level
Normalizated to β-Tubulin
k.
p-4EBP1 protein level
Normalizated to β-Actin
l.
4EBP1 protein level
Normalizated to β-Actin
CO
DDT
*
**
***

Figure S4

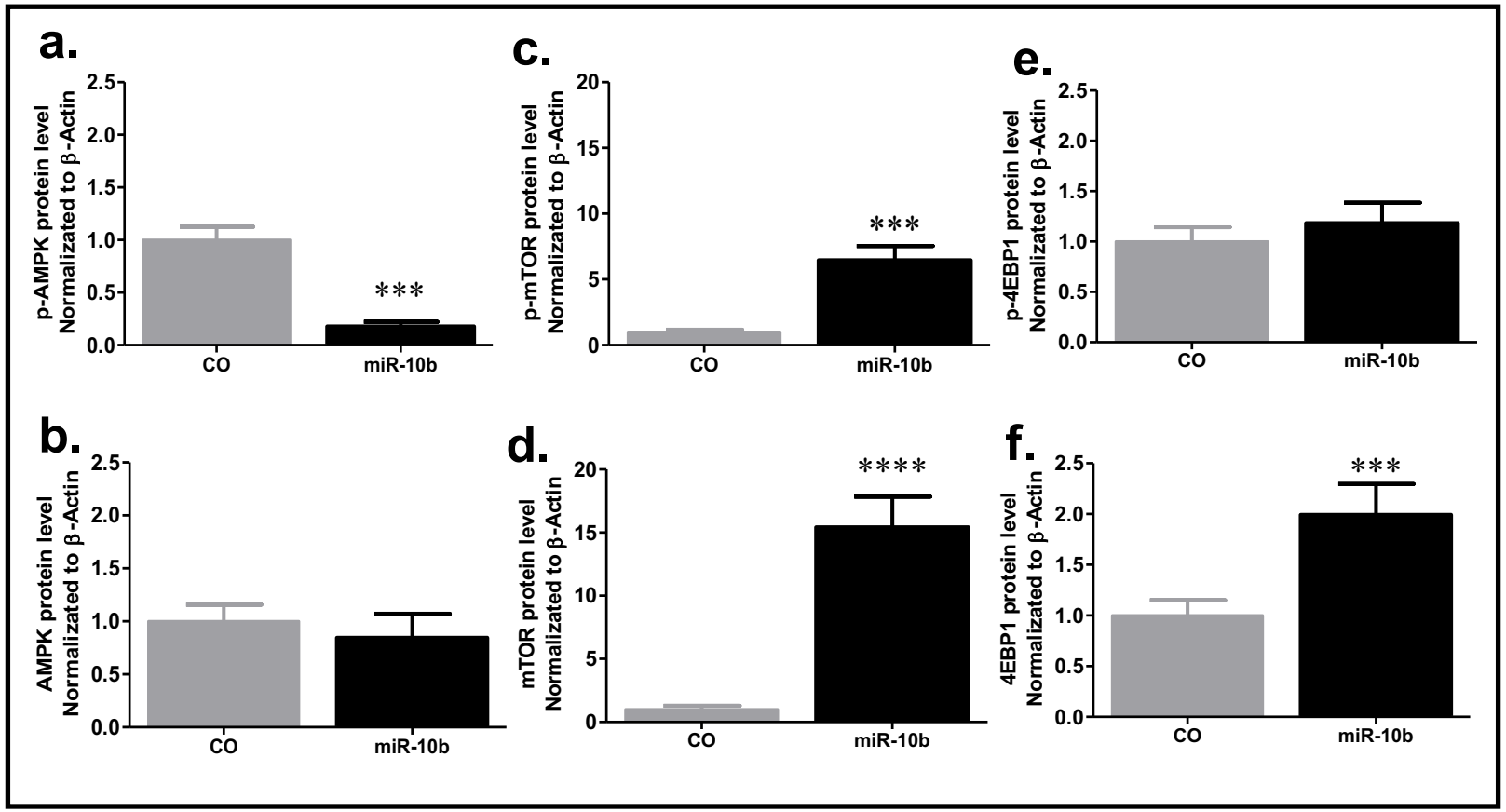
a.
p-AMPK protein level
Normalizated to β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
***
CO
miR-10b
c.
p-mTOR protein level
Normalizated to β-Actin
20
15
10
5
0
***
CO
miR-10b
e.
p-4EBP1 protein level
Normalizated to β-Actin
2.5
2.0
1.5
1.0
0.5
0.0
CO
miR-10b
b.
AMPK protein level
Normalizated to β-Actin
2.5
2.0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

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).