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## The tissue-specific effects of different $17\beta$ -estradiol doses reveal the key sensitizing role of AF1 domain in $ER\alpha$ activity

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1                   **The tissue-specific effects of different 17 $\beta$ -estradiol doses reveal the key**  
2                   **sensitizing role of AF1 domain in ER $\alpha$  activity**

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**HIGHLIGHTS**

- Some biological response to E2 displays biphasic dose relationship.
- ER $\alpha$ AF1 plays a key sensitizing role in ER $\alpha$  activity.
- Differences and even contradictory conclusions could be explained by differences of E2 doses.

Journal Pre-proof

57

58 **ABSTRACT**

59 17 $\beta$ -Estradiol (E2) action can be mediated by the full-length estrogen receptor alpha (ER $\alpha$ 66),  
60 but also by the AF1 domain-deficient ER $\alpha$  (ER $\alpha$ 46) isoform, but their respective sensitivity  
61 to E2 is essentially unknown. To this aim, we first performed a dose response study using  
62 subcutaneous home-made pellets mimicking either metestrus, proestrus or a pharmacological  
63 doses of E2, which resulted in plasma concentrations around 3, 30 and 600 pM, respectively.  
64 Analysis of the uterus, vagina and bone after chronic exposure to E2 demonstrated dose-  
65 dependent effects, with a maximal response reached at the proestrus- dose in wild type mice  
66 expressing mainly ER $\alpha$ 66. In contrast, in transgenic mice harbouring only an ER $\alpha$  deleted in  
67 AF1, these effects of E2 were either strongly shifted rightward (10 to 100-fold) and/or  
68 attenuated, depending on the tissue studied. Finally, experiments in different cell lines  
69 transfected with ER $\alpha$ 66 or 46 also delineated varying profiles of ER $\alpha$  AF1 sensitivity to E2.  
70 Altogether, this work emphasizes the importance of dose in the tissue-specific actions of E2  
71 and demonstrates the key sensitizing role of AF1 in ER $\alpha$  activity.

72

73 **Keywords:** Estrogen receptor alpha (ER $\alpha$ ), activation function (AF), dose response, tissue-  
74 specific

75

## 76 1. Introduction

77 During her lifetime, a woman is exposed to varying levels of estrogens. At puberty,  
78 the onset of the menstrual cycle leads to a cyclical production of estrogens with large  
79 fluctuations in estrogen levels. Pregnancy is marked by an extensive increase in estrogens  
80 levels whereas menopause, the consequence of the cessation of estrogen production by  
81 ovaries in women, results in a sharp decrease in the main estrogen 17- $\beta$  estradiol (E2) levels.  
82 These hormonal variations translate into changes not only in sex targets organs involved in  
83 sex secondary characters and reproduction, but also in many other non-reproductive tissues.  
84 In particular, estrogens play an important role in the growth and maturation of bone as well as  
85 in the regulation of bone turnover in adult. The importance of estrogen for skeletal health is  
86 well known and represents an important research area. Indeed, estrogen deficiency is a major  
87 risk for development of osteoporosis and leads to increased fracture risk, while estrogen  
88 treatment reduces this risk (Almeida *et al.*, 2017). Unfortunately, long-term estrogen therapy  
89 is also associated with serious side-effects, mainly increased risk of breast cancer and of deep  
90 venous thrombosis (Valera *et al.*, 2018).

91 Mouse models have already contributed to elucidation of the mechanisms of action of  
92 estrogens *in vivo* (Guillaume *et al.*, 2017; Hewitt and Korach, 2018; Rooney and van der  
93 Meulen, 2017). In general, mice are ovariectomized to eliminate the main source of  
94 endogenous estrogens, and to allow studying the effects of doses of exogenous E2 delivered  
95 by various devices, such as subcutaneous pellets. A large range of pellets that deliver estrogen  
96 from 0.01 mg/pellet (*i.e.*, 0.8  $\mu$ g/kg/day) to 5 mg/pellet (*i.e.*, 2000  $\mu$ g/kg/day) are available,  
97 and very different doses are used in the literature, often without justification of the dose  
98 employed. Furthermore, estrogens have frequently been reported to exert a hormetic or  
99 biphasic dose-response relationship, characterized by low-dose stimulation and high-dose  
100 inhibition (Strom *et al.*, 2011; Duarte-Guterman *et al.*, 2015). One example of such a

101 dichotomous effect was reported in ischemic stroke; whereas some studies have shown a  
102 neuroprotective effect of estrogen, others have reported neurotoxicity, the differences being  
103 attributed to the dose (Strom *et al.*, 2009).

104 The different responses to E2 are initiated by their binding to the estrogen receptors  
105 (ERs), ER $\alpha$  and ER $\beta$ , which belong to the nuclear receptor superfamily and are structurally  
106 organized into 6 functional domains (A to F). The E domain supports hormone binding, which  
107 induces conformational changes that are required for ER transcriptional activity through the  
108 modulation of two activation functions (AFs), AF1 and AF2, located in the A/B and E  
109 domains, respectively. In addition to the full-length 66-kDa (ER $\alpha$ 66) isoform, a 46-kDa ER $\alpha$ -  
110 isoform (ER $\alpha$ 46), lacking the N-terminal portion (domains A/B), and thereby AF-1, has been  
111 reported to be expressed in various cell types such as human osteoblasts (Denger *et al.*, 2001),  
112 macrophages (Murphy *et al.*, 2009), and vascular endothelial cells (Murphy *et al.*, 2009), but  
113 also in cancer cells (Chantalat *et al.*, 2016). Mechanisms regulating both the expression of  
114 ER $\alpha$ 46 and its functions remain essentially unknown. ER $\alpha$  can be generated by either  
115 alternative splicing, proteolysis, or an alternative initiation of translation via an internal  
116 ribosome entry site (IRES) (Chantalat *et al.*, 2016).

117 ER-mediated transcriptional regulation involves either a direct interaction of ER with  
118 specific estrogen-responsive elements (EREs) located in enhancers or near the promoter  
119 region of target genes, or an indirect mechanism (tethered) via protein/protein interactions  
120 with other transcriptional factors (Hamilton *et al.*, 2017). ER $\alpha$ AF1 and ER $\alpha$ AF2 have been  
121 shown to play crucial roles in the transcriptional effects of ER $\alpha$  through the recruitment of  
122 coactivators. In cultured cells, the respective roles of ER $\alpha$ AF1 and ER $\alpha$ AF2 appear to depend  
123 on the state of cell differentiation (Merot *et al.*, 2004). Using mice selectively deficient in  
124 ER $\alpha$ AF1 or ER $\alpha$ AF2, we previously demonstrated that ER $\alpha$ AF2 is absolutely required for all  
125 nuclear effects of ER $\alpha$  in response to E2 whereas the role of ER $\alpha$ AF1 is highly tissue

126 dependent (Abot *et al.*, 2013; Arnal *et al.*, 2013; Billon-Gales *et al.*, 2009; Borjesson *et al.*,  
127 2011).

128 However, the potential role of the E2 dose on the different biological effects mediated by the  
129 wild-type full length ER $\alpha$ -66 or the AF1/AB domain-deficient ER $\alpha$  has not been evaluated  
130 nor taken into account in most *in vivo* studies.

131 In this work, we describe the effect of a 3-week exposure of ovariectomized mice to 3  
132 doses of E2 mimicking metestrus (low dose), proestrus (medium dose) or a supra-  
133 pharmacological dose on three major tissues: uterus, vagina and bone. This experimental  
134 paradigm allowed us to precisely compare tissue responses to these 3 doses. An additional  
135 very high dose of E2 was used in transgenic mice harbouring an ER $\alpha$  deleted for AF1  
136 (ER $\alpha$ AF1<sup>0</sup>) and in its littermate wild type mice.

137

## 138 **2. Material and Methods**

### 139 **2.1 Mice**

140 All procedures involving experimental animals were performed in accordance with the  
141 principles and guidelines established by the National Institute of Medical Research and were  
142 approved by the local Animal Care and Use Committee. The investigation conforms to the  
143 directive 2010/63/EU of the European parliament. Mice were housed in cages in groups of  
144 four to six and kept in a specific pathogen-free and temperature-controlled facility on a 12-  
145 hour light to dark cycle. Each experimental group included at least six animals. Female  
146 C57BL/6J mice were purchased from Charles River Laboratories. ER $\alpha$ AF-1<sup>0</sup> mice were  
147 generated as previously described (Billon-Gales *et al.*, 2009). Mice were anesthetized by  
148 intraperitoneal injection of ketamine (25 mg/kg) and xylazine (10 mg/kg), and ovariectomized  
149 or sham operated at 4 weeks of age, before puberty, to avoid any endogenous estrogen  
150 exposure. After 2 weeks of recovery, mice were implanted subcutaneously with pellets  
151 releasing either vehicle (cholesterol) or various amounts of E2 for 21 days. To deliver  
152 increasing doses of estradiol over prolonged periods of time, various amounts of E2 (E2758  
153 Sigma-Aldrich) and cholesterol (C3045 Sigma-Aldrich) were thoroughly mixed in powder  
154 form and compacted to obtain pellets (20 mg total) that were then implanted subcutaneously  
155 into ovariectomized mice as previously described (Hsieh *et al.*, 1998). Low (LD-E2), medium  
156 (MD-E2), high (HD-E2) and very high (VHD-E2) doses correspond respectively to  
157 weight/weight ratio of E2/cholesterol: 1/20000, 1/2500, 1/250 and 1/25.

### 158 **2.2 Hormone assays**

159 Plasma E2 levels were assayed by GC/MS, as described previously (Giton *et al.*, 2015) with  
160 minor modifications. Saline water / twice charcoal dextran stripped aged female rabbit serum  
161 (1/1, v/v) was used as the matrix for calibrators and quality control (QC) standards. Briefly,  
162 each sample (serums, calibration standards, quality controls, and blank matrix) was collected



163 in an 8 ml borosilicate tube. A spiking solution of deuterated steroid internal standard (IS) (50  
164  $\mu\text{l}$  containing 10 pg of E2-d4, except for blank matrix) (CDN Isotopes, Inc., Point-Claire,  
165 Canada), and 3 ml of 1-chlorobutane were added to each sample. After fast centrifugation, the  
166 upper organic phase was collected on conditioned Hypersep SI 500mg SPE minicolumn  
167 (Thermo Scientific, Rockwood, USA). The column and adsorbed material were then washed  
168 with ethyl acetate / hexane (6 ml; 1/9, v/v). The second fraction containing E2 was eluted  
169 using ethyl acetate / hexane (4 ml; 1/1, v/v), then evaporated at 60°C to dryness. E2 was  
170 derivatized with pentafluorobenzoyl chloride (PFBC) (103772-1G, Sigma-Aldrich, Steinheim,  
171 Germany). Final extracts were reconstituted in isooctane, then transferred into conical vials  
172 for injection into the GC system (GC-2010 Plus, Shimadzu, Japan) using a 50%  
173 phenylmethylpolysiloxane VF-17MS capillary column (20m x 0.15mm, internal diameter,  
174 0.15 $\mu\text{m}$  film thickness) (Agilent Technologies, Les Ulis, France). A TQ8050 (Shimadzu,  
175 Japan) triple quadrupole mass spectrometer equipped with a chemical ionization source and  
176 operating in Q3 single ion monitoring (SIM) mode was used for detection. The reagent gas for  
177 the NCI detection was methane. The GC was performed in pulsed splitless mode with a 1 min  
178 pulsed splitless-time. The oven temperature was initially 150°C for 0.50 min, further  
179 increased to 305°C at 20°C/min and held at 305°C for 3.60 min, and then to 335°C at  
180 30°C/min and held at 335°C for 1.7 min. The injection port and transfer line temperatures  
181 were respectively 290 and 280°C. The flow-rate of helium (carrier gas) was maintained  
182 constant at 0.96 ml/min. The mass spectrometer CI source temperature was 220°C. The  
183 linearity of steroid measurement was confirmed by plotting the ratio of the steroid peak  
184 response / internal standard (IS) peak response to the concentration of E2 for each calibration  
185 standard. Accuracy, target ions, corresponding deuterated internal control, range of detection,  
186 low limit of quantification (LLOQ), and intra & inter assay CVs of the quality control are  
187 reported in Supplemental Table 1.

### 188 **2.3 Determination of total plasma cholesterol**

189 Mice were anesthetized and blood samples were collected from the retro-orbital venous  
190 plexus. Total plasma cholesterol was assayed using the CHOD-PAD kit (Horiba ABX,  
191 Montpellier, France).

### 192 **2.4 Histological analysis**

193 Paraffin-embedded transverse sections (4  $\mu\text{m}$ ) from formalin-fixed uterine or vaginal  
194 specimens were stained as previously described with anti-Ki67 (Abot *et al.*, 2013) (RM-9106;  
195 Thermo-scientific). Sections were examined after digitization using a NanoZoomer Digital  
196 Pathology<sup>®</sup>. To examine the proliferative effects of each treatment, the ratio of Ki67-positive  
197 epithelial cell/total cell number from two microscopic fields of measurement at x20  
198 magnification for each uterine and vaginal section was evaluated. For uterus, the luminal  
199 epithelial height (LEH) of endometrium is the mean of 10 measurements.

### 200 **2.5 Peripheral quantitative computed tomography (pQCT):**

201 Computerized tomography scans were performed using the pQCT XCT RESEARCH M  
202 (version 4.5B, Norland Stratec, Germany) with a voxel size of 70  $\mu\text{m}$ , as previously described  
203 (Windahl *et al.*, 1999). To measure trabecular volumetric bone marrow density (BMD), the  
204 scan was positioned in the distal metaphysis of the femur at a distance corresponding to 3.4%  
205 of the total femur length in the proximal direction from the distal growth plate. This area  
206 contains trabecular and cortical bone and the trabecular region was defined as the inner 45%  
207 of the total cross-sectional area. The cortical bone parameters were analyzed in the mid-  
208 diaphyseal region of femur (Vidal *et al.*, 2000).

### 209 **2.6 Cell culture and transfection**

210 HeLa, HepG2 and MDA-MB231 cell lines were maintained in DMEM (Invitrogen)  
211 supplemented with 10% fetal calf serum (FCS) (Biowest) and antibiotics (Invitrogen) at 37 °C in  
212 5% CO<sub>2</sub>. Transfections were carried out using jetPEI reagent according to manufacturer's

213 instructions (Polyplus transfection). One day before transfection, cells were plated in 24-well  
214 plates at 50% confluence. One hour prior to transfection, the medium was replaced with phenol  
215 red-free DMEM (Invitrogen) containing 2.5% charcoal-stripped FCS (Biowest). Transfection was  
216 carried out with 100 ng of the reporter gene C3-LUC, 100 ng of CMV- $\beta$ Gal internal control and  
217 50 ng of expression vectors pCR-ER $\alpha$  or pCR-ER $\alpha$ AF1<sup>0</sup> (Merot *et al.*, 2004). Following an  
218 incubation overnight, cells were treated for 24 h with ethanol (vehicle control) or increasing  
219 concentration of E2. Cells were then harvested and luciferase and  $\beta$ -galactosidase assays were  
220 performed as previously described (Merot *et al.*, 2004).

## 221 **2.7 Western Blot**

222 One  $\mu$ g of pCR-ER $\alpha$ WT or pCR-ER $\alpha$ AF1<sup>0</sup> plasmid were transcribed and translated using  
223 TNT<sup>®</sup> quick system Promega. Total proteins were separated on a 10% SDS/PAGE gel and  
224 transferred to a nitrocellulose membrane. Primary antibody against ER $\alpha$  was purchased from  
225 Santa Cruz Biotechnology (SP1 ABCAM, 1/500). Revelation was performed using an HRP-  
226 conjugated secondary antibody and visualized by enhanced chemoluminescence (ECL)  
227 detection according to the manufacturer's instructions (Amersham Biosciences/GE  
228 Healthcare), using ChemiDoc Imaging System (Bio-Rad).

## 229 **2.8 Statistics**

230 Statistics analysis were performed using GraphPad Prism software (version 6, GraphPad  
231 Software, Inc.). To test the different doses of E2, a 1-way ANOVA was performed, followed  
232 by a Fisher's LSD's multiple comparison post test. To test the respective roles of E2 treatment  
233 and genotype, a 2-way ANOVA was performed. When a significant interaction was observed  
234 between the 2 factors, the difference between genotype and treatment was evaluated by a  
235 Fisher's LSD's multiple comparison post test.  $P < 0.05$  was considered statistically significant.

236

### 3. Results

#### 3.1 Dose-response profile to E2 is tissue specific in wild type mice.

We first studied *in vivo* the tissue-specific response to different doses of long-term E2 administration (3 weeks) on ovariectomized C57Bl/6 mice (**Figure 1A**). Plasma estradiol measurement allowed us to determine the E2/cholesterol ratio that mimicked either metestrus (low dose, LD-E2 ~1 pg/ml: 3pM) and proestrus (medium dose, MD-E2~10 pg/ml: 30pM) as previously reported (Nilsson *et al.*, 2015), or a pharmacological dose (high dose HD-E2 ~200 pg/ml: 600pM) (**Figure 1B**). In mice on a chow diet, E2 treatment had no impact on body mass (**Figure 1C**) or plasma cholesterol content (**Figure 1D**), regardless of the dose administered. The pharmacological dose led to high level of E2 especially in view of the absence of SHBG in mice. Importantly, in mice receiving the high dose of E2, fraction of E2 was converted into estrone (E1) and became detectable in the plasma (**Supplementary Table 2**). By contrast, the level of estrone in mice supplemented with LD-E2 or MD-E2 is below the threshold of 10 pg/ml.

Wet weight of the uterus represents the classic endpoint for measuring estrogenicity. As expected, ovariectomy led to uterine atrophy compared to intact mice, and all E2 doses increased uterine weight (**Figure 2A**). The maximal response in terms of weight gain was reached with the MD-E2, with an attenuated response observed using the HD-E2 (**Figure 2A**). We next evaluated the level of uterine epithelial proliferation using Ki67 staining. Epithelial proliferation in response to E2 was maximal at the medium dose, whereas the pharmacological dose of E2 led to a much lower epithelial proliferation; this represented a much greater decline than that reflected by uterine weight (**Figures 2B and C**). Accordingly, analysis of the height increase of uterine epithelial cells (LEH) as well as stromal proliferation and density confirmed that the medium dose is required to obtain strongest E2 response (**Table 1**).

262 Although the evaluation of morphological changes in the vagina is less used to assess  
263 estrogenic exposure in the mouse compared to measurements of uterine parameters, this tissue  
264 is another major target for estrogenic action. A maximal response of vagina in terms of weight  
265 gain was observed using the medium dose of E2, and it then plateaued with the medium or  
266 high dose of E2 (**Figure 2D**). By contrast, a low dose was sufficient to induce maximal  
267 epithelial proliferation (**Figures 2E and F**).

268 We next analyzed dose response to E2 treatment in bone, a major non-reproductive  
269 target of estrogens. As expected, ovariectomy significantly altered both cortical (**Figure 3A**)  
270 and trabecular (**Figure 3B**) bone compartments, but did not impact bone length (**Figure 3C**).  
271 Treatment with the lowest dose of E2 was sufficient to restore cortical thickness and cortical  
272 bone mineral content (**Figure 3A**) as well as trabecular bone mineral density (**Figure 3B**). As  
273 observed in reproductive tissues, the medium dose of E2 induced maximal tissue response  
274 thereafter reaching a plateau, with no additional elevation using the pharmacological dose.

275 Altogether, these results demonstrated that using pellets designed to release E2 levels  
276 similar to those encountered during metestrus (low dose) and proestrus (medium dose), the  
277 low dose of E2 stimulated 3 target tissues (*i.e.*, vagina, uterus and bone), whereas the medium  
278 dose was necessary to elicit a maximal effect.

### 279 **3.2 Involvement of ER $\alpha$ AF1 action in mediating E2 response is dose dependent**

280 Our results highlight that response to E2 is dose-dependent, with differences between tissues.  
281 In addition, we previously demonstrated that the role of ER $\alpha$ AF-1 is also tissue-specific, with  
282 a crucial role played in uterus and trabecular bone, but not in cortical bone (Abot *et al.*, 2013;  
283 Borjesson *et al.*, 2011). We first compared the dose response to E2 on an ERE-driven reporter  
284 gene (C3-LUC) in cells expressing either the wild type receptor (ER $\alpha$ 66) or the isoform  
285 having a deletion of AF1 (ER $\alpha$ AF1<sup>0</sup> = ER $\alpha$ 46) (**Figure 4A**) that were introduced into three  
286 different cell lines by transient transfection (**Figures 4B-D**). Expression vectors encoding

287 wild type ER $\alpha$  and ER $\alpha$ AF1<sup>0</sup> were expressed at similar level after transfection in these  
288 different cell types (**Supplementary Figure 1**). In addition to the previously reported cell-  
289 specific differences in the transactivation efficiency between both receptors (Merot *et al.*,  
290 2004), we show here the need for higher doses of E2 for ER $\alpha$ AF-1<sup>0</sup> than for ER $\alpha$ WT to  
291 induce the activity of the reporter gene. The EC<sub>50</sub> was about 10 to 100 fold higher for ER $\alpha$ AF-  
292 1<sup>0</sup> than for ER $\alpha$ WT, depending of the cell-type (**Figures 4B-D**). In addition, even in higher  
293 dose, the maximum response was also much lower using ER $\alpha$ AF-1<sup>0</sup> (**Figure 4B, C**), except  
294 for MDA-MB-231 cells (**Figure 4D**).

295 We then decided to systematically assess the role of ER $\alpha$ AF-1 in the estrogenic response. To  
296 this end, we used ER $\alpha$ AF1<sup>0</sup> mice expressing a 49-kDa truncated protein lacking the A domain  
297 and motifs constituting ER $\alpha$  AF-1 in the B domain (**Figure 5A**) (Billon-Gales *et al.*, 2009).  
298 Since some previous studies have used high doses of estrogens *in vivo*, until 5 mg E2/ pellet  
299 for 60 day release (Pedram *et al.*, 2014), we have decided to add a very high dose (VHD-E2:  
300 ~2000pg/ml *i.e* 2048 pg/ml  $\pm$  242) to evaluate the role of AF-1 function *in vivo*. We  
301 previously reported that ER $\alpha$ AF1<sup>0</sup> mice treated with pellets from Innovative Research (0.1  
302 mg E2/pellet: 60 day release) presented a moderate uterine hypertrophy that was essentially  
303 due to stromal edema, with minimal epithelial proliferation compared to WT mice (Abot *et*  
304 *al.*, 2013). Here, we confirm that no uterine hypertrophy was observed in ER $\alpha$ AF1<sup>0</sup> mice in  
305 response to LD and MD of E2 (**Figure 5B**). However, HD-E2 or VHD-E2 induced similar  
306 increase of uterine weight in ER $\alpha$ AF1<sup>0</sup> and ER $\alpha$ WT mice, demonstrating that the deletion of  
307 the ER $\alpha$  AF1 function can be fully compensated by a 10 to 100-fold increase in the E2 dose  
308 for some parameters such as uterine weigh (**Figure 5B**). For several other parameters, such as  
309 endometrium epithelial proliferation (**Figure 5C**) and vagina weight gain (**Figure 5D**), E2  
310 action was not only shifted rightward but the magnitude of the response was also decreased,  
311 remaining below the full effect observed in control mice, even at the VHD-E2

312 pharmacological dose. In addition, we demonstrate for the first time a role of ER $\alpha$ AF1 in  
313 vaginal epithelial proliferation, which as for uterine epithelial proliferation, is highly  
314 dependent of E2 dose (**Figure 5E**).

315 Finally, we reconsidered the question of the role of ER $\alpha$ AF1 in bone, and, as previously  
316 described, we demonstrated that ER $\alpha$ AF1 is dispensable for cortical response to E2 regardless  
317 of the dose used, as revealed by the absence of interaction between genotype and E2 treatment  
318 (**Figures 6A and B**). It should be however noted a significant effect of genotype due to a  
319 smaller cortical thickness and a smaller cortical BMC in ER $\alpha$ AF1<sup>0</sup> mice, independently on  
320 E2. By contrast, ER $\alpha$ AF1 is required for the increase of bone mineral density (BMD) in  
321 response to E2 in the trabecular compartment regardless of the dose (**Figure 6B**). We  
322 confirmed that E2 has no impact on bone length in both WT and ER $\alpha$ AF1<sup>0</sup> mice but  
323 interestingly in ovariectomized mice, bone lengths from ER $\alpha$ AF1<sup>0</sup> were smaller than in WT  
324 mice, as previously reported in older ER $\alpha$ AF1<sup>0</sup> mice (Borjesson *et al.*, 2012) (**Figure 6C**).  
325 Altogether, these data definitely demonstrate that ER $\alpha$ AF1 is necessary for the full E2  
326 response in uterus, vagina and the trabecular bone compartment. However, depending on the  
327 phenotypic feature, ER $\alpha$ AF1 deficiency can be partially or fully overcome by using high E2  
328 levels.

#### 329 **4. Discussion**

330 To understand the physiology of estrogens, as well as to optimize the use of female  
331 sex hormones in medicine, it is crucial to precisely delineate the sensitivity of the tissues to  
332 estrogen levels. It is a common practice to ovariectomize mice to eliminate the main source of  
333 endogenous estrogens, and subsequently to administer E2 exogenously. Subcutaneous  
334 implantation of slow-release pellets is the most convenient and efficient way to deliver  
335 estrogen to mice; it avoids the stress associated with daily subcutaneous injections. In our  
336 study, the low dose of E2 (metestrus level) stimulated vagina, uterus and bone tissues in

337 ovariectomized mice, whereas the medium dose (proestrus level) was necessary to elicit a  
338 maximal effect. However, use of a supraphysiological dose (HD-E2) did not confer any  
339 further stimulation compared to the medium dose in these tissues. The HD of E2 even induced  
340 a lesser response than the MD of E2 in the uterus, but not in bone nor in the vagina. There are  
341 numerous examples of estrogen hormesis, *i.e.* lowering activity levels while increasing doses,  
342 concerning a wide variety of endpoints, including cerebral (Strom *et al.*, 2011; Strom *et al.*,  
343 2009; Strom *et al.*, 2010), mammary gland differentiation (Vandenberg *et al.*, 2006) and  
344 insulin sensitivity (Gonzalez *et al.*, 2002). High concentrations of E2 can also lead to  
345 increased levels of metabolites, such as estrone (**Supplementary Table 2**), which can also  
346 influence the biological responses through either dependent or independent ER mechanisms  
347 (Thomas and Potter, 2013).

348 Remarkably, despite a significant effect on cortical thickness and BMC as well as on  
349 trabecular BMD, E2 even at a very high dose had no impact on bone length in our  
350 experimental conditions. This result differs from previous studies that reported a reduced bone  
351 length and growth plate width using high dose of E2 in older mice (Borjesson *et al.*, 2012;  
352 Borjesson *et al.*, 2010). These apparent discrepancies underline the importance of the age of  
353 ovariectomy, age of mouse studied, and timing of E2 supplementation.

354 E2 effects are mediated by hormone binding to ER $\alpha$ , ER $\beta$  or GPR30 (Miller *et al.*,  
355 2017). ER $\alpha$  also exists as two isoforms, designated ER $\alpha$ 66 and ER $\alpha$ 46, based on their  
356 respective molecular weight, and both isoforms were reported to regulate E2-mediated  
357 proliferation in the mouse uterus (Abot *et al.*, 2013). The expression of ER $\alpha$ 46 in the rat  
358 uterus was demonstrated even before the use of antibodies against ER $\alpha$  (Faye *et al.*, 1986).  
359 Importantly, we recently demonstrated that ER $\alpha$ 46 is frequently and sometimes abundantly  
360 expressed in ER $\alpha$ -positive breast tumors, but the mechanisms of its generation as well as  
361 pathophysiological significance of its expression are essentially unknown (Chantalat *et al.*,



2016). Furthermore, the specific signalling events associated with this AF1-deficient isoform as well as its interactions with the full length ER $\alpha$ 66 remain unclear. Given the complexity of estrogen-mediated actions, which goes far beyond the single-receptor situation on which the linear dose-response model is based, it is not surprising that E2 can elicit hormetic responses. In particular, varied affinities for distinct receptors having different intrinsic activity levels would essentially lead to a “mixture” effect, where the summation of their individual dose-response curves would lead to composite behaviour that could be non-monotonic (Miller *et al.*, 2017). In particular, we previously proposed that ER $\alpha$ 46 counteracts ER $\alpha$ 66 on E2-induced cell proliferation (Abot *et al.*, 2013; Penot *et al.*, 2005).

The structural difference between ER $\alpha$ 66 and ER $\alpha$ 46, according to our current knowledge, resides mainly in the absence of AF1 in ER $\alpha$ 46 (Chantalat *et al.*, 2016). Accordingly, both receptors exhibit different transactivation efficiencies in a cell-specific manner (Merot *et al.*, 2004). It has been proposed that ER $\alpha$ 46 could have a repressive role in AF1-permissive contexts through interference with ER $\alpha$ 66 binding to DNA (Penot *et al.*, 2005). The present study deepens the comparison between both ER $\alpha$  forms by revealing a major difference in their transcriptional sensitivity to the dose of E2. To reach the maximal level of ligand-dependent transcriptional activity, ER $\alpha$ 46 requires an E2 dose 10 to 100 times higher than does ER $\alpha$ 66. Several studies have shown that N-terminal truncated forms of ER $\alpha$ , including ER $\alpha$ 46, have an affinity for E2 which is similar to that found for the wild-type ER $\alpha$ 66, indicating that the hormone binding domain is able to function independently of the N-terminal half of the ER (Kumar *et al.*, 1986; Carlson *et al.*, 1997; Lin *et al.*, 2013). However, differences in ER $\alpha$  binding to DNA and in coactivator and/or corepressor recruitment could explain the distinctively lower sensitivity of ER $\alpha$ 46 *versus* ER $\alpha$ 66 to the dose of E2. We previously reported that although the qualitative overall recruitment of cofactors is quite similar between ER $\alpha$ 46 and ER $\alpha$ 66, there is a quantitative differential

387 modulation with some preference for cofactor binding to ER $\alpha$ 66 over ER $\alpha$ 46 (Chantalat *et al.*,  
388 2016). ER $\alpha$ 46 is also able to recruit transcriptional repressors in a ligand-independent manner  
389 (Metivier *et al.*, 2004). In accordance with the specific binding of coregulators, we reported  
390 that some genes were specifically regulated by either the ER $\alpha$ 46 or ER $\alpha$ 66 isoforms,  
391 demonstrating that the absence of ER $\alpha$ AF1 affects specific transcriptional programs rather  
392 than leading to an overall decreased activity (Chantalat *et al.*, 2016) .

393         Using mice harbouring an ER $\alpha$  deleted for AF1 (ER $\alpha$ AF1<sup>0</sup>), we previously  
394 demonstrated that ER $\alpha$ AF1 is necessary to induce uterine hypertrophy, suggesting that  
395 ER $\alpha$ 46 is not able to mediate E2 responses in uterus (Abot *et al.*, 2013). Here, we  
396 demonstrate that with a HD of E2, the increase in uterine weight is similar between ER $\alpha$ AF1<sup>0</sup>  
397 and ER $\alpha$ WT, although an attenuated response is observed in terms of epithelial proliferation.  
398 By contrast, physiological LD or MD doses of E2 leading to levels encountered during the  
399 estrous cycle were unable to induce a uterine response in ER $\alpha$ AF1<sup>0</sup> mice in contrast to  
400 ER $\alpha$ WT mice. These results demonstrate that the involvement of ER $\alpha$ AF1 in the E2  
401 response is highly dependent on ligand dose, and that different signalling pathways regulate  
402 epithelial proliferation and uterine weight gain. Similarly, the response to E2 in vagina and  
403 trabecular bone are dependent of ER $\alpha$ AF-1, since this response is not only shifted rightward  
404 but also decreased in intensity, suggesting that these tissues can be used as an index of  
405 ER $\alpha$ AF-1-dependent estrogenicity.

406         There is also evidence that amino acid phosphorylation located in the ER $\alpha$ AF-1  
407 domain, regulates numerous aspects of ER $\alpha$  function, including protein stability, hormone  
408 binding, receptor dimerization, DNA binding, protein-protein interactions, and transcription  
409 activity (Orti *et al.*, 1992; Lannigan, 2003; Weigel and Moore, 2007). In particular, Ser118  
410 has been implicated in ER $\alpha$  mediated transcriptional regulation since a partial or complete

411 attenuation of E2-induced transcription has been observed in ER $\alpha$ S118A transfected cells  
412 (Ali *et al.*, 1993; Duplessis *et al.*, 2011). Appropriate transgenic mouse studies should be  
413 developed to determine the importance of ER $\alpha$  Ser118 phosphorylation *in vivo*.

414

## 415 **5. Conclusion**

416 We describe here the dose-effect of E2 on sexual targets and bone thanks to a simple, reliable  
417 and unexpansive slow release device. Our results demonstrate that the estrogenic effect on  
418 tissues is highly dependent on the dose used. Although the precise role of ER $\alpha$ AF1 both in  
419 physiology and in pathology remains to be clarified, the present work reveals that ER $\alpha$ AF1  
420 provides an important layer of regulation through the sensitization of the response to  
421 estrogens. The role of ER $\alpha$ AF1 can be even more spectacular in the action of some selective  
422 ER modulators (SERMs) (Arao and Korach, 2019). For instance, tamoxifen selectively  
423 activates ER $\alpha$ AF1, and this activation is required for the beneficial effects of this SERM on  
424 arteries (Fontaine *et al.*, 2013) and metabolism (Guillaume *et al.*, 2017), while at the same  
425 time it prevents the recurrence of breast cancer. Whereas antagonist activity of tamoxifen is  
426 essentially through the ER $\alpha$ AF2 blockade, molecular mechanism of its partial agonist activity  
427 that involved ER $\alpha$ AF1 activation is not fully understood (Arao and Korach, 2019). Here, we  
428 report that importance of ER $\alpha$ AF1 domain in estrogenic response depend on both considered  
429 cell type/ tissues and ligand dose, two parameters which could be determinant in the agonist  
430 activity of E2 or SERM that control ER $\alpha$ AF-1-mediated physiological response.

431

432

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**588 FIGURE LEGEND**

**589 Figure 1: Plasma estradiol (E2) and cholesterol concentrations in mice implanted with**  
**590 E2/cholesterol pellets.**

591 Pellets releasing low dose (LD), medium dose (MD) or high dose (HD) of E2 were implanted  
592 subcutaneously into ovariectomized mice for a period of 3 weeks (A). Plasma E2 (B), body  
593 weight (C) and cholesterol content (D) of the mice were evaluated.

**594 Figure 2: E2 dose response analysis of uterine and vaginal parameters**

595 Pellets releasing low dose (LD), medium dose (MD) or high dose (HD) of E2 were implanted  
596 subcutaneously into ovariectomized mice for a period of 3 weeks and uterus (A, B, C) and  
597 vagina (D, E, F) were analyzed. Uterine (A) and vagina (D) weight. Percentage of uterine (B)  
598 and vagina (E) Ki67-positive epithelial cells. Results are expressed as means  $\pm$  SEM. To test  
599 effect of E2 exposure, one-way ANOVA and a Fisher's LSD's post test were performed (E2  
600 exposure *versus* placebo \*P < 0.05, \*\*\*P < 0.001, difference in E2 concentration  $\dagger\dagger\dagger$ P<0.001  
601 n=10/12 mice/group). Representative Ki-67 detection in transverse uterus (C) and vagina (F)  
602 sections.

**603 Figure 3: E2 dose response analysis of bone parameters**

604 Pellets releasing low dose (LD), medium dose (MD) or high dose (HD) of E2 were implanted  
605 subcutaneously into ovariectomized mice for a period of 3 weeks and femurs were analyzed.  
606 Cortical thickness and cortical bone mineral content (BMC) (A) in the mid-diaphyseal part of  
607 femur. Trabecular bone mineral density (BMD) in the distal metaphyseal part of femur (B)  
608 and Bone length (C). Results are expressed as means  $\pm$  SEM. To test effect of E2 exposure,  
609 one-way ANOVA and a Fisher's LSD's post test were performed (E2 exposure *versus* placebo  
610 \*\*P < 0.01, \*\*\*P < 0.001, difference in E2 concentration  $\dagger\dagger\dagger$ P<0.001, n=6/7 mice/group).

**611 Figure 4: Dose response of E2 transcriptional activity in cultured cells expressing ER $\alpha$ 66**  
**612 or ER $\alpha$ 46.**



613 Schematic representation of the wild-type ER $\alpha$ 66 (ER $\alpha$ AF1<sup>+/+</sup>) and ER $\alpha$  (ER $\alpha$ AF1<sup>0</sup>) isoforms  
614 and expression of these isoforms obtained from in vitro translation pCR-ER $\alpha$ WT or pCR-  
615 ER $\alpha$ AF1<sup>0</sup> vectors performed with the TNT Coupled Reticulocyte Lysate System (A). HepG2  
616 (B), HeLa (C) and MDA-MB231 (D) cells were transiently transfected with the C3-Luc  
617 reporter constructs in the presence of pCR-ER $\alpha$ WT, pCR-ER $\alpha$ AF1<sup>0</sup>, or empty pCR vectors.  
618 Cells were treated with the indicated dose of E<sub>2</sub> or vehicle (Ctrl) for 24 h. Normalized  
619 luciferase activities were expressed as fold increase above values measured with empty pCR  
620 and vehicle. Data correspond to the mean values  $\pm$  SEM of at least three separate transfection  
621 experiments. Two-way ANOVA followed by Fisher's LSD's multiple comparison test were  
622 performed (ER $\alpha$ AF1<sup>+/+</sup> versus ER $\alpha$ AF1<sup>0</sup>: \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001).

623 **Figure 5: The role of ER $\alpha$ AF1 in uterine and vagina is dependent of E2 dose**

624 Schematic representation of the wild-type ER $\alpha$ AF1<sup>+/+</sup> and ER $\alpha$ AF1<sup>0</sup> mice constructs (A).  
625 Pellets releasing low dose (LD), medium dose (MD), high dose (HD) or very high dose  
626 (VHD) of E<sub>2</sub> were implanted subcutaneously into ER $\alpha$ AF1<sup>0</sup> ovariectomized mice during 3  
627 weeks. Uterus (B, C) and vagina (D, E) were analyzed. Uterine (B) and vagina (D) weight.  
628 Percentage of uterine (C) and vagina (E) Ki67-positive epithelial cells. Representative Ki-67  
629 detection in transverse uterus sections. Results are expressed as means  $\pm$  SEM. Two-way  
630 ANOVA followed by Fisher's LSD's multiple comparison test were performed (ER $\alpha$ AF1<sup>+/+</sup>  
631 versus ER $\alpha$ AF1<sup>0</sup>: \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, n=6/8 mice/group).

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633

634 **Figure 6: E2 dose response analysis of trabecular and cortical bone parameters in wild-**  
635 **type ER $\alpha$ AF1<sup>+/+</sup> and ER $\alpha$ AF1<sup>0</sup> mice.**

636 Pellets releasing low dose (LD), medium dose (MD), high dose (HD) or very high dose  
637 (VHD) of E2 were implanted subcutaneously to ER $\alpha$ AF1<sup>0</sup> ovariectomized mice for a period  
638 of 3 weeks and femurs were analyzed. Cortical thickness and cortical bone mineral content  
639 (BMC) in the mid-diaphyseal part of femur. 2-way ANOVA test revealed no significant  
640 interaction, the overall effect of the treatment and genotype was analyzed (A). Trabecular  
641 bone mineral density (BMD) in the distal metaphyseal part of femur (B) and Bone length (C).  
642 Results are expressed as means  $\pm$  SEM. Since two-way ANOVA test revealed a significant  
643 interaction between the 2 factors (E2 exposure and genotype), a Fisher's LSD's multiple  
644 comparison test were performed (ER $\alpha$ AF1<sup>+/+</sup> versus ER $\alpha$ AF1<sup>0</sup>: \*\*P < 0.01, \*\*\*P < 0.001,  
645 n=6/8 mice/group) (B, C).

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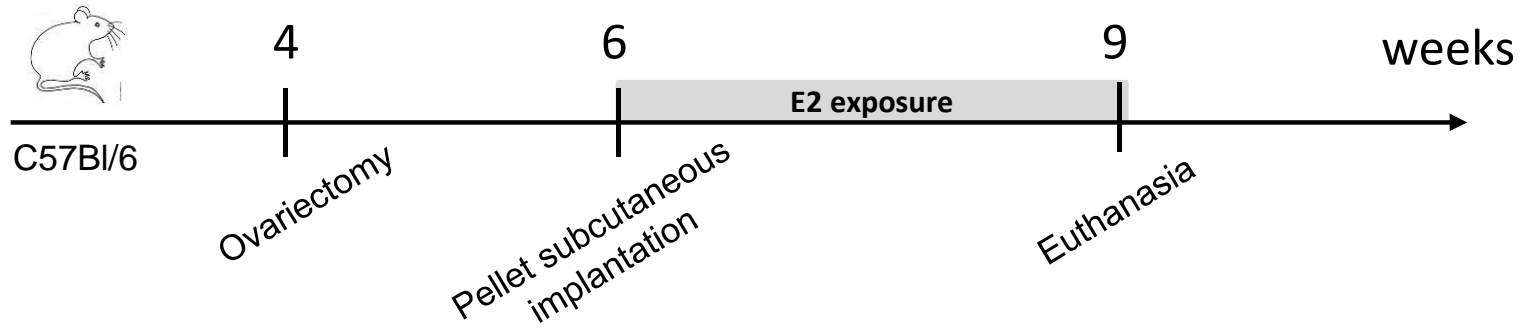
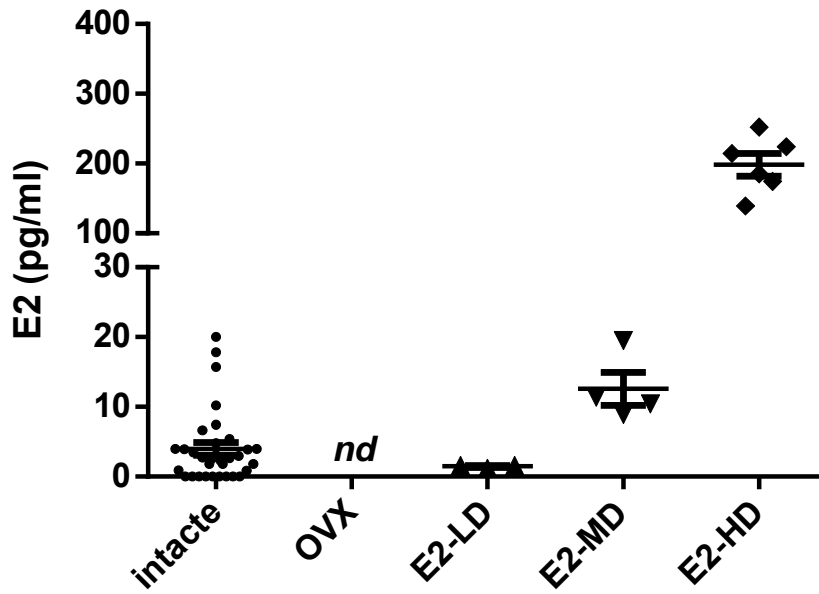
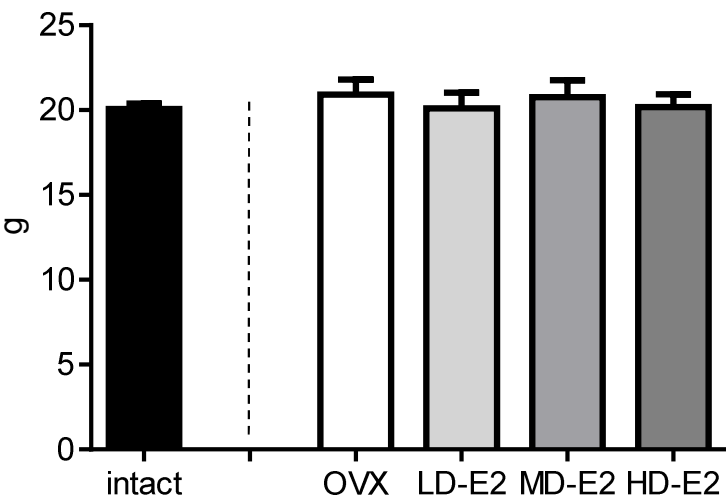
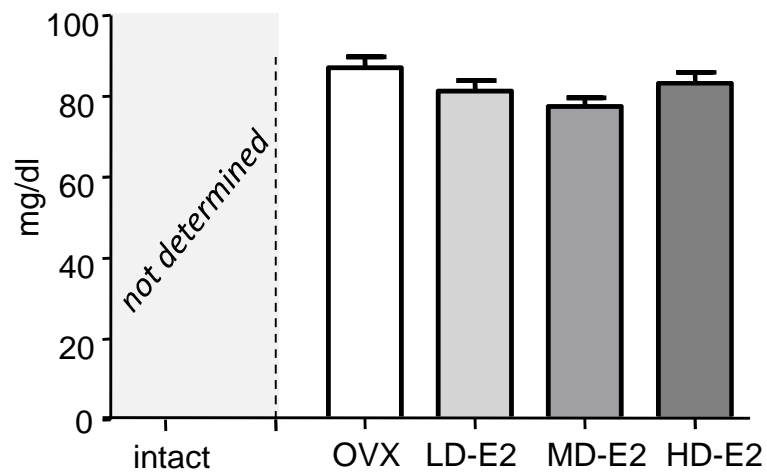
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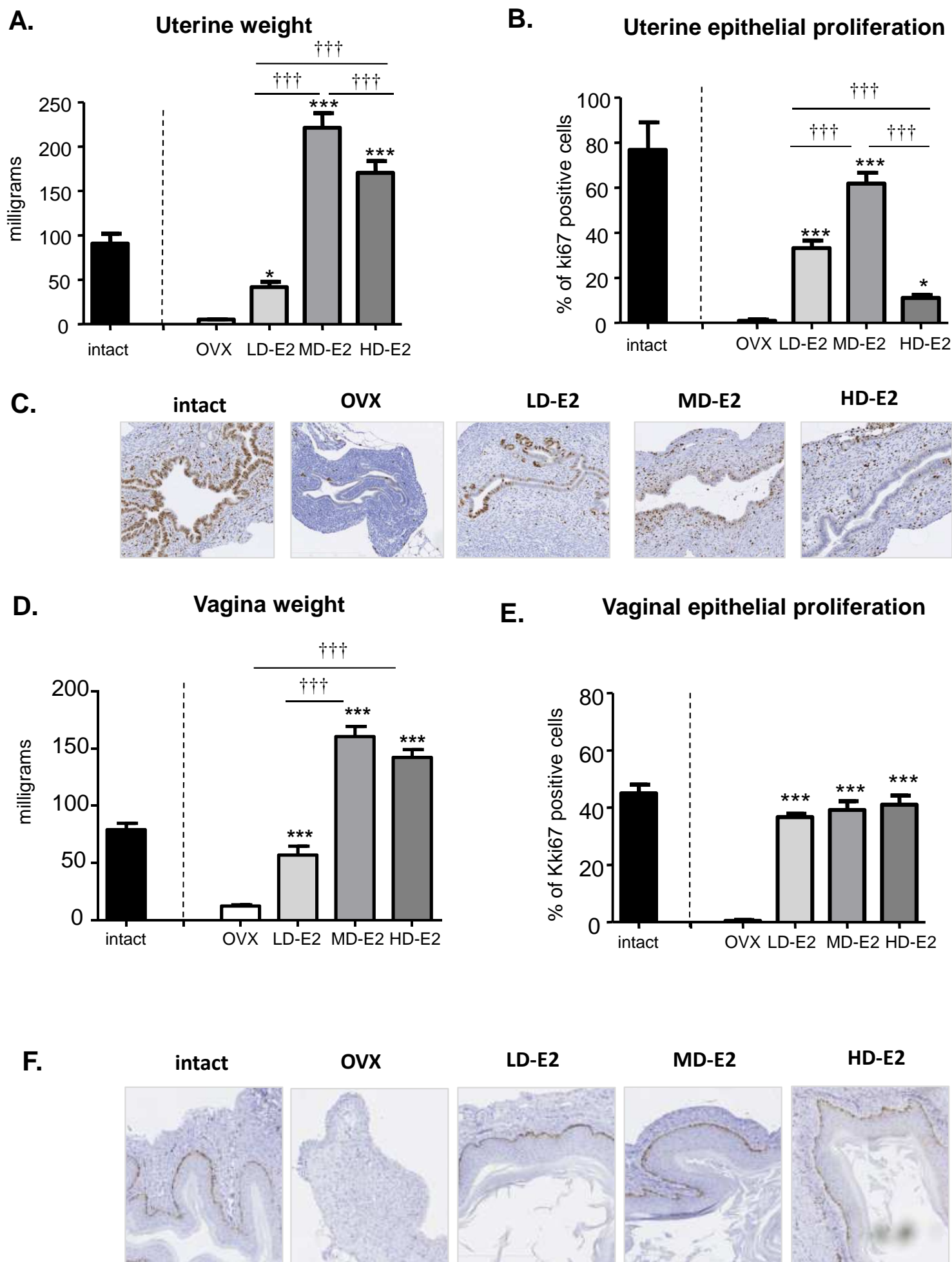
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650

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652

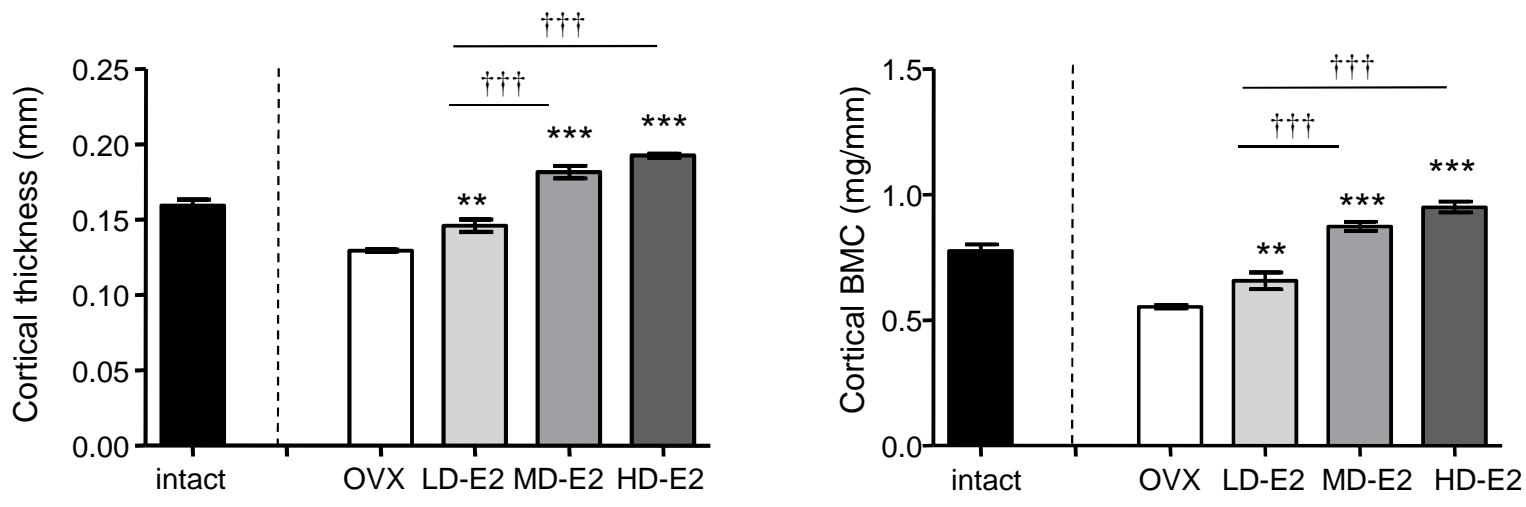
**A.****B.****Plasma E2****C.****Body weight****D.****Plasma Cholesterol****Figure 1**



**Figure 2**

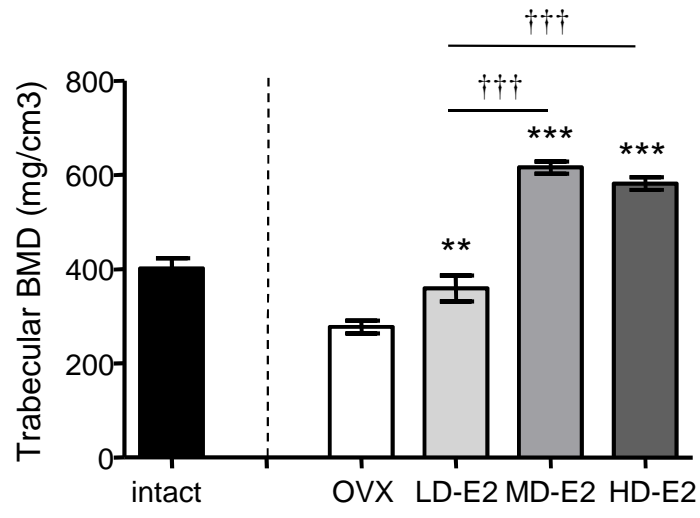
**A.**

**Cortical compartment**



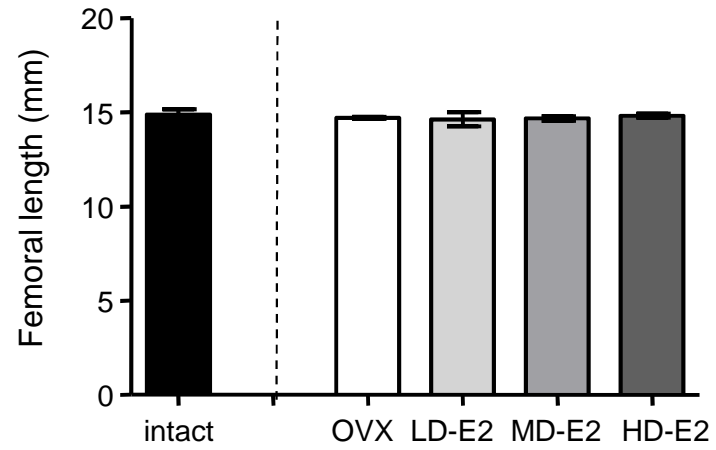
**B.**

**Trabecular compartment**

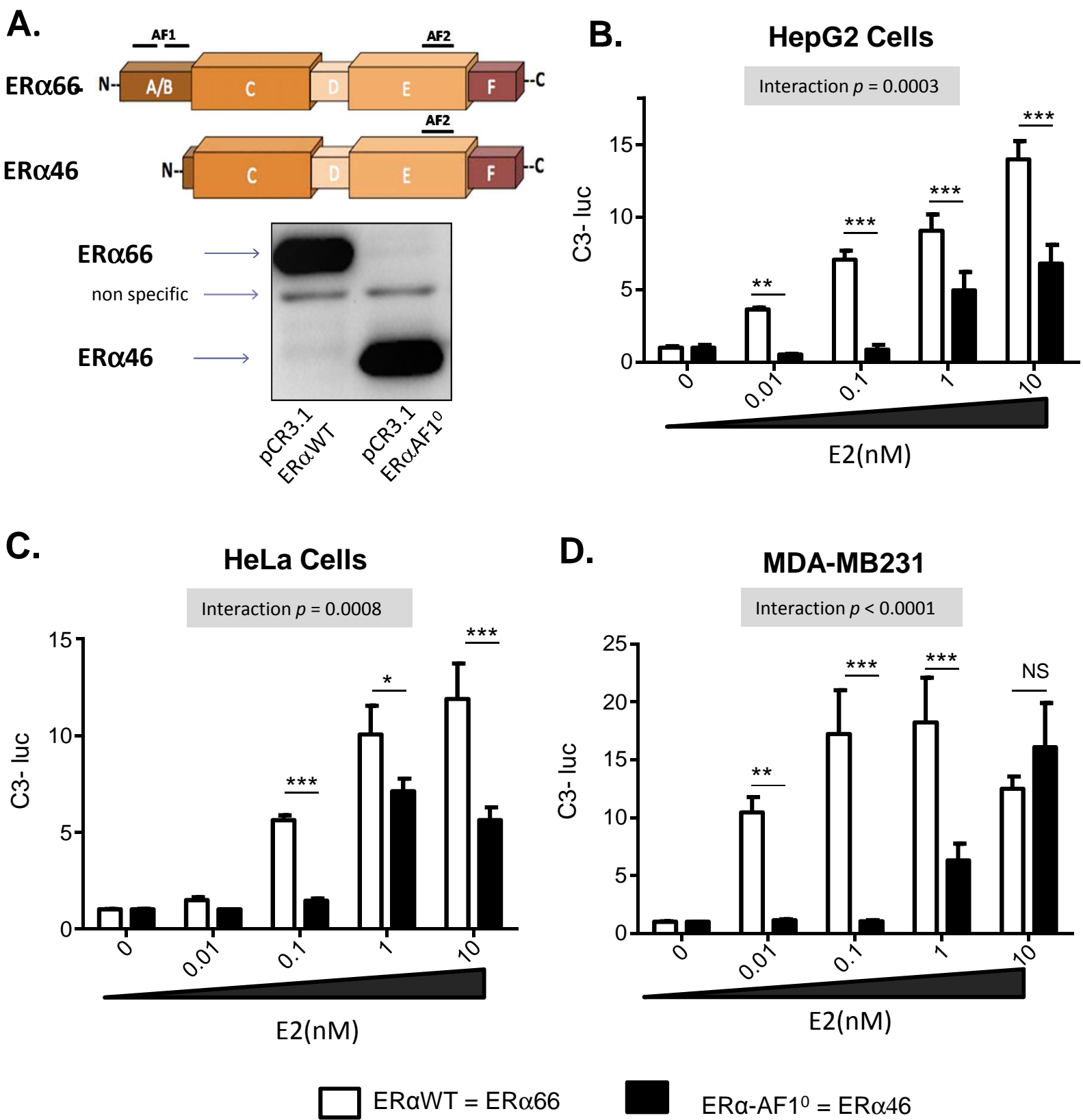


**C.**

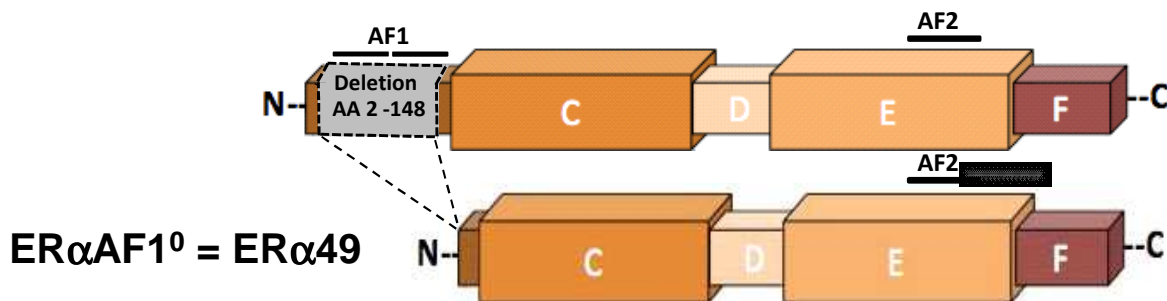
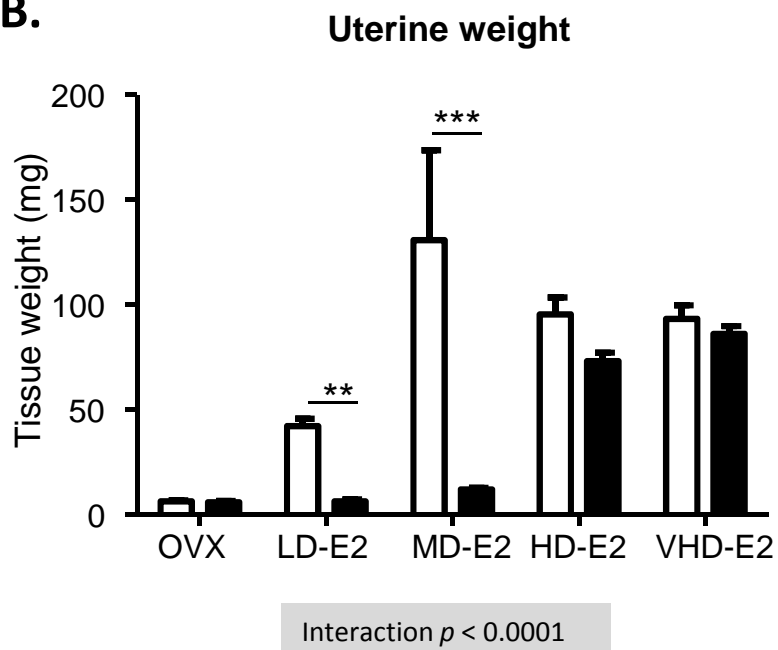
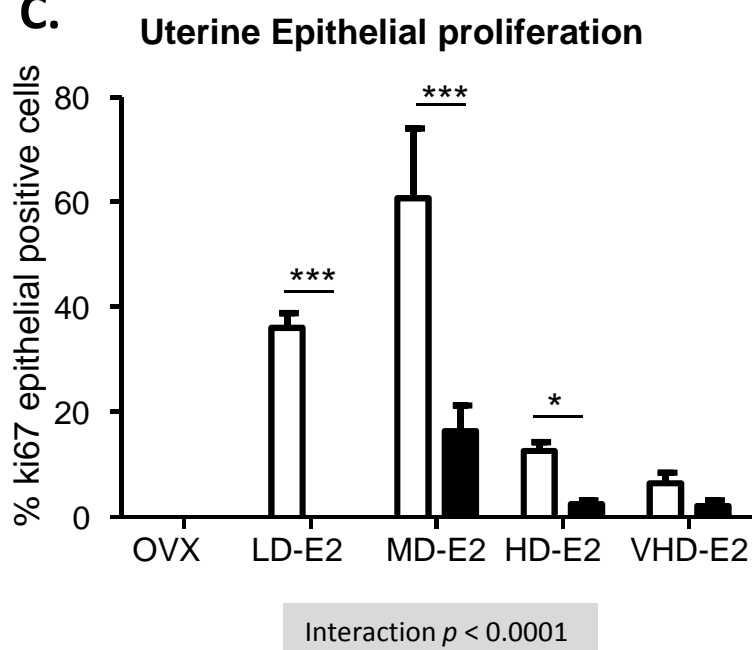
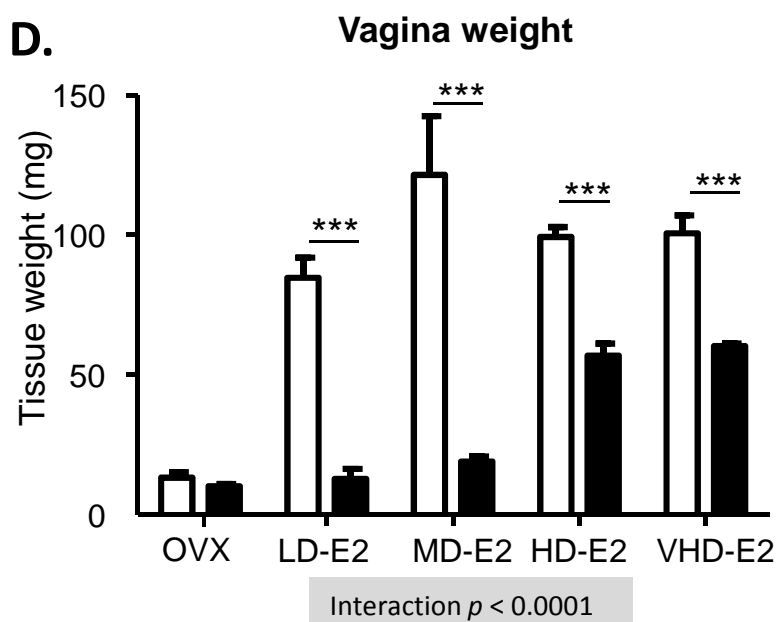
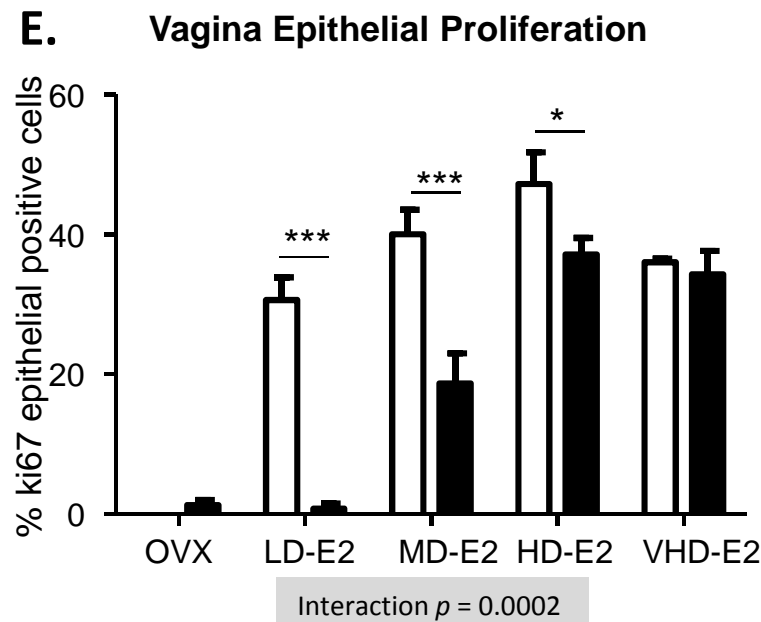
**Bone length**



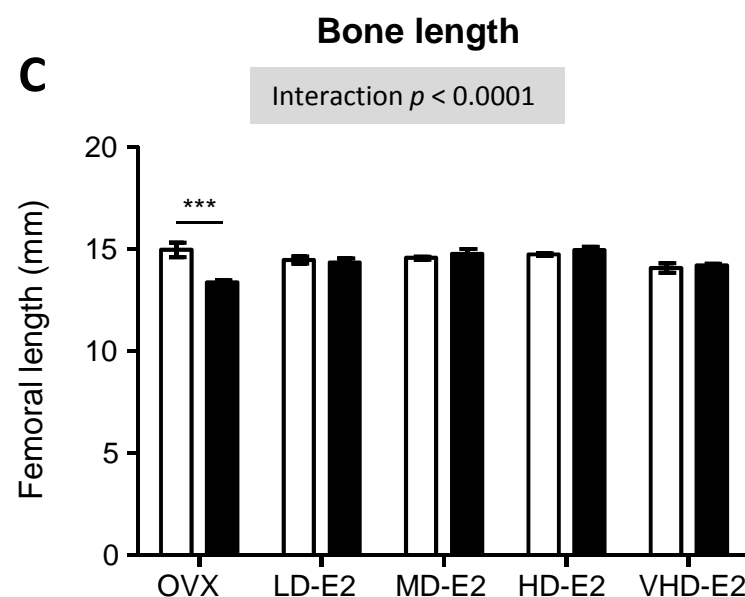
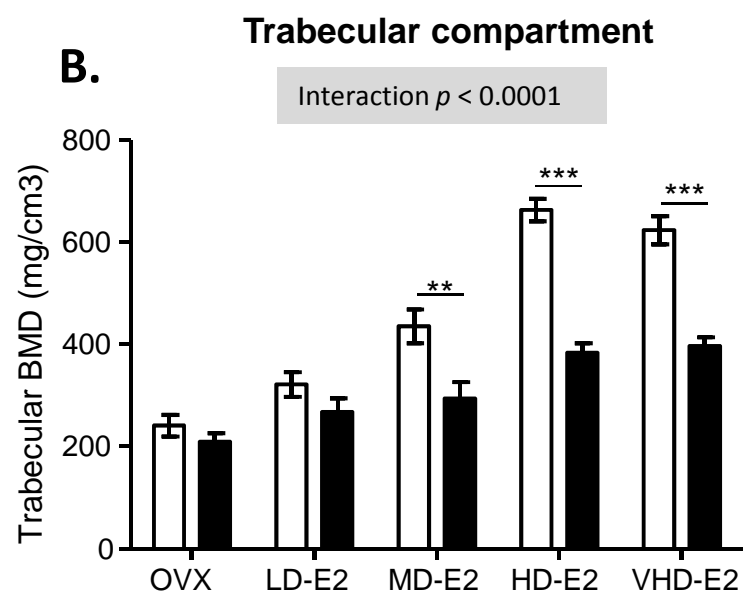
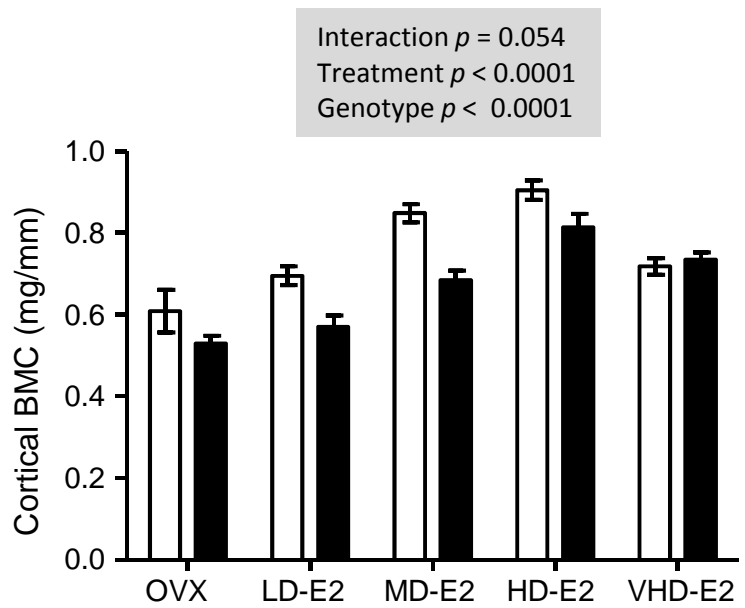
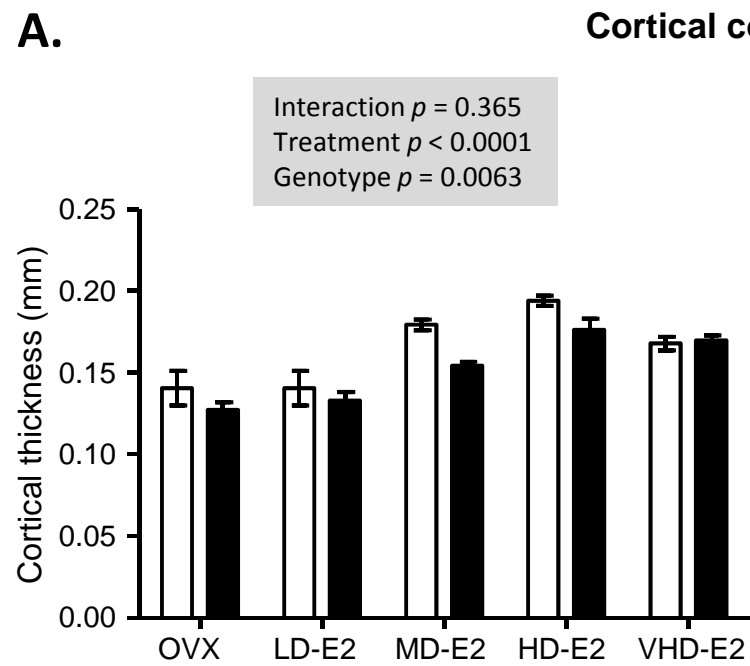
**Figure 3**



**Figure 4**

**A.****B.****C.****D.****E.**
 ER $\alpha$ -AF1<sup>+/+</sup>

 ER $\alpha$ -AF1<sup>0</sup>
**Figure 5**



□ ER $\alpha$ -AF1<sup>+/+</sup>

■ ER $\alpha$ -AF1<sup>0</sup>

**Figure 6**



	OVX	LD-E2	MD-E2	HD-E2
<b>Uterine LEH (<math>\mu\text{m}</math>)</b>	12.1 +/- 0.6	20.2 +/- 1.2 **	37.6 +/- 1.9 ***	40.5 +/- 2.1 ***
<b>Uterine Stromal Density</b>	257 +/- 1.14	116.7 +/- 44.6 ***	87 +/- 35.5 ***	72.7 +/- 34.5 ***
<b>Uterine Stromal proliferation (%Ki67positive cells)</b>	0.3 +/- 0.5	8 +/- 5.6	42.1 +/- 11 ***	21.6 +/- 8 ***

Results are expressed as means  $\pm$  SEM. To test the respective roles of each treatment, one-way ANOVA and a Fisher's LSD's *post test* were performed (treatment versus placebo \*\*, P < 0.01, \*\*\*, P < 0.001)

**Table 1:** Analysis of uterine parameters in C57Bl/6 mice

Accuracy (%)	Analyte	Target ion analyte /IS (amu)	Range (pg/ml) R <sup>2</sup>	Mean (pg/ml) 17 runs <i>Intra- &amp; Inter assay CVs (%)</i>			
				LLOQ	Low QC	Middle QC	High QC
94-107	E2	660/664	0.2- 56.0 0.9994	0.22 17.3 -19.6	2.87 2.9-4.2	6.07 2.6-3.7	12.88 2.3-3.33

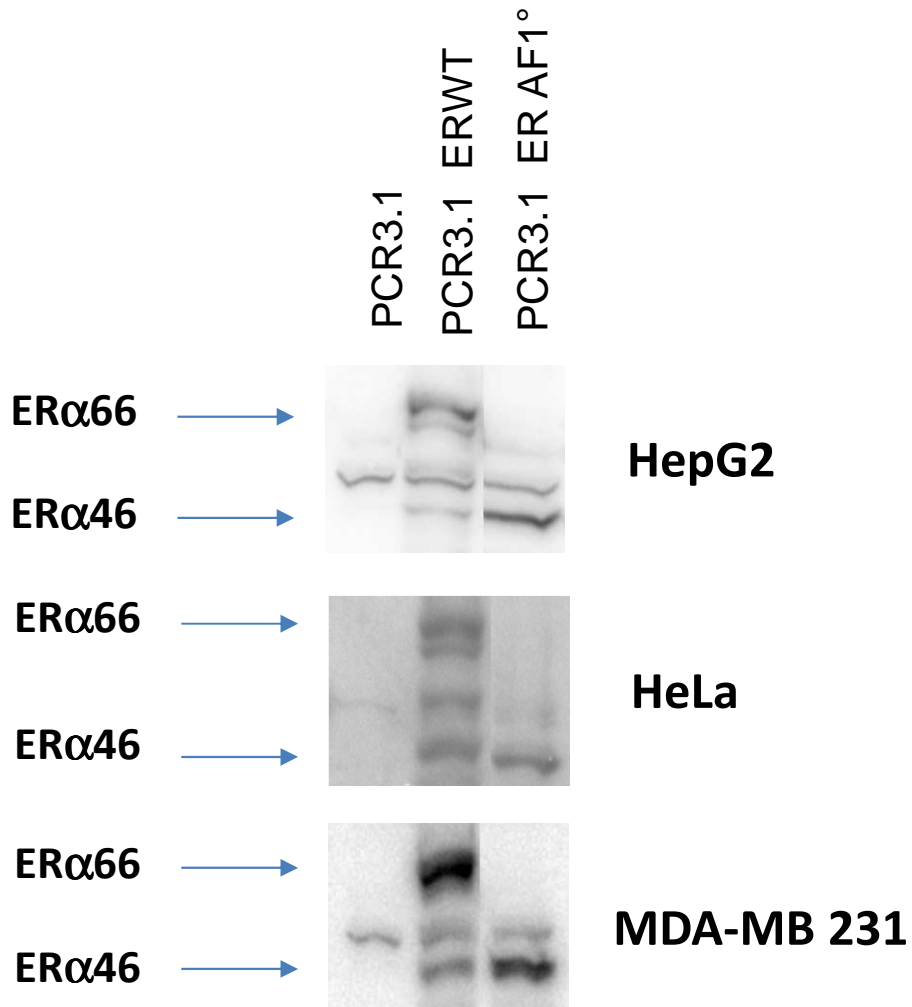
*LLOQ : low limit of quantification; QC : quality control*

**Supplementary Table 1 : GC/MS plasma analytical control validation**

	<b>OVX</b>	<b>LD-E2</b>	<b>MD-E2</b>	<b>HD-E2</b>
<b>Estrone (E1)</b>	< 10pg/ml	< 10pg/ml	< 10pg/ml	22,5 +/- 5,4

Results are expressed as means  $\pm$  SEM.

**Supplementary Table 2: GC/MS plasma Estrone (E1) dosage**



**Supplementary Figure 1:** Western blot analysis was performed on an equal amount of cellular extracts prepared from cells transfected with empty vectors (lane 1) expression vectors encoding wild type (lane 2), or ERαAF1<sup>0</sup> mutant (lane 3).

**HIGHLIGHTS**

- Some biological response to E2 displays biphasic dose relationship.
- ER $\alpha$ AF1 plays a key sensitizing role in ER $\alpha$  activity.
- Differences and even contradictory conclusions could be explained by differences of E2 doses.