The tissue-specific effects of different 17β-estradiol doses reveal the key sensitizing role of AF1 domain in ERα activity

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HIGHLIGHTS

- Some biological response to E2 displays biphasic dose relationship.
- ERαAF1 plays a key sensitizing role in ERα activity.
- Differences and even contradictory conclusions could be explained by differences of E2 doses.

ABSTRACT

17β-Estradiol (E2) action can be mediated by the full-length estrogen receptor alpha (ERα66), but also by the AF1 domain-deficient ERα (ERα46) isoform, but their respective sensitivity to E2 is essentially unknown. To this aim, we first performed a dose response study using subcutaneous home-made pellets mimicking either metestrus, proestrus or a pharmacological doses of E2, which resulted in plasma concentrations around 3, 30 and 600 pM, respectively. Analysis of the uterus, vagina and bone after chronic exposure to E2 demonstrated dose-dependent effects, with a maximal response reached at the proestrus- dose in wild type mice expressing mainly ERα66. In contrast, in transgenic mice harbouring only an ERα deleted in AF1, these effects of E2 were either strongly shifted rightward (10 to 100-fold) and/or attenuated, depending on the tissue studied. Finally, experiments in different cell lines transfected with ERα66 or 46 also delineated varying profiles of ERα AF1 sensitivity to E2. Altogether, this work emphasizes the importance of dose in the tissue-specific actions of E2 and demonstrates the key sensitizing role of AF1 in ERα activity.

Keywords: Estrogen receptor alpha (ERα), activation function (AF*)*, dose response, tissue-specific

1. Introduction

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

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

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

The different responses to E2 are initiated by their binding to the estrogen receptors (ERs), ERα and ERβ, which belong to the nuclear receptor superfamily and are structurally organized into 6 functional domains (A to F). The E domain supports hormone binding, which induces conformational changes that are required for ER transcriptional activity through the modulation of two activation functions (AFs), AF1 and AF2, located in the A/B and E domains, respectively. In addition to the full-length 66-kDa (ERα66) isoform, a 46-kDa ERα-isoform (ERα46), lacking the N-terminal portion (domains A/B), and thereby AF-1, has been reported to be expressed in various cell types such as human osteoblasts (Denger *et al.*, 2001), macrophages (Murphy *et al.*, 2009), and vascular endothelial cells (Murphy *et al.*, 2009), but also in cancer cells (Chantalat *et al.*, 2016). Mechanisms regulating both the expression of ERα46 and its functions remain essentially unknown. ERα can be generated by either alternative splicing, proteolysis, or an alternative initiation of translation via an internal ribosome entry site (IRES) (Chantalat *et al.*, 2016).

ER-mediated transcriptional regulation involves either a direct interaction of ER with specific estrogen-responsive elements (EREs) located in enhancers or near the promoter region of target genes, or an indirect mechanism (tethered) via protein/protein interactions with other transcriptional factors (Hamilton *et al.*, 2017). ERαAF1 and ERαAF2 have been shown to play crucial roles in the transcriptional effects of ERα through the recruitment of coactivators. In cultured cells, the respective roles of ERαAF1 and ERαAF2 appear to depend on the state of cell differentiation (Merot *et al.*, 2004). Using mice selectively deficient in ERαAF1 or ERαAF2, we previously demonstrated that ERαAF2 is absolutely required for all nuclear effects of ERα in response to E2 whereas the role of ERαAF1 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 ERα-66 or the AF1/AB domain-deficient ERα 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 ERα deleted for AF1
136 (ERαAF1^{0}) and in its littermate wild type mice.

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2. Material and Methods

2.1 Mice

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

2.2 Hormone assays

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

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

2.3 Determination of total plasma cholesterol

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

2.4 Histological analysis

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

2.5 Peripheral quantitative computed tomography (pQCT):

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

2.6 Cell culture and transfection

HeLa, HepG2 and MDA-MB231 cell lines were maintained in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Biowest) and antibiotics (Invitrogen) at 37 ºC in 5% CO_2. Transfections were carried out using jetPEI reagent according to manufacturer's

instructions (Polyplus transfection). One day before transfection, cells were plated in 24-well plates at 50% confluence. One hour prior to transfection, the medium was replaced with phenol red-free DMEM (Invitrogen) containing 2.5% charcoal-stripped FCS (Biowest). Transfection was carried out with 100 ng of the reporter gene C3-LUC, 100 ng of CMV-βGal internal control and 50 ng of expression vectors pCR-ERα or pCR-ERαAF-1^0 (Merot *et al.*, 2004). Following an incubation overnight, cells were treated for 24 h with ethanol (vehicle control) or increasing concentration of E2. Cells were then harvested and luciferase and β-galactosidase assays were performed as previously described (Merot *et al.*, 2004).

2.7 Western Blot

One µg of pCR-ErαWT or pCR-ERαAF1^0 plasmid were transcribed and translated using TNT® quick system Promega. Total proteins were separated on a 10% SDS/PAGE gel and transferred to a nitrocellulose membrane. Primary antibody against ERα was purchased from Santa Cruz Biotechnology (SP1 ABCAM, 1/500). Revelation was performed using an HRP-conjugated secondary antibody and visualized by enhanced chemoluminescence (ECL) detection according to the manufacturer's instructions (Amersham Biosciences/GE Healthcare), using ChemiDoc Imaging System (Bio-Rad).

2.8 Statistics

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

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**).

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

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

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

3.2 Involvement of ERαAF1 action in mediating E2 response is dose dependent

Our results highlight that response to E2 is dose-dependent, with differences between tissues. In addition, we previously demonstrated that the role of ERαAF-1 is also tissue-specific, with a crucial role played in uterus and trabecular bone, but not in cortical bone (Abot *et al.*, 2013; Borjesson *et al.*, 2011). We first compared the dose response to E2 on an ERE-driven reporter gene (C3-LUC) in cells expressing either the wild type receptor (ERα66) or the isoform having a deletion of AF1 (ERαAF1^0 = ERα46) (**Figure 4A**) that were introduced into three different cell lines by transient transfection (**Figures 4B-D**). Expression vectors encoding

wild type ERα and ERαAF1^0 were expressed at similar level after transfection in these different cell types (**Supplementary Figure 1**). In addition to the previously reported cell-specific differences in the transactivation efficiency between both receptors (Merot *et al.*, 2004), we show here the need for higher doses of E2 for ERαAF-1^0 than for ERαWT to induce the activity of the reporter gene. The EC_{50} was about 10 to 100 fold higher for ERαAF-1^0 than for ERαWT, depending of the cell-type (**Figures 4B-D**). In addition, even in higher dose, the maximum response was also much lower using ERαAF-1^0 (**Figure 4B, C**), except for MDA-MB-231 cells (**Figure 4D**).

We then decided to systematically assess the role of ERαAF-1 in the estrogenic response. To this end, we used ERαAF1^0 mice expressing a 49-kDa truncated protein lacking the A domain and motifs constituting ERα AF-1 in the B domain (**Figure 5A**) (Billon-Gales *et al.*, 2009). Since some previous studies have used high doses of estrogens *in vivo,* until 5 mg E2/ pellet for 60 day release (Pedram *et al.*, 2014), we have decided to add a very high dose (VHD-E2: ~2000pg/ml *i.e* 2048 pg/ml ± 242) to evaluate the role of AF-1 function *in vivo*. We previously reported that ERαAF1^0 mice treated with pellets from Innovative Research (0.1 mg E2/pellet: 60 day release) presented a moderate uterine hypertrophy that was essentially due to stromal edema, with minimal epithelial proliferation compared to WT mice (Abot *et al.*, 2013). Here, we confirm that no uterine hypertrophy was observed in ERαAF1^0 mice in response to LD and MD of E2 (**Figure 5B**). However, HD-E2 or VHD-E2 induced similar increase of uterine weight in ERαAF1^0 and ERαWT mice, demonstrating that the deletion of the ERα AF1 function can be fully compensated by a 10 to 100-fold increase in the E2 dose for some parameters such as uterine weigh (**Figure 5B)**. For several other parameters, such as endometrium epithelial proliferation (**Figure 5C**) and vagina weight gain (**Figure 5D**), E2 action was not only shifted rightward but the magnitude of the response was also decreased, remaining below the full effect observed in control mice, even at the VHD-E2

pharmacological dose. In addition, we demonstrate for the first time a role of ERαAF1 in vaginal epithelial proliferation, which as for uterine epithelial proliferation, is highly dependent of E2 dose (**Figure 5E**).

Finally, we reconsidered the question of the role of ERαAF1 in bone, and, as previously described, we demonstrated that ERαAF1 is dispensable for cortical response to E2 regardless of the dose used, as revealed by the absence of interaction between genotype and E2 treatment (**Figures 6A and B)**. It should be however noted a significant effect of genotype due to a smaller cortical thickness and a smaller cortical BMC in ERαAF1^0 mice, independently on E2. By contrast, ERαAF1 is required for the increase of bone mineral density (BMD) in response to E2 in the trabecular compartment regardless of the dose (**Figure 6B**). We confirmed that E2 has no impact on bone length in both WT and ERαAF1^0 mice but interestingly in ovariectomized mice, bone lengths from ERαAF1^0 were smaller than in WT mice, as previously reported in older ERαAF1^0 mice (Borjesson *et al.*, 2012) (**Figure 6C**). Altogether, these data definitely demonstrate that ERαAF1 is necessary for the full E2 response in uterus, vagina and the trabecular bone compartment. However, depending on the phenotypic feature, ERαAF1 deficiency can be partially or fully overcome by using high E2 levels.

4. Discussion

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

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

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

E2 effects are mediated by hormone binding to ERα, ERβ or GPR30 (Miller *et al.*, 2017). ERα also exists as two isoforms, designated ERα66 and ERα46, based on their respective molecular weight, and both isoforms were reported to regulate E2-mediated proliferation in the mouse uterus (Abot *et al.*, 2013). The expression of ERα46 in the rat uterus was demonstrated even before the use of antibodies against ERα (Faye *et al.*, 1986). Importantly, we recently demonstrated that ERα46 is frequently and sometimes abundantly expressed in ERα-positive breast tumors, but the mechanisms of its generation as well as 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 ERα66 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 ERα46 counteracts ERα66 on E2-induced cell proliferation (Abot *et al.*, 2013; Penot *et al.*, 2005).

The structural difference between ERα66 and ERα46, according to our current knowledge, resides mainly in the absence of AF1 in ERα46 (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 ERα46 could have a repressive role in AF1-permissive contexts through interference with ERα66 binding to DNA (Penot *et al.*, 2005). The present study deepens the comparison between both ERα 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, ERα46 requires an E2 dose 10 to 100 times higher than does ERα66. Several studies have shown that N-terminal truncated forms of ERα, including ERα46, have an affinity for E2 which is similar to that found for the wild-type ERα66, 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 ERα binding to DNA and in coactivator and/or corepressor recruitment could explain the distinctively lower sensitivity of ERα46 *versus* ERα66 to the dose of E2. We previously reported that although the qualitative overall recruitment of cofactors is quite similar between ERα46 and ERα66, there is a quantitative differential

modulation with some preference for cofactor binding to ERα66 over ERα46 (Chantalat *et al.*, 2016). ERα46 is also able to recruit transcriptional repressors in a ligand-independent manner (Metivier *et al.*, 2004). In accordance with the specific binding of coregulators, we reported that some genes were specifically regulated by either the ERα46 or ERα66 isorforms, demonstrating that the absence of ERαAF1 affects specific transcriptional programs rather than leading to an overall decreased activity (Chantalat *et al.*, 2016) .

Using mice harbouring an ERα deleted for AF1 (ERαAF1^0), we previously demonstrated that ERαAF1 is necessary to induce uterine hypertrophy, suggesting that ERα46 is not able to mediate E2 responses in uterus (Abot *et al.*, 2013). Here, we demonstrate that with a HD of E2, the increase in uterine weight is similar between ERαAF1^0 and ERαWT, although an attenuated response is observed in terms of epithelial proliferation. By contrast, physiological LD or MD doses of E2 leading to levels encountered during the estrous cycle were unable to induce a uterine response in ERαAF1^0 mice in contrast to ERαWT mice. These results demonstrate that the involvement of ERαAF1 in the E2 response is highly dependent on ligand dose, and that different signalling pathways regulate epithelial proliferation and uterine weight gain. Similarly, the response to E2 in vagina and trabecular bone are dependent of ERαAF-1, since this response is not only shifted rightward but also decreased in intensity, suggesting that these tissues can be used as an index of ERαAF-1-dependent estrogenicity.

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

attenuation of E2-induced transcription has been observed in ERαS118A transfected cells (Ali *et al.*, 1993; Duplessis *et al.*, 2011). Appropriate transgenic mouse studies should be developed to determine the importance of ERα Ser118 phosphorylation *in vivo*.

5. Conclusion

We describe here the dose-effect of E2 on sexual targets and bone thanks to a simple, reliable and unexpansive slow release device. Our results demonstrate that the estrogenic effect on tissues is highly dependent on the dose used. Although the precise role of ERαAF1 both in physiology and in pathology remains to be clarified, the present work reveals that ERαAF1 provides an important layer of regulation through the sensitization of the response to estrogens. The role of ERαAF1 can be even more spectacular in the action of some selective ER modulators (SERMs) (Arao and Korach, 2019). For instance, tamoxifen selectively activates ERαAF1, and this activation is required for the beneficial effects of this SERM on arteries (Fontaine *et al.*, 2013) and metabolism (Guillaume *et al.*, 2017), while at the same time it prevents the recurrence of breast cancer. Whereas antagonist activity of tamoxifen is essentially through the ERαAF2 blockade, molecular mechanism of its partial agonist activity that involved ERαAF1 activation is not fully understood (Arao and Korach, 2019). Here, we report that importance of ERαAF1 domain in estrogenic response depend on both considered cell type/ tissues and ligand dose, two parameters which could be determinant in the agonist activity of E2 or SERM that control ERαAF-1-mediated physiological response.

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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 ± 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 †††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 ± 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 †††P<0.001, n=6/7 mice/group).

611 **Figure 4: Dose response of E2 transcriptional activity in cultured cells expressing ERα66**
612 **or ERα46.**

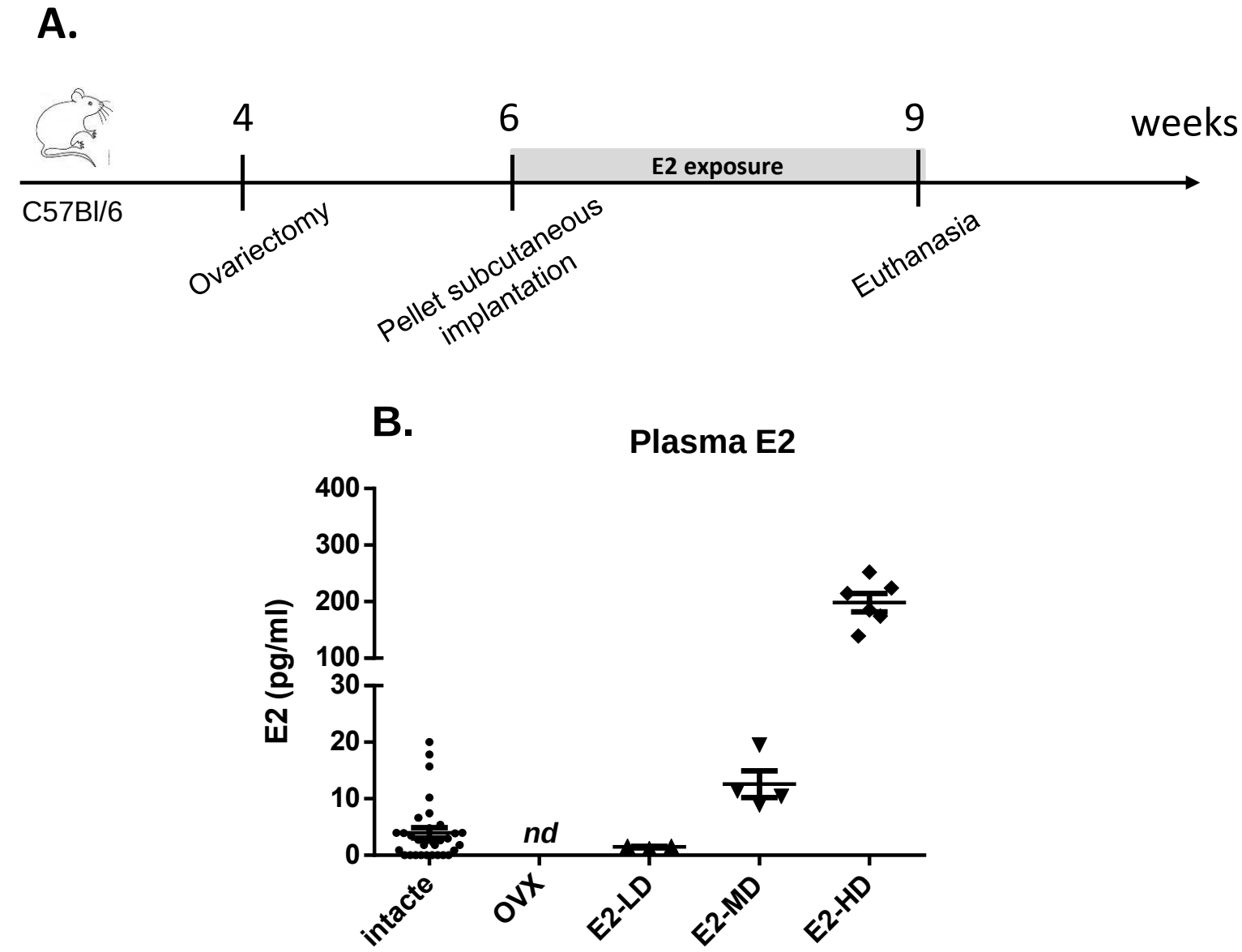
Schematic representation of the wild-type ERα66 (ERαAF1$^{+/+}$) and ERα (ERαAF1^{0}) isoforms and expression of these isoforms obtained from in vitro translation pCR-ERαWT or pCR-ERαAF1^{0} vectors performed with the TNT Coupled Reticulocyte Lysate System (**A**). HepG2 (**B**), HeLa (**C**) and MDA-MB231 (**D**) cells were transiently transfected with the C3-Luc reporter constructs in the presence of pCR-ERαWT, pCR-ERαAF1^{0}, or empty pCR vectors. Cells were treated with the indicated dose of E_2 or vehicle (Ctrl) for 24 h. Normalized luciferase activities were expressed as fold increase above values measured with empty pCR and vehicle. Data correspond to the mean values ± SEM of at least three separate transfection experiments. Two-way ANOVA followed by Fisher's LSD's multiple comparison test were performed (ERαAF1$^{+/+}$ *versus* ERαAF1^{0}: *P < 0.05, **P < 0.01, ***P < 0.001).

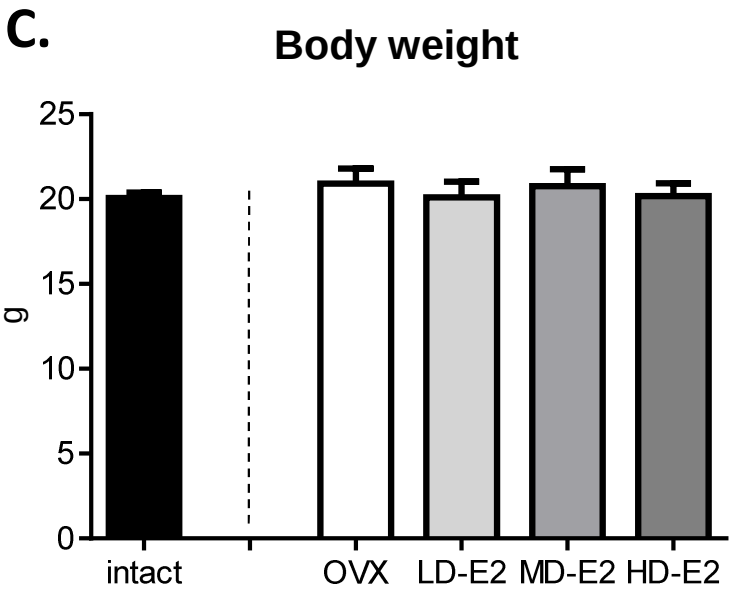
Figure 5: The role of ERαAF1 in uterine and vagina is dependent of E2 dose

Schematic representation of the wild-type ERαAF1$^{+/+}$ and ERαAF1^{0} mice constructs (**A**). Pellets releasing low dose (LD), medium dose (MD), high dose (HD) or very high dose (VHD) of E2 were implanted subcutaneously into ERαAF1^{0} ovariectomized mice during 3 weeks. Uterus **(B, C)** and vagina (**D, E**) were analyzed. Uterine **(B)** and vagina **(D)** weight. Percentage of uterine (**C**) and vagina (**E**) Ki67-positive epithelial cells. Representative Ki-67 detection in transverse uterus sections. Results are expressed as means ± SEM. Two-way ANOVA followed by Fisher's LSD's multiple comparison test were performed (ERαAF1$^{+/+}$ *versus* ERαAF1^{0}: *P < 0.05, **P < 0.01, ***P < 0.001, n=6/8 mice/group).

Figure 6: E2 dose response analysis of trabecular and cortical bone parameters in wild-type ERαAF1$^{+/+}$ and ERαAF1^{0} mice.

Pellets releasing low dose (LD), medium dose (MD), high dose (HD) or very high dose (VHD) of E2 were implanted subcutaneously to ERαAF1^{0} ovariectomized mice for a period of 3 weeks and femurs were analyzed. Cortical thickness and cortical bone mineral content (BMC) in the mid-diaphyseal part of femur. 2-way ANOVA test revealed no significant interaction, the overall effect of the treatment and genotype was analyzed (**A**). Trabecular bone mineral density (BMD) in the distal metaphyseal part of femur **(B)** and Bone length (**C**). Results are expressed as means ± SEM. Since two-way ANOVA test revealed a significant interaction between the 2 factors (E2 exposure and genotype), a Fisher's LSD's multiple comparison test were performed (ERαAF1$^{+/+}$ *versus* ERαAF1^{0}: **P < 0.01, ***P < 0.001, n=6/8 mice/group) (**B, C**).


A.
4
6
9
weeks
E2 exposure
C57Bl/6
Ovariectomy
Pellet subcutaneous implantation
Euthanasia
B.
Plasma E2
E2 (pg/ml)
400
300
200
100
30
20
10
0
nd
intacte
OVX
E2-LD
E2-MD
E2-HD


C.
Body weight
g
25
20
15
10
5
0
intact
OVX
LD-E2
MD-E2
HD-E2

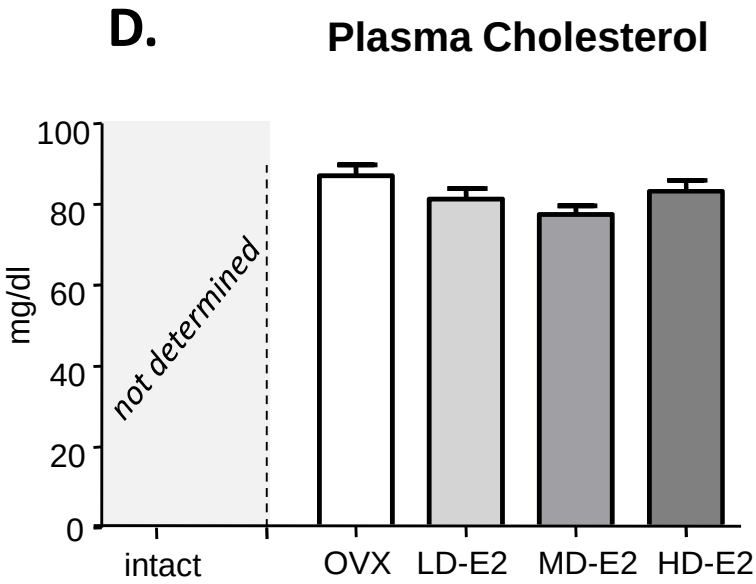

D.
Plasma Cholesterol
mg/dl
100
80
60
40
20
0
not determined
intact
OVX
LD-E2
MD-E2
HD-E2

Figure 1

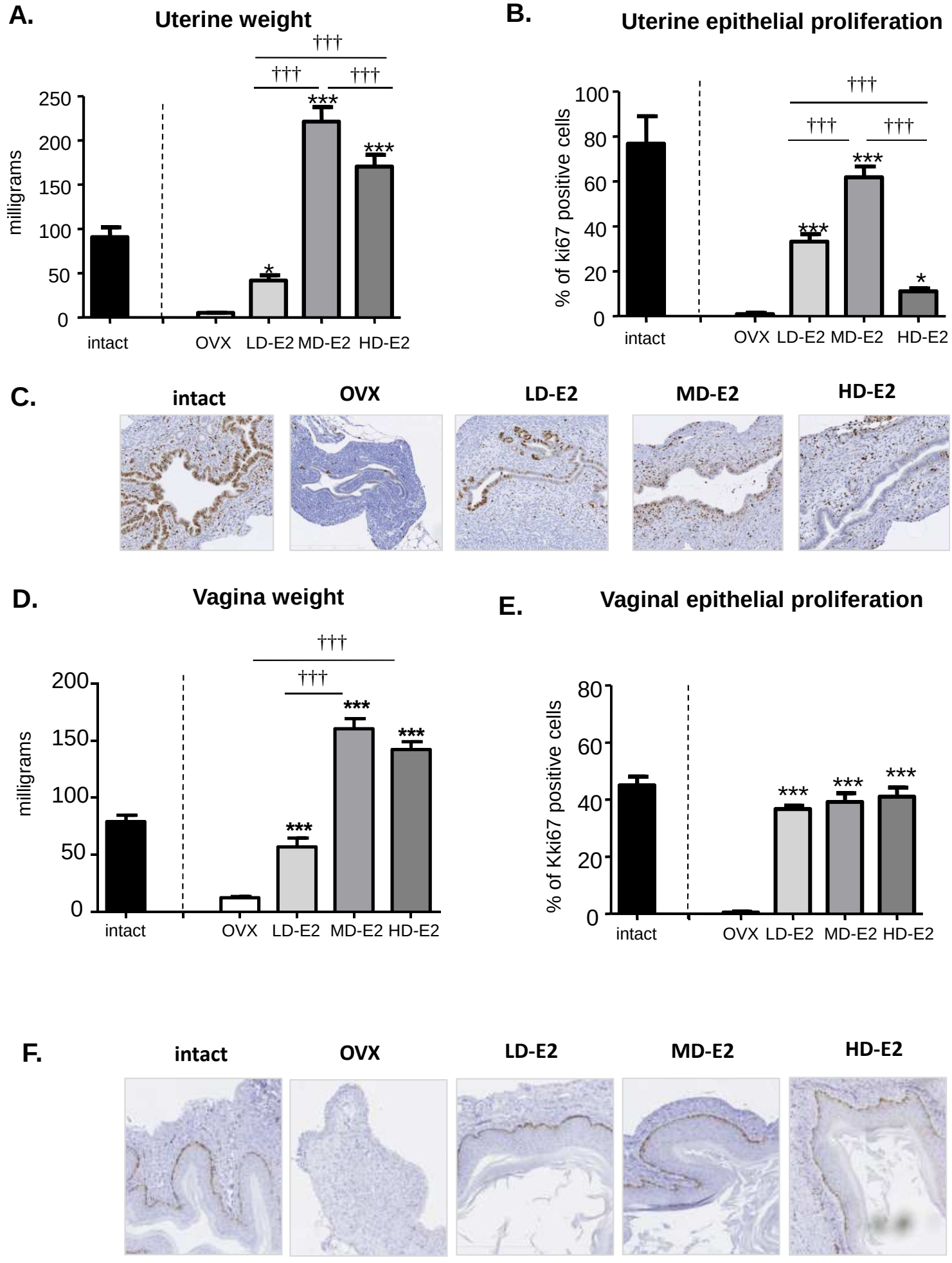

A.
Uterine weight
milligrams
250
200
150
100
50
0
intact
OVX
LD-E2
MD-E2
HD-E2
†††
†††
†††
***
***
*
B.
Uterine epithelial proliferation
% of ki67 positive cells
100
80
60
40
20
0
intact
OVX
LD-E2
MD-E2
HD-E2
†††
†††
†††
***
***
*
C.
intact
OVX
LD-E2
MD-E2
HD-E2
D.
Vagina weight
milligrams
200
150
100
50
0
intact
OVX
LD-E2
MD-E2
HD-E2
†††
†††
***
***
***
E.
Vaginal epithelial proliferation
% of Kki67 positive cells
80
60
40
20
0
intact
OVX
LD-E2
MD-E2
HD-E2
***
***
***
F.
intact
OVX
LD-E2
MD-E2
HD-E2

Figure 2

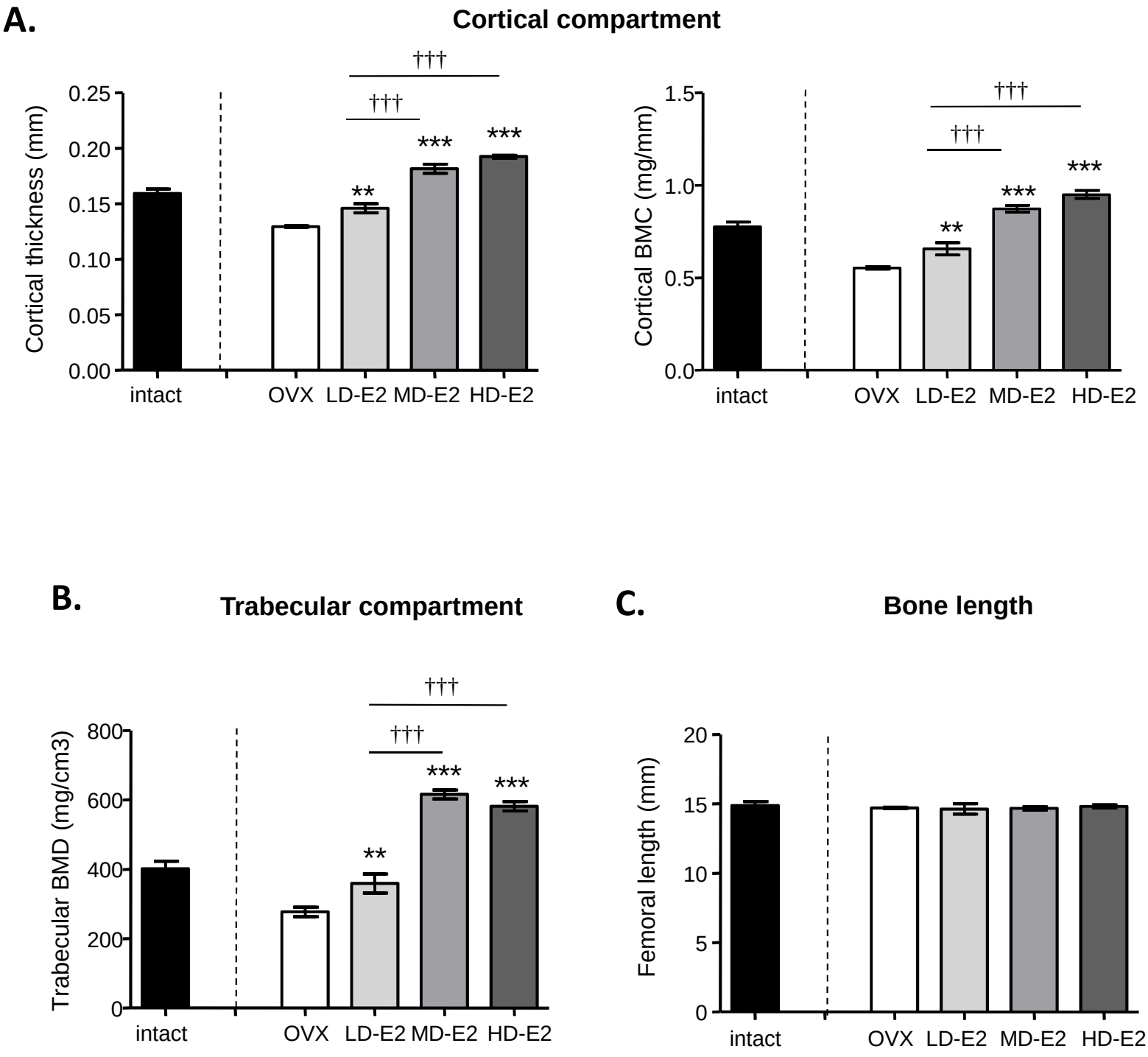

A.
Cortical compartment
Cortical thickness (mm)
0.25
0.20
0.15
0.10
0.05
0.00
†††
†††
**
***
***
intact
OVX
LD-E2
MD-E2
HD-E2
Cortical BMC (mg/mm)
1.5
1.0
0.5
0.0
†††
†††
**
***
***
intact
OVX
LD-E2
MD-E2
HD-E2
B.
Trabecular compartment
Trabecular BMD (mg/cm3)
800
600
400
200
0
†††
†††
**
***
***
intact
OVX
LD-E2
MD-E2
HD-E2
C.
Bone length
Femoral length (mm)
20
15
10
5
0
intact
OVX
LD-E2
MD-E2
HD-E2

Figure 3

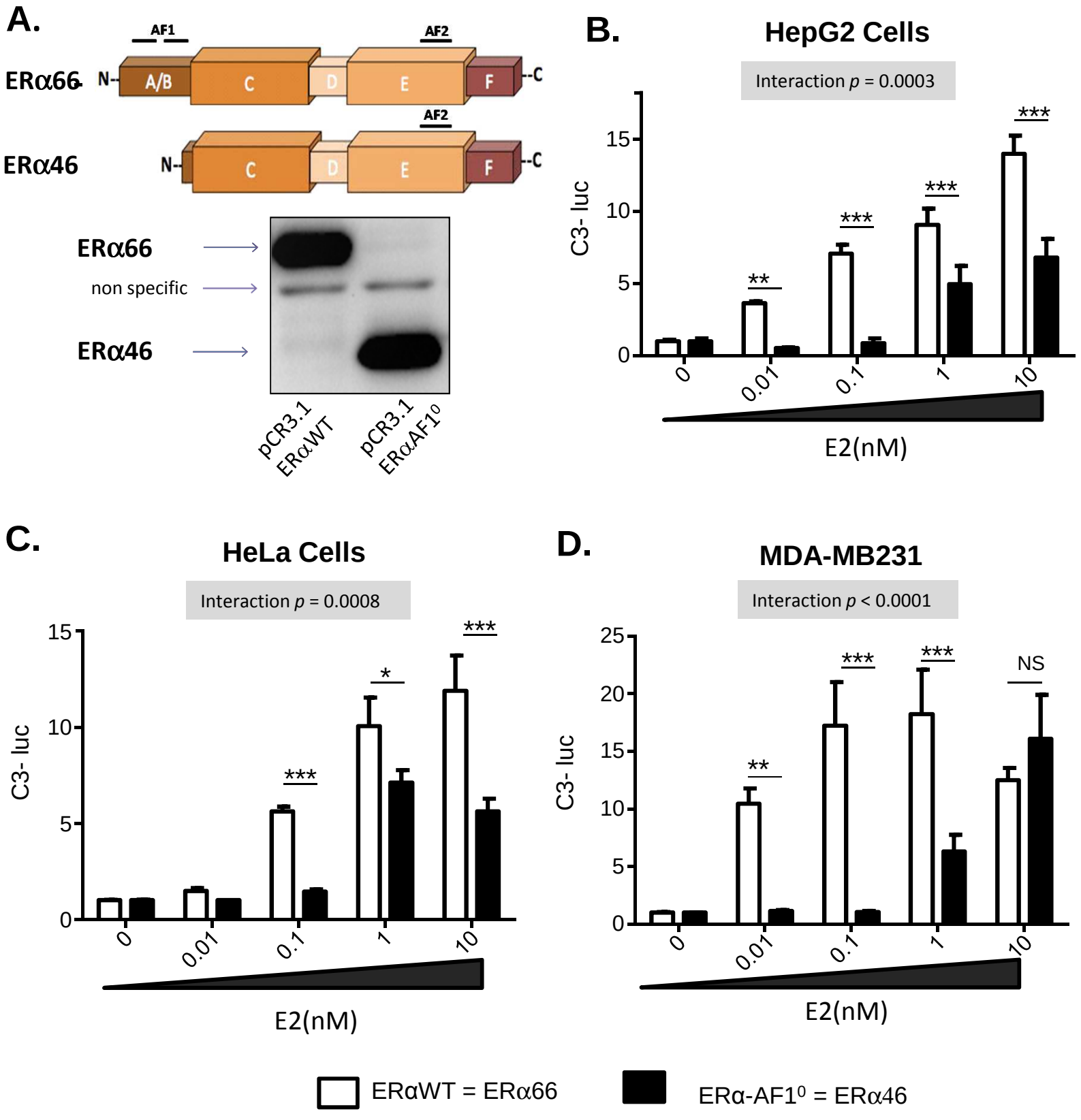

A.
AF1
AF2
ERα66
N-
A/B
C
D
E
F
-C
ERα46
ERα66
non specific
ERα46
pCR3.1 ERαWT
pCR3.1 ERαAF1⁰
B.
HepG2 Cells
Interaction p = 0.0003
C3- luc
E2(nM)
C.
HeLa Cells
Interaction p = 0.0008
C3- luc
E2(nM)
D.
MDA-MB231
Interaction p < 0.0001
C3- luc
E2(nM)
NS
ERαWT = ERα66
ERα-AF1⁰ = ERα46

Figure 4

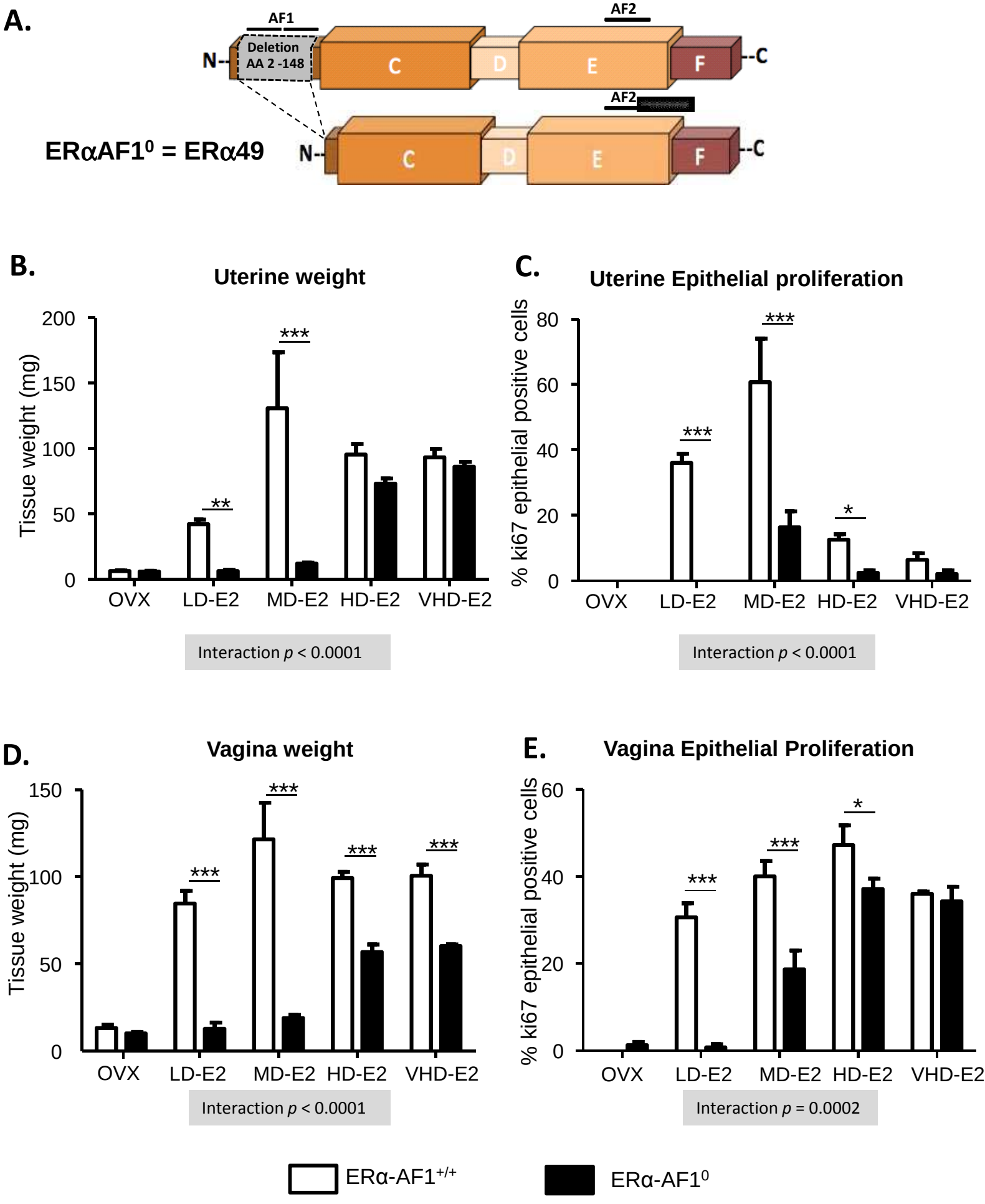

A.
AF1
Deletion
AA 2 -148
AF2
N--
C
D
E
F
--C
ERαAF1⁰ = ERα49
B.
Uterine weight
Tissue weight (mg)
200
150
100
50
0
**
***
OVX
LD-E2
MD-E2
HD-E2
VHD-E2
Interaction p < 0.0001
C.
Uterine Epithelial proliferation
% ki67 epithelial positive cells
80
60
40
20
0
***
*
Interaction p < 0.0001
D.
Vagina weight
150
100
50
0
E.
Vagina Epithelial Proliferation
60
40
20
Interaction p < 0.0001
Interaction p = 0.0002
ERα-AF1+/+
ERα-AF1⁰

Figure 5

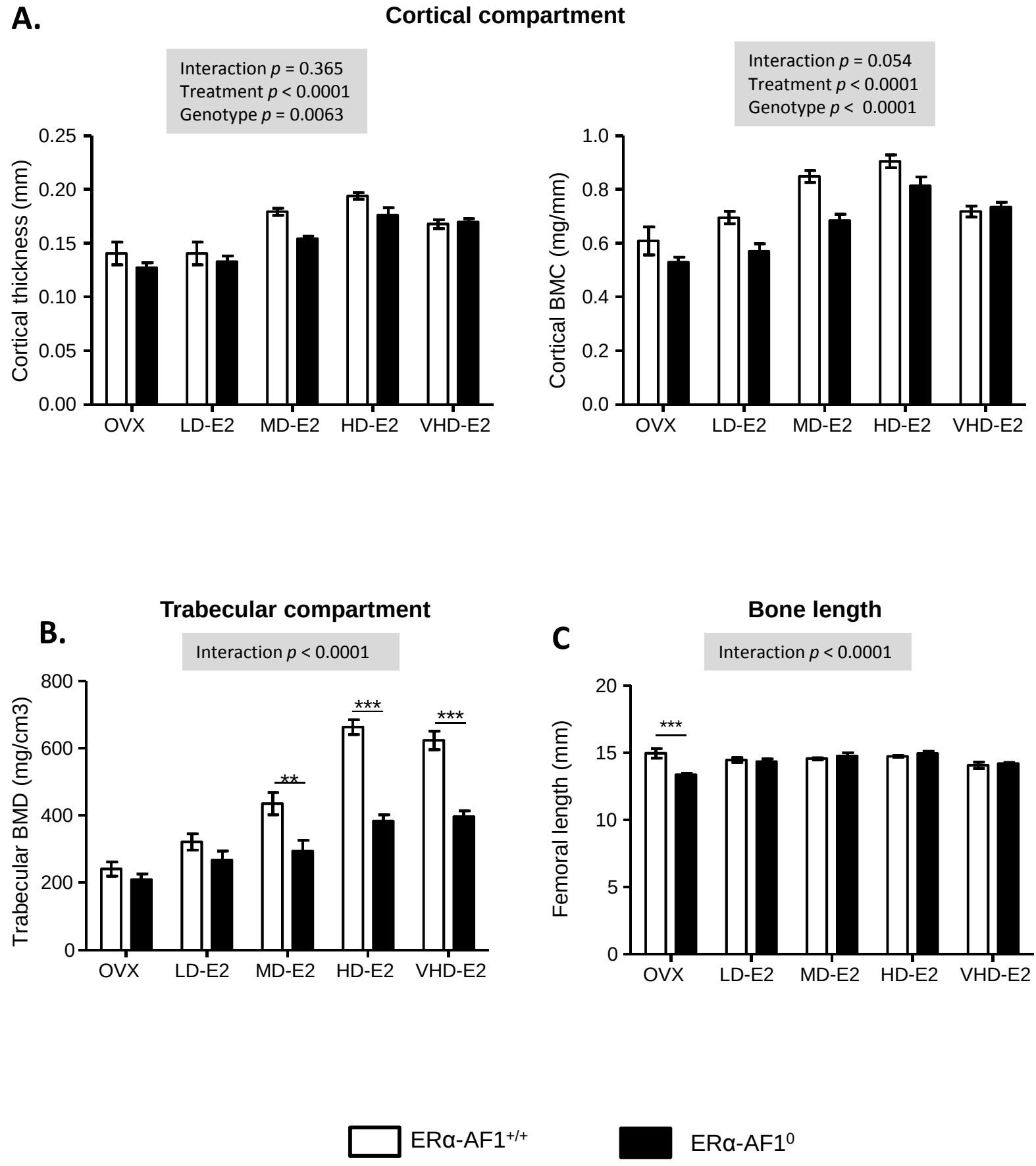

A.
Cortical compartment
Interaction p = 0.365
Treatment p < 0.0001
Genotype p = 0.0063
Cortical thickness (mm)
0.25
0.20
0.15
0.10
0.05
0.00
OVX
LD-E2
MD-E2
HD-E2
VHD-E2
Interaction p = 0.054
Treatment p < 0.0001
Genotype p < 0.0001
Cortical BMC (mg/mm)
1.0
0.8
0.6
0.4
0.2
0.0
B.
Trabecular compartment
Interaction p < 0.0001
Trabecular BMD (mg/cm3)
800
600
400
200
0
**
***
***
C
Bone length
Interaction p < 0.0001
Femoral length (mm)
20
15
10
5
0
***
ERα-AF1+/+
ERα-AF10

Figure 6

	OVX	LD-E2	MD-E2	HD-E2
Uterine LEH (µm)	12.1 +/- 0.6	20.2 +/- 1.2 **	37.6 +/- 1.9 ***	40.5 +/- 2.1 ***
Uterine Stromal Density	257 +/- 1.14	116.7 +/- 44.6 ***	87 +/- 35.5 ***	72.7 +/- 34.5 ***
Uterine Stromal proliferation (%Ki67positive cells)	0.3 +/- 0.5	8 +/- 5.6	42.1 +/- 11 ***	21.6 +/- 8 ***

Results are expressed as means ± 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

Accucary (%)	**Analyte**	**Target ion analyte /IS (amu)**	**Range (pg/ml) R^2**	**Mean (pg/ml) 17 runs** ***Intra- & Inter assay CVs (%)***			
				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

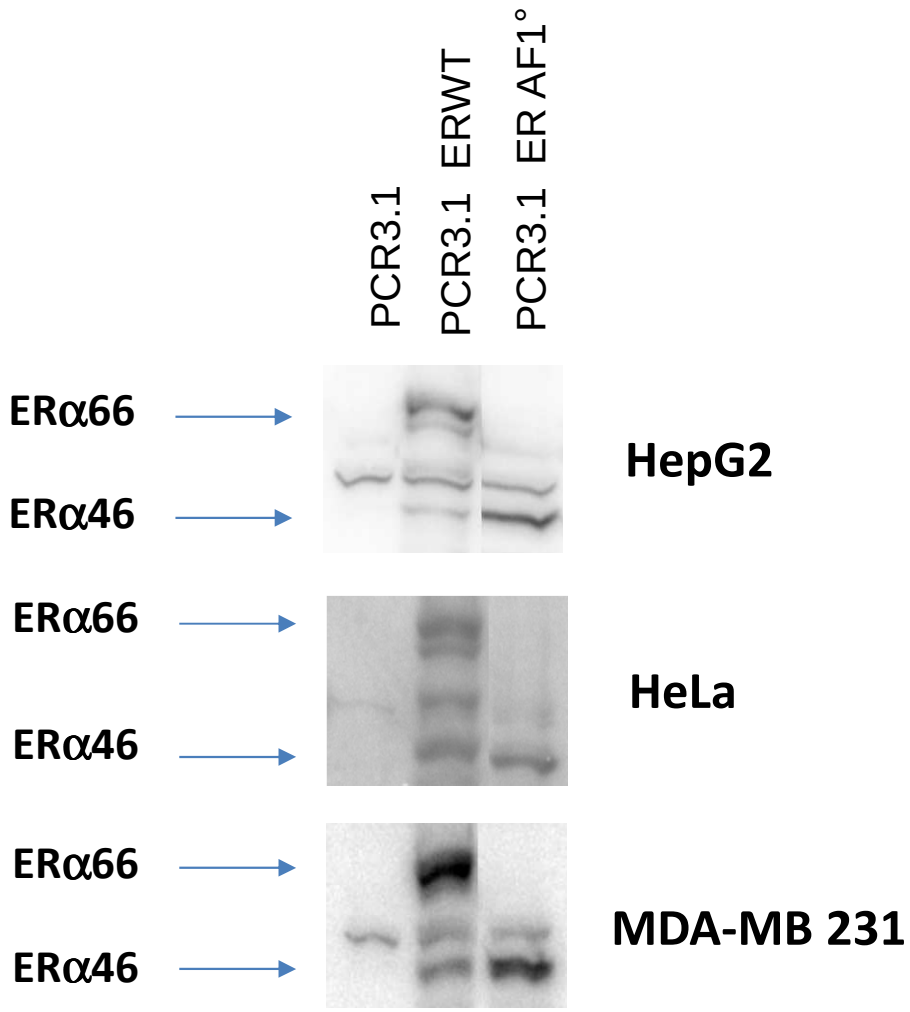
LLOQ : low limit of quantification; QC : quality control

Supplementary Table 1 : GC/MS plasma analytical control validation

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

Results are expressed as means ± 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 ERαAF1^0 mutant (lane 3).

HIGHLIGHTS

- Some biological response to E2 displays biphasic dose relationship.
- ERαAF1 plays a key sensitizing role in ERα activity.
- Differences and even contradictory conclusions could be explained by differences of E2 doses.