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Phosphorylation site S122 in estrogen receptor α has a tissue-dependent role in female mice

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Abstract

Estrogen treatment increases bone mass and reduces fat mass but is associated with adverse effects in postmenopausal women. Knowledge regarding tissue-specific estrogen signaling is important to aid the development of new tissue-specific treatments. We hypothesized that the posttranslational modification phosphorylation in estrogen receptor alpha (ERα) may modulate ERα activity in a tissue-dependent manner. Phosphorylation of site S122 in ERα has been shown in vitro to affect ERα activity, but the tissue-specific role in vivo is unknown. We herein developed and phenotyped a novel mouse model with a point mutation at the phosphorylation site 122 in ERα (S122A). Female S122A mice had increased fat mass and serum insulin levels but unchanged serum sex steroid levels, uterus weight, bone mass, thymus weight, and lymphocyte maturation compared to WT mice. In conclusion, phosphorylation site S122 in ERα has a tissue-dependent role with an impact specifically on fat mass in female mice. This study is the first to demonstrate in vivo that a phosphorylation site in a transactivation domain in a nuclear steroid receptor modulates the receptor activity in a tissue-dependent manner.

KEYWORDS
adipose tissue, bone, estrogen, posttranslational modifications

Abbreviations: aBMD, total body areal BMD; BV/TV, bone volume/total volume; Ct.Th., cortical thickness; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; PR, progesterone receptor; PTMs, posttranslational modifications; S122A, serine to alanine substitution of ERα; Tb.N., trabecular number; Tb.Sp, trabecular separation; Tb.Th., trabecular thickness; WT, wild type.

Claes Ohlsson and Karin L. Gustafsson are contributed equally to this work.

1 | INTRODUCTION

Estrogens, the female sex hormones, are well known to play a major role in the development and maintenance of reproductive organs. However, estrogens are also involved in the regulation of several other organ systems in both males and females including the skeleton, where they affect both bone growth and adult bone metabolism.[1-3] Estrogens also affect adipose tissue metabolism and play a role in the regulation of the immune system.[4-7] Estrogen deficiency, as seen following menopause, is a major trigger for developing osteoporosis, which is characterized by low bone mass and deteriorated skeletal microarchitecture, leading to increased risk of fractures.[1,8,9] Diminished estrogen levels are also associated with increased risk of obesity at menopause and increased risk of developing metabolic syndrome.[5,6,10-12] Estrogen treatment protects against low bone mass and decreases the risk of fractures, and studies also show that hormone replacement therapy can decrease the risk of developing different metabolic disorders after menopause.[10,13] However, estrogen is not a suitable treatment option due to adverse effects, including increased risk of cancer in reproductive tissues and venous thrombosis.[8,14-16] Thus, new tissue-specific treatment options, with no risk of side effects, are needed. The development of such drugs requires knowledge regarding estrogen signaling mechanisms in different tissues.

The effects of estradiol, the predominant and most biologically active estrogen, are mainly mediated by the estrogen receptors alpha (ERα) and beta (ERβ). ERα has been shown to be the primary receptor mediating the effects of estradiol in many organ systems including the skeleton, the immune system, and adipose tissue.[17-23]

Estrogen receptor signaling triggered by ligand-binding can be divided into genomic (nuclear) and nongenomic (extra-nuclear) signaling pathways.[24] The genomic pathway involves the nuclear translocation of the activated ER-ligand complex, which acts as a transcription factor and leads to the modulation of gene transcription. In contrast, the nongenomic pathway is initiated at the plasma membrane and involves more rapid effects. These rapid effects include the activation of various kinase signaling cascades, altered ion fluxes, and induction of enzyme activities, which in turn can modify the function of the cell directly, or lead to the activation of various transcription factors leading to altered gene transcription.[25,26] In addition to this ligand-dependent activation, the ERs can also be activated in a ligand-independent way.[27] Unliganded ERs can exert genomic effects by modulating gene transcription after activation by selected post-translational modifications (PTMs), and they can also, after activation by PTMs, induce extra-nuclear effects by interaction with other cytoplasmic signaling molecules.

ERα can undergo several different PTMs, including acetylation, methylation, palmitoylation, and phosphorylation.[24,28,29] The most common PTM, phosphorylation, is the reversible addition of a phosphoryl group from adenosine triphosphate (ATP) to serine, threonine, or tyrosine residues in the protein and results in altered function of the protein. Phosphorylation of ubiquitously expressed proteins has been shown to differ between cell-types, thus, this PTM may provide a means to fine-tune the function of proteins in a tissue-dependent manner via different phosphorylation patterns.[30]

Phosphorylation of ERα has been shown to modulate the interaction of the receptor with other proteins, including coregulatory factors and the transcriptional machinery, thereby affecting the ability of both liganded and unliganded ERα to modulate gene transcription.[31,32] Phosphorylation has also been shown to modify the function of the receptor by affecting its hormone sensitivity, nuclear localization, DNA binding, and protein stability.[31,33-39] There are several phosphorylation sites in ERα, and the most well-characterized is S118 (S122 in mouse).[38] This site is found in the activation function 1 (AF-1), the N-terminal transactivation domain of ERα. Phosphorylation at this site in human breast cancer tissue, evaluated in ex vivo experiments, has been reported to be associated with disease prognosis and to be correlated to resistance to tamoxifen treatment.[40,41] Furthermore, the importance of this phosphorylation site has been proven by in vitro studies in several different cell types including COS-1 cells (a monkey kidney cell line), HeLa cells (a human cervical cancer cell line), and MCF-7 cells (a human breast cancer cell line) in which serine 118 has been changed into alanine, an amino acid that cannot be phosphorylated.[32,42] The presence of phosphorylation of serine 122 (S122) in mouse has been demonstrated using phospho-specific antibodies,[43-46] and the serine to alanine substitution at site 122 in the murine ERα (S122A) has been shown to affect the signaling of the receptor in vitro.[47] Interestingly, the inability of the human ERα to be phosphorylated at site 118 leads to decreased estradiol-induced gene transcription in some,[34] but not all,[48] cell-types. Thus, the importance of this phosphorylation site may differ in a tissue-dependent manner.

Phosphorylation is a common PTM, not only in ERα, but also in other nuclear steroid receptors including the androgen and the progesterone receptors.[49] However, even though the importance of different phosphorylation sites in these nuclear steroid receptors has been studied in various cell lines, the role of a specific phosphorylation site in a nuclear steroid receptor has, as of yet, not been demonstrated in vivo. The aim of this study was, therefore, to investigate the physiological role of phosphorylation site S122 in ERα, corresponding to the human S118, in vivo in different mouse tissues, using mice with an S122A amino acid substitution.

2 | MATERIALS AND METHODS

2.1 | Animals

To generate S122A mice with a point mutation in ERα at phosphorylation site S122 leading to an amino acid shift from serine to alanine (S122A), a targeting vector was constructed as follows: a point mutation (T > G) resulting in the amino acid change S122A was introduced in a 752-bp fragment including ERα exon 2 and part of intron 2-3 (modified arm, MA) (Figure 1A). This fragment was subcloned into a vector containing a floxed neomycin resistance cassette (Neo) together with a 5′ homology arm (5′HA, 3.9 kb) and 3′ homology arm (3′HA, 3.7 kb) (Figure 1A). The linearized construct was electroporated into C57BL/6NTac mouse embryonic stem (ES) cells (BD10). After selection, targeted clones were identified by PCR using 5′ and 3′ external primers (Supporting Figure S1A,B). The targeted allele was further confirmed by Southern blot with a Neo probe (two 5′ digests and two 3′ digests, Supporting Figure S1A,C,D) and a 3′ external probe (three 3′ digests, Supporting Figure S1A,C,E). The targeted ES clone BD10-27 (Supporting Figure S1) was microinjected into *C57BL/6NTac* blastocysts. Resulting chimaeras were bred with a Cre deleter line (to excise the floxed Neo cassette) and germ-line transmission was achieved. The insertion of the point mutation, leading to the S122A amino acid substitution, was confirmed by DNA sequencing (Figure 1B). Female ERαS122A$^{\pm}$ mice were bred with male ERαS122A$^{\pm}$ mice to generate homozygous S122A mice and wild-type (WT) littermate controls. We observed a normal litter size, a normal sex balance, and the genotypes were distributed according to Mendel′s law of inheritance. Primers used for genotyping and sequencing of S122A mice were 5′-GGGAAGTCAGAAAGGATTCTTTGGCAG-3′ and 5′-GGTTCCTTGCTAGCACAGGCCAC-3′. The mice were housed in a standard animal facility under controlled temperature (22°C) and photoperiod (12 h of light and 12 h of darkness) and fed pellet diet (Harlan 2016) and water ad libitum. Three-month-old S122A mice and littermate controls were anesthetized with Ketanest/Dexdomitor (Pfizer/Orion Pharma, Sollentuna, Sweden), bled, and euthanized by cervical dislocation. Uterus, fat depots, liver, thymus, bone marrow, and flushed femur and tibia (cortical bone) were collected from female mice and stored at −80° for further analysis, and long bones and vertebrae L5 were dissected, fixated in 4% paraformaldehyde for 2 days and then stored in 70% ethanol for further analysis. Fat depots from three-month-old male S122A mice and WT littermate controls were also collected and weighed. All experimental procedures involving animals were approved by the Gothenburg Ethical Committee for Animal Research.

2.2 | Simple western

To prepare protein, snap-frozen uteri from three-month-old female S122A and WT littermates were homogenized in RIPA-buffer with a mixture of protease inhibitors (Complete Mini EDTA-free; Roche Diagnostics, Solna, Sweden) and phosphatase inhibitors (PhosSTOP; Roche

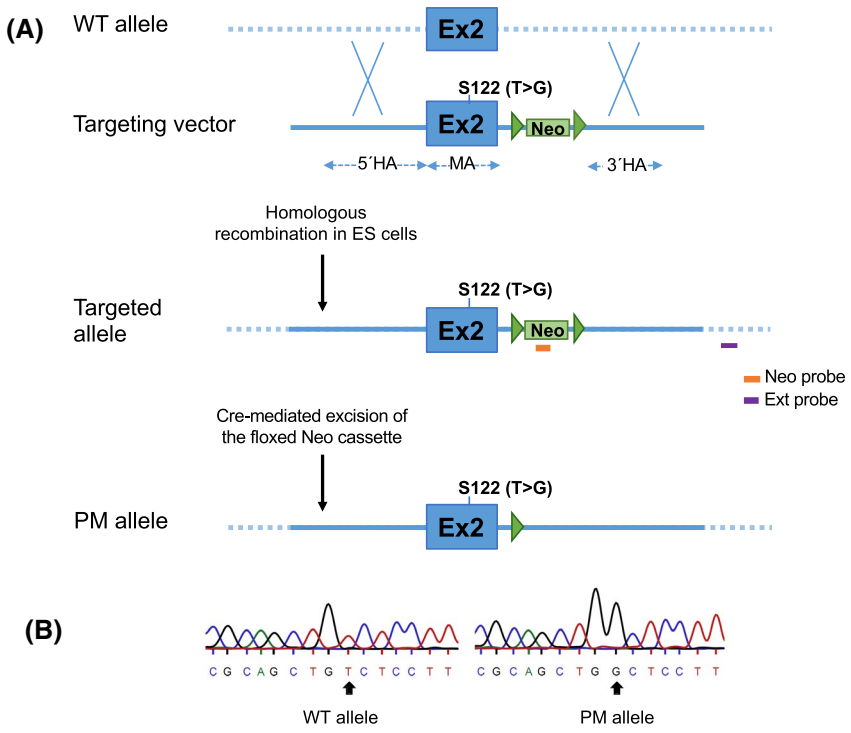

FIGURE 1 Generation of estrogen receptor (ER) α mice with an S122A mutation. Schematic illustration showing the targeting vector with the point mutation (PM) in exon 2 (Ex2) used for the generation of the PM allele (A). The insertion of the PM (T > G) was confirmed by sequencing (B). HA; homologous arm, MA; modified arm, Neo; floxed neomycin resistance cassette

Diagnostics). Protein levels of total ERα were measured with WES (ProteinSimple, Santa Clara, CA, USA) and protein levels of ERα, phosphorylated specifically at serine 122, were measured with JESS (ProteinSimple) according to the manufacturer's protocol. The rabbit polyclonal ERα antibody (ab75635; Abcam, Cambridge, UK) diluted 1:10, the polyclonal pERα (Ser118) antibody (ab32396, Abcam) diluted 1:50, and the rabbit monoclonal β-actin antibody (#7970S; Cell signaling Technology, Leiden, Netherlands) diluted 1:50 were used as primary antibodies followed by the anti-rabbit Detection Module (ProteinSimple). Data were analyzed using the Compass Software (ProteinSimple). In an additional experiment, uteri were collected from two- to eight-month-old ovariectomized S122A female mice injected intraperitoneally with 17β-estradiol, dissolved in ethanol and diluted in PBS (2 μg/mouse; Sigma-Aldrich, final ethanol concentration 2%) or vehicle (ethanol in PBS, final ethanol concentration 2%) 6 hours before sacrifice one week after ovariectomy. The rabbit polyclonal pAKT antibody (#9271, Cell signaling Technology) diluted 1:50 and the rabbit polyclonal AKT antibody (#44-609G, Thermo Fisher, Waltham, MA, USA) diluted 1:125, were used as primary antibodies.

2.3 | Real-time PCR

RNA was isolated from snap-frozen uteri, gonadal fat, and flushed mid-diaphyseal cortical bone from long bones (tibia and femur) using TRIzol reagent (Thermo Fisher) followed by the RNeasy Mini Kit (Qiagen, Sollentuna, Sweden). Amplifications were performed using the Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher) and Assay-on-Demand primer and probe sets (Thermo Fisher), labeled with the reporter fluorescent dye FAM. Predesigned primers and probe labeled with the reporter fluorescent dye VIC, specific for 18S ribosomal RNA, were included in the reaction as an internal standard. The assay identification numbers were *Esr1 (encoding estrogen receptor alpha);* Mm00433147_m1, *Pgr (encoding progesterone receptor);* Mm00435628_m1, *Muc1 (encoding mucin-1);* Mm00449604_m1, *Igfbp3 (encoding insulin-like growth factor-binding protein 3);* Mm01187817_m1, *Bglap (encoding osteocalcin);* Mm03413826_mH, *Runx2 (encoding runt-related transcription factor 2);* Mm00501580_m1, *Lep (encoding leptin);* Mm00434759_m1, *Cebpa (encoding CCAAT/enhancer-binding protein alpha);* Mm01265914_s1, *Adipoq (encoding adiponectin);* Mm00456425_m1, *Pparg (encoding peroxisome proliferator-activated receptor gamma);* Mm00440940_m1, *Il1b (encoding interleukin 1 beta);* Mm00434228_m1, *Tnf (encoding tumor necrosis factor alpha);* Mm00443258_m1, and 18S; 4310893E.

2.4 | Serum analyses

Serum levels of 17β-estradiol, testosterone, dihydrotestosterone (DHT), and progesterone (P) in three-month-old female S122A and WT mice, were measured in a single run by GC-MS/MS, as described previously.[50] Serum samples from three-month-old female S122A and WT mice were pooled two and two for measurements of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels by time-resolved immunofluorometric assays as previously described.[51,52] Non-detectable levels of 17β-estradiol and LH were set to half LOQ (Limit Of Quantitation). Serum leptin levels were measured using a Mouse Leptin ELISA kit (Cat# 90 030, Crystal Chem, Zaandam, Netherlands) and insulin levels were measured with an Ultra-Sensitive Mouse Insulin ELISA kit (Cat# 90 080, Crystal Chem).

2.5 | Dual-energy X-Ray absorptiometry (DXA)

Analyses of total body areal bone mineral density (aBMD), lean mass, and fat percent were performed using a Lunar PIXImus mouse densitometer (Wipro GE Healthcare). Isoflurane inhalation (Baxter Medical AB, Kista, Sweden) was used as anesthesia in five-week-old female mice and intraperitoneal injection of Ketanest/Dexdomitor (Pfizer/Orion Pharma) was used as anesthesia in three-month-old female mice. In an additional experiment, in vivo DXA analyses of female S122A and WT controls were performed at 6, 9, and 12 months of age under anesthesia by isoflurane inhalation (Baxter Medical AB). Fat percent was also analyzed by DXA in three-month-old male S122A mice and four-month-old female ERαKO, ERαAF1KO, and ERαAF2KO mice, generated as previously described.[53]

2.6 | High-resolution microcomputed tomography (μCT)

High-resolution microcomputed tomography (μCT) analyses were performed on the vertebrae L5 and femur using an 1172 model μCT (Bruker microCT, Aartselaar, Belgium) as previously described.[54] Briefly, in vertebrae, the trabecular bone in the vertebral body caudal of the pedicles was selected for analysis within a conforming volume of interest (cortical bone excluded) commencing at a distance of 4.5 μm caudal of the lower end of the pedicles, and extending a further longitudinal distance of 328 μm in the caudal direction. In the femur, the trabecular bone proximal to the distal growth plate was selected for analyses within a conforming volume of interest (cortical bone excluded), commencing at a distance of 650 μm from the growth plate and extending a further longitudinal distance of 134 μm in the proximal direction. The

cortical measurements were performed in the mid-diaphyseal region of femur starting at a distance of 5.2 mm from the growth plate and extending a further longitudinal distance of 134 μm in the proximal direction.

2.7 | Cell preparation and flow cytometry

Bone marrow cells were harvested from femur using a syringe with 5 mL of PBS. Pelleted cells were resuspended in Tris-buffered 0.83% NH_4Cl solution to lyse erythrocytes, washed in PBS, resuspended in FACS buffer (PBS supplemented with 10% FCS (Thermo Fisher) and 0.1% NaN_3) and counted using an automated cell counter (Sysmex Europe GmbH, Norderstedt, Germany). Thymi were dissected and mashed through 70 μm filters, washed in PBS, resuspended in FACS buffer, and counted using an automated cell counter (Sysmex). Bone marrow cells were stained with APC-conjugated anti-CD3 (BioLegend, San Diego, CA, USA) and PE-conjugated anti-CD19 (Becton Dickinson, Franklin Lakes, USA), and thymocytes were stained with APC-conjugated anti-CD4 and PE-conjugated anti-CD8 (BioLegend). Samples were run on a FACSVerse (Becton Dickinson) and FlowJo software version 10.4.2 (Tree Star, Ashland, USA) was used for data analysis.

2.8 | DNA sequencing

The PCR product generated from the genotyping primers, containing the mutated DNA site, was purified using the QIAquick PCR purification kit (Qiagen). The size of the purified product was verified by gel electrophoresis and sent to Eurofins (Germany) for sequencing.

2.9 | Histology

Formalin-fixed mesenteric fat tissue from three-month-old female S122A and WT controls was weighed and thereafter dehydrated, paraffin-embedded, and cut in 5-μm-thick longitudinal sections. The sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (Histocenter, Gothenburg, Sweden). Analysis of adipocyte size was performed in a double-blinded manner by two independent observers, using the Adipocyte Tool in the ImageJ software with simple segmentation in three different areas per section (one section per animal).

2.10 | Statistical analyses

Values are given as mean ± SEM. The statistical difference between the two groups was calculated using Student's *t*-test and $P < .05$ was considered statistically significant.

3 | RESULTS

To investigate if phosphorylation site S122 in ERα is involved in the physiological regulation of different tissues in vivo, we used female S122A mice, in which ERα cannot be phosphorylated at site S122 due to a point mutation, and compared them to WT littermates. The presence of ERα specifically phosphorylated on S122 in uteri of WT littermates and the absence of phosphorylation at this site in the S122A females were confirmed using a phospo-specific antibody only detecting ERα phosphorylated on serine 122 (Figure 2A, Supporting Figure S2A).

3.1 | The ability to phosphorylate site S122 is dispensable for normal feedback regulation of sex steroids, uterus weight, and immunological parameters in female mice

ERα mRNA expression was normal in uterus, gonadal fat, and cortical bone in three-month-old S122A female mice compared to WT littermates (Figure 2B) and protein levels of ERα in uterus were similar between S122A mice and WT controls (Figure 2C, Supporting Figure S2B). The weight of the uterus, a highly estrogen-sensitive organ, was similar between three-month-old S122A and WT female mice (Table 1), and analysis of the estrogen-regulated genes PR, IGFBP-3, and mucin-1 in uterine tissue, revealed no differences in gene expression levels between S122A and WT control mice (Supporting Table S1). Furthermore, analysis of the level of p-AKT in relation to total AKT in uterus showed that acute estrogen treatment (6h) was able to induce the phosphorylation of AKT in S122A females (Supporting Figure S4). S122A mice had normal serum levels of estradiol and testosterone compared to WT controls (Figure 2D-E), and serum levels of LH, FSH, P, and DHT were also similar between S122A females and WT controls (Supporting Table S2). Thymus weight and cellularity (the number of thymocytes) were similar between genotypes (Table 1). Furthermore, flow cytometry analysis of T cell maturation in thymus showed no differences in $CD4^+$, $CD8^+$ or double positive ($CD8^+CD4^+$) T cells between S122A mice and WT controls (Table 1). In addition, S122A and WT mice displayed the same frequency of $CD3^+$ T cells and $CD19^+$ B cells in bone marrow (Table 1). Liver weights were also unaffected in S122A females compared to WT controls (Table 1).

3.2 | The ability to phosphorylate site S122 in ERα is dispensable for the physiological regulation of the skeleton in female mice

DXA analyses at five weeks and three months of age (Table 2) showed that total body areal BMD (aBMD) was similar between

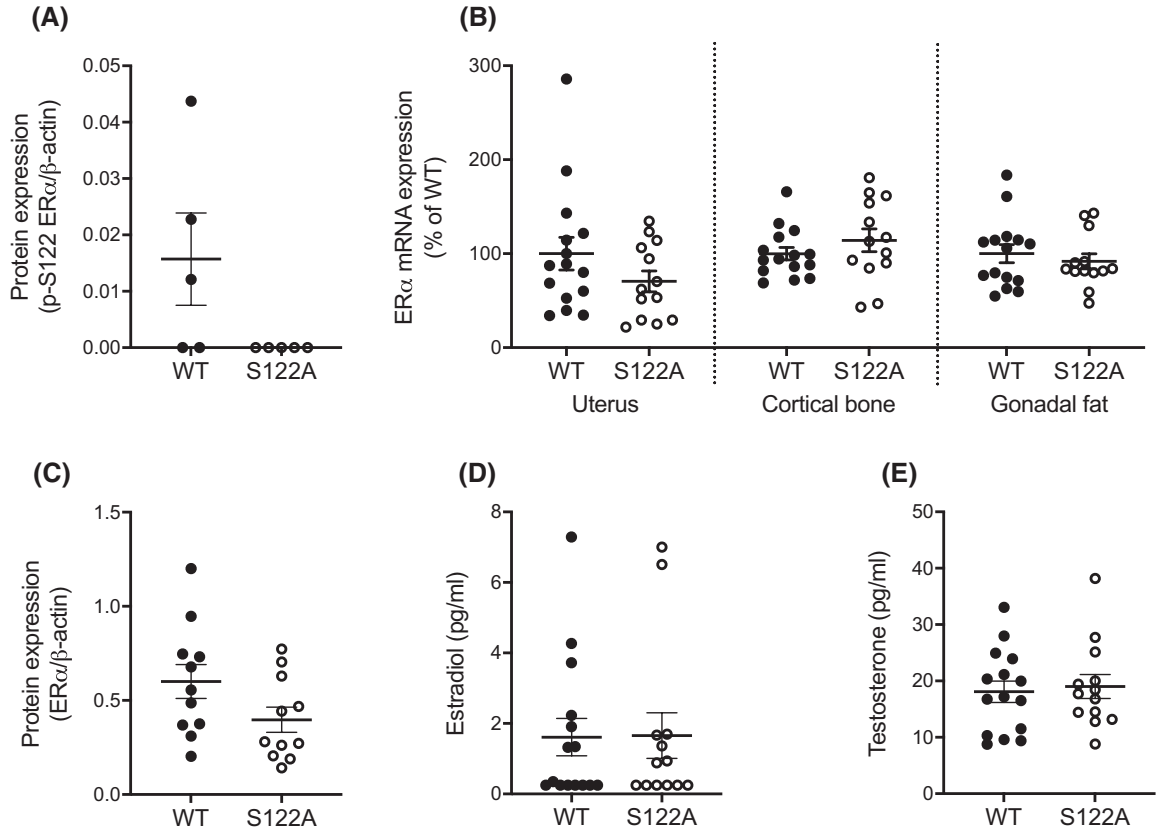

FIGURE 2 Mutation of phosphorylation site S122 in ERα has no impact on ERα mRNA expression or protein levels. Three-month-old female S122A and wild-type (WT) mice were used to verify the model by measuring p-S122 ERα protein levels in uterus using Simple Western (A). ERα mRNA expression was measured in uterus, cortical bone, and gonadal fat with Real-Time PCR (B), ERα protein expression was measured in uterus with Simple Western (C), and serum levels of 17β-estradiol (d), and testosterone (E) were measured with GC-MS/MS in three-month-old female S122A and wild-type (WT) mice. Non-detectable levels of 17β-estradiol were set to half LOQ (Limit Of Quantitation). Values are given as mean ± SEM. [n = 5-15]. Student's *t*-test, WT vs S122A mice

S122A females and WT controls and femur length (Table 3) at three months of age showed no difference between genotypes. High-resolution μCT analysis of femur in three-month-old S122A and WT mice showed that both cortical bone parameters (cortical thickness, endosteal circumference, and periosteal circumference) (Figure 3A, Table 3) and trabecular bone volume/total volume (BV/TV) (Figure 3B) were similar between S122A and WT mice. Furthermore, structural analysis of trabecular bone in femur (trabecular number; Tb.N., trabecular thickness; Tb.Th., and trabecular separation; Tb.Sp) (Table 3) showed no differences between S122A and WT mice. Similarly, the trabecular bone parameters (BV/TV, Tb.N., Tb.Th. and Tb.Sp) in vertebrae L5 did not differ between S122A and WT mice (Table 3). In vivo DXA analyses at 6, 9, and 12 months of age showed that total body aBMD was similar between WT and S122A females also at older ages (Figure 3C).

3.3 | Phosphorylation site S122 in ERα is involved in the regulation of body fat mass in female mice

Interestingly, shortly after sexual maturation, at three months of age, gonadal, and retroperitoneal fat depots were significantly increased in female S122A mice compared to WT littermates (Figure 4A,B), and the mesenteric fat was also increased in S122A mice (+26.3 ± 10.1%, $P < .05$) compared to WT littermates. Histological analysis of mesenteric fat showed that S122A mice had an increased adipocyte size compared to WT controls (Figure 4C-E). Three-month-old S122A mice also showed a strong tendency of the increased percentage of fat as measured by DXA, while no tendency was observed before sexual maturation at five weeks of age (Table 2). Furthermore, leptin levels were increased in three-month-old S122A mice compared to WT mice (Figure 4F), while insulin levels were unchanged at this age (Figure 4G). In accordance to the increased serum leptin levels, leptin gene expression was increased in gonadal fat tissue in S122A females compared to WT controls (Supporting Table S1). Leptin expression was also assessed in cortical bone, where no difference between S122A and WT controls was found (Supporting Table S1). No significant differences in expression of PPARγ, CEBPA or adiponectin in gonadal fat were detected between S122A females and WT controls, and the expression of the inflammatory cytokines TNFα and IL1b was also unchanged between the two genotypes (Supporting Table S1).

TABLE 1 Organ weights and immunological parameters in S122A mice

	WT	S122A
Uterus weight/BW (mg/g)	3.62 ± 0.41	2.81 ± 0.19
Liver weight/BW (mg/g)	47.33 ± 0.40	46.57 ± 0.53
Thymus weight/BW (mg/g)	2.53 ± 0.08	2.47 ± 0.07
Thymus cellularity (10^6 cells)	41.22 ± 2.95	42.40 ± 2.62
Bone marrow (% of living cells)		
T cells ($CD3^+$)	3.30 ± 0.11	3.34 ± 0.10
B cells ($CD19^+$)	11.53 ± 0.36	11.40 ± 0.27
Thymus (% of living cells)		
T cells ($CD4^+$)	7.29 ± 0.76	7.69 ± 0.92
T cells ($CD8^+$)	2.33 ± 0.08	2.26 ± 0.10
T cells (DP; $CD4^+$ $CD8^+$)	86.21 ± 1.44	86.59 ± 0.94

Note: Organ weights and flow cytometry analysis of bone marrow and thymus in three-month-old female S122A and wild-type (WT) littermates. Values are given as mean ± SEM. [n = 9-15]. Student's *t*-test, WT vs S122A mice. BW; body weight, DP; double positive.

TABLE 3 Skeletal analysis of S122A mice

	WT	S122A
Femur		
Bone length (mm)	15.1 ± 0.06	15.2 ± 0.06
Trabecular number (mm^{-1})	3.80 ± 0.13	3.50 ± 0.12
Trabecular thickness (μm)	45.7 ± 0.6	46.7 ± 1.1
Trabecular separation (μm)	125 ± 1.0	128 ± 0.9
Endosteal circumference (mm)	3.59 ± 0.03	3.63 ± 0.04
Perosteal circumference (mm)	4.80 ± 0.03	4.86 ± 0.04
Vertebrae L5		
BV/TV (%)	23.6 ± 0.3	24.1 ± 0.5
Trabecular number (mm^{-1})	5.51 ± 0.09	5.49 ± 0.08
Trabecular thickness (μm)	43.0 ± 0.4	43.9 ± 0.5
Trabecular separation (μm)	147 ± 2.2	148 ± 2.4

Note: High-resolution μCT analyses of femur and lumbar vertebra (L5) in three-month-old female S122A and wild-type (WT) littermates. Values are given as mean ± SEM. [n = 13-15]. Student's *t*-test, WT vs S122A mice. BV/TV; bone volume/total volume.

TABLE 2 Body composition in mice lacking phosphorylation site S122 in ERα

	5 weeks		3 months	
	WT	S122A	WT	S122A
Total body aBMD (mg/cm^2)	39.6 ± 0.4	40.3 ± 0.4	49.0 ± 0.4	48.6 ± 0.4
Body weight (g)	16.1 ± 0.2	16.6 ± 0.4	21.3 ± 0.3	19.7 ± 0.5
Fat percent (%)	13.4 ± 0.4	14.1 ± 0.5	14.9 ± 0.5	$17.9 \pm 1.5^{p=0.06}$
Lean mass (g)	11.7 ± 0.2	12.0 ± 0.3	16.3 ± 0.2	16.6 ± 0.4

Note:: Body weight and DXA measurements in five-week-old and three-month-old female S122A and wildtype (WT) littermates. Values are given as mean ± SEM. [n = 13-15]. Student's *t*-test, WT vs S122A mice.

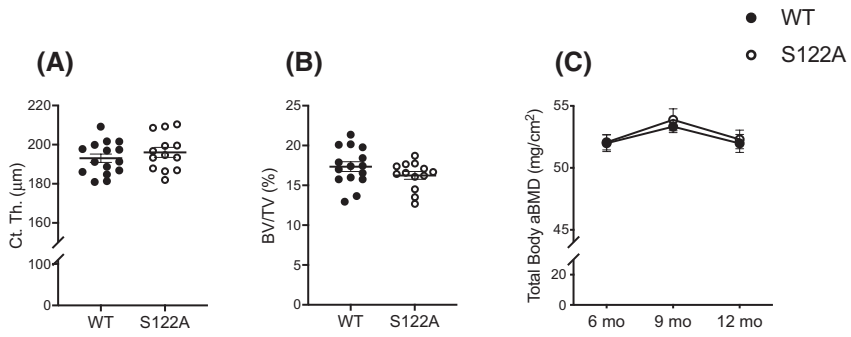

FIGURE 3 The ability to phosphorylate site S122 in ERα is dispensable for the physiological regulation of the skeleton in female mice. Cortical thickness (Ct. Th.) (A), and trabecular bone volume per total volume (BV/TV) (B) were analyzed by high-resolution μCT in femur at three months of age. Total body areal bone mineral density (aBMD) measured by DXA in female S122A and WT mice at 6, 9, and 12 months of age (C). Values are given as mean ± SEM. [n = 8-15]. Student's *t*-test, WT vs S122A mice

To further investigate these metabolic findings, we measured body weight and performed in vivo DXA in an additional study at 6, 9, and 12 months of age. Body weights at all these time points were significantly increased in S122A compared to WT mice (Figure 4H). The increased body weight of S122A mice at 6 months of age and onwards was

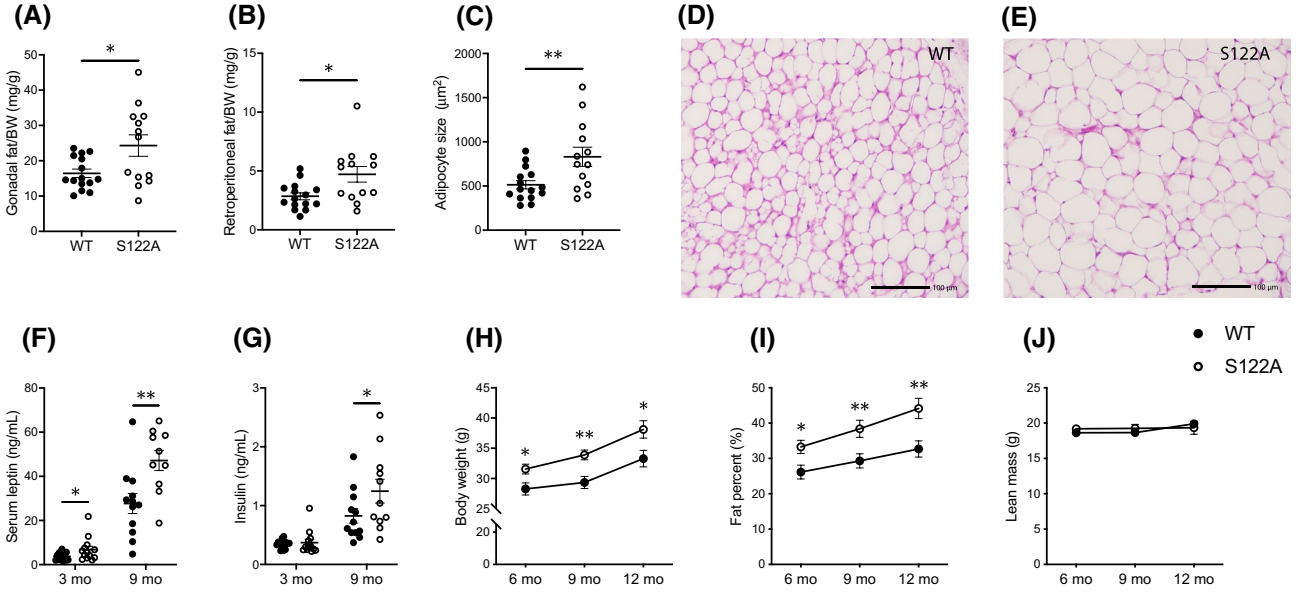

FIGURE 4 Mutation of phosphorylation site S122 in ERα increases body weight, fat mass, and insulin levels in female mice. Gonadal (A) and retroperitoneal (B) fat depot weights per body weight, adipocyte size (C), and representative pictures of adipocytes in WT (D) and S122A (E) mice, at three months of age. Serum leptin levels (F) and insulin levels (G) at three and nine months of age. Body weight (H), percent fat (I), and lean mass (J) measured by DXA, at 6, 9, and 12 months of age. Values are given as mean ± SEM. [n = 8-15]. *$P < .05$, **$P < .01$, Student's *t*-test, WT vs S122A mice.

accompanied by an increased percent of fat (Figure 4I), while lean mass was unchanged (Figure 4J) compared to WT mice. In line with the increased percent fat, serum levels of leptin and insulin were increased in nine-month-old S122A mice as compared to WT controls (Figure 4F-G). Body weight, weights of gonadal and retroperitoneal fat depots, and fat percent, measured by DXA, were also assessed in three-month-old male mice and no differences were seen between S122A males and WT controls (Supporting Figure S3).

We also assessed fat percent in female mice with total ERα inactivation (ERαKO) and other ERα mutant strains. Fat percent in female mice lacking the ERα AF-1 domain (ERαAF1KO) was increased to a similar extent as seen in S122A females compared to WT littermate controls (24 ± 8% and 27 ± 7%, respectively) at 4-6 months of age (Supporting Table S3). The fat percent increased by 35 ± 10% in total ERαKO mice and by 49 ± 12% in mice lacking the ERα AF-2 domain (ERαAF2KO) mice compared to WT littermate controls (Supporting Table S3). There were no significant differences between the increases in fat percent between any of the analyzed ERα mutant strains.

4 | DISCUSSION

Estrogen deficiency following menopause is coupled with increased risk of developing different diseases including osteoporosis, autoimmune diseases such as rheumatoid arthritis, and different metabolic disorders.[1,5,6,8,10,11] Estrogen treatment has a protective role in these diseases but is not a suitable treatment option due to adverse effects.[8,14-16] It is, therefore, important to increase our knowledge about estrogen signaling mechanisms in different tissues to be able to develop new tissue-specific drugs avoiding these adverse effects. We show, for the first time, that the inactivation of a specific phosphorylation site in ERα results in tissue-specific effects in female mice. The mouse model we have used has a point mutation at site S122 (corresponding to the human S118), leading to an amino acid shift that renders ERα unable to be phosphorylated at this specific site. We demonstrate that the ability to phosphorylate ERα at site S122 is essential for the normal regulation of metabolism in female mice, with affected fat mass and altered insulin levels, while it is dispensable for the normal regulation of the skeleton and for the immunological parameters investigated. Thus, targeting of specific posttranslational modifications sites in ERα, such as phosphorylation site S122, can result in tissue-specific estrogenic effects.

Phosphorylation of the human ERα at site S118 has been shown to be mediated by several different kinase pathways in vitro, including MAPK and CDK7.[38] S118 phosphorylation is stimulated by E2, but also by growth factors such as epidermal growth factor (EGF), leading to the ligand-independent activation of ERα,[55] and has been shown, in vitro, to affect several aspects of ERα function including coactivator binding, protein stability, and gene transcription.[38] The skeleton is an estrogen-sensitive tissue and we and others have shown that functional ERα signaling is crucial for the normal regulation of the skeleton.[17-19,21] Considering the important role that ERα signaling has in this tissue, and that the S118A (human) and S122A (murine) amino acid substitutions have been shown to affect ERα function in vitro, we hypothesized

that inhibiting the ability to phosphorylate site S122 in the murine ERα would have an impact on the regulation of the skeleton in vivo.

However, a thorough examination of the skeleton in females displayed no changes in bone mass or structural skeletal parameters when comparing S122A females with WT mice. Thus, the physiological regulation of the skeleton is not dependent on phosphorylation at site S122 in ERα. S118 is the most well-characterized phosphorylation site in the human ERα, but there are other phosphorylation sites in the vicinity of S118 and in vitro studies have shown that the inactivation of more than one phosphorylation site (eg, S104, S106, and S118) appears to have a larger impact on transcriptional function in human cells compared to the inactivation of a single site.[32,56,57] It might, therefore, be of interest to study the inactivation of S122 in combination with the inactivation of other phosphorylation sites in ERα in vivo in female mice and see if combined inactivation might affect the skeleton.

ERα signaling is involved in the negative feedback regulation of sex steroid levels and inactivation of ERα leads to dramatically increased serum levels of sex steroid.[18,58] We, therefore, examined sex steroid levels to determine whether phosphorylation site S122 in ERα in female mice is involved in this feedback system. Both testosterone and estradiol levels were comparable between S122A and WT mice, demonstrating that phosphorylation site S122 in female mice is not required for the normal negative feedback regulation of serum sex steroid levels.

One of the most estrogen-sensitive tissues in the body is the uterus and we and others have shown that lack of ERα leads to impaired uterine growth and unresponsiveness to estrogen treatment.[53,59] Uterus weight and transcription of estrogen-regulated genes in uterine tissue were normal in three-month-old females, suggesting that the S122 phosphorylation site in ERα is not involved in the physiological regulation of uterine growth. In addition, estrogen is able to induce the activation of intracellular signaling pathways including the PI3K-AKT pathway,[60] and we found that estrogen still was able to induce the phosphorylation of AKT in uterine tissue of S122A mice, suggesting that this phosphorylation site is not required for the activation of the PI3K-AKT signaling pathway in uterus. Although uterine weight and gene regulation appeared normal in the S122A female, we cannot rule out the possibility that reproduction may be affected by the lack of phosphorylation at site S122 given the pivotal role ERα has, not only in uterus, but also in reproductive organs, including the mammary gland. The lack of investigation of the reproductive status is a limitation to the present study.

Estrogens have a large impact on the regulation of the immune system. B lymphopoiesis in bone marrow is increased in estrogen-deficient states and decreased by estrogen treatment.[20,61,62] T lymphocytes are also affected by estrogen and thymus weight and cellularity are increased following ovariectomy.[61,63] Estrogen treatment leads to decreased thymus weight and decreased frequency of immature T cells (double-positive $CD4^+CD8^+$ cells), while the frequency of mature single-positive $CD4^+$ and $CD8^+$ T cells is increased. We sought to determine whether the S122A amino acid substitution in ERα affects lymphopoiesis in bone marrow or thymus. No effects on lymphocyte frequencies in bone marrow were found and thymus weight, cellularity, and T cell maturation, determined by frequencies of double-positive and single-positive T cells, in thymus were also unaffected by the S122A mutation. Thus, the ability to phosphorylate site S122 in females is not required for the normal regulation of B and T lymphopoiesis in female mice.

Menopause is associated with alterations in body composition and energy homeostasis, with increased visceral fat and insulin resistance as a consequence, and the frequency of metabolic syndrome is higher in postmenopausal compared to premenopausal women.[12] Effects of estrogen on body weight and fat mass have been extensively studied in rodents, and impaired estrogen signaling, either by removal of the ligand by ovariectomy, or global removal of ERα, the main mediator of estrogenic effects in adipose tissue,[23] leads to an increase in body weight and visceral fat depots.[17] Studies have shown that adipocyte-specific ERα deletion leads to increased fat mass,[64] demonstrating that estrogen has direct effects in fat cells. However, ERα deletion in other cell-types including muscle cells,[65] myeloid cells,[66] and neuronal cells[67] also results in increased fat mass, demonstrating that ERα-mediated regulation of fat mass is complex and involves estrogen signaling in several different tissues. In our study, we show that female mice lacking the possibility to phosphorylate site S122 in ERα had increased body weight and fat mass compared to WT littermates. Gene expression of leptin was increased in fat, but not in cortical bone, in S122A females compared to WT controls, a finding strengthening the tissue-specific role of the S122A mutation. Furthermore, analysis of male S122A mice showed no effect on fat mass when compared to WT controls, demonstrating that the inability to phosphorylate site S122 in ERα results in a sex-specific effect on fat mass. This sex-specificity is not seen in total ERαKO mice, where both females and males display increased adiposity.[68] Reasons for this discrepancy between ERα models may be that both female and male total ERαKO mice have a disturbed sex steroid feedback regulation with elevated sex steroid levels, which can affect adipose tissue regulation.[58,69] In the S122A model, no elevation of sex steroid levels is seen that can mask the potential sex-specific role of lacking phosphorylation at site S122 in ERα. There are also reports of differences in ERα expression in adipose tissue, and structures in the brain involved in the regulation of metabolism, between the sexes, indicating that the sensitivity for changes in ERα function can differ between sexes regarding the regulation of adiposity[70,71] and deletion of ERα in specific hypothalamic nuclei has also been shown to result in a greater impact in females compared to males.[67] Interestingly, the increase in fat mass in female S122A mice,

as demonstrated by increased weights of dissected fat depots, fat percent by DXA, and serum leptin levels, was seen after sexual maturation. Furthermore, adult female mice (9 months old) had increased serum levels of insulin suggesting insulin resistance. Thus, our data suggest that the ability to phosphorylate site S122 in ERα is of importance for preventing the development of metabolic disorders such as increased adiposity and insulin resistance in females. The S122 site is situated in the AF-1 domain of ERα and analysis of female ERαAF1KO mice revealed a similar increase in relative body fat levels compared to WT littermate controls as seen in S122A. Furthermore, when comparing the relative increase in fat percent between S122A and total ERαKO females and mice lacking the AF-2 domain (ERαAF2KO), no significant differences were detected. This suggests that phosphorylation site S122 is responsible for the main part of the effect of ERα on fat mass. A recent publication demonstrated that site S118 in the human ERα is involved in ERα turnover and that an S118A mutation prevented ERα degradation. Thus, it is possible that a normal ERα turnover is essential for the physiological regulation of fat mass, while this might be compensated for in other estrogen-responsive tissues.[39]

We have evaluated the consequences of the S122A amino acid substitution, rendering the ERα incapable to be phosphorylated at this site, in various tissues in vivo. The sequence surrounding site S122 in the murine ERα is highly homologous to site S118 in the human ERα and lack of phosphorylation in S118A mutated receptors have been verified in humans.[72,73] Previous studies have shown evidence of phosphorylation at site S122 in mice using phospho-specific antibodies, and we here verify the occurrence of phosphorylation at site S122 in WT mice, and show that the S122A amino acid shift results in lack of phosphorylation at this site also in the murine ERα. A limitation of the study is the lack of analyses on metabolism including food and water intake, physical activity, and oxygen consumption, which would increase the understanding of the mechanism behind the effect of the S122A substitution on adipose tissue and metabolism. Furthermore, in this study, we have investigated the role of phosphorylation at site S122 in mice in vivo under physiological conditions. Treatment of ovariectomized mice with estrogen may uncover other tissue-specific effects. The lack of a treatment experiment is a limitation of the current study and this research question will be interesting to address in the future.

Total ERα inactivation has been shown to lead to increased bone mass and this is suggested to be caused by disturbed sex steroid feedback. However, a strong association between body weight and bone mass has been shown in mice[74] and there is, therefore, a possibility that the lack of a skeletal phenotype in the S122A female mice might be caused by a compensatory effect of the increased body weight. However, for the total ERα knockout mice, it has been demonstrated that the increased bone mass is mainly caused by the elevation of androgen levels acting via the androgen receptors,[75] making this possibility less likely.

In vitro human studies have demonstrated that the importance of phosphorylation site S118 differs between cell types[34,48] and it has also been shown that protein phosphorylation varies between tissues.[30] Thus, increased knowledge regarding the importance of phosphorylation site S122, corresponding to site S118 in humans, in vivo may aid in the development of tissue-specific estrogenic drugs that can be used in various estrogen-associated diseases. In this study, we demonstrate the importance of a specific phosphorylation site in a transactivation domain in a nuclear steroid receptor for the first time in vivo and show that phosphorylation site S122 in ERα has a tissue-dependent role and is essential for the normal regulation of fat mass in female mice.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

KLG, CO, and MKL conducted the study design. KLG, MKL, HF, PH, VL, SMS, UI, KS, AT, AA, AIB, and MP, were responsible for the acquisition of data and KLG, MKL, and CO performed the analysis and interpretation of data. MKL, KLG, and CO wrote the main manuscript text and KLG and MKL prepared the figures. All authors reviewed the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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