

# Health benefits attributed to $17\alpha$ -estradiol, a lifespan-extending compound, are mediated through estrogen receptor $\alpha$

Shivani N Mann<sup>1,2,3</sup>, Niran Hadad<sup>4</sup>, Molly Nelson Holte<sup>5</sup>, Alicia R Rothman<sup>1</sup>, Roshini Sathiaseelan<sup>1</sup>, Samim Ali Mondal<sup>1</sup>, Martin-Paul Agbaga<sup>3,6,7</sup>, Archana Unnikrishnan<sup>3,8</sup>, Malayannan Subramaniam<sup>5</sup>, John Hawse<sup>5</sup>, Derek M Huffman<sup>9</sup>, Willard M Freeman<sup>2,10,11</sup>, Michael B Stout<sup>1,2,3\*</sup>

<sup>1</sup>Department of Nutritional Sciences, University of Oklahoma Health Sciences Center, Oklahoma City, United States; <sup>2</sup>Oklahoma Center for Geroscience, University of Oklahoma Health Sciences Center, Oklahoma City, United States; <sup>3</sup>Harold Hamm Diabetes Center, University of Oklahoma Health Sciences Center, Oklahoma City, United States; <sup>4</sup>The Jackson Laboratory, Bar Harbor, United States; <sup>5</sup>Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, United States; <sup>6</sup>Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, United States; <sup>7</sup>Dean McGee Eye Institute, University of Oklahoma Health Sciences Center, Oklahoma City, United States; <sup>8</sup>Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, United States; <sup>9</sup>Department of Molecular Pharmacology, Albert Einstein College of Medicine, New York, United States; <sup>10</sup>Genes & Human Disease Research Program, Oklahoma Medical Research Foundation, Oklahoma City, United States; <sup>11</sup>Oklahoma City Veterans Affairs Medical Center, Oklahoma City, United States

\*For correspondence:  
michael-stout@ouhsc.edu

**Competing interests:** The authors declare that no competing interests exist.

**Funding:** See page 21

**Received:** 03 June 2020

**Accepted:** 07 December 2020

**Published:** 08 December 2020

**Reviewing editor:** Rochelle Buffenstein, Calico Life Sciences, LLC, United States

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**Abstract** Metabolic dysfunction underlies several chronic diseases, many of which are exacerbated by obesity. Dietary interventions can reverse metabolic declines and slow aging, although compliance issues remain paramount.  $17\alpha$ -estradiol treatment improves metabolic parameters and slows aging in male mice. The mechanisms by which  $17\alpha$ -estradiol elicits these benefits remain unresolved. Herein, we show that  $17\alpha$ -estradiol elicits similar genomic binding and transcriptional activation through estrogen receptor  $\alpha$  (ER $\alpha$ ) to that of  $17\beta$ -estradiol. In addition, we show that the ablation of ER $\alpha$  completely attenuates the beneficial metabolic effects of  $17\alpha$ -E2 in male mice. Our findings suggest that  $17\alpha$ -E2 may act through the liver and hypothalamus to improve metabolic parameters in male mice. Lastly, we also determined that  $17\alpha$ -E2 improves metabolic parameters in male rats, thereby proving that the beneficial effects of  $17\alpha$ -E2 are not limited to mice. Collectively, these studies suggest ER $\alpha$  may be a drug target for mitigating chronic diseases in male mammals.

## Introduction

Aging is the leading risk factor for most chronic diseases, many of which are associated with declines in metabolic homeostasis (López-Otín *et al.*, 2013). Metabolic detriments associated with advancing age are further exacerbated by obesity (Villareal *et al.*, 2005; Waters *et al.*, 2013), which has risen

substantially in the older population (>65 years) over the past several decades (Flegal et al., 2010; Flegal et al., 2016). Moreover, obesity in mid-life has been shown to accelerate aging mechanisms and induce phenotypes more commonly observed in older mammals (Bischof and Park, 2015; Horvath et al., 2014; Nevalainen et al., 2017; Yang et al., 2009; Whitmer et al., 2005a; Whitmer et al., 2005b; Dye et al., 2017). These observations have led many to postulate that obesity may represent a mild progeria syndrome (Salvestrini et al., 2019; Tzanetakou et al., 2012; Pérez et al., 2016; Tchkonja et al., 2010; Stout et al., 2017a). Although it is well established that dietary interventions, including calorie restriction, can reverse obesity-related metabolic sequelae, many of these strategies are not well tolerated in older patients due to concomitant comorbidities (Villareal et al., 2005; Jensen et al., 2014). Compliance issues across all age groups also remain a paramount hurdle due to calorie restriction adversely affecting mood, thermoregulation, and musculoskeletal mass (Dirks and Leeuwenburgh, 2006). These adverse health outcomes demonstrate the need for pharmacological approaches aimed at curtailing metabolic perturbations associated with obesity and aging.

17 $\alpha$ -estradiol (17 $\alpha$ -E2) is one of the more recently studied compounds to demonstrate efficacy for beneficially modulating obesity- and age-related health outcomes. The NIA Interventions Testing Program (ITP) found that long-term administration of 17 $\alpha$ -E2 extends median lifespan of male mice in a dose-dependent manner (Strong et al., 2016; Harrison et al., 2014). Our group has been exploring potential mechanisms by which 17 $\alpha$ -E2 may improve healthspan and extend lifespan in a sex-specific manner. We have found that 17 $\alpha$ -E2 administration reduces calorie intake and regional adiposity in combination with significant improvements in a multitude of systemic metabolic parameters in both middle-aged obese and old male mice without inducing deleterious effects (Stout et al., 2017b; Steyn et al., 2018; Miller, 2020). Other groups have also determined that lifelong administration of 17 $\alpha$ -E2 beneficially modulates metabolic outcomes, including glucose tolerance, mTORC2 signaling, and hepatic amino acid composition and markers of urea cycling, which were reported to be dependent upon the presence of endogenous androgens (Garratt et al., 2017; Garratt et al., 2018). Additionally, multiple lifespan extending compounds, including 17 $\alpha$ -E2, exhibit similar modifications in liver function (Tyshkovskiy et al., 2019). In all, recent studies by several independent laboratories strongly indicate that the lifespan-extending effects of 17 $\alpha$ -E2 are at least associated with, if not dependent on, metabolic improvements.

Despite the mounting evidence demonstrating that 17 $\alpha$ -E2 improves numerous health parameters, the signaling mechanism(s) and primary tissues through which 17 $\alpha$ -E2 elicits these benefits remain unknown. Although 17 $\alpha$ -E2 is a naturally occurring enantiomer to 17 $\beta$ -estradiol (17 $\beta$ -E2), it has been postulated that 17 $\alpha$ -E2 signals through a novel uncharacterized receptor (Toran-Allerand, 2005; Toran-Allerand et al., 2002; Toran-Allerand et al., 2005; Green and Simpkins, 2000) as opposed to classical estrogen receptors  $\alpha$  (ER $\alpha$ ) and  $\beta$  (ER $\beta$ ), which is due to 17 $\alpha$ -E2 having significantly reduced binding affinity for ER $\alpha$  and ER $\beta$  as compared to 17 $\beta$ -E2 (Edwards and McGuire, 1980; Korenman, 1969; Littlefield et al., 1990; Anstead et al., 1997). For this reason, 17 $\alpha$ -E2 is often referred to as a non-feminizing estrogen (Green and Simpkins, 2000; Engler-Chiurazzi et al., 2017; Kaur et al., 2015). A few studies have suggested that a novel but uncharacterized estrogen receptor, termed ER-X, may mediate 17 $\alpha$ -E2 actions in the brain (Toran-Allerand, 2005; Toran-Allerand et al., 2002; Toran-Allerand et al., 2005; Green and Simpkins, 2000), although more recent studies supporting this hypothesis are lacking in the literature. Similarly, no reports to date have directly tested whether the doses of 17 $\alpha$ -E2 shown to improve healthspan and lifespan in mice are mediated through ER $\alpha$  and/or ER $\beta$ .

There is a multitude of data in the diabetes and metabolism literature demonstrating that ER $\alpha$  is a regulator of systemic metabolic parameters. Although most of these studies have historically been performed in female mammals, more recent studies have demonstrated that ER $\alpha$  also plays a critical role in modulating metabolism in male mammals. For instance, Allard and colleagues recently demonstrated that genomic actions of ER $\alpha$  regulate systemic glucose homeostasis in mice of both sexes and insulin production and release in males (Allard et al., 2019). Other studies have also determined that hepatic steatosis and insulin sensitivity, and therefore the control of gluconeogenesis, are regulated through FOXO1 in an ER $\alpha$ -dependent manner in male mice (Yan et al., 2019). Furthermore, hepatocyte-specific deletion of ER $\alpha$  was sufficient to abrogate similar estrogen-mediated metabolic benefits (Guillaume et al., 2019; Qiu et al., 2017; Meda et al., 2020). Given that several reports have linked the administration of 17 $\alpha$ -E2 to improvements in metabolic homeostasis, we

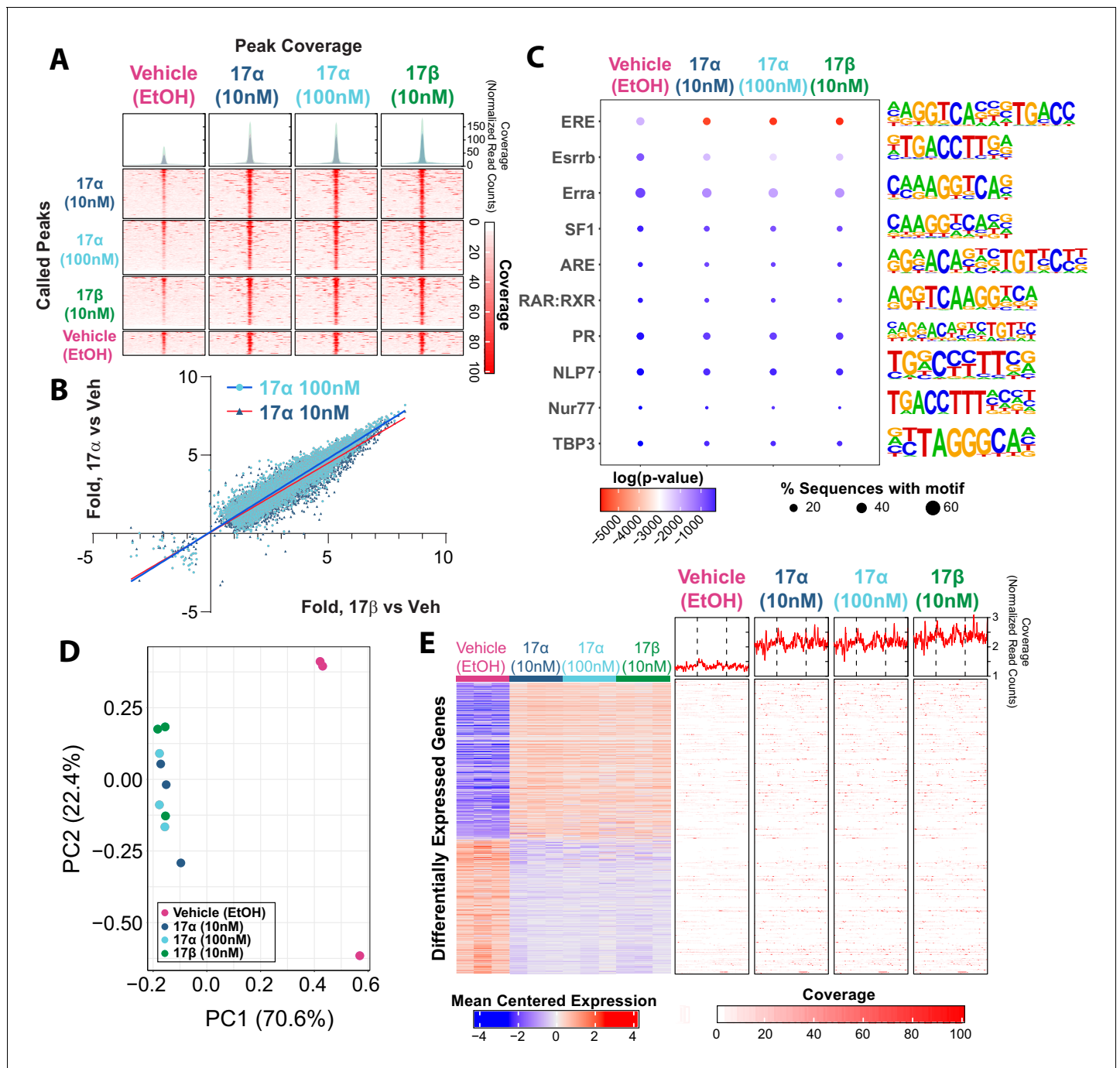
hypothesized that  $17\alpha$ -E2 signals through ER $\alpha$  to modulate hepatic function and systemic metabolism, thereby potentially contributing to the lifespan-extending effects of  $17\alpha$ -E2.

The work outlined in this report sought to determine if ER $\alpha$  is the primary receptor by  $17\alpha$ -E2 signals and modulates health parameters in mice. We initially determined that  $17\alpha$ -E2 and  $17\beta$ -E2 elicit similar genomic actions through ER $\alpha$ . Given that no studies to date have tested the potential role of ER $\alpha$  in modulating  $17\alpha$ -E2-mediated effects in vivo, we treated obese wild type (WT) and ER $\alpha$  knockout (ER $\alpha$  KO) littermate mice with  $17\alpha$ -E2 to determine if the ablation of ER $\alpha$  could attenuate  $17\alpha$ -E2-induced benefits on adiposity, metabolic homeostasis, and hepatic function. We found that the ablation of ER $\alpha$  completely attenuated all beneficial metabolic effects of  $17\alpha$ -E2. Follow-up studies in male WT rats undergoing hyperinsulinemic-euglycemic clamps revealed that  $17\alpha$ -E2 modulates hepatic insulin sensitivity following acute exposure. Given the established connection between the hypothalamus and liver in the modulation of hepatic insulin sensitivity (Könner *et al.*, 2007; Ruud *et al.*, 2017; Poci *et al.*, 2005a; Poci *et al.*, 2005b; Dodd *et al.*, 2018), coupled with our data demonstrating ER $\alpha$ -dependency of  $17\alpha$ -E2 actions on metabolic parameters, we speculate that  $17\alpha$ -E2 acts through ER $\alpha$  in the liver and/or hypothalamus to improve metabolic homeostasis in male mammals.

## Results

### **$17\alpha$ -E2 and $17\beta$ -E2 similarly modulate genomic binding and transcriptional activity of ER $\alpha$**

Ligand-mediated ER $\alpha$  dimerization leads to nuclear translocation and transcriptional activity. Previous work has shown that  $17\alpha$ -E2 and  $17\beta$ -E2 can bind to ER $\alpha$  with different affinities (Edwards and McGUIRE, 1980; Korenman, 1969; Littlefield *et al.*, 1990; Anstead *et al.*, 1997), yet potential differences in resultant genomic binding and transcriptional activity between the two ligands remains unexplored. We assessed ER $\alpha$  DNA binding and transcriptional induction following exposure to  $17\beta$ -E2 (10 nM) or  $17\alpha$ -E2 (10 nM or 100 nM) in U2OS cells that stably express ER $\alpha$  following doxycycline induction. We chose to use these cells because they do not endogenously express any form of ER $\alpha$  or ER $\beta$  and have been extensively utilized to elucidate the effects of ER $\alpha$  and ER $\beta$  agonists and antagonists on gene expression (Monroe *et al.*, 2003; Monroe *et al.*, 2005). ChIP-sequencing revealed peaks of ER $\alpha$  genomic binding in all conditions, that when compared, are qualitatively similar across treatments (Figure 1A). Statistically significant differences in ER $\alpha$  binding were determined by negative binomial regression with a Wald's pairwise post-hoc comparison (false discovery rate correction, FDR < 0.05). A total of 21,443 peaks were found to have a significant pairwise post-hoc comparison between vehicle and  $17\alpha$ -E2 and/or  $17\beta$ -E2 treated cells. No statistically significant differences between  $17\alpha$ -E2 and  $17\beta$ -E2-treated groups were observed.  $17\alpha$ -E2 and  $17\beta$ -E2 not only induced ER $\alpha$  binding at the same genomic locations but also to similar magnitudes. Comparing the levels of increased or decreased ER $\alpha$  binding (as compared to vehicle control) between treatments demonstrates the consistency of ER $\alpha$  genomic binding regardless of the agonist (Figure 1B, Supplementary file 1). The degree of increased or decreased ER $\alpha$  binding was highly similar between 10 nM  $17\alpha$ -E2 and 10 nM  $17\beta$ -E2, (Pearson's  $r = 0.95$ ,  $p < 0.001$ ) and 100 nM  $17\alpha$ -E2 and 10 nM  $17\beta$ -E2 (Pearson's  $r = 0.96$ ,  $p < 0.001$ ). As expected, ER $\alpha$ -binding sites were enriched for estrogen response elements (ERE), estrogen-related receptor beta (Esrrb), and estrogen-related receptor alpha (Esrra). Other common motifs found within ER elements, including steroidogenic factor-1 (SF1) (Lin *et al.*, 2007), and motif elements of known interacting partners, including retinoid acid receptor:retinoid X receptor (RAR:RXR) (Lee *et al.*, 1998), were also enriched (Figure 1C). In addition, we observed enrichment of androgen response elements (ARE) in ER $\alpha$  peaks (Figure 1C). Of particular relevance, many of the top enriched motifs identified contained the ERE consensus sequence TTGAC (Supplementary file 2). Following motif enrichment, we performed pairwise differential motif enrichment across all groups to determine if a specific agonist or agonist concentration caused a differential enrichment of any motifs, as would be suggestive of differential genomic binding. No differential motif binding was observed across treatment groups indicating that both  $17\alpha$ -E2 and  $17\beta$ -E2 cause ER $\alpha$  to bind to the same types of genomic elements (Hypergeometric test, FDR < 0.05).



**Figure 1.** 17α-E2 and 17β-E2 elicit similar genomic binding and transcriptional profiles through ERα. (A) Heatmap representing normalized genome-wide DNA binding by ERα via ChIP sequencing analyses centered according to peak summits for each treatment group and compared to each other group. (B) Differential binding was identified between vehicle and 17α-E2 or 17β-E2 treatment groups, but no differences were identified between 17α-E2 and 17β-E2-treated groups (negative binomial regression, followed by Wald test for pairwise comparisons, FDR < 0.05). Fold change in binding relative to vehicle control was compared between 17α-E2 treatments and 17β-E2. (C) Motif enrichment analysis, filtered for mammalian and non-overlapping motif groups, showing the top 10 non-redundant enriched sequence motifs across treatment groups (hypergeometric test, FDR < 0.05), (D) PCA plot of transcriptional profiles by RNA sequencing analyses demonstrating clustering of 17α-E2 and 17β-E2 treatment groups together, opposite from Vehicle-treated group along the first principle component, and (E) Heatmap representing differentially expressed genes (negative binomial regression, followed by Wald test for pairwise comparisons, FDR < 0.05) by RNA sequencing analyses (left) and ERα binding patterns within the gene body ±5 kb flanking regions of these genes via ChIP sequencing (right). Significant differential pairwise expression was observed only between 17α-E2 and 17β-E2 treatment groups and vehicle-treated control. These studies utilized U2OS-ERα cells treated with low dose (10 nM) 17α-E2, high dose (100 nM) 17α-E2, 17β-E2 (10 nM), or vehicle (EtOH). n = 3/group.

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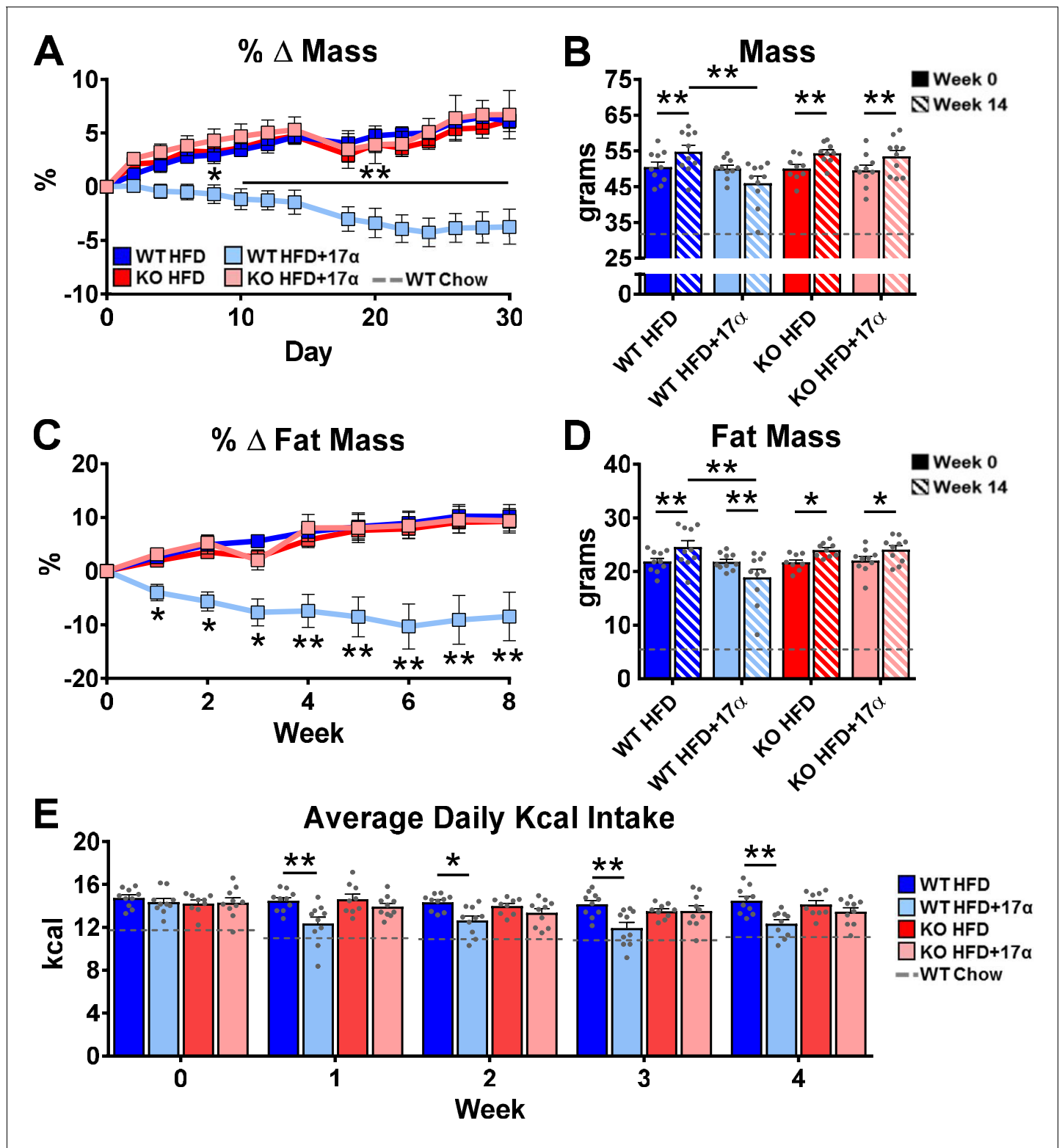
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**Figure supplement 1.** 17 $\alpha$ -E2 and 17 $\beta$ -E2 elicit similar ER $\alpha$  binding profile.

Next, we examined potential differences in transcriptional responses between treatment groups using RNA-sequencing. Principle component analysis based on the entire transcriptome revealed that all samples exposed to either 17 $\alpha$ -E2 or 17 $\beta$ -E2 clustered together, whereas vehicle-treated cells remained distinctly separated from treated cells on the first principle component, which explains the majority of the variance in transcription (70.6%) (**Figure 1D**). These data suggest that treatment vs vehicle is the primary covariate explaining variance in transcriptional profiles, not the specific agonist. Next, differential expression was assessed between all groups using a negative binomial regression model with a Wald pairwise post-hoc test. No genes were found to be differentially regulated (FDR < 0.05) between the estrogen treatments. Yet, compared to vehicle-treated cells, treatment of U2OS cells with either 10 nM or 100 nM 17 $\alpha$ -E2 or 10 nM 17 $\beta$ -E2 resulted in nearly identical gene suppression and activation signatures (**Figure 1E**, left). Additionally, both 17 $\alpha$ -E2 or 17 $\beta$ -E2 treatment conditions resulted in higher ER $\alpha$  DNA binding affinity to gene bodies of these differentially expressed transcripts compared to vehicle treatment, and no differences were observed between 17 $\alpha$ -E2 and 17 $\beta$ -E2 conditions (**Figure 1E**, right) (negative binomial regression with Wald pairwise post-hoc). These findings led us to postulate that 17 $\alpha$ -E2 may be the signaling through ER $\alpha$  to modulate health parameters in male mice. As such, we subsequently sought to determine if the ablation of ER $\alpha$  in vivo would mitigate the effects of 17 $\alpha$ -E2.

### ER $\alpha$ ablation attenuates 17 $\alpha$ -E2-mediated benefits on metabolic parameters in male mice in vivo

To induce obesity and metabolic perturbations in male mice, we administered high-fat diet (HFD) for several months prior to initiating 17 $\alpha$ -E2 treatment. Control mice remained on HFD, whereas 17 $\alpha$ -E2-treated mice were switched to an identical HFD containing 17 $\alpha$ -E2. Almost immediately after 17 $\alpha$ -E2 treatment began, male WT mice displayed significant reductions in mass (**Figure 2A–B**) and adiposity (**Figure 2C–D**). This is aligned with our previous reports demonstrating that 17 $\alpha$ -E2 administration quickly reduces body mass and adiposity (**Stout et al., 2017b; Steyn et al., 2018; Miller, 2020**), which we have linked to hypothalamic regulation of anorexigenic signaling pathways (**Steyn et al., 2018**). Indeed, male WT mice in the current study also displayed robust declines in calorie consumption during the first 4 weeks of treatment (**Figure 2E**). Conversely, all these benefits were completely abolished in male mice lacking ER $\alpha$  (ER $\alpha$  KO), thereby confirming that 17 $\alpha$ -E2 definitively acts through ER $\alpha$  to modulate feeding behaviors, mass, and adiposity in male mice. Given the close association between adiposity and metabolic homeostasis, coupled with our previous work demonstrating the ability of 17 $\alpha$ -E2 to improve metabolic parameters (**Stout et al., 2017b; Steyn et al., 2018**), we also assessed several metabolic variables in these studies. Similar to the mass and adiposity data described above, male WT mice receiving 17 $\alpha$ -E2 displayed significant improvements in fasting insulin (**Figure 3B**), HbA1C (**Figure 3C**), and glucose tolerance (**Figure 3D–E, Figure 3—figure supplement 1**), whereas male ER $\alpha$  KO mice receiving 17 $\alpha$ -E2 failed to recapitulate these findings. Interestingly, despite the masses of the male WT 17 $\alpha$ -E2 treatment group being nearly 15 grams greater than those of the male WT chow-fed controls, glucose tolerance was essentially identical between these groups, thereby indicating that 17 $\alpha$ -E2 restores metabolic flexibility in the presence of obesity in male mice (**Figure 3D–E, Figure 3—figure supplement 1**). We also evaluated the effects of 17 $\alpha$ -E2 on metabolic parameters in female WT and ER $\alpha$  KO mice provided a standard chow diet. In contrast to the males, we chose not to subject female WT and ER $\alpha$  KO mice to HFD because female ER $\alpha$  KO mice spontaneously develop obesity due to the ablation of ER $\alpha$  (**Manrique et al., 2012; Vidal et al., 1999**). Given that the female ER $\alpha$  KO mice are already in a challenged state, HFD would further exacerbate mass and adiposity differences between ER $\alpha$  KO and WT female mice. We found that 17 $\alpha$ -E2 failed to elicit improvements in mass, adiposity, calorie consumption, or metabolic parameters in female mice of either genotype (**Figure 3—figure supplement 2**). The positive effects of 17 $\alpha$ -E2 in male mice led us to speculate that the liver may play a key role in modulating 17 $\alpha$ -E2-mediated effects on systemic metabolic homeostasis. Importantly, several

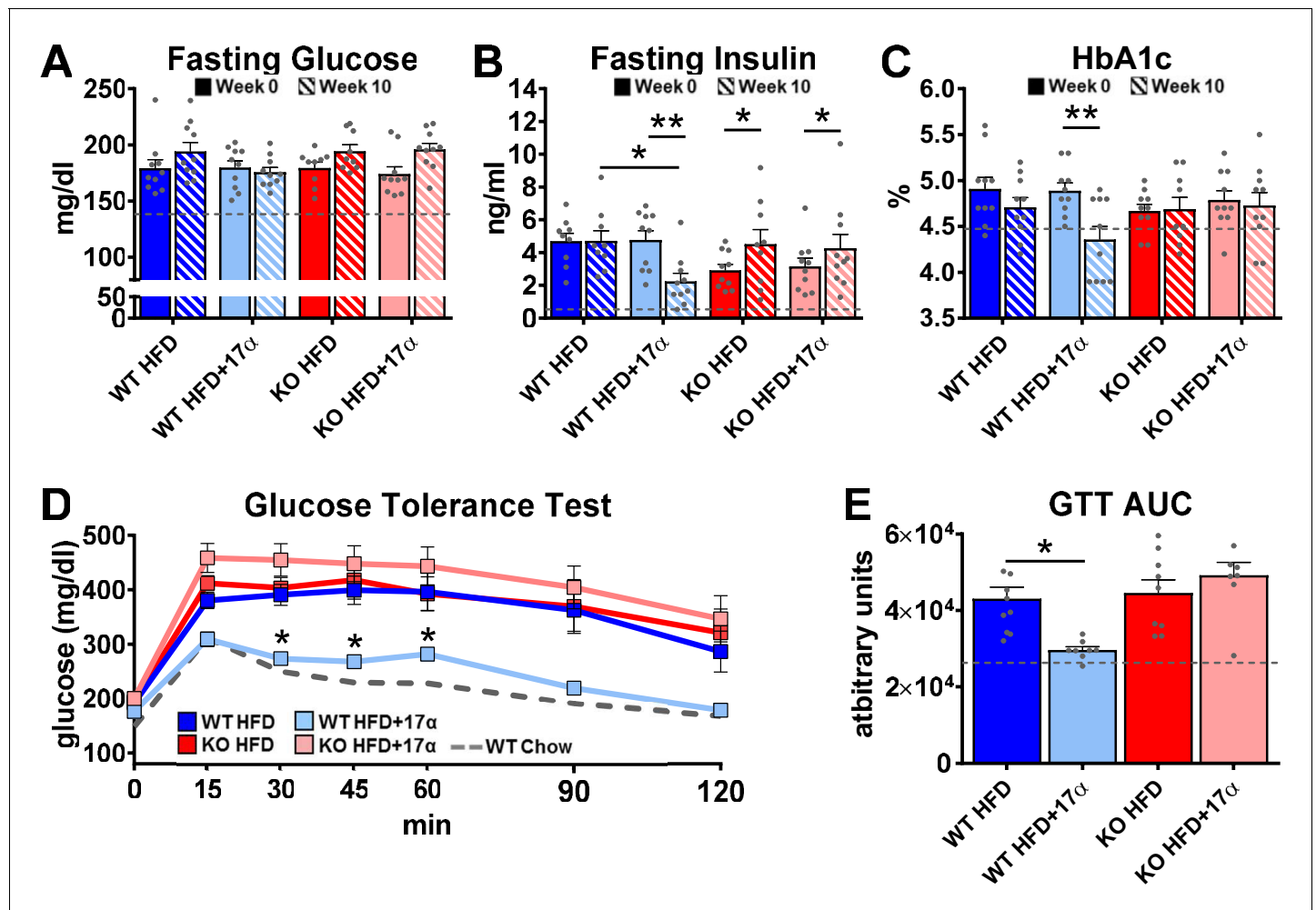


**Figure 2.** ERα is required for 17α-E2 to reduce mass, adiposity, and calorie intake in male mice. (A) Percent change in mass (mean ± SEM, two-way repeated measures ANOVA with Holm-Sidak post-hoc; \*p<0.05, \*\*p<0.005 between WT HFD and WT HFD+17α), (B) Mass at baseline (week 0; solid) and week 14 (striped) (mean ± SEM, two-way repeated measures ANOVA with Holm-Sidak post-hoc; \*p<0.05, \*\*p<0.005), (C) Percent change in fat mass (mean ± SEM, two-way repeated measures ANOVA with Holm-Sidak post-hoc; \*p<0.05, \*\*p<0.005), (D) Fat mass at baseline (week 0; solid) and week 14 (striped) (mean ± SEM, two-way repeated measures ANOVA with Holm-Sidak post-hoc; \*p<0.05, \*\*p<0.005), and (E) Average daily calorie intake per week in WT and ERα KO mice provided 45% HFD (TestDiet 58V8)±17α-E2 (14.4ppm) (mean ± SEM, two-way repeated measures ANOVA with Holm-Sidak post-hoc; \*p<0.05, \*\*p<0.005). *Figure 2 continued on next page*

Figure 2 continued

Holm-Sidak post-hoc; \* $p < 0.05$ , \*\* $p < 0.005$ ). Age-matched, male WT, chow-fed (TestDiet 58YP) mice were also evaluated as a normal-weight reference group and the corresponding means are depicted as dashed gray lines.  $n = 10$  (WT HFD), 10 (WT HFD+17 $\alpha$ ), 9 (KO HFD), 10 (KO HFD+17 $\alpha$ ), 12–15 (WT Chow).

studies have implicated hepatic ER $\alpha$  in the regulation of glucose homeostasis, insulin sensitivity, and crosstalk with hypothalamic neurons that modulate metabolism and feeding behavior (Meda et al., 2020; Torre et al., 2017; Barros and Gustafsson, 2011).



**Figure 3.** 17 $\alpha$ -E2 reverses obesity-related metabolic dysfunction in male WT, but not ER $\alpha$  KO, mice. (A) Fasting glucose (mean  $\pm$  SEM, two-way repeated measures ANOVA), (B) Fasting insulin (mean  $\pm$  SEM, two-way repeated measures ANOVA with Holm-Sidak post-hoc; \* $p < 0.05$ , \*\* $p < 0.005$ ), and (C) glycosylated hemoglobin (HbA1c) at baseline (week 0; solid) and week 14 (striped) in WT and ER $\alpha$  KO mice provided 45% HFD (TestDiet 58V8) $\pm$ 17 $\alpha$ -E2 (14.4 ppm) (mean  $\pm$  SEM, two-way repeated measures ANOVA with Holm-Sidak post-hoc; \*\* $p < 0.005$ ). (D) Glucose tolerance testing (GTT; 1 mg/kg) (mean  $\pm$  SEM, two-way repeated measures ANOVA with Holm-Sidak post-hoc; \* $p < 0.05$  between WT HFD and WT HFD+17 $\alpha$ ), and (E) GTT AUC during week 10 of the study (mean  $\pm$  SEM, two-way ANOVA with Holm-Sidak post-hoc; \* $p < 0.05$ ). Age-matched, male WT, chow-fed (TestDiet 58YP) mice were also evaluated as a normal-weight reference group and the corresponding means are depicted as dashed gray lines.  $n = 9$ –10 (WT HFD), 8–10 (WT HFD+17 $\alpha$ ), 9–10 (KO HFD), 8–10 (KO HFD+17 $\alpha$ ), 12–15 (WT Chow).

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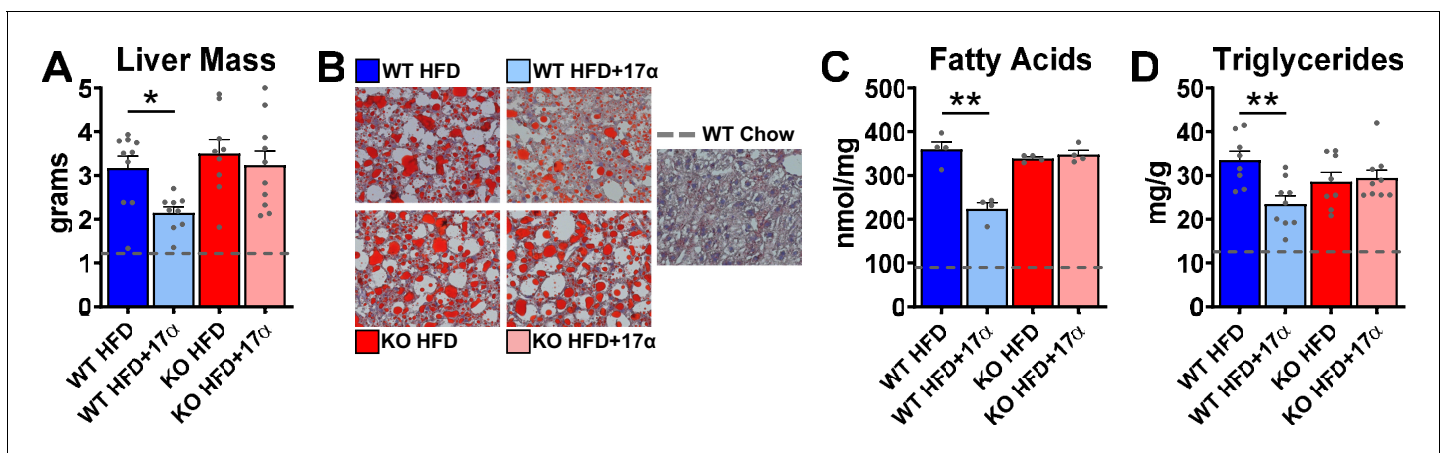
**Figure supplement 1.** 17 $\alpha$ -E2 reverses obesity-related metabolic dysfunction in male WT, but not ER $\alpha$  KO, mice.

**Figure supplement 2.** 17 $\alpha$ -E2 fails to alter metabolic parameters in WT or ER $\alpha$  KO female mice.

## 17 $\alpha$ -E2 improves liver disease pathology in an ER $\alpha$ -dependent manner in male mice

We previously reported that 17 $\alpha$ -E2 alters hepatic lipid deposition and DNA damage responses in male mice through unknown mechanisms (Stout et al., 2017b). In the current study, we sought to determine if these findings are mediated through ER $\alpha$ . We found that 17 $\alpha$ -E2 significantly reduced liver mass and steatosis in male WT, but not ER $\alpha$  KO mice, as evidenced by reductions in oil-red-O positivity, fatty acid content, and triglyceride accumulation (Figure 4, Figure 4—figure supplement 1). These observations were accompanied by significant alterations in gene expression associated with de novo lipogenesis (fatty acid synthase [*Fasn*]) and  $\beta$ -oxidation (peroxisome proliferator-activated receptor alpha [*Ppara*]; sterol regulatory element binding transcription factor 1 [*Srebf1*]) (Figure 4—figure supplement 1). These findings are similar to previous reports showing that 17 $\beta$ -E2 acts through ER $\alpha$  to modulate the expression and activity of genes that regulate hepatic lipid metabolism (Della Torre et al., 2016; Stubbins et al., 2012; Zhang et al., 2013). Interestingly, despite seeing overall reductions in hepatic fatty acid content with 17 $\alpha$ -E2 treatment in male WT mice (Figure 4C), we also observed elevations in specific fatty acids in these mice as compared to WT HFD controls. Notably, arachidonic acid (AA, 20:4n6) and docosahexaenoic acid (DHA, 22:6n3), both of which are precursors for eicosanoid, resolvins, and protectin production (Szefel et al., 2015; Kohli and Levy, 2009), were found to be increased by 17 $\alpha$ -E2 treatment in male WT mice (Figure 4—figure supplement 2). Our findings are aligned with a previous report by Garratt et al. showing that 17 $\alpha$ -E2 increases AA and DHA in liver (Garratt et al., 2018). None of the 17 $\alpha$ -E2-mediated changes in fatty acid profiles were observed in male ER $\alpha$  KO mice receiving 17 $\alpha$ -E2. In response to the elevations in AA and DHA with 17 $\alpha$ -E2 treatment, we also assessed circulating eicosanoids. We found that 17 $\alpha$ -E2 treatment also mildly altered several circulating eicosanoid concentrations in male WT mice (Supplementary file 3). Many of these have been linked to changes in inflammatory signaling (Kiss et al., 2010; Gilroy et al., 2016), although the role they are playing in 17 $\alpha$ -E2-mediated effects of on metabolism and/or aging remain unclear.

Due to the association between obesity-related hepatic steatosis and the onset of fibrosis, we assessed collagen deposition by trichrome staining and found that 17 $\alpha$ -E2 reduced this in male WT, but not ER $\alpha$  KO, mice (Figure 5A). We also observed significant suppression of several transcripts associated with liver fibrosis in male WT mice receiving 17 $\alpha$ -E2, including collagen type 1 alpha 1



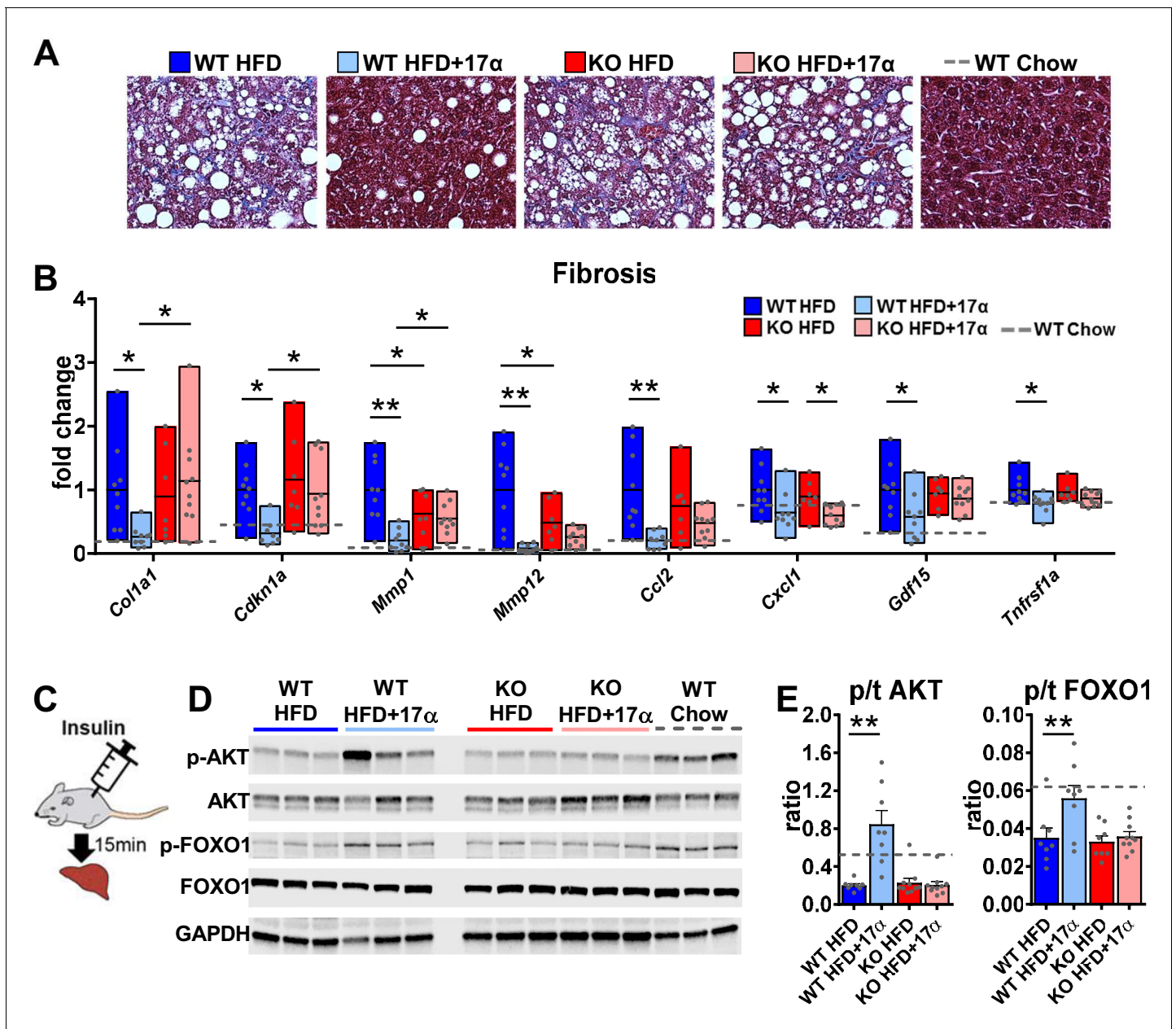
**Figure 4.** 17 $\alpha$ -E2 reverses obesity-related hepatic steatosis in an ER $\alpha$ -dependent manner in male mice. (A) Liver mass (mean  $\pm$  SEM, two-way ANOVA with Holm-Sidak post-hoc; \* $p$ <0.05), (B) Representative liver oil-red-O staining, (C) Liver fatty acids (mean  $\pm$  SEM, two-way ANOVA with Holm-Sidak post-hoc; \*\* $p$ <0.005), and (D) Liver triglycerides in WT and ER $\alpha$  KO mice provided 45% HFD (TestDiet 58V8) $\pm$ 17 $\alpha$ -E2 (14.4ppm) for 14 weeks (mean  $\pm$  SEM, two-way ANOVA with Holm-Sidak post-hoc; \*\* $p$ <0.005). Age-matched, male WT, chow-fed (TestDiet 58YP) mice were also evaluated as a normal-weight reference group and the corresponding means are depicted as dashed gray lines.  $n$  = 4–10 (WT HFD), 4–9 (WT HFD+17 $\alpha$ ), 4–9 (KO HFD), 4–10 (KO HFD+17 $\alpha$ ), 4–15 (WT Chow).

The online version of this article includes the following figure supplement(s) for figure 4:

**Figure supplement 1.** 17 $\alpha$ -E2 alters markers of lipid and glucose homeostasis predominantly through ER $\alpha$  in male mice.

**Figure supplement 2.** 17 $\alpha$ -E2 alters the hepatic fatty acid profile in male WT, but not ER $\alpha$  KO, mice.





**Figure 5.** 17 $\alpha$ -E2 reverses obesity-related liver fibrosis and insulin resistance in an ER $\alpha$ -dependent manner in male mice. (A) Representative liver Masson's trichrome staining for collagen and (B) Liver transcriptional markers of fibrosis in WT and ER $\alpha$  KO mice provided 45% HFD (TestDiet 58V8) $\pm$  17 $\alpha$ -E2 (14.4ppm) for 14 weeks (box plots depict total range of fold changes in gene expression with mean shown as a horizontal black line, Benjamini–Hochberg multiple testing correction, two-way ANOVA with Holm–Sidak post-hoc; \* $p$ <0.05, \*\* $p$ <0.005). (C) Schematic of in vivo insulin stimulation (2mU/g) in fasting mice, (D) Representative liver immunoblots, and (E) Quantification of phospho/total (p/t) AKT (pS473) and FOXO1 (pS256) in WT and ER $\alpha$  KO mice provided 60% HFD (TestDiet 58Y1) $\pm$ 17 $\alpha$ -E2 (14.4ppm) for 12 weeks (mean  $\pm$  SEM, Benjamini–Hochberg multiple testing correction, two-way ANOVA with Holm–Sidak post-hoc; \*\* $p$ <0.005). Age-matched, male WT, chow-fed (TestDiet 58YP) mice were also evaluated as a normal-weight reference group and the corresponding means are depicted as dashed gray lines.  $n$  = 7–10 (WT HFD), 8–9 (WT HFD+17 $\alpha$ ), 7–10 (KO HFD), 10 (KO HFD +17 $\alpha$ ), 7–11 (WT Chow).

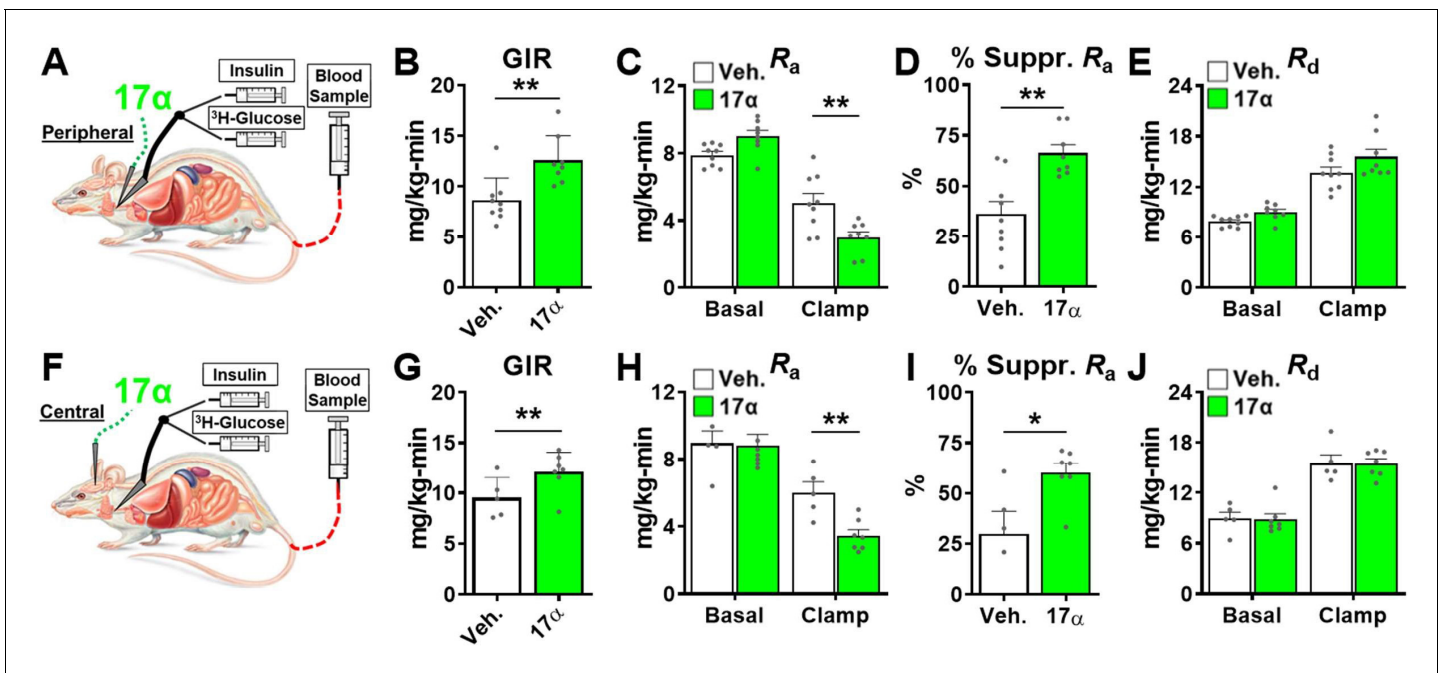
(*Col1a1*) (Hayashi et al., 2014; Lua et al., 2016), cyclin-dependent kinase inhibitor 1A (*Cdkn1a*) (Crary and Albrecht, 1998; Yang et al., 2020), matrix metalloproteinase 1 (*Mmp1*) (Lichtinghagen et al., 2003), matrix metalloproteinase 12 (*Mmp12*) (Madala et al., 2010), monocyte chemoattractant protein 1 (*Ccl2*) (Glass et al., 2018; Baeck et al., 2012), C-X-C motif chemokine ligand 1 (*Cxcl1*) (Yang et al., 2017), growth differentiation factor 15 (*Gdf15*) (Koo et al., 2018), and

TNF receptor superfamily member 1A (*Tnfrsf1a*) (*Grattagliano et al., 2019; Figure 5B*). Transcripts shown to be predictive of hepatic insulin resistance (follistatin [*Fst*], inhibin subunit beta E [*Inhbe*], insulin receptor substrate 2 [*Irs2*]) (*Tao et al., 2018; Parks et al., 2015*) and gluconeogenic plasticity (phosphoenolpyruvate carboxykinase 1 [*Pck1*], pyruvate kinase [*Pkm*]) (*Xiong et al., 2011*) were also beneficially modulated by 17 $\alpha$ -E2 in male WT mice (*Figure 4—figure supplement 1*). To confirm that 17 $\alpha$ -E2 improves hepatic insulin sensitivity, we also evaluated phosphorylation status of AKT and FOXO1 in livers from male WT and ER $\alpha$  KO mice following the administration of an insulin bolus (*Figure 5C*). We found dramatic improvements in phosphorylated AKT (pS473) and FOXO1 (pS256) in male WT mice treated with 17 $\alpha$ -E2 (*Figure 5D–E*), whereas these benefits were not observed in male ER $\alpha$  KO mice. Our findings are aligned with previous reports demonstrating that hepatic ER $\alpha$  plays a critical role in regulating insulin sensitivity in the liver of male mice (*Yan et al., 2019; Guillaume et al., 2019; Qiu et al., 2017; Zhu et al., 2014*). Collectively, these findings suggest that the liver is highly responsive to 17 $\alpha$ -E2 and that hepatic ER $\alpha$  is likely the signaling mechanism by which 17 $\alpha$ -E2 prevents and/or reverses steatosis, fibrosis, and insulin resistance.

Despite our findings demonstrating that 17 $\alpha$ -E2 reduces calorie intake and improves liver disease parameters in male mice in an ER $\alpha$ -dependent manner, it has historically been unclear if the benefits attributed to 17 $\alpha$ -E2 occur primarily due to long-term reductions in calorie intake. Moreover, it remains unclear if 17 $\alpha$ -E2 acts in a tissue-specific manner and if these observations would also occur in other mammalian species. To address these questions, we subsequently evaluated the effects of acute 17 $\alpha$ -E2 administration during hyperinsulinemic-euglycemic clamps in male WT outbred rats. These experiments allowed us to evaluate tissue-specific insulin-sensitivity following acute 17 $\alpha$ -E2 exposure, thereby circumventing long-term effects of the compound including reductions in calorie intake.

### Acute 17 $\alpha$ -E2 administration improves hepatic insulin sensitivity in male rats

The hyperinsulinemic-euglycemic clamp is the gold-standard for assessing insulin action in vivo (*Ayala et al., 2010*). Animals are fasted overnight prior to receiving a constant infusion of insulin and a variable infusion of [ $3\text{-}^3\text{H}$ ] glucose to maintain a euglycemia throughout the clamping period. Blood samples are frequently obtained to assess glucose concentration and adjust glucose infusion rates (GIRs) to maintain euglycemia, thereby allowing the calculation of insulin sensitivity to be done. Our first set of experiments in male rats sought to determine if acute peripheral infusions of 17 $\alpha$ -E2 modulates metabolic parameters during hyperinsulinemic-euglycemic clamps (*Figure 6A*). We found that acute peripheral administration of 17 $\alpha$ -E2 significantly increased systemic insulin responsiveness as compared to vehicle controls, which is indicated by increased GIRs (*Figure 6B*). These studies also determined that peripheral 17 $\alpha$ -E2 administration robustly suppressed hepatic gluconeogenesis as compared to vehicle controls ( $R_a$ ; *Figure 6C–D*), whereas glucose disposal rates ( $R_d$ ; *Figure 6E*) were essentially identical between groups under clamped conditions. These data indicate that 17 $\alpha$ -E2 beneficially modulates metabolic parameters independent of reductions in calorie intake and adiposity. Furthermore, these findings strongly suggest that the liver is a primary site where 17 $\alpha$ -E2 acts to improve metabolic homeostasis due to gluconeogenesis being tightly controlled by hormonal actions on hepatocytes (*Zhang et al., 2018*). However, it is also well established that the hypothalamus can directly modulate gluconeogenesis in the liver through hepatic innervation (*Timper and Brüning, 2017*); therefore, we sought to determine if acute intracerebroventricular (ICV) delivery of 17 $\alpha$ -E2 (*Figure 6F*) could modulate metabolic parameters similarly to that observed during peripheral 17 $\alpha$ -E2 administration. Interestingly, we found that central administration of 17 $\alpha$ -E2 essentially phenocopied the effects of peripheral 17 $\alpha$ -E2 infusion with regard to GIRs and suppression of hepatic gluconeogenesis (*Figure 6G–I*). These findings suggest that 17 $\alpha$ -E2 likely acts through hypothalamic neurons to regulate hepatic gluconeogenesis. Indeed, agouti-related peptide/neuropeptide Y (AgRP/NPY) and pro-opiomelanocortin (Pomc) neurons are known to regulate hepatic glucose production (*Könner et al., 2007; Ruud et al., 2017; Pocai et al., 2005a; Pocai et al., 2005b; Dodd et al., 2018*) and both neuronal populations express ER $\alpha$  (*Smith et al., 2013; Skinner and Herbison, 1997; Xu et al., 2011; Acosta-Martinez et al., 2007; Stincic et al., 2018; Kelly and Rønnekleiv, 2015; Smith et al., 2014*). Collectively, the hyperinsulinemic-euglycemic clamp studies revealed that 17 $\alpha$ -E2 definitively modulates metabolic homeostasis in an acute manner and suggests



**Figure 6.** Acute delivery of  $17\alpha$ -E2 improves hepatic insulin sensitivity. (A) Schematic of peripheral  $17\alpha$ -E2 infusions (or vehicle) during hyperinsulinemic-euglycemic clamps, (B) glucose infusion rates (GIR) (mean  $\pm$  SEM, unpaired Student's t-test; \*\* $p < 0.005$ ), (C) rate of glucose appearance ( $R_a$ ; hepatic glucose production) (mean  $\pm$  SEM, unpaired Student's t-test on Clamp; \*\* $p < 0.005$ ), (D) % suppression of hepatic glucose production (mean  $\pm$  SEM, unpaired Student's t-test; \*\* $p < 0.005$ ), and (E) rate of glucose disappearance ( $R_d$ ; peripheral glucose disposal) in 6 month old, male, FBN-F1 hybrid rats (mean  $\pm$  SEM, unpaired Student's t-test on Clamp). (F) Schematic of ICV (central)  $17\alpha$ -E2 infusions (or vehicle) during hyperinsulinemic-euglycemic clamps, (G) GIR (mean  $\pm$  SEM, unpaired Student's t-test; \* $p < 0.05$ ), (H)  $R_a$  (mean  $\pm$  SEM, unpaired Student's t-test on Clamp; \*\* $p < 0.005$ ), (I) % suppression glucose production (mean  $\pm$  SEM, unpaired Student's t-test; \* $p < 0.05$ ), and (J)  $R_d$  in 6-month-old, male, FBN-F1 hybrid rats (mean  $\pm$  SEM, unpaired Student's t-test on Clamp).  $n = 5$ –9 (Veh.), 7–8 ( $17\alpha$ ).

that the liver and hypothalamus are two primary sites of action for the regulation of metabolic parameters by  $17\alpha$ -E2.

## Discussion

$17\alpha$ -E2 has recently been found to increase median lifespan in male mice through uncharacterized mechanisms (Strong et al., 2016; Harrison et al., 2014). We and others have shown that metabolic improvements by  $17\alpha$ -E2 may underlie the lifespan extending effects. In these studies, we sought to determine the role of ER $\alpha$  in  $17\alpha$ -E2-mediated transcriptional effects in vitro and metabolic effects in vivo. Although previous studies have shown that  $17\alpha$ -E2 has limited binding affinity for ER $\alpha$ , it remains unclear if  $17\alpha$ -E2 can induce transcriptional and physiological alterations in this manner. Given the close association between metabolic improvements and ER $\alpha$  activity, we hypothesized that  $17\alpha$ -E2 signals through ER $\alpha$  to elicit beneficial health outcomes. In these studies, we utilized U2OS cells stably expressing ER $\alpha$  and ER $\alpha$  global knockout mice to assess the involvement of this receptor in mediating  $17\alpha$ -E2 effects. Results from these studies demonstrate that ER $\alpha$  plays a pivotal role in  $17\alpha$ -E2-mediated effects on genomic activity and metabolism. Moreover, these data suggest that ER $\alpha$  may be a target for the treatment of aging and chronic diseases in males.

Given the similarities between the metabolic benefits observed in vivo with  $17\alpha$ -E2 treatment and the established body of literature linking ER $\alpha$  activity to systemic metabolic regulation (Barros and Gustafsson, 2011), we utilized a well-established cell line model to globally assess the ER $\alpha$  cistrome and transcriptome following  $17\alpha$ -E2 and  $17\beta$ -E2 treatment. We found that, regardless of dose,  $17\alpha$ -E2 and  $17\beta$ -E2 elicited the same pattern of ER $\alpha$  genomic binding loci and these loci shared the same DNA motif enrichments. Additionally, activation and suppression of gene expression were similar with both  $17\alpha$ -E2 and  $17\beta$ -E2 exposure and were independent of dosage. This provides strong evidence that  $17\alpha$ -E2 is signaling through ER $\alpha$  to elicit beneficial outcomes, which is contrary to

what other reports have suggested (*Harrison et al., 2014; Garratt et al., 2017; Garratt et al., 2018; Toran-Allerand et al., 2002; Toran-Allerand et al., 2005; Toran-Allerand, 2005*). Toran-Allerand et al. reported that  $17\alpha$ -E2 signals through a novel receptor in the brain, which they termed ER-X (*Toran-Allerand et al., 2002; Toran-Allerand, 2005*). Although our findings appear to dispute this notion, several reports have shown that ER $\alpha$  exists and functions as multiple alternatively spliced variants (*Flouriot et al., 1998; Wang et al., 2005; Taylor et al., 2010; Zhang et al., 2016; Lin et al., 2013*). Therefore, we speculate that ER-X may have been a truncated, alternatively spliced, form of ER $\alpha$ , which nonetheless causes the same genomic and transcriptomic effects. These findings led us to investigate how ER $\alpha$  may modulate  $17\alpha$ -E2-induced benefits in vivo using ER $\alpha$  global KO mice.

In alignment with our previous reports (*Stout et al., 2017b; Steyn et al., 2018*),  $17\alpha$ -E2 reduced calorie intake, body mass, adiposity, and obesity-related metabolic perturbations in male WT mice. Conversely,  $17\alpha$ -E2 failed to elicit these beneficial effects in ER $\alpha$  KO mice, further supporting our hypothesis that ER $\alpha$  is the receptor by which  $17\alpha$ -E2 signals to induce beneficial metabolic outcomes. These observations are similar to how ER $\alpha$  is known to mediate the actions of endogenous estrogens on metabolic parameters in females (*Barros and Gustafsson, 2011*). In particular,  $17\beta$ -E2 acts through ER $\alpha$  to regulate systemic insulin sensitivity, lipid distribution, thermogenesis, and hypothalamic anorexigenic pathways (*Barros and Gustafsson, 2011; Stincic et al., 2018; López and Tena-Sempere, 2015*). The loss of endogenous estrogen action due to menopause in humans or ovariectomy (OVX) in rodents eliminates these beneficial effects and elicits metabolic perturbations (*Stefanska et al., 2015*). Moreover, OVX following sexual maturation has also been shown to reduce lifespan in female mice (*Benedusi et al., 2015*), indicating that endogenous estrogens regulate lifespan in females; which we surmise is at least partially mediated through ER $\alpha$ .

In the current study,  $17\alpha$ -E2 failed to induce beneficial metabolic effects in female mice of either genotype, which we postulate is due to endogenous  $17\beta$ -E2 saturating ER $\alpha$  in female WT mice, thereby limiting the potential benefits of  $17\alpha$ -E2 treatment. This interpretation is supported by our recent report showing that OVX renders WT female mice responsive to the beneficial effects of  $17\alpha$ -E2 on adiposity and bone mass (*Mann et al., 2020*), both of which are regulated by ER $\alpha$  activity (*Heine et al., 2000; Khosla and Monroe, 2018*). In males, very few studies have evaluated the role of ER $\alpha$  in metabolism, although a few recent reports have suggested that ER $\alpha$  plays tissue-specific roles, particularly in the liver, by regulating glucoregulatory pathways (*Allard et al., 2019; Yan et al., 2019; Guillaume et al., 2019; Qiu et al., 2017; Meda et al., 2020; Zhu et al., 2014*). These studies, coupled with our current findings, led us to speculate that  $17\alpha$ -E2 may be signaling through ER $\alpha$  in the liver to reverse metabolic disease and potentially extend healthspan and/or lifespan in males.

The liver is a major regulator of systemic metabolic homeostasis. Obesity and advancing age often promote a variety of liver conditions, including steatosis, fibrosis, and insulin resistance; all of which are associated with hallmarks of aging (*Hunt et al., 2019*), including cellular senescence (*Ogrodnik et al., 2017*), epigenetic alterations (*Horvath et al., 2014*), and dysregulated nutrient-sensing (*López-Otín et al., 2013*). We have previously shown that  $17\alpha$ -E2 can reduce hepatic steatosis, hepatic insulin resistance, and hepatocyte DNA damage through unknown mechanisms (*Stout et al., 2017b*). In the present study, we sought to determine if these findings are mediated through ER $\alpha$ . We found that  $17\alpha$ -E2 dramatically reduced liver mass and lipid content. As expected, these observations were not seen in ER $\alpha$  KO mice, providing further support for the hypothesis that  $17\alpha$ -E2 regulates systemic metabolic parameters through ER $\alpha$ . Interestingly, our findings suggest that  $17\alpha$ -E2 suppresses de novo lipogenesis and increases  $\beta$ -oxidation, predominantly in an ER $\alpha$ -dependent manner. This is aligned with previous reports showing that  $17\beta$ -E2 can modulate hepatic lipid dynamics through both genomic and non-genomic actions (*Pedram et al., 2016*), leading to altered expression of rate limiting enzymes that control de novo lipogenesis (*Zhang et al., 2013*) and  $\beta$ -oxidation (*Camporez et al., 2013*). Reports have also shown that  $17\beta$ -E2 can increase triglyceride export, thereby decreasing hepatic lipid deposition (*Zhu et al., 2013*). Although we did not directly assess cholesterol profiles in these studies, we speculate that  $17\alpha$ -E2 may partially reduce hepatic steatosis by increasing VLDL synthesis and/or triglyceride incorporation into VLDL. Additional studies will be needed to confirm how  $17\alpha$ -E2 alters hepatic lipoprotein dynamics.

Hepatic steatosis promotes liver fibrosis, which exacerbates hepatic insulin resistance (*Kim et al., 2015*). Endogenous estrogens and hormone replacement therapies in post-menopausal women

have been shown to serve a protective role on liver function (*Stubbins et al., 2012; Camporez et al., 2013; Zhu et al., 2013; Rossi et al., 2004; Brussaard et al., 1997*). Additionally, male humans are at a higher risk of developing hepatic steatosis and fibrosis as compared to age-matched females (*Guy and Peters, 2013; GBD 2017 Cirrhosis Collaborators and Collaborators, 2020*). In addition to reducing hepatic lipid deposition in male WT mice in the current studies, 17 $\alpha$ -E2 also dramatically suppressed transcriptional and histological markers of hepatic fibrosis in an ER $\alpha$ -dependent manner. We also determined that 17 $\alpha$ -E2 improved hepatic insulin sensitivity in male WT, but not ER $\alpha$  KO, mice. Several transcriptional markers associated with hepatic insulin resistance were suppressed in male WT mice receiving 17 $\alpha$ -E2, whereas this suppression was almost entirely absent in ER $\alpha$  KO mice. Subsequent studies employing insulin stimulation prior to euthanasia also revealed that 17 $\alpha$ -E2 robustly increased liver AKT and FOXO1 phosphorylation in male WT mice, indicating a reversal of obesity-related hepatic insulin resistance and increased control of gluconeogenesis. These findings clearly demonstrate that 17 $\alpha$ -E2 modulates hepatic insulin sensitivity in an ER $\alpha$ -dependent manner. These observations are aligned with previous reports showing that 17 $\beta$ -E2 acts through ER $\alpha$  to improve glucoregulation (*Meda et al., 2020; Zhu et al., 2013*). This provides further support that 17 $\alpha$ -E2 is eliciting metabolic improvements through ER $\alpha$  that are specific to the liver. Therefore, hepatic ER $\alpha$  may be a promising target for the development of therapeutics to alleviate metabolic disease in males. Future studies utilizing cell-type-specific ablation of ER $\alpha$  in the liver will be needed to unravel these possibilities.

Despite the robust effects of 17 $\alpha$ -E2 on liver function, it remained unclear if 17 $\alpha$ -E2 directly modulates hepatic insulin sensitivity or if these benefits were a secondary response to prolonged reductions in calorie intake, adiposity, and lipid redistribution. To test this, we performed hyperinsulinemic-euglycemic clamps, in conjunction with acute infusions of 17 $\alpha$ -E2 in male WT rats. We found that peripheral infusions of 17 $\alpha$ -E2 improved hepatic insulin sensitivity almost immediately, as evidenced by a greater suppression of hepatic glucose production in rats receiving 17 $\alpha$ -E2 as compared to vehicle controls. Additionally, we did not observe improvements in glucose disposal, thereby indicating that 17 $\alpha$ -E2 does not acutely increase systemic insulin-stimulated glucose uptake. This observation is aligned with recent literature demonstrating limited involvement of ER $\alpha$  in skeletal muscle insulin sensitivity (*Iñigo et al., 2020*). These data indicate that 17 $\alpha$ -E2 primarily alters systemic metabolic homeostasis through the modulation of hepatic gluconeogenesis, which is known to account for 76–87% of glucose production in the body (*Cherrington et al., 1994*). Although these studies are suggestive of direct actions in the liver, 17 $\alpha$ -E2 also has the ability to cross the blood brain barrier and elicit responses in the hypothalamus (*Steyn et al., 2018*). Given that the hypothalamus can regulate hepatic glucose production (*Könner et al., 2007; Ruud et al., 2017; Poci et al., 2005a; Poci et al., 2005b; Dodd et al., 2018; Timper and Brüning, 2017*), we also evaluated systemic insulin sensitivity following central administration of 17 $\alpha$ -E2. These experiments essentially phenocopied the results of the peripheral 17 $\alpha$ -E2 infusions, suggesting that the suppression of hepatic gluconeogenesis by 17 $\alpha$ -E2 is at least partially mediated by the hypothalamus. A multitude of studies have shown that the arcuate nucleus (ARC) of the hypothalamus plays a critical role in the regulation of hepatic gluconeogenesis through autonomic regulation and vagus nerve activity (*Ruud et al., 2017; Poci et al., 2005a; Zhang et al., 2018; Brandt et al., 2018*). Multiple neuronal populations within the ARC are known to be involved in the regulation of hepatic glucose production, including Pomc (*Dodd et al., 2018*) and AgRP/NPY (*Könner et al., 2007; Ruud et al., 2017; Poci et al., 2005a; Poci et al., 2005b*). Similarly, we have previously shown that the effects of 17 $\alpha$ -E2 on calorie intake and adiposity are dependent upon functional Pomc neurons, thereby providing evidence that 17 $\alpha$ -E2 can act through the hypothalamus to mediate systemic metabolic parameters (*Steyn et al., 2018*). However, in the absence of functional Pomc neurons, 17 $\alpha$ -E2 was still able to reduce fasting glucose and insulin, suggesting that 17 $\alpha$ -E2 modulates peripheral metabolism through multiple mechanisms, which may include alternative neuronal populations. Given that both Pomc (*Xu et al., 2011*) and AgRP/NPY (*Smith et al., 2013; Skinner and Herbison, 1997; Acosta-Martinez et al., 2007; Stincic et al., 2018; Kelly and Rønnekleiv, 2015; Smith et al., 2014; Sar et al., 1990*) neurons express ER $\alpha$ , regulate systemic metabolic parameters, and modulate feeding circuitry in a coordinated counter-regulatory fashion, it remains unclear whether 17 $\alpha$ -E2 is altering hepatic and systemic metabolic parameters through Pomc and/or AgRP/NPY neurons. Interestingly, a recent report from Debarba et al. demonstrated that 17 $\alpha$ -E2 increased hypothalamic ER $\alpha$  expression in the ARC (*Debarba, 2020*), which further suggests that 17 $\alpha$ -E2 signals through

ER $\alpha$  in the hypothalamus. Future studies utilizing hypothalamic cell-type-specific ER $\alpha$  KO models will be needed to disentangle which populations of neurons are required for 17 $\alpha$ -E2 to control food intake, peripheral glucose homeostasis, and insulin sensitivity.

Collectively, our findings strongly suggest that 17 $\alpha$ -E2 acts through ER $\alpha$  in the liver and/or hypothalamus to modulate metabolic parameters. However, our findings are in contrast to other reports suggesting that 17 $\alpha$ -E2 elicits health benefits by modulating androgen metabolism (Garratt et al., 2017; Garratt et al., 2018; Garratt and Stout, 2018). Garratt et al. reported that responsiveness to 17 $\alpha$ -E2 was significantly attenuated in castrated male mice (Garratt et al., 2018), which the authors proposed may indicate 17 $\alpha$ -E2 acts as a 5 $\alpha$ -reductase inhibitor (Schriefers et al., 1991) to prevent the conversion of testosterone into dihydrotestosterone (DHT). 17 $\alpha$ -E2 is known to be a mild 5 $\alpha$ -reductase inhibitor that is prescribed as a topical treatment for androgenetic alopecia (Orfanos and Vogels, 1980). 5 $\alpha$ -Reductase inhibition could conceivably elicit beneficial metabolic effects by either reducing the concentration of DHT, which has been shown to decrease adiposity (Movérare-Skrtec et al., 2006; Bolduc et al., 2004), or by promoting greater aromatization of testosterone to 17 $\beta$ -E2 (Veldhuis et al., 2009), which has been linked to improvements in metabolic parameters (Rubinow, 2017). If true, this would imply that the benefits of 17 $\alpha$ -E2 are occurring in an indirect manner. However, the dose of 17 $\alpha$ -E2 used in the vast majority of these studies, does not induce dramatic feminization of the sex hormone profiles in male mice (Stout et al., 2017b), which leads us to speculate that 17 $\alpha$ -E2 is acting in a direct manner through ER $\alpha$  rather than indirectly through androgen modulation. Furthermore, studies in male rodents (Livingstone et al., 2015; Dowman et al., 2013) and humans Wei et al., 2019 demonstrate that 5 $\alpha$ -reductase inhibition or deficiency increases insulin resistance and hepatic steatosis and fibrosis, which are contradictory to the effects of 17 $\alpha$ -E2 treatment in all of our studies utilizing male mice (Stout et al., 2017b; Steyn et al., 2018; Miller, 2020; Sidhom et al., 2020). Despite these contrasting observations, the studies by Garratt et al. do provide important insights into the interconnected and underappreciated relationship between androgen- and estrogen-signaling pathways and their roles in metabolism and aging. For instance, several recent reports have demonstrated interactions between the androgen receptor (AR) and ER $\alpha$  (D'Amato et al., 2016; Panet-Raymond et al., 2000; Peters et al., 2009), which suggests that modulation of one may affect function of the other. Additional factors to consider when comparing and contrasting our studies from those of Garratt et al. are differences in the length of study, age, and obesity status of the mice, and counterregulatory and/or compensatory effects of castration. Notably, it is plausible that 17 $\alpha$ -E2 could be inducing metabolic benefits and lifespan-extending effects through several distinct mechanisms, including direct actions through ER $\alpha$ , suppression of DHT production, and/or aromatization of testosterone. Future studies will be needed to discern the potentially interdependent nature of 17 $\alpha$ -E2 actions on ER $\alpha$  and androgen metabolism in metabolic improvement and lifespan extension.

There are a few notable caveats to our studies. First, we utilized constitutive global ER $\alpha$  KO mice, which have been shown to display varying degrees of compensatory ER $\beta$  activity due to the absence of ER $\alpha$  during development (Sánchez-Criado et al., 2012; Rosenfeld et al., 1998). However, if compensatory ER $\beta$  expression was playing a role in our study, we likely would not see a complete attenuation of 17 $\alpha$ -E2-mediated effects. As such, the results of our studies clearly indicate that ER $\alpha$  is the primary receptor by which 17 $\alpha$ -E2 signals. Another potential concern of the model is that ER $\alpha$  KO mice are known to have elevated endogenous testosterone levels (Gould et al., 2007), although the studies by Garratt et al. would suggest that higher testosterone levels could potentially render the male mice more responsive to 17 $\alpha$ -E2, whereas we observed the opposite. Future studies utilizing inducible Cre models to knockdown ER $\alpha$  post-sexual development may be considered if it is determined that Cre induction and subsequent ER $\alpha$  ablation is consistent throughout multiple organ systems, which has been shown to be inconsistent in other reports (Murray et al., 2012). Despite these minor concerns related to the model, the use of the constitutive global ER $\alpha$  KO was undoubtedly the best option for these studies. However, it must also be noted that female mice present a greater phenotypic response than males to the ablation of ER $\alpha$ , thereby exacerbating obesity and metabolic dysfunction which makes comparisons to female WT mice as well as their male littermates problematic (Manrique et al., 2012; Vidal et al., 1999). For this reason, we chose not to provide HFD to female mice in these studies. Regardless, 17 $\alpha$ -E2 still failed to elicit beneficial responses in female mice of either genotype (WT or ER $\alpha$  KO), which is aligned with previous reports demonstrating a lack of effect of 17 $\alpha$ -E2 in intact females (Garratt et al., 2017; Garratt et al., 2018). We also

recently reported that OVX renders female mice responsive to several of the benefits conferred by  $17\alpha$ -E2 treatment in male mice (Mann *et al.*, 2020). These observations, coupled with the genomic data presented herein, support the hypothesis that endogenous  $17\beta$ -E2 actions on ER $\alpha$  diminishes potential benefits of  $17\alpha$ -E2 in intact female mice. However, future studies will be needed to definitively determine if female mice subjected to diet-induced obesity will display responsiveness to  $17\alpha$ -E2 once severe metabolic dysfunction has emerged. Lastly, the current studies were relatively short in duration and it remains unclear if metabolic improvements with  $17\alpha$ -E2 treatment are required for the lifespan extension effects of the compound. Although several other studies have evaluated the long-term effects of  $17\alpha$ -E2 (Strong *et al.*, 2016; Harrison *et al.*, 2014; Garratt *et al.*, 2017; Garratt *et al.*, 2018), a shorter treatment duration was effective for testing our hypothesis in these studies. Similarly, given the close relationship between metabolic homeostasis, sex hormones, and longevity (López-Otín *et al.*, 2013; Barros and Gustafsson, 2011), we surmise that future studies evaluating the effects of ER $\alpha$  on male lifespan in the presence or absence of  $17\alpha$ -E2 will be needed. Although our current report does not provide direct evidence that ER $\alpha$  modulates the lifespan extending effects of  $17\alpha$ -E2, it does provide insight into the involvement of hepatic and/or hypothalamic ER $\alpha$  on  $17\alpha$ -E2-mediated metabolic effects in male mice.

In summary, the data presented herein are the first to show that  $17\alpha$ -E2 and  $17\beta$ -E2 induce nearly identical ER $\alpha$  chromatin association patterns and transcriptional activity. Moreover, we demonstrate that the metabolic benefits of  $17\alpha$ -E2 in male mice are ER $\alpha$ -dependent. We also provide evidence that strongly suggests  $17\alpha$ -E2 acts through the liver and hypothalamus to regulate metabolic homeostasis in male mice. These effects were mirrored by studies in male WT rats receiving  $17\alpha$ -E2, indicating that  $17\alpha$ -E2 can modulate metabolism almost instantaneously and that these effects are not limited to a single mammalian species. Future studies will be needed to confirm that  $17\alpha$ -E2 acts predominantly through ER $\alpha$  in a cell-type-specific manner in the liver and hypothalamus to modulate systemic metabolic homeostasis. It is also imperative that we determine if ER $\alpha$  exclusively modulates the lifespan-extending effects of  $17\alpha$ -E2 in male mice. Another potential avenue of investigation that remains unresolved is whether  $17\alpha$ -E2 acts through ER $\alpha$  in a genomic or non-genomic manner to modulate health parameters. Potential interactions between androgen and estrogen signaling must also be considered when evaluating the effects of  $17\alpha$ -E2 on metabolism and lifespan. These studies will provide additional insight into mechanisms of metabolic improvement and lifespan extension by  $17\alpha$ -E2. Our studies provide critical insight into the molecular mechanisms by which  $17\alpha$ -E2 elicits metabolic benefits in males, which were previously unknown and may underlie its lifespan-extending effects.

## Materials and methods

### Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent ( <i>M. musculus</i> )	B6N(Cg)-Esr1tm4.2Ksk/J	The Jackson Laboratory	Stock No:026176; RRID:IMSR_JAX:026176	ER $\alpha$ ( <i>Esr1</i> ) KO mice
Cell line ( <i>Homo sapien</i> )	U2OS Cells	ATCC	HTB-96; RRID:CVCL_0042	PMID:15802376 PMID:14505348
Antibody	anti-FLAG M2 (Mouse monoclonal)	Sigma-Aldrich	F1804	IP: 1 uL per pull-down (1 mg/mL)
Commercial assay or kit	Protein G Dynabeads	Applied Biosystems/ThermoFisher Scientific	10003D	IP: 30 uL per IP
Chemical compound, drug	$17\alpha$ -E2	Steraloids, Inc	E0870-000	
Chemical compound, drug	Novolin R 100 U/ml	Novolin		2mU/g
Other (diet)	Chow; TestDiet 58YP	TestDiet	TestDiet 58YP	
Other (diet)	HFD; TestDiet 58V8	TestDiet	TestDiet 58V8	HFD 45% by kcal
Other (diet)	HFD; TestDiet 58Y1	TestDiet	TestDiet 58Y1	HFD 60% by kcal

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Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Commercial assay or kit	Mouse Ultrasensitive Insulin ELISA	ALPCO	Cat# 80-INSMSU-E01; RRID:AB_2792981	
Commercial assay or kit	Free Glycerol Agent	Sigma-Aldrich	Sigma F6428	
Commercial assay or kit	Triglyceride Reagent	Sigma-Aldrich	Sigma F6428	
Commercial assay or kit	Glycerol Standard	Sigma-Aldrich	Sigma G1394	
Antibody	anti-pS473 AKT (Rabbit polyclonal)	Abcam	Cat# ab81283; RRID:AB_2224551	WB: (1:3000)
Antibody	Anti-pan-AKT (Rabbit polyclonal)	Abcam	Cat# ab179463; RRID:AB_2810977	WB (1:10000)
Antibody	Anti-pS256 FOXO1 (Rabbit polyclonal)	Abcam	Cat# ab131339; RRID:AB_11159015	WB (1:1000)
Antibody	Anti-FOXO1a (Rabbit polyclonal)	Abcam	Cat# ab52857; RRID:AB_869817	WB (1:1000)
Antibody	Anti-GAPDH (Rabbit polyclonal)	Abcam	Cat# ab9485; RRID:AB_307275	WB (1:2500)
Antibody	Anti-Rabbit IgG, IRDye 800 CW	LI-COR	Cat# 926-32211; RRID:AB_621843	WB (1:15000)
Commercial assay or kit	TaqMan Gene Expression Master Mix	Applied Biosystems/ThermoFisher Scientific	4369542	
Sequenced-based reagent	qPCR primer <i>Mmp1</i>	Integrated DNA Technologies	Mm.PT.58.42286812 Ref Seq: NM_008607(1)	Exon 5–6
Sequenced-based reagent	qPCR primer <i>Mmp12</i>	Integrated DNA Technologies	Mm.PT.58.31615472 Ref Seq: NM_008605(1)	Exon 8–9
Sequenced-based reagent	qPCR primer <i>Ccl2</i>	Integrated DNA Technologies	Mm.PT.58.42151692 Ref Seq: NM_011333(1)	Exon 1–3
Sequenced-based reagent	qPCR primer <i>Srebfl1</i>	Integrated DNA Technologies	Mm.PT.58.8508227 Ref Seq: NM_011480(1)	Exon 1–2
Sequenced-based reagent	qPCR primer <i>Pck1</i>	Integrated DNA Technologies	Mm.PT.58.11992693 Ref Seq: NM_011044(1)	Exon 3–4
Sequenced-based reagent	qPCR primer <i>Cdkn1a</i>	Integrated DNA Technologies	Mm.PT.58.17125846 Ref Seq: NM_007669(1)	Exon 2–3
Sequenced-based reagent	qPCR primer <i>Ppara</i>	Integrated DNA Technologies	Mm.PT.58.9374886 Ref Seq: NM_001113418(2)	Exon 8–9
Sequenced-based reagent	qPCR primer <i>Cxcl1</i>	Integrated DNA Technologies	Mm.PT.58.42076891 Ref Seq: NM_008176(1)	Exon 2–4
Sequenced-based reagent	qPCR primer <i>Col1a1</i>	Integrated DNA Technologies	Mm.PT.58.7562513 Ref Seq: M_007742(1)	Exon 1–2
Sequenced-based reagent	qPCR primer <i>Tnfrsf1a</i>	Integrated DNA Technologies	Mm.PT.58.28810479 Ref Seq: NM_011609(1)	Exon 5–7
Software, algorithm	SigmaPlot 12.5	Systat Software	RRID:SCR_003210	statistical analyses
Software, algorithm	ImageJ	ImageJ	RRID:SCR_003070	histological quantification
Software, algorithm	Image Studio	LI-COR	RRID:SCR_015795	western blot quantification
Software, algorithm	RStudio	GenomicAlignments DiffBind DESeq2 GenomicRanges	RRID:SCR_000432	Peak Calling Differential expression Differential binding
Software, algorithm	Bowtie2 MACS2 Bedtools Samtools Picard-tools Trimmomatic	Bowtie2 MACS2 Bedtools Samtools Picard-tools Trimmomatic		Alignment, Peak Calling, trimming, duplicate identification



## U2OS cells

U2OS osteosarcoma cells stably expressing flag-tagged ER $\alpha$  (U2OS-ER $\alpha$ ) under the control of doxycycline (dox)-inducible promoter (*Monroe et al., 2003*) were utilized for the studies described here. U2OS cells were originally purchased from ATTC and were authenticated using IDEXX BioAnalytics (Westbrook, ME). Cells were also regularly checked for mycoplasma contamination using a PCR-based mycoplasma detection kit from SouthernBiotech (Birmingham, AL) and were confirmed to be negative. U2OS-ER $\alpha$  cells were cultured in phenol-free  $\alpha$ MEM medium supplemented with 10% HyClone<sup>TM</sup> charcoal/dextran stripped FBS (GE Healthcare Life Sciences, Pittsburgh, PA), 1% antibiotic/antimycotic, 5 mg/L blasticidin S, and 500 mg/L zeocin in a humidified 37°C incubator with 5% CO<sub>2</sub>. Cells were plated in 12-well plates in the presence of doxycycline to induce ER $\alpha$  expression. The following day, cells were treated for 24 hr with 17 $\beta$ -E2 (10 nM) or 17 $\alpha$ -E2 (10 nM and 100 nM) (Steraloids, Newport, RI) in charcoal-stripped FBS-containing media.

## ChIP-sequencing

To evaluate patterns of ER $\alpha$  binding agonized by 17 $\alpha$ -E2 vs 17 $\beta$ -E2, we performed ChIP-Sequencing. U2OS-ER $\alpha$  cells were harvested 24 hr post-treatment and chromatin immunoprecipitation was performed as previously described (*Reese et al., 2018; Nelson et al., 2006*). Briefly, ER $\alpha$  was immunoprecipitated overnight at 4°C using 10  $\mu$ g of Flag antibody (clone M2, Sigma-Aldrich, St. Louis, MO). Complexes bound to the antibody were captured with protein G Dynabeads (Thermo Fisher Scientific, Waltham, MA), extensively washed, and reverse cross-linked at 65°C overnight. DNA isolation was performed by phenol/chloroform extraction and was used for ChIP-sequencing library preparation. Libraries were sequenced using paired-end 100 bp reads on the Illumina HiSeq 4000 (GSE151039). Reads were aligned to the human genome (hg19, <https://genome.ucsc.edu/cgi-bin/hgGateway>) using bowtie2 (*Langmead and Salzberg, 2012*) and duplicated reads were flagged with Picard-tools (<http://broadinstitute.github.io/picard/>). ER $\alpha$ -binding peaks were called using MACS2 (*Zhang et al., 2008*) with recommended settings. Peak genomic location, breadth of coverage, and peak summit location were determined using MACS2. NarrowPeak files containing peak information were used to determine differential ER $\alpha$  binding. First, peaks were centralized around the summit and 250 bp flanking regions were added to the summit location to generate equal 500 bp regions across all experimental groups. Peak files were then used to extract read counts from the aligned de-duplicated BAM file using samtools (*Li et al., 2009*), read counts were then normalized to total library sequencing depth. To determine differential binding, the R package diffbind was utilized (*Ross-Innes et al., 2012*). Normalized read counts were log<sub>2</sub> transformed and normalized across all experimental groups. Differential binding between treatment groups was determined using negative binomial regression models utilized in the R package DESeq2, statistical significance for pairwise comparisons between experimental groups was determined using Wald test. To account for multiple comparisons, we used Benjamini-Hochberg multiple testing correction (False-discovery rate, FDR). Motif analysis was performed using HOMER with standard settings to identified motifs. Peak regions called for each treatment group were analyzed to identify enriched motifs relative to the entire genome. For pairwise differential motif enrichment or depletion between experimental groups, we utilized a hypergeometric test with the number of sequences with a motif from each group and total number of peaks as total sample size. Motifs that appeared in less than five sequences between both pairwise test groups were removed. Benjamini-Hochberg multiple testing correction was utilized to control for multiple testing (FDR < 0.05).

## RNA-sequencing

U2OS-ER $\alpha$  cells were harvested 24 hr post-treatment and RNA was extracted using Trizol and DNase cleanup. RNA libraries were prepared with Illumina's TrueSeq RNA-seq library prep according to manufacturer protocol. Libraries were sequenced with 150 bp paired-end reads on the Illumina 4000 platform (Illumina, San Diego, CA) (GSE151039). Sequence quality control was performed with fastQC, Paired reads were trimmed using trimmomatic, and were aligned to the hg19 genome using STAR (*Dobin et al., 2013*). Differential expression was determined using previously described methods (*Hadad et al., 2019*). In brief, gene counts were determined with the R package GenomicAlignments 'summarizeOverlap' function. Gene counts were then transformed using regularized log

transformation and normalized relative to library size using the DESeq2 (Love et al., 2014) R package. Differential expression was determined using negative binomial generalized linear model using counts ~ treatment model. We performed pairwise differential expression between all experimental groups using Wald test. All comparisons were corrected for multiple testing using Benjamini-Hochberg multiple testing correction method. Differential expression significance threshold was set to FDR corrected  $p < 0.05$ .

### Animal study 1

To determine if ER $\alpha$  is the primary receptor by which 17 $\alpha$ -E2 signals to elicit metabolic benefits in vivo, we utilized male global ER $\alpha$  KO and WT littermate mice. Mice were acquired from Dr. Kenneth Korach (National Institute of Environmental Health Sciences [NIEHS]) and were also bred at OUHSC by pairing ER $\alpha$  heterozygous KO mice (JAX; strain #026176). Mice were fed a 45% high-fat diet (HFD) (TestDiet 58V8, 35.5% CHO, 18.3% PRO, 45.7% FAT) from TestDiet (Richmond, IN) for 4 months prior to study initiation to induce obesity and metabolic perturbations. Additionally, age-matched, male WT, chow-fed mice were maintained on TestDiet 58YP (66.6% CHO, 20.4% PRO, 13.0% FAT) throughout the entire study as a healthy-weight reference group. Mice were individually housed with ISO cotton pad bedding, cardboard enrichment tubes, and nestlets at  $22 \pm 0.5^\circ\text{C}$  on a 12:12 hr light-dark cycle. Unless otherwise noted, all mice had ad libitum access to food and water throughout the experimental timeframe. At the conclusion of the fattening period, all mice (age: 6–8 months) receiving HFD were randomized within genotype by age, body mass, fat mass, calorie intake, fasting glucose, fasting insulin, and glycosylated hemoglobin (HbA1C) into HFD or HFD+17 $\alpha$  (TestDiet 58V8 + 17 $\alpha$ -E2, 14.4ppm; Steraloids, Newport, RI) treatment groups for a 14-week intervention. Body mass and calorie intake were assessed daily for the first 4 weeks, followed by body mass and body composition (EchoMRI, Houston, TX) on a weekly basis. At 10 weeks post-treatment, mice were fasted for 5–6 hr and fasting glucose, fasting insulin, HbA1C, and glucose tolerance were assessed. At the conclusion of the study (14 weeks post treatment), mice were euthanized with isoflurane in the fasted state (5–6 hr). Blood was collected into EDTA-lined tubes by cardiac puncture, and plasma was collected and frozen. Tissues were excised, weighed, flash frozen, and stored at  $-80^\circ\text{C}$  unless otherwise noted. Small sections of liver were fixed in 4% paraformaldehyde in preparation for paraffin- or cryo-embedding for future analyses. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at OUHSC.

### Animal study 2

Although previous studies [Strong et al., 2016; Harrison et al., 2014; Garratt et al., 2017; Garratt et al., 2018] have demonstrated minimal effects of 17 $\alpha$ -E2 in female mice, we thought it prudent to determine if the ablation of ER $\alpha$  would alter female responsiveness to 17 $\alpha$ -E2. Female WT and ER $\alpha$  KO mice were acquired from Dr. Kenneth Korach (National Institute of Environmental Health Sciences [NIEHS]). Female mice were maintained on Chow TestDiet 58YP (66.6% CHO, 20.4% PRO, 13.0% FAT) and were not subject to HFD feeding due to ER $\alpha$  KO female mice naturally displaying an obesity phenotype. Mice were individually housed with ISO cotton pad bedding, cardboard enrichment tubes, and nestlets at  $22 \pm 0.5^\circ\text{C}$  on a 12:12 hr light-dark cycle. Unless otherwise noted, all mice had ad libitum access to food and water throughout the experimental timeframe. At age 9–11 months, female mice were randomized within genotype by age, body mass, fat mass, calorie intake, fasting glucose, fasting insulin, and glycosylated hemoglobin (HbA1C) into Chow or Chow +17 $\alpha$ -E2 (TestDiet 58YP + 17 $\alpha$ -E2, 14.4ppm; Steraloids, Newport, RI) treatment groups. The study was terminated following a 4-week intervention due to a lack of responsiveness to 17 $\alpha$ -E2. At the conclusion of the study, mice were euthanized with isoflurane in the fasted state (5–6 hr). Blood was collected into EDTA-lined tubes by cardiac puncture, and plasma was collected and frozen. Tissues were excised, weighed, flash frozen, and stored at  $-80^\circ\text{C}$ . All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at OUHSC.

### Animal study 3

To assess insulin sensitivity within the liver, an additional cohort of ER $\alpha$  KO and WT littermate mice were bred from mice acquired from Jackson Laboratory (JAX; strain #026176), which were generated from identical founder strains in the laboratory of Dr. Korach at NIEHS. Male ER $\alpha$  KO and WT

mice were fed a 60% high-fat diet (HFD; TestDiet 58Y1, 20.3% CHO, 18.1% PRO, 61.6% FAT) for 4 months prior to study initiation to induce obesity and metabolic perturbations. Additionally, as was done in Animal Study 1, age-matched, male WT, chow-fed mice were maintained on TestDiet 58YP (66.6% CHO, 20.4% PRO, 13.0% FAT) throughout the entire study as a healthy-weight reference group. Mice were group housed with corncob bedding, cardboard enrichment tubes, and nestlets at  $22 \pm 0.5^\circ\text{C}$  on a 12:12 hr light-dark cycle. Unless otherwise noted, all mice had ad libitum access to food and water throughout the experimental timeframe. At the conclusion of the fattening period, all mice (age: 6 months) receiving HFD were randomized within genotype by body mass, fat mass, calorie intake, fasting glucose, and fasting insulin into HFD or HFD+17 $\alpha$  (TestDiet 58Y1 + 17 $\alpha$ -E2, 14.4ppm; Steraloids, Newport, RI) treatment groups for a 12-week intervention. Prior to being euthanized, mice were fasted (5–6 hr) and IP injected with insulin (Novolin R 100 U/ml; 2mU/g) to assess insulin activity and sensitivity in tissue as previously described ([Lu et al., 2012](#)). Each mouse was euthanized with isoflurane 15 min following their insulin injection. Blood was collected into EDTA-lined tubes by cardiac puncture, and plasma was collected and frozen. Tissues were excised, weighed, flash frozen, and stored at  $-80^\circ\text{C}$  unless otherwise noted. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at OUHSC.

#### Animal study 4

Hyperinsulinemic-euglycemic clamp experiments, the gold-standard for assessing insulin sensitivity, were performed in male rats to determine if 17 $\alpha$ -E2 can acutely modulate insulin sensitivity and glucose homeostasis. FBN-F1 hybrid male rats were acclimated to the animal facilities within the Einstein Nathan Shock Center for 2 weeks prior to undergoing surgeries in preparation for hyperinsulinemic-euglycemic clamp studies. Rats were fed Purina 5001 (58.0% CHO, 28.5% PRO, 13.5% FAT) and were individually housed with corncob bedding at  $22 \pm 0.5^\circ\text{C}$  on a 14:10 hr light-dark cycle with ad libitum access to food and water. All surgeries were conducted under 2% isoflurane. For clamp studies incorporating central infusions, rats underwent two surgical procedures. First, stereotactic placement of a steel-guide cannula (Plastics One, Roanoke, VA) reaching the 3rd ventricle was performed. The implant was secured in place with dental cement and animals were treated with analgesic as needed. Approximately 14 days later, animals underwent a second surgical procedure to place indwelling catheters into the right internal jugular vein and the left carotid artery, which was also performed for animals undergoing only peripheral clamp studies. Hyperinsulinemic-euglycemic clamp studies incorporating peripheral 17 $\alpha$ -E2 infusions were performed as previously described ([Einstein et al., 2010](#)). For studies employing peripheral infusions of 17 $\alpha$ -E2, 17 $\alpha$ -E2 was diluted in sterile saline to a final concentration of 30 ng/ $\mu\text{l}$ . Beginning at  $t = 0$  min animals received a primed-continuous infusion of saline or 30 ng/ $\mu\text{l}$  17 $\alpha$ -E2 provided as a 3  $\mu\text{g}$  bolus at a rate of 20  $\mu\text{l}/\text{min}$  over 5 min, followed by a continuous infusion at a rate of 0.06 ml/hr over 235 min (9.4 ng/hr) for a maintenance dose of 7  $\mu\text{g}$  (total dose 10  $\mu\text{g}$ ). Hyperinsulinemic-euglycemic clamp studies with intracerebroventricular (ICV) infusions were performed as previously described ([Huffman et al., 2016a](#)). 17 $\alpha$ -E2 powder (Steraloids, Newport, RI) was dissolved in DMSO at a concentration of 10 mg/ml and stored at  $-80^\circ\text{C}$ . For ICV infusions, 17 $\alpha$ -E2 was diluted in artificial cerebral spinal fluid (ACSF) to a final concentration of 2 ng/ $\mu\text{l}$ . Beginning at  $t = 0$  min, animals received a primed-continuous ICV infusion of ACSF (Veh.) or 17 $\alpha$ -E2 (17 $\alpha$ ) provided as a 15 ng bolus at a rate of 1  $\mu\text{l}/\text{min}$  over 7.5 min, followed by a continuous infusion of 56.5 ng at a rate of 0.08  $\mu\text{l}/\text{hr}$  over 6 hr (9.4 ng/hr) and a total dose of 71.5 ng. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Einstein College of Medicine.

#### In vivo metabolic analyses in mice

To evaluate the effects of 17 $\alpha$ -E2 on metabolic parameters in vivo, we performed several assessments of glucose homeostasis. Unless otherwise noted, all experiments requiring fasting conditions were performed in the afternoon, 5–6 hr following the removal of food at the beginning of the light-cycle as outlined elsewhere ([Ayala et al., 2010](#)). To ensure fasting conditions, mice were transferred to clean cages containing ISO cotton padding and clean cardboard enrichment tubes. Non-terminal blood was collected via tail snip. Fasting glucose was evaluated using a Bayer Breeze 2 Blood Glucose Monitoring System (Bayer Global, Leverkusen, Germany). Fasting insulin was evaluated using a Mouse Ultrasensitive Insulin ELISA from Alpco (Salem, NH). HbA1c was assessed by A1C-Now

Monitoring kits (Bayer, Whippany, NJ). Glucose tolerance tests were performed following a 5 hr fast using an intraperitoneal filtered dextrose injection of 1 g/kg body mass (*Huffman et al., 2016b*). Blood glucose was measured immediately pre-injection (time 0) and at 15, 30, 60, 90, and 120 min post-injection.

### Liver histology

To evaluate the effects of  $17\alpha$ -E2 treatment on lipid accumulation and fibrosis, we evaluated fixed liver tissue. Tissues were fixed in 4% PFA for 24 hr, cryo-embedding samples were transferred to 30% sucrose for 72 hr and embedded in OCT, paraffin-embedding samples were transferred to 1X PBS for 48 hr, then to 70% ethanol until embedding. Liver oil-red-O and Masson's trichrome staining were performed by the Oklahoma Medical Research Foundation Imaging Core Facility using previously reported methodology (*Leonard et al., 2018; Mehlem et al., 2013*). Oil-red-O (ORO, Sigma-Aldrich, St. Louis, MO) and H and E counterstaining were performed on cryo-embedded tissues, and were imaged within 6 hr of staining. Red lipid stain was blindly quantified from 10 images per animal using ImageJ software and presented as a lipid to total tissue ratio. Masson's trichrome staining was performed on paraffin embedded liver tissue and was used for qualitative purposes. In brief, slides were stained with Weigert's Iron Hematoxylin (Sigma-Aldrich, St. Louis, MO), washed, stained with Biebrich Scarlet-Acid Fusion (Sigma-Aldrich, St. Louis, MO), washed, stained with Phosphomolybdic Acid-Phosphotungstic Acid, and then stained with Aniline Blue (Sigma-Aldrich, St. Louis, MO).

### Liver triglycerides

We evaluated the effects of  $17\alpha$ -E2 treatment on triglyceride accumulation in the liver. Liver samples (~100 mg) were homogenized on ice for 60 s in 10X (v/w) Cell Signaling Lysis Buffer (Cell Signaling, Danvers, MA) with protease and phosphatase inhibitors (Boston BioProducts, Boston, MA). Total lipid was extracted from the homogenate using the Folch method with a 2:1 chloroform-methanol mixture (*Folch et al., 1957*). Lipid was dried down using a nitrogen drier at room temperature, and resuspended in 100  $\mu$ l of 3:1:1 tert-butyl alcohol-methanol-Triton X-100 solution. Final triglyceride concentrations were determined using a spectrophotometric assay with a 4:1 Free Glycerol Agent/Triglyceride Agent solution (Sigma Triglyceride and Free-Glycerol reagents, St. Louis, MO) as previously described (*Stout et al., 2011*).

### Liver fatty acids

We evaluated the effects of  $17\alpha$ -E2 on hepatic fatty acid content. Liver samples (~50 mg) were homogenized and on ice for 60 s in 10X (v/w) Cell Signaling Lysis Buffer (Cell Signaling, Danvers, MA) with protease and phosphatase inhibitors (Boston BioProducts, Boston, MA). Total lipid was extracted using a modified Bligh and Dyer method (*Bligh and Dyer, 1959*) (Sigma-Aldrich, St. Louis, MO). Of 15:0 and 17:0 internal standards, 50 nmol were added and acid hydrolysis/methanolysis was done to generate fatty acid methyl esters (FAMES) (*Agbaga et al., 2018*). FAMES were identified as previously described by GC-MS (*Agbaga et al., 2018*). A 6890N gas chromatograph with flame ionization detector (GC-FID) (Agilent Technologies) was used to quantify FAMES (*Yu et al., 2012*). Standards 15:0 and 17:0 were used to compare and determine sample concentrations. Data is represented as the relative mole percent of each fatty acid.

### Plasma eicosanoids

We evaluated the effects of  $17\alpha$ -E2 treatment on circulating eicosanoids (*Supplementary file 3*). Plasma eicosanoid analyses were performed by the UCSD Lipidomics Core as described previously (*Quehenberger et al., 2010*). Eicosanoids were isolated from plasma, extracted, separated using liquid chromatography, and analyzed with mass spectrometry (MDS SCIEX 4000 Q Trap; Applied Biosystems, Foster City, CA) (*Quehenberger et al., 2010*).

### Real-time PCR

To evaluate alterations in gene expression following  $17\alpha$ -E2 treatment, we performed qPCR for genes related to fibrosis, lipid metabolism, insulin resistance, and glucose metabolism in the liver. Total RNA was extracted using Trizol (Life Technologies, Carlsbad, CA) and was reverse transcribed to cDNA with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City,

CA). Real-time PCR was performed in a QuantStudio 12K Flex Real Time PCR System (ThermoFisher Scientific, Waltham, MA) using TaqMan Gene Expression Master Mix (Applied Biosystems/ThermoFisher Scientific, Waltham, MA) and predesigned gene expression assays with FAM probes from Integrated DNA Technologies (Skokie, Illinois). Target gene expression was expressed as  $2^{-\Delta\Delta CT}$  by the comparative CT method (Livak and Schmittgen, 2001) and normalized to the expression of TATA-Box Binding Protein (TBP) in liver.

## Western blotting

To determine if 17 $\alpha$ -E2 altered hepatic insulin sensitivity, we evaluated phosphorylation status of AKT and FOXO1 following an insulin bolus. Liver was homogenized in RIPA Buffer (Cell Signaling, Danvers, MA) with protease and phosphatase inhibitors (Boston Bioproducts, Boston, MA). Total protein was quantified using BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Proteins were separated on an Any kD Criterion TGX Stain-Free Protein Gel (Biorad, Hercules, CA) at 75V for 150 min in Running Buffer (Cell Signaling, Danvers, MA) and transferred to a 0.2  $\mu$ m pore size nitrocellulose membrane, (Biorad, Hercules, CA) at 75V for 90 min on ice. Primary antibodies used were pS256 FOXO1 (Abcam ab131339, 1:1000), FOXO1a (Abcam ab52857, 1:1000), pS473 AKT (Abcam ab81283, 1:3000), pan-AKT (Abcam ab179463, 1:10000), GAPDH (Abcam ab9485, 1:2500). Primary antibody detection was performed with IRDye 800CW Infrared Rabbit (LI-COR Biotechnology, Lincoln, NE) at 1:15,000 concentration. GAPDH was diluted in 5% dry milk (Cell Signaling, Danvers, MA), all other antibodies were diluted in 5% BSA (Cell Signaling, Danvers, MA). Blot imaging was done on Odyssey Fc Imaging System (LI-COR Biotechnology, Lincoln, NE) with a two-minute exposure time at 800 $\lambda$ , and protein detection and quantification were performed using Image Studio Software (LI-COR Biotechnology, Lincoln, NE).

## Statistical analyses

Results are presented as mean  $\pm$  SEM unless otherwise stated with *p* values less than 0.05 considered to be significant unless otherwise specified. Analyses of differences between groups were performed by two-way ANOVA, two-way repeated measures ANOVA, or Student's *t*-test where appropriate using SigmaPlot 12.5 Software. A Benjamini-Hochberg multiple testing correction was applied to the *F* test result to correct for the number of transcripts, proteins, and fatty acids analyzed.

## Acknowledgements

We thank Dr. Kenneth Korach at the National Institute of Environmental Health Sciences for providing ER $\alpha$  KO and WT littermate mice. We also thank Dr. Lora Bailey-Downs, Richard Brush, and Michael Sullivan for technical support. This work was supported by the National Institutes of Health (R00 AG51661 and R01 AG069742 to MBS, T32 AG052363 to SNM, R01 EY030513 to M-PA, and R01 AG059430 to WMF), Veterans Affairs (I01B  $\times$  003906 to WMF) and pilot research funding from the Harold Hamm Diabetes Center (MBS and SNM), Einstein Nathan Shock Center (P30 AG038072) of Excellence in the Basic Biology of Aging (MBS), and OUHSC Lipidomics Core (P30 EY012190).

## Additional information

### Funding

Funder	Grant reference number	Author
National Institutes of Health	R00 AG51661	Michael B Stout
Harold Hamm Diabetes Center	Pilot Research Funding	Shivani N Mann Michael B Stout
National Institutes of Health	R01 AG069742	Michael B Stout
National Institutes of Health	R01 AG059430	Willard M Freeman
Veterans Affairs Oklahoma City	I01BX003906	Willard M Freeman
University of Oklahoma Health	P30 EY012190	Martin-Paul Agbaga

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Sciences Center

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National Institutes of Health	R01 EY030513	Martin-Paul Agbaga
National Institutes of Health	T32 AG052363	Shivani N Mann
Einstein Nathan Shock Center	P30 AG038072	Michael B Stout

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The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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### Author contributions

Shivani N Mann, Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft, Project administration, Writing - review and editing; Niran Hadad, Software, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing - review and editing; Molly Nelson Holte, Formal analysis, Validation, Methodology; Alicia R Rothman, Formal analysis, Validation, Writing - review and editing; Roshini Sathiaselan, Formal analysis, Investigation, Methodology; Samim Ali Mondal, Formal analysis, Investigation, Writing - review and editing; Martin-Paul Agbaga, Archana Unnikrishnan, Formal analysis, Investigation, Methodology, Writing - review and editing; Malayannan Subramaniam, Formal analysis, Supervision, Investigation, Methodology, Writing - review and editing; John Hawse, Formal analysis, Supervision, Validation, Investigation, Methodology, Writing - review and editing; Derek M Huffman, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing - review and editing; Willard M Freeman, Data curation, Software, Formal analysis, Supervision, Investigation, Visualization, Writing - review and editing; Michael B Stout, Conceptualization, Resources, Data curation, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Methodology, Writing - original draft, Project administration, Writing - review and editing

### Author ORCIDs

Willard M Freeman  <http://orcid.org/0000-0001-7027-999X>

Michael B Stout  <https://orcid.org/0000-0002-9996-9123>

### Ethics

Animal experimentation: This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All of the animals were handled according to approved institutional animal care and use committee (IACUC) protocols (#19-063-SEAH) of the University of Oklahoma Health Science Center.

### Decision letter and Author response

Decision letter <https://doi.org/10.7554/eLife.59616.sa1>

Author response <https://doi.org/10.7554/eLife.59616.sa2>

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## Additional files

### Supplementary files

- Supplementary file 1. Pairwise statistical comparisons of ER $\alpha$  binding. Negative binomial regression Wald post-hoc comparison test, FDR < 0.05. n = 3/group.
- Supplementary file 2. ER $\alpha$  binding motif analysis. Motif analysis was performed using HOMER with standard settings with the significance threshold set to FDR corrected p < 0.05. Peak regions called for each treatment group were analyzed to identify enriched motifs relative to the entire genome. Hypergeometric test was used to test enrichment. Only motifs with FDR corrected p < 0.05 were reported as significant. For pairwise differential motif enrichment or depletion across experimental groups, we utilized the hypergeometric test by using the number of sequences with motif from each group and total number of peaks as total sample size. Motifs that appear in less than five sequences between both test groups were removed. Benjamini-Hochberg multiple testing correction was utilized to control for false discovery rate (FDR < 0.05).

- Supplementary file 3. Circulating eicosanoid levels (pmol/ml). 17 $\alpha$ -E2 mildly alters the circulating eicosanoid profile in obese middle-aged male mice. WT mice were provided 45% HFD (TestDiet 58V8) $\pm$ 17 $\alpha$ -E2 (14.4ppm) for 14 weeks. Age-matched, male WT, chow-fed (TestDiet 58YP) mice were also evaluated as a normal-weight reference group. All data are presented as mean  $\pm$  SEM and were analyzed by Student's t-test with the WT Chow group being excluded from statistical comparisons. n = 5–7.
- Transparent reporting form

### Data availability

Sequencing data has been deposited in GEO under accession code GSE151039.

The following dataset was generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Stout M, Hawse J, Freeman W, Hadad N, Mann S	2020	Assessment of transcriptional ER $\alpha$ activity following exposure to 17 $\alpha$ -E2 and 17 $\beta$ -E2	<a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE151039">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE151039</a>	NCBI Gene Expression Omnibus, GSE151039

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