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# High-fat diet and estrogen impacts the colon and its transcriptome in a sex-dependent manner

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There is a strong association between obesity and colorectal cancer (CRC), especially in men, whereas estrogen protects against both the metabolic syndrome and CRC. Colon is the first organ to respond to high-fat diet (HFD), and estrogen receptor beta (ER $\beta$ ) can attenuate CRC development. How estrogen impacts the colon under HFD and related sex differences has, however, not been investigated. To dissect this, mice were fed control diet or HFD for 13 weeks and administered receptor-selective estrogenic ligands for the last three weeks. We recorded impact on metabolism, colon crypt proliferation, macrophage infiltration, and the colon transcriptome. We found clear sex differences in the colon transcriptome and in the impact by HFD and estrogens, including on clock genes. ER $\alpha$ -selective activation reduced body weight and generated systemic effects, whereas ER $\beta$ -selective activation had local effects in the colon, attenuating HFD-induced macrophage infiltration and epithelial cell proliferation. We here demonstrate how HFD and estrogens modulate the colon microenvironment in a sex- and ER-specific manner.

Obesity represents a worldwide public-health issue and epidemiological studies highlight a strong association between body mass index (BMI) and risk of colorectal cancer (CRC)<sup>1–4</sup>. A high-fat diet (HFD) increases this risk, and the incidence of CRC is increasing among young adults due to altered life-style factors<sup>5</sup>, but exactly how is not clear. It is known that inflammatory bowel disease (IBD) increases the risk of CRC, and that both obesity and HFD induce a general low-grade inflammatory state<sup>6,7</sup>. The colon is the first organ to respond to HFD<sup>8</sup>, with reported increase of inflammation, PPAR $\delta$  signaling, stem cell activity, and altered gut microbiota<sup>9–16</sup>, which may all contribute to initiation of CRC. Interestingly, while obesity is a risk factor for both sexes, this association appears stronger among men<sup>17</sup>. Men also have a higher incidence of CRC<sup>18,19</sup>. A role for sex hormones is supported by epidemiological studies (reviewed in<sup>20</sup>), and especially estrogen appears protective against CRC<sup>21,22</sup>. Numerous clinical studies have also indicated that estrogen protects against several aspects of obesity and the metabolic syndrome<sup>23,24</sup>, as well as impacts the immune system (reviewed in<sup>25</sup>).

The biological action of estrogen is mediated by three receptors, the nuclear receptors ER $\alpha$  (ESR1) and ER $\beta$  (ESR2), and the transmembrane G protein-coupled estrogen receptor 1 (GPER1). These receptors are expressed in both sexes, with a tissue-specific expression pattern. Both ER $\alpha$  and ER $\beta$  have been implicated to elicit anti-obesogenic effects<sup>26–29</sup>. Whereas ER $\alpha$  clearly improves the metabolic profile, the role of ER $\beta$  in this respect is controversial<sup>30</sup>. Most of the CRC protective effects, however, have been linked to ER $\beta$ . Although its mRNA expression in colon is low, treatment with ER $\beta$ -selective agonist, for example, resulted in anti-inflammatory and anti-tumorigenic effects in CRC mouse models<sup>31–34</sup>. Furthermore, loss of ER $\beta$  results in pro-inflammatory environment and increased tumor development in both APC<sup>Min/+</sup> and colitis-associated tumor mouse model<sup>35,36</sup>. Notably, intestinal-specific deletion of ER $\beta$  increased tumor development and strongly enhanced inflammatory signaling in the colon of both male and female mice<sup>37</sup>. In fact, inflammatory signaling itself was shown to enhance transactivation of ER $\beta$ <sup>37</sup>. It is therefore plausible that this ER $\beta$  mechanism could protect against HFD-induced colonic inflammation. We hypothesize that estrogenic signaling, through ER $\alpha$  (systemic effects by improving overall metabolism), ER $\beta$ , or both; can attenuate the effect of HFD in colon, with possible sex differences.

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In order to test this, and to characterize the impact of estrogen signaling on the colon microenvironment under HFD, we performed a detailed analysis of mice of both sexes. In order to compare sex differences and to obtain data comparable to premenopausal women, where obesity is thought to increase the incidence of CRC, none of the female mice in this study were ovariectomized. Mice were fed either a control diet (CD) or a HFD for 13 weeks, and administered different estrogenic ligands for the last three weeks. Importantly, by analyzing corresponding effects on the full colon transcriptome, we generated an unbiased overview of alterations mediated by HFD and estrogens. We observed sex differences in the colon transcriptome and in its response to HFD. In particular, ER $\beta$  opposed the HFD impact on colonic inflammation, epithelial cell proliferation and regulation of circadian rhythmicity. Altogether, we present the novel finding that estrogen signaling is indeed partially protective against HFD-induced alterations of the colon microenvironment, in part directly through intestinal ER $\beta$ . These findings open up new insights into prevention of obesity-associated CRC.

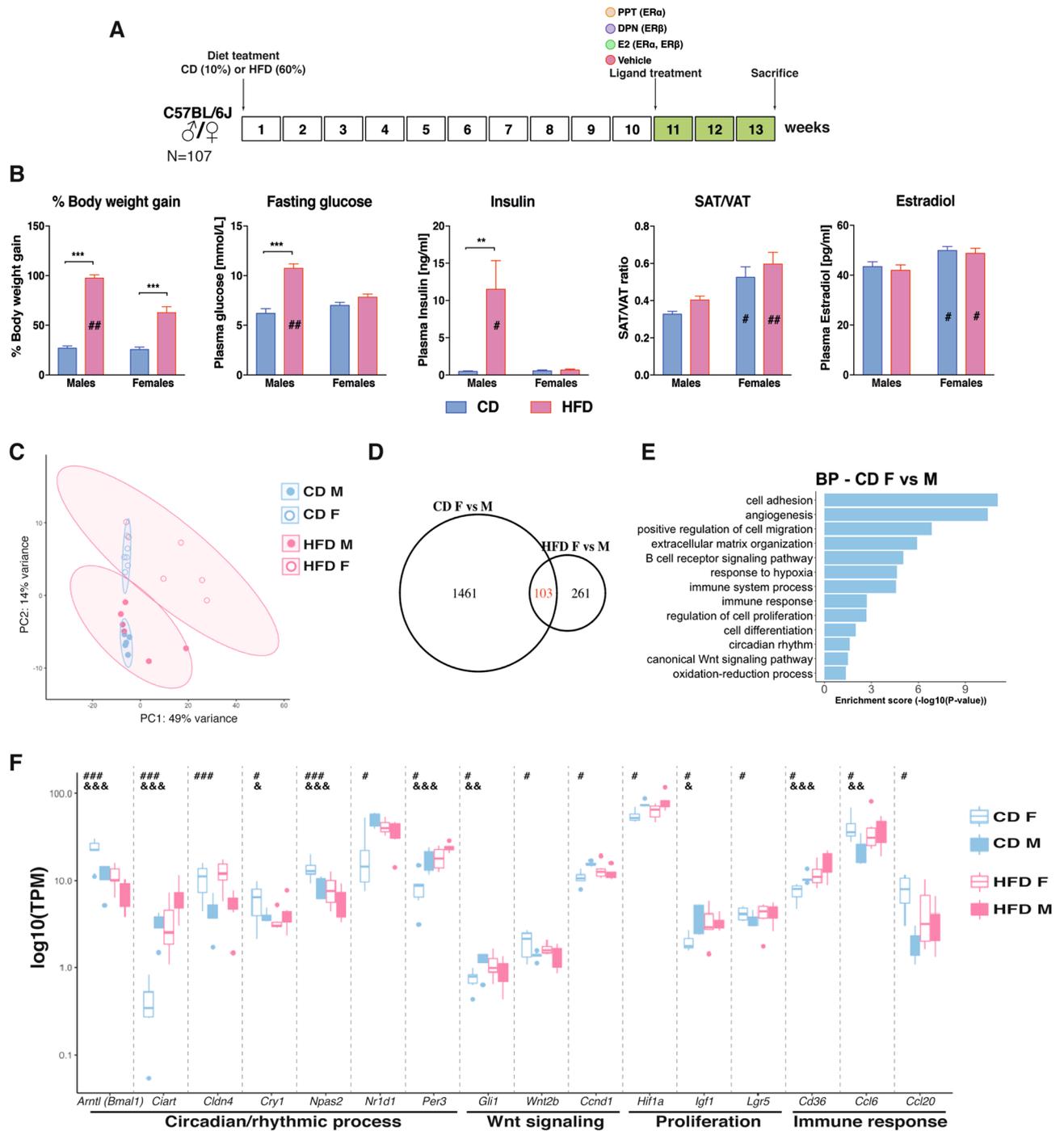
## Results

**Sex impacts obesity, fat distribution and fasting glucose levels.** The impact of sex and 13-week HFD were characterized according to standard metabolic and endocrinologic measurements, including weight gain, fat distribution, fasting glucose, insulin, and circulating estrogen levels. HFD-induced weight gain was significantly higher in males compared to females (Fig. 1B). Obese males further presented significantly increased blood glucose level (after 6-h fasting) and insulin level (2-h fasting), which was not observed in obese females (Fig. 1B). These results support previous observations that obese males are more susceptible to glucose intolerance and insulin resistance compared to females<sup>38,39</sup>. As also noted previously<sup>39</sup>, the fat distribution was sex-dependent. Males had more visceral white adipose tissue (VAT) and less subcutaneous white adipose tissue (SAT) in relation to total fat during both dietary conditions, compared to females (Supplementary Fig. 1A). As a consequence, males had a substantially lower SAT/VAT ratio (Fig. 1B). A low SAT/VAT ratio has been correlated to poor metabolic outcomes. As expected, female mice had significantly higher circulating estradiol levels compared to males during both CD and HFD (Fig. 1B). Circulating estradiol levels were not significantly impacted by obesity (Fig. 1B). This contradicts one previous study which found increasing circulating estradiol levels upon HFD<sup>30</sup>. However, obesity is primarily known to increase local levels of estrogen in adipose tissue, which express aromatase<sup>40</sup>. We did not measure local adipose tissue estradiol levels. Overall, these results and sex differences replicate previous findings.

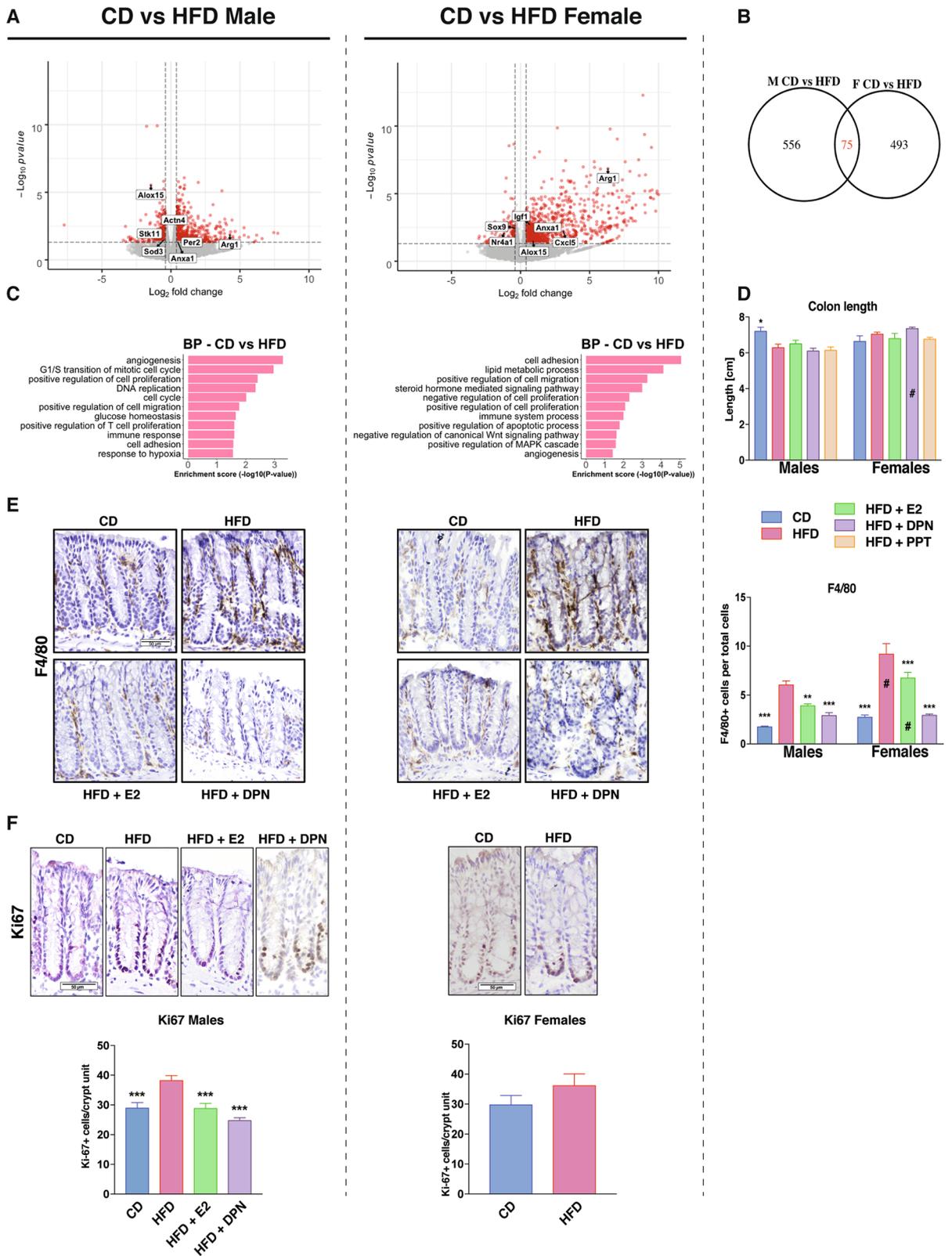
**The colon transcriptome exhibits clear sex differences.** To specifically study sex differences in the colon, we performed RNA-seq of colon tissue from males and females. As evident from the principal component analysis (PCA) plot, the colon transcriptomes exhibited clear sex differences, especially during CD (Fig. 1C). Gene expression analysis also identified 1564 significantly differentially expressed genes (DEG) between male and female colon under CD (Fig. 1D). While the CD colon transcriptome clustered tightly together, the HFD was more spread out (Fig. 1C). As a result, the sex difference under HFD was less distinct, but 364 DEGs were still revealed (Fig. 1D). For animals fed a CD, enrichment analysis for biological processes (BP) disclosed that the genes responsible for the sex differences were related to immune response (e.g. *Cd36*, *Ccl6* and *Ccl20*), cell proliferation (e.g. *Hif1a*, *Igf1* and *Lgr5*) and canonical Wnt signaling (e.g. *Ccnd1*, *Gli1* and *Wnt2b*, Fig. 1E,F). Although the mice had been sacrificed at the same time of the day, we found sex differences in expression of circadian genes, including the clock genes *Arntl* (*Bmal1*), *Npas2*, *Cry1*, *Per3*, and *Nr1d1/Rev-Erba* (Fig. 1E,F). Over one fourth (28%) of the sex differences after HFD were also present under CD (Fig. 1D). The most overrepresented genes among these stable DEGs (103) belonged to the circadian/rhythmic pathway (e.g. *Bmal1*, *Ciart*, *Cldn4* and *Npas2*, Fig. 1F). Thus, we report for the first time that mice fed CD, which lacks phytoestrogens, display clear sex differences in the colon transcriptome.

**HFD modulates the colon transcriptome differently in males and females.** In order to explore how HFD impacted male and female colon, we compared the transcriptomes under HFD with corresponding expression under CD, for males and females separately. We identified 568 HFD-modulated DEGs in females and 631 in males (Fig. 2A). We noted that a larger proportion of genes was upregulated overall, but especially in females (85%, versus 51% in males), and that females exhibited stronger upregulation. Most of the modified genes differed between the sexes, only 75 genes responded to HFD in both (Fig. 2B). Among these common genes were, again, those related to the circadian rhythm (*Bmal1*, *Ciart*, *Npas2* and *Per3*, Fig. 1F), oxidation–reduction process (*Alox15*), and inflammatory response (e.g. *Arg1* and *Anxa1*, Fig. 2A). Both sexes altered expression of genes with functions in cell adhesion, cell proliferation, angiogenesis, migration, and immune system (Fig. 2C and Supplementary Table 2), but different genes were altered in males and females. Regulated genes involved in cell cycle (e.g. *Stk11* and *Wee1*), hypoxia (e.g. *Actn4* and *Sod3*) and glucose homeostasis (e.g. *Stk11*) were particularly enriched in males, whereas regulation of apoptosis (e.g. *Nr4a1*), inflammatory response (e.g. *Cxcl5*), MAPK cascade (e.g. *Igf1*) and Wnt signaling (e.g. *Sox9*) were enriched in females (Fig. 2A,C and Supplementary Table 2). Thus, while both sexes presented alterations within similar biological functions, including the immune system and cell adhesion, other changes appeared tied to sex, such as more pronounced changes of cell cycle and hypoxia in males, and of the lipid metabolism, steroid hormone and Wnt signaling in females. All together our data demonstrate, for the first time, that sex impacts the gene expression response to HFD in colon.

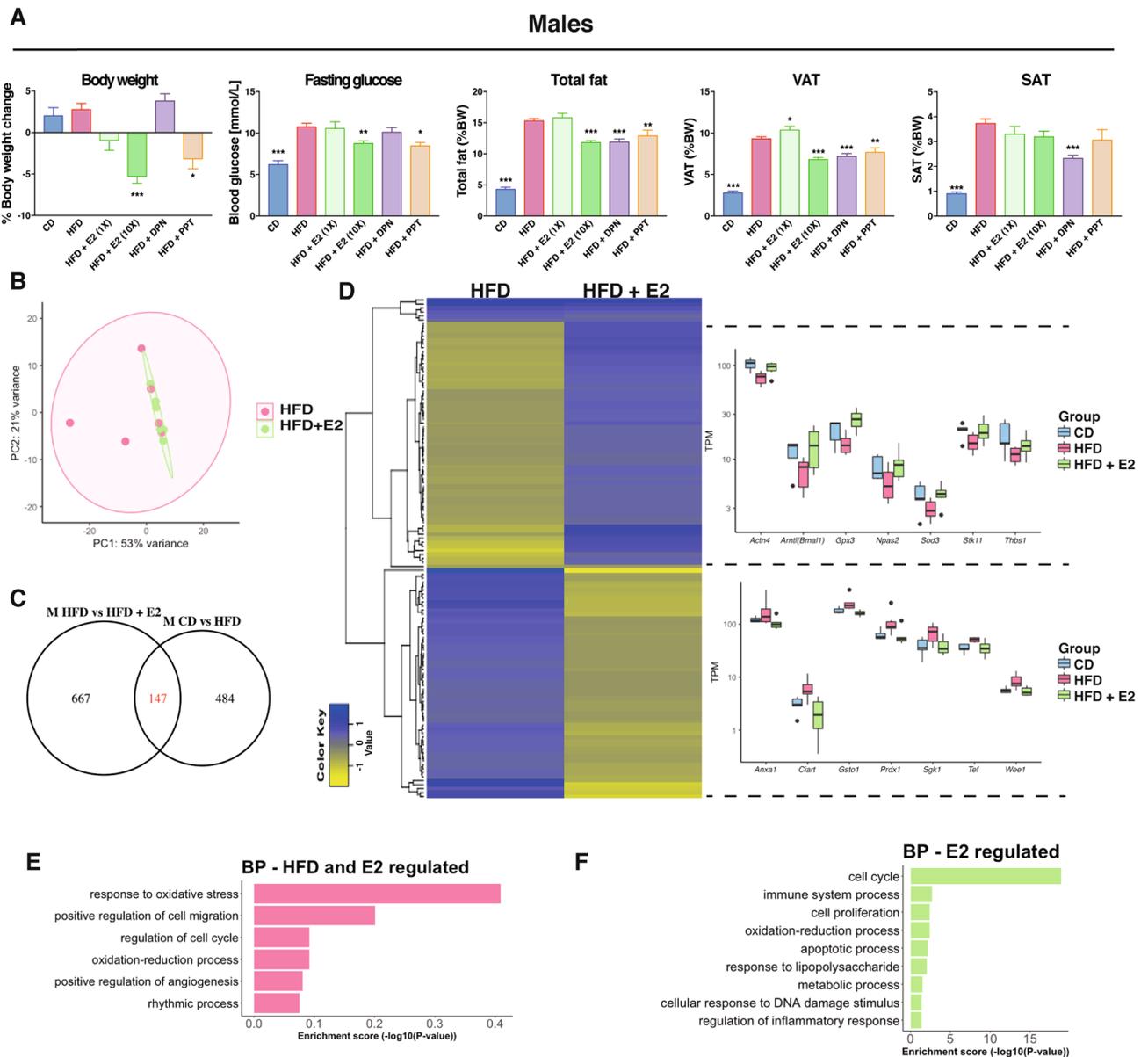
**HFD increases colon epithelial inflammation and proliferation in males.** The above colon transcriptome analysis indicated that the immune system was impacted by HFD and that, especially in males, the cell cycle and hypoxia were modulated. A shortened colon is indicative of colonic inflammation, and we found that males but not females fed an HFD presented a significantly shorter colon (Fig. 2D). Next, we explored markers



**Figure 1.** The male metabolic profile is more sensitive to HFD and the colon transcriptome exhibits sex differences independent of diet. (A) Experimental study design. Mice of both sexes were given CD or HFD for 13 weeks and injected with different estrogenic ligands or vehicle for the last 3 weeks prior to sacrifice. (B) Percentage of BW gain, fasting glucose level, insulin level, SAT/VAT ratio, and plasma estradiol levels. (C) Principal component analysis of RNA-seq samples for male (unfilled) and female (filled) mice fed a CD (blue) or HFD (red) (n = 5–6). (D) Venn diagram comparing differentially expressed genes in colon between male and female mice fed a CD or a HFD. Genes were considered as differentially expressed when  $p$  value < 0.05 and  $\log_2FC > |0.4|$ . (E) Biological process enrichment analysis based on genes differentially expressed between the sexes during CD. (F) Boxplot of TPM values of genes differentially expressed between sexes and/or dietary conditions. The results are presented as mean  $\pm$  SEM. Two-way ANOVA followed by fisher's LSD test, \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001. # indicate significant sex differences during CD, ## during HFD and ### during both dietary conditions; & indicate significantly difference between CD and HFD in females, && in males, and &&& in both sexes. Blue color indicates CD and pink HFD.



**Figure 2.** The colon transcriptome responds to HFD in a sex-dependent manner. (A) Volcano plots show HFD-regulated genes in males (left) and females (right). Genes were considered differentially expressed when  $p < 0.05$  and  $\log_2FC > |0.4|$ , indicated in red. (B) Venn diagram comparing differentially expressed genes in colon between CD and HFD in male and female mice. (C) Biological process enrichment analysis of HFD-regulated genes, separated by sex. (D) Colon length measured in cm. (E) Macrophage infiltration measured by F4/80<sup>+</sup> IHC and quantified by stained cells per total cells ratio. (F) Intestinal epithelial cell proliferation measured with Ki67 IHC and quantified by number of positive cells per crypt unit.  $n = 5-15$  per group. Results are presented as mean  $\pm$  SEM. One-way and two-way ANOVA with uncorrected Fisher's LSD test. \*Indicate significant differences compared to HFD + vehicle, \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . #Indicate sex differences.



**Figure 3.** Estrogen treatment impacts the metabolic profile and colon gene expression in males. (A) BW change during treatment, fasting glucose level, total fat mass (reported to BW), VAT, and SAT (reported to BW) for males on a CD, HFD, and HFD treated with E2, DPN and PPT. (B) PCA of RNA-seq data for males fed a HFD with and without E2 treatment. (C) Venn diagram comparing HFD-regulated genes in males with E2-regulated genes in males under a HFD. (D) Heatmap of the homogeneously regulated common genes (regulated by both HFD and E2 under HFD) and boxplots of TPM values of some genes. Biological process enrichment analysis of the (E) HFD-induced genes opposed by estrogen signaling and (F) estrogen-regulated genes under HFD feeding ( $n = 5-15$  per group). Results are presented as mean  $\pm$  SEM. One-way ANOVA with uncorrected Fisher's LSD test. \* Indicate significant differences compared to HFD + vehicle, \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

for macrophages in histological sections using immunohistochemistry (IHC). We found that mice of both sexes exhibited a significantly increased F4/80<sup>+</sup> macrophage infiltration when fed HFD, but females had a stronger infiltration than males (Fig. 2E). Males on the other hand, presented significantly increased proliferation of crypt cells after HFD, which females did not (Fig. 2F). This reveals that HFD results in clear sex-dependent functional defects in the colon, in accordance with the above noted transcriptional differences.

**Estrogen improves the metabolic profile of obese males via ER $\alpha$ .** Several studies have shown that estradiol (E2) improves the metabolic profile of HFD-fed rodents of both sexes<sup>41,42</sup>. Further, knockout of ER $\alpha$  results in obese mice<sup>26</sup>. In our study, neither E2 nor ER $\alpha$ -selective ligand PPT treatment for the last 3 weeks (Fig. 1A) affected the body weight (BW)-gain in females compared to vehicle treatment (Supplementary Fig. 1C,D). However, male mice showed a dose-dependent BW loss during E2 treatment, with up to 5% weight reduction (0.5 mg/kg BW, Fig. 3A and Supplementary Fig. 1C). This was accompanied by a significant reduc-

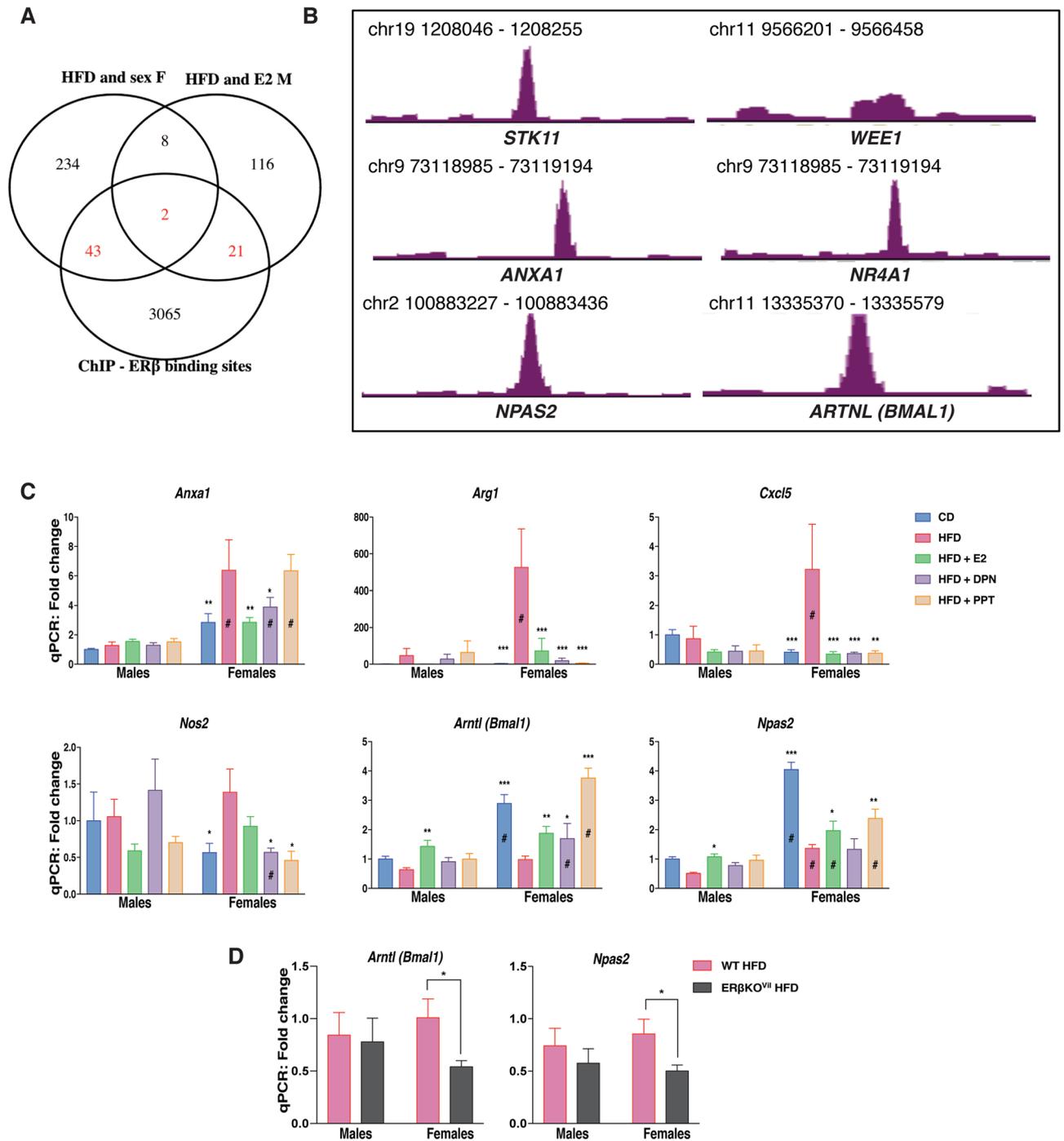
tion of total fat and decreased blood glucose level upon fasting, not seen in females (Fig. 3A and Supplementary Fig. 1D). These effects were also evident with the selective ER $\alpha$  ligand PPT (Fig. 3A). Thus, our experimental set-up confirms that exogenous E2 via ER $\alpha$  impacts obesity, fasting glucose, total fat and VAT ratios in males.

**Estrogen impacts the obese colon transcriptome.** Next, we wanted to determine if E2 treatment impacted the colon, and specifically if it could modulate the HFD-induced colon inflammation signature. We focused on males, who had exhibited a shortening of the colon upon HFD and had shown a significant E2-mediated impact on their metabolic profile. The RNA-seq data, visualized in a PCA plot, showed that estrogen-treated HFD-fed male colon clustered tighter together compared to vehicle treated (Fig. 3B), just as CD samples did (Fig. 1C). We found that E2 treatment modified the expression of 814 genes in distal colon. Among these E2-regulated genes, we noted enrichment of cell cycle and cell proliferation (e.g. *Cdk1*, *Melk*, *Mki67*), and immune system process (e.g. *Alcam*, *Irf7* and *Bcl6*) pathways (Fig. 3F). Of these, 147 genes were also impacted by HFD (compared to CD), nearly all (97%) in the opposite direction (Fig. 3C,D). BP enrichment analysis revealed that genes regulated by both HFD and E2 were involved in cell cycle and rhythmic processes (Fig. 3E). Two homogeneous (regulated similarly in at least three animals from each group) clusters of DEG appeared, illustrated by a heatmap in Fig. 3D. One cluster (61 genes) was downregulated by HFD and then upregulated by E2, such as *Actn4*, *Bmal1*, *Gpx3*, *Npas2*, *Sod3*, *Stk11* and *Thbs1* (Fig. 3D, upper right). The second cluster included 58 genes that were upregulated by HFD and downregulated by E2, and comprised genes such as *Anxa1*, *Clart*, *Gsto1*, *Prdx1*, *Sgk1*, *Tef* and *Wee1* (Fig. 3D, lower right). qPCR supported these regulations. This demonstrates that both sex and estrogen have a substantial impact on the colon transcriptome, that estrogen may have a strong effect on proliferation and clearly opposes a fraction of the HFD-induced expression.

**Estrogen reduces immune cell recruitment and proliferation of the HFD colon.** Since we noted that E2 treatment could attenuate signs of inflammation and proliferation at the gene expression level, we investigated whether E2 impacted the length of colon, infiltration of macrophages, or proliferation. E2 did not significantly impact the colon length but did reduce F4/80<sup>+</sup> macrophage infiltration in both sexes (Fig. 2D,E). We also noted a significant reduction of positive Ki67-stained cells upon E2 treatment of HFD males (Fig. 2F). These data clearly demonstrate that E2 impacts the colon, both in terms of immune cell recruitment and colonic epithelial cell proliferation.

**ER $\beta$ -selective agonist impacts fat distribution and reduces colonic macrophages infiltration and cell proliferation.** To investigate ER $\beta$ -mediated effects, we included treatment with a ligand selective for ER $\beta$  (DPN, Fig. 1A). DPN treatment did not result in weight reduction, in accordance with data assigning this phenotype to ER $\alpha$ . On the contrary, DPN-treated males and females exhibited a non-significant trend of increased weight gain, which was accompanied by a significant increase of fasting blood glucose levels in females but not males (Fig. 3A and Supplementary Fig. 1D). This also demonstrates that the ligands in the concentrations used in this set up retained receptor selectivity. Although the effects by DPN on weight were not significant in males, we noted clear reductions of total fat and VAT by DPN treatment, in a scale similar or higher than what was achieved with PPT treatment (Fig. 3A). Further, a reduction in SAT was specific for ER $\beta$  ligand activation (Fig. 3A). We did not see these effects in females where, on the contrary, VAT was increased by DPN (Supplementary Fig. 1D). This suggests that while obesity and HFD-induced BW can be decreased only through ER $\alpha$ , ER $\beta$  activation appears to modify the ratio of total fat, VAT, and SAT in males, and increase fasting glucose and VAT in females. Furthermore, estrogen through ER $\beta$  (E2 and DPN) opposed the HFD-induced Ki67 proliferation in males (Fig. 2F). The increased F4/80<sup>+</sup> macrophage infiltration upon HFD was also significantly reduced by estrogen through ER $\beta$  (E2 and DPN) in both sexes (Fig. 2E). Our data thus establish that while an improved metabolic profile is mediated via ER $\alpha$ , ER $\beta$  impacts the colon by attenuating HFD-induced colonic proliferation in males and macrophage infiltration in both sexes.

**ER $\beta$  regulates gene expression in the colon.** As shown above, E2 treatment opposed a set of HFD-induced colonic gene expression in males. This could be an indirect consequence of systemic effects (e.g. ER $\alpha$  improving the overall metabolism) or direct local effects through ERs expressed in the colon. We have previously shown that ER $\beta$  is expressed in intestinal epithelial at low levels, whereas there is not sufficient support that ER $\alpha$  is expressed in the colon<sup>43</sup>. While we did not sequence the colon transcriptome of DPN-treated mice, we evaluated the E2-mediated response of ER $\beta$ -target genes (as predicted from our ER $\beta$  chromatin immunoprecipitation (ChIP)-seq data from human CRC cell lines, with transduced ER $\beta$ , GSE149979) in the male colon transcriptome. This resulted in identification of 23 genes, which were regulated by both HFD and E2, and also have cis-chromatin ER $\beta$ -binding sites (Fig. 4A). These included the cell cycle genes *Stk11* and *Wee1* and the inflammatory related gene *Anxa1* among others (Fig. 4B). Moreover, we compared the female-only HFD-regulated genes (potential female endogenous E2 regulation). This comprised of 287 genes of which 45 had ER $\beta$ -binding sites, including the orphan nuclear receptor *Nr4a1* (*Nur77*, Fig. 4A,B). qPCR analysis of colon tissue corroborated the HFD-induced upregulation of *Anxa1* gene expression and that this was indeed attenuated by both E2 and DPN (ER $\beta$ ) but not by PPT in females, whereas it was not affected in males (Fig. 4C). Further, the HFD-induction of the M2 macrophage marker *Arg1* was also stronger in females, and this was blocked by both ER $\alpha$  and ER $\beta$  activation (Fig. 4C). In addition, we have previously shown that ER $\beta$  can cross-talk with and regulate NF $\kappa$ B signaling in both human colon cell lines and mouse in vivo colon (ER $\beta$ -intestinal knockout mice)<sup>37</sup>. In line with this, we here observed that HFD-induced expression of several NF $\kappa$ B target genes (*Cxcl5*, *Nos2*) in females, which were blocked by both ER $\beta$  (DPN) and ER $\alpha$  (PPT). Our data suggest that systemic activation of ER $\alpha$  improves the overall metabolism, which helps oppose obesity-mediated colon dysfunction. ER $\beta$ , on the other hand, appears



**Figure 4.** Estrogen through both ER $\alpha$  and ER $\beta$  can modulate the expression of specific sets of genes under HFD in both sexes. (A) Venn diagram of the E2-regulated genes under HFD in males and the HFD and sex regulated genes in females compared to ER $\beta$ -binding chromatin sites in two different CRC cell lines (SW480 and HT29). (B) ER $\beta$ -binding sites revealed by ChIP-seq in CRC cell lines. (C) qPCR measurement of gene expression in the colon of female and male mice on a CD, HFD and HFD treated with E2, DPN and PPT (n = 5–15). (D) qPCR measurement of gene expression in colon of wild type and intestinal-specific ER $\beta$  KO mice fed a HFD. The results are presented as mean  $\pm$  SEM. Two-way ANOVA with uncorrected Fisher's LSD test. \* Indicate significant differences compared to HFD + vehicle, \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . #Indicate sex differences.

to locally downregulate proliferation and inflammatory genes (*Cxcl5* and *Nos2*) in the colon. This data provides evidence that E2 through systemic and local effects via ER $\alpha$  and ER $\beta$  can partially attenuate inflammatory effects in the colon.

**Sex, hormones, and diet influence regulation of circadian clock genes in colon.** The circadian rhythm is essential for intestinal homeostasis. Disruptions can increase intestinal permeability, proliferation, exacerbate colitis, modulate microbiota composition, and impact the immune system<sup>44–47</sup>. HFD impacts the circadian rhythmicity in both liver and adipose tissue, but very little is known about the impact of HFD on the circadian rhythm in the colon epithelium. Above, we identified sex differences in the expression of clock genes with higher expression of *Bmal1*, *Npas2*, and *Cry1* in female colon and higher expression of *Per3* and *Nr1d1* (*Rev-ErbA*) in male colon (Fig. 1F). HFD downregulated *Bmal1* and *Npas2* and upregulated *Per3* in both sexes, downregulated *Cry1* specifically in females and upregulated *Per2* specifically in males (Fig. 1E, 2A). In addition to exhibiting sex differences in their expression levels, ER ligands could oppose the HFD-induced downregulation of both *Bmal1* and *Npas2* in both sexes (Fig. 3D, 4C). ChIP-seq indicated that ER $\beta$  is a regulator of the clock genes *Bmal1* and *Npas2* (Fig. 4B). In males, both *Bmal1* and *Npas2* were upregulated in the colon by estrogen, but significant regulation by ER $\beta$  (DPN) were not observed (Fig. 4D). However, we could confirm that the circadian clock gene *Bmal1* indeed was upregulated by DPN (ER $\beta$ ) and, also, by E2 and PPT (ER $\alpha$ ) in females (Fig. 4B). This evidence points towards local regulation of clock genes by ER $\beta$  in the colon. The circadian rhythm of the colon has essential impacts on the body metabolism. If this is impacted by ER $\beta$ , as our study shows, this has substantial implications for our understanding of how hormones affect the metabolism. To provide conclusive evidence of direct ER $\beta$ -mediated regulation of *Bmal1* and *Npas2* in the in vivo female colon, we fed intestinal-specific ER $\beta$  knockout mice HFD. We observed a significant decrease in the expression of both *Bmal1* and *Npas2* in female colon lacking intestinal ER $\beta$  (Fig. 4D). This was not observed in males. Our results demonstrate, that the expression of genes in colon, including clock genes, has clear sex differences, is impacted by HFD in both sexes, and is directly regulated by intestinal epithelial ER $\beta$  in the colon of females.

## Discussion

Our objective with this study was to determine whether estrogen could improve the colon microenvironment and thereby attenuate the negative effects of HFD and, if so, to dissect this mechanism and identify sex differences. Several studies have suggested that both ER $\alpha$  and ER $\beta$  can improve the metabolic syndrome, a risk factor for CRC<sup>26–28</sup>. Other studies support a protective role of ER $\beta$  against CRC (reviewed in<sup>19</sup>). The role of ER $\beta$ , or ER $\alpha$ , in the colon during HFD-induced obesity has, however, not been investigated. We here demonstrate that estrogen indeed modulates the colon microenvironment in mice fed HFD, and that ER $\alpha$ - and ER $\beta$ -selective ligands can in part counteract different facets of HFD-induced phenotypes, including at the level of colonic gene expression.

As the metabolic data show, males appeared more sensitive to HFD, and the higher-dose E2 and ER $\alpha$ -selective activation improved their glucose metabolism, in line with previous studies<sup>38,41</sup>. Although both doses of E2 injections resulted in similar plasma concentrations, the low-dose E2 did not significantly impact glucose metabolism. It is possible that the higher dose resulted in increased local concentrations in certain tissues, especially as lipophilic steroid hormone can be sequestered in the visceral adipose tissue, or that it was higher at certain time points, not reflected in the plasma concentrations at the point of sacrifice. Some studies have also reported a protective effect through ER $\beta$  against HFD-induced obesity, but the data are conflicting. Foryst-Ludwig et al. found that ER $\beta$  protected against HFD-induced weight gain but still elicited pro-diabetogenic effects<sup>30</sup>. We could not confirm that ER $\beta$  protected against HFD-induced weight gain, but we did find that its activation (E2 and DPN, not PPT) impaired glucose levels in females (Supplementary Fig. 1D).

Our study detail several other significant sex differences. In particular, a notable sex difference in the colon transcriptome of mice (CD), which was not expected. As this diet did not contain phytoestrogens, which standardized soy-based chow diets are rich in, this enabled us to observe the impact of endogenous estrogen signaling without external interference. The sex differences that were observed in both dietary conditions (CD and HFD) were related to hypoxia, immune response, cell proliferation, circadian rhythm and Wnt signaling. Thus, our data bring in a role of sex hormones in the basal regulation of these pathways in colon. Intriguingly, we found that estrogen treatment in males indeed regulated the immune response, cell proliferation and rhythmic processes in colonic tissue.

Interestingly, HFD-induced pro-inflammatory modifications are known to occur earlier in the colon than in the adipose tissue. These changes are characterized by increased infiltration of pro-inflammatory macrophages and by epithelia permeability in the colon<sup>8</sup>. Estrogen treatment has been shown to elicit an anti-inflammatory response in the colon of mouse models for colitis<sup>32,48</sup>. Our transcriptomic data reveal that both sexes' immune response were altered upon HFD feeding, but with clear sex differences. This was supported by a significant increase of F4/80<sup>+</sup> macrophage infiltration in the colon upon HFD in both sexes, but with a significantly higher overall macrophage count in females. The F4/80<sup>+</sup> macrophage infiltration was significantly reduced by ER $\beta$  activation (E2 and DPN) in both males and females. In addition, our mechanistic data from ChIP-seq in CRC cell lines demonstrated that ER $\beta$  could bind to cis-regulatory chromatin regions of both *Anxa1* and the orphan nuclear receptor *Nr4a1* (*Nur77*). *Anxa1* is known to recruit monocytes<sup>49</sup> and *Nur77* can regulate the immune system and suppress DSS-induced colitis<sup>50</sup>. In females, we found that ER $\beta$  activation could reduce the expression of *Anxa1* and markers for anti-inflammatory M2 (*Arg1*) and pro-inflammatory M1 (*Nos2*) macrophages. Thus, our study delineates how ER $\beta$  contributes to anti-inflammatory effects mediated by estrogen in the in vivo colon. ER $\beta$  is not detected in macrophages, but is expressed in intestinal epithelial cells<sup>51,52</sup>. Cross-talk between the colonic epithelial cells and macrophages has been demonstrated in IBD<sup>53</sup> and with HFD in an obesity context<sup>8</sup>. Although the etiology of obesity and IBD is different, both are characterized by gut inflammation with common inflammatory pathways including TNF $\alpha$ , IL6, and IL1b<sup>54–57</sup>. We have recently shown in a colitis-induced CRC mouse model that ER $\beta$  in intestinal epithelial cells can downregulate the TNF $\alpha$ /NF $\kappa$ B signaling<sup>37</sup>. Together, our data suggest that specific activation of intestinal ER $\beta$  may have a beneficial impact to modulate gut inflammation in both pathologies.

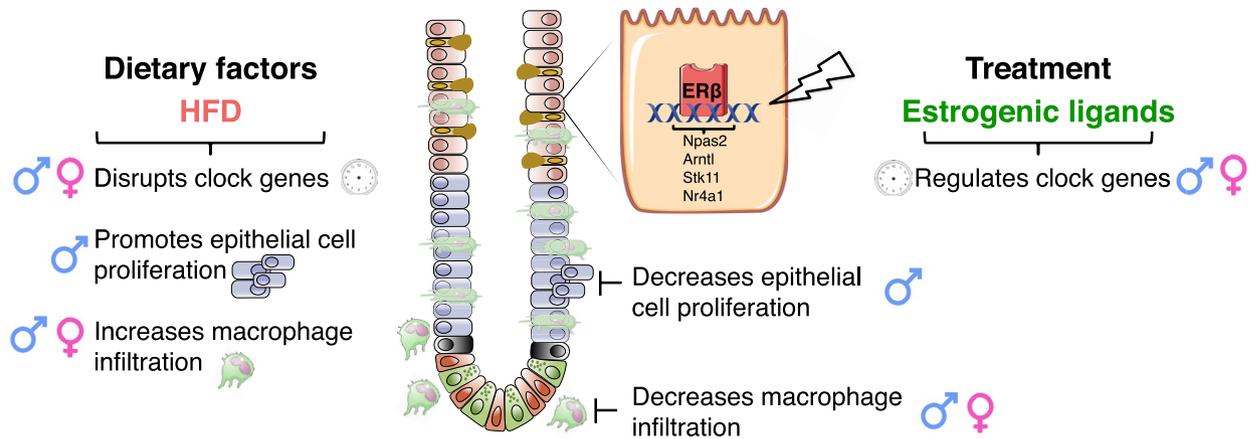
Obesity is a well-known risk factor for CRC, and HFD-induced obesity exacerbates colon tumor development in mice by enhancing colonic cell proliferation<sup>58</sup>. We reveal that the colon transcriptome, which responded to HFD-feeding in a sex-dependent manner, were particularly enriched for pathways involved in cell proliferation. HFD clearly increased and E2 treatment opposed the HFD-induced proliferation, both at the transcriptome and functional (Ki67) level, in males. While we saw a trend of HFD-increased proliferation in females, this was not significant and we assume that they may be protected through their already higher levels of endogenous estrogen. Previous in vitro results have demonstrated anti-proliferative effects by exogenously expressed ER $\beta$  in CRC cell lines<sup>59–61</sup>. In line with these data, we found a significant decrease in HFD-induced cell proliferation upon ER $\beta$  activation (DPN). These results demonstrate that ER $\beta$  can protect against the HFD-induced cell proliferation in males, and potentially undermine the promotion of colon tumor development.

The most unexpected finding was that key circadian clock genes were differentially expressed between the sexes in the colon, during both dietary conditions. The circadian clock regulates critical processes involved in inflammation and proliferation. The circadian rhythm has been shown to be dysregulated in both obesity and IBD<sup>62,63</sup>. HFD feeding is known to impact the circadian rhythm in the hypothalamus, liver and fat tissue, and to disrupt the eating behavior. However, timed HFD-feeding can reset the circadian metabolism and help prevent obesity and metabolic disorders<sup>64,65</sup>, indicating a major contribution of the circadian rhythm in the pathogenesis of the disease. The core circadian clock is regulated by different factors including sex hormones such as estrogen and there are evidences of sex-dependent regulation<sup>66</sup>. In breast cancer cell lines, ER $\alpha$  was shown to increase the protein and mRNA expression of Clock<sup>67</sup>, indicating the potential of estrogen receptors to regulate clock genes at the tissue level. However, the impact of HFD and estrogen on the circadian rhythm in the colon epithelium has not been investigated previously. Here we show that mice fed HFD, of both sexes, alter expression of key clock genes *Npas2* and *Bmal1*, and that estrogen can modulate this expression. Estrogen is also known to impact the feeding behavior<sup>68</sup>. The food intake was not successfully monitored in this study, due to problems with the HFD breaking into powder, and we cannot exclude the possibility that some of the effects mediated by estrogen could be through decreased diet consumption. However, our mechanistic data demonstrated that ER $\beta$  could bind to cis-regulatory chromatin regions of both *Npas2* and *Bmal1*. This suggests that ER $\beta$  acts locally in the colon and directly regulates these genes, and we could confirm this using our intestinal-specific ER $\beta$  knockout mice. The expression of colonic *Bmal1*, which was enhanced by selective-ER $\beta$  activation in females, was indeed significantly decreased upon colon epithelia ER $\beta$  knockout in female mice on a HFD. It is worth noting that ER $\alpha$  activation by PPT also regulated the expression of *Npas2* and *Bmal1* in the colon. Available data do not support significant expression of ER $\alpha$  in the colon<sup>51</sup>, and we therefore assume that these effects are systemic through its effect on the overall metabolism. However, we did notice a two-fold increase of colonic ER $\alpha$  mRNA levels in female colon upon HFD-feeding (data not shown), and we cannot completely exclude local effects of ER $\alpha$  in colon, possibly via immune cells. The ER $\beta$ -binding chromatin site by *Bmal1* included an estrogen response element (ERE), which ER $\alpha$  should also be able to bind, if expressed. However, the *Npas2* ER $\beta$ -binding site was indicated to be through tethering with JUN/AP-1. Since the two ERs differ in their protein interactions, because of less conserved AF1 and AF2 domains, this site is more likely to be specific for ER $\beta$ . It should be emphasized that although all mice were sacrificed at the same time of the day, this was not a circadian experiment, and we also did not monitor the estrous cycle of the females. Hence, while our results highlight the implication of ER $\beta$  in the regulation of core clock genes in the colon, further experiments investigating different zeitgeber times are needed to conclude its impact on their rhythmicity. Additionally, further studies are needed to investigate the impact of different estrous phases on the colon transcriptome, since our results in females presents the average response of diet, independent of the estrous cycle.

In conclusion, our data provide evidence that the colon microenvironment responds to HFD in both sexes, but with apparent sex differences, as illustrated in Fig. 5. Both sexes present an altered expression of core clock genes, although this was stronger in females, and increased macrophage infiltration. Males, on the other hand, showed significant effects on epithelial cell proliferation, increased by HFD and decreased through ER $\beta$ . All of the above functions play important roles in the pathogenesis of HFD-induced metabolic disorders and colitis. Entirely novel findings in our study demonstrate that estrogen signaling can partially modulate the colon microenvironment during HFD, and that core clock genes, colonic cell proliferation and macrophage infiltration can be modulated by estrogen via ER $\beta$  in this context, in a sex-dependent manner. Intestinal-specific ER $\beta$  knockout mice and CHIP-seq data demonstrate that ER $\beta$  in the colon is a regulator of both *Bmal1* and *Npas2*. This study opens up new insights, where ER $\beta$  exhibits beneficial action against deleterious effects of diet-induced obesity on colon microenvironment.

## Methods

**Animal experiment and tissue collection.** Five to six-week old male and non-ovariectomized female C57BL/6J mice were obtained from in-house breeding. Mice lacking ER $\beta$  specifically in the epithelial cells in the intestine were generated by crossing ER $\beta^{\text{lox/lox}}$  mice (B6.129X1- Esr2<sup>tm1Gust</sup>, Jan-Åke Gustafsson's laboratory) with the transgenic mice bearing Cre recombinase expressed under the control of the enterocyte-specific Villin promoter (B6.SJL-Tg(Vil-cre)<sup>997</sup> Gum/J; Jackson Laboratory, Bar Harbor, ME). Littermates (ER $\beta^{\text{lox/lox}}$ ) lacking the Cre allele were used as controls (referred to as WT). The genotype was confirmed with standard PCR protocol. Animals were housed under controlled environment at 20 °C with a 12-h light–dark cycle. The animals were fed HFD (D12492, 60% kcal fat, Research Diet) or a control diet corresponding to a matched low-fat diet (D12450J, 10% kcal fat, Research Diet) and water was provided ad libitum. For the treatment with estrogenic ligands, at 10 weeks of diet the mice were injected *i.p.* every other day for a total of 9 injections with 0.05<sup>69</sup> (N = 10 for males and 11 for females) or 0.5 mg/kg<sup>70</sup> (N = 10 for males) BW for 17 $\beta$ -estradiol (E2, Sigma-Aldrich), 2.5 mg/kg BW for 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)triphenoxy (PPT, Tocris, N = 5 per group for both



**Figure 5.** Schematic illustration; proposed model for estrogen regulation of the colon microenvironment during HFD-induced obesity. The HFD-induced impaired colonic circadian clock genes and increased cell proliferation was opposed by ER $\beta$  in males. The HFD-induced increase in macrophage infiltration was repressed by ER $\beta$  activation in both females and males.

females and males)<sup>71</sup>, 5 mg/kg BW for 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN, Tocris, N = 10 for males and 9 for females)<sup>71</sup>, or vehicle. The ligands were prepared in a solution of 40% PEG400, 5% DMSO and 55% water. Mice were fasted for 6 h prior the measurement of the glucose level. Briefly, a small piece of the tail was cut with a scalpel, a drop of blood was removed, and glucose level immediately measured with a OneTouch Ultra glucometer (AccuChek Sensor, Roche Diagnostics). Mice were dissected and fat pads from the abdominal and posterior subcutaneous regions carefully removed and weighted. Total fat (TF) was calculated as the sum of all fat depots including gonadal fat, retroperitoneal fat, omental fat and inguinal/gluteal fat depots. Visceral adipose (VAT) comprised of the sum of gonadal fat, retroperitoneal fat and omental fat. The subcutaneous fat (SAT) comprised of the inguinal/gluteal fat depots. All experiments were performed in accordance with ARRIVAL guidelines and EU Directive 2010/63/EU, for the care and use of laboratory animals. The local ethical committee of the Swedish National Board of Animal Research (Stockholm ethical committee, N230/15) approved all experimental protocols. Colons were harvested, washed, measured and opened along the long axis. The colons were then either embedded in OCT (Tissue-tek, Sakura) and prepared for IHC analyses using the “swiss roll” method, or snap frozen in liquid nitrogen for further analysis.

**ELISA.** Blood was collected by cardiac puncture at sacrifice using EDTA-treated tubes and plasma was collected after centrifugation and stored at -80 for further analysis. Estradiol levels were measured in mouse plasma by mouse/rat estradiol kit (ES180S-100, Calbiotech) according to manufacturer’s instructions. Plasma samples were diluted 1:5 for vehicle treated CD and HFD and 1:12 for E2 treated mice with assay diluent. Insulin level was measured in plasma by mouse/rat Elisa kit (EZRMI-13K) according to manufacturer’s instructions with plasma samples dilution of 1:2. Calculations were performed using standard controls provided with the kit.

**IHC.** Colonic samples embedded in OCT were cryo-sectioned at 8  $\mu$ m thickness. Sections were fixed in 4% formaldehyde for 10 min and endogenous peroxidase activity was blocked with 3% hydrogen peroxidase in 50% methanol and PBS for 30 min. Slides were blocked with 5% normal goat serum for 30 min at 4 °C and blocked for unspecific avidin/biotin binding (DAKO). The sections were incubated overnight at 4 °C with the primary antibodies, anti-F4/80\* (1:100, cat# MCA497R, lot# 1608, RRID: AB\_323279, Bio-Rad), anti-Ki67 (1:100, SP6, cat# MA5-14520, lot# TC2542944A, RRID AB\_10979488, Invitrogen) in 0.1% IGEPAL in PBS. Negative controls without primary antibodies were used for each slide. The sections were incubated with appropriate biotin conjugated anti IgG secondary antibodies (1:500) for 1 h at room temperature (RT), and then incubated with avidin–biotin complex (ABC, Thermofisher) for 1 h at RT. Sections were developed with Liquid DAB + (3,3-diaminobenzidine) Substrate Chromogen System (DAKO) and counterstained with Mayer’s Hematoxylin (Sigma-Aldrich). After dehydration in ethanol and xylene, the slides were cover-slipped using Pertex (Histolab). Ten to fifteen random images from three fully stained sections per animal were randomly taken using a BX53 light microscope and CAM-SC50 camera (Olympus). Positive stained cells per crypts were counted using QuPath and approximately 30 crypts per animal were analyzed.

**RNA isolation and qPCR.** Frozen colon distal tissue and colon epithelial layer were homogenized with a tissue lyser (Qiagen, Chatsworth, CA). Total RNA was isolated with QIAzol and purified using miRNeasy Mini Kit (Qiagen, Chatsworth, CA) according to the standard protocol and on-column DNase treatment was applied. Quantitative and qualitative analyses of the RNA were performed with NanoDrop 1000 spectrophotometer and Agilent TapeStation 2200 (Agilent Technologies, Palo Alto, CA), respectively. All samples had RNA integrity > 6.5. One microgram RNA was reversed transcribed using iScript cDNA synthesis kit (Biorad) according to standard protocol. Ten nanogram of cDNA were used to perform qPCR in the CFX96 Touch System (Biorad), with iTaq universal SYBR Green supermix (Biorad) as recommended by the supplier. Samples were

run in duplicates and the relative gene expression was calculated as the mean per group using the  $\Delta\Delta C_t$  method, normalized to the geometric mean of three reference genes (*Actb*, *Eef2* and *Tbp*). Primer sequences are provided in supplementary table 1.

**RNA-seq.** RNA from colon tissue was prepared from 6 biological replicates, from male and female fed with CD and HFD, and males fed with HFD and treated with E2 (0.5 mg/kg BW). Library preparation (Illumina RiboZero) and sequencing (Illumina NovaSeq6000) was performed at Sweden's National Genomics Infrastructure (NGI). At least 17 M paired-end reads (2 × 51 bp in length) were generated for each sample, and mapped against mouse genome (GRCm38) using STAR. FeatureCounts and StringTie were used to generate gene counts and TPM values. DESeq2 was used to calculate differentially expressed genes (DEG) with raw counts as input and the Benjamini–Hochberg procedure was used to estimate FDR. Genes were considered as significantly differentially expressed if  $p$  value < 0.05 and  $\log_2 FC > |0.4|$ . Gene ontology/biological function was performed with DAVID bioinformatics website.

**Statistical analysis.** GraphPad Prism was used for statistical analysis (GraphPad Software Inc, La Jolla, CA). The results are presented as mean ± SEM. A (two-tailed) Welch's  $t$ -test was used for comparison between two groups. One-way analysis of variance (ANOVA) was used for comparison between multiple conditions followed by fisher's LSD test. Two-way ANOVA was used for comparison between multiple conditions in two different groups followed by fisher's LSD test. All conditions were compared to HFD vehicle and a  $p$  value < 0.05 was considered being statistically significant (\* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001).

### Data availability

Gene expression data are deposited in the NCBI Gene Expression Omnibus database [GSE149811].

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## Author contributions

L.H. and A.A. designed the in vivo study; L.H., A.A., R.I., M.B., C.S. and M.K.-A. performed all the in vivo experiments; A.A. and M.B. performed the histology experiments; R.I. performed ChIP experiments; L.H., and A.A. performed the qPCR experiments; L.H. performed the RNA-seq analyses; L.H., A.A. and C.W. interpreted results; L.H., R.I., and A.A. prepared figures; L.H., A.A. and C.W. wrote manuscript; M.K.-A. advised on the metabolic parameters; A.A. and C.W. initiated, coordinated the study and supervised. All authors discussed the results and approved final version of manuscript.

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## Competing interests

The authors declare no competing interests.

## Additional information

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