Estrogen receptor α (ERα)-binding super enhancers drive key mediators that control uterine estrogen responses in mice

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ABSTRACT

Estrogen receptor α (ERα) modulates gene expression by interacting with chromatin regions that are frequently distal from the promoters of estrogen-regulated genes. Active chromatin–enriched “super-enhancer” (SE) regions, mainly observed in in vitro culture systems, often control production of key cell type–determining transcription factors. Here, we defined super-enhancers that bind to ERα in vivo within hormone-responsive uterine tissue in mice. We found that SEs are already formed prior to estrogen exposure at the onset of puberty. The genes at SEs encoded critical developmental factors, including retinoic acid receptor α (RARA) and homeobox D (HOXD). Using high-throughput chromosome conformation capture ((Hi-C) along with DNA sequence analysis, we demonstrate that most SEs are located at a chromatin loop end and that most uterine genes in loop ends associated with these SEs are regulated by estrogen. Although the SEs were formed before puberty, SE-associated genes acquired optimal ERα-dependent expression after reproductive maturity, indicating that pubertal processes that occur after SE assembly and ERα binding are needed for gene responses. Genes associated with these SEs affected key estrogen-mediated uterine functions, including transforming growth factor β (TGFβ) and LIF interleukin 6 family cytokine (LIF) signaling pathways. To the best of our knowledge, this is the first identification of SE interactions that underly hormonal regulation of genes in uterine tissue and optimal development of estrogen responses in this tissue.

INTRODUCTION

Estrogen hormones are intricately involved in key molecular events that underlie development of female reproductive tract tissues, optimizing responses to ovarian hormones essential for successful reproduction later in life (1). Many studies of estrogen response mechanisms have utilized in vitro cell culture models, which have advanced understanding, but which are limited in their application to more biological contexts. In vivo models of hormone response include the rodent female reproductive tract, which is highly hormone responsive, facilitating study of mechanisms of tissue development and hormone response. For example, female reproductive tissues of estrogen receptor α (ERα)-null and estrogen deficient Cyp19 (aromatase) KO mouse models, both of which resemble their WT littersmates at birth, lack pubertal uterine development and thus exhibit a hypoplastic uterine phenotype (1-4). These experimental characteristics duplicate those found clinically in patients with insensitivity syndromes due to mutations in their ESR1 or CYP19 genes (5-8). Estrogen’s activity involves interaction with its nuclear receptor, ERα, which localizes to enhancer regions of chromatin, driven by high affinity for estrogen response element (ERE) DNA motifs. Enhancer regions that ERα interacts
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with are often distal from promoters of estrogen regulated genes (1,9) and current models incorporate a mechanism of chromatin looping to facilitate contacts between enhancer and promoter regions (10,11). Earlier studies have noted that some enhancers have particularly high enrichment of indicators of active chromatin; these are classified as “super enhancers” (12,13). Frequently these super enhancers control production of key transcription factors that characterize a particular cell type (12-14). In mice, prior to production of ovarian hormones, which begins with the onset of estrus cyclicity at puberty (post-natal day 28), the pre-pubertal uterus is hypoplastic, has ERα in all cells and exhibits rapid transcriptional and growth responses of all uterine cell types to exogenous estradiol (E2) (15-17). The response, however, differs from what is observed in an ovariectomized adult mouse uterus, where the uterus response is restricted to only the epithelial cells (15,17). Through the process of pubertal maturation, the reproductive tract acquires optimal reproductive functionality (3,15,16,18,19). The molecular details of this important process have not been explored, therefore we sought to define the estrogen-dependent enhancer landscape of the developing mouse uterus by analyzing uterine chromatin isolated from pre-pubertal (21-days old) or from adult (10-weeks old) mice that were ovariectomized (ovexed) to remove the source of endogenous E2, and then treated for one hour with vehicle (V) or with E2 to gain insights into molecular components and responses impacted during development of a hormonally regulated tissue.

RESULTS

Defining uterine super-enhancers that bind ERα

We defined super enhancers (SE) that bind ERα in pre-pubertal uterine samples from mice treated for 1h with vehicle (V) or with E2, as well as in uterine samples from ovexed adult mice treated for 1h with V or E2 as described in the methods. Briefly, ERα binding enhancers were identified using ERα ChIP seq data by stitching together regions with more than one ERα peak within 12500 bp in at least one of the samples. The 4634 enhancer stretches containing multiple ERα binding sites were then classified as “typical” or “super” enhancers (TE or SE, respectively) by plotting ranked H3K27Ac signal of each; enhancers beyond the slope=1 elbow of the curve (Figure 1A) were considered SE. Therefore, locations ERα ChIP-seq peaks from all four sample types were classified as either single peaks (Figure 1B) or were within enhancer regions with multiple peaks (Figure 1B). The multi peak enhancer regions were then classified as super enhancers or typical enhancers (Figure 1B). Several hundred SE were identified in each sample type (Figure 1A). Comparison of the enhancers called in each sample indicates that many SE overlap across the sample types (Figure 1A and Figure 2), and most that are SE, but not in all samples, are ranked in the top 25% of enhancers (Figure 1A, red lines in heatmap below each curve). The SE in the pre-pubertal V treated mice are present before the cells have been exposed to ovarian E2 beginning at puberty, whereas the SE in the ovexed adult samples represent super enhancers after the uterus has undergone pubertal maturation. For each super enhancer curve, genes located at the top 10 SE are listed. We noted that three of the genes at SE (Zmiz1, Ncor2 and Rara) were among the 10 highest ranked in all samples (Figure 1C). Comparison of the SE using heatmaps of ERα and H3K27Ac ChIP-seq signals centered on each of the 2431 ERα-binding sites that is within a SE in at least one of the four samples (Figure 2), indicates the H3K27Ac signal intensities of adult and 21-days old are similar in V and E2 treated samples, respectively, showing that the SE seen in pre-pubertal samples, prior to pubertal uterine development, are largely unchanged by pubertal development. E2 leads to increased ERα binding at each site, as well as increased H3K27Ac signal flanking SE ERα binding locations. Interestingly, although E2 also increases ERα binding at the 21894 non-SE ERα binding sites (TE and single ERα binding sites), H3K27Ac signal is more static (Figure 2), with only the highest ranked ERα binding sites exhibiting E2 dependent H3K27Ac signal increase. Some researchers use H3K4Me1 ChIP-seq signal intensity, also associated with active enhancers, to classify SE.
Using H3K4Me1 signal to classify uterine enhancers revealed that 65-87% of SE by H3K4Me1 were also classified as SE using H3K27Ac (Table S1). Additionally, >98% of SE by H3K4Me1 signal were within the top quartile of H3K27Ac signal. Locations of SE called using H3K27Ac in the adult ovexed 1h E2 uterus relative to genes and chromosomes is illustrated in Figure S1.

Chromatin interactions in uterine tissue

Distal enhancers can exert transcriptional control by physically contacting gene promoters through “looping” mechanisms (10). It is not known whether contacts are dynamically formed in order to exert regulatory signals between distal enhancers and promoters, or if loops are pre-formed, and the activity of the promoters is mediated via dynamic transcription factor interactions, or whether a combination of mechanisms occurs (20). Assigning ERα interacting regions to regulated genes is important for understanding how ERα binding to distal enhancers regulates gene promoters, therefore, we analyzed ovariectomized adult mouse uterus one hour after treatment with V, E2, or progesterone (P4) using Hi-C. We used the Juicer tool to call loops from the Hi-C data of each treatment as well as differential loops in pairwise comparisons. (Figure 3A) We observed little variability between replicates or between different treatments suggesting that loops are not altered globally by the hormone treatments we administered (Figure 3A). Therefore, we combined the loops from all WT samples into an “atlas” of uterine loops. Then, we used cohesin subunit SMC1a ChIP-seq to assess interactions and potential impacts of hormone treatment. Since cohesin slides along chromatin until it contacts a pair of converging CTCF sites (10,21), we compared SMC1a peaks at loops anchors selected to have cohesin at both ends as a surrogate for the presence of a loop in the sample. We examined SMC1A signal intensity, both at selected loop anchors and at other locations, and compared V and E2 treated samples (Figure 3B). When we compared V vs. E2 SMC1a signal intensity we did not observe a notable impact of E2 treatment (Figure 3B) at selected loop anchors, or at other locations, suggesting once again, that loops are pre formed and not impacted by our hormone treatments. To assess whether specific transcription factors might be associated with V vs. E2 loops, we analyzed the DNA motifs associated with SMC1a peaks that were located at both ends of loops. The CTCF motif is highly enriched in SMC1a peaks at loop anchors of both V and E2 samples (Table 1). The ERα motif ERE and ERα “tethering” factor motif SP1 are both selectively enriched at SMC1a peaks of the E2 sample. These observations suggest E2-induced mechanisms in which ERα and associated transcription factors such as SP1 interact with pre-formed chromatin loops. Consistent with this mechanism, we note that Hi-C analysis of uterine tissue from mice lacking ERα shows that loops are present and do not differ from WT samples any more than the variation observed between replicates (Figure 3A).

Super-enhancers are predominantly located in loop ends and are associated with uterine genes

Next, we evaluated the relationships between SE and chromatin loops, and whether SE and uterine genes are brought into proximity in 3D space. For this part of our analysis we focused on the adult ovexed E2 treated samples. First, we examined average H3K27Ac signal per loop at all loop ends or “anchors” (Figure 4A), which indicated this histone modification was centered on ends of loops. By further comparing loop anchors overlapping TE vs. SE, we observed significantly more H3K27Ac signal at TE-associated loop anchors than at than at all loop anchors (p<0.0001; Figure 4A). Signal was further enriched at SE-associated loop anchors relative to all or to TE-associated loop anchors (p<0.0001), reflecting the more robust enhancer activity, and confirming these regions as SE. Of the 281 SE identified in adult E2 treated tissue, 94% (263) were at the end of a loop, suggesting they were likely to contact genes in distal regions (Figure 4B). To get an indication of the gene regulation that might occur by looping between genes and SEs, we selected genes that either were at one of the SEs in a loop end, or that were at the other end of a loop that formed from a SE. Altogether, we found this entailed 1600 genes (Figure 4C). Our previous studies have indicated that ERα
interaction with chromatin occurs within 1h, and that resulting gene regulation occurs subsequently (9,22). Therefore, we used RNAseq of uterine RNA from ovariectomized mice treated with V or treated with E2 for 2h or 6h to determine whether these 1600 ERα-binding SE associated genes were transcribed and E2 regulated in the uterus. Indeed, 963 of these 1600 SE-associated genes are expressed (TPM≥1 in at least one treatment condition) in uterine cells (Figure 4C). Overall, 9975 uterine genes are regulated by E2 (TPM≥1, adjusted p value<0.01) in at least one treatment condition (Table S2). 569 of the 963 uterine SE-associated genes are regulated by estrogen (Figure 4D and Table S3). This suggests that ERα-binding SE may be involved in regulation of 5-6% of E2 responsive uterine genes. The remaining E2 regulated uterine genes are likely to be mediated by ERα interactions at TE or at single ERα binding sites as we have previously demonstrated that the ERα is required for uterine E2 genomic response (4,22). To assess how the 569 SE associated estrogen regulated uterine genes impact biological functions, we used Ingenuity Pathway Analysis. Genes involved with promotion of gene transcription, cell survival, genitourinary tract development and suppression of mortality and apoptosis were apparent (Table 2). Notably, upstream regulators with known roles in uterine function, such as estrogen and cytokines, including LIF, a critical mediator of embryo implantation (23), as well as IGF1 and TGFβ(24-32), are identified as activating upstream regulators (Table 3). Therefore, these ERα-binding SE associated genes are enriched for important uterine processes.

**Super-enhancer associated genes acquire estrogen responsiveness during post-pubertal development**

To assess whether post-pubertal development impacted E2 regulation of the SE associated genes identified above, we analyzed gene regulation in 21 day old and ovariectomized adult samples from WT mice and mice with deletion of deletion of the ERα gene (ERαKO). We treated the mice for 2h with V or E2 and isolated uterine RNA for microarray. We observed 245 SE associated genes were differentially expressed after 2h treatment with E2. Hierarchical clustering reveals that the responses are ERα-dependent (Figure S2, Table S4). What is surprising is that, despite the presence of the SE prior to pubertal development (Figures 1 and 2), estrogen regulation is much more robust in adult ovariectomized samples (Figure S2, Table S4), indicating that these genes acquire the transcriptional response as a result of pubertal development.

**Super-enhancers are associated with key uterine factors**

Our analysis has indicated an association between ERα-mediated responses and looping between SE and estrogen regulated genes. Because genes at SE often encode factors critical for specific cell types (14), we evaluated examples of four genes at SE that are potentially important for uterine development and E2 transcriptional response and that are among the top ranked SE (Figure 1A). Retinoic acid receptor a (Rara) is a ligand dependent transcription factor in the nuclear receptor superfamily (33). Homeobox (Hox) transcription factors are key mediators of anterior-to-posterior developmental patterning of tissues (34). Ncor2 (SMRT, Nuclear receptor corepressor 2) interacts with and activates histone deacetylases (HDACs), thereby reducing transcriptional activity (35). Zinc finger MIZ domain-containing protein 1 (Zmiz1) is a novel transcription factor. We first used RT-PCR of RNA samples from pre-pubertal (21 day old) or adult ovariectomized uteri to examine the impact of pubertal development on expression of these four genes. All genes showed increases in E2 response in adult tissues versus pre-pubertal tissues (Figure 5A).

Hi-C analysis indicates distal chromatin loops with SEs at Rara (Figure 5Ba), the Hoxd cluster (Figure S3), Ncor2 (Figure S4A) and Zmiz1 (Figure S4B) genes. We wondered whether the expression of genes that either directly coincide with the SEs or are within loops would be impacted by E2. Therefore, we looked at expression and estrogen regulation of genes within loops formed at these four SE. We used our RNA seq data to evaluate the levels and E2 regulation of these genes. (Table 4). We assessed the relative level of expression of each gene by
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computing the average number of transcripts per million (TPM) of nine uterine RNA samples (3 replicates each of uterine RNA from animals treated with V or with E2 for 2 or 6 hours). We examined the E2 regulation of each gene based on fold change of E2 2h RNA vs. V RNA or E2 6h RNA vs. V RNA. The Rara SE forms loops that include several genes (Table 4, Figure 5Ba), but only Rara and Igfbp4 were regulated by E2 (TPM≥1, FDR p-value<0.01; Table 4). The Hoxd cluster SE coincides with the coding genes for Hoxd 8, 9, 10 and 11 and contacts two additional SEs (Figure S3A). Some of genes within loops that include the SE are regulated by E2 (Table 4).

There is a loop between the Hoxd SE and a third SE nearly 30 MB away, in the Wilms Tumor 1 (Wt1) gene (Figure S3B). Thus, the Hoxd cluster SE appears to be at the core of a very large 3D chromatin structure of SEs. Two SE are localized at the Ncor2 coding sequence (Figure S4A). Ncor2 is one of the most highly expressed genes in the loops that include the SE within it. (Figure S4A, Table 4). The Zmiz1 SE loops with two distal SEs (Figure S4B). Zmiz1 and several other genes within the loop are expressed (Figure S4B, Table 4).

To experimentally determine whether a SE ERα binding site can impact expression of genes within Hi-C defined chromatin loops, we examined expression levels of genes within loops from a previously described SE distal from the Igf1 gene (36). The Igf1 SE forms a loop that includes Tym-ps, Dram1, Pmch, Parphp, Nup37 and Ccdg53 within it (Figure 5Bb). Pmch and Tym-ps are not expressed (average TPM<1); Dram1, Igf1 and Nup37 are regulated by E2 (Table 4). In our previous study we disrupted the Igf1 distal SE by deleting one of its ERα binding sites (36); using this IGF1enh4KO model we examined whether the distal SE is important for E2 regulation of genes in the loops that emanate from it. RT-PCR analysis of uterine RNA samples from IGF1enh4KO and WT littermates shows that although E2 regulation of Igf1 itself was affected by disrupting the SE (Figure 5C), no change in expression of Dram1, Nup37 or Parphp occurred, indicating the deleted site selectively impacts only Igf1 expression.

Mouse uterus super-enhancers differ from those in breast cancer cells

To evaluate how the mouse uterine SEs might be related to SE in other E2 responsive cells, we compared our findings to a similar analysis that defined SE that bind ERα in the MCF-7 breast cancer cell line grown in hormone deprived media (37). We compared the genes that are closest to 227 MCF-7 SE to a list of genes closest to 281 uterine tissue SE (Figure 6). After removing genes that do not have both mouse and human orthologs (Figure 6A), about 10% of the SE genes were common between the mouse uterus and MFC-7 cell systems (Figure 6B). The small number of SE located at genes shared in MCF7 cells and uterine tissue is likely due to differences in the biological responses E2 elicits in a normal E2 responsive tissue (mouse uterus) vs. an immortalized breast cancer derived cell line.

DISCUSSION

The importance of E2 and ERα in uterine maturation is emphasized by the observation of hypoplastic uterine tissue in adult aged ERα-null mice (4), which circulate high levels of E2, but have no receptor protein, and in mice lacking Cyp19, that are therefore unable to synthesize E2 (2,38), but that have normal levels of ERα. We defined the estrogen-dependent enhancer landscape of the developing mouse uterus and observed that overall, most of the 21-day old and adult SE had similar characteristics; in general, both ERα and H3K27Ac signals are increased by E2 at the SE (Figure 2). Although E2 increases ERα signal of TE and single ERα binding sites as well, H3K27Ac is less impacted by E2 (Figure 2), with only the highest ranked ERα binding sites showing an increase. Overall, this suggests that the enhancer landscape necessary for uterine maturation is already in place by postnatal day 21.

It was clear, however, from our gene regulation analyses, that pubertal development leads to more robust regulation of genes associated with these SE (Figure S2).

Comprehensive analysis of ERα interaction with chromatin using ChIP-seq has revealed that ERα
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binding regions are often distal from coding genes, which has led to a need to understand the 3D structure of chromatin within ERα expressing cells. We noted that all ERα-binding loop ends had significantly more H3K27Ac signal, centered on the loop anchor, than loop anchors in general (Figure 4A), especially those at super enhancers. This highlights the importance of ERα in the mechanism of activation via these 3D structures. We did note that H3K27Ac signal is greater at loop ends with TE (Figure 4A), indicating a significant role of these ERα-binding regions in E2 response as well. How responses mediated by single ERα-binding sites or by TE vs SE impact uterine development and biological processes will be an important focus of future study. Our previous work characterized one such gene mechanism by deleting an ERα-binding site in a super enhancer distal from the Igf1 gene, resulting in loss of E2 induction of the coding transcript (36). Deleting one of the five distal ERα binding sites did not impact E2-dependent ERα binding to the four remaining distal sites, nor to a site near the promoter, however E2-dependent recruitment of the histone acetyltransferase p300 and the cohesin subunit SMC1a was decreased at neighboring sites, and acetyltransferase p300 and the cohesin subunit dependent recruitment of the histone to a site near the promoter, however E2 -ERα distal enhancer. The transcription is governed by a critical site in the coding mRNA transcription was lost (36). Thus, E2 dependent recruitment of factors necessary to optimize enhancer-promoter mediated Igf1 transcription is governed by a critical site in the distal enhancer. The Igf1 distal SE also forms chromatin loops that include other genes, some of which are also regulated by E2, however, deletion of the ERα-binding site did not change the E2 response of these other genes, indicating the primary target of the SE in this case is the Igf1 gene. Whether this relationship between SE and regulated distal genes is generally seen, or whether cases in which expression of multiple genes is impacted by a single SE remains to be studied.

In this study, we identified SE either in or looping to other well-known highly E2-responsive genes, including Greb1, Lif, Fos, Cebpb, Cyr61 and Inhbb (Table S3). The Greb1 interacting super enhancer identified here was the focus of a previous study indicating the potential role of distal ERα binding regions in Greb1 regulation (39). The same ERα binding region was utilized to model mechanisms of distal interaction in a recent study (40), highlighting a role for the steroid receptor coactivator, SRC-3, in optimizing contacts between enhancer and promoter regions via interaction with an intronic SRC-3 binding sequence (40). Prior to E2 treatment, the enhancer and promoter are held in proximity to each other through their contacts with SRC-3 bound to the intron. E2 treatment leads to SRC-3 dependent formation of the enhancer-promoter transcriptional complex (40), and consequently, increased Greb1 transcription. The formation of structures such as the SRC-3/intron/enhancer/promoter complex may be a mechanism utilized by enhancer-promoter interactions.

We note that most of the SE identified in adult ovex E2 treated samples were at a loop end (263 of 281; Figure 4B) and were likely to be at or form a loop to genes expressed in the uterus (Figure 4C). Many of these SE-associated genes are involved in transcription and in pathways important for uterine function, such as p53 (41) and Tgfβ (26) signaling, therefore these SE are associated with important mediators of uterine maturation and function. We did not observe a SE at the Esr1 gene, although ERα is detected in all uterine cells and SE associated genes are often characteristic of a particular cell type. Perhaps we should have expected that ERα is not driven by a SE, as we know that the ERα null mice develops all uterine cell types normally (4,42). We did see that Esr1 was found to be one of the genes closest to an ERα-binding SE in both the MCF-7 cell and our uterus datasets (Figure 6C). When we examined the impact of E2 on expression of the 963 SE associated uterine genes, 569 were regulated by E2, a small portion of the 9975 uterine genes that are regulated by E2 (Figure 4D and Tables S2 and S3). These SE-associated DEG are involved with activation of TGFβ and estrogen responses, and with cytokine pathways, including LIF, a cytokine that is critical for embryo implantation (23) (Table 3).

Our finding of a SE overlapping the uterine Rara gene confirms the association of SE with key genes that determine cell fate (12,13), as critical roles for retinoic acid (RA) signaling in uterine
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development have been described. RARα is activated by retinol derivatives (Vitamin A), and disruption of retinol signaling, either by disrupting the Rara gene or with retinol deficient diet, impacts female reproductive tract development (43,44). RA is required for Mullerian duct development, and for maintenance of epithelia of adult female reproductive tracts (45). Mechanistically, retinoic acid signaling influences uterine vs. vaginal epithelial differentiation (46), therefore precise regulation of RA signaling mediators is important for normal development and function. This is partly achieved via regulation of enzymes involved in retinoic acid synthesis and degradation (47), but clearly RAR expression is needed to mediate the signal, suggesting that SE in the Rara gene plays an important role in female reproductive tract development and function. Additionally, inappropriate perinatal estrogen exposures are known to disrupt the normal patterning of female reproductive tract mesenchyme, but this effect can be prevented by co-treatment with RA (45), illustrating an interplay between estrogen and RA signaling during female reproductive tract development. Evaluation of the Rara SE showed that it is within chromatin loops that include two members of the DNA replication complex Cdc6 and Top2a (Figure 5Ba, Table 4), which may indicate a role for the Rara SE in expression of genes involved in growth responses to E2.

There are four Hox clusters in the mouse genome (Hoxa, Hoxb, Hoxc, and Hoxd) and Hox9-13 are believed to have arisen from a single ancestral gene (48). The Hoxa cluster, in particular, has been shown to play a key role in reproductive tract development and function (34). We were interested, then, to note that one of the top ranked pre-pubertal SE was located in the Hoxd cluster. Hoxa factors are expressed in a progressive pattern in the developing female reproductive tract, with Hoxa9 in the oviduct, Hoxa10 in the uterus, and Hoxa11 in the cervix (34). Hoxd9, 10 and 11 are expressed similarly to their Hoxa counterparts in the female reproductive tract (49) (Figure S5A). A role for Hoxd in uterine function has been described using complementation studies with Hoxa and Hoxd deleted mice (49). In addition, a recent study evaluated the impact of deletion of one allele each of Hoxa9,10,11, Hoxc9,10,11, and Hoxd9,10,11 on uterine development. This study indicated that the combined deletion of one allele each of these nine Hox genes greatly decreased uterine gland development (50), further showing a role for Hoxd genes in uterine function. Although the Hoxa cluster has been very thoroughly described and characterized for its role in uterine function, we did not find any SE at this gene cluster (Figure S5B). We do observe chromatin loops between the Hoxa cluster and a region more than 1MB from the Hoxa cluster that includes SEs (Figure S5B).

Ncor2, also called Silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), was originally described as a corepressor for RAR and TR, mainly through its interactions with these receptors and its associated HDAC activity (35). Further study has revealed its dual role as a gene-selective co-activator and co-repressor for ERα (51,52). A role for NCOR2 in the uterus has not been investigated, but considering its potential to regulate responses of TR, RAR and ERα, it is noteworthy that we see two SEs in the Ncor2 gene (Figure S4A). Recently, it was shown that removing Ncor2 disrupts formation of a retinoid gradient in mouse embryos, and leads to perturbation of HOXC expression patterns, greatly impacting proper development (53). The disruptions observed incorporate developmental pathways involving three SE associated uterine genes (Ncor2, Rara and Hoxd), suggesting these SEs might underlie processes important for pubertal development of uterine cells.

Zmiz1 is a member of the PIAS (protein inhibitor of activated STAT) family, and was originally identified due to its role in prostate cancer cells as a co-activator that facilitates androgen receptor (AR) sumoylation (54). ZMIZ1 regulates activities of certain transcription factors, several of which are important for uterine development and function, including p53 (41), Notch (55), SMAD (24), STAT (56) and AR (57). Its essential role is highlighted by the finding that its deletion in mice causes embryonic lethality (12). Embryonic fibroblasts isolated from Zmiz1 null mouse embryos fail to proliferate normally (58,59), emphasizing its critical cellular role. A
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missense mutation in ZMIZ1 has been identified from endometroid cancer samples (Catalog of Somatic Mutations in Cancer; cancer.sanger.ac.uk (60)), and additionally, decreased ZMIZ1 expression (61) has been reported in adenomyosis biopsies, indicating an essential role for ZMIZ1 in human uterine health as well. We could readily detect Zmiz1 in the mouse uterus (Figure 5A, Table 4). Its expression was recently noted in rat uterine stromal cells, with down-regulation observed in response to circadian synchronization using the glucocorticoid agonist dexamethasone (62). SE-dependent expression of Zmiz1 could ensure optimal uterine responses mediated by pathways it regulates, however the embryonic lethality of the global deletion prevents evaluation of uterine function with current models. We plan to examine its role in uterine tissue via conditional deletion of its expression.

Our approach of identifying Super Enhancers in developing uterine tissue is validated by observations that some SE are at genes encoding factors known to be critical for uterine development and function, such as RARα. The location of most Super Enhancers at ends of chromatin loops, and the uterine expression and developmental acquisition of E2 regulation of genes either at SEs or connected by looping to a SE indicate an important role for these enhancers. Indeed, regulatory pathways known to be involved in uterine development and function are impacted by these SE-associated E2 responsive genes. These important regulatory regions likely serve to optimize appropriate, efficient and timely responses to circulating E2 during each estrous cycle and in preparation for establishing pregnancy.

Experimental Procedures

Animals

All mice were used in accordance with an NIEHS approved animal study protocol and using the 2015 edition of the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals. For E2 response experiments, intact 21-day old or ovariectomized (ovex) adult (10+ week old) female C57bl6/J mice were purchased from Charles River Laboratories (Raleigh, NC). ERαKO mice (4) (also called Ex3αERKO) and their WT and heterozygous (ERα +/-) littermates were produced in our contract colony at Taconic Farms (Albany NY). IGF1enh4 KO mice and their WT littermates were produced in our colony at NIEHS and ovariectomized at 10 weeks of age. Genotypes were determined from an ear biopsy by Transnetyx (Cordova TN). 21-day old females were used the week they were received; ovariectomized females were housed for 10-14 days before the experiments to allow endogenous ovarian hormones to diminish. There was no blinding and mice were randomly assigned to treatment groups. Mice were given a single intraperitoneal injection of 250 ng E2 (Research Plus Inc., Barnegat, NJ) dissolved in 0.1 ml of normal saline, which is a 10 µg/kg dose. Some were injected subcutaneously with 1 mg P4 (Sigma) dissolved in 100 µl sesame oil (Sigma). Control vehicle (V) animals were injected with 0.1 ml normal saline. Uterine tissue was collected 1 hour, 2 hours, 6 hours or 24 hours after the injections and was snap frozen in liquid nitrogen. RNA was isolated and cDNA synthesized for real time RT-PCR as previously described (22,36). Primer sequences are listed in Table S5. For gene expression studies, at least 3 animals per group were used based on an at least 2-fold change, with CV=0.2 and 90% power, tested by 2-way ANOVA.

Identification of Super Enhancers

Super-enhancers were identified based on the method described in Bojesuk et al (63). First, 1h V and 1h E2 ERα peak calls with high stringency were called by HOMER (parameters: -fdr 0.00001 -F 12 -style factor) for the ovexed adult and 21-day old samples, then combined via BEDtools mergeBed (v2.24.0), entailing >24K ERα binding locations altogether. BEDtools mergeBed (v2.24.0) was then re-run, this time to merge all peak calls within 12.5kb. This set was subsequently filtered to retain only those regions with more than one contributing called peak, resulting in 4634 ERα binding enhancer regions,
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each with multiple ERα binding locations. Each enhancer region was scored by counting the number of overlapping uniquely-mapped non-duplicates reads with BEDtools multiBamCov (v2.24.0); reads in this calculation were H3K27ac or H3K4Me1 ChIP-seq data after extension of mapped read length to 200nt. After normalizing to 20 million reads, the input-subtracted signal was determined per region. The signal curve was plotted as H3K27ac or H3K4Me1 signal versus region rank and re-scaled to span 0 to 1 on both axes. Definition of each enhancer region containing multiple ERα binding sites as a ‘typical enhancer’ or ‘super enhancer’ was determined according to the elbow of the signal curve where slope=1.

SMC1a ChIP-seq Analysis

Processing of the SMC1a ChIP-seq data was performed as previously described (36). Peak calls were made by HOMER v4.10.3 via findPeaks with parameter ‘-style factor’. The de novo motif analysis was done with HOMER v4.10.3 findMotifsGenome.pl with parameter ‘-size given’, for consistency, query regions (SMC1a peaks at loop ends) were re-sized to 300bp centered on the midpoint of the called peaks.

RNA-seq Analysis

Processing of the RNA-seq data was performed as previously described (28). Read counts per gene were determined by Subread featureCounts v1.5.0-p1 (64) with parameters ‘-s2 -Sfr -p’ for gene models based on RefSeq transcripts as downloaded from the UCSC Table Browser on February 9, 2015. Differential gene analysis was performed with DESeq2 v1.15.1 (65). Genes were considered expressed in the uterus if TPM≥1 in at least one condition. Genes were considered differentially expressed by applying a FDR cutoff of ≤0.01. SE-associated genes that were expressed or differentially expressed (FDR<0.01) in the uterus were identified using Partek List Manager Tool to find RefSeq gene symbols common to both sets.

Microarray

Two sets of uterine RNA samples were isolated. The first set was obtained from ovariectomized adult ERαKO females and their WT littermates treated for 2h with V or E2 as above. The second set was obtained from 21-day old ERαKO females and their WT littermates (produced by timed matings of heterozygous (ERα +/-) males and females) treated for 2h with V or E2. RNA was assessed by microarray as previously described (66) and analyzed in Partek using ANOVA to find DEG and then filtering for signal intensity >100 in at least one sample and then filtering for super enhancer associated genes. Data from ovariectomized adults is deposited in GEO (GSE100131), and data from 21-day old mice under GSE148006.

Hi-C and Data Analysis

The Hi-C analysis was based on previously described samples (36) (V-1, E2 and P4-1 in Figure 3A) together with additional samples. Samples V-2 and ERαKO V were processed by Arima Genomics (San Diego CA), and libraries were sequenced by the NIEHS Molecular Genomics Core, as described for the previous study (36). Samples V-3 and P4-2 were processed and sequenced by Active Motif, Inc (Carlsbad, CA) using the Arima Genomics Hi-C kit. The Juicer (v1.5.6) platform was used for processing the Hi-C samples as previously described (36, 67). The QC metrics from the Juicer output are shown in Table S6. Chromatin loops were identified with the Juicer hiccup utility, an algorithm for finding chromatin loops, by searching for clusters of contact matrix entries with enriched contact frequency relative to local background, at default parameters (67). Simple overlap assessment via BEDtools intersectBed was used to determine localization of super enhancers and SMC1a peaks at loop ends. The “atlas” of mouse uterine chromatin loops was generated by combining Juicer loop calls from the six individual WT samples, whereby all loops with both ends overlapping were collapsed.

Data Availability Statement
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Microarray, ChIP-seq, RNA-seq and some of the Hi-C data were previously described and were deposited as GEO GSE100131 (68), GSE56501 (9) and GSE125972 (36). Hi-C and microarray data from additional samples are deposited in GSE147843 and GSE148006.

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CONFLICT OF INTEREST

None of the authors have any conflicts to declare
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Figure 1. Defining super enhancers in mouse uterine tissue
A: Ranked H3K27Ac signal at 4634 regions with >1 ERα-binding sites within 12500 bp regions from uterine samples of 21 day old or adult ovexed mice treated for 1h with vehicle (V) or E2 (E2 1h). The plots show the distribution of the re-scaled scores per sample. The red points beyond the elbow of the curve, where slope=1, are classified as super enhancers. The enhancer status (typical = grey, super = red) for each peak in each of the 4 samples in the same order as the corresponding plot above it is shown below each curve.
B: Proportion of ERα ChIP-seq peaks that are single peaks, or that are in regions with multiple ERα ChIP-seq peaks within 12.5 KB that were classified as super enhancers in at least one sample or typical enhancers (not a super enhancer in any sample) in the above analysis.
C: Genes that are colocalized with the 10 top ranked SE regions of each sample are listed. Top 10 SE shared by all 4 samples are in red text, SE that are in the top 10 in all samples except 21 day E2 are in purple text, SE in the top 10 only in ovex samples are highlighted in yellow, SE that are in the top 10 only in V sample are in green text, SE that are in the top 10 only in E samples are in blue text.

Figure 2. Estrogen increases ERα and H3K27Ac enrichment at super enhancers
Heatmap of ERα ChIP-seq and H3K27Ac ChIP-seq signal centered on 2431 ERα peaks that are at super enhancers (top) or 21894 ERα peaks that are not at super enhancers but are at typical enhancers or are single ERα binding sites (bottom) in uterine chromatin samples from Adult ovex or 21 day old female mice treated with saline vehicle (V) or with E2 for 1 hour. For each set, enhancers are in order of ERα signal in the adult E2 sample. Each panel shows ChIP-seq signal at +/- 1kb relative to the ERα peak midpoint, with signal depth-normalized to 20M uniquely-mapped non-duplicate reads per sample.

Figure 3. E2/ERα does not alter uterine loops
A. Juicer output showing number of loops called from each sample in parentheses and differential loops called between indicated pairs. Minimal differences are seen between replicates (V-1, V-2, V-3; P4-1, P4-2) as well as between vehicle (V) and hormone treatment (E2 or P4), or between mice lacking ERα (ERαKO) and wildtype litter mates (V-2).
B. An “atlas” of uterine loops was built from the six combined WT sample Hi-C datasets. This collapsed set of loops was then filtered to retain only those with SMC1a ChIP-seq peaks at both loop ends using either V or E2-treated SMC1a ChIP-seq data. The heatmap shows SMC1a signal centered on V (left) or E2 (right) SMC1a peaks that are (top) or are not (bottom) at loop ends of selected loops. Each panel shows ChIP-seq signal at +/- 1kb relative to the SMC1a peak midpoint, with signal depth-normalized to 20M uniquely-mapped non-duplicate reads per sample.

Figure 4: Association of SE with uterine gene expression
A. Plot of normalized H3K27Ac signal in 1h E2 adult ovexed sample per called loop end from the same sample. Curves are from all called loop ends, loop ends at typical enhancers (TE), and loop ends at SE
B. Proportion of adult E2 1h SE that are in E2 1h called loop ends
C. Proportion of 1600 SE-associated genes (at adult E2 1h SEs that overlap a loop end OR at the other end of the SE loop) that are detected in uterine V, E2 2h or E2 6h samples (TPM ≥1).
D. Proportion of 963 SE associated uterine genes differentially expressed (DEG; p<0.01) 2h or 6h after E2 treatment vs. V treatment.

Figure 5 Uterine factors at SE
A. RT-PCR of uterine RNA isolated from 21 day old or ovex adult mice treated for 2h with vehicle (V) or with E2. a. Rara, b. Hoxd11, c. Ncor2, d. Zmiz1; *p<0.05 vs V; +p<0.01 vs 21 day old.
B. Chromatin loops between genes at SE and distal transcripts. SE are indicated by black blocks. Chromatin loops from uterine Hi-C samples are indicated by thin black lines. UCSC Genome Browser screenshots of: a. Rara (Chr11:mm10; chr11:98,854,783-99,160,103) near the SE (yellow highlight) at the Rara transcript. b. Igf1 (Chr10; mm10 chr10:87,635,437-88,361,810) near the SE (yellow highlight) distal from Igf1. ERα binding site that was deleted in IGF1enh4KO is outlined in red.
C. Disrupting ERα-binding site in SE distal from Igf1 prevents E2 induction of Igf1 but does not alter expression of other genes that contact the SE or that are within the loop. RT-PCR of RNA from ovariectomized IGF1enh4KO or WT littermates that were treated with V (0) or with E2 for 2, 6 or 24 hours. a. Dram1, b. Igf1, c. Nup37, d. Parpbp. *p<0.05 vs. V; +p<0.05 vs WT.

Figure 6: Genes closest to SE in the uterus differ from those in MCF-7 breast cancer cells.
A. Genes closest to 281 mouse uterus or 227 MCF-7 cell (human) SE were evaluated for whether they had an ortholog in the other species. Only genes with both mouse and human orthologs were used in the subsequent comparison.
B. 25 of the genes closest to mouse uterus or MCF-7 cell SE were shared, and are listed in C.
C. Table listing the gene symbols and Entrez gene IDs of the 25 shared genes from the analysis in B.
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| V SMC1A peaks at loop ends | Motif (best match) | RNAseq average TPM | Enrichment target/Bkg* | P-value |
|----------------------------|--------------------|--------------------|------------------------|---------|
|                            | CTCF               | 75.4               | 33.03                  | 1e-2224 |
|                            | RORC               | 23.0               | 16.00                  | 1E-31   |
|                            | MYB                | 10.7               | 14.00                  | 1E-21   |
|                            | ZFX                | 52.2               | 12.00                  | 1E-17   |
|                            | LIN54              | 14.6               | 11.00                  | 1E-18   |
|                            | SMAD3              | 36.5               | 10.00                  | 1E-10   |
|                            | VDR                | 2.5                | 6.00                   | 1E-22   |
|                            | RXRB               | 42.0               | 5.67                   | 1E-16   |
|                            | RUNX2              | 1.8                | 5.00                   | 1E-09   |
|                            | HIF1A              | 168.3              | 4.00                   | 1E-09   |
|                            | Jaspar PB0032.1    | unknown            | 2.87                   | 1E-24   |
|                            | FOXJ1              | 4.0                | 2.78                   | 1E-23   |
|                            | CEBPa              | 18.9               | 2.31                   | 1E-15   |

| E2 SMC1A peaks at loop ends | Motif (best match) | RNAseq average TPM | Enrichment target/Bkg* | P-value |
|-----------------------------|--------------------|--------------------|------------------------|---------|
|                             | Jaspar SD0002.1    | unknown            | 16.00                  | 1.00E-16|
|                             | CTCF               | 75.4               | 13.32                  | 1E-3306 |
|                             | HIC1               | 22.8               | 13.00                  | 1.00E-13|
|                             | SOX17              | 35.1               | 12.00                  | 1.00E-24|
|                             | SP1                | 97.6               | 10.00                  | 1.00E-10|
|                             | EWS-ERG-fusion(ETS)| 13.2               | 7.67                   | 1.00E-16|
|                             | ZBTB3              | 5.1                | 6.00                   | 1.00E-09|
|                             | ERE                | 187.5              | 5.05                   | 1.00E-47|
|                             | REL                | 6.8                | 4.50                   | 1.00E-06|

Table 1 HOMER analysis showing de novo motifs in SMC1a peaks that overlap with anchors of loops with SMC1a at both ends (Figure 3B) in V (top) or E2 (bottom) treated samples. Average TPM (average of all E2 2h E2 6h transcripts per million.) of transcription factors that bind to motifs. * Ratio of % of target with motif to % of background with motif.
Table 2 Biological functions and diseases associated with 569 E2 regulated uterine genes identified in Figure 4D

| Diseases or Functions Annotation                      | p-value   | Activation z-score 2h | Activation z-score 6h |
|--------------------------------------------------------|-----------|-----------------------|-----------------------|
| Morbidity or mortality                                 | 7.47E-08  | -5.82                 | -3.22                 |
| Activation of DNA endogenous promoter                  | 9.63E-11  | 4.30                  | 2.47                  |
| Cell movement                                          | 3.50E-10  | 3.99                  | 3.86                  |
| Migration of cells                                     | 3.89E-08  | 3.92                  | 3.41                  |
| Microtubule dynamics                                   | 8.93E-07  | 3.85                  | 2.41                  |
| Development of body trunk                              | 5.71E-10  | 3.48                  | 2.30                  |
| Outgrowth of cells                                     | 1.07E-07  | 3.28                  |                       |
| Cell survival                                          | 4.54E-08  | 3.07                  |                       |
| Organization of cytoskeleton                           | 7.52E-09  | 3.06                  |                       |
| Organization of cytoplasm                              | 9.02E-09  | 3.06                  |                       |
| Size of body                                           | 2.39E-07  | 3.06                  |                       |
| Cell movement of endothelial cells                     | 8.57E-07  | 2.92                  | 2.62                  |
| Formation of vessel                                    | 4.97E-06  | 2.73                  | 2.25                  |
| Phosphorylation of protein                             | 1.19E-06  | 2.71                  | 2.25                  |
| Apoptosis                                              | 1.70E-10  | -2.53                 | -2.28                 |
| Development of genitourinary system                    | 1.30E-09  | 2.51                  | 2.18                  |
Table 3 Upstream regulators enriched in 569 E2 regulated uterine genes identified in Figure 4D

| Upstream Regulator | Activation z-score 2h | Activation z-score 6h | p-value of overlap |
|--------------------|-----------------------|-----------------------|--------------------|
| TGFB1*             | 5.32                  | 5.27                  | 1.77E-11           |
| beta-estradiol     | 4.63                  | 4.54                  | 1.16E-12           |
| LIF                | 3.12                  | 2.29                  | 3.01E-05           |
| Cytokine*          | 3.06                  | 3.06                  | 1.3E-06            |
| PGR                | 2.92                  | 2.92                  | 5.14E-07           |
| Ige                | 2.62                  | 2.07                  | 2.86E-05           |
| GDF2               | 2.61                  | 2.61                  | 2.44E-06           |
| ERK1/2*            | 2.50                  | 2.62                  | 2.84E-05           |
| IGF1               | 2.35                  | 4.41                  | 3.49E-06           |
| Alpha catenin      | -2.51                 | -2.11                 | 1.22E-10           |

*Includes

TGFB: TGFB1, TGFB3, SMAD3

cytokines: CSF1,CSF2,EDN1,IFNG,IL1,IL17A,IL1A,IL1B,IL2,IL3,IL4,IL6,OSM,TNF,TNFSF11

ERK signaling: EGF,ERBB2,MAPK3,MAPK8,MAPK9,P38 MAPK
Table 4: RNA seq values for genes within loops of each SE;

SE genes are highlighted in yellow

| Gene Symbol | average TPM | fold change: E2, 2h vs. V | fold change: E2, 6h vs. V |
|-------------|-------------|--------------------------|--------------------------|
| Rara        | 5.0         | 1.36                     | 1.84                     |
| Gld3        | 0.1         | 1.32                     | 1.11                     |
| Igfbp4      | 328.6       | -1.08                    | -1.49                    |
| Rara        | 28.3        | 1.85                     | 2.11                     |
| Tns4        | 10.2        | -1.24                    | 1.96                     |
| Top2a       | 69.3        | 1.42                     | 1.46                     |
| Wipf2       | 8.9         | 1.07                     | 1.02                     |

Hoxd cluster

| Gene Symbol | average TPM | fold change: E2, 2h vs. V | fold change: E2, 6h vs. V |
|-------------|-------------|--------------------------|--------------------------|
| Atf2        | 43.4        | 1.06                     | -1.01                    |
| Atp5g3      | 256.7       | 1.18                     | 2.22                     |
| Chn1        | 6.0         | -1.06                    | 2.49                     |
| Chn10s3     | 0.7         | -1.09                    | -1.39                    |
| Cir1        | 35.3        | -1.16                    | -3.53                    |
| Gpr155      | 9.2         | -1.24                    | -2.62                    |
| Hagl1       | 0.2         | -1.88                    | -2.75                    |
| Hoxd3       | 0.1         | -1.29                    | -2.05                    |
| Hoxd3as1    | 7.3         | -1.48                    | -2.37                    |
| Hoxd1       | 35.3        | 1.26                     | -1.1                     |
| Hoxd4       | 39.5        | -1.43                    | -2.95                    |
| Hoxd8       | 71.7        | -1.02                    | 1.37                     |
| Hoxd9       | 45.5        | -1.15                    | -1.48                    |
| Hoxd10      | 73.0        | -1.22                    | -1.65                    |
| Hoxd11      | 23.8        | -1.05                    | -1.23                    |
| Hoxd12      | 0.0         | -1.04                    | 1                       |
| Hoxd13      | 0.0         | 1.2                      | -1.08                    |
| Lnp         | 17.4        | 1.03                     | 1.07                     |
| LOC102634401| 0.2         | -1.92                    | -2.49                    |
| Mtx2        | 79.7        | 1.09                     | 1.54                     |
| Ola1        | 50.9        | 1.08                     | 3.14                     |
| Wipf1       | 14.2        | -1.06                    | -1.15                    |

Ncor2

| Gene Symbol | average TPM | fold change: E2, 2h vs. V | fold change: E2, 6h vs. V |
|-------------|-------------|--------------------------|--------------------------|
| Fam101a     | 0.9         | 2.11                     | 1.01                     |
| Scarb1      | 25.1        | 1.06                     | -1.65                    |
| Ncor2       | 29.2        | 1.47                     | -1.08                    |

Zmiz1

| Gene Symbol | average TPM | fold change: E2, 2h vs. V | fold change: E2, 6h vs. V |
|-------------|-------------|--------------------------|--------------------------|
| 4930572O13Rik| 0.5        | -1.19                    | -2.71                    |
| 4931406H21Rik| 24.7       | 2.64                     | -1.02                    |
| Mir3075     | 25.9        | 2.02                     | 1.09                     |
| Polr3a      | 35.0        | 1.05                     | 1.51                     |
| Rps24       | 2395.9      | 1.04                     | -1.08                    |
| Zmiz1       | 55.8        | 2.31                     | 1.45                     |

Igf1

| Gene Symbol | average TPM | fold change: E2, 2h vs. V | fold change: E2, 6h vs. V |
|-------------|-------------|--------------------------|--------------------------|
| Ccdc53      | 34.0        | -1.05                    | -1.17                    |
| Dram1       | 29.3        | 1.11                     | 2.14                     |
| Igf1        | 182.5       | 1.6                      | 10.84                    |
| Nup37       | 27.3        | 1.04                     | 1.49                     |
| Parbp       | 2.3         | -1.11                    | -1.02                    |
| Pmch        | 0.1         | -1.19                    | -1.38                    |
| Tyms-ps     | 0.2         | -1.41                    | 2.74                     |
### Figure 1

**A.**

![Graphs showing H3AC2e-Signal rank for different samples.](image)

**B.**

**Locations of ERα peaks**

Total=24,325 ERα peaks

- **554 Super Enhancers** (2431 ERα peaks)
- **4080 Typical Enhancers** (9983 ERα peaks)
- **Single ERα peaks** (11901 ERα peaks)

**C.**

| SE Rank | ovx E   | ovx V   | 21dE   | 21d V  |
|---------|---------|---------|--------|--------|
| 1       | Zmiz1   | Rara    | Zmiz1  | Rara   |
| 2       | Cmklr1  | Zmiz1   | Ncor2  | Zmiz1  |
| 3       | Ncor2   | Mir1191b| Rara   | Mir1191b|
| 4       | Rara    | Hoxd9-11| Socs3  | Ncor2  |
| 5       | Gm14216 | Steap3  | Isyna1 | Nfix   |
| 6       | Ksr1    | Gse1    | Gpr146 | Mrps18a|
| 7       | Gse1    | Gm35608 (ncRNA) | Gm14216 | Traf3ip2|
| 8       | Zfp469  | Ncor2   | Ksr1   | Hoxd9-11|
| 9       | Htra3   | 9330198N18Rik | Mir7653, Nek8, Rab34, Rpl23a, Snord42a, Snord42b, Snord4a, Tcld1, Traf4 | Malat1 |
| 10      | Mir1191b| Hspg2   | Ptges  | Gm35608 (ncRNA) |

*Note: The table lists the different samples and their associated genes. The graph in **A** displays the H3AC2e-Signal rank for different samples, while **B** shows the locations of ERα peaks with various categories.*
Figure 2
| Sample (# of loops) | V-1 | V-2 | P4-2 (11752) |
|--------------------|-----|-----|-------------|
| V-1 (20019)        |     |     |             |
| V-2 (16387)        | 639 |     |             |
| V-3 (9385)         | 1328| 219 |             |
| E2 (24139)         | 631 |     |             |
| P4-1 (23005)       | 392 | 1014|             |
| ERαKO V (21829)    |     | 739 |             |

**Figure 3**
A. 

B. Locations of SE relative to loop ends

C. SE associated genes transcribed in uterus

D. E2 regulation of SE associated uterine genes

Figure 4
**Figure 5**

A. Relative levels of Rara, Hoxd11, Ncor2, and Zmiz1 in 21 day old Ovex adults, compared to adult Ovex, are shown. Levels are relative to the Veh/E2,2h group.

B. Uterine Loops diagrams showing ERα enhancers for Igf1 and Parpbp genes in the context of 1h E2 ERα ChIP-seq data.

C. Graphs illustrating the levels of Dram1, Igf1, Nup37, and Parpbp in response to IGF1enh4KO treatment over time.
A. Genes nearest 281 mouse uterus SEs
Genes nearest 227 MCF-7 SEs
Total=456
with ortholog (325)
no ortholog (131)

B. 25 genes
230 mouse SE genes
325 MCF-7 SE genes

C. mouse human
Cops8|108679  COPS8|10920
Cuedc1|103841  CUEDC1|404093
Cxxc5|67393  CXXC5|51523
Esr1|13982  ESR1|2099
Gse1|382034  GSE1|23199
Hes1|15205  HES1|3280
Jund|16478  JUND|3727
Nanos3|244551  NANOS3|342977
Nek8|140859  NEK8|284086
Nhp2|52530  NHP2|55651
Nr2f2|11819  NR2F2|7026
Pbx1|18514  PBX1|5087
Pgpep1|66522  PGPEP1|54858
Pttn1|19246  PTPN1|5770
Rab34|19376  RAB34|83871
Rad51b|19363  RAD51B|5890
Rai1|19377  RAI1|10743
Rara|19401  RARA|5914
Rmnd5b|66089  RMND5B|64777
Rxra|20181  RXRA|6256
Socs3|12702  SOCS3|9021
Sulf2|72043  SULF2|55959
Tlcd1|68385  TLCD1|116238
Traf4|22032  TRAF4|9618
Zmynd8|228880  ZMYND8|23613
Estrogen receptor α (ERα)-binding super enhancers drive key mediators that control uterine estrogen responses in mice

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