Transcriptional Regulation of Mammary Alveolar Proliferation and Differentiation during Early Pregnancy

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This work was supported by a grant from the NICHD 1R01HD104606-01 to ERA and a Susan Komen ASPIRE grant to JGL.

Running title: E2F5 functions as a mammary tumor suppressor.

Keywords: E2F transcription factor, E2F5, mammary gland, development, tumor

Abstract

Proliferation and differentiation of the mammary gland during pregnancy is regulated by a wide variety of factors. Using gene expression data, we have predicted that the E2F5 transcription factor has a role in the mammary gland during pregnancy. Using CUT&RUN for E2F5 in combination with gene expression data revealed that there were a number of E2F5 target genes associated with gene expression in early pregnancy, suggesting a critical role. This prediction was then functionally tested through analysis of mice with an ablation of E2F5 in the mouse mammary epithelium using a Floxed E2F5 in combination with MMTV-Cre. This revealed a striking delay in alveolar proliferation and differentiation at the early stages of pregnancy. The significance of this effect was lost in both a second pregnancy and by the mid-point of pregnancy in single parous females. Analysis of E2F5 targets revealed overlap but a slight divergence from the consensus E2F sequence and that E2F5 was bound to the promoter of many genes involved in cellular proliferation, including known regulators of pregnancy associated alveolar expansion such as Stat6. However, progesterone stimulation of cells in a reporter assay revealed that it counteracts the repression of Stat6 by E2F5, leading to expression of Stat6. Together these data reveal the complexity of the transcriptional regulation of alveolar proliferation in early pregnancy.

Introduction

The mammary gland is a unique organ in that much of the morphogenetic development occurs post-embryonically. The mammary gland remains relatively underdeveloped until puberty when hormonal signals signal the rudimentary gland to form a branched ductal tree. Upon reaching the terminus of the fat pad, the branched epithelium then achieves a static phase with only small estrus associated changes. With the induction of pregnancy, the mammary epithelium undergoes rapid proliferation and differentiation, resulting in the formation of secretory alveoli associated with the ductal network for milk production. This process is tightly regulated by hormonal cues and transcription factors that control cell proliferation, differentiation, and apoptosis [1]. Understanding the molecular mechanisms that drive mammary development, particularly during pregnancy, provides crucial insights into both normal glandular physiology and pathological conditions such as breast cancer.

The transcriptional control of mammary gland biology has been investigated with key studies examining the interplay between well known mammary gland transcription factors such as Stat5 [2, 3] and Stat 3[4]. However, the precise control of mammary development during pregnancy will be dependent on transcriptional control beyond the Stat pathway. For instance, the E2F family of transcription factors plays a pivotal role in controlling cell cycle progression, differentiation, and apoptosis[5] and may well regulate a portion of these functions. E2F proteins are divided into activators (E2F1, E2F2, and E2F3a) and repressors (E2F3b, E2F4, E2F5, E2F6-8), with each subfamily playing distinct roles in regulating gene expression during various cellular processes [6]. In the context of mammary gland development, the role of E2F transcription factors has been a subject of growing interest. Several studies have demonstrated

that members of the E2F family regulate mammary gland development[7-9] and breast cancer[10, 11]. However, the specific role of E2F5 in pregnancy remains poorly understood.

Emerging evidence suggests that E2F5 may play a role in the differentiation of mammary epithelial cells during pregnancy with initial evidence in bioinformatic predictions [8]. Traditionally classified as a repressor, E2F5 has been implicated in regulating development of several tissue types, including the nervous system[12, 13], gastrointestinal system[12], and male reproductive system[14, 15]. In the mammary gland, E2F5 expression has been detected during key stages of development and prior work has demonstrated a ductal delay during pubertal outgrowth [8], and its potential involvement in alveolar differentiation has yet to be fully characterized. Our study aims to elucidate the role of E2F5 in mammary alveolar formation during pregnancy, using a mammary conditional knockout mouse model to evaluate the effects of E2F5 deficiency during early pregnancy.

To investigate the role of E2F5 in mammary development, we utilized an MMTV-Cre driven mammary conditional knockout of E2F5 to study its effects during pregnancy. We used CUT&RUN to identify E2F5 genomic targets in a mouse mammary epithelial cell line. CUT&RUN is a method that allows precise mapping of protein-DNA interactions in a native chromatin context[16], providing insights into the direct regulatory targets of E2F5. In addition, we performed bulk mRNA sequencing from whole mammary glands, assessed proliferation by PCNA immunohistochemistry, and conducted reporter assays. By understanding the target genes and pathways regulated by E2F5, we aim to clarify the mechanisms through which E2F5 contributes to mammary gland development during pregnancy.

Results

Expression and activity of E2F5 in the mammary gland during pregnancy

We analyzed the expression of E2F5 using public RNA-seq data from mammary glands across stages of development, including virgin, pregnancy day 6, pregnancy day 18, lactation day 1, and lactation day 10 [17-19]. Expression of E2F5 from whole mammary glands remained relatively steady across these conditions (Figure 1A). Next, we analyzed public RNA-seq data where fluorescence-activated cell sorting (FACS) was used to isolate basal and luminal cells from dissociated mammary glands at different stages: virgin, pregnancy day 18.5, and lactation day 2 [19]. We found that E2F5 expression levels increased during pregnancy, particularly in luminal cells, compared to the virgin and lactating stages (Figure 1B), suggesting that the bulk RNAseq in Figure 1A had some of this signal masked by other cell types.

To identify E2F5 target genes that were relevant to mammary gland biology we performed CUT&RUN in the HC11 mouse mammary epithelial cell line. The E2F5 Cut and Run target genes were then explored in differentially expressed genes from virgin versus pregnancy day 6 mammary glands that were up- and down-regulated. The Venn diagram in Figure 1C shows that there are 437 up-regulated E2F5 targets and 246 down-regulated targets, suggesting that E2F5 might act as an important transcriptional activator in the context of mammary development during pregnancy. Many of the up-regulated E2F5 target genes are known to be associated with proliferation and differentiation during pregnancy (Figure 1D). Figure 1E shows a volcano plot of differentially expressed genes between virgin and pregnancy day 6, with the E2F5 targets labeled, providing an overview of how E2F5 targets are distributed among significantly up- and down-regulated genes during early pregnancy. Notably, many of the labeled E2F5 targets fall

into the significantly up-regulated category, suggesting the hypothesis that E2F5 acts as a transcriptional activator during mammary development in pregnancy.

Loss of E2f5 in the mammary gland impairs alveolar development during early pregnancy

To investigate the functional role of E2F5 in mammary alveolar development during pregnancy, we compared mammary gland morphology in E2F5 conditional knockout (CKO) mice and their littermate controls (E2F5 f/f) during early pregnancy (day 5). Early adult mammary wholemounts from the E2F5 CKO mice demonstrated a normal ductal architecture prior to the onset of pregnancy (Figure 2A,B Top). With the induction of pregnancy, we observed that E2F5 CKO mice had delayed mammary alveolar development during early pregnancy, as shown by the whole mounts and histology taken from pregnancy day 5 in E2F5 f/f compared to E2F5 CKO (Figures 2A, 2B). Specifically, alveolar formation was impaired in the E2F5 CKO mice, indicating a critical role for E2F5 in alveolar development during early pregnancy. This was seen in both the whole mounts were reduced alveolar budding was observed as well as through histology. We next examined proliferation within the mammary gland by performing immunohistochemistry (IHC) to stain for proliferating cell nuclear antigen (PCNA) in formalin-fixed paraffin-embedded (FFPE) mammary gland sections from pregnancy day 5. Comparing E2F5 f/f and E2F5 CKO mice, we observed fewer PCNA-positive cells in the CKO mammary tissue compared to E2F5 f/f (Figure 2C), suggesting impaired proliferation during early pregnancy in the absence of E2F5. This reduction in proliferation aligns with the impaired alveolar development observed in the whole mount and histology analyses.

To quantify alveolar formation, we measured the pixel density of the lateral side branches in whole mounts. We observed significantly less pixel density in CKO compared to f/f whole mounts at pregnancy day 5 ($p = 0.002$, Figure 2D), suggesting reduced alveolar proliferation in

the absence of E2F5. These findings indicate that E2F5 is required for proper alveolar development during early pregnancy.

E2F5 is indispensable for mammary development in later stages of pregnancy

Given prior work demonstrating differences between first and second pregnancies [20], we sought to test for this response in the E2F5 CKO mice. At pregnancy day 5 in the second pregnancy, we observed slightly fewer alveoli in E2F5 CKO glands compared to controls from whole mounts (Figure 3A) and H&E staining (Figure 3B). However, this difference was not as striking as the initial pregnancy. While we previously reported that there were no differences in E2F5 knockout mice for lactation [8], we then sought to test the latter stages of pregnancy for alterations that persisted. At pregnancy day 14, we observed no significant difference between the two genotypes, suggesting that the effect of E2F5 loss on alveolar development may be compensated for by other E2F transcription factors.

E2F5 binds the promotors of genes involved in cellular proliferation and differentiation

To precisely define the mechanisms associated with E2F5 and alveolar proliferation and differentiation, we performed CUT&RUN in HC11 mouse mammary epithelial cells to identify chromatin occupancy of E2F5, H3K4me3, a histone modification associated with actively transcribed gene promoters[21-23], and H3K27me3, a modification associated with down-regulation of nearby genes[24, 25]. We performed CUT&RUN using two replicates per target. Principal component analysis (PCA) analysis suggests that the reads obtained from replicates were highly similar (Figure 4A). We then performed peak calling to obtain consensus peaks for each target using the SEACR software package[26] and did motif discovery and similarity analysis using MEME-ChIP[27]. Following de-novo motif discovery, we found that the E2F5 motif is distinct from the known E2F consensus motif of TTTCCCGC[28, 29] (Figure 4B).

However, motif similarity analysis using Tomtom from the MEME-suite identified overlap between the discovered E2F5 motif and the E2F consensus motif within called peaks (Figure 4C). Using IGV to visualize E2F5 and H3K4me3 occupancy, we found that E2F5 was bound to the promoter of many known E2F target genes involved in cellular proliferation (Figure 4D).

Progesterone signaling and E2F5 cooperate to regulate genes involved in mammary development during early pregnancy

Using the promoter binding data for E2F5, we tested whether E2F5 regulates known regulators of mammary development in early pregnancy. We found that E2F5 binds the promoter of STAT6 (Figure 5A). STAT6 is known to positively regulate cellular proliferation and survival in the mammary gland during early pregnancy[30]. To test whether E2F5 was an activator or repressor of Stat6 we performed a luciferase reporter assay in 293T cells. Our results suggest that E2F5 acts to repress STAT6 transcription with a dramatic reduction of the signal associated with the Stat6 promoter-Luciferase construction alone compared to with the overexpression of E2F5 (Figure 5B). Given the hormonal control of pregnancy, we then asked if progesterone signaling reverses E2F5 repression of STAT6, leading to transactivation. To test this, we performed a STAT6 reporter assay in MCF7 cells, which express the progesterone receptor, with and without E2F5 overexpression and treated with either vehicle (ethanol) or a progesterone receptor agonist (R5020). This suggests that progesterone receptor signaling counteracts repression of STAT6 by E2F5, leading to transactivation of the STAT6 promoter in the presence of both activated progesterone receptor and E2F5 (Figure 5C).

Methods

CUT&RUN and Sequencing Library Preparation

CUT&RUN was performed using the CUTANA ChIC/CUT&RUN kit (Epicypher, version 3), following the user manual version 3.1. Briefly, cells were bound to canavalin-A magnetic beads, permeabilized with 0.01% digitonin, and 5 x 10^5 HC11 cells per target (n=2 per target) were incubated at 4C on a nutator with 0.5 ug of antibody overnight in 50 ul of antibody buffer. Cells were washed and pAG-Mnase was added to the cells in 50 ul of permeabilization buffer and incubated for 10 min at room temperature. Cells were washed again and CaCl2 was added to activate Mnase and incubated at 4C on a nutator for 2 hours. The reaction was stopped with EGTA STOP buffer. E. coli DNA was included in the STOP buffer as a spike-in control. Following purification, DNA was quantified using the Qubit dsDNA HS kit. CUT&RUN sequencing libraries were prepared using the CUTANA CUT&RUN Library Prep kit (Epicypher, version 1), following the user manual version 1.4. Briefly, 5 ng of CUT&RUN DNA per target replicate were added at equal volumes for end repair for 20 minutes at 20C. Following adapter ligation for 15 min at 20C and U-excision for 15 min at 37C, DNA was purified using SPRIselect magnetic beads (Beckman Coulter). Next, library amplification was performed for 14 cycles of indexing PCR, followed by PCR cleanup using 1X volume of SPRIselect beads. Sequencing libraries were analyzed by TapeStation (Agilent) and sequenced on the NovaSeq 6000 (Illumina) at 2x50 bp for 8-10 million reads per library.

CUT&RUN Analysis

Raw FASTQ files were processed using the nf-core CUT&RUN pipeline (nf-core/cutandrun v3.2.2). Adapter and quality trimming was performed using Trim Galor v0.6.6. Post-trim quality

control was performed using FastQC v0.12.1. Trimmed FASTQ reads and spike-in reads were aligned to mm10 and K12-MG1655, respectively, using Bowtie2 v2.4.4. Peaks were called using SEACR v1.3 and consensus peaks were merged using bedtools v2.31.0. Genomic tracks were visualized using IGV desktop v2.16.1**.**

Whole Mounts and Histology

Wholemounts were stained using carmine alum using standard methods [31] and samples for H&E were fixed in 10% formalin and processed / stained using standard methods using the MSU Physiology Histopathology core facility.

Cell Culture

HC11 cells (ATCC #CRL-3062) were obtained from the ATCC and cultured RPMI 1640 medium ATCC modification (Gibco #A1049101) with 10% fetal bovine serum (FBS) (Gibco #A5256701) and 1% Antibiotic-Antimycotic (anti-anti) (Gibco #15240062). MCF7 (ATCC #HTB-22) and 293T (ATCC #CRL-3216) cells were obtained from the ATCC and cultured in DMEM medium (Gibco #11995065) with 10% FBS and 1% anti-anti.

Antibodies

For IHC, rabbit monoclonal anti-PCNA (Clone D3H8P; Cell Signaling #13110-15; RRID:AB_2636979) was used at a 1:4000 dilution. For CUT&RUN, rabbit IgG (Epicypher #13-0042; RRID:AB_2923178), rabbit monoclonal anti-H3K27me3 (Invitrogen #MA5-11198; RRID:AB_1100074), rabbit monoclonal anti-H3K4me3 (Epicypher #13-0041;

RRID:AB_3076423), and rabbit polyclonal anti-E2F5 (Invitrogen #PA585578; RRID:AB_2792718) were used at 0.5 ug per sample replicate in 50 uL of volume.

Animal Experiments

All animal husbandry and use was in compliance with local, national and institutional guidelines. Ethical approval for the study was approved by Michigan State University Animal Care & Use Committee (IACUC) under AUF 06/18-084-00. The MMTV-Cre [32] and E2F5 conditional knockout mice [8] were previously described.

RNA Sequencing Analysis

Raw sequencing reads and metadata were obtained from the Gene Expression Omnibus (GEO) using the nf-core/fetchngs pipeline. Raw FASTQ files were processed using the nf-core/rnaseq pipeline. Adapter and quality trimming was performed using Trim Galore. Trimmed FASTQ files were aligned to the mouse GRCm39 reference genome using STAR. RNA sequencing transcripts were quantified using Salmon. Quantified transcripts were analyzed using the nf-core/differentialabundance pipeline. Differential expression analysis was performed using DESeq2 and EnhancedVolcano from Bioconductor in R. Integration of CUT&RUN and RNA sequencing analysis was performed using ChIPpeakAnno and ggVennDiagram. RNA sequencing normalized expression plots were made using ggplot2.

Reporter Assays

All reporter assays were performed in 96-well plates with 8 to 10 replicates per condition. No replicates were removed from the analysis. The STAT6-promoter luciferase reporter was made by cloning ~1 kb of DNA upstream of the coding region into the empty LightSwitch reporter vector (SwitchGear Genomics #32021). For the 293T reporter assay, HA-E2F5 (Addgene

#24213) or vector control (Addgene #16440) was co-transfected with the empty luciferase reporter vector or the STAT6-promoter luciferase reporter using Lipofectamine3000 (Invitrogen #L3000008) following the manufacturers protocol. After an overnight incubation, the transfection medium was aspirated, and fresh medium was added to the cells. After 48 hours post-transfection, the luciferase reporter assay was performed using the LightSwitch Luciferase Assay Kit (SwitchGear Genomics #32031) following the manufacturer's protocol and the samples were imaged using the Tecan Spark for 2 seconds per replicate. For the MCF7 reporter assay, HA-E2F5 or vector control was co-transfected with the STAT6-promoter luciferase reporter using Lipofectamine3000 (Invitrogen #L3000008). 24-hours later, the cells were treated with either vehicle (ethanol) or 20 nM R5020. After 24-hours, the luciferase reporter assay was performed using the LightSwitch Luciferase Assay Kit (SwitchGear Genomics #32031) following the manufacturer's protocol and the samples were imaged using the Tecan Spark for 2 seconds per replicate.

Statistics

For alveoli quantification using pixel density, we performed a two-tailed T-Test. For differential expression analysis, DESeq2 calculates p-values using the Wald test and BH-adjusted p-value (padj) is calculated using the Benjamini Hochberg method. We analyzed reporter assays using a one-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) post-hoc test for pairwise comparisons.

Data Availability

Public datasets were obtained from the Gene Expression Omnibus (GEO) database under the following accession codes: GSE60450, GSE115369, GSE127140, GSE231440.

Discussion

Here we demonstrate that E2F5 plays a significant role in mammary gland development during pregnancy, particularly during early pregnancy when epithelial proliferation is critical. The results from the conditional knockout mouse model suggest that the absence of E2F5 delays mammary alveolar development, resulting in fewer alveoli during early pregnancy. This phenotype was apparent through both whole mount analysis and histological examination. Furthermore, the observed decrease in PCNA-positive cells indicates impaired proliferation in the absence of E2F5, aligning with the reduced alveolar development phenotype.

Interestingly, the effect of E2F5 loss on alveolar development was less pronounced during later stages of pregnancy. At pregnancy day 14 we observed no substantial differences between E2F5 CKO and control mice, suggesting that other compensatory mechanisms, such as redundancy among E2F family members, might mitigate the loss of E2F5 at later stages of pregnancy. The compensation by other E2F family members has previously been observed in the mammary gland [9] as well as numerous other experimental settings [33, 34]. These findings indicate that E2F5 is particularly important for the initiation of alveolar proliferation and differentiation during early pregnancy but may be dispensable or compensated for by other factors during subsequent stages of mammary development.

Prior work has also established the epigenetic regulation of pregnancy, with effects being more robust in a second pregnancy [20]. Given the epigenetic memory and the compensatory mechanisms of the other E2F family members in mammary gland biology, one might hypothesize that instead of observing effects on alveolar expansion in a second pregnancy, the compensatory mechanisms may already be primed. As observed in the second pregnancy with the lack of E2F5, there were less significant phenotypic effects, consistent with this hypothesis.

The promoter binding of the CUT&RUN analysis revealed that E2F5 bound to the promoters of genes associated with cellular proliferation and differentiation, as evidenced by the co-localization of E2F5 with H3K4me3, an active transcription mark. Many of the identified E2F5 targets were significantly upregulated during early pregnancy, supporting the hypothesis that E2F5 acts as a transcriptional activator in this context. The overlap between the discovered E2F5 motif and the known E2F consensus motif further suggests that E2F5 functions similarly to other E2F transcription factors in promoting the expression of genes involved in cell cycle progression.

We also identified a functional relationship between E2F5 and progesterone signaling in regulating mammary gland development. The data suggest that E2F5 directly represses STAT6 transcription, a known regulator of proliferation and cell survival in the mammary gland. However, when progesterone signaling is activated, this repression is counteracted, leading to transactivation of the STAT6 promoter. Our proposed model, illustrated in Figure 6, highlights two possible mechanisms for this reversal of repression: either E2F5 is displaced from the STAT6 promoter by competitive binding or E2F5 cooperates with activated progesterone receptor or a downstream co-factor to activate STAT6 transcription. This finding highlights a cooperative mechanism between E2F5 and progesterone signaling, suggesting that E2F5-mediated repression may be context-dependent and influenced by hormonal signaling during pregnancy. The interaction between E2F5 and progesterone signaling may provide a means of fine-tuning gene expression to ensure proper timing of alveolar proliferation and differentiation during early pregnancy.

Overall, this study provides new insights into the transcriptional control of mammary gland development and proliferation during pregnancy. The findings indicate that E2F5 is important for early alveolar development and suggest a cooperative role between E2F5 and progesterone

signaling in regulating gene expression during this process. Future studies should explore the potential compensatory roles of other E2F family members during later stages of mammary development and investigate how E2F5 activity is modulated by hormonal cues to ensure proper mammary gland function. Understanding these regulatory mechanisms could provide valuable insights into breast cancer, where dysregulation of E2F transcription factors and hormonal signaling pathways are known to contribute to tumor development and progression.

References

1. Hennighausen, L. and G.W. Robinson, *Information networks in the mammary gland.* Nat Rev Mol Cell Biol, 2005. **6**(9): p. 715-25.
2. Philp, J.A., T.G. Burdon, and C.J. Watson, *Differential activation of STATs 3 and 5 during mammary gland development.* FEBS Lett, 1996. **396**(1): p. 77-80.
3. Santos, S.J., S.Z. Haslam, and S.E. Conrad, *Signal transducer and activator of transcription 5a mediates mammary ductal branching and proliferation in the nulliparous mouse.* Endocrinology, 2010. **151**(6): p. 2876-85.
4. Chapman, R.S., et al., *Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3.* Genes Dev, 1999. **13**(19): p. 2604-16.
5. DeGregori, J. and D.G. Johnson, *Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis.* Curr Mol Med, 2006. **6**(7): p. 739-48.
6. Trimarchi, J.M. and J.A. Lees, *Sibling rivalry in the E2F family.* Nat Rev Mol Cell Biol, 2002. **3**(1): p. 11-20.
7. Andrechek, E.R., et al., *Patterns of cell signaling pathway activation that characterize mammary development.* Development, 2008. **135**(14): p. 2403-13.
8. To, B., et al., *Insight into mammary gland development and tumor progression in an E2F5 conditional knockout mouse model.* Oncogene, 2024. **43**(46): p. 3402-3415.
9. To, B. and E.R. Andrechek, *Transcription factor compensation during mammary gland development in E2F knockout mice.* PLoS One, 2018. **13**(4): p. e0194937.
10. Yoon, S.O., S. Shin, and A.M. Mercurio, *Ras stimulation of E2F activity and a consequent E2F regulation of integrin alpha6beta4 promote the invasion of breast carcinoma cells.* Cancer Res, 2006. **66**(12): p. 6288-95.
11. Macaluso, M., et al., *pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 multimolecular complexes mediate the transcription of estrogen receptor-alpha in breast cancer.* Oncogene, 2003. **22**(23): p. 3511-7.
12. Dagnino, L., et al., *Expression patterns of the E2F family of transcription factors during mouse nervous system development.* Mech Dev, 1997. **66**(1-2): p. 13-25.
13. Lindeman, G.J., et al., *A specific, nonproliferative role for E2F-5 in choroid plexus function revealed by gene targeting.* Genes Dev, 1998. **12**(8): p. 1092-8.
14. Danielian, P.S., R.A. Hess, and J.A. Lees, *E2f4 and E2f5 are essential for the development of the male reproductive system.* Cell Cycle, 2016. **15**(2): p. 250-60.
15. Xie, H., et al., *E2f5 is a versatile transcriptional activator required for spermatogenesis and multiciliated cell differentiation in zebrafish.* PLoS Genet, 2020. **16**(3): p. e1008655.
16. Skene, P.J. and S. Henikoff, *An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites.* Elife, 2017. **6**.
17. Lee, H.K., et al., *Progressing super-enhancer landscape during mammary differentiation controls tissue-specific gene regulation.* Nucleic Acids Res, 2018. **46**(20): p. 10796-10809.

18. Lee, H.K., et al., *Redundant and non-redundant cytokine-activated enhancers control Csn1s2b expression in the lactating mouse mammary gland.* Nat Commun, 2021. **12**(1): p. 2239.
19. Lee, H.K., et al., *Cell-specific and shared regulatory elements control a multigene locus active in mammary and salivary glands.* Nat Commun, 2023. **14**(1): p. 4992.
20. Dos Santos, C.O., et al., *An epigenetic memory of pregnancy in the mouse mammary gland.* Cell Rep, 2015. **11**(7): p. 1102-9.
21. Santos-Rosa, H., et al., *Active genes are tri-methylated at K4 of histone H3.* Nature, 2002. **419**(6905): p. 407-11.
22. Guenther, M.G., et al., *A chromatin landmark and transcription initiation at most promoters in human cells.* Cell, 2007. **130**(1): p. 77-88.
23. Barski, A., et al., *High-resolution profiling of histone methylations in the human genome.* Cell, 2007. **129**(4): p. 823-37.
24. Cao, R., et al., *Role of histone H3 lysine 27 methylation in Polycomb-group silencing.* Science, 2002. **298**(5595): p. 1039-43.
25. Pasini, D., et al., *Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity.* EMBO J, 2004. **23**(20): p. 4061-71.
26. Meers, M.P., D. Tenenbaum, and S. Henikoff, *Peak calling by Sparse Enrichment Analysis for CUT&RUN chromatin profiling.* Epigenetics Chromatin, 2019. **12**(1): p. 42.
27. Machanick, P. and T.L. Bailey, *MEME-ChIP: motif analysis of large DNA datasets.* Bioinformatics, 2011. **27**(12): p. 1696-7.
28. Kheradpour, P. and M. Kellis, *Systematic discovery and characterization of regulatory motifs in ENCODE TF binding experiments.* Nucleic Acids Res, 2014. **42**(5): p. 2976-87.
29. Morgunova, E., et al., *Structural insights into the DNA-binding specificity of E2F family transcription factors.* Nat Commun, 2015. **6**: p. 10050.
30. Khaled, W.T., et al., *The IL-4/IL-13/Stat6 signalling pathway promotes luminal mammary epithelial cell development.* Development, 2007. **134**(15): p. 2739-50.
31. Tolg, C., M. Cowman, and E.A. Turley, *Mouse Mammary Gland Whole Mount Preparation and Analysis.* Bio Protoc, 2018. **8**(13): p. e2915.
32. Andrechek, E.R., et al., *Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3444-9.
33. Andrechek, E.R., *HER2/Neu tumorigenesis and metastasis is regulated by E2F activator transcription factors.* Oncogene, 2015. **34**(2): p. 217-25.
34. Kong, L.J., et al., *Compensation and specificity of function within the E2F family.* Oncogene, 2007. **26**(3): p. 321-7.

Figure Legends

Figure 1. Expression and activity of E2f5 in the mammary gland during pregnancy.

(A) Normalized expression of E2F5 in whole mammary glands at different stages of development: virgin, pregnancy day 6 (P6), pregnancy day 18 (P18), lactation day 1 (L1), and lactation day 10 (L10). (B) Normalized expression of E2F5 in basal and luminal cells isolated from mammary glands at different stages: virgin, pregnancy day 18.5 (Preg day 18.5), and lactation day 2 (Lac day 2). (C) Venn diagram showing overlap between E2f5 CUT&RUN target genes and differentially expressed genes in early pregnancy (virgin vs. P6). (D) Normalized expression of several up-regulated E2f5 target genes known to be associated with proliferation and differentiation during pregnancy. (E) Volcano plot of differentially expressed genes between virgin and P6, with E2f5 targets labeled.

Figure 2. Alveolar development is delayed during early pregnancy in E2F5 conditional knockout mice.

Paired mammary wholemounts (A) and H&E staining (B) from 12 weeks virgin adult E2F5 CKO, day 5 pregnant E2F5 f/f and day 5 pregnant E2F5 CKO mice shown an impairment on alveoli formation during pregnancy on mice lacking E2F5 in mammary epithelium. (C) PCNA expression in pregnant mice shows reduced proliferative activity in E2F5 CKO mice. (D) Alveoli quantification shown reduced proliferative activity in mice lacking E2F5 in mammary epithelium ($p=0.0025$).

Figure 3. Alveolar impair phenotype is conserved in early pregnancy during subsequent gestation cycles.

(A) Early pregnancy in E2F5 CKO females undergoing a second gestation shows reduced number of alveoli in comparison with E2F5 f/f group. Upon day 14 of pregnancy, E2F5 CKO females shows non phenotypic differences in comparison with control groups. (B) Matched histological sections shown evidence of early but no late pregnancy defects in E2F5 CKO females.

Figure 4. Identification of E2F5 target genes in HC11 mouse mammary epithelial cells.

(A) Principal component analysis (PCA) plot showing clustering of CUT&RUN data based on chromatin occupancy for E2F5, H3K4me3, H3K27me3, and IgG across two replicates each. (B) De novo motif discovery using MEME-ChIP revealed a motif that is distinct from the known E2F consensus motif. (C) Motif similarity analysis using Tomtom identified overlap between the discovered E2F5 motif and the known E2F consensus motif. (D) Integrative Genomics Viewer (IGV) tracks showing E2F5 and H3K4me3 occupancy at the promoters of known cell cycle regulatory genes, including CDKN2A, CDKN2B, CDKN2C, CDK4, and CCND1, supporting the role of E2F5 in regulating genes involved in cellular proliferation.

Figure 5. E2F5 occupancy at the STAT6 promoter and its impact on transcriptional regulation.

(A) Integrative Genomics Viewer (IGV) tracks showing E2F5, H3K4me3, and IgG CUT&RUN signal at the STAT6 promoter in HC11 mouse mammary epithelial cells. (B) Luciferase reporter assay in 293T cells showing that E2F5 represses STAT6 promoter activity. (C) STAT6 promoter-luciferase reporter assay of MCF7 cells treated with vehicle or R5020 and co-transfected with CMV-HA-E2F5 or CMV-Neo-Bam (empty vector).

Figure 6. Proposed model of E2F5 and progesterone receptor (PR) interaction at the STAT6 promoter.

In the absence of progesterone signaling, E2F5 binds to the STAT6 promoter and represses its transcription (left panel). Upon activation of PR, E2F5-mediated repression is relieved, leading to transcriptional activation of STAT6. This could occur either by displacement of E2F5 from the STAT6 promoter or through cooperation between E2F5 and activated PR at the STAT6 promoter (right panel).

Figure One

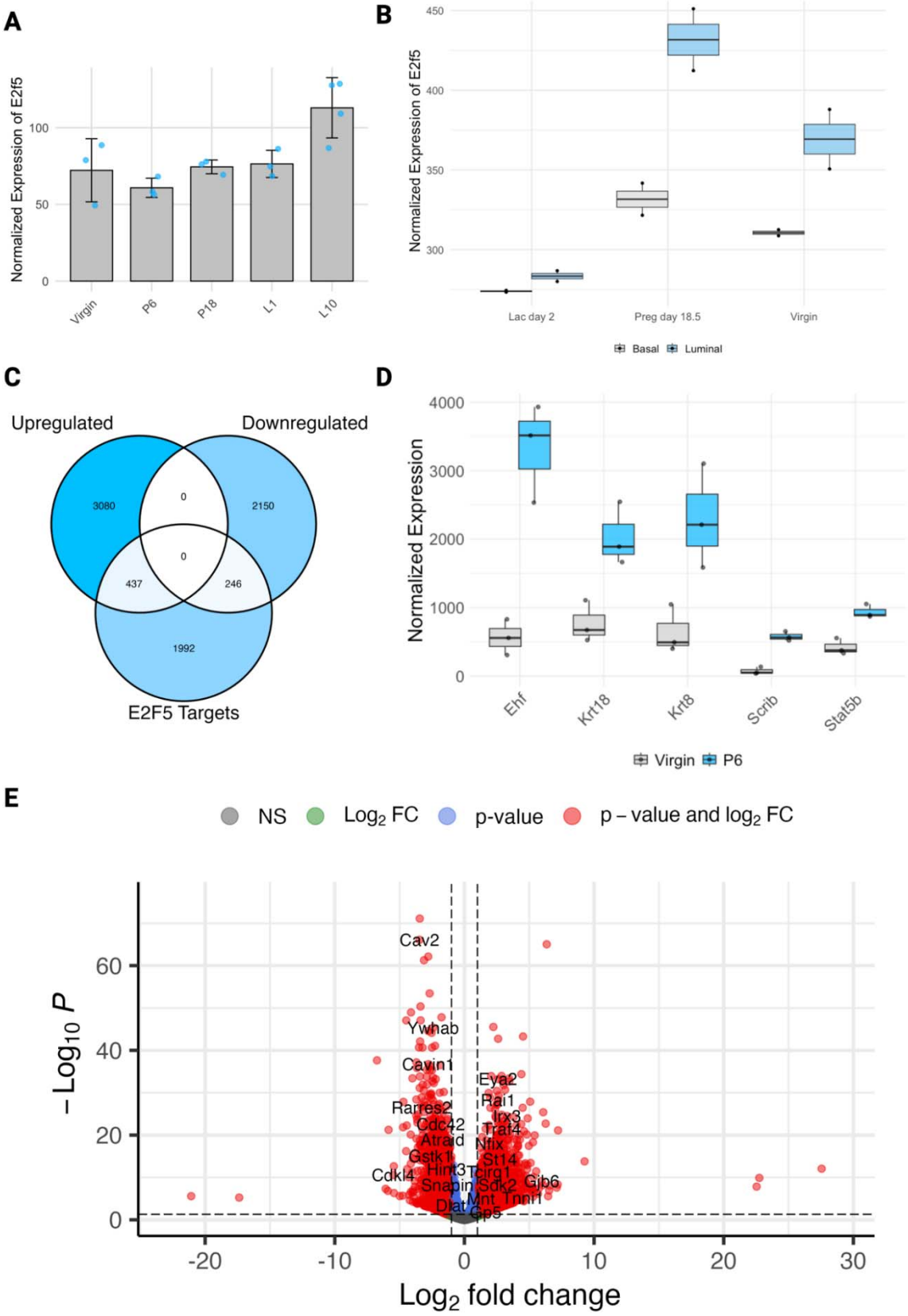
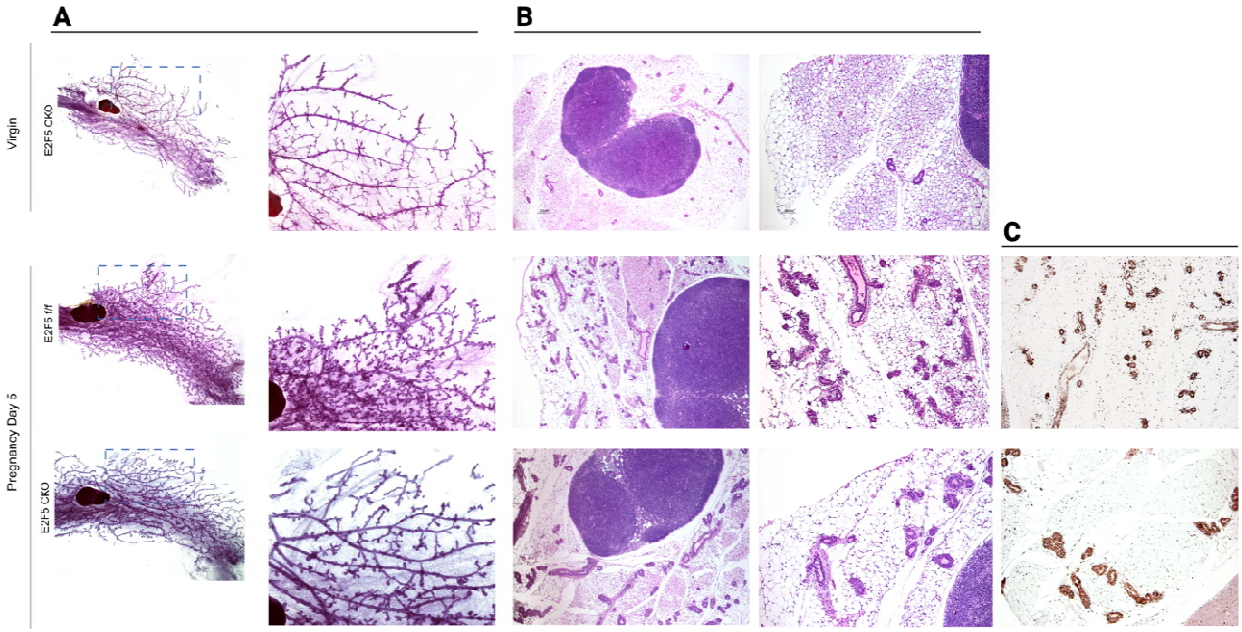

A
Normalized Expression of E2f5
100
50
0
Virgin
P6
P18
L1
L10
B
Normalized Expression of E2f5
450
400
350
300
Lac day 2
Preg day 18.5
Virgin
Basal
Luminal
C
Upregulated
Downregulated
3080
0
2150
0
437
246
1992
E2F5 Targets
D
Normalized Expression
4000
3000
2000
1000
0
Ehf
Krt18
Krt8
Scrib
Stat5b
Virgin
P6
E
NS
Log2 FC
p-value
p – value and log2 FC
– Log10 P
60
40
20
0
Cav2
Ywhab
Cavin1
Rarres2
Cdc42
Atraid
Gstk1
Cdkl4
Hint3
Snapin
Dlat
Eya2
Rai1
Irx3
Traf4
Nfix
St14
Tcirg1
Sdk2
Gjb6
Mnt
Tnnt1
Gp5
-20
-10
0
10
20
30
Log2 fold change

Figure Two


A
B
C
Virgin
E2F5 CKO
Pregnancy Day 5
E2F5 f/f
E2F5 CKO

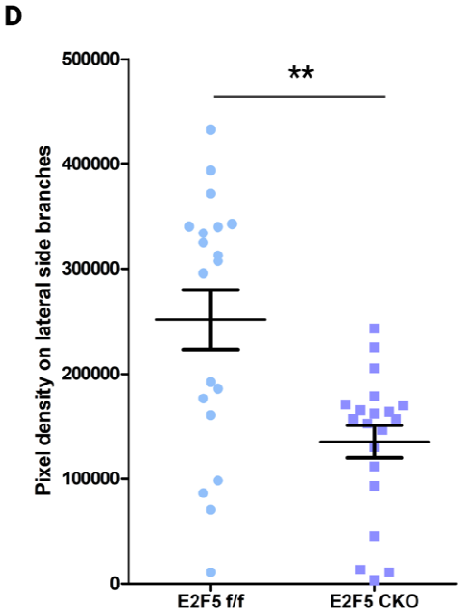

D
**
Pixel density on lateral side branches
500000
400000
300000
200000
100000
0
E2F5 f/f
E2F5 CKO

Figure Three

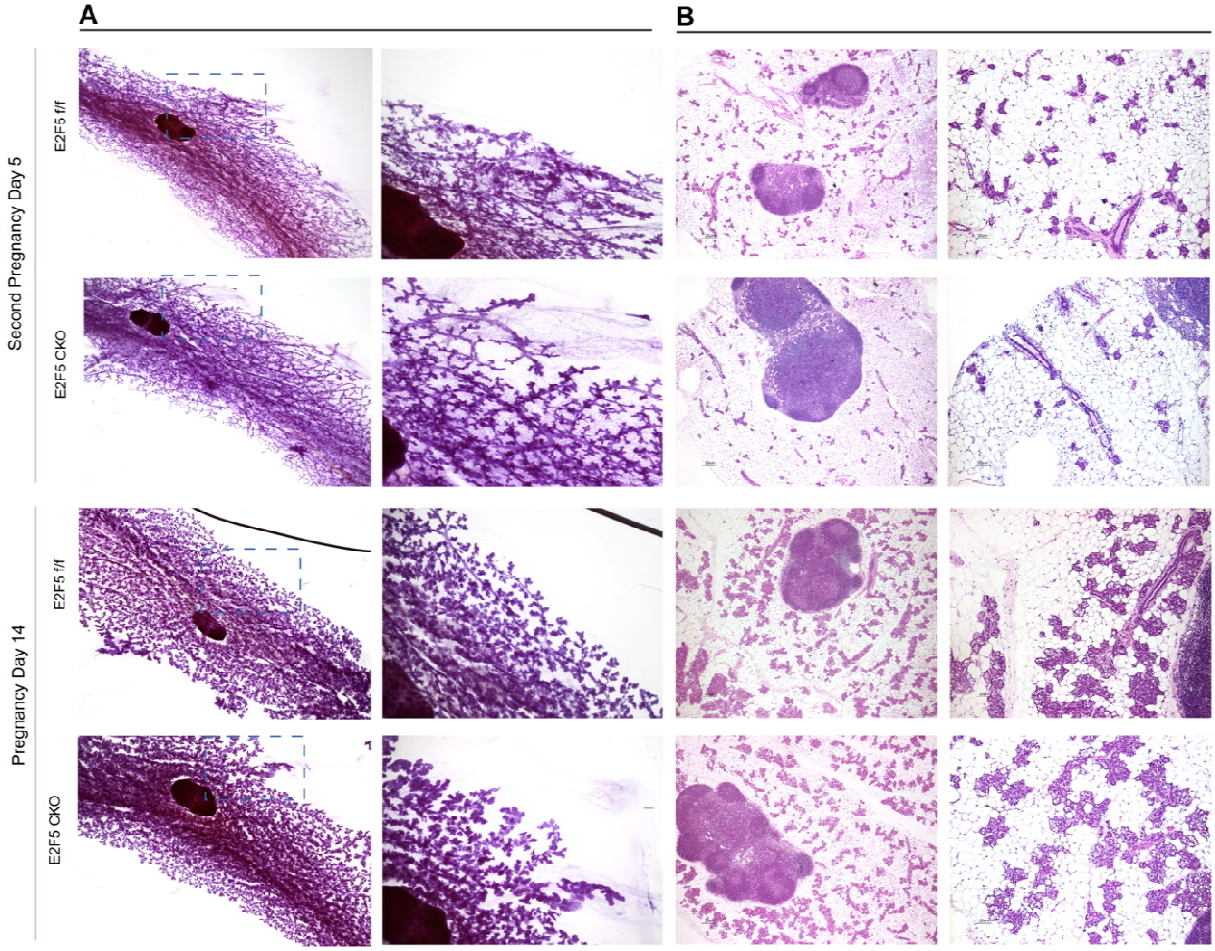


Figure Four

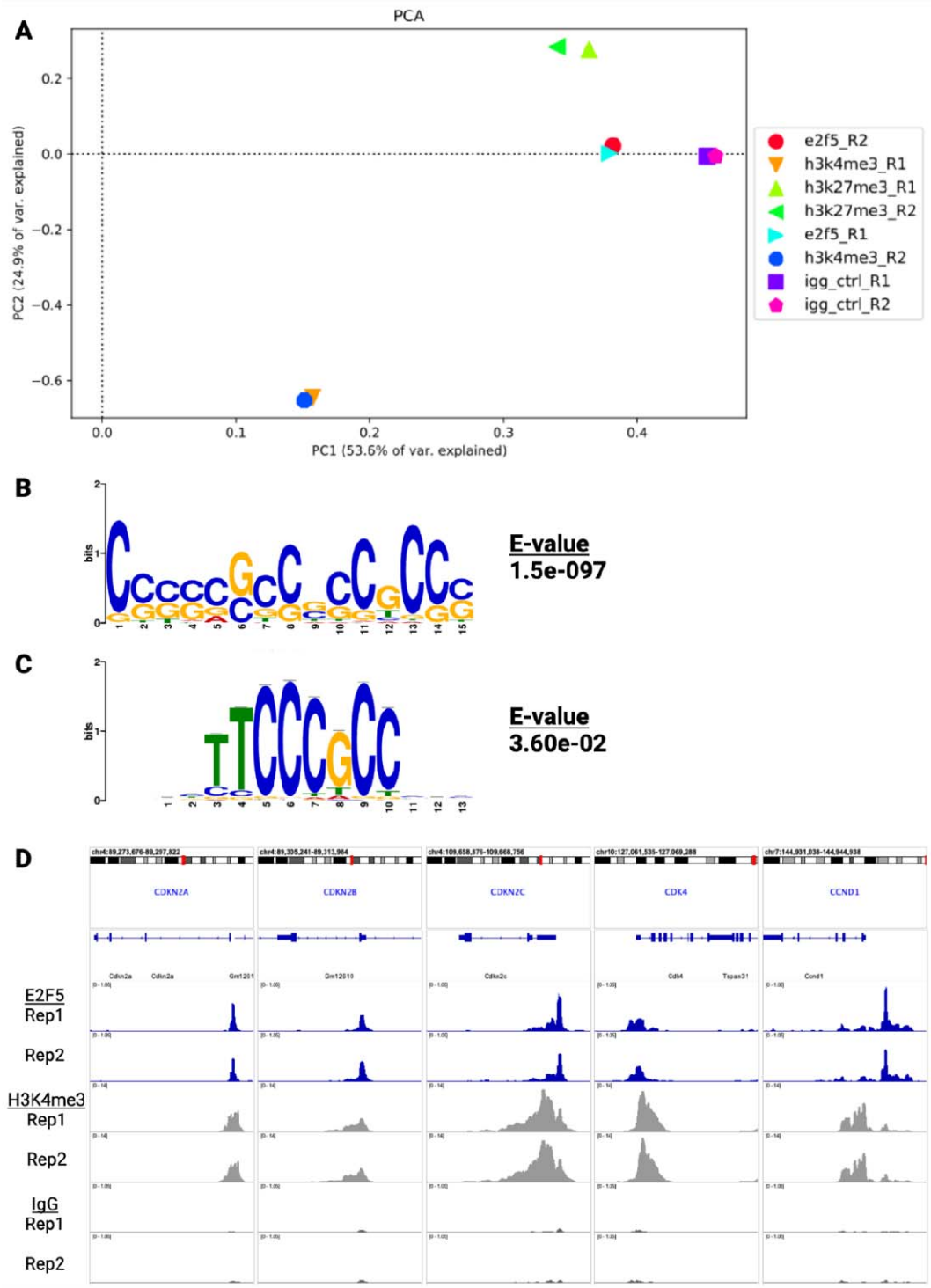
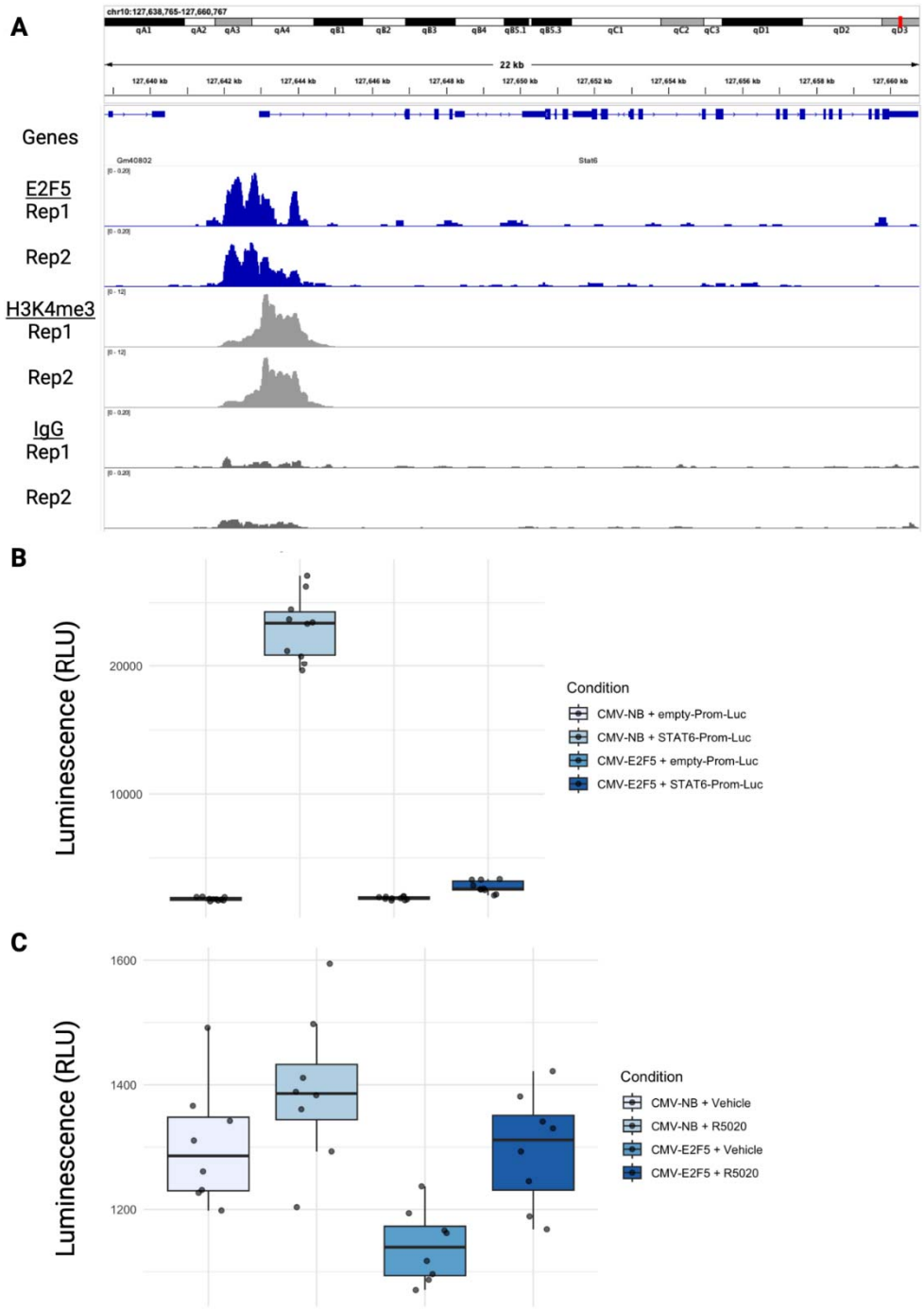

A
PCA
PC2 (24.9% of var. explained)
PC1 (53.6% of var. explained)
e2f5_R2
h3k4me3_R1
h3k27me3_R1
h3k27me3_R2
e2f5_R1
h3k4me3_R2
igg_ctrl_R1
igg_ctrl_R2
B
E-value
1.5e-097
C
E-value
3.60e-02
D
CDKN2A
CDKN2B
CDKN2C
CDK4
CCND1
E2F5
Rep1
Rep2
H3K4me3
Rep1
Rep2
IgG
Rep1
Rep2

Figure Five


A
chr10:127,638,765-127,660,767
22 kb
Genes
Gm40802
Stat6
E2F5
Rep1
Rep2
H3K4me3
Rep1
Rep2
IgG
Rep1
Rep2
B
Luminescence (RLU)
20000
10000
Condition
CMV-NB + empty-Prom-Luc
CMV-NB + STAT6-Prom-Luc
CMV-E2F5 + empty-Prom-Luc
CMV-E2F5 + STAT6-Prom-Luc
C
Luminescence (RLU)
1600
1400
1200
Condition
CMV-NB + Vehicle
CMV-NB + R5020
CMV-E2F5 + Vehicle
CMV-E2F5 + R5020

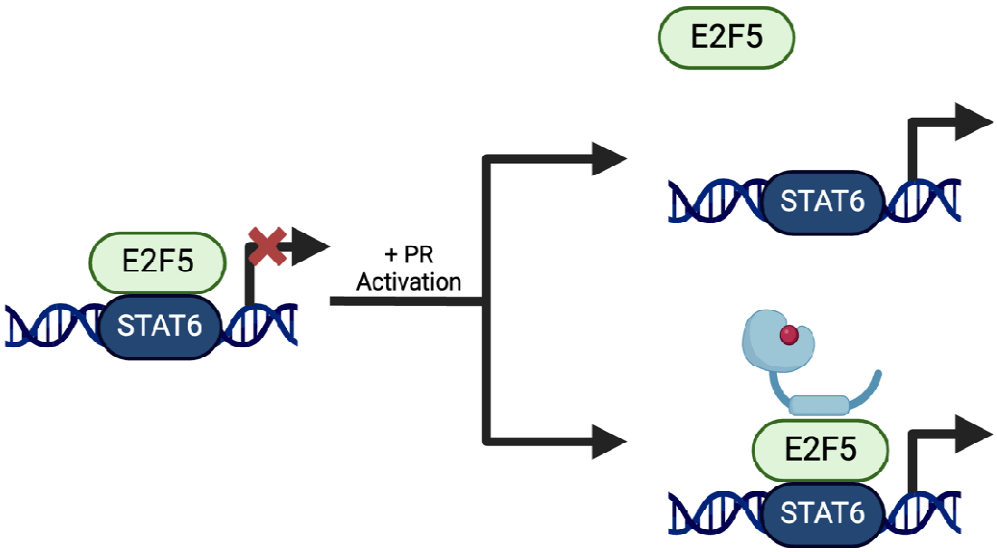



Figure Six