Navigating progestrone-driven gene regulatory networks in the mammary gland

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Chapter 1

General introduction

3D genome organization, epigenetic regulation, and spatiotemporal control of gene expression

The genome encodes the full complement of information necessary for the development, differentiation, and homeostasis of an organism. However, gene expression is not uniform or constitutive; rather, it is tightly regulated in a spatiotemporal manner by a complex network of transcriptional and epigenetic regulatory mechanisms. This regulation ensures that genes are expressed at the right time, at the right place. While only approximately 1% of the human genome encodes proteins, the remaining 99% comprises a vast array of non-coding elements that play essential roles in gene regulation. These include enhancers, silencers, insulators, enhancer RNAs (eRNAs), microRNAs and long non-coding RNAs (lncRNAs). Far from being "junk DNA", these regulatory elements are integral to the precise control of transcriptional programs that underlie cellular identity and function[1,2].

Enhancers play a pivotal role in the precise spatiotemporal regulation of gene expression. They are defined as short, non-coding DNA sequences bound by sequence-specific transcription factors (TFs) and co-factors[3,4]. In response to intrinsic cues or external stimuli, they converge on their target gene promotor, bringing transcription factors essential for transcription initiation or silencing[3,4]. Enhancers may reside in intergenic regions, introns, or even within coding exons. A typical gene is regulated by multiple enhancers, which can be located at variable distances, ranging from a few base pairs to over a megabase away, relative to their target promoter[5]. The ability of enhancers to regulate target gene expression often depends on long-range chromatin interactions, bringing enhancers into physical proximity with promoters despite large genomic distances[6]. However, identifying functionally relevant enhancers and accurately linking them to their target genes remains a major challenge. While epigenomic signatures such as chromatin accessibility (as determined by ATAC-seq), H3K4me1 (a mark of poised enhancers), and H3K27ac (a mark of active enhancers) are commonly used to predict enhancer regions, these features alone do not confirm enhancer function or target specificity[7,8]. Moreover, candidate enhancers are frequently assigned to the nearest gene, but this approach is error-prone; studies estimate that 33–73% of enhancers bypass the closest gene, instead regulating more distal genes[9–11]. As such, accurate enhancer–promoter mapping requires high-resolution chromatin conformation capture techniques, such as 4C, Hi-C, 5C, ChIA-PET, and HiChIP, which can identify physical interactions between regulatory elements and promoters at a region of interest or at

genome-wide scale[12]. Nevertheless, the detection of a chromatin contact does not, in and of itself demonstrate functional relevance[4]. Additional experimental validation**,** such as CRISPR-based enhancer perturbation or reporter assays, is essential to confidently link enhancers to their target gene and demonstrate enhancer activity.

The three-dimensional (3D) organization of the genome imposes an additional layer of regulatory control over gene expression. Regulatory interactions between enhancers and promoters are often constrained within topologically associated domains (TADs), megabase-scale genomic regions characterized by a high frequency of internal chromatin contacts[13–15]. These domains are typically demarcated by boundaries enriched for the architectural proteins CCCTC-binding factor (CTCF) and the Cohesin complex, which serve to insulate regulatory units and prevent aberrant enhancer–promoter interactions across neighboring domains[13,14,16] (**Fig1A**). This spatial compartmentalization restricts the regulatory search space of enhancers, thereby ensuring specificity and fidelity in transcriptional programs[17]. Within TADs, smaller chromatin loops bring enhancers into proximity with their target promoters, forming a dynamic and flexible regulatory architecture[9,18] (**Fig1B**). Long-distance chromatin loops spanning over 100 kilobases are often also mediated by CTCF and Cohesin, to help stabilize these interactions[19].

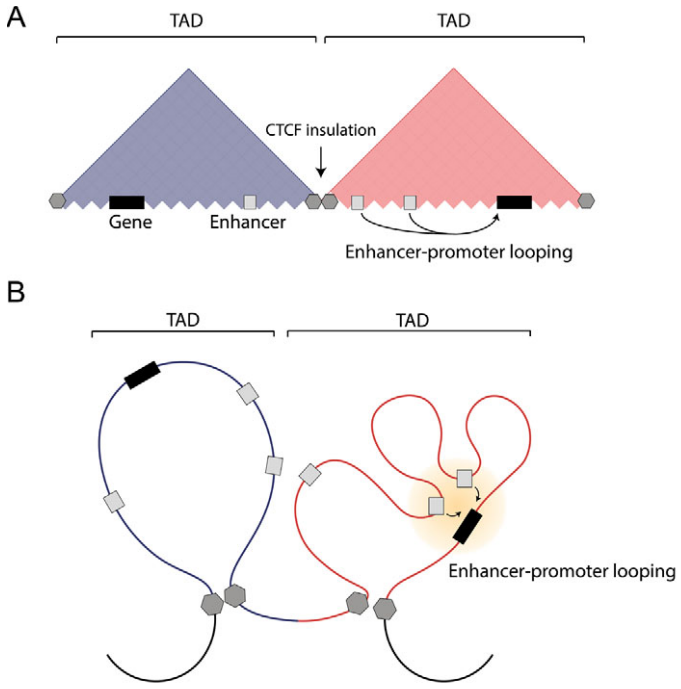

Fig1 (left). Schematic presentation of the 3D genome architecture
A) Schematic representation of a Hi-C contact map showing topologically associated domain (TAD) organization. Blue and red colors represent different TADs that are insulated by CTCF boundaries. Enhancer promotor looping generally occurs within a TAD. B) Illustration of how DNA folding within a TAD facilitates enhancer promotor contact in the region presented in (A). Adapted from Robson *et al.*[3].

Progesterone and the progesterone receptor

Progesterone (P4) is a key ovarian steroid hormone essential for female physiology[20]. P4 orchestrates a broad spectrum of reproductive functions and exerts its effects on diverse target tissues, including the ovaries, uterus, mammary gland, and brain[20–25]. P4 mediates its biological effects through the progesterone receptor (PR), a member of the nuclear steroid hormone receptor family, which also includes the estrogen receptor (ER), glucocorticoid receptor (GR), androgen receptor (AR), and mineralocorticoid receptor (MR)[26]. These receptors constitute a unique class of ligand-activated transcription factors that integrate extracellular hormonal signals with transcriptional regulation of gene expression[27]. Two major PR isoforms exist that are transcribed from the same gene, PR-A and PR-B. They share identical DNA-binding and ligand-binding domains but differ in their N-terminal regions[28].

PR-B contains an additional activation function domain that is not present in PR-A. Although the specific functions of each isoform remain incompletely understood, they regulate distinct gene expression programs in a tissue-specific manner. Most cells express both isoforms, and they can exert different transcriptional effects within the same cellular context. Generally, PR-A plays a dominant role in the uterus and ovaries[29], whereas PR-B is more prominent in the mammary gland[30], where it acts as a more potent transcriptional activator and is essential for P4-induced proliferative responses[31–34]. Since PR is an ER target gene, and thus downstream of estrogen signaling, and ER signaling is heavily involved in breast cancer, progesterone has long been seen as the 'other' reproductive hormone[35]. As a result, PR is the lesser-studied counterpart of ER, with fewer dedicated tools and studies[36].

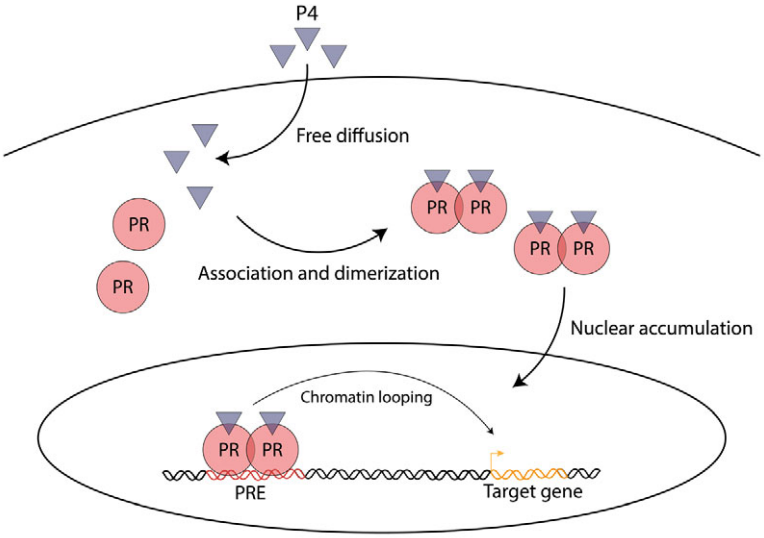

Fig2. Schematic overview of progesterone receptor signaling

Progesterone (P4) can freely diffuse into the cell. Upon progesterone receptor (PR) binding, PR dimerizes and accumulates in the nucleus. Here it can bind to progesterone responsive elements (PREs) and regulate its target genes by 3D enhancer promoter looping.

Molecular mechanisms of progesterone driven gene regulation

Following ligand binding, PR undergoes a conformational change, dimerizes, and accumulates in the nucleus, where it binds to DNA at specific progesterone response elements (PREs) to modulate gene expression (**Fig2**). The canonical PRE consists of an inverted palindrome (ACAnnnTGT), but this can vary, and alternative PRE sites have been described[37]. Additionally, PR can be recruited to DNA indirectly through protein–protein interactions with other transcription factors, thereby regulating genes that lack identifiable PREs[38,39]. Genome-wide studies have identified over 25,000 PR binding sites associated with the regulation of more than 2,000 genes[40,41]. Most of these binding sites do not contain a full consensus PRE but instead feature one or more half-sites[40]. PR binding typically occurs at distal regulatory regions, often located more than 30 kb from target gene promoters. These regions are frequently marked by enhancer-associated histone modifications such as H3K4me1 and H3K27ac[40,42,43].

Many PR binding sites are located in nucleosome-enriched (i.e. closed) regions of the chromatin prior to hormone exposure[40,43]. Upon activation, PR recruits chromatin remodelers and co-factors to these sites, facilitating local chromatin opening and transcriptional initiation[27,40,44]. Notably, PR binding to DNA is not stable; rather, PR–

chromatin interactions are transient and dynamic, particularly during the early phases after hormone exposure, when chromatin remodeling occurs[44]. Higher-order genome architecture further contributes to progesterone-mediated gene regulation. While progesterone signaling activation does not globally reorganize topologically associating domains (TADs), many progesterone-responsive genes are clustered within hormone-responsive TADs, where genes tend to be co-regulated in a coordinated manner[45]. This suggests that TADs act as regulatory compartments that facilitate enhancer–promoter communication in response to hormonal signals. Although considerable progress has been made in identifying PR target genes and subsequent activation of cellular functions, the molecular mechanisms that underlie PR's engagement with the genome remain incompletely understood. In particular, how PR signaling integrates with the 3D genome organization to drive precise, context-dependent gene expression is an area of active investigation.

Mammary gland development and physiology

The mammary gland is a unique mammalian exocrine organ that primarily functions to produce and secrete milk to nourish offspring. The mammary gland predominantly develops postnatally and is capable of undergoing multiple cycles of expansion and regression during pregnancy, lactation, and involution. At birth, a rudimentary epithelial ductal tree originating from the nipple is embedded in the fat pad (**Fig3, birth**). Until puberty, the mammary gland grows isometrically in proportion to overall body growth[46]. During puberty, mammary ductal development accelerates under the influence of the ovarian hormones estrogen (E2) and progesterone (P4). This hormonally driven process, resulting in branching morphogenesis, is directed by terminal end buds (TEBs), which form at the tips of invading ducts[47–50]. TEBs contain highly proliferative cells that drive elongation of the ductal tree, a process most active between postnatal days 28 and 42 (P28–P42, 4-8 weeks after birth) in mice[50,51] (**Fig3, puberty**). When the growing ductal tree reaches distal parts of the fat pad, TEBs regress[51]. By the end of puberty, the ductal network occupies approximately 60% of the fat pad, with additional space reserved for further expansion during pregnancy[52] (**Fig3**, **adult virgin**).

Upon sexual maturity, the mammary gland undergoes cyclical morphological changes driven by fluctuating levels of E2 and P4 during the estrous cycle, leading to the repeated formation and regression of short tertiary side branches[53–55]. Full blossoming of the mammary ductal tree, however, occurs during pregnancy, when sustained high

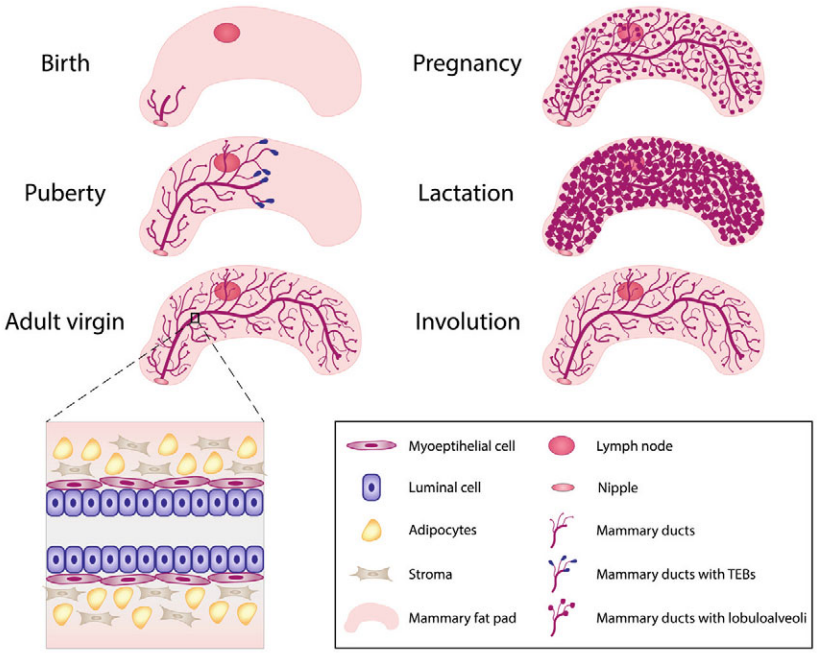

Fig3. Illustration of the different stages of mammary gland development

At birth the mammary fat pad only contains a rudimentary ductal tree (Birth). Under the influence of ovarian hormones, this tree grows out into the fat pad during puberty. This outgrowth is directed by terminal end buds (TEBs) (Puberty). At the end of puberty, the mammary ductal network has reached the periphery of the gland (Adult Virgin). The mammary ducts consist of a bilayered epithelium of luminal and myoepithelial cells that are surrounded by adipocytes and other stromal cells. During pregnancy, extensive side branching as well as the formation of alveolar buds takes place (Pregnancy). In preparation for lactation, alveolar buds are transformed into milk-secreting large lobuloalveolar structures (Lactation). Following weaning, the involution process is triggered restoring the mammary gland to a pre-pregnancy state.

levels of E2 and P4 promote significant tissue remodeling. While E2 predominantly drives ductal elongation during puberty[48], P4 is critical during pregnancy for the induction of extensive side branching and the formation of alveolar buds[56] (**Fig3, pregnancy**). In preparation for lactation, P4 synergizes with prolactin, to promote the outgrowth/terminal differentiation of alveolar buds into milk-secreting large lobuloalveolar structures[57] (**Fig3, lactation**). Following weaning, the mammary gland undergoes involution, a regression process that restores the tissue to a pre-pregnancy state[58] (**Fig3, involution**).

The adult mammary gland is composed of multiple cell types, including epithelial, adipose, fibroblast, immune, and vascular (blood and lymphatic) cells. The ducts are composed of a bilayered epithelium composed of apically oriented luminal cells, encompassing both luminal progenitors and hormone-sensing mature luminal cells,

and basally oriented myoepithelial cells (**Fig3**). Myoepithelial cells are able to contract to help stimulate milk flow to the nipple during lactation[52]. During pregnancy, the luminal progenitor cells can rapidly expand and differentiate into alveolar cells that are ready to secrete milk at parturition[59]. The main non-epithelial cell type present in the mouse mammary gland are the adipocytes, which can serve as a fat reserve during milk production[60,61] and provide endocrine functions[61,62] (**Fig3**). Additionally, stromal fibroblasts secrete growth factors and manage the composition of the mammary extra cellular matrix[63–65] (**Fig3**). Extensive molecular and functional crosstalk exists between these different cell types and populations at each of the developmental stages of the tissue.

Progesterone signaling in the mammary gland

In the mammary gland, estrogen and progesterone receptors are predominantly expressed in mature luminal, or hormone-sensing, epithelial cells. In the adult gland, approximately 40% of ductal epithelial cells express PR[66–68]. Notably, PR-positive cells are largely non-proliferative and are interspersed among proliferative PR-negative cells, suggesting that progesterone regulates epithelial expansion through paracrine mechanisms[66–68]. Indeed, PR-positive cells translate systemic progesterone signals into local paracrine cues that stimulate proliferation in neighboring PR-negative cells and activate mammary stem and progenitor populations[69]. Two well-characterized paracrine targets of PR in the adult mammary gland are *Wnt4* and *Tnsf11* (RANKL)[30,70–73]. WNT4 primarily acts on myoepithelial cells by activating CTNNB1-dependent WNT signaling, thereby promoting side branching during early pregnancy[70], partly through the maintenance and regulation of the stem cell pool[73,74]. In contrast, RANKL is thought to mainly signal to neighboring luminal progenitor cells to induce proliferation and is essential for PR-driven side branching and alveologenesis[30,71]. While we have decent knowledge about the progesterone-progesterone receptor targets in the mammary gland, the mechanisms by which progesterone regulates their expression at the chromatin and DNA level remain incompletely understood.

Similarities and differences of the mouse mammary gland and human breast

The mouse mammary gland is a widely used model to study developmental mechanisms due to its suitability for *in vivo* experimental manipulation. However, the relevance of the mouse mammary gland as a model for the human breast is often questioned due to physiological and anatomical differences between the two species. Indeed,

there are notable differences, such as irregular estrous cycles in mice[75], differences in stromal composition[76] and differences in hormone levels and hormone metabolism[77]. Nonetheless, the overall steroid hormone requirement, regulation and function during development is conserved.

Until recently, gene expression profiling of E2 and P4 responses in mouse and human models show poor overlap[35], suggesting species-specific differences in downstream hormonal response. However, as most human studies have relied on cultured breast cancer cells in comparison to *in vivo* studies for normal, non-transformed mouse mammary glands, these discrepancies may at least be partly explained by differences in models used. More recently, short-term *ex vivo* studies using human breast tissue have shown that progesterone-induced proliferation and downstream targets of progesterone closely mirror those observed in the mouse mammary gland[78], suggesting a stronger cross-species similarity than previously recognized[35].

Aim and outline of this thesis

It has been nearly 95 years since progesterone was first discovered and characterized[79]. Nowadays, its crucial role in the female reproductive system and mammary gland development is well established. Yet, many fundamental questions remain unresolved, and progesterone is just emerging from the shadow of its counterpart, estrogen. In particular, the molecular mechanisms by which the progesterone receptor engages with chromatin, cooperates with co-factors, and modulates target gene expression within the context of 3D genome architecture remain incompletely understood. The rapid development of advanced omics technologies now presents a unique opportunity to address these open questions. The central aim of this thesis is to gain novel insights into the chromatin-level mechanisms through which progesterone signaling regulates its target genes. A second aim is to better understand how spatiotemporal gene expression is regulated by enhancer sequences.

A major limitation in the field has been the lack of sensitive assays to detect and visualize PR pathway activity. To address this, **chapter 2** describes the development of new tools for quantifying PR signaling, including optimized progesterone response element (PRE) luciferase constructs and PRE-GFP reporters for single-cell imaging. Additionally, we define optimized conditions and cell lines that allow robust detection of PR activity and its downstream transcriptional response. We use these tools and conditions in **chapter 3**, where we focus on the previously unexplored tissue-specific

regulation of *Wnt4*, a key PR target gene in the mammary gland. We dissect and compare the mouse and human enhancer landscapes controlling *Wnt4* expression and identify a 3D chromatin hub that governs its regulation. Our results reveal a critical role for GRHL transcription factors in modulating *Wnt4* expression, and we propose a model of two-step priming and transcriptional regulation of *Wnt4* in both mouse and human contexts. **Chapter 4** extends this analysis to investigate the spatiotemporal regulation of *Wnt4* across multiple mouse tissues, including the embryonic and adult kidney and lung, in comparison to the mammary gland. We map the enhancer networks that fine-tune *Wnt4* expression and functionally interrogate the role of a conserved enhancer element via *in vivo* deletion studies. In **chapter 5**, we identify GRHL2 as a physical interactor and transcriptional co-regulator of PR. Through integrated genomic and functional analyses, we unravel the coordinated transcriptional activity of PR and GRHL2, illustrating how their cooperation, along with 3D genome architecture, shapes PR-driven gene regulation. Finally, **chapter 6** summarizes the findings of this thesis and discusses their broader implications for understanding progesterone-mediated gene regulation and enhancer biology.

Declaration of the use of generative AI

In this scientific work, no generative AI tools were used to generate content. During the writing process, ChatGPT was used to improve writing style of the first draft. The output was critically reviewed and partly re-written by the author. The author takes full responsibility for the final text, content, claims and references in this work.

References

1. ENCODE Project Consortium, Moore, J.E., Purcaro, M.J., Pratt, H.E., Epstein, C.B., Shoresh, N., Adrian, J., Kawli, T., Davis, C.A., Dobin, A., *et al.* (2020). Expanded encyclopaedias of DNA elements in the human and mouse genomes. Nature *583*, 699–710. https://doi.org/10.1038/s41586-020-2493-4.
2. Shen, Y., Yue, F., McCleary, D.F., Ye, Z., Edsall, L., Kuan, S., Wagner, U., Dixon, J., Lee, L., Lobanenkov, V.V., *et al.* (2012). A map of the cis-regulatory sequences in the mouse genome. Nature *488*, 116–120. https://doi.org/10.1038/nature11243.
3. Robson, M.I., Ringel, A.R., and Mundlos, S. (2019). Regulatory Landscaping: How Enhancer-Promoter Communication Is Sculpted in 3D. Mol. Cell *74*, 1110–1122. https://doi.org/10.1016/j.molcel.2019.05.032.
4. Furlong, E.E.M., and Levine, M. (2018). Developmental enhancers and chromosome topology. Science *361*, 1341–1345. https://doi.org/10.1126/science.aau0320.
5. Sagai, T., Masuya, H., Tamura, M., Shimizu, K., Yada, Y., Wakana, S., Gondo, Y., Noda, T., and Shiroishi, T. (2004). Phylogenetic conservation of a limb-specific, cis-acting regulator of Sonic hedgehog (Shh). Mamm. Genome Off. J. Int. Mamm. Genome Soc. *15*, 23–34. https://doi.org/10.1007/s00335-033-2317-5.
6. Vermunt, M.W., Zhang, D., and Blobel, G.A. (2019). The interdependence of gene-regulatory elements and the 3D genome. J. Cell Biol. *218*, 12–26. https://doi.org/10.1083/jcb.201809040.
7. Catarino, R.R., and Stark, A. (2018). Assessing sufficiency and necessity of enhancer activities for gene expression and the mechanisms of transcription activation. Genes Dev. *32*, 202–223. https://doi.org/10.1101/gad.310367.117.
8. Creyghton, M.P., Cheng, A.W., Welstead, G.G., Kooistra, T., Carey, B.W., Steine, E.J., Hanna, J., Lodato, M.A., Frampton, G.M., Sharp, P.A., *et al.* (2010). Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proc. Natl. Acad. Sci. U. S. A. *107*, 21931–21936. https://doi.org/10.1073/pnas.1016071107.
9. Sanyal, A., Lajoie, B.R., Jain, G., and Dekker, J. (2012). The long-range interaction landscape of gene promoters. Nature *489*, 109–113. https://doi.org/10.1038/nature11279.
10. Li, G., Ruan, X., Auerbach, R.K., Sandhu, K.S., Zheng, M., Wang, P., Poh, H.M., Goh, Y., Lim, J., Zhang, J., *et al.* (2012). Extensive promoter-centered chromatin interactions provide a topological basis for transcription regulation. Cell *148*, 84–98. https://doi.org/10.1016/j.cell.2011.12.014.
11. Mifsud, B., Tavares-Cadete, F., Young, A.N., Sugar, R., Schoenfelder, S., Ferreira, L., Wingett, S.W., Andrews, S., Grey, W., Ewels, P.A., *et al.* (2015). Mapping long-range promoter contacts in human cells with high-resolution capture Hi-C. Nat. Genet. *47*, 598–606. https://doi.org/10.1038/ng.3286.
12. Yao, L., Berman, B.P., and Farnham, P.J. (2015). Demystifying the secret mission of enhancers: linking distal regulatory elements to target genes. Crit. Rev. Biochem. Mol. Biol. *50*, 550–573. https://doi.org/10.3109/10409238.2015.1087961.
13. Dixon, J.R., Selvaraj, S., Yue, F., Kim, A., Li, Y., Shen, Y., Hu, M., Liu, J.S., and Ren, B. (2012). Topological domains in mammalian genomes identified by analysis of chromatin interactions. Nature *485*, 376–380. https://doi.org/10.1038/nature11082.
14. Nora, E.P., Lajoie, B.R., Schulz, E.G., Giorgetti, L., Okamoto, I., Servant, N., Piolot, T., van Berkum, N.L., Meisig, J., Sedat, J., *et al.* (2012). Spatial partitioning of the regulatory

landscape of the X-inactivation centre. Nature *485*, 381–385. https://doi.org/10.1038/nature11049.
15. Sexton, T., Yaffe, E., Kenigsberg, E., Bantignies, F., Leblanc, B., Hoichman, M., Parrinello, H., Tanay, A., and Cavalli, G. (2012). Three-dimensional folding and functional organization principles of the Drosophila genome. Cell *148*, 458–472. https://doi.org/10.1016/j.cell.2012.01.010.
16. Phillips-Cremins, J.E., Sauria, M.E.G., Sanyal, A., Gerasimova, T.I., Lajoie, B.R., Bell, J.S.K., Ong, C.-T., Hookway, T.A., Guo, C., Sun, Y., *et al.* (2013). Architectural protein subclasses shape 3D organization of genomes during lineage commitment. Cell *153*, 1281–1295. https://doi.org/10.1016/j.cell.2013.04.053.
17. Symmons, O., Uslu, V.V., Tsujimura, T., Ruf, S., Nassari, S., Schwarzer, W., Ettwiller, L., and Spitz, F. (2014). Functional and topological characteristics of mammalian regulatory domains. Genome Res. *24*, 390–400. https://doi.org/10.1101/gr.163519.113.
18. Rao, S.S.P., Huntley, M.H., Durand, N.C., Stamenova, E.K., Bochkov, I.D., Robinson, J.T., Sanborn, A.L., Machol, I., Omer, A.D., Lander, E.S., *et al.* (2014). A 3D map of the human genome at kilobase resolution reveals principles of chromatin looping. Cell *159*, 1665–1680. https://doi.org/10.1016/j.cell.2014.11.021.
19. Kubo, N., Ishii, H., Xiong, X., Bianco, S., Meitinger, F., Hu, R., Hocker, J.D., Conte, M., Gorkin, D., Yu, M., *et al.* (2021). Promoter-proximal CTCF binding promotes distal enhancer-dependent gene activation. Nat. Struct. Mol. Biol. *28*, 152–161. https://doi.org/10.1038/s41594-020-00539-5.
20. Lydon, J.P., DeMayo, F.J., Funk, C.R., Mani, S.K., Hughes, A.R., Montgomery, C.A., Shyamala, G., Conneely, O.M., and O'Malley, B.W. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. *9*, 2266–2278. https://doi.org/10.1101/gad.9.18.2266.
21. Navot, D., Anderson, T.L., Droesch, K., Scott, R.T., Kreiner, D., and Rosenwaks, Z. (1989). Hormonal manipulation of endometrial maturation. J. Clin. Endocrinol. Metab. *68*, 801–807. https://doi.org/10.1210/jcem-68-4-801.
22. Neville, M.C., McFadden, T.B., and Forsyth, I. (2002). Hormonal regulation of mammary differentiation and milk secretion. J. Mammary Gland Biol. Neoplasia *7*, 49–66. https://doi.org/10.1023/a:1015770423167.
23. De Nicola, A.F., Gonzalez Deniselle, M.C., Garay, L., Meyer, M., Gargiulo-Monachelli, G., Guennoun, R., Schumacher, M., Carreras, M.C., and Poderoso, J.J. (2013). Progesterone protective effects in neurodegeneration and neuroinflammation. J. Neuroendocrinol. *25*, 1095–1103. https://doi.org/10.1111/jne.12043.
24. Salehnia, M., and Zavareh, S. (2013). The effects of progesterone on oocyte maturation and embryo development. Int. J. Fertil. Steril. *7*, 74–81.
25. Brisken, C., and Scabia, V. (2020). 90 YEARS OF PROGESTERONE: Progesterone receptor signaling in the normal breast and its implications for cancer. J. Mol. Endocrinol. *65*, T81–T94. https://doi.org/10.1530/JME-20-0091.
26. Frigo, D.E., Bondesson, M., and Williams, C. (2021). Nuclear receptors: from molecular mechanisms to therapeutics. Essays Biochem. *65*, 847–856. https://doi.org/10.1042/EBC20210020.
27. Dilworth, F.J., and Chambon, P. (2001). Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. Oncogene *20*, 3047–3054. https://doi.org/10.1038/sj.onc.1204329.

28. Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., and Chambon, P. (1990). Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J. *9*, 1603–1614. https://doi.org/10.1002/j.1460-2075.1990.tb08280.x.
29. Mulac-Jericevic, B., Mullinax, R.A., DeMayo, F.J., Lydon, J.P., and Conneely, O.M. (2000). Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. Science *289*, 1751–1754. https://doi.org/10.1126/science.289.5485.1751.
30. Mulac-Jericevic, B., Lydon, J.P., DeMayo, F.J., and Conneely, O.M. (2003). Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. Proc. Natl. Acad. Sci. U. S. A. *100*, 9744–9749. https://doi.org/10.1073/pnas.1732707100.
31. Graham, J.D., Mote, P.A., Salagame, U., van Dijk, J.H., Balleine, R.L., Huschtscha, L.I., Reddel, R.R., and Clarke, C.L. (2009). DNA replication licensing and progenitor numbers are increased by progesterone in normal human breast. Endocrinology *150*, 3318–3326. https://doi.org/10.1210/en.2008-1630.
32. Jacobsen, B.M., Richer, J.K., Sartorius, C.A., and Horwitz, K.B. (2003). Expression profiling of human breast cancers and gene regulation by progesterone receptors. J. Mammary Gland Biol. Neoplasia *8*, 257–268. https://doi.org/10.1023/b:jomg.0000010028.48159.84.
33. Boonyaratanakornkit, V., McGowan, E., Sherman, L., Mancini, M.A., Cheskis, B.J., and Edwards, D.P. (2007). The role of extranuclear signaling actions of progesterone receptor in mediating progesterone regulation of gene expression and the cell cycle. Mol. Endocrinol. Baltim. Md *21*, 359–375. https://doi.org/10.1210/me.2006-0337.
34. Skildum, A., Faivre, E., and Lange, C.A. (2005). Progesterone receptors induce cell cycle progression via activation of mitogen-activated protein kinases. Mol. Endocrinol. Baltim. Md *19*, 327–339. https://doi.org/10.1210/me.2004-0306.
35. Brisken, C. (2013). Progesterone signalling in breast cancer: a neglected hormone coming into the limelight. Nat. Rev. Cancer *13*, 385–396. https://doi.org/10.1038/nrc3518.
36. Ali, S., Balachandran, K., and O'Malley, B. (2020). 90 Years of progesterone: Ninety years of progesterone: the "other" ovarian hormone. J. Mol. Endocrinol. *65*, E1–E4. https://doi.org/10.1530/JME-20-0145.
37. Ceballos-Chávez, M., Subtil-Rodríguez, A., Giannopoulou, E.G., Soronellas, D., Vázquez-Chávez, E., Vicent, G.P., Elemento, O., Beato, M., and Reyes, J.C. (2015). The chromatin Remodeler CHD8 is required for activation of progesterone receptor-dependent enhancers. PLoS Genet. *11*, e1005174. https://doi.org/10.1371/journal.pgen.1005174.
38. Clarke, C.L., and Graham, J.D. (2012). Non-overlapping progesterone receptor cistromes contribute to cell-specific transcriptional outcomes. PloS One *7*, e35859. https://doi.org/10.1371/journal.pone.0035859.
39. Mohammed, H., Russell, I.A., Stark, R., Rueda, O.M., Hickey, T.E., Tarulli, G.A., Serandour, A.A., Birrell, S.N., Bruna, A., Saadi, A., *et al.* (2015). Progesterone receptor modulates ERα action in breast cancer. Nature *523*, 313–317. https://doi.org/10.1038/nature14583.
40. Ballaré, C., Castellano, G., Gaveglia, L., Althammer, S., González-Vallinas, J., Eyras, E., Le Dily, F., Zaurin, R., Soronellas, D., Vicent, G.P., *et al.* (2013). Nucleosome-driven transcription factor binding and gene regulation. Mol. Cell *49*, 67–79. https://doi.org/10.1016/j.molcel.2012.10.019.
41. Zaurin, R., Ferrari, R., Nacht, A.S., Carbonell, J., Le Dily, F., Font-Mateu, J., de Llobet Cucalon, L.I., Vidal, E., Lioutas, A., Beato, M., *et al.* (2021). A set of accessible enhancers enables the initial response of breast cancer cells to physiological progestin concentrations. Nucleic

Acids Res. *49*, 12716–12731. https://doi.org/10.1093/nar/gkab1125.
42. Yin, P., Roqueiro, D., Huang, L., Owen, J.K., Xie, A., Navarro, A., Monsivais, D., Coon, J.S., Kim, J.J., Dai, Y., *et al.* (2012). Genome-wide progesterone receptor binding: cell type-specific and shared mechanisms in T47D breast cancer cells and primary leiomyoma cells. PloS One *7*, e29021. https://doi.org/10.1371/journal.pone.0029021.
43. Dinh, D.T., Breen, J., Akison, L.K., DeMayo, F.J., Brown, H.M., Robker, R.L., and Russell, D.L. (2019). Tissue-specific progesterone receptor-chromatin binding and the regulation of progesterone-dependent gene expression. Sci. Rep. *9*, 11966. https://doi.org/10.1038/s41598-019-48333-8.
44. Rayasam, G.V., Elbi, C., Walker, D.A., Wolford, R., Fletcher, T.M., Edwards, D.P., and Hager, G.L. (2005). Ligand-Specific Dynamics of the Progesterone Receptor in Living Cells and during Chromatin Remodeling In Vitro. Mol. Cell. Biol. *25*, 2406–2418. https://doi.org/10.1128/MCB.25.6.2406-2418.2005.
45. Le Dily, F., Baù, D., Pohl, A., Vicent, G.P., Serra, F., Soronellas, D., Castellano, G., Wright, R.H.G., Ballare, C., Filion, G., *et al.* (2014). Distinct structural transitions of chromatin topological domains correlate with coordinated hormone-induced gene regulation. Genes Dev. *28*, 2151–2162. https://doi.org/10.1101/gad.241422.114.
46. Flux, D.S. (1954). Growth of the mammary duct system in intact and ovariectomized mice of the CHI strain. J. Endocrinol. *11*, 223–237. https://doi.org/10.1677/joe.0.0110223.
47. Ruan, W., and Kleinberg, D.L. (1999). Insulin-like growth factor I is essential for terminal end bud formation and ductal morphogenesis during mammary development. Endocrinology *140*, 5075–5081. https://doi.org/10.1210/endo.140.11.7095.
48. Vandenberg, L.N., Wadia, P.R., Schaeberle, C.M., Rubin, B.S., Sonnenschein, C., and Soto, A.M. (2006). The mammary gland response to estradiol: monotonic at the cellular level, non-monotonic at the tissue-level of organization? J. Steroid Biochem. Mol. Biol. *101*, 263–274. https://doi.org/10.1016/j.jsbmb.2006.06.028.
49. Rillema, J.A. (1994). Development of the mammary gland and lactation. Trends Endocrinol. Metab. TEM *5*, 149–154. https://doi.org/10.1016/1043-2760(94)90011-6.
50. Williams, J.M., and Daniel, C.W. (1983). Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. Dev. Biol. *97*, 274–290. https://doi.org/10.1016/0012-1606(83)90086-6.
51. Paine, I.S., and Lewis, M.T. (2017). The Terminal End Bud: the Little Engine that Could. J. Mammary Gland Biol. Neoplasia *22*, 93–108. https://doi.org/10.1007/s10911-017-9372-0.
52. Macias, H., and Hinck, L. (2012). Mammary gland development. Wiley Interdiscip. Rev. Dev. Biol. *1*, 533–557. https://doi.org/10.1002/wdev.35.
53. Andres, A.C., and Strange, R. (1999). Apoptosis in the estrous and menstrual cycles. J. Mammary Gland Biol. Neoplasia *4*, 221–228. https://doi.org/10.1023/a:1018737510695.
54. Cole, H.A., and Parkes, A.S. (1997). The mammary gland of the mouse, during the œstrous cycle, pregnancy and lactation. Proc. R. Soc. Lond. Ser. B Contain. Pap. Biol. Character *114*, 136–161. https://doi.org/10.1098/rspb.1933.0077.
55. Fata, J.E., Chaudhary, V., and Khokha, R. (2001). Cellular turnover in the mammary gland is correlated with systemic levels of progesterone and not 17beta-estradiol during the estrous cycle. Biol. Reprod. *65*, 680–688. https://doi.org/10.1095/biolreprod65.3.680.
56. Brisken, C., Park, S., Vass, T., Lydon, J.P., O'Malley, B.W., and Weinberg, R.A. (1998). A paracrine role for the epithelial progesterone receptor in mammary gland development. Proc. Natl. Acad. Sci. U. S. A. *95*, 5076–5081.

57. Brisken, C., Kaur, S., Chavarria, T.E., Binart, N., Sutherland, R.L., Weinberg, R.A., Kelly, P.A., and Ormandy, C.J. (1999). Prolactin controls mammary gland development via direct and indirect mechanisms. Dev. Biol. *210*, 96–106. https://doi.org/10.1006/dbio.1999.9271.
58. Stein, T., Salomonis, N., and Gusterson, B.A. (2007). Mammary gland involution as a multi-step process. J. Mammary Gland Biol. Neoplasia *12*, 25–35. https://doi.org/10.1007/s10911-007-9035-7.
59. Hennighausen, L., and Robinson, G.W. (2005). Information networks in the mammary gland. Nat. Rev. Mol. Cell Biol. *6*, 715–725. https://doi.org/10.1038/nrm1714.
60. Gregor, M.F., Misch, E.S., Yang, L., Hummasti, S., Inouye, K.E., Lee, A.-H., Bierie, B., and Hotamisligil, G.S. (2013). The role of adipocyte XBP1 in metabolic regulation during lactation. Cell Rep. *3*, 1430–1439. https://doi.org/10.1016/j.celrep.2013.03.042.
61. Hovey, R.C., and Aimo, L. (2010). Diverse and active roles for adipocytes during mammary gland growth and function. J. Mammary Gland Biol. Neoplasia *15*, 279–290. https://doi.org/10.1007/s10911-010-9187-8.
62. Bartley, J.C., Emerman, J.T., and Bissell, M.J. (1981). Metabolic cooperativity between epithelial cells and adipocytes of mice. Am. J. Physiol. *241*, C204-208. https://doi.org/10.1152/ajpcell.1981.241.5.C204.
63. Howard, B.A., and Lu, P. (2014). Stromal regulation of embryonic and postnatal mammary epithelial development and differentiation. Semin. Cell Dev. Biol. *25–26*, 43–51. https://doi.org/10.1016/j.semcdb.2014.01.004.
64. Wiseman, B.S., and Werb, Z. (2002). Stromal effects on mammary gland development and breast cancer. Science *296*, 1046–1049. https://doi.org/10.1126/science.1067431.
65. Simian, M., Hirai, Y., Navre, M., Werb, Z., Lochter, A., and Bissell, M.J. (2001). The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. Dev. Camb. Engl. *128*, 3117–3131. https://doi.org/10.1242/dev.128.16.3117.
66. Ismail, P.M., Li, J., DeMayo, F.J., O'Malley, B.W., and Lydon, J.P. (2002). A novel LacZ reporter mouse reveals complex regulation of the progesterone receptor promoter during mammary gland development. Mol. Endocrinol. Baltim. Md *16*, 2475–2489. https://doi.org/10.1210/me.2002-0169.
67. Shyamala, G., Chou, Y.-C., Louie, S.G., Guzman, R.C., Smith, G.H., and Nandi, S. (2002). Cellular expression of estrogen and progesterone receptors in mammary glands: regulation by hormones, development and aging. J. Steroid Biochem. Mol. Biol. *80*, 137–148. https://doi.org/10.1016/s0960-0760(01)00182-0.
68. Silberstein, G.B., Van Horn, K., Shyamala, G., and Daniel, C.W. (1996). Progesterone receptors in the mouse mammary duct: distribution and developmental regulation. Cell Growth Differ. Mol. Biol. J. Am. Assoc. Cancer Res. *7*, 945–952.
69. Brisken, C., and Duss, S. (2007). Stem cells and the stem cell niche in the breast: an integrated hormonal and developmental perspective. Stem Cell Rev. *3*, 147–156. https://doi.org/10.1007/s12015-007-0019-1.
70. Brisken, C., Heineman, A., Chavarria, T., Elenbaas, B., Tan, J., Dey, S.K., McMahon, J.A., McMahon, A.P., and Weinberg, R.A. (2000). Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. Genes Dev. *14*, 650–654.
71. Brisken, C., Ayyannan, A., Nguyen, C., Heineman, A., Reinhardt, F., Tan, J., Dey, S.K., Dotto, G.P., and Weinberg, R.A. (2002). IGF-2 is a mediator of prolactin-induced morphogenesis in the breast. Dev. Cell *3*, 877–887. https://doi.org/10.1016/s1534-5807(02)00365-9.

72. Cao, Y., Bonizzi, G., Seagroves, T.N., Greten, F.R., Johnson, R., Schmidt, E.V., and Karin, M. (2001). IKKalpha provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. Cell *107*, 763–775. https://doi.org/10.1016/s0092-8674(01)00599-2.
73. Rajaram, R.D., Buric, D., Caikovski, M., Ayyanan, A., Rougemont, J., Shan, J., Vainio, S.J., Yalcin-Ozuysal, O., and Brisken, C. (2015). Progesterone and Wnt4 control mammary stem cells via myoepithelial crosstalk. EMBO J. *34*, 641–652. https://doi.org/10.15252/embj.201490434.
74. Joshi, P.A., Jackson, H.W., Beristain, A.G., Di Grappa, M.A., Mote, P.A., Clarke, C.L., Stingl, J., Waterhouse, P.D., and Khokha, R. (2010). Progesterone induces adult mammary stem cell expansion. Nature *465*, 803–807. https://doi.org/10.1038/nature09091.
75. Caligioni, C.S. (2009). Assessing reproductive status/stages in mice. Curr. Protoc. Neurosci. *Appendix 4*, Appendix 4I. https://doi.org/10.1002/0471142301.nsa04is48.
76. Howard, B.A., and Gusterson, B.A. (2000). Human breast development. J. Mammary Gland Biol. Neoplasia *5*, 119–137. https://doi.org/10.1023/a:1026487120779.
77. Chow, J.D.Y., Simpson, E.R., and Boon, W.C. (2009). Alternative 5'-untranslated first exons of the mouse Cyp19A1 (aromatase) gene. J. Steroid Biochem. Mol. Biol. *115*, 115–125. https://doi.org/10.1016/j.jsbmb.2009.03.010.
78. Tanos, T., Sflomos, G., Echeverria, P.C., Ayyanan, A., Gutierrez, M., Delaloye, J.-F., Raffoul, W., Fiche, M., Dougall, W., Schneider, P., *et al.* (2013). Progesterone/RANKL is a major regulatory axis in the human breast. Sci. Transl. Med. *5*, 182ra55. https://doi.org/10.1126/scitranslmed.3005654.
79. Corner, G.W., and Allen, W.M. (1929). Physiology of the corpus luteum. Am. J. Physiol.-Leg. Content *88*, 326–339. https://doi.org/10.1152/ajplegacy.1929.88.2.326.