Estrogen Receptor-Mediated Gene Transcription and Cistrome

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Abstract The discovery of the estrogen receptor 60 years ago radically transformed the field of hormonal signaling and led to the recognition of ER as a prototype nuclear receptor that primarily functions as a transcription factor. In this chapter, we will first describe the conserved domain architecture of ER and its regulation through various modifications by diverse intracellular pathways. We will then discuss the history and most recent advancement in the understanding of ER regulation of target genes at both individual gene and whole genome levels. A number of new concepts emanated from these studies, including ER cistrome, pioneer factors, chromosome looping and enhancer RNA, etc. and their potential impact on the fight against breast cancer therapeutic resistance all will be discussed in detail in this chapter.

Keywords ER domain structure · Posttranslational modification (PTM) · Cistrome · Pioneer factors · Chromosome looping · Enhancer RNA

1 Introduction

The paradigm shifting discovery of estrogen receptor (ER) by Dr. Elwood Jensen in 1958 revolutionized the popular view that estrogen functions through affecting enzymatic activities [1, 2]. Instead, the steroid hormone estrogen can directly diffuse through the plasma membrane to interact with its intracellular receptor to elicit its biological functions in the nucleus [3, 4]. Not until 10 years later, Dr. Bert O'Malley further discovered that the primary function of ERα is to regulate the expression of a subset of mRNAs [5, 6]. The cloning of ERα further established its

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role as a ligand-dependent transcription factor and continued to open up a whole nuclear receptor field [7–9]. Since its discovery, the estrogen receptor has been extensively studied both for its own function and as a model for our understanding of other nuclear receptor family members. We will focus on ERα in this chapter as ERβ will be discussed in chapter "Estrogen Receptor β and Breast Cancer." We will first briefly discuss the basic ERα domain structure and its regulation by posttranslational modifications such as phosphorylation, ubiquitination, acetylation, and methylation. We are then putting our main emphasis on the current understanding of how ERα binds to specific estrogen regulatory element (EREs) to regulate its target gene expression and the recent advancement in utilizing state-of-the-art approaches to map its genome-wide binding sites (cistrome). New concepts and insights emerging from these genome-wide studies that included the roles for pioneer factors, chromosome looping, and enhancer RNAs (eRNAs) in ERα-mediated transcription will be discussed in detail. Finally, we will discuss how cellular signaling pathways affect the ERα cistrome and how these studies could provide not only new insights into molecular mechanisms underlying ERα-mediated transcription and antiestrogen resistance but may also provide potential new avenues for the development of innovative strategies for overcoming therapeutic resistance.

2 Estrogen Receptor α Domain Structure

Estrogen receptor α is 595 amino acids long and contains six functional domains including two transactivation domains (Fig. 1). The activation function 1 (AF1 domain, aa 51–149) is responsible for ligand-independent interaction with many cofactors to drive gene transcription [10, 11]. AF-1 is a common target for growth factor-driven phosphorylation cascades, allowing diverse inputs to affect the activity of estrogen receptor α. The DNA-binding domain (DBD, aa 183–246),

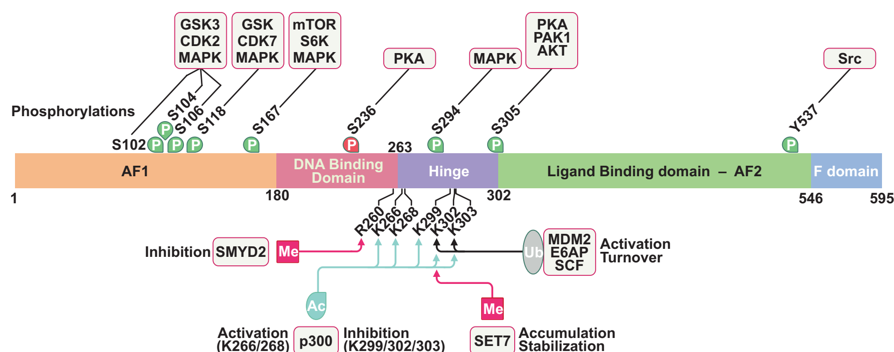

Fig. 1 Domain structure and modification landscape of estrogen receptor α. Layout of the domains of ERα with selected phosphorylation (activation/green, repression/red), methylation, acetylation, and ubiquitination sites. The responsible enzymatic pathways and effects on estrogen receptor functions are also shown

which follows AF1, is highly conserved between ERα and other nuclear receptors and contains two zinc fingers which specify DNA-binding sequences [12]. DNA binding is further stabilized by amino acids through 282, indicating that the following domain also plays a role in DNA binding [13]. Amino acids 263–302 constitute the hinge domain which also contains the nuclear localization sequence (NLS) of ERα [14]. The hinge domain has been shown to be important for optimal synergy between the AF1 and AF2, possibly by allowing them to interact with shared cofactors [15]. The C-terminus of ERα is made up of the ligand-binding domain (LBD) that can also act as a transactivation domain (AF2) and the c-terminal F domain. The ligand-binding domain of ERα is bound by protein folding chaperones such as HSP90 and is released after ligand binding [16]. Crystal structures of the ERα ligand-binding domain show that it is made of 12 helixes [11]. Helix 10 is primarily responsible for the dimer interface, while the 12th helix acts as an activation gatekeeper. When bound to estrogen, helix 12 adopts an open conformation allowing for the binding of coactivator proteins. Interestingly when bound to inhibitors such as tamoxifen, helix 12 prevents this opening to cover the sites where coactivators typically bind and allows for the binding of corepressors [17, 18]. Finally, the extreme c-terminal F domain of ERα appears to play a role in dimerization and cofactor binding as well, but its role is not very well defined [17, 19, 20].

3 Regulation of ERα Activity by Posttranslational Modifications

3.1 Phosphorylation

The activity of ERα has been found to be regulated by a number of posttranslational modifications such as phosphorylation, ubiquitination, acetylation, and methylation (Fig. 1). To date, more than 15 phosphorylation sites on ERα have been characterized, with many of them located in the AF-1 domain. Phosphorylation of Serine 118 is among the best-studied posttranslational modification of the AF-1 domain. The initial characterization of pS118 found that estrodiol (E2) treatment can increase the levels of pS118, while later studies found it is also induced by growth factors (such as EGF or IGF-1) even in the absence of hormone [21, 22]. S118 has also been found to be targeted by several additional pro-growth pathways including the MAP kinase, CDK7, and glycogen synthase kinase (GSK) to promote ERα-mediated transactivation [23]. To understand how pS118 affects ERα function mechanistically, many groups examined its role in affecting protein-protein interactions. By using yeast as a model, it was found that mutation of 118 in human ERα led to a dramatic reduction in ERα homodimerization [24]. In human cell lines, phospho-Serine 118 has been found to mediate interactions with important ERα coactivators such as p300 [23, 25]. Additionally, ERα S118A mutant shows reduced recruitment to nonclassical sites that require protein-protein interactions with other transcription factors such as AP-1 as described later in this chapter.

Most ERα-AF-1 phospho-target sites are similar to S118 in that they are targeted by pro-growth pathways and serve to increase the activation potential of ERα. A cluster of serines, S102, 104, and 106, are also targeted by pathways including MAPK, GSK3, and Cdk2, and mutation of these sites to alanine reduces transactivation activity of ERα [23]. S167 is a target of the MAPK, mTOR, and S6K pathways, and phosphorylation of this residue increases transactivation from the AF-1 domain of ERα [23]. Outside of the AF-1 domain, serine 236, in the second zinc finger of the DNA-binding domain, is targeted by the PKA (protein kinase A) pathway. This phosphorylation appears to inhibit activation, as mutating this residue to glutamic acid (a phosphoserine mimic) prevented homodimer formation, DNA binding, and the ability to activate reporter gene expression [26, 27]. PKA and PAK1 and AKT phosphorylate ERα at serine 305, which has been found to promote cofactor binding and transactivation [23, 28]. In addition, serine 305 phosphorylation has also been shown to moderate modifications on other residues of ERα such as S118 phosphorylation and K303 acetylation [29]. Importantly, high PAK1 protein expression and S305 phosphorylation in breast cancers have been found to correlate with sensitivity to tamoxifen [23, 28]. Interestingly, S294 can be phosphorylated by MAPK, which activates ERα through increased ubiquitination at nearby lysines as will also be discussed below. In addition to all these serine phosphorylations, a tyrosine residue in the F domain (Tyr537) has been found to be phosphorylated by Src kinase to regulate the dimerization ability and transcription [23]. Phosphorylation mapping by mass spectrometry has uncovered additional phosphorylation sites, but their functions and the pathways responsible for their phosphorylation remain to be explored [30].

3.2 *Ubiquitination*

Interestingly, the half-life of ERα is significantly decreased after the administration of E2 (3–5 days versus 3–5 h), suggesting that ERα turnover is an important step in the cellular response to estrogens [31]. ERα is degraded by the ubiquitin proteasome system after treatment by E2, linking its degradation to its transcriptional function [31, 32]. This relationship was supported by the discovery that treating cells with MG132, a proteasome inhibitor, reduced expression of E2-responsive reporter genes, despite stabilization of ERα at protein levels [33]. Further studies indicated that E2 induction results in a cyclical recruitment of ERα and its cofactors on the TFF1 promoter [34, 35], a pattern that was abrogated by MG132 treatment [36]. The lysines responsible for ubiquitin-dependent degradation, 302 and 303, were found to be essential for E2-induced degradation, while mutation of these lysines to alanines led to both stabilization of ERα and a reduction in its transcriptional activities [37]. Knockdown experiments have found that several E3-ubiquitin ligases, including MDM2, E6AP, and SCF, are responsible for ubiquitination of ERα [38–40]. As discussed previously, phosphorylation of ERα on S294 allows

ERα to be recognized by SKP2, a member of the SCF ubiquitin ligase complex. SKP2 recognizes the phosphorylation site, bridging the E2 ubiquitin ligase SCF complex and ERα [41]. This has been found to be particularly important in late response genes such as E2F-1 [40]. Inhibition of MAPK or mutation of S294 to alanine reduced ubiquitination of ERα and transcription of its target genes. In addition, ERα coactivators, including AIB1 and SRC-1, have also been shown to be able to enhance ubiquitination of ERα and recruit the proteasomal subunit LMP2, respectively [42, 43]. Conversely, ERα function can be inhibited by the deubiquitinating enzyme OTUB1. As might be expected, OTUB1 overexpression drastically reduces ubiquitination and activation of ERα, while knockdown of OTUB1 led to higher rates of ubiquitination and transcription [44]. Together, these studies indicate that ERα turnover is a highly regulated step required for its transactivation activities.

3.3 Acetylation and Methylation

Estrogen receptor α activity can also be regulated by acetylation and methylation on these ubiquitination sites (K302, K303) as well as other lysines. Protein acetyltransferases such as p300 and P/CAF are well-characterized ERα cofactors that acetylate histones and activate transcription. Wang and colleagues found that p300 but not P/CAF can also directly acetylate ERα preferentially at lysines 302 and 303 with additional sites such as lysine 299 [45]. Interestingly, mutation of these lysines to arginine, glutamic acid, or threonine did not alter the expression of ERα but affected both ERα-mediated transactivation and the sensitivity to E2 stimulation [45]. In addition, p300 has also been found to acetylate K266 and 268 to increase ERα DNA binding and transactivation [46]. Furthermore, ERα is also known to recruit a number of histone methyltransferases and demethylases. It is therefore not surprising that ERα itself is also subjected to regulation by methylation and demethylation. Through a functional screening of different methyltransferase catalytic domains, SMYD2 was found to be able to methylate ERα at lysine 266. Knockdown of SMYD2 increased E2-induced transcription of target genes and recruitment of ERα to enhancer elements, while also leading to marked increases in acetyl-K266 and 268. Meanwhile, the lysine demethylase LSD1 acts as the demethylase of K266/268. Knockdown of LSD1 was shown to increase levels of methyl-K266 and a subsequent decrease in the acetyl-K266 [47]. ERα K302 has been shown to be a target of the SET7 methyltransferase, and methylation of K302 has been found to prevent ERα polyubiquitination and degradation and enhance the accumulation of ERα at target genes [48]. Together, these studies indicate that additional modifications such as acetylation and methylation could provide further mechanisms to regulate ERα functions through diverse signaling inputs, which is important given that ERα can directly feed from hormone and signaling stimulation and quickly translate into transcriptional regulations.

4 Estrogen Receptor α Binding Modes

Once activated, ERα recognizes and binds to specific sequences on DNA to regulate its target gene transcription. ERα can either directly bind to DNA at specific sequences in enhancer elements in a "classical" mode or by tethering with other transcription factors such as AP-1 that themselves are recruited in a sequence-specific manner in a "nonclassical" mode. In the classical mode, ERα binds to specific DNA motifs called estrogen response elements (ERE) to regulate its target gene expression. EREs are palindromic or near-palindromic sequences and can function as typical enhancer elements in both directions and in a distance-independent manner. The ERE was first discovered on the *Xenopus* vitellogenin genes A1, A2, B1, and B2 as the estrogen-responsive and estrogen receptor-binding regulatory elements [49]. Subsequently, human EREs have been identified in other estrogen-induced target genes such as TFF-1/pS2, GREB1, Cathepsin D, TGFα, etc. The consensus ERE core sequence has been deduced to be composed of GGTCAnnnTGACC in which n can be any nucleotide (Fig. 2a, b) [49]. These sites are bound by mirrored homodimers, in which the two zinc fingers in the DNA-binding domain are essential for ERα recognition of the proper ERE. The first zinc finger contains a helix structure that is inserted into the major groove to identify the proper sequence. Three amino acids (Glu203, Gly204, and Ala207) with the proximal box (P-box) have been shown to be particularly important for sequence

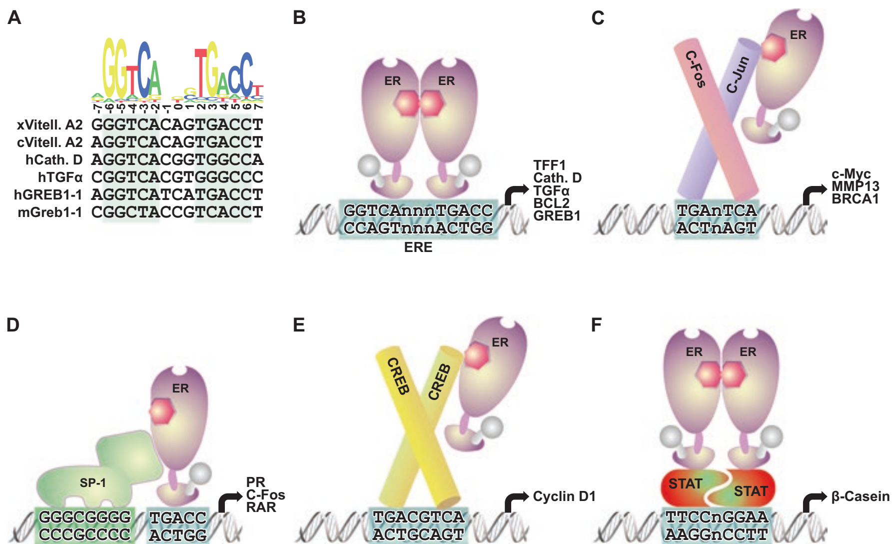

Fig. 2 Schematics of classical and nonclassical ERα binding modes. (**a**) Estrogen receptor consensus binding sequence with representative ERE sites. (**b**) Classical binding of an estrogen receptor dimer to the canonical estrogen response element, (**c**–**f**) nonclassical tethering of ERα binding with AP-1 (**c**), SP-1 (**d**), CREB (**e**), and STAT (**f**) to their respective response elements. Genes known to be regulated by enhancer elements are shown for each element

determination. The second zinc finger promotes ERα dimerization as a mechanism for recognition of the spacing between the half sites [11, 12, 50, 51].

While many E2-responsive genes are activated from EREs, the nonclassical recruitment ERα onto target gene promoters represents an additional layer of complexity [52]. In this nonclassical mode, ERα may or may not bind DNA directly but instead is recruited through interacting with a variety of other DNA-binding transcription factors, such as AP-1, SP-1, cAMP-like elements, and STAT dimers (Fig. 2c–f). For example, ERα is able to transactivate through the AP-1 site through interacting with AP-1 dimer (Fos and Jun proteins), regulating genes including matrix metalloproteinase 13 and BRCA1 (Fig. 2c) [53, 54]. The in vitro interaction studies have mapped the interaction site with Jun to the central hinge region of ERα, while interaction between ERα and Fos was undetected [55]. C-Myc is another well-known ERα target regulated by a combined AP-1 site with a half-ERE, indicating a possible ERα monomer interaction with the DNA in this configuration. Deletion of either enhancer element reduced estrogen inducibility of a reporter gene from this enhancer [56, 57]. Other nonclassical activation sites function in similar ways to AP-1 sites. Well-known SP-1-recruited ERα target genes include progesterone receptor, c-Fos, and the retinoic acid receptor [58, 59]. Similar to the c-Myc enhancer, these enhancer elements also include half-EREs and require binding of both SP-1 and ERα for full expression of the target genes. Other response elements can also function by similar mechanisms through proteins such as the cAMP-response element binding protein (CREB) and STATs to regulate the target genes such as cyclin D1 or β-casein, respectively [52]. Overall, this nonclassical mode of action of ERα in mediating gene transcription greatly expands the repertoire and complexity of estrogen receptor α-regulated target genes and transcriptional programs.

5 Dynamic and Cyclic Recruitment of ERα Cofactors in ERα-Mediated Gene Transcription

Once estrogen-bound and activated, ERα recognizes and binds to these specific EREs to recruit diverse transcriptional cofactors to regulate its target gene expression. These cofactors play essential roles in regulating the expression of ERα target genes by facilitating the recruitment and/or the function of the RNA polymerase II and general transcription machinery [60, 61]. The better-characterized class of nuclear receptor-interacting coactivators includes ATP-dependent chromatin-remodeling SWI/SNF complexes and histone-modifying enzymes including p160/SRC family members (SRC1, 2, and 3), p300/CBP, PGC-1, PRMT1, CARM1, HDACs, and LSD1, among many others [62, 63]. These coactivators have intrinsic enzymatic activities (e.g., ATP-dependent remodeling functions, histone acetyltransferase, methyltransferase, deacetylases, demethylase, etc.) and are thought to act, at least in part, through chromatin remodeling or histone modifications to open

up the chromatin structure that in turn facilitate the recruitment and function of the general transcription machinery [64, 65]. Furthermore, ERα-dependent transcription also requires another class of coactivators to send the signal directly to the general transcription machinery to activate transcription [66–68]. Among these coactivators, Mediator has recently emerged as the main bridge for direct communication between ERα and RNA polymerase II through direct interaction between ERα and MED1 subunit of the Mediator complex [69–74]. Moreover, a number of other ERα cofactors have also recently been reported to play roles in transcription elongation, splicing, etc., further linking ERα to not only transcription initiation but also these other processes in regulating target gene expression [62, 64]. Interestingly, studies found that the recruitment of these diverse cofactors by ERα is a rather dynamic process, occurring in a sequential and cyclical fashion. It was first reported by Shang et al. that ERα promoter occupancy peaked first followed by p160, p300, MED1, and then RNA pol II upon estrogen stimulation and released in a cyclic mode by using a kinetic chromatin IP method [35]. This phenomenon was further confirmed by a number of other studies using the similar and additional approaches [34, 75–77]. Although the exact functional significance of this cyclic recruitment has just started to be deciphered and will be extensively discussed in the next chapter (Chapter "Structural Studies with Coactivators for the Estrogen Receptor"), it is consistent with the findings that the degradation of ERα and its cofactor is required for their optimal activation of target genes. It further reflects the precise and tightly controlled nature of the ERα-mediated target gene transcription at multiple levels.

6 Genome-Wide Analyses of Estrogen Receptor α Binding Cistrome

With the completion of the Human Genome Project and development of computational tools, scientists began to search for EREs near promoter regions to identify potential ERα target genes [78–80]. One such genome-wide analysis of consensus or near consensus EREs found over 70K potential EREs in the human and mouse genomes [81]. By eliminating the EREs that are not conserved among species, they identified 660 genes with one or more conserved ERE in their proximal promoter regions (708 conserved EREs in total) [81]. Most of these conserved EREs were located in the 0 to +2-kb region; but there are also a significant number of conserved EREs (24.6%) mapped to between −5 and −10 kb of the transcriptional start sites. Further, gel shift and chromatin IP experiments were able to confirm the binding of ERα to most of these ERE sites both in vitro and in vivo. Interestingly, another study also combining computational prediction and experimental validation estimated the total ERα direct binding EREs to be between 5000 to 10,000 [82].

The combination of chromatin IP with high-throughput sequencing methodologies like DNA microarray (ChIP-on-chip) and next-generation sequencing (ChIP-seq) has further accelerated the identification of genome-wide ERα-binding

cistrome. By using the ChIP-on-chip method, Carroll et al. examined the ERα binding sites, initially on chromosomes 21 and 22, and later expanded on the whole genome [83, 84]. Overall, they identified 3665 ERα binding sites using a stringent threshold with a false discovery rate of ~1%. Interestingly while a majority of RNA pol II (67%) bound at proximal promoter regions (−800 bp to +200 bp), only 4% of ERα binding sites could be mapped to these 1-kb regions. Instead, the majority of ERα binding sites were found in intronic or distal upstream locations within 100 kb of the transcription start site. These studies also revealed enrichment of binding sites for ERα cofactors like FOXA1, C/EBP, and OCT adjoining ERE sequences in the ERα binding regions. Interestingly, although there is a strong negative correlation of ERE and AP-1 elements, C/EBP, Oct, and Forkhead transcription factors showed equal likelihood of occurrence with both motifs, suggesting that these factors are important for both classical and nonclassical ERα activation.

Since then, a number of additional genome-wide characterizations of the ERα cistrome have been carried out using varying approaches and cell lines [85–92]. For example, Lin et al. performed ChIP-PET (paired-end tags) experiments and identified 1234 high-confidence ERα binding sites, approximately 95% of which were farther than 5 kb from a TSS [86]. Of these regions, most (~71%) contained at least one ERE-like sequence, and a large minority (~25%) contained putative half-EREs with the rest containing no discernible ERE sequences [86]. In addition to FOXA1 sites, they also found several more enriched motifs like Sp1 and PAX3 motifs in the ERα binding sites. However, they found that only 22–24% of ERα binding sites were conserved among vertebrates [86, 93]. Joseph et al. [92] mapped the ERα cistrome using ChIP-seq and found the majority of ERα binding sites were located in the intragenic regions (39.5%) and 5′-distal (17.4%) and 3′-distal regions (14.3%), with only 9.3% found in promoter regions. While not all of the ERα binding sites identified were consistent among these experiments, a common theme that emerged is that ERα binds preferentially not to the promoter regions but to distal enhancer regions to regulate its target gene expression in response to estrogen stimulation [94].

7 ERα Genome-Wide Binding and Pioneer Factors

These genome-wide analyses of ERα binding sites have also revealed another previously unexpected mode of action for ERα-mediated gene transcription in terms of the pioneer factors. As discussed above, in one of the first chromosome-wide mappings of ERα binding studies, ChIP analysis was combined with the use of tiled oligonucleotide microarrays that cover the entire non-repetitive chromosomes 21 and 22 at a 35-bp resolution [83]. This analysis revealed 57 ERα binding sites within 32 clusters in chr 21 and 22, far less than the 5500 predicted elements, suggesting that ERE sequences alone are insufficient to predict ERα binding sites. Further in-depth analysis of the surrounding sequences of these ERα binding sites for enriched

motifs revealed that Forkhead factor binding sites were present in over half of the 57 ERα binding regions. Subsequent ChIP experiments have verified the FOXA1 binding to these sites, and siRNA-mediated knockdown of FOXA1 has confirmed the requirement for FOXA1 in both ERα recruitment and target gene expression [83]. FOXA1 is known to be a pioneer factor that can interact with and open compact chromatin due to its structural similarity to linker histones [95]. Subsequent studies confirmed the requirement of FOXA1 for ERα-chromatin interactions and transcription in multiple breast cancer cell lines [96]. Together, these studies have therefore established a new model for ERα-mediated transcription in which pioneer factor FOXA1 binds to the chromatin prior to estrogen treatment and functions to guide and provide accessibility for the binding of ERα to regulate the expression these target genes upon estrogen stimulation (Fig. 3).

Since the discovery of FOXA1, additional proteins such as TLE, Ap2γ, and PBX1 have also been reported to be able to act as pioneer factors. Like FOXA1, the Groucho/transducin-like enhancer of split (TLE) proteins are known to interact with chromatin independent of other factors. Holmes et al. explored the role of TLE proteins in ERα-mediated transcriptional activation and functions [97]. Similar to FOXA1, they found that TLE1 is associated with ERα binding sites with or without estrogen treatment. Additionally, knockdown of TLE1 affected 45% of all ERα

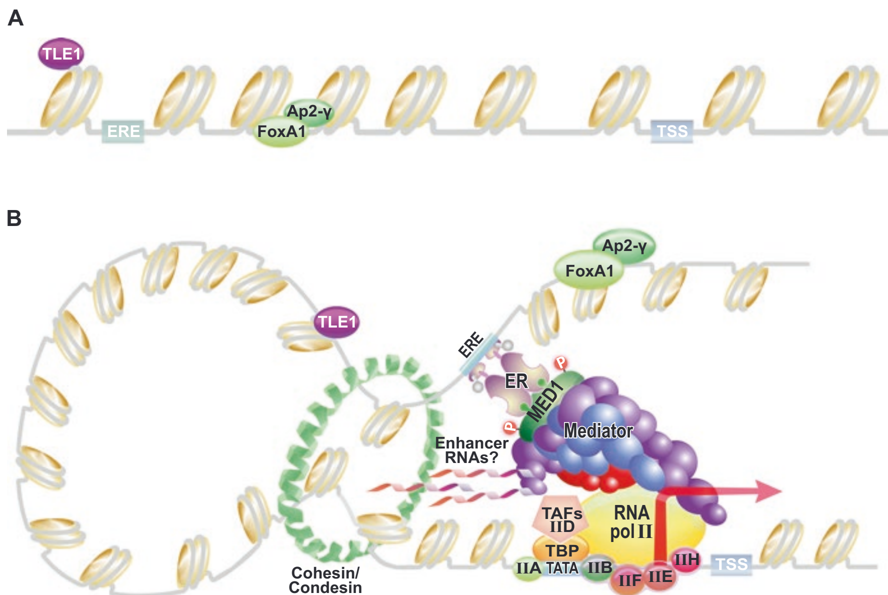

Fig. 3 Molecular model of ERα-mediated gene transcription. (**a**) Prior to estrogen treatment, pioneer factors such as FoxA1, AP2-γ, and TLE1 are present near estrogen response elements to facilitate ERα binding. (**b**) After estrogen treatment, ERα binds to enhancer elements and recruits cofactors (e.g., MED1/Mediator and SRC-1), as well as condensin/cohesin and enhancer RNAs to form chromosome loop to activate transcription by RNA Pol II and the general transcription factors (GTFs)

binding events and significantly impaired both ERα-mediated gene transcription and cell proliferation. Interestingly, TLE1 knockdown does not affect the binding of FOXA1, and most of the ERα binding events affected by TLE1 knockdown are on those target sites not cobound by FOXA1. In another study, Tan et al. observed that AP-2 motifs are enriched in the ERα binding sites [98]. They demonstrated that AP-2γ binds to ERα binding sites in a ligand-independent manner, while perturbation of AP-2γ expression significantly impaired ERα binding, long-range chromatin interactions, and target gene transcription. Unlike TLE1, AP-2γ colocalizes with FoxA1 on ERα binding sites that are associated with long-range chromatin interactions, and their functions are mutually dependent. More recently, PBX1 (pre-B-cell leukemia homeobox 1) has also been implicated to function as a pioneer factor promoting an ERα-dependent transcriptional program favorable to drive breast cancer progression and metastasis [99, 100].

8 ERα-Driven Chromosome Looping

Given that ERα predominantly binds to distal enhancers upon estrogen stimulation, an important question is raised as to how ERα regulates target gene expression from tens to hundreds of kilobases away. One proposed model hypothesized that PolII might be recruited to enhancers and then translocate along the DNA until it hits the promoter and transcriptional start site to initiate transcription. A second model hypothesized that transcription factors and cofactors could curve the DNA to bring enhancers and promoters close to one another in a three-dimensional space through a process called "chromosome looping." Recently developed methods and technologies such as chromosome conformation capture (3C) [101, 102] have provided crucial evidence supporting the latter model. Basic 3C methods and their derivatives such as ChIP-3C, 4C, 5C, and 6C approaches usually involve chemically cross-linked chromatin treated with restriction enzymes or sonication similar to ChIP [103]. Unlike ChIP, the DNA fragments are religated allowing sequences connected by protein bridges to be ligated together. These novel combined fragments can then be identified through PCR or sequenced and mapped to the genome. By using such technologies, chromosome looping has been detected and confirmed between the promoter and enhancer of ERα target genes such as TFF1 and GREB1 [83, 84, 104].

Using a 3C-based technique called ChIA-PET, Fullwood and colleagues generated a genome-wide map of the ERα-bound chromatin interactome [105]. The ChIA-PET approach enriches cross-linked DNA-protein complexes through ChIP after sonication to shear the chromatin. The immunoprecipitated DNA fragments are then ligated to paired-end tags (PET) which can be ligated more easily and also bypasses the use of restriction enzymes to reduce the inherent bias caused by only allowing digestion at specific sites. Through this approach, they identified a total of 1451 intrachromosomal interactions and a small set of 15 interchromosomal interactions. They also found many nearby duplex interactions (1036) in which two

anchor loci are interconnected to form a complex interaction. Interestingly, they found that most (86%) of the duplex interactions span a genomic region of less than 100 kb, while 13% span 100 kb to 1 Mb; however, the complex interactions have genomic spans in the range of 100 kb to 1 Mb. They were able to verify these results by additional ChIA-PET using a different antibody against ERα and further experimental confirmation by ChIP-qPCR, 3C, ChIP-3C, 4C, FISH, etc. Overall, the study found that most high-confidence remote ERα binding sites are indeed anchored through long-range chromosome interactions, supporting chromosome looping as a primary mechanism for ERα-mediated transcription.

ER-driven loop formation has been found to involve a number of additional proteins including cohesin, condensin, as well as some ERα cofactors (e.g., Mediator, LSD1) [106]. Cohesin and condensin are evolutionarily conserved protein complexes which form ring-like structures to keep DNA strands organized in close proximity during replication in preparation for mitosis [107]. Interestingly, it was found that they also play important roles in mediating the chromosomal looping between distal enhancers and promoters. Cohesin has been found to be involved in repressing gene expression through promoting CTCF-mediated insulator function, but it has also been found to work independently of CTCF to promote gene looping. Schmidt et al. [108] found that in MCF7 cells, cohesin can be recruited to distal EREs in an estrogen-dependent manner. Further, ERα and cohesin co-recruitment was more successful at predicting estrogen-responsive genes than ERα binding alone. Importantly, knocking down the cohesin subunit, Rad21, in MCF7 cells leads to a decrease in looping and expression of ERα target genes [109]. Similarly, the condensin 1 and 2 subunits NCAPG and NCAPH2 have been shown to be required for optimal activation of ERα target genes such as GREB1 and FOXC1. These subunits are localized to ERα-bound EREs in an estrogen-dependent manner and are required for looping of ERE enhancers and TSSs [104]. Several lines of evidence also support the involvement of the ERα coactivator Mediator complex in chromosome looping. The Mediator complex was shown to interact with the cohesin-loading protein Nipbl on the ERα target gene enhancers and promoters. In addition, RNAi-mediated knockdown of condensin subunits in MCF7 cells can also reduce the recruitment of ERα cofactor and Mediator subunit MED1 [104, 110, 111]. Moreover, it has been shown that in castration-sensitive prostate cancer cells, MED1 phosphorylation and activation are required for androgen receptor-dependent chromosome looping [112]. Although direct evidence for MED1 in ERα-mediated chromosome looping still remains to be seen, a similar mechanism is likely to exist. In addition, another recent study has also supported the involvement of another ERα cofactor LSD1 in ERα-mediated long-range chromatin interactions and looping [113]. Together these studies support chromosome looping as a key step involved in ERα-mediated transactivation from ERα-bound distal enhancers.

9 Enhancer RNA and ERα-Mediated Transcription

While chromosome looping provides an elegant model for how distal enhancers can feed into PolII activity at promoters to regulate ERα-mediated transcription, interestingly global ChIP experiments also found that these distal enhancer regions are highly enriched in active transcription marks and have high levels of RNA polymerase II recruitment [114]. Using the global run-on sequencing (GRO-seq) method to examine nascent transcripts, it has been found that noncoding RNAs are actively transcribed from the enhancer region in a regulated manner. These noncoding RNAs have been termed enhancer RNAs (eRNAs), although it has been debated as whether these are functional or merely indicative of active enhancers [115]. Comparing enhancer RNA (eRNA) expression data with data gathered from ChIP and chromosome conformation capture (3C) experiments across multiple tissue types, several patterns emerged. First, eRNAs are usually long and transcribed in both directions from the enhancer elements. These eRNAs tend to be expressed from enhancers with high levels of H3K4mono- and di-methylation, but not trimethylation, and high H3K27 acetylation. In addition, eRNA levels are highly correlated with the transcription levels of the cognate gene [116], and highly expressed enhancers participate in more chromosomal looping and have higher POLII occupancy levels. Functionally, knocking down eRNAs has been found not to reduce POLII recruitment to the enhancer elements, but does reduce RNA Pol II recruitment to the gene body and subsequent gene transcription [117]. Together, these data support that eRNAs could play important roles in gene expression and regulation.

Interestingly, estrogen-responsive enhancers are not only enriched with RNA Pol II and the activating histone mark H3K27ac but also express higher levels of enhancer RNAs (Fig. 3) [104, 118, 119]. The expression of these eRNAs, like the genes they regulate, also depends on estrogen stimulation. Li et al. [118] found that knockdown of ERα-driven enhancer RNAs causes a sharp decrease in the level of cognate mRNA expression, while tethering enhancer RNAs to an engineered enhancer region was sufficient to increase expression from that enhancer. The mechanism by which eRNAs impact expression of their cognate mRNAs is unclear but may involve chromosome looping. In vitro binding assays showed that E2-responsive eRNAs, but not a control RNA, could pull down cohesin subunits RAD21 and SMC3. Further, knockdown of enhancer RNAs caused a significant reduction in estrogen-induced localization of these cohesin subunits to the respective enhancer elements and caused a reduction in enhancer-promoter looping at ERα target genes such as NRIP1 and GREB1 [118]. These studies support the hypothesis that eRNAs may enhance transcription by stabilizing cohesins at promoter-enhancer looping sites. However, in another study, it was found that treatment of a global transcription inhibitor flavopiridol did not inhibit ERα-mediated chromosome looping or recruitment of coactivators in MCF7 cells [120]. Different conclusions from these studies could be due to different methods used (RNAi-mediated knockdown or transcription inhibition, respectively), but it is clear that

further understanding of molecular mechanisms of eRNA functions and their binding partners could provide deeper understanding into ERα-mediated transcription and functions.

10 Growth Factor Signaling Regulation of ERα Cistrome

Interestingly, studies have found that the ERα genome-wide binding cistrome can also undergo reprogramming in response to other signaling pathways such as EGF, PKA, and AKT. As we have discussed earlier in this chapter, the transcriptional activity of ERα can be regulated by distinct posttranslational modifications such as phosphorylation. However, how such changes impacted the ERα cistrome was not known. In one early study, Bhat-Nakshatri et al. investigated this by focusing on the phosphorylation of ERα by the serine/threonine kinase AKT/PKB [121], which is aberrantly activated in ~50% of human malignancies and plays key roles in antiestrogen resistance. Using ChIP-on-chip, they compared ERα binding sites in MCF7s transduced with a retroviral control vector (MCF-7p) or constitutively active AKT (MCF-7AKT). They found that while the control MCF7 cells and the AKT-overexpressing cells displayed a similar number of ERα binding sites (4349 and 4359, respectively), about 40% of ERα binding sites were unique to either cell line. This suggested that AKT activation has a significant effect on ERα cistrome. Importantly, they found a similar percentage of differentially expressed genes between these two cell types. These AKT-induced estrogen-regulated genes were linked to transforming growth factor β (TGF-β), NF-κB, and E2F pathways, which are known to be involved in tumor progression and antiestrogen resistance. These results highlight a unique role of AKT in modulating estrogen signaling that changes the landscape of ERα binding to the genome and the expression pattern of its downstream target genes.

Another such example is the regulation of the ERα cistrome by protein kinase A. PKA can phosphorylate ERα at two target sites Ser236 and Ser305. Importantly, the predominant phosphorylation of ERα is at position S305 and is known to induce a conformational arrest of ERα upon tamoxifen treatment, causing tamoxifen to act as an agonist and inducing breast cancer cell growth [122]. In fact, immunodetection of pS305 in tumor sections has been successfully used to identify tamoxifen-resistant breast cancer patients. To determine the effects of pS305 on ERα genome-wide binding, De Leeuw et al. [123] activated PKA by treating cells with forskolin and then performed ChIP-seq using anti-ERα-pS305 antibody. They found that ERα-pS305 shows 3327 binding events, of which only about 912 overlap with previously reported ERα binding sites. In addition, there was a striking enrichment for ERα-pS305 for promoter regions, 3′-UTRs and 5′-UTRs, in contrast to the total ERα that generally prefers distal enhancers. Further, only a subset of these PKA-induced pS305 binding sites overlap with above EGF-induced ERα binding sites, suggesting distinct ERα cistrome patterns can form through activation of different kinase pathways. By integrating ERα-pS305 chromatin binding and gene expression

analyses, De Leeuw and colleagues have identified a 26-gene signature of ERα-pS305 targets that significantly correlate with poor disease outcome in breast cancer patients [123].

ERα is also known to be stimulated by a variety of growth factors such as epidermal growth factor (EGF) in breast cancer cells. To assess the impact of EGF on the ERα cistrome, Lupien et al. compared EGF and estrogen-induced ERα cistromes in MCF7 breast cancer cells [124]. They found that both estrogen and EGF can induce ERα recruitment to many of the same sites as expected but EGF activation can also induce ERα recruitment to a significant number of unique sites. For the shared estrogen and EGF-induced ERα cistromes, there is an enrichment in Forkhead (FKH) motif binding sites, which is consistent with the central role of FoxA1 as a pioneer factor for ERα recruitment. However, the EGF-unique sites were instead enriched for AP-1 binding sites, suggesting a nonclassical tethering binding mode through AP-1 family members. Interestingly, the EGF-induced ERα cistrome is enriched in genes that are overexpressed in ERBB2-positive human breast cancers, and these EGF-specific ERα target genes are significantly associated with poor patient outcomes such as metastasis, recurrence, death, and high grade. These data are significant since hormone-refractory tumors are often dependent on the overexpression of the EGFR or ERBB2 and this study supported a new strategy to overcome such resistance through blocking both estrogen- and growth factor-stimulated activities. Together, the above studies not only highlight the dynamic regulation of the ERα cistrome by diverse signaling pathways but also reveal novel molecular mechanisms underlying antiestrogen resistance and potential new avenues for the development of innovative strategies to overcome such resistance.

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