The first decade of estrogen receptor cistromics in breast cancer

Koen D Flach and **Wilbert Zwart**

Division of Molecular Pathology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

Correspondence should be addressed to W Zwart
Email
w.zwart@nki.nl

Abstract

The advent of genome-wide transcription factor profiling has revolutionized the field of breast cancer research. Estrogen receptor α (ERα), the major drug target in hormone receptor-positive breast cancer, has been known as a key transcriptional regulator in tumor progression for over 30 years. Even though this function of ERα is heavily exploited and widely accepted as an Achilles heel for hormonal breast cancer, only since the last decade we have been able to understand how this transcription factor is functioning on a genome-wide scale. Initial ChIP-on-chip (chromatin immunoprecipitation coupled with tiling array) analyses have taught us that ERα is an enhancer-associated factor binding to many thousands of sites throughout the human genome and revealed the identity of a number of directly interacting transcription factors that are essential for ERα action. More recently, with the development of massive parallel sequencing technologies and refinements thereof in sample processing, a genome-wide interrogation of ERα has become feasible and affordable with unprecedented data quality and richness. These studies have revealed numerous additional biological insights into ERα behavior in cell lines and especially in clinical specimens. Therefore, what have we actually learned during this first decade of cistromics in breast cancer and where may future developments in the field take us?

Key Words

- breast cancer
- cistromics
- estrogen receptor
- ChIP-seq

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Introduction

Breast cancer is the most prevalent form of cancer in women, with approximately 1.7 million annual new diagnoses (Ferlay *et al.* 2015). Despite the improvement of breast cancer treatment, still over half a million women die of this disease every year (Ferlay *et al.* 2015). Approximately 70% of breast tumors are estrogen receptor α (ERα) positive, and tumor cell proliferation is thought to be dependent on the activity of this hormone-mediated transcription factor (Hayashi *et al.* 2003, Dahlman-Wright *et al.* 2006).

The first evidence for a link between estrogens (produced in the ovaries) and breast cancer was reported by George Thomas Beatson in 1896 with a case report describing a premenopausal breast cancer patient with metastatic disease (Beatson 1896). Although not aware of the exact mechanisms of hormonal action in human physiology, Beatson was familiar with a procedure performed in cattle in which lactation after giving birth can be extended by removal of the ovaries. Inspired by this phenomenon, Beatson performed a bilateral oophorectomy on his patient, which initially resulted in a complete remission of the disease (Beatson 1896, Thomson 1902). The protein responsible for this clinical benefit was found almost 80 years later, with the seminal discovery of the estrogen receptor (ER) in 1973 by Elwood Jensen (Jensen & DeSombre 1973). After first being cloned

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in 1985 (Walter *et al.* 1985), in 1986 a complementary DNA clone of the translated mRNA of the ER from MCF-7 human breast cancer cells was sequenced and upon expression gave rise to a functional protein (Greene *et al.* 1986).

Today, ERα is recognized as the major drug target in hormonal breast cancer. In the adjuvant treatment of ERα-positive disease, receptor inhibition is achieved by either a direct blockage of ERα activation through competitive inhibition of estradiol association using tamoxifen (Katzenellenbogen *et al.* 1985, Jordan & Murphy 1990, Arpino *et al.* 2009) or by preventing estrogen synthesis using aromatase inhibitors (Fabian 2007). Despite the extensive use of these treatment modalities in adjuvant therapy, a significant number of patients still develop a recurrence (Early Breast Cancer Trialists' Collaborative *et al.* 2011). Although cross-resistance between the different endocrine therapy options can occur, patients that relapse on one type of endocrine therapy can still benefit from a different treatment modality (Vergote *et al.* 2006, Wang *et al.* 2009, Yoo *et al.* 2011), suggesting that multiple resistance mechanisms can exist that may be treatment selective. In order to directly administer the right drug to the right patient, it is vital to increase our knowledge about ERα functioning as well as its selective responses to prolonged exposure to hormonal agents.

Even though ERα inhibitors have been used in the clinic since the early 1980s, the direct mode of ERα's genomic action on a genome-wide scale has remained elusive for many years. With the initial development of ChIP-on-chip (chromatin immunoprecipitation coupled with tiling array) technologies, this situation changed dramatically with the interrogation of ERα action for the first time on a human chromosome-wide scale (Carroll *et al.* 2005). With the development of massive parallel high-throughput sequencing techniques, a full genome coverage of ERα became possible (and importantly affordable) through ChIP sequencing (ChIP-seq) (Welboren *et al.* 2009). Now, 10 years after the first unbiased and systemic assessment of ERα-binding sites in human cell lines, we will discuss what we have learned from the cistromics of ERα and where future developments might take us.

ER complex formation and its mode of action

ERα is activated through the association of its natural ligand estradiol with the receptors' ligand-binding domain, which enables dissociation from chaperone protein Hsp90 (Catelli *et al.* 1985, Pratt & Toft 1997, Devin-Leclerc *et al.* 1998) and facilitates ERα-chromatin interactions (Kumar & Chambon 1988). Initial ChIP-on-chip experiments have shown ERα to mainly bind enhancer regions (Carroll *et al.* 2005). Computational DNA sequence motif analyses of ERα-binding sites resulted in the identification of a number of upstream transcription factors that facilitate the binding of ERα to the chromatin, including pioneer factor FOXA1 (Carroll *et al.* 2005, Hurtado *et al.* 2011) and putative pioneer factors PBX1 (Magnani *et al.* 2011) and AP-2γ (Tan *et al.* 2011) (Fig. 1). Pioneer factors can associate with compacted chromatin and trigger enhancer competency by decondensing the chromatin, facilitating the binding of additional chromatin-binding factors (Zaret & Carroll 2011, Jozwik & Carroll 2012). Additionally, ERα-cooperating transcription factor GATA3 is capable of mediating enhancer accessibility at ERα regulatory regions and has properties similar to FOXA1 (Kong *et al.* 2011, Theodorou *et al.* 2013). Besides binding directly to the DNA, ERα can also associate with the chromatin via other transcription factors, a mechanism also known as tethering, including RUNX1 (Stender *et al.* 2010) and AP-1 (Umayahara *et al.* 1994, Kushner *et al.* 2000, Cheung *et al.* 2005).

After activation, ERα undergoes a conformational change (Paige *et al.* 1999), forming a coactivator-binding pocket at the receptors' carboxy-terminus (Shiau *et al.* 1998). This interaction surface subsequently leads to the recruitment of the members of the p160 coactivator family: SRC1 (NCOA1; Onate *et al.* 1995), SRC2 (NCOA2, TIF2, GRIP1; Voegel *et al.* 1996, Hong *et al.* 1997), and SRC3 (NCOA3, p/CIP, AIB1, ACTR; Anzick *et al.* 1997, Chen *et al.* 1997, Torchia *et al.* 1997, Suen *et al.* 1998). The binding of these SRCs to the coactivator-binding pocket of activated ERα has been described to occur both in a competitive manner (exclusive recruitment of one type of SRC) (Shiau *et al.* 1998, Margeat *et al.* 2001, Carraz *et al.* 2009) and in a joint manner, possibly through heterodimerization (Zhang *et al.* 2004). Reports on the exact stoichiometry within the p160/ERα complex are conflicting, describing a single p160 to associate with an ERα dimer (Margeat *et al.* 2001) or two SRCs per active ERα complex (Zhang *et al.* 2004, Yi *et al.* 2015), although both situations might occur side to side (Zhang *et al.* 2004). Recently, it has been shown, for SRC3, that these ERα interactions occur in a monomeric fashion, where two ligand-bound ERα monomers individually recruit one SRC3 protein, after which an ERα dimer (binding two SRC3 molecular) associates with a single p300 protein (Yi *et al.* 2015). The p160 composition of the ERα transcriptional

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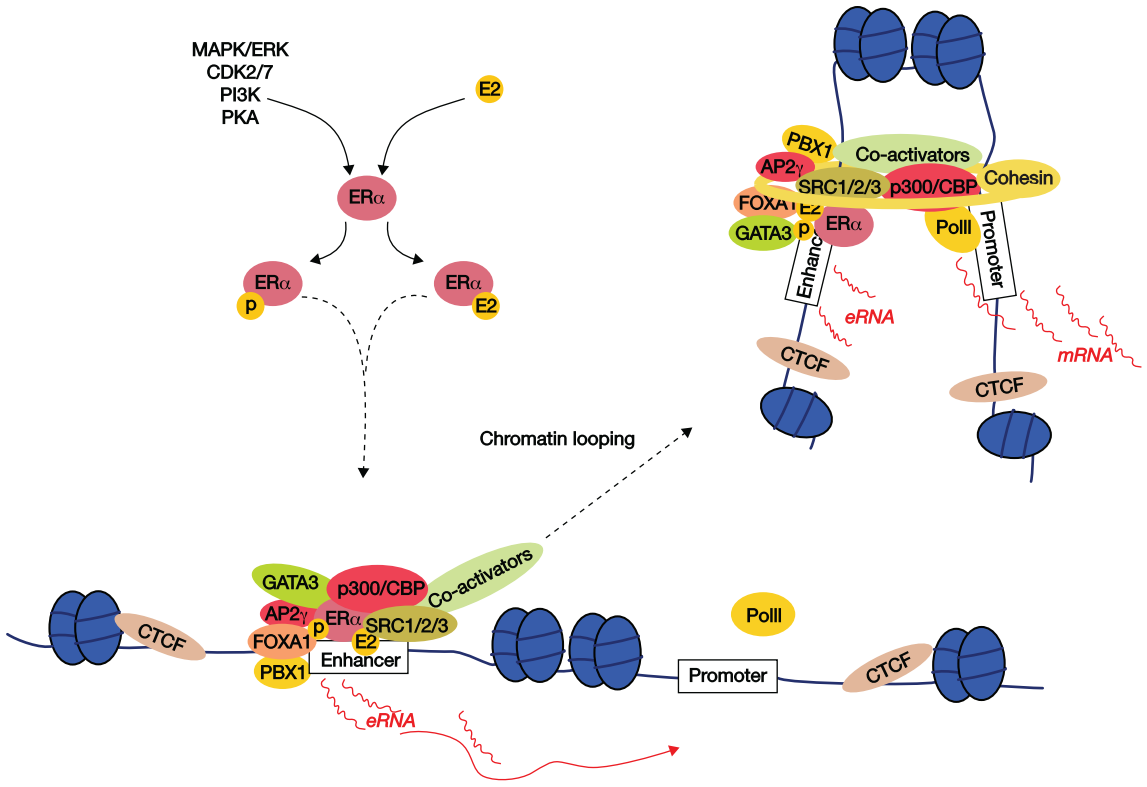

Figure 1
The ER α transcriptional complex pathway. When activated by its natural ligand estradiol or by direct phosphorylations, ERα binds to enhancers made accessible by pioneer factors (*e.g.* FOXA1). A transcriptional complex, including p300, CBP, SRC1-2-3, and other coactivators, is assembled and enhancer RNAs are transcribed. After cohesin-stabilized chromatin looping to associated gene promoters, RNA polymerase II is recruited and an active transcriptional complex is formed, capable of transcribing associated genes.

complex influences its genomic binding preferences on a genome-wide scale, consequently resulting in an altered transcriptional repertoire (Zwart *et al.* 2011) and altered phenotypic behavior (Fig. 2).

After ERα binding, p160 proteins can subsequently recruit other essential proteins for transcriptional regulation, including p300 and CBP (McKenna *et al.* 1999), which can modify chromatin accessibility through their acetyltransferase activity (Ogryzko *et al.* 1996). In order to further modify the chromatin toward a transcription favorable landscape, histone modifiers CARM1 (Chen *et al.* 1999*a*, Stallcup *et al.* 2000) and JMJD2B (Kawazu *et al.* 2011, Shi *et al.* 2011) and members of the SWI/SNF chromatin remodeling complex, including BAF57, are recruited (Belandia *et al.* 2002).

With the recent discovery of estradiol-induced enhancer RNAs (eRNAs) at a set of ~1200 ERα-bound enhancer elements (Hah *et al.* 2013, Li *et al.* 2013), an additional layer of ERα biology was revealed. This eRNA production is not just limited to ERα-bound enhancers but is, for example, also apparent for the androgen receptor (AR; Wang *et al.* 2011*a*) and p53 (Melo *et al.* 2013). DNAse I sensitivity assays demonstrated that eRNAs are capable of regulating genomic access of the transcriptional complex to regulatory regions (Mousavi *et al.* 2013). eRNAs found at ERα-binding sites strongly correlated with the enrichment of a number of genomic features associated with enhancers and enhancer looping to target gene promoters (Hah *et al.* 2013). The physiological relevance of eRNAs in ERα biology was further stipulated by the observation that knockdown of a subset of eRNAs (*e.g.* GREB1 enhancer) reduced the transcription of coding gene transcripts, as well as reducing promoter–enhancer interactions as shown by chromosome conformation capture (3C; Li *et al.* 2013), although conflicting 3C results have also been described (Hah *et al.* 2013). Hah and coworkers found that inhibition of eRNA production by flavopiridol, a CDK9 inhibitor blocking transcriptional elongation, did not affect other indicators of enhancer activity or estradiol-dependent promoter–enhancer looping (Hah *et al.* 2013), leaving the exact role of eRNAs somewhat elusive. These

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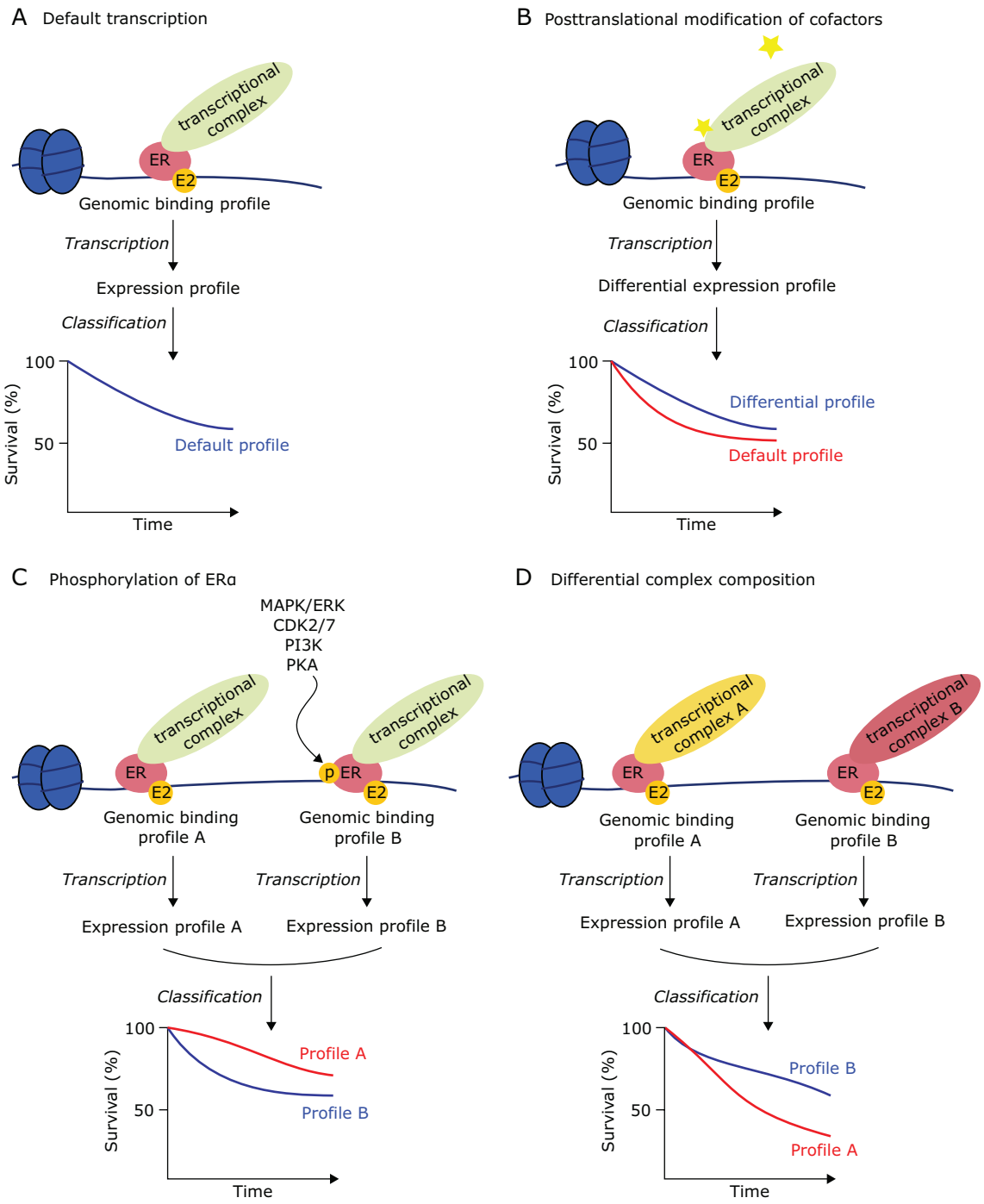

Figure 2
ERα transcriptional complex composition, genomic profile, and transcriptional output. Illustration of ERα-induced transcription, where the genomic binding profile of ERα's transcriptional complex leads to induced transcription and an expression profile on the basis of which a classification profile can be made (A). These genomic, transcriptional, and classification profiles can be altered by posttranslational modifications of cofactors (B), phosphorylations on ERα itself (C), and the composition of the transcriptional complex (D).

eRNA-associated promoter–enhancer interactions, also known as chromosomal looping structures, have been described to promote ERα-regulated gene transcription and seem to be stabilized by Cohesin (Fullwood *et al.* 2009, Schmidt *et al.* 2010, Li *et al.* 2013). Although these observations hint toward an important role for eRNAs

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in ERα-regulated transcription, only a subset of eRNAs has yet been investigated thoroughly, with conflicting roles in chromosomal looping, leaving the exact physiological roles for them currently elusive.

After ERα has recruited its cofactors, an active transcriptional complex can be formed by RNA polymerase II recruitment and transcription of responsive genes can be initiated (Glass *et al*. 1997) (Fig. 1). When treated with tamoxifen, the ligand-binding domain of ERα adopts an alternative conformation, impairing the docking of p160 proteins to ERα, preventing the correct assembly of the transcriptional complex (Shiau *et al*. 1998).

The genome-wide kinetics with which the ERα complex assembles on the chromatin is not yet fully understood. By using ChIP at three ERα-responsive gene promoters, Shang and coworkers have reported that ERα and a number of its coactivators associate on these estrogen responsive promoters in a cyclic fashion and that these cycles of ERα-complex assembly are followed by transcription (Shang *et al*. 2000). This cyclic recruitment of ERα and its coregulators could be confirmed by others, who reported cofactor recruitment to be preceded by histone deacetylases and nucleosome-remodeling complexes at the TFF1 promoter (Metivier *et al*. 2003). These data imply that transcriptional activation of ERα-responsive genes may require both activating and repressive epigenetic processes. Although both papers state that ERα-induced transcriptional activation occurs in a cyclic fashion, both papers only investigated the dynamic nature of ERα on a couple of sites and a comprehensive overview of ERα dynamics on a genome-wide scale is currently lacking. Furthermore, whether this cyclic ERα complex assembly occurs only on promoters, as studied in both papers, or whether it is also apparent at ERα-bound enhancers remains unclear.

ERα cistromics in breast cancer cell lines

Initially, most reports on ERα chromatin interactions, its dynamics and recruitment of coregulators were centred on single binding site-based analyses, often limited to the TFF1 promoter. With the technological development of tiling arrays, ERα genomic interactions could reliably be assessed on a chromosome-wide scale (Carroll *et al*. 2005). As technology progressed, this approach was quickly succeeded by massive parallel sequencing technologies, enabling the interrogation of ERα sites on a genome-wide scale in a cost-effective manner (Welboren *et al*. 2009). These initial reports resulted in a huge paradigm shift, completely changing the way we think about ERα genomics. These studies illustrated that even though most pioneering studies on ERα genomics exclusively interrogated promoters, this genomic behavior of ERα clearly represents an exception. In fact, only a small proportion of about 5% of ERα-binding sites was found at gene promoters; a characteristic feature that has been validated by others (Carroll *et al*. 2005, Zwart *et al*. 2011) and is also apparent for other nuclear receptors, including AR (Yu *et al*. 2010) and glucocorticoid receptor (GR; Reddy *et al*. 2009). Approximately 95% of all ERα-binding sites are found at distal *cis*-regulatory elements (hence designated as 'cistromics' (Lupien *et al*. 2008)) that were later recognized as enhancer regions. These regions are putative regulatory elements and might not all be functional. Recently, a CRISPR-Cas9 screen has been used to functionally asses ERα enhancer elements and their effect on cell proliferation (Korkmaz *et al*. 2016). Out of the 99 ERα-bindings sites that were targeted, the deletion of only three of them affected cell proliferation, further illustrating that only a subset of ERα-bindings sites at cistromics might actually be functionally involved in cell proliferation processes.

The discovery of enhancer preference for ERα-binding repositioned the classical promoter-centered ERα studies considerably on the level of physiological extrapolation. Chromatin interaction analysis by paired-end tag (ChIA-PET) sequencing analyses, which enable the identification of long-range chromatin interactions, illustrated that the distal enhancer-associated ERα-bindings sites were found to loop to anchor genes through connections with proximal ERα-binding sites, suggesting that ERα functions by bringing genes together for coordinated transcriptional regulation by extensive chromatin looping (Fullwood *et al*. 2009). At the GREB1 and TFF1 loci, this chromatin looping was dependent on ERα expression and was inducible by estradiol stimulation (Pan *et al*. 2008, Fullwood *et al*. 2009). Probing the 3D architecture of the genome by coupling proximity-based ligation with massively parallel sequencing (Hi-C; Lieberman-Aiden *et al*. 2009) yielded similar ERα-mediated enhancer–promoter interactions (Mourad *et al*. 2014). These sites of chromatin looping highly correlated with CCCTC-binding factor (CTCF)-binding sites, suggesting CTCF to play a key role defining the boundaries of chromosomal territories and influence gene expression through cross talk between promoters and regulatory elements (Splinter *et al*. 2006, Botta *et al*. 2010, Handoko *et al*. 2011). Besides ERα, these chromatin loops have also been observed for other nuclear transcription factors, including AR (Wang *et al*. 2005) and GR (Hakim *et al*. 2009).

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On the transcriptomic level, the use of global nuclear run-on sequencing (GRO-seq; Core *et al*. 2008) analysis increased our understanding of ERα-regulated transcription by identifying primary and immediate estrogen-induced effects as opposed to steady-state transcript level analyses (Hah *et al*. 2011). GRO-seq demonstrated that estrogen is able to regulate the activity of all three RNA polymerases and led to the discovery of previously undetected estrogen-regulated intergenic transcripts (Hah *et al*. 2011). Transcription profiling by GRO-seq could be used for the prediction of *de novo* enhancers across various cell types (Hah *et al*. 2013). In combination with RNA-seq, GRO-seq was able to annotate long noncoding RNAs (lncRNAs) and characterized the lncRNA transcriptome in MCF-7 breast cancer cells, including over 700 previously unannotated lncRNAs (Sun *et al*. 2015). Furthermore, GRO-seq analysis at ERα enhancers revealed the existence of estradiol-induced unidirectional and bidirectional eRNAs, which were strongly correlated with enhancer–promoter looping (Hah *et al*. 2013). The described role of these intergenic transcripts in enhancer–promoter looping (Fullwood *et al*. 2009, Schmidt *et al*. 2010, Li *et al*. 2013) and the fact that one promoter can be involved in multiple enhancer-associated loops (Fullwood *et al*. 2009, Mourad *et al*. 2014) might explain the seemingly large discrepancy between the number of ERα-regulated genes (approximately 2000; Zwart *et al*. 2011) in relation to the number of ERα-binding sites in the same cell line (>10,000; Welboren *et al*. 2009, Hurtado *et al*. 2011).

Due to technical limitations in the ChIP-seq protocol, the resolution of DNA-binding analyses is typically quite limited with events being mapped with ±300 base pairs. Further refinement of the ChIP-seq procedure has led to the implementation of lambda exonuclease digestion in the protocol (ChIP-exo), enabling high-resolution mapping of chromatin binding and identification of unique transcription factor binding sites that could not be identified by ChIP-seq (Rhee & Pugh 2011, 2012, Serandour *et al*. 2013). The addition of exonucleases also results in the degradation of contaminating DNA, effectively lowering the required depth of sequencing coverage.

Apart from forming the foundations of *cis*-regulatory gene regulation, chromatin looping and eRNA action, genome-wide profiling analyses of ERα sites can also lead to the identification of additional transcription factor motifs often co-enriched at ERα sites and proximal to estrogen response elements. These motif analyses revealed the presence of Forkhead binding motifs at roughly 50% of ERα-bindings sites (Carroll *et al*. 2005). This observation led to the discovery that FOXA1 is essential for chromatin accessibility at ERα sites and crucial for ERα binding and functionality (Carroll *et al*. 2005, Hurtado *et al*. 2011). More recently, this same approach has been used to identify other putative pioneer factors for ERα, including PBX1 that can guide ERα to a specific subset of sites (Magnani *et al*. 2011). When investigating the motifs of ERα-bindings sites identified by ChIA-PET, Tan and coworkers found that approximately 40% of these binding sites contained the AP-2 motif (Tan *et al*. 2011). They next demonstrated that transcription factor AP-2γ can bind to these ERα-bindings sites in a ligand-independent manner and there is a functional interplay between AP-2γ and FOXA1 (Tan *et al*. 2011).

Besides the interplay between ERα and its pioneer factors and coregulators, it is becoming increasingly apparent that a complex interplay exists between different steroid hormone receptor family members. The AR, a transcription factor classically known for its oncogenic role in prostate cancer, is expressed in 84–95% of the ERα-positive breast cancers (Niemeier *et al*. 2010, Qi *et al*. 2012, Chia *et al*. 2015) and is usually associated with a favorable outcome (Peters *et al*. 2009, Castellano *et al*. 2010, Hu *et al*. 2011). Exogenous overexpression of AR inhibits ERα transactivation activity and estrogen-induced cell growth (Ando *et al*. 2002, Peters *et al*. 2009), which may be explained by a direct competition between ERα and AR at binding the same genomic regions (Peters *et al*. 2009). This notion was further strengthened by ChIP analysis showing AR recruitment to the progesterone receptor (PR) promoter in T47D cells (Peters *et al*. 2009).

Another steroid hormone receptor family member known for its coexpression and favorable outcome in ERα-positive breast cancers is the PR (Pichon *et al*. 1980, Blows *et al*. 2010). Progesterone induces the association of PR with ERα, thereby regulating ERα–chromatin interactions and transcriptional activity, providing mechanistic insights behind the clinical implications of PR status in ERα-positive tumors (Mohammed *et al*. 2015).

The GR, in the presence of dexamethasone, is able to associate with similar binding regions as ERα, and GR stimulation leads to reduced transcription of key ERα target genes (Meyer *et al*. 1989, Karmakar *et al*. 2013). This direct protein–protein interaction between GR and ERα can play an important role in the GR-mediated growth inhibition of ERα-positive cells (Karmakar *et al*. 2013). Besides this general inhibitory role of GR, gene-specific regulation with both cooperation and antagonism

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has also been described (Bolt *et al*. 2013). Apart from direct physical interactions between nuclear receptors, nuclear receptors can also inhibit each other's activity through cross-interference ("squelching"), where direct competition for cofactor recruitment can inhibit nuclear receptor activity without associating with the same genomic regions (Cahill *et al*. 1994, Lopez *et al*. 1999).

Cistromics of ERα coregulators

To date, several studies have compiled an overview of ERα coregulators and interacting proteins, with numbers varying around 17 (Foulds *et al*. 2013) to 108 (Mohammed *et al*. 2013). The p160 protein family members are reproducibly and consistently identified as part of the ERα complex, for which a level of mutual exclusivity has been described for ERα binding (Shiau *et al*. 1998, Margeat *et al*. 2001, Carraz *et al*. 2009). With the recent finding that an activated ERα dimer can bind one p300 protein (Yi *et al*. 2015) and p300 and CBP have a substantial overlap of ~70% in binding sites (Zwart *et al*. 2011), it is not unlikely that a level of mutual exclusivity between p300 and CBP also exists. As a direct consequence thereof, the composition of ERα complexes can differ between different sites on a genome-wide scale, with potentially far-reaching consequences on gene expression profiles. Cistromic analyses of the p160 family members illustrated that even though most genomic sites are shared among SRC1, SRC2, and SRC3, distinct subsets of sites were identified where gene expression was selectively responsive to one specific p160 protein, as part of the ERα complex (Zwart *et al*. 2011). Interestingly, the gene profile under the control of ERα with exclusively SRC3 binding (devoid of SRC1 or SRC2) had prognostic potential and enabled the identification of breast cancer patients with a poor outcome after tamoxifen treatment (Zwart *et al*. 2011). This link between SRC3 gene targets and tamoxifen treatment is in line with previous reports describing increased SRC3 expression, in combination with increased ERBB2 expression, to correlate with a poor tamoxifen response (Osborne & Schiff 2003, Shou *et al*. 2004, Hurtado *et al*. 2008, Zhao *et al*. 2009). Another ERα-interacting protein that can affect ERα complex formation and gene expression is the transcriptional regulator RIP140 (Rosell *et al*. 2014). Genes under the specific control of RIP140 (identified by siRNA experiments) could be used to classify tamoxifen-treated patients on clinical outcome (Rosell *et al*. 2014). Both RIP140 and the p160 family members further stipulate the observation that the composition of the transcriptional complex may differ on a genome-wide scale, which could have direct physiological consequences on the level of transcriptional output and clinical response (Fig. 2).

ERα phosphorylations and genome-wide effects on ERα action

Besides the composition of the transcriptional complex, phosphorylations on ERα can also regulate the transcriptional activity of the receptor and play a crucial role in endocrine resistance (Joel *et al*. 1998, de Leeuw *et al*. 2013). These phosphorylation events mainly revolve around serine residues 104/106 (Thomas *et al*. 2008), 118 (Kok *et al*. 2009), 167 (Yamashita *et al*. 2005), 236 (Atsriku *et al*. 2009), and 305 (Michalides *et al*. 2004). The kinases involved in phosphorylation of ERα at s104/106 include CDK2 and ERK1/2 (Rogatsky *et al*. 1999, Thomas *et al*. 2008); at s118 ERK1/2, EGFR, and IGF1R (Park *et al*. 2005, Santen *et al*. 2009); at s167 AKT and CK2 (Arnold *et al*. 1994, Campbell *et al*. 2001); at s236 PKA (Chen *et al*. 1999*b*); and at s305 PAK1 and PKA (Wang *et al*. 2002, Michalides *et al*. 2004). The clinical implications of these phosphorylations remain not fully understood, where higher expressions of s118 and s167 phosphorylations are generally but not uniformly associated with a favorable outcome in patients on tamoxifen therapy (Murphy *et al*. 2004, 2011, Jiang *et al*. 2007, Yamashita *et al*. 2005, 2008), whereas the s305 phosphorylation is associated with a poor clinical outcome (Kok *et al*. 2011, Murphy *et al*. 2011). Furthermore, s118 phosphorylation expression appears to be a predictive biomarker for tamoxifen response (Murphy *et al*. 2004, Kok *et al*. 2009). Recently, the phosphorylation on the 594 threonine (t594) residue of ERα was found to play a key role in the regulatory interaction of ERα with 14-3-3 proteins (De Vries-van Leeuwen *et al*. 2013). This t594 phosphorylation resulted in decreased estradiol-stimulated ERα dimerization, reduced ERα–chromatin interactions, and reduced gene expression (De Vries-van Leeuwen *et al*. 2013).

The spectrum of ERα phosphorylation events appears to be able to dictate differential transcriptional programs of ERα, as exemplified by the PKA-induced s305 phosphorylation that redirects ERα to differential transcriptional start sites, translating into a 26-gene expression classifier that identified patients with a poor clinical outcome after tamoxifen treatment (de Leeuw *et al*. 2013). Additionally, it was found that stimulation of ERα by EGF, which induces s118 phosphorylation

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(Bunone *et al*. 1996), led to a distinct cistromic landscape and induced a unique set of genes, compared to estradiol stimulation (Lupien *et al*. 2010). Stimulation of ERα by AKT, capable of inducing s167 phosphorylation (Campbell *et al*. 2001), also mediated changes in ERα chromatin binding and altered its transcriptional output (Bhat-Nakshatri *et al*. 2008), further indicating that specific phosphorylations of ERα may yield distinct genomic actions and may target unique locations throughout the genome (Fig. 2). Although the binding patterns of some of the phosphorylated ERα forms are known, a complete and comparative overview is still lacking. Furthermore, multiple reports have studied ERα cistromics upon activation of a specific cellular signaling cascade, including the previously mentioned AKT or EGF, where it still remains elusive which specific variable is actually responsible for the altered ERα behavior.

Besides the effect direct ERα phosphorylations can have on ERα's genomic landscape and transcriptional activity, posttranslational modifications of coregulators can also influence ERα action. Where ERα-bound SRC3 binding is predominantly enhancer bound, phosphorylated SRC3 at Ser543 (pSRC3) was selectively found at promoters of ERα-regulated genes (Zwart *et al*. 2015). pSRC3 functioned as an independent prognostic factor as well as a predictive marker for tamoxifen treatment, potentially enabling the identification of patients with a good clinical outcome without receiving adjuvant therapy (Zwart *et al*. 2015). Additionally, SRC2 can be phosphorylated at Ser736 through the MAPK pathway, increasing SRC2 interactions with p300 and CBP, further facilitating SRC2 recruitment to the ERα complex (Lopez *et al*. 2001). These posttranslational modifications on coregulators further illustrate the intrinsic complexity and flexibility of ERα transcription complex formation, where multiple cell signaling cascades converge to collaboratively fine-tune ERα action on a genome-wide scale (Fig. 2).

Cistromic analyses in clinical samples and potential clinical applications

Over recent years, the transition has been made from studying ERα cistromics in cell lines toward genomic interrogation of ERα sites in clinical specimens. Obviously, in contrast to cell lines, clinical samples cannot be readily manipulated and represent heterogeneous populations of multiple cell types. Even with this difference between tumors and cell lines, the cistromic information obtained from both settings yields quite similar conclusions. When looking at ERα, most well-described ERα-binding sites found in MCF-7 cells (Carroll *et al*. 2005, Welboren *et al*. 2009) such as enhancer regions proximal to RARA, GREB1, XBP1, and TFF1 are also observed in tumor specimens (Ross-Innes *et al*. 2012). Not only for ERα but also for its coregulators, the overlap of chromatin binding in cell lines versus clinical specimens was considerably high. For example, SRC3–pS543 ChIP-seq analyses showed 51% overlap in binding sites between MCF-7 cells and an ER+/PR+ breast tumor, being on the same order of magnitude as found between two tumor samples (61% overlap; Zwart *et al*. 2015).

The first analyses of ERα-binding patterns in clinical samples directly illustrated the added value of assessing ERα binding in clinical specimens (Ross-Innes *et al*. 2012), where differential ERα-binding sites found between tumors could stratify patients on outcome (Ross-Innes *et al*. 2012). A more recent study identified ERα–chromatin-binding patterns in primary breast tumors that enabled patient classification on their response to aromatase inhibition in the metastatic setting (Jansen *et al*. 2013). This same report analyzed profiles for H3K27me3, resulting in a gene classifier that seemed to outperform other prognostic classifiers, including Oncotype DX (Cobleigh *et al*. 2005) and PAM50 (Parker *et al*. 2009). As the classification potential of these genes was only partially preserved in a cohort of tamoxifen-treated patients, this suggests some treatment selectivity for patient classification. Both studies demonstrate clear advantages of studying ERα cistromic analyses in clinical specimens, with the potential to facilitate tailored therapy selection and enable patient stratification on outcome.

Although these cistromic classifiers made use of associated gene profiles, it remains largely unknown which genes in these classifiers are now the driving force behind any prognostic or predictive effect. Fine-tuning these classifiers toward optimized gene sets and further biological investigation of these genes could reveal the biologically most relevant genes for disease progression and might lead to novel biological insights in ERα biology as well as potentially novel drug targets.

As the main function of ERα is to activate its downstream target genes involved in tumor progression, ERα cistromic analyses may yield novel drug targets. A key example for this line of thought can be found in Myc, representing one of the best-studied ERα-responsive genes (Dubik *et al*. 1987, Dubik & Shiu 1988, Watson *et al*. 1991) and widely accepted as a potent novel drug target in cancer (Soucek *et al*. 2008, Albihn *et al*. 2010).

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Besides targeting ERα-regulated genes to inhibit its stimulatory effect, ERα cofactors also receive increasing attention as potential drug targets. Small-molecule inhibitors against both SRC1 and SRC3 (Wang *et al.* 2011*b*, 2014) or SRC3 alone (Yan *et al.* 2014), as well as a stimulator for SRC3 activity (Wang *et al.* 2015), have been recently identified and proved successful in inhibiting breast cancer cell proliferation *in vitro* as well as in xenograft mouse models. Such novel therapeutic options could revolutionize endocrine therapeutic drug design, not aiming at blocking the receptor itself, but targeting the proteins required for receptor action. As in case of endocrine therapy resistance ERα can still remain a driver (Vergote *et al.* 2006, Wang *et al.* 2009, Yoo *et al.* 2011), such novel inhibitors have the potency to remain effective after progression on currently available endocrine therapies.

Even though promising, at the moment there are no cistromic classifiers being used in the clinic. One of the major practical limitations is the typically low amounts of available tumor tissue. Although initially challenging, continuing technical developments, including single-tube linear DNA amplification method (Shankaranarayanan *et al.* 2011) and the combination of a high-sensitivity ChIP assay with new library preparation procedures (Adli *et al.* 2010), have now greatly increased the applicability of ChIP-seq on limited amounts of tissue. Another example of these developments is the incorporation of carrier chromatin that can be removed before library preparation, improving ChIP efficiency while limiting background signal (Zwart *et al.* 2013). Furthermore, a great promise for the future of ChIP-seq on limited tumor material might be found in the combination of microfluidics and DNA barcoding and sequencing, which have recently enabled the generation of ChIP-seq data at a single-cell resolution (Rotem *et al.* 2015).

Discussion

Within 10 years, ERα genomics has gone from single locus to genome wide and toward single cell. Initial reports on ERα cistromics in breast cancer have revolutionized the way we think about ERα action and ERα-responsive genes. By far, most transcriptional effects found regulated by ERα are represented as eRNAs. With conflicting reports about the role of eRNAs in chromosomal looping, a comprehensive overview of eRNA action, and with this to a certain degree a functional overview of ERα-enhancer action, is currently lacking. As ERα seems to function mostly through chromatin loops, it is not unlikely that ERα enhancers and a subset of responsive eRNAs are functionally involved in such looping structures.

In ERα-positive breast cancer cell lines and tumors, many thousands of ERα-binding sites can be found, of which a large number are shared between them. This could imply a selection pressure throughout human evolution for the maintenance of these ERα sites throughout the human genome. As technological development continues, future studies will further elucidate the functional relevance of all these ERα sites and identify the genomic regions responsible for proliferative potential.

Clearly, our knowledge on ERα genomic regulation in breast cancer has increased exponentially over the last decade. A major factor in this is the parallel development of novel technologies and computational tools, which not only enable us to generate genomic data with an unprecedented level of data richness and detail but also enable us to process and understand the data. Now, with novel technologies on genome editing (*e.g.* CRISPR Cas9) and single-cell ChIP-seq analyses, the second decade of cistromics in breast cancer will no doubt unveil another layer of unprecedented complexity in breast cancer and may lead us toward a comprehensive understanding of the disease with its full genomic complexity and diversity.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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