Chromatin Reprogramming in Breast Cancer

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Abstract

Reprogramming of the chromatin landscape is a critical component to the transcriptional response in breast cancer. Effects of sex hormones such as estrogens and progesterone have been well described to have a critical impact on breast cancer proliferation. However, the complex network of the chromatin landscape, enhancer regions, and mode of function of steroid receptors (SRs) and other transcription factors (TFs), is an intricate web of signaling and functional processes that is still largely misunderstood at the mechanistic level. In this review, we describe what is currently known about the dynamic interplay between TFs with chromatin and the reprogramming of enhancer elements. Emphasis has been placed on characterizing the different modes of action of TFs in regulating enhancer activity, specifically, how different SRs target enhancer regions and reprogram chromatin in breast cancer cells. In addition, we discuss current techniques employed to study enhancer function at a genome-wide level. Further, we have noted recent advances in live cell imaging technology. These single cell approaches enable the coupling of population based assays with real-time studies to address many unsolved questions about SRs and chromatin dynamics in breast cancer.

INTRODUCTION

Transcriptional regulation is one of the most important biological processes in organisms, contributing to every aspect of health and disease. The regulation of gene expression is a complex process involving a multitude of proteins, including transcription factors (TFs), cofactors and RNA polymerases. These proteins bind and interact with specific regulatory elements termed enhancers on chromatin. Here the transcriptional regulation activity is influenced through a number of different processes (Long, et al. 2016; Voss and Hager

2014). The enhancer landscape and the accessibility of chromatin at enhancers is constantly changing during cellular development and differentiation (Chronis, et al. 2017). Interestingly, the enhancer landscape in all developed cells appears to represent subsets of a general population of enhancers identified in embryonic stem cells (Stergachis, et al. 2013). In many cases, TFs reprogram the chromatin landscape by altering the chromatin accessibility, thereby influencing the activity and recruitment of other TFs, cofactors, and RNA polymerases. The cofactors recruited by TFs can possess a variety of enzymatic properties, such as histone-modifying and ATP-dependent chromatin remodeling activities. These events have a crucial role in regulating chromatin accessibility (Long et al. 2016; Murakami, et al. 2017; Perissi and Rosenfeld 2005; Yi, et al. 2017). Reprogramming ensures accurate regulatory TF binding and subsequent regulation of cell-type specificity. Abnormal alterations in the activity of TFs can aberrantly program the chromatin landscape leading to many different disease states (Smith and Shilatifard 2014). The most drastic examples of abnormal alterations of TF binding and chromatin accessibility occurs in cancers (Denny, et al. 2016; Qu, et al. 2017). Breast cancer is no exception, with alterations occurring in chromatin accessibility, TF action, and regulation (D'Antonio, et al. 2017; Jeselsohn, et al. 2015; Rheinbay, et al. 2017; Toy, et al. 2017). Furthermore, the development of mammary epithelial cells to breast carcinoma can create novel enhancers unique to the progression of this cancer subtype (Stergachis et al. 2013). It has been shown that deletion of single nucleotide polymorphisms (SNPs) containing the enhancer that up-regulates MYC in intestinal cancer results in decrease of MYC expression and resistance to tumorigenesis (Sur, et al. 2012). This highlights the importance of enhancers in cancers (Sur and Taipale 2016). Furthermore, this mechanism is not exclusive to cancer, as SNPs at nuclear receptor regulatory regions can influence metabolic disease risk and subsequent effectiveness of therapeutics (Soccio, et al. 2015). In addition to SNPs, a large number of genomic alterations and mutation occur in cancers (Beroukhim, et al. 2010; Bignell, et al. 2010; Rheinbay et al. 2017). For example, a mutational hotspot at the FoxA1 promoter region leads to increased protein expression that can influence the action of other TFs in breast cancer (Rheinbay et al. 2017). In addition, FoxA1 can drive enhancer reprogramming during development of pancreatic cancer (Roe, et al. 2017). In the last year, it has also been recognized that not only direct DNA binding TFs, but also recruited cofactors can drive chromatin reprogramming. This has prominently been described with chromatin remodeling complexes whose action can drastically change the chromatin landscape in various cancers (Boulay, et al. 2017; Kadoch, et al. 2017; Pulice and Kadoch 2016). In addition, many other cofactors, such as histone modification writers, readers, and erasers are misregulated in cancers (Dawson 2017; Kim and Roberts 2016; Lonard and O'Malley 2016). Due to these observations, there is a growing interest to develop therapeutics targeting cofactors rather than TFs (Bennett and Licht 2017; Illendula, et al. 2015; Lasko, et al. 2017; Ribich, et al. 2017; Song, et al. 2016).

The discovery and history of nuclear receptors, including SRs, has been extensively reviewed by others (Chawla, et al. 2001; Lazar 2017; Mangelsdorf, et al. 1995). Here, we will focus on the reprogramming of the breast cancer chromatin landscape through steroid receptors (SRs). As the female sex hormone estrogen plays a predominate role in breast cancer growth, emphasis will be placed on the estrogen receptor (ER) and its cooperative

action with other TFs. Furthermore, we will review the current knowledge of how TFs bind and interact with chromatin to reprogram the chromatin landscape.

BREAST CANCER

Breast cancer is the leading cause of cancer related death in women. One in 8 women will develop the disease in their lifetime in the United States alone. In addition, there will be 230,000 new cases diagnosed and approximately 40,000 women succumbing to the disease each year (Siegel, et al. 2011). The epithelial cells that line the lobules or ducts are the predominant site for breast cancer initiation. These first detectable lesions are neoplastic growths confined within individual ducts, considered pre-invasive and termed *in situ* carcinoma. Invasive carcinoma is the next stage in breast cancer development. Here the cells breach the basement membrane and invade the surrounding breast stromal tissue (Roses 1999). The last stage in the progression of the disease is metastasis of the invasive cells. During this stage, the cells can migrate from the primary tumor site, via the blood stream or lymphatic system, where they transplant into the lymph nodes or other organs. It is well documented that 17β-estradiol (E2), an active metabolite of estrogen, is required for the development, growth, and homeostatic maintenance of normal and malignant breast tissue. Historically, it has been determined that the removal of the ovaries suppressed the growth of breast cancer (Beatson 1896; Mauvais-Jarvis, et al. 1990; Wittliff 1984). This was first reported in 1896, when a bilateral oophorectomy was performed in a premenopausal patient, resulting in a complete remission of the disease (Beatson 1896). Many years later the estrogen receptor (ER) was discovered (Jensen and DeSombre 1973) and the association to the removal of the ovaries and tumor remission could be attributed to the dependence of E2 on breast cancer growth. This discovery was quickly followed by the first cloning of ER (Walter, et al. 1985) and then isolation of a complementary DNA clone from translated mRNA of ER. This isolation was from the MCF-7 human breast cancer cells, which manifest functional expression of the protein (Greene, et al. 1986).

The steroid receptors (SRs) that mediate the effects of steroid hormones, such as E2, are involved in the progression and prognosis of hormone associated cancers. The estrogen receptor (ER) is expressed in approximately 50–88% of all breast cancers, with the progesterone receptor (PR) expressed in 45–82% (McGuire 1978; Rosa, et al. 2008). Primary diagnosis of breast cancer is subtyped by ER, PR, and the human epidermal growth factor 2 (HER2) expression to determine current treatment approaches. Given the known role of ER in breast cancer development, it is primarily utilized as a therapeutic target in the clinic. PR is a well described E2 regulated gene, and its dependence on ER signaling is utilized solely as a marker of a functional ER (Creighton, et al. 2009). At present, the standard of care for patients with ER positive breast cancers is to inhibit the receptors functionality. This is achieved by active competition of ER with antagonists such as tamoxifen. Alternatively, the use of aromatase inhibitors results in ER signaling inhibition by blocking the catalytic processes of estrogen production (Arpino, et al. 2009). Generally, these treatments are effective short term; however, 30–50% of ER positive tumors display resistance to these therapies and generally all metastatic ER positive breast cancer acquire resistance (Arpino et al. 2009; Mouridsen, et al. 2003). Two major trials, the Women's Health Initiative (WHI) and the Million Women Study, investigated the effects of estrogen

and progestin on breast cancer incidence. Both studies looked at the use of hormone replacement therapy (HRT) (i.e. estrogen only) and combined hormone replacement therapy (cHRT) (i.e. estrogen and progestin in combination). The WHI concluded that women on cHRT had an increased risk of invasive breast cancer compared to placebo treated women, with an incidence of 0.38% and 0.3% respectively (Rossouw, et al. 2002). Similar findings were found in the Million Women Study, concluding that women on HRT or cHRT were at a higher risk of developing breast cancer compared to women that had not used, or were not currently using either of these therapies (Beral 2003). However, these results have remained controversial over the years. Recently there has been the suggestion that HRT may have no effect on breast cancer incidence in younger women and could provide a level of protection in older women (Santen 2014). The level of proposed protection in older women has been suggested to be a result of estrogen-induced apoptosis in breast tumors (Jordan 2015; Santen 2014) This finding contrasts historic and current data and is an avenue of importance that needs to be further explored.

For the SR family, the androgen receptor (AR) and the glucocorticoid receptor (GR) each have a multitude of functions in human biology and disease progression. In addition to ER and PR, AR and GR have also been implicated in breast cancer progression. Specifically, AR is found to be expressed in up to 85% of primary breast tumors (Honma, et al. 2012; Qi, et al. 2012). Tumors expressing AR/ER/PR present with a better prognosis (Garreau, et al. 2006) compared to AR positive ER/PR negative tumors (Lin Fde, et al. 2012). Further, the patients with AR/ER/PR positive tumors have smaller tumor size, lower Ki-67 expression (marker of proliferation), and better disease-free survival compared to AR negative ER/PR positive tumor patients (Hu, et al. 2011; Qi et al. 2012). More recently the role of GR in mediating breast cancer development has begun to immerge. It appears the effect of GR on breast cancer is dependent on the expression of ER. Specifically, there has been an association with chemo-resistance and a short disease free survival period in triple negative breast cancer (breast cancer which lacks expression of ER, PR, and HER2 growth factor) (Pan, et al. 2011). However, cancers that express ER, PR, and have high GR expression, demonstrate an increased overall disease free survival (Pan et al. 2011). It is important to note that the overall levels of circulating endogenous ligand of the SRs change throughout the female monthly menstrual cycle. In addition, levels of estrogen are markedly decreased once a female goes through menopause. It is becoming clear that the role of SR signaling in breast cancer progression is complex and likely involves a crosstalk between multiple TFs. To fully understand the complexity behind these SR signaling pathways we need to increase our knowledge of the genetic elements that they bind, and the modes of action by which ER, PR, AR and GR collaborate at these enhancer elements. Advances in our understanding of the genomic responses of these factors will undoubtedly lead to improved patient therapies.

TECHNIQUES USED TO STUDY CHROMATIN DYNAMICS

The chromatin landscape predominately defines the genomic response of TFs, with the accessibility of enhancer elements effecting the gene transcription response of a given stimulus. Enhancers serve as the critical regulatory elements of the cells; characterization their functional activity states can be achieved by multiple population based assays (Figure 1). In the last 10 years these techniques have become available to almost all scientists (Soon,

et al. 2013). These methods can be used to characterize a wide-range of chromatin landscape properties including chromatin accessibility, nucleosome mapping, TF occupancy and long range chromatin conformations (Maston, et al. 2012). For transcriptional regulation of an enhancer region, the chromatin landscape must be accessible to the TF attempting to elucidate its response.

Open chromatin mapping

The most widely utilized assay for measuring the DNA accessibility is the DNase hypersensitivity assay followed by sequencing (DNase-seq) (Figure 1A) (Boyle, et al. 2008). This technique was based on the initial concept that nucleases can preferentially cut chromatin at regions with disrupted nucleosome structure [DNase I hypersensitive sites (DHSs)] (Keene, et al. 1981; McGhee, et al. 1981). Mapping DHSs in breast cancer can be used to identify chromatin sites that influence cancer development (D'Antonio et al. 2017). Formaldehyde assisted isolation of regulatory elements (FAIRE)-seq is an alternative technique whereby chromatin is cross-linked using formaldehyde, sonicated, and then phenol-chloroform extracted. The fragments in the aqueous phase are considered to derive from accessible regions and are sequenced (Gaulton, et al. 2010). In general, FAIRE-seq and DNase-seq can identify the same open chromatin sites (Song, et al. 2011) however, there are also unique regions identified by both techniques. More recently, an assay for transposase-accessible chromatin (ATAC)-seq has been developed. This alternative technique assays for accessible chromatin using the hyperactive Tn5 transposase (Buenrostro, et al. 2013). Here, Tn5 will cut and insert sequencing adapters to open chromatin sites, which enables direct deep sequencing and mapping of genome-wide chromatin accessibility from the sample.

ATAC-seq is gaining favor over DNase- and FAIRE-seq, due to the small amount of starting material required and the much faster time to effect the assay. It can also be used for frozen as well as fresh tissue samples (Corces, et al. 2017). The limits of DNase-seq and ATAC-seq have been extensively explored. Both these assays have been performed on single-cells (Buenrostro, et al. 2015; Cooper, et al. 2017); in addition to measuring chromatin accessibility, DNase-seq and ATAC-seq data can be utilized to measure TF footprints (Baek, et al. 2017; Buenrostro et al. 2013). The short region within an accessible enhancer sequence that is bound by a TF is sometimes protected from enzymatic attack. This level of protection provides a TF footprint (Galas and Schmitz 1978; He, et al. 2014; Neph, et al. 2012; Sung, et al. 2016). If the DNA cut fragments are sequenced with enough depth the number of cuts per base pair (no. of cuts/bp) can be determined. The footprint can be quantified by an aggregation cut count plot across the motif and motif flanking regions (Baek and Sung 2016) (Figure 1A [bottom]). These TF footprints have been described as a signature of TF binding to the protected site from a single experiment (Siersbaek, et al. 2014; Stergachis, et al. 2014). However, we and others have recently demonstrated that many TFs lack a detectable footprint (Baek et al. 2017; He et al. 2014; Sung et al. 2016; Sung, et al. 2014). The "absence" of these footprints is likely related to the rapid exchange observed for many transcription factors on chromatin in living cells (see below; Live Cell Imaging).

Nucleosome mapping

Characterization of chromatin accessibility is valuable tool assessing the status of active chromatin landscape. However, the techniques mentioned above do not provide adequate information on the structure of chromatin, specifically, the positions of nucleosomes. This can be achieved with genome-wide nucleosome mapping by digestion of the chromatin with micrococcal nuclease (MNase) (Schones, et al. 2008). This nuclease preferentially attacks linker regions between nucleosomes (Figure 1B). Thus, sequencing the DNA fragments insensitive to MNase digestion produces a map of nucleosome positions, or the lack thereof. Traditional mapping of nucleosome positions around promoters has been used to determine gene activity, as active promoters tend to be nucleosome depleted. For TF binding events, nucleosome content can be used to evaluate whether factors can bind to nucleosomal DNA (Ballare, et al. 2012; Iwafuchi-Doi, et al. 2016). This approach has been used as a rationale to determine whether TFs act as pioneer factors (further details below). Although MNase-seq is a powerful technique to map nucleosome positions, there are potential drawbacks. MNase digestion has a sequence preference, with digestion occurring at A/T rich regions over G/C rich regions. Furthermore, due to exonuclease activity of the MNase, nucleosome position can be altered due to concentration of MNase utilized in the experiments (Chereji, et al. 2017; Lai and Pugh 2017; Voong, et al. 2017). To overcome this issue, MNase-seq experiments are often performed at differing concentrations of enzymes. This facilitates a more accurate evaluation of nucleosome positions (Iwafuchi-Doi et al. 2016). In addition to MNase-seq, several other techniques, such as ATAC-seq and RICC-seq (using ionizing radiation) have the capabilities to map nucleosome positions (Buenrostro et al. 2013; Risca, et al. 2017). Furthermore, an increased resolution of nucleosome positions can be achieved using chemical cleavage mapping of nucleosome centers (Voong, et al. 2016; Voong et al. 2017).

Transcription factor and modification mapping

One of the most highly used techniques to study TF binding to chromatin to date is chromatin immunoprecipitation (ChIP) (Orlando and Paro 1993; Solomon, et al. 1988). This technique (Figure 1C) uses crosslinking (via formaldehyde), DNA fragmentation (sonication), and immunoprecipitation to capture all the occupying sites for a given protein, such as TF, cofactor or histone modification (Massie and Mills 2008). Before the wide availability of deep sequencing, chromosome-wide mapping of TF action, such as ER, was mapped using the ChIP-on-chip (ChIP coupled with tiling array) technique (Carroll, et al. 2005). Soon after the discovery of this technique ChIP was coupled with deep sequencing (ChIP-seq) (Johnson, et al. 2007; Robertson, et al. 2007), allowing the genome-wide mapping of TFs (including ER) binding events (Welboren, et al. 2009). Due to the ease of accessibility to deep sequencing platforms, ChIP-seq has become the standard technique to characterize the genome-wide occurrence of a given protein (Furey 2012). However, some of the binding events mapped by ChIP-seq can arise from non-specific enrichment, creating a phenomenon termed "Phantom peaks" (Jain, et al. 2015). Thus, several controls are needed to discriminate between a real binding and a false peak. As a result, a substantial number of modifications to the technique have been introduced, improving and expanding the output of the data. The usage of exonuclease digestion with bound protein of interest protecting the binding site (ChIP-exo) increases the resolution to a single nucleotide level (Rhee and Pugh

2011). Furthermore, ChIP-seq can be performed on single-cells (Rotem, et al. 2015), and coupling mass spectrometry to ChIP enables the detection of interactomes at the chromatin level (Mohammed, et al. 2013; Rafiee, et al. 2016). The ChIP-seq technique is by no means restricted to cell lines and fresh tissue samples, as ChIP-seq can also be performed from fixed clinical samples (Cejas, et al. 2016) and core needle biopsy samples (Zwart, et al. 2013). Thus, recent developments in sequencing techniques now enable researchers to perform both open chromatin techniques (Corces et al. 2017; Jin, et al. 2015), as well as TF binding mapping from clinical samples (Cejas et al. 2016), as well as older cataloged material.

Chromatin conformation mapping

After the realization that many TFs binding events are located at far distances from promoters, several methods to characterize long-range chromosomal interactions were developed (Bernstein, et al. 2012; Davies, et al. 2017; Thurman, et al. 2012). Most of these techniques are based on the digestion and ligation of interacting sites, termed chromosome conformation capture (3C) (Figure 1D) (Davies et al. 2017; Dekker, et al. 2002). However, newer techniques are emerging that allow the study of genome-wide interaction of these sites (Beagrie, et al. 2017) such as genome-wide 3C, called Hi-C, (Lieberman-Aiden, et al. 2009) and chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) (Fullwood, et al. 2009). While these techniques are similar, Hi-C is used to map all DNA-DNA interactions while ChIA-PET uses ChIP to pre-select specific TF interacting sites. Initially, Hi-C was capable of 1 megabase resolution (Lieberman-Aiden et al. 2009), while ChIA-PET could identify individual interactions on a kilobase resolution due to antibody selection (Li, et al. 2010). However, improvements in Hi-C technique (in situ Hi-C) have enabled detection of interactions in 1 kilobase resolution (Rao, et al. 2014). This detailed resolution was achieved by performing the DNA-DNA proximity ligation in intact nucleic and deeply sequencing the data. Higher resolution in Hi-C can also be increased by focusing on certain interaction such as promoters via capture enriched Hi-C (Javierre, et al. 2016; Schoenfelder, et al. 2015). Both Hi-C and ChIA-PET techniques have been used to study chromatin architecture in breast cancer. Hi-C data clearly shows that interactions differ between mammary epithelial and breast cancer cells (Barutcu, et al. 2015). Furthermore, ER (Fullwood et al. 2009) and RNA polymerase II (Li, et al. 2012) ChIA-PET in breast cancer cells indicates that they are anchored to promoters through long-range interactions.

Live cell fluorescence imaging

All the above-mentioned assays are valuable tools to characterize the action of TFs. However, they suffer from two major drawbacks; the assays average signals across populations of heterogeneous cells and rely on dead cells. While biochemical and population-based assays suggest that TFs are assembled in a well-ordered manner to chromatin, live cell fluorescent imaging has indicated a more stochastic assembly (Coulon, et al. 2013; Stasevich and McNally 2011). This difference arises due to the temporal resolution of biochemical and population-based assays, which cannot resolve the dynamic binding of TFs that occurs in live cells (Hager, et al. 2009). In the past fifteen years, several fluorescent microscopy techniques, such as fluorescent recovery after photobleaching (FRAP) and fluorescent correlation spectroscopy (FCS), have been used to resolve the

dynamic action of TFs (Mueller, et al. 2013). Many early FRAP experiments indicated that TF binding to chromatin occurs in the range of seconds (McNally, et al. 2000; Stenoien, et al. 2001). Furthermore, the rapid exchange of TFs with chromatin can influence transcriptional output (Karpova, et al. 2008; Stavreva, et al. 2004). Although FRAP and FCS can be used to resolve milli-sec to min range dynamic processes, both techniques are restricted to molecular populations in single cells. Hence, FRAP data will mostly represent diffusing TFs rather than bound ones. Recent advancements in imaging technologies (Chen, et al. 2014; Gebhardt, et al. 2013; Tokunaga, et al. 2008), protein tags (Gautier, et al. 2008; Los, et al. 2008) and fluorescent dyes (Grimm, et al. 2015), have enabled direct measurement of TF action at the single-molecule level (Mazza, et al. 2012). This technique, known as single-molecule tracking (SMT) or single-particle tracking (SPT), utilizes bright and stable fluorophores, and EMCCD cameras to resolve fluorescent signals originating from single fluorophores (Presman, et al. 2017). Several recent reviews have extensively covered the details and challenges of SMT (Liu, et al. 2015; Liu and Tjian 2018; Manzo and Garcia-Parajo 2015; Vera, et al. 2016; von Diezmann, et al. 2017). Single-molecules in general can be divided into two modes, bound and unbound states (Figure 1E) (Paakinaho, et al. 2017). It has been suggested that unbound molecules that can be captured on a single-frame but not tracked represent diffusing molecules (Figure 1F). These diffusing molecules can be classified into several types of diffusion (Izeddin, et al. 2014; Mazza et al. 2012).

Many investigators in the field have adopted an empirical method to describe the dynamics of bound molecules. This method involves fitting the dwell time data to alternate exponential distributions, and choosing the model that provides the best fit (Ball, et al. 2016; Chen et al. 2014; Hansen, et al. 2017; Kieffer-Kwon, et al. 2017; Kilic, et al. 2015; Loffreda, et al. 2017; Morisaki, et al. 2014; Schmidt, et al. 2016; Sugo, et al. 2015; Zhen, et al. 2016). The most frequent models currently invoked argue for 2 or 3 component distributions for the bound fraction. In a two component version, fast bound molecules are proposed to represent non-specific binding. The TF scans the genome attempting to find its specific binding sites remaining bound only for short period of time (Figure 1E–F) (Chen et al. 2014; Elf, et al. 2007; Paakinaho et al. 2017). In contrast, slow bound molecules remain bound for longer periods (5–15 sec) and are proposed to represent TF binding to specific response element (Figure 1E–F). In support of this interpretation, mutating the DNA-binding domain of a TF drastically reduces or abolishes the slow bound population of single-molecules (Chen et al. 2014; Morisaki et al. 2014; Paakinaho et al. 2017; Sugo et al. 2015). Work in breast cancer cell lines has indicated that chromatin binding of SRs is a very dynamic process (Swinstead, et al. 2016a). Interestingly, the pioneer factor FoxA1 (to be described below) also displays rapid dynamics in breast cancer cells. Essentially all TFs studied so far show dynamic action at the single-molecule level in live cells (Chen et al. 2014; Goldstein, et al. 2017a; Mazza et al. 2012; Morisaki et al. 2014; Paakinaho et al. 2017; Sugo et al. 2015; Teves, et al. 2016; Zhen et al. 2016), while structural proteins such as CTCF show much slower binding dynamics (Hansen et al. 2017). It should be emphasized that current interpretations for single molecule tracking are not based on rigorous thermodynamic models. It is likely that more accurate descriptions of real time TF/chromatin interactions will emerge. However, current results clearly demonstrate that TF action in general is a very rapid and dynamic process. Eventually population-based and live cell fluorescent microscopy perspectives

should be resolved within a single comprehensive model (Paakinaho et al. 2017). We are already beginning to see this combination with the development of synthetic techniques such as ATAC-see (Chen, et al. 2016).

ENHANCER REPROGRAMMING MODES OF TRANSCRIPTION FACTORS

Enhancers are short stretches of regulatory elements generally located distal to promoters. During transcriptional regulation TFs reprogram enhancers by altering chromatin accessibility thereby influencing the recruitment of other factors including RNA polymerases. Thus, the initial enhancer reprogramming determines the transcriptional outcomes (Schaffner 2015; Smith and Shilatifard 2014). To reprogram enhancers and regulate transcription, TFs must be able to access enhancer regions on chromatin. Current models envisage TFs binding and enhancer reprogramming through five major modes (Figure 2) (Long et al. 2016; Spitz and Furlong 2012; Voss and Hager 2014).

1. Cooperative transcription factor binding

It has been generally proposed that TFs require cooperative action of two of more factors to gain access to binding sites in closed chromatin regions (Figure 2A), through direct or indirect interactions (Long et al. 2016; Spitz and Furlong 2012). These events are thought to be ATP-dependent, requiring the recruitment of chromatin remodeling complexes enabling an alteration to chromatin accessibility. The direct interaction model relies on a physical contact or simultaneous binding between the cooperative TFs. Furthermore, TF pairs can have closely proximal recognition motifs on DNA influencing TF concomitant binding (Jolma, et al. 2015; Morgunova and Taipale 2017). In the case of indirect interaction, cooperativity occurs without apparent physical contact but with TFs binding in close proximity to each other. For some TFs, the indirect interaction mode can be classified as pioneer action (to be described). It is likely that both direct and indirect cooperative action can occur simultaneously. For example, it was recently described that during reprogramming of somatic cells to pluripotency (Takahashi and Yamanaka 2006), Oct4, Sox2, and Klf4 interact cooperatively to reprogram enhancers for pluripotency (Chronis et al. 2017). However, this cooperative action can function in the pioneer factor mode to induce the binding of somatic TFs. Further, during T cell activation, two pairs of TFs can cooperatively act to reprogram a different set of enhancers (Bevington, et al. 2016). In this case, enhancer priming (to be described) is regulated by the cooperative action of ETS-1 and RUNX1, while activation of inducible enhancer is regulated by AP-1 and NFAT. Interestingly, integration of several genomic and interactome datasets can identify several layers of cooperative TF action in liver (Dubois-Chevalier, et al. 2017) and during adipogenesis (Siersbaek et al. 2014). Finally, cooperative TF binding most likely influences enhancer activity since it is seemingly dependent on the other TFs in the immediate vicinity rather than an actual TF binding event (Grossman, et al. 2017).

2. Pioneer factor binding

It has been postulated that most TFs act in a cooperative manner. However, pioneer factors have been described as a small class of unique TFs that can penetrate chromatin on their own and assist the binding of non-pioneer proteins to the chromatin (Figure 2B) (Drouin

2014; Zaret and Carroll 2011). This group includes Oct4, Sox2, Klf4 (Soufi, et al. 2015), factors that reprogram somatic cells to pluripotency, and GATA and FoxA family members (Cirillo, et al. 2002). In addition, Pax7 can act as pioneer factor in pituitary melanotrope cells (Mayran, et al. 2018). Their capability to bind and remodel nucleosomes is the major characteristic of a pioneer factor (Zaret and Mango 2016). In the case of Oct4, Sox2 and Klf4, these factors can recognize their motifs on the surface of nucleosomes (Soufi et al. 2015), and increase the binding of other non-pioneer factors. In comparison, FoxA also has capability to bind to core histones (Cirillo et al. 2002); however, the structure of FoxA's DNA-binding domain resembles that of linker histone H1 (Zaret and Carroll 2011). This suggests that FoxA can efficiently displace H1 from the chromatin thus maintaining accessible chromatin (Iwafuchi-Doi et al. 2016). Pax7 can bind to heterochromatin regions and slowly promote chromatin opening providing epigenetic memory (Mayran et al. 2018). Most interesting is the proposal that the action of pioneer factors occurs in an ATP-independent fashion (Cirillo et al. 2002). However recent discoveries suggest, at least for Oct4 and GATA pioneer factors, that ATP-dependent chromatin remodeling complexes are required for efficient pioneer factor function during enhancer reprograming (King and Klose 2017; Swinstead, et al. 2016b; Takaku, et al. 2016). Interestingly, the pioneer activity of FoxA2, Oct4 and GATA4 is not increased by ectopic expression of the factor (Donaghey, et al. 2018). However, the activity of FoxA2 is increased when coexpressed together with GATA4, suggesting that pioneer factors might operate in cooperative manner.

Pioneer factors, particularly FoxA1, have been shown to be exceptionally important for SR recruitment (Lupien and Brown 2009; Zaret and Carroll 2011). A major fraction of chromatin binding for both ER and AR depends on the pioneer activity of FoxA1 (Hurtado, et al. 2010; Lupien, et al. 2008; Robinson, et al. 2011), with GATA3 also playing a pioneer role for ER in breast cancer (Theodorou, et al. 2012). In the case of GR, AP-1 functions as a pioneer factor for a significant fraction of the receptor binding events (Biddie, et al. 2011; John, et al. 2011). While it has been suggested that SRs themselves can function as pioneer factors, the mechanism behind the SR "pioneer activity" appears to be more complex than the classically described process (Sahu, et al. 2011; Swinstead et al. 2016a).

3. Dynamic Assisted loading

Based on *in vitro* experiments it is expected that TFs that bind to the same DNA sites would in fact compete for binding. However, it was shown *in vivo* that two SRs failed to compete for the same binding site; rather one receptor assisted the binding of the secondary receptor (Voss, et al. 2011). These findings led to the proposal of a new model, termed "dynamic assisted loading." In this model, one TF binds to a closed chromatin site, induces chromatin remodeling though ATP dependent processes, and thereby assists the binding of the second TF to the site (Figure 2C). It is further suggested that competition does not occur at the assisted loading sites *in vivo* due to the rapid interaction of TFs with chromatin in live cells (Paakinaho et al. 2017; Swinstead et al. 2016a). Furthermore, some TFs have been shown to be mobile during chromatin remodeling reactions (Li, et al. 2015; Nagaich, et al. 2004). In this scenario, the initiating TF inducing chromatin remodeling is actively displaced by the remodeler before the binding of the assisted factor. The assisted loading model differs from the cooperative and pioneer model in several distinct ways (Swinstead et al. 2016b). In

comparison to the cooperative model, the two factors do not physically interact. In addition, assisted loading can be a symmetric event, with one factor acting as an initiator at some sites, while the other acts as initiator at different sites. This bimodal symmetry highlights the difference of assisted loading from the pioneer model. Furthermore, ATP-dependent chromatin remodeling factors are crucial for assisted loading, while the classically described pioneer model suggests ATP-independent action.

Although assisted loading occurs in symmetric manner, it also occurs in an enhancer-specific manner. This is a major mechanism of enhancer reprograming for SRs (Goldstein et al. 2017a; Grontved, et al. 2013; Swinstead et al. 2016a) and other nuclear receptors (Madsen, et al. 2014; Soccio et al. 2015) in various cellular contexts. For example, GR will assist the binding of ER at a subset of enhancers while ER will assist the binding of GR at another subset of enhancers (Miranda, et al. 2013). In addition to nuclear receptors, other TFs have been described to also operate through the assisted loading mechanism (Goldstein, et al. 2017b; Zhu, et al. 2015).

4. Tethering

An alternative model for TF function at enhancers involves a tethering event, whereby one TF can access an assessable site by physically tethering to another TF (Figure 2D). Genome-wide ChIP-seq studies alone are insufficient to determine if a binding event is a classical direct binding event or a tethering paradigm. Consequently, the DNA that is immunoprecipitated by the antibody of interest will represent sites of direct binding events, as well as sites of protein-protein interaction. DNA binding motif identification is frequently used to distinguish between these modes of action.

Classically, the anti-inflammatory action of GR is thought to be the consequence of GR tethering to pro-inflammatory factors thereby inhibiting their action (Cain and Cidlowski 2017; Petta, et al. 2016). However, recent results suggest that tethering might not be as prominent a mode of action as was previously believed (Oh, et al. 2017; Uhlenhaut, et al. 2012). Furthermore, GR can tether to other TFs without inflammatory stimuli to influence their action (Langlais, et al. 2012). In the case of classical ER binding events, the receptor binds to an estrogen response element (ERE); however, there are a number of identified sites missing the canonical ERE, suggesting a tethering phenomenon. Specifically, in MBA-MD-231 cells breast cancer cells transfected with wild type ER or DBD mutant ER incapable of binding to EREs, there is a clear difference between the transcription profiles of the two receptors (Stender, et al. 2010). This segregation was used to identify direct ER binding or tethering events. As tethered sites were enriched for the RUNX motif in the absence of an ERE, it was concluded that ER tethers RUNX to mediate DNA-independent gene regulation (Stender et al. 2010). It is important to note that the tethering event is largely different from the dynamic assisted loading model. Specifically, during dynamic assisted loading there is a presence of binding response elements of both TFs (initiating and secondary) (Swinstead et al. 2016a). However, there can be collaboration between the tethering and the dynamic assisted loading model. At a population of ER sites that are assisted by GR, AP-1 is tethered to ER facilitating the binding of ER at a number of assisted loading sites (Miranda et al. 2013). However, because formaldehyde crosslinks both DNA-

protein and protein-protein interactions, the tethering mode can only be proposed but not confirmed by ChIP assays. Alternative techniques should be used, such as expression of DBD mutant TFs (Langlais et al. 2012; Stender et al. 2010). In addition, the improved resolution obtained by ChIP-exo can be used to distinguish direct DNA binding and tethering events (Starick, et al. 2015). More sophisticated techniques are being harnessed to address these issues. Previously, UV-laser cross-linking has been used in vitro (Nagaich et al. 2004), to study direct DNA binding of TFs. Interestingly, very recently it has been shown that UV-laser cross-linking can be coupled to ChIP (Steube, et al. 2017) to resolve tethering events from direct DNA binding. Future development of this technique will further help to distinguish protein-protein from protein-DNA interactions.

5. Enhancer priming

The selection and function of TF binding events can prime the enhancer for a resultant transcriptional response (Figure 2E). Enhancer priming is prevalent in differentiation. Specifically, a TF can bind, altering a poised enhancer to an activated enhancer through histone H3 modifications. This results in a recruitment of a secondary factor (Heinz, et al. 2015). This has been most clearly shown in the differentiation of hematopoietic cells, where lineage-determining factors, such as PU.1, prime enhancers for macrophage or B cell differentiation (Heinz, et al. 2010). Eventually these primed enhancers will serve as a platform for signal-dependent TF binding driving differentiation to a specific direction. Furthermore, H3K4 methylation and enhancer transcription seem especially important for enhancer priming (Heinz et al. 2010; Kaikkonen, et al. 2013). Another example of enhancer priming is illustrated in the phenomenon of T cell memory acquisition. Here, activation of T cells induce NCAT and AP-1 binding, resulting in a number of new DHS site and subsequent recruitment of ETS-1 and RUNX1. Interestingly, the DHSs remained stable long after T cell activation, maintaining open chromatin regions at active enhancer regions (Bevington et al. 2016). The dynamic assisted loading model can be extended further with evidence for enhancer priming. The dynamic crosstalk between two factors through this interaction results in alteration of H3K27ac and an associated recruitment of P300 (Goldstein et al. 2017b).

ENHANCER REPROGRAMMING BY STEROID RECEPTORS IN BREAST CANCER

Specifically relevant to breast cancer, a number of investigators are beginning to examine the mechanistic processes of SR recruitment to enhancer elements. Many of the enhancer reprogramming modes are utilized by SRs in breast cancer. These studies have explored the different modes of action a SR can have on the chromatin landscape and the consequential output for gene regulation and transcription profiles. Cooperative action among SRs is beginning to appear as a major mode of enhancer reprogramming in breast cancer cells (see next section for details). However, other pathways can cooperatively reprogram enhancers with SRs. Growth factors, independently or in cooperation with ER, can reprogram the enhancer landscape influencing the ER cistrome in breast cancer cells (Lupien, et al. 2010). Interestingly, in non-tumorigenic mammary cells, growth factors and GR can act in a cooperative and antagonistic manners to reprogram enhancers and gene regulation (Enuka,

et al. 2017). In addition to growth factors, inflammatory pathways can reprogram the ER enhancer landscape potentially influencing clinical outcome of breast cancer (Franco, et al. 2015), or endocrine resistance (Stender, et al. 2017). Thus, it is expected that other signaling pathways will cooperatively influence SR enhancer reprogramming. In addition, mutations in ER (ESR1) arising from endocrine resistance, can reprogram ER binding (Jeselsohn, et al. 2018; Martin, et al. 2017; Toy et al. 2017), influencing receptor action on chromatin. In the case of tethering, Carroll and colleagues reported that activated PR could reprogram the ER enhancer landscape, contributing to an inhibition of breast cancer tumor growth under the dual treatment conditions. These newly acquired ER sites under the dual activation of both receptors was proposed to be through a tethering event, whereby PR and ER physically interact with cofactors and FoxA1 at binding sites. It was proposed that there is a lack of a classical ERE at these unique sites (Mohammed, et al. 2015). Myers and colleagues have also suggested that cell type specific ER and GR binding events that lack a strong canonical response element represent tethering events (Gertz, et al. 2013). Interestingly, this model also suggests that these SR tethered enhancers are primed by other TFs. These results imply that in some cases SRs can have only a minor effect on enhancer reprogramming as they bind to already accessible chromatin. This is in line with the pioneer factor model, where SRs binding is dictated by other TFs (Biddie et al. 2011; Hurtado et al. 2010; John et al. 2011; Theodorou et al. 2012). As indicated above, the main property of a pioneer factor is the capability to bind histones at closed chromatin sites (Drouin 2014; Zaret and Carroll 2011).

In this vein, SRs can target nucleosomes in breast cancer cells transforming them to bona fide pioneer factors. It has been suggested that the vast majority of ER and PR binding regions are largely nucleosome rich (Ballare et al. 2012; He, et al. 2012) (Figure 3A–B). ER binding in breast cancer cells is largely marked by H3K4me2 implying nucleosome rich binding regions (He et al. 2012) (Figure 3A). However, whether ER binds to nucleosomes without a H3K4me2 mark or how chromatin remodelers influence these processes is unknown. In the case for PR, Beato and colleagues described PR binding events at PRE enhancer regions rich with nucleosomes. The majority of sites are DNase I hypersensitive and hormone activation results in displacement of H1 and H2A/H2B dimers (Ballare et al. 2012) (Figure 3B). Further it has been described that Brg1 is largely involved in the resultant gene transcriptional response at enhancer regions (Ceballos-Chavez, et al. 2015). Thus, two important factors for breast cancer development, ER and PR, can both act as pioneer factors, and at least ER can regulate the binding of other factors by this pioneer activity (Swinstead et al. 2016a). For GR, initial genome-wide studies indicated frequent binding at sites with pre-existing DNase hypersensitivity. This accessibility is often interpreted as areas lacking nucleosomes (John et al. 2011) (Figure 3C). However, we have recently completed high resolution nucleosome positioning studies, and discovered that many DHS elements retain modified nucleosomes. This has led to a refined view of the chromatin structures present at responsive GR enhancers (Johnson, et al. 2018). In this view, they can be located either in pre-existing nucleosome depleted regions or within a nucleosome (Figure 3C). The nucleosomal depleted GR enhancers are already marked with Brg1, a chromatin remodeling factor, and flanked by H2A.Z. However, GR sites that are rich with nucleosomes can be segregated further into i) DNase I hypersensitive sites associated with Brg1, or ii) sites

insensitive to DNase I and lacking Brg1, suggestive of a true GR pioneer function by dynamic assisted loading (Johnson et al. 2018) (Figure 3C). Thus, although GR action differs from that of ER and PR, it can also act as a pioneer factor targeting nucleosomal chromatin sites. The association of Brg1 and SR transcriptional responses has been well characterized (Swinstead et al. 2016b). However, an in-depth understanding of how these different enhancer region states relate specifically to SR binding and transcriptional response in breast cancer remains incomplete.

COLLABORATIVE CROSSTALK OF STEROID RECEPTOR BINDING EVENTS

Classically ER and PR binding events in breast cancer have been studied as single receptor binding events. It is becoming apparent that i) SRs collaborate with each other by the various mechanisms described above, and ii) there is an increasing appreciation for the importance of AR and GR signaling in breast cancer. The role of activated PR and the collaboration with ER signaling, and consequences for breast cancer growth is becoming well documented (Daniel, et al. 2015; Finlay-Schultz, et al. 2017; Hegde, et al. 2016; Mohammed et al. 2015; Singhal, et al. 2016). PR influences ER genomic recruitment (Mohammed et al. 2015; Singhal et al. 2016) potentially influencing decisions on breast cancer therapies. Interestingly, ER/PR crosstalk can be defined by PR isoforms, wherein PR-A inhibits while PR-B redistributes chromatin binding of ER (Singhal, et al. 2018). In addition, recently it was shown that PR can decrease the expression of proteins needed for translation, such as tRNAs in breast cancer (Finlay-Schultz et al. 2017). This decrease of tRNAs will restrict the translation of ER-regulated genes related for breast cancer growth. This highlights the importance of looking at these factors in a common setting. In addition, AR has been described to facilitate ER binding at a number of loci with enzalutamide, an AR antagonist, attenuating the response (D'Amato, et al. 2016). Furthermore, AR can collaborate with ER enhancing its transcriptional activity in aromatase inhibited breast cancer cells (Rechoum, et al. 2014). Lastly, the role of GR and ER crosstalk is emerging in the field, with several studies suggesting GR can induce or repress a number of ER binding events (Miranda et al. 2013; Yang, et al. 2017). Post-translational modifications of GR, such as those mediated by small ubiquitin-related modifier (SUMO; SUMOylation) is seemingly important in the repression of ER (Yang et al. 2017). However, this repression was only shown for a few SR-regulated loci. On a genome-wide level GR SUMOylation fine-tunes GR chromatin occupancy (Paakinaho, et al. 2014), suggesting that SUMOylation can have an even wider effect on SR crosstalk. Conversely, activated ER can result in enrichment of GR at proximal promoter regions, with increased GR chromatin association at ER, FOX, and AP-1 binding response regions (West, et al. 2016). The profiling of the SR landscape in human male breast cancer tumors indicate extensive overlap between ER, PR, AR, and GR binding (Severson, et al. 2018). This suggests that the interplay of SRs is also important in primary patient samples. Finally, analysis of nuclear receptor networks in breast cancer cells has revealed not only SRs interactions, but also complex interactions among nuclear receptors and other TFs related to breast cancer growth (Kittler, et al. 2013).

While many studies have started to uncover the concomitant crosstalk of SRs in breast cancer biology, what is still unclear are the mechanisms associated with the collaboration. Whether these events occur through the pioneer factor model, dynamic assisted loading,

tethering, or direct binding events associated with other cofactors is still poorly understood at the level of molecular mechanism.

CONCLUSION

To date the clear majority of studies investigating TF signaling in breast cancer have focused on individual SR binding events. This is not a representative capture of the biologically important functions in the human body. Above we have provided an in-depth description of the dual collaboration of TFs on enhancer regulation. Lacking in the field of SR biology is a comprehensive understanding of the collaborative crosstalk of multiple SRs and other TFs at any given time, and the underlying mechanisms associated with these events. In addition, as many SRs in breast cancer cells can act as pioneer factors, existing models should be refined. It is becoming more apparent that there is no certain set of pioneer factors, but rather multiple TFs that in certain settings possess pioneering activities.

As we more fully understand the different types of SR binding events in breast cancer and underlying chromatin landscape, we can assess the direct effects on gene transcriptional profiles. These events are critical to driving breast cancer progression and proliferation. This information will assist in the understanding of the complex and in-depth systems associated with TF biology in breast cancer and the effects that the chromatin landscape has on these events.

Acknowledgments

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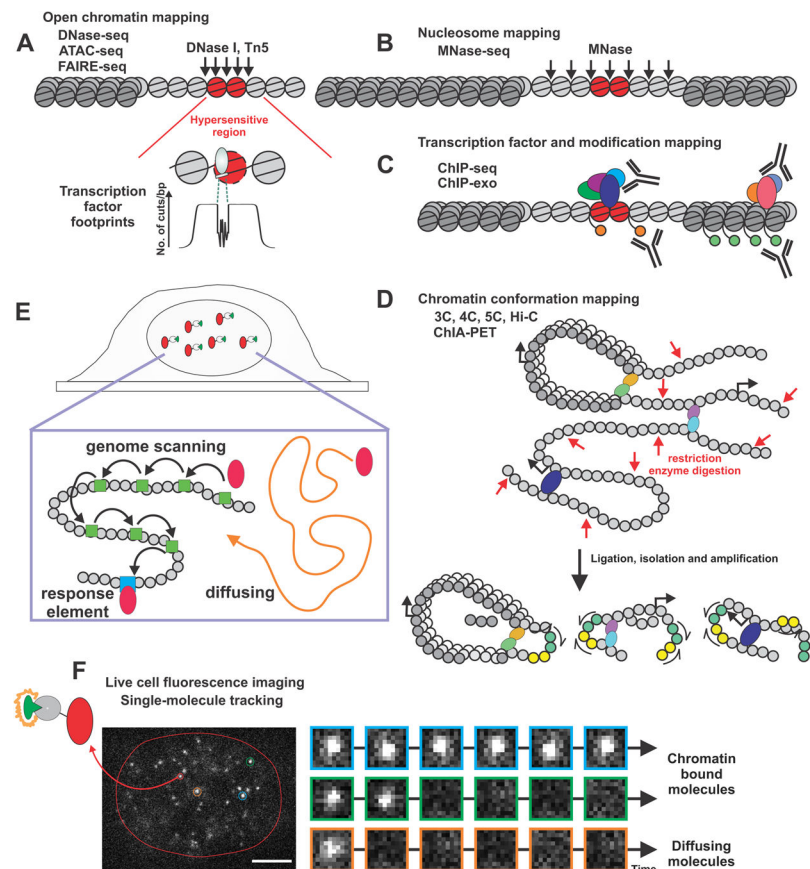
Figure 1.
Illustration of current techniques utilized to study enhancer elements through population based assays and single molecule approaches. (A) Mapping of open chromatin accessibility via DNase- ATAC- or FAIRE-seq. Nuclease such as DNase I or transposase such as Tn5 can target hypersensitive region (black arrows). The hypersensitive region is marked in red with the ability to detect the TF footprint at this region. The protection by the TF in the footprint is usually counted as number of cuts per bp (No. of cuts/bp). (B) Mapping of nucleosome positioning using MNase-seq. Micrococcal nuclease (MNase) preferentially cuts DNA strand between nucleosomes (black arrows) enabling chromatin structure mapping. (C) Mapping of the genomic location of TF (colored ovals) and chromatin modifications (small orange or green circles) by ChIP-seq and ChIP-exo. The TF or histone modification of interest is targeted by an antibody located at open or closed regions of chromatin. (D) The capture of chromatin interactions via 3C, 4C, 5C, Hi-C and ChIP-PET. Illustrations demonstrates the restriction enzyme digestion, ligation, isolation, and amplification interaction events. Depending on the technique used different degree of interaction can be captured and measured. (E) Capture of single-molecules bound to a fluorescent tag demonstrating TF (red oval) diffusing in the nucleus of the cell (orange arrow), scanning the genome (green square), and binding to a response element (RE) (blue square). (F) Live cell fluorescent imaging of a TF with post-translational labeling tag upon activation via a fluorescent ligand can be tracked in the nucleus and a residence time of the bound molecules

can be determined. Orange represents a diffusing molecule, green a fast bound molecule and blue a TF binding at a response element (RE) termed slow bound. White scale bar 5 μm.
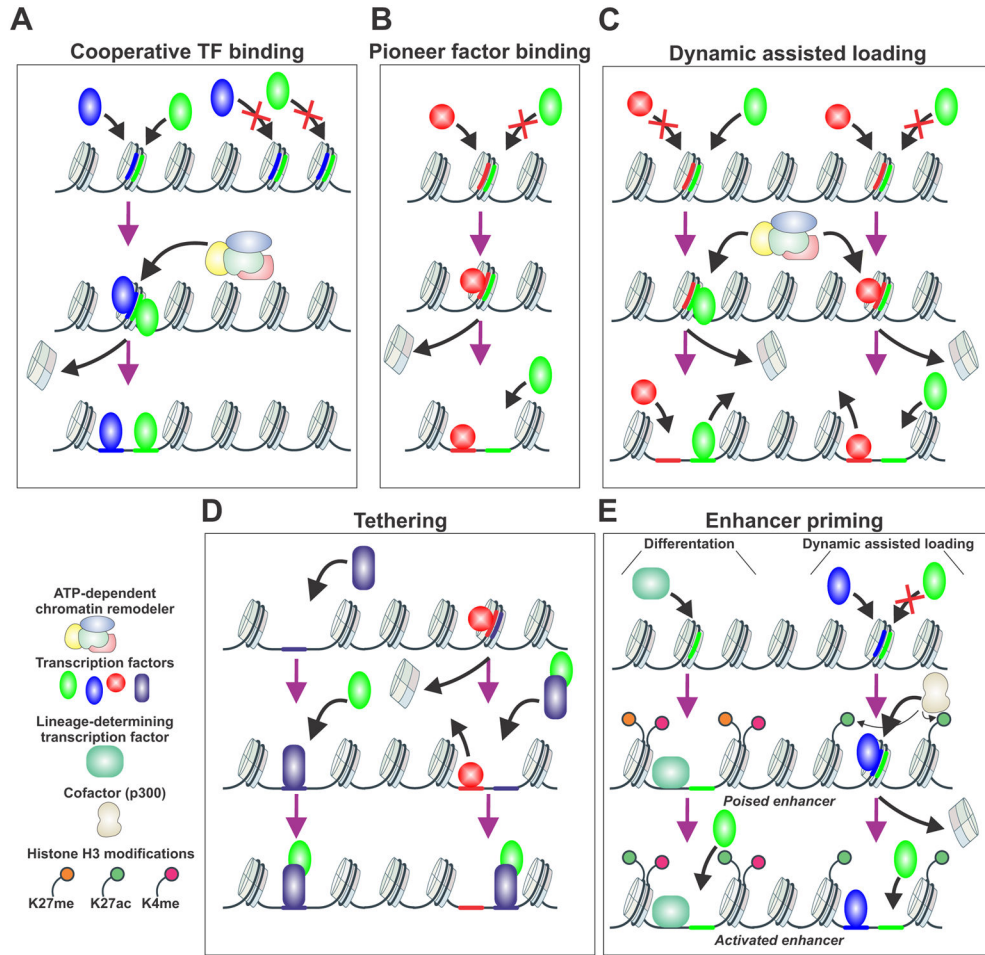
Figure 2.
Illustration of enhancer reprogramming models. (A) Cooperative TF binding model. The binding region of interest is inaccessible until two TF concomitantly binding, recruit chromatin remodeling factors resulting in an accessible region. (B) Pioneer factor binding. Pioneer factors such as FoxA1 bind to closed regions of chromatin, expel the nucleosomes, allowing binding of a secondary factor in the absence of ATP-dependent processes. (C) Dynamic Assisted Loading. The initiating factor binds to a closed chromatin region, upon recruitment of chromatin remodeling factors the secondary factor can binding to a region previously deemed inaccessible. This is usually a bimodal switch between two TF depending on the chromatin landscape and the enhancer region. (D) The tethering model. One TF binds to a chromatin region with the secondary TF recruited upon binding or initially tether to the first TF. (E) Enhancer priming. This mechanism is functional in differentiation or dynamic assisted loading. The bound lineage-determining TF can alter the chromatin landscape by changing enhancer accessibility and histone modifications. Conversely in assisted loading the initiating factor binding actives enhancer region by increasing active histone modifications inducing the recruitment of the secondary factor to these sites. In both cases, poised enhancer state exists between the inactive and active enhancer states.
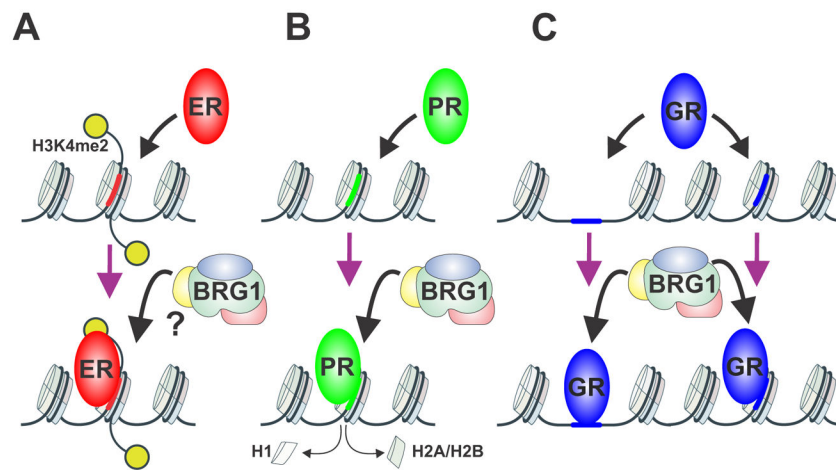
Figure 3.
Nucleosomal enhancer reprogramming by steroid receptors in breast cancer. (A) ER is bound to regions largely marked by H3K4me2 and rich in nucleosomes. ER potentially recruits chromatin remodelers to increase chromatin accessibility. (B) PR binds to nucleosome rich regions, recruits the Brg1 chromatin remodeler, resulting in a hypersensitive site and displacement of H1 and H2A/H2B dimers. (C) GR can bind to nucleosome depleted sites or sites enriched with a nucleosome. The nucleosomal depleted GR enhancers are marked with Brg1. GR sites that are rich with nucleosomes are suggestive of dynamic assisted loading or the pioneer model.