Nuclear receptors and chromatin: an inducible couple

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Abstract

The nuclear receptor (NR) family comprises 48 transcription factors (TFs) with essential and diverse roles in development, metabolism and disease. Differently from other TFs, NRs engage with well-defined DNA-regulatory elements, mostly after ligand-induced structural changes. However, NR binding is not stochastic, and only a fraction of the cognate regulatory elements within the genome actively engage with NRs. In this review, we summarize recent advances in the understanding of the interactions between NRs and DNA. We discuss how chromatin accessibility and epigenetic modifications contribute to the recruitment and transactivation of NRs. Lastly, we present novel evidence of the interplay between non-coding RNA and NRs in the mediation of the assembly of the transcriptional machinery.

Key Words
- nuclear receptors
- chromatin
- epigenetics
- oestrogen receptors
- gene expression

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Introduction/overview

Nuclear receptors (NRs) are evolutionarily related DNA-binding transcription factors (TFs) (Gronemeyer *et al.* 2004, Chambon 2005, Evans 2005). They regulate many aspects of mammalian physiology, including metabolism, development and reproduction (Mangelsdorf *et al.* 1995, Willy *et al.* 1995, Chawla *et al.* 2001), priming the activation of multiple genes to achieve accurate, synchronized and coherent functional responses. Most NRs regulate gene transcription in response to a ligand activation process, being activated by several hormones, vitamins, lipophilic metabolites and dietary lipids. NRs are also able to recruit a variety of classical TFs, co-regulators and proteins of the basal transcriptional machinery to target gene promoters and coordinate the whole process of gene transcription. The NR superfamily has a high potential for drug targeting, because the lipophilic ligands of NRs can easily pass through biological membranes. Consequently, NRs are the object of intensive translational research, which has resulted in their establishment as drug targets for several pathological conditions. Currently, 13% of all FDA-approved drugs target NRs (Overington *et al.* 2006). These include thiazolidinediones targeting the peroxisome proliferator-activated receptor (PPAR) in type II diabetes patients, steroids targeting the glucocorticoid receptor (GR) in inflammatory diseases, tamoxifen targeting the oestrogen receptor-α (ERα) in breast cancer and antiandrogens targeting the androgen receptor (AR) in prostate cancer.

In 1985, the GR was the first NR to be cloned (Hollenberg *et al.* 1985), followed by the ER and thyroid hormone receptor (THR; Walter *et al.* 1985, Greene *et al.* 1986, Sap *et al.* 1986). To date, the NR superfamily comprises 48 members in humans. NRs mediate gene transcription via distinct mechanisms, including both classical genomic transactivation and transrepression activities. Furthermore, NRs can also modulate different pathways in a non-genomic fashion, and their activities can be influenced by post-translational modifications

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(e.g. acetylation, phosphorylation, sumoylation and ubiquitination), making them targets of other cellular pathways. In this review, we mostly focus on the genomic activities of NRs.

Despite their broad range of activities, NRs share a common structural organization. They consist of an N-terminal regulatory domain, containing the ligand-independent activation function 1 (AF1; Wärnmark *et al.* 2003) that usually has a very weak transcriptional activity (with the notable exception of the AR), but is capable of synergizing with another activation domain, activation function 2 (AF2), present in the ligand-binding domain (LBD; see below). The most conserved domain is the DNA-binding domain (DBD), which contains the P-box, responsible for direct DNA interaction and DNA-binding specificity. It consists of two zinc fingers, which recognize specific NR-responsive elements (NRREs) in the DNA-regulatory regions of their target genes. NRs can bind to NRREs located in the proximity of target gene promoters and to more distal elements such as enhancer regions, as monomers, homodimers (e.g. GR, ER and AR) or heterodimers with the retinoid X receptor (RXR) (e.g. farnesoid X receptor (FXR), liver X receptor (LXR) and others). NRREs consist of two hexameric core half-site motifs, separated by a variable number of nucleotides. The bipartite elements can form direct, inverted or everted repeats (Mangelsdorf & Evans 1995). The DBD also contains a dimerization interface (Zechel *et al.* 1994, Germain *et al.* 2006, Bain *et al.* 2007), which can also be the site of post-translational modifications, resulting in different effects for different NRs. In some NRs, DBD phosphorylation results in decreased DNA binding (e.g. THR, Tzagarakis-Foster & Privalsky (1998) and ER, Chen *et al.* (1999)); in others, it increases DNA binding and co-activator recruitment (e.g. FXR, Gineste *et al.* 2008). The DBD and the C-terminal LBD are connected by a flexible domain called hinge region, which houses a nuclear localization sequence (Giguère *et al.* 1986). The LBD presents with a structure forming a hydrophobic pocket for ligand recognition and lodging. It also contains the AF2 domain, the action of which is dependent on the presence of bound ligand (Wärnmark *et al.* 2003), as well as motifs responsible for dimerization and co-regulator-binding regions.

For the majority of NRs, ligand binding is the critical event shifting their function from an inactive to an active state by inducing a conformational change in the LBD (Bourguet *et al.* 2000). The active conformation allows the second step of NR activation: recruitment of co-regulatory complexes. In recent years, it has become clear that many of these co-factors function as chromatin-remodelling factors and can catalyse histone modifications (Kouzarides 2007, Suganuma & Workman 2008). Small modifications of ligand structure seem to affect the co-activator-binding interface, providing a molecular basis for NR–ligand binding specificity and potency (Wu *et al.* 2003). The LBD is also a target for post-translational modifications, which govern a variety of cellular functions, including NR activity, DNA-binding affinity, ligand sensitivity, receptor stability and subcellular distribution. Therefore, NR post-translational modifications can ultimately result in either increased or decreased target gene expression (Balasubramaniyan *et al.* 2012) or in transrepression of other pathways (such as sumoylation of PPARγ (Pascual *et al.* 2005) and FXR, leading to NF-κB transrepression (Vavassori *et al.* 2009)).

NR classification

NRs can be classified in several ways. Based on ligand-binding properties, they can be divided into three classes: hormone NRs, metabolic NRs and orphan NRs (Table 1). Hormone NRs are often located in the cytosol. Once bound to their ligands, they translocate to the nucleus, usually as homodimers, and bind to their cognate NRREs. The members of this subfamily include the AR, GR and progesterone receptor (PR) and ligands include hormonal lipids. Upon ligand binding, some hormone NRs bind to the DNA on accessible chromatin at regulatory sites in the

Table 1 Classification of some members of the nuclear receptor superfamily. NRs can be classified in several ways. Based on ligand-binding property, they can be divided into hormone, metabolic and orphan NRs. Hormone NRs generally bind to the DNA as homodimers. On the contrary, most of the metabolic NRs bind to DNA as heterodimers with their obligate partner RXR. Orphan NRs can work as either heterodimers or monomers. For a more complete classification, see Nuclear Receptors Nomenclature Committee (1999)

Hormone NRs ligands: hormones	Metabolic NRs ligands: dietary lipids	Orphan NRs ligands: unknown
ERα,β	RXRα,β,γ	COUP-TFI,II,III
GR	PPARα,β,γ	NUR77, NURR1, NOR1
MR	LXRα,β	GCNF
AR	FXRα,β	LRH1
PR	PXR/SXR	Rev-ERBα,β
RARα,β,γ	CAR	SHP
THRα,β		THR2,3,4
VDR		HNF4α,β,γ
ECR		DAX1

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target genes, such as ER and GR; others bind to chromatin and attract co-activator factors that have chromatin-remodelling properties, as in the case of ER and PR (Vicent *et al*. 2011). DNA binding and chromatin remodelling then enhance the recruitment and/or function of the general transcription machinery (Fondell *et al*. 1996, Lefterova *et al*. 2008).

Metabolic receptors are typically located in the nucleus, bound to the DNA mostly as heterodimers with the RXR. In the absence of ligands, they are often complexed on the chromatin with co-repressor proteins. In this instance, ligand binding to NRs causes the dissociation of co-repressor and recruitment of co-activator proteins. Additional proteins of the basal transcriptional machinery, including RNA polymerase II, are then recruited to the NR–DNA complex to start the transcription process. The members of this subfamily include the FXR, LXR, PPARs and others, while ligands for this subgroup encompass dietary lipids. The third subgroup of NRs is the so-called family of orphan receptors, for which regulatory ligands are still unknown or may not exist (true orphans) or for which candidates have only recently been identified (adopted orphans). Members include the chicken ovalbumin upstream transcription factors (COUP-TFs), reverse ERBA-α/-β, liver receptor homologue-1 (LRH1) and others.

In this review, we summarize current knowledge regarding the bidirectional crosstalk between NRs and the chromatin landscape in the context of gene regulation.

Genomic features of NR binding

Similarly to most TFs, NRs modulate transcription by binding to well-defined DNA sequences (NRREs) interspersed in the genome (Kininis & Kraus 2008). NRs can also bind to DNA via tethering mechanisms; for example, ERα can 'piggyback' on AP1, MAF and RUNX1 TFs (Stender *et al*. 2010, Heldring *et al*. 2011). However, for the sake of clarity, we focus only on direct binding events. NRREs are extremely well conserved and share a significant homology. Concordantly, NR DBDs share extensive homologies (Kininis & Kraus 2008, Willis & Griffin 2011). Despite this, a recent NR binding survey has identified dramatically distinct topological differences in regulatory element selection (Kittler *et al*. 2013). Kittler *et al*. (2013) used a tag-based ChIP-seq approach to circumvent the noise created by different antibody efficiencies and characterized the binding profile of 24 NRs. Several NRs appear to have a strong preference for distal binding (ERα, RORC, AR and PR). Others are found in proximity to promoter elements (testicular receptors TR2L, TR2S, TR4, LXRα and THRα (Kittler *et al*. 2013). Interestingly, only 32% of over 2×10^5 individual genomic loci have been found to recruit a single NR, suggesting that NRs exhibit extensive cooperative binding (Voss *et al*. 2011). For example, ten of the 24 NRs assayed are recruited to the *CCND1* promoter. These genomic regions with multiple NR-binding sites (HOT regions) exhibit a significant increase in features normally associated with active regulatory elements, including epigenetic modifications and increased chromatin accessibility (Kittler *et al*. 2013). HOT regions are reminiscent of super-enhancers described, for example, in embryonic stem cells (Whyte *et al*. 2013). In this case, several regions termed super-enhancers span significantly large stretches of DNA (median size 8 kb) and recruit large arrays of TFs (e.g. OCT4, SOX2, NANOG, KLF4 and ESRRB). Not surprisingly, breast cancer-relevant genes are enriched with HOT regions (e.g. *CCND1* and *MYC* genes; Kittler *et al*. 2013). These data suggest that combinatorial binding patterns may play an unexpected role in hormone-dependent cancer and other physiological processes regulated by NRs.

Chromatin accessibility predisposes regulatory elements to NR binding

NRs invariably bind to regulatory elements to control cell type-specific transcription in response to external stimuli. The search for putative regulatory elements, such as promoters and enhancers, has been simplified by the recent development of epigenomics (ENCODE Project Consortium *et al*. 2007, 2012). Cell type-specific regulatory elements can be classified using several epigenetic modifications, including chromatin accessibility (Ernst *et al*. 2011). With its nucleosome-rich structure, chromatin represents the first barrier for TF binding. In agreement with this, regulatory elements are commonly found in nucleosome-depleted pockets, a conformation thought to be essential for DNA motif recognition and binding (Thurman *et al*. 2012). Uniquely among other TFs, several NRs bind to DNA only after conformational changes induced by their ligands (Kininis & Kraus 2008). This raises the fundamental question about pre-induction and post-induction chromatin determinants of NR binding (Magnani & Lupien 2013) (Fig. 1).

Chromatin accessibility pre-induction

Most of our understanding of NR–chromatin interaction comes from dissecting ERα, GR and AR signalling. Initial

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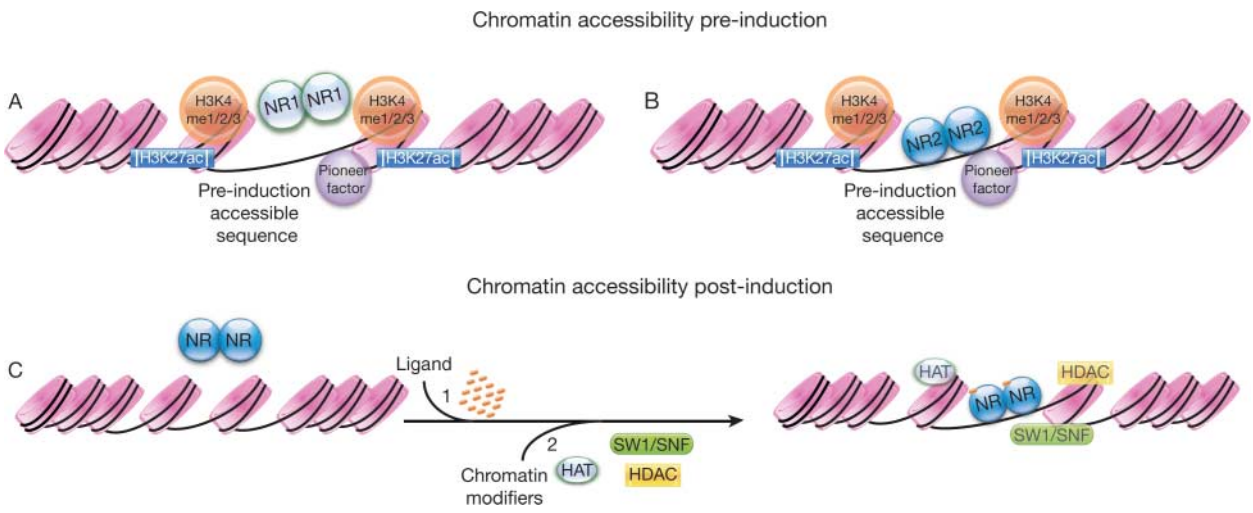

Figure 1

Schematic representation of nuclear receptors and chromatin accessibility. Nuclear receptors bind to responsive elements present in the DNA with different mechanisms. Chromatin accessibility and histone modifications contribute to NR binding via regulatory element bookmarking before ligands mediate activation (A). In some instances, NRs bind even in the absence of ligands while in complex with other proteins (B). Upon ligand activation, several NRs can guide further modifications of the chromatin landscape by recruiting histone modifiers and chromatin-remodelling complexes (C).

studies considering a few loci of GR-bound chromatin found some sites to be constitutively accessible using DNase I hypersensitivity site (DHS) assays and others to be remodelled upon ligand stimulation (John *et al*. 2008). However, a follow-up genome-wide screen using DHS assays coupled with next-generation sequencing (DHS-seq) revealed that the majority (95%) of GR binding occurs at pre-induction accessible sequences. These data demonstrate that chromatin accessibility can pre-select regulatory elements for GR binding. Adult cells present unique accessibility profiles (Thurman *et al*. 2012), suggesting that cell type-specific effects of NR activation could be partly mediated by the chromatin topology (John *et al*. 2008).

Nucleosome occupancy plays a different role in the context of AR binding at enhancers. After stimulation with dihydrotestosterone (DHT), He *et al*. (2012) observed the destabilization of a central nucleosome while the flanking nucleosomes remained occupied. In agreement with this, the central nucleosome is built around a modified histone protein called H2.A.Z, normally associated with fragile nucleosomes (Jin *et al*. 2009). Another study confirmed and expanded these observations and described a significant correlation between changes in DHS and AR-mediated transcription, suggesting that AR binding relieves nucleosome occupancy and potentially increases access for collaborating factors (Tewari *et al*. 2012). However, a later report suggested an intermediate model in which enhancers recruiting AR are in a stable equilibrium between nucleosome occupancy and nucleosome-free state in uninduced cells. Androgen treatment then destabilizes this equilibrium in favour of the more accessible nucleosome-free state (Andreu-Vieyra *et al*. 2011).

The situation is less clear-cut for ERα. Using formaldehyde-assisted isolation of regulatory element (FAIRE) assays, a technique that allows enrichment of nucleosome-depleted regions (Giresi *et al*. 2007), Hurtado *et al*. (2010) reported that a substantial amount of ERα can bind to more compacted chromatin regions. More importantly, they found that a significant proportion of accessible sites are present before oestrogen induction and they can recruit ERα (Hurtado *et al*. 2010). Identical conclusions can be drawn from a study that profiled chromatin accessibility before and after oestradiol (E2) stimulation, again using FAIRE assays (Joseph *et al*. 2010). These data are in substantial agreement with a more recent work that profiled ERα chromatin accessibility using DHS assays (He *et al*. 2012). In addition, it was noted that ERα binding that leads to a substantial increase in chromatin accessibility clusters closer to oestrogen-responsive genes, suggesting that basal accessibility state can be further modified (discussed below, He *et al*. (2012)).

Chromatin accessibility post-induction

In the case of ligand-mediated DNA binding, nucleosome remodelling appears to continue even if the chromatin is

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already in an accessible structure (He *et al*. 2012). Post-induction remodelling may have functional consequences for NR crosstalk. The assisted loading model predicts that binding of a NR may increase the chances of binding of a second NR via chromatin remodelling (Stratmann & Schibler 2011). ERα directly or indirectly recruits several chromatin-remodelling complexes on the chromatin after oestrogen stimulation (Belandia *et al*. 2002, Jeong *et al*. 2009). Similarly, GR is associated with SWI/SNF complexes, proteins involved in ATP-mediated nucleosome remodelling (reviewed elsewhere, King *et al*. (2012)). Accordingly, GR activation using dexamethasone can increase binding of E2-induced ERα at proximal chromatin locations (Voss *et al*. 2011, Miranda *et al*. 2013). Very similar observations were made with regard to the PR (Grøntved & Hager 2012). Furthermore, another recent report has identified local interactions between ERα and LRH1 (Lai *et al*. 2013). Depletion of LRH1 was found to cause decreased ERα binding, while LRH1 overexpression was found to facilitate ERα recruitment in the absence of ERα protein levels changes, again supporting the notion of assisted loading. These data are consistent with the transient nature of the interaction of NRs with the chromatin. Indeed, ERα and the molecular machinery that accompanies ERα binding can contact the chromatin at relatively predictable intervals in synchronized MCF7 cells (Métivier *et al*. 2003, 2008, Reid *et al*. 2003). Although speculative, it is conceivable that activated NRs could also influence binding of other TFs. Collectively, these data highlight the importance of chromatin accessibility in dictating NR binding and how NRs modulate post-induction remodelling processes.

Induction-independent

Ligand-mediated activation does not always lead to chromatin binding. For example, several NRs are constitutively bound to the chromatin even in the absence of ligands. While there is evidence for agonist-dependent changes in binding of some hormone NRs to chromatin (as described above), the influence of agonist binding on DNA occupancy for metabolic NRs is complex and not fully understood. ChIP-seq analysis of mouse liver chromatin for FXR-binding sites revealed that the majority are located in distal intergenic regions or introns and fewer sites are localized in more proximal promoter regions (Chong *et al*. 2010). The high distribution of binding sites within intergenic and intronic regions is consistent with similar reports for other steroid and metabolic NRs, including PPARγ (Lefterova *et al*. 2008, Nielsen *et al*. 2008), ERα (Carroll *et al*. 2005) and AR (Bolton *et al*. 2007). Chong *et al*. (2010) found no statistically significant difference in genome-wide binding of FXR in the livers of a control group fed a normal chow diet vs a group fed chow supplemented with GW4064, the potent synthetic FXR agonist, suggesting that FXR binding is unaffected by ligand stimulation. These data are in substantial agreement with another genome-wide FXR binding study carried out in WT and obese mice, revealing a ligand-independent co-occupancy of FXR and RXR (Lee *et al*. 2012). The same pattern was observed in another genome-wide binding site study, in mouse liver and intestine, where it was shown that FXR was already bound to its own REs in the regulatory regions of FXR target genes (Thomas *et al*. 2010). Although further studies need to be carried out, and different ligands studied, these results support the concept that metabolic NRs are bound to the DNA regardless of the presence of the ligand at accessible regulatory elements.

However, it is important to underline that these data might also be influenced by the basal FXR activation by bile acids, the FXR endogenous ligands. Nevertheless, the binding intensity was found to be boosted upon treatment with GW4064, in agreement with other *in vitro* studies in a liver cell line, HepG2, where it was shown that FXR binds to its REs weakly in the absence of ligands, and this binding is increased upon ligand treatment (Rizzo *et al*. 2005, Fang *et al*. 2008). Once again, this effect might be due to a ligand-switch effect, and it would be interesting to dissect the binding patterns of FXR upon treatment with natural ligands. Noteworthily, as pointed out by Chong *et al*. (2010), although bioinformatics analysis revealed over 1.7 million sites in the mouse genome matching the canonical FXR-REs, a very small percentage ($<0.1\%$) were shown to be occupied by FXR in their study. These results further support the idea that NRs are able to bind to their REs, although RE specificity is not sufficient. To achieve fully efficient NR–DNA binding, specific conditions are needed, including specific nucleosome positioning and epigenetic modifications able to alter chromatin structure and DNA accessibility.

Histone post-translational modifications and their role in NR recruitment

Epigenetic modifications have a well-characterized function during normal development where they bookmark regulatory elements to maintain heritable gene expression (Bernstein *et al*. 2006, Christophersen & Helin 2010, Ernst & Kellis 2010, Heinz *et al*. 2010, Ernst *et al*. 2011,

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Zhu *et al*. 2013). Only recently have we started to understand the relationship between histone modifications and NR activity. Several studies have shown direct interactions between the activation of NRs and the remodelling of chromatin through histone modifications. Analogously to chromatin remodelling, histone modifications play different roles and can be divided into pre-induction and post-induction.

Pre-induction histone modifications

Regulatory elements are characterized by an epigenetic grammar where combinatorial patterns of epigenetic modifications are associated with active, poised or inactive promoters and enhancers (Ernst & Kellis 2010, Heinz *et al*. 2010, Ernst *et al*. 2011). For example, active enhancers are commonly marked by histone H3 lysine K4 mono- and/or dimethylation (H3K4me1/2) (Lupien *et al*. 2008, Ernst & Kellis 2010). On the other hand, nucleosomes near the transcription start sites of active promoters are enriched with histone H3 lysine K4 trimethylation (H3K4me3; Bernstein *et al*. 2006, Thurman *et al*. 2012). NRs, similar to many other TFs, interpret the chromatin to find their cognate binding sequences (Magnani & Lupien 2013). Therefore, preloaded epigenetic modifications can contribute to the reduction of the number of potential binding sites by 'flagging' active regulatory elements. For example, Lupien *et al*. (2008) found that binding of sex steroid NRs ERα and AR significantly correlated with H3K4me2 patterns in breast and prostate cancer cell lines respectively. As expected, breast and prostate cells are characterized by markedly different H3K4me2 histone mark layouts in agreement with cell type specificity of enhancer elements (Ernst *et al*. 2011, Zhu *et al*. 2013). The H3K4me2 as well as the H4K4me1 signal does not change in response to oestrogen stimulation in breast cancer cells (Lupien *et al*. 2008, Joseph *et al*. 2010, Magnani *et al*. 2011*a*), suggesting that histone methylation is independent of and precedes ERα binding. Similarly, in prostate cancer cells, H3K4me1/2 is found at active enhancers capable of recruiting AR before DHT stimulation in support of its role as a bookmarking modification (Wang *et al*. 2009). Limited functional studies assessing the effect of H3K4me removal on ERα signalling (Lupien *et al*. 2008, Magnani *et al*. 2011*a*) using overexpression of a histone lysine (K)-specific demethylase 1 (LSD1) indeed found a significant loss in ERα binding.

Interaction between epigenetic modification and NR binding is in some cases mediated by a group of proteins called pioneer factors (Magnani *et al*. 2011*b*, Zaret & Carroll 2011, Magnani & Lupien 2013). Pioneer factors are a functionally defined family of proteins, including structurally unrelated TFs, capable of binding compacted chromatin (for extensive reviews, see Magnani *et al*. 2011*b*, Zaret & Carroll 2011). These factors can relieve chromatin compaction during lineage commitment, to maintain accessibility at cell type-specific enhancers (Cirillo & Zaret 1999). Pioneer factors can also act as molecular beacons for other TFs; for example, the forkhead family members FoxD3 and FoxA1 mark the liver-specific Alb1 enhancer starting from early embryonic development, preceding C/EBP, Ey and NFF1 binding and Alb1 expression (Zaret *et al*. 2008). Similarly, the homeobox pre-B-cell leukaemia transcription factor (PBX1) was found to precede MyoD recruitment during muscle development (Berkes *et al*. 2004). Accumulating evidence suggests that pioneer factors are preloaded on the chromatin at ERα-recruiting regulatory elements before ligand activation and contribute to increased chromatin accessibility (Jozwik & Carroll 2012). Depletion of pioneer factors including FOXA1 and PBX1 has a significant negative impact on ERα–chromatin interaction (Lupien *et al*. 2008, Magnani *et al*. 2011*a*). It is not clear whether all NRs are associated with these proteins; however, it appears that different NRs share pioneer factors as in the case of ERα, AR and retinoic acid receptor gamma (RARγ) and the pioneer factors FoxA1 and the trans-acting T-cell-specific transcription factor (GATA3) (Lupien *et al*. 2008, Hua *et al*. 2009). Pioneer factors lack protein domains typically interacting with modified histones (e.g. bromo- or cromodomains), and although some data suggest that pioneer factors can recognize specific histone modifications (Lupien *et al*. 2008, Magnani *et al*. 2011*a*), more studies are needed to establish the exact epigenetic-pioneer factor–NR hierarchy (Magnani & Lupien 2013).

Post-induction histone modifications

Upon ligand binding, ERα recruits a large cohort of accessory proteins as co-activators and co-repressors (O'Malley & Kumar 2009, Mohammed *et al*. 2013). Interestingly, several chromatin modifiers belong to this class, including the histone acetyl transferase (HAT) p300 (Kraus & Kadonaga 1998), arginine methyltransferase CARM1 (Lee *et al*. 2005), histone deacetylase HDAC2 (Mohammed *et al*. 2013) and histone demethylase KDM1A (aka LSD1) (Hu *et al*. 2008). Similarly, the H3K9me3 histone demethylase KDM3A is recruited by AR and PPARγ at NRREs (Yamane *et al*. 2006, Tateishi *et al*. 2009). There is a functional significance in HAT recruitment to the

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chromatin as acetylation of histones destabilizes the nucleosome structure by neutralizing the basic charge of the lysine (Yamane *et al*. 2006, Tateishi *et al*. 2009). In addition, acetylated lysine acts as molecular bait for bromodomain-containing proteins, including SWI/SNF chromatin-remodelling complexes, another group of NR co-factors (Trotter & Archer 2008). Not surprisingly, chromatin remodellers and HAT proteins seem to trail very closely ERα binding dynamics in synchronized cells stimulated with E2 (Métivier *et al*. 2003).

Histone-modifying proteins also interact with other NRs. Known interactors of GR include HDAC2 (Ito *et al*. 2000), HDAC1 (Qiu *et al*. 2011) and CBP/p300 (Pfitzner *et al*. 1998). Assembly of such multi-tasking complexes is compatible with the dual roles of NRs as activators and repressors. It is also likely that co-activator/co-repressors are cell type specific, considering the diverse number of cellular processes controlled by NRs.

As described for hormone NRs, once activated by its own natural ligands (bile acids), FXR recruits a number of co-activator complexes able to remodel the chromatin structure and facilitate the assembly of the transcriptional machinery to activate target gene expression. Anantha-narayanan *et al*. (2004) demonstrated with ChIP assay experiments that upon ligand binding, a simultaneous increase in FXR and CARM1 occupation occurs at the FXR target *BSEP* (*ABCB11*) (bile salt export pump) gene promoter. This binding was found to correspond with increased deposition of Arg-17 methylation and Lys-9 acetylation of histone H3 within the FXREs of BSEP, leading to increased *BSEP* gene transactivation. Furthermore, FXR can be methylated by Set7/9 within its hinge region at lysine-206. ChIP assays demonstrated the recruitment of Set7/9 and the presence of the associated H3K4 monomethylation activation mark at the loci of two of the known FXR hepatic targets, the small heterodimer partner *NR0B2* (*SHP*) and *BSEP*. This significantly enhanced the transcription of these two FXR target genes (Balasubramaniyan *et al*. 2012). Similar data for AR (Ko *et al*. 2011) and ER (Subramanian *et al*. 2008) indicate that this mechanism could also possibly stabilize FXR and promote its interaction with its heterodimerization partner RXR and the FXRE (Balasubramaniyan *et al*. 2012). Furthermore, recruitment of chromatin-remodelling proteins and NR-dependent gene transcription is a very dynamic process and different co-regulators and chromatin-remodelling proteins can occupy different REs of the same NR. In the hepatic FXR/SHP pathway, FXR senses elevated intrahepatic bile acid levels and indirectly suppresses the expression of the key-limiting enzyme of the bile acid synthesis pathway, CYP7A1, via SHP. Chromatin immunoprecipitation and chromatin remodelling studies in both HepG2 cells and mouse liver have shown that upon treatment with FXR agonists, the transcription activator Brg-1 is recruited to the *SHP* promoter and it interacts with FXR, resulting in accessible chromatin, thereby enhancing FXR-dependent transactivation of *SHP*. On the other hand, Brm is recruited to both *SHP* and *CYP7A1* promoters, resulting in inactive, inaccessible chromatin (Miao *et al*. 2009). This indicates that chromatin remodellers Brm and Brg-1 have distinct functions in the regulation of two key genes within a single physiological pathway, by differentially modulating accessibility at SHP- and FXR-regulatory elements. In summary, these data demonstrate how NR binding facilitates the deposition of histone modifications, transiently altering the epigenetic profile of regulatory elements while locally increasing chromatin accessibility for the transcriptional machinery.

Epigenetic modification contributes to stimulus-specific activation of regulatory elements

NRs respond to a large array of molecules of diverse nature. Additionally, individual NRs can be promiscuous in terms of ligands. For example, metabolic NRs participate in complex, overlapping transcriptional regulation networks involving metabolic homoeostasis. Some of these receptors, such as the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), are promiscuous with respect to the structurally wide range of ligands that act as agonists. In fact, they can bind to metabolites that are also ligands for other NRs, such as bile acids or steroid hormones and others (Kliewer & Willson 2002). This could be due to the presence of multiple LBDs, a very large binding pocket, or a binding site with flexibility to alter size and shape depending on the size of the ligand (reviewed in Krasowski *et al*. (2011)). In this context, the ERα has been the object of a substantial amount of work considering its role in breast cancer (Ali *et al*. 2011). ERα binding localization (its cistrome) has a significant impact on gene expression (Krum *et al*. 2008). Importantly, the ERα cistrome partially varies dependent on the type of ligand. Tamoxifen is one of the key molecules used in the treatment of breast cancer (Jordan 2003). Tamoxifen competes with E2 for the LBD of ERα and imparts a specific tridimensional structure not compatible with the recruitment of co-activators, at least in some tissues (Shang & Brown 2002). Recent evidence suggests that tamoxifen

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alters the ERα–DNA binding profile (Welboren *et al*. 2009). In agreement, cells that acquire resistance to tamoxifen recruit ERα at a distinct set of loci compared with that of non-resistant cells (Ross-Innes *et al*. 2012). ERα can also be activated via growth factor-mediated phosphorylation induced by, for example, tyrosine kinase receptors such as the epidermal growth factor receptor (Kato *et al*. 1995). Genes regulated by phosphorylated ERα are distinct from oestrogen-responsive genes (Lupien *et al*. 2010). Importantly, oestrogen and EGF can induce ERα recruitment at three classes of enhancers: enhancers bound with stimulation with either oestrogen or EGF, enhancers bound exclusively following oestrogen treatment and enhancers bound exclusively following EGF treatment. These data are in agreement with ligand-specific NR binding (Lupien *et al*. 2010). However, the first class of regulatory elements (e.g. recruiting ERα in response to both stimuli) still exhibits a ligand-specific response as transcription is induced by only one ligand. This apparent contradiction is resolved taking into account ERα-mediated post-induction epigenetic modifications. Indeed, a fraction of these shared sites acquire stimulus-specific histone acetylation (H3K18ac; Lupien *et al*. 2010) and correlate with EGF- or oestrogen-regulated genes. A recent study probing different classes of ERα ligands has demonstrated that very minute changes in the chemical structure have significant effects on ERα-regulated cellular processes such as proliferation (Srinivasan *et al*. 2013). These results were observed even in response to different allosteric interactions of the same ligand (Srinivasan *et al*. 2013). Although the ERα cistrome was not assessed, it is conceivable that each ligand induces topologically different binding events.

Functional role of non-coding RNA in NR signalling

We have previously mentioned that a large class of NRs exhibit a significant tendency towards binding enhancers (Kittler *et al*. 2013). Recent work pioneered by studies on X-chromosome inactivation has highlighted a central role for long non-coding RNAs (lncRNAs) in epigenetic silencing and enhancer activation (Calabrese *et al*. 2012). Enhancer RNAs (eRNAs) are a class of lncRNAs with a potential role in the mediation of epigenetic modifications as well as interactions with TFs (Gupta *et al*. 2010, Kogo *et al*. 2011, Calabrese *et al*. 2012). Enhancer RNAs are typically bidirectional short RNAs transcribed by RNA polymerase II and centred around epigenetically defined enhancer elements (H3K4me1/2 and H3K27ac; Gupta *et al*. 2010, Kim *et al*. 2010, Kogo *et al*. 2011). They have been implicated in several molecular processes including polycomb-mediated repression (Tsai *et al*. 2010), metastatic progression (Gupta *et al*. 2010) and enhancer activity (Orom & Shiekhattar 2011). Nonetheless, only recently, scientists have been able to elucidate their functional role partly through the use of NR-dependent cell lines (Orom & Shiekhattar 2011, Li *et al*. 2013). ER binding is thought to promote enhancer–promoter looping to activate the transcriptional machinery at target genes (Perillo *et al*. 2008, Fullwood *et al*. 2009, Li *et al*. 2012, 2013). The Rosenfeld group has described a strong relationship between E2–ERα-induced eRNA production and gene regulation (Li *et al*. 2013). Using siRNA and locked nucleic acid antisense oligonucleotides, the group was able to show that eRNA disruption suppresses E2–ERα-mediated transcription by destabilizing long-distance interactions between promoters and enhancers (Li *et al*. 2013). The production of eRNAs substantially correlates with numerous markers of enhancer activation, including post-induction epigenetic modifications and increased chromatin accessibility (Hah *et al*. 2013). Interestingly, eRNA production does not seem to influence pre-induction histone modifications such as H3K4me1 and H3K4me3 (Hah *et al*. 2013). However, Hah *et al*. (2013) suggest that eRNA production may be occurring after the assembly of the transcriptional machinery on the chromatin and is decoupled by promoter–enhancer interaction. While we are just beginning to understand eRNAs, their interaction with NRs seems to be conserved as demonstrated by orphan NRs Rev-ERBα and Rev-ERBβ. In this case, Rev-Erb receptors function as strong repressors by suppressing eRNA transcription at lineage-specific enhancers (Lam *et al*. 2013). Altogether, these data suggest that NR activity at enhancers is far more complex that initially postulated.

Future directions

NRs have been studied extensively for over 30 years; nevertheless, we are still gathering new information about the way they function. Several aspects are yet to be defined, including how NRs cooperate physically and mechanistically with each other and the DNA structure. For example, in hormone-dependent cancers, it will be crucial to establish the role of understudied NRs and understand whether they could replace the classical sex hormone receptors once anti-cancer drugs have targeted ERα and AR. It will also be important to determine the role of unliganded NRs in development as well as disease. An intriguing study has found that unliganded PR forms a repressor complex with a non-coding RNA and several

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epigenetic silencers including histone demethylases LSD1, KDM5B and histone deacetylases HDAC1/2 (Vicent *et al*. 2013). In other words, unactivated PR maintains repressed chromatin structures at progesterone target gene enhancers, while the epigenetic landscape is reversed upon the interaction of PR with its ligand (Vicent *et al*. 2013). These data further highlight the complex interaction between non-coding RNA, epigenetic modifications, NRs and transcription. More questions concerning NR binding topology would need an answer, such as how heterogeneous is the binding in normal and diseased tissues and how targeted therapies can alter NR binding profiles. Considering that enhancer elements are inherently variable between individuals (Kasowski *et al*. 2013), it is reasonable to expect that NRs will also target different regions in different patients. Indeed, a recent study that profiled ERα binding in breast cancer patients has found little consensus between individuals for most of the ERα-binding regions (Ross-Innes *et al*. 2012). Furthermore, in a study of hepatic genomic binding of ligand-activated FXR in healthy and obese mice, Lee *et al*. (2012) demonstrated that almost 50% of the sites were unique to either healthy or obese mice and that the altered FXR binding led to direct repression of many of these unique targets. Finally, it has been shown that the H3K4me3 epigenetic mark, essential for the transactivation of a number of hepatic FXR target genes, is reduced at their FXREs in a mouse model of cholestasis (Ananthanarayanan *et al*. 2004, Lee *et al*. 2012). Whether this is a reflection of an intrinsic heterogeneity in the epigenetic landscape of diseased tissues still requires clarification.

Ultimately, in the last few years, there has been a surge in studies addressing the fundamental role of chromatin and epigenetics in directing transcription. The inducible nature of NRs and the study of their interaction with the DNA allow us to mechanistically explore the dynamic nature of transcription and how diseases alter TF–chromatin crosstalk. Further understanding of these processes will contribute to the discovery of new relevant clinical implications (e.g. location-specific chromatin interactions as a prognostic factor) and the development of novel and more targeted treatments. Considering the importance of NRs in development, metabolism and disease and the high potential for pharmacological manipulation, we foresee new breakthroughs in the coming years.

Declaration of interest

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

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