

CONTROLLING NUCLEAR RECEPTORS: THE CIRCULAR LOGIC OF COFACTOR CYCLES

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Abstract | Nuclear receptors regulate many biologically important processes in development and homeostasis by their bimodal function as repressors and activators of gene transcription. A finely tuned modulation of the transcriptional activities of nuclear receptors is crucial for determining highly specific and diversified programmes of gene expression. Recent studies have provided insights into the molecular mechanisms that are required to switch between repression and activation functions, the combinatorial roles of the multiple cofactor complexes that are required for mediating transcriptional regulation, and the central question of how several different signalling pathways can be integrated at the nuclear level to achieve specific profiles of gene expression.

ORPHAN RECEPTOR

A subclass of nuclear receptors that were originally identified as orphans because the ligand was unknown.

Precise spatial and temporal patterns of gene expression are crucial for the normal development of all organisms. Orchestrating these patterns requires the coordination of numerous regulatory events and mechanisms that mediate both the repression and activation of specific target genes at specific times and places during development. DNA-binding transcription factors, non-DNA-binding coregulators and general components of the basal RNA polymerase machinery are essential to regulate transcription and achieve the correct patterns of gene expression. Although there are many variations in their functions and in the highly specific strategies of regulation, several general themes and common rules have emerged.

Nuclear receptors provide an interesting model to study the specific, as well as the more general, mechanisms of transcriptional regulation; they are highly regulated DNA-binding transcription factors that are directly modulated by ligand binding and can both activate and repress gene expression. Here, we review several recent advances in our understanding of nuclear-receptor-mediated transcriptional regulation by focusing on the dedicated mechanisms that regulate the switch from gene repression to gene

activation and that further modulate transcriptional activity. The complexity of regulatory proteins and the many ways of modulating their recruitment and their activity tightly regulates this apparently simple, but fascinating, recruitment event.

The nuclear receptor superfamily: an overview

The mammalian nuclear receptor superfamily comprises more than 45 transcription factors, many of which regulate gene expression in a ligand-dependent manner. Members of the nuclear receptor superfamily include: receptors for steroid hormones, such as the oestrogen receptor (ER), the androgen receptor (AR) and the glucocorticoid receptor (GR); receptors for non-steroidal ligands, such as the thyroid hormone receptor (TR) and the retinoic acid receptor (RAR); as well as receptors that bind diverse products of lipid metabolism such as fatty acids and prostaglandins (peroxisome proliferator activated receptors (PPARs) and liver X receptors (LXR))^{1,2}. The nuclear receptor superfamily also includes many so-called ORPHAN RECEPTORS for which regulatory ligands are still unknown or for which candidates have only recently been identified by screening strategies that

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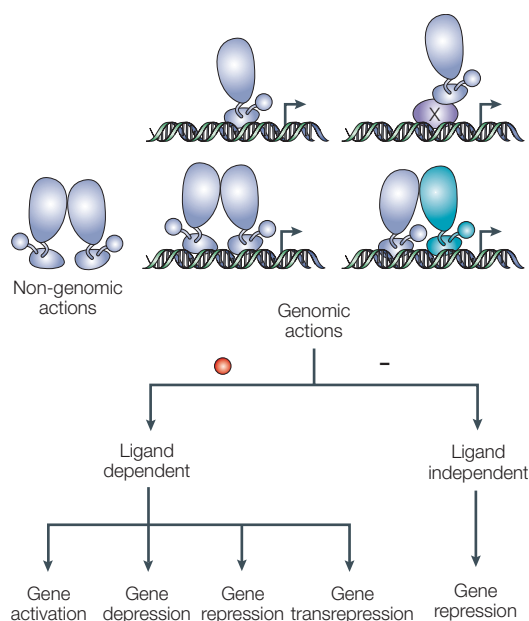


Figure 1 | Mechanisms of transcriptional regulation by nuclear receptors. Ligand-inducible nuclear receptors modulate transcription through several distinct mechanisms, which include both activation and repression activities. These activities can be genomic or non-genomic (a description of rapid steroid actions that activate signalling pathways and that do not directly influence gene expression can be found in REF. 144), ligand dependent or independent, and can mediate gene repression, the release of gene repression, gene activation, or gene transrepression (when they negatively regulate transcription indirectly through another transcription factor 'X'). Furthermore, nuclear receptors can bind to their DNA-response elements as a single unit (monomer; for example, steroidogenic factor-1 (SF1)), or associated with another identical receptor (homodimer; for example, ER-ER), or with a receptor of a different type (heterodimer; for example, RAR-RXR). ER, oestrogen receptor; RAR, retinoic acid receptor; RXR, retinoic X receptor.

have uncovered physiological and pharmacological ligands³.

Remarkably, the sequence of the *Caenorhabditis elegans* genome has revealed that the nuclear receptor family, with more than 200 members, constitutes the largest family of transcriptional regulators encoded by this genome, which indicates that these proteins have an important role in the environmental adaptation of nematodes⁴. The sequencing of the human genome has so far led to the identification of 48 nuclear receptors, which have diverse, crucial roles in the regulation of growth, development and homeostasis. Comparative analysis among human, nematode and insect genome sequences has shown that divergent evolutionary pathways have defined separate phylogenetic lineages so that most of the *C. elegans* nuclear receptors diverge from those found in humans and flies⁵.

Nuclear receptor proteins have a characteristic, modular structure, which includes a highly conserved DNA-binding domain (DBD) and ligand-binding domain (LBD)^{1,2}. However, despite the structural

conservation, members of the nuclear receptor family are functionally extremely flexible in regulating transcription. For example, they can bind directly to specific response elements in DNA, either as a monomer or as homo- and heterodimers. Or, they can regulate transcription indirectly through other classes of DNA-bound transcription factors. Also, a subset of receptors, which includes TR and RAR, can actively repress target genes in the absence of ligand binding, whereas on ligand binding, all nuclear receptors usually become potent transcriptional activators. Conversely, several nuclear receptors have been shown to inhibit transcription in a ligand-dependent manner — either by binding to negative response elements or by antagonizing the transcriptional activities of other classes of transcription factors, an effect known as transrepression^{1,2,6,7} (FIG. 1).

Coregulators: corepressor and coactivators

Nuclear receptors carry out their many different transcriptional functions through the recruitment of a host of positive and negative regulatory proteins, referred to as coactivators or corepressors, respectively⁸ (FIG. 2). These regulatory cofactors are not exclusive to nuclear receptors, and are used in a similar manner by numerous other DNA-binding transcription factors. The molecular mechanism that regulates the alternative interactions of the nuclear receptor with either class of cofactors has been decoded by crystallographic studies⁹. For a detailed description of how these alternative interactions are directly regulated by specific changes in the receptor conformation, see BOX 1.

In general, unliganded nuclear receptors preferentially interact with corepressors to mediate repression, whereas liganded receptors are transcriptional activators owing to their ability to recruit coactivator proteins. However, a few exceptions have been identified, as some corepressors, such as LCoR (ligand-dependent nuclear-receptor corepressor), RPI140 (receptor-interacting protein-140) and REA (repressor of oestrogen-receptor activity)^{10,11}, can bind to nuclear receptors in a ligand-dependent manner and compete with coactivators by displacing them. These findings indicate the existence of specific regulatory mechanisms that use similar, but reverse, approaches to attenuate the function of agonist-bound receptors. Furthermore, there are coregulatory factors, such as the ATP-dependent chromatin remodelling complexes SWI/SNF, which have been associated with the regulation of both gene activation and gene repression^{12–16}. Therefore, it is important to consider that the role of a given coregulatory protein can be context dependent and, hence, it is not always possible to assign a functional outcome on the basis of the recruitment of a specific cofactor.

Alternatively, coregulators can also be classified into two main groups according to their mode of action. The first group contains factors that covalently modify histones (for example, by ACETYLATION/deacetylation, METHYLATION/demethylation, phosphorylation/dephosphorylation or UBIQUITYLATION/deubiquitylation), a process that follows a precise and combinatorial code.

ACETYLATION

The enzymatic process of adding an acetyl group to a Lys residue on histone tails or on other proteins.

METHYLATION

The enzymatic process of adding a methyl group to a Lys or an Arg residue on histone tails or other proteins. Alternatively, methyl groups can be added to DNA itself on cytosine bases.

UBIQUITYLATION

A three-step enzymatic process that covalently conjugates the small protein ubiquitin to a Lys residue on a protein substrate. Ubiquitin can be added as a monomer or as a polymer to form long chains.

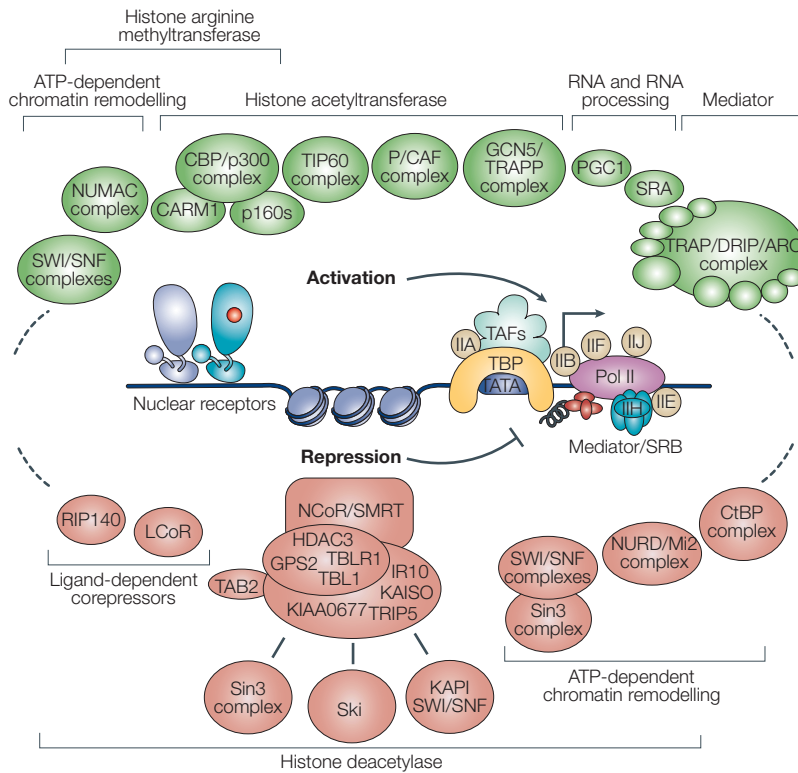


Figure 2 | Coactivator and corepressor complexes are required for nuclear-receptor-mediated transcriptional regulation. The regulation of a general transcription unit by nuclear receptors requires a vast number of coregulatory complexes that have various functions and enzymatic activities. Coactivator complexes (green) include factors that contain ATP-dependent chromatin remodelling activity^{14,38,52}, histone arginine methyltransferases^{145–147}, histone acetyltransferases^{103,148,149}, as well as factors that are involved in RNA processing^{20,150} and components of the so-called Mediator complex that mediate the interaction with the RNA polymerase II (pol II) machinery^{151–154}. Conversely, corepressors (red) include ATP-dependent chromatin remodelling complexes^{21,155–159}, basal corepressors, such as NCoR and SMRT, which function as platforms for the recruitment of several subcomplexes that often contain histone deacetylase activity^{13,27,88,122,123}, and specific corepressors, such as LCoR and RIP140, which are surprisingly able to recruit general corepressors on ligand induction¹⁰. This schematic representation is useful to underline the numerous regulatory complexes that are involved in nuclear-receptor-mediated regulation; however, it is important to keep in mind that it does not illustrate the dynamics of their recruitment to the regulated transcription unit (see FIG. 3). IIA, IIB, IIE, IIF, IIH, IIJ, general transcription factors A, B, E, F, H, J; HDAC, histone deacetylase; LCoR, ligand-dependent nuclear-receptor corepressor; NCoR, nuclear-receptor corepressor; RIP140, receptor-interacting protein-140; SMRT, silencing mediator of retinoic acid and thyroid hormone receptors; TAF, TBP-associated factor; TBP, TATA-binding protein.

The second group includes ATP-dependent chromatin remodelling factors that modulate promoter accessibility to transcription factors and to the basal transcriptional machinery^{17–19}. However, this broad division is not fully inclusive as there are numerous cofactors that do not have any known direct effects on chromatin structure or modification, but instead function in the assembly, the recruitment or the release of coregulatory complexes.

Furthermore, a precise classification is complicated by the fact that new coregulators are continually being discovered and these include factors that were not expected to serve such functions, as exemplified by the RNA transcript for the steroid-receptor-RNA activator-1 (SRA1) coactivator²⁰, the NAD/NADH

sensor CtBP (C-terminal binding protein of E1A)^{21–23}, and several ACTIN-binding proteins^{24–29}. This indicates that transcriptional regulation cannot be considered as an independent, chromatin-based process, but rather should be considered as coupled to many other cellular events that are carried out by several distinct groups of factors. For an exhaustive list of known coregulators, see REFS 30–33.

In conclusion, the general concept that emerges from the uncovering of this complicated network of coregulators is that an unexpectedly large array of proteins and enzymatic activities converges at promoters, defining what could be considered a ‘cofactor code’, which is characterized by distinct patterns of cofactor recruitment and by their regulated enzymatic activities. The HISTONE CODE, which is determined by specific combinations of covalent histone modifications¹⁸, is a consequence as well as a determinant of this cofactor code, as histones are crucial targets for the enzymatic activities of cofactors, but also have a key role in specifying cofactor recruitment on the basis of the ‘reading’ of the histone code by specific chromatin-binding domains.

Coregulators in multiprotein complexes

The ability of corepressors and coactivators to associate in several different diverse complexes further increases the complexity of their transcriptional regulatory roles and allows a temporal- and tissue-specific modulation of the combinatorial cofactor and histone codes for specific, single genes. The existence of numerous platform coregulatory proteins, each capable of several interactions, has a fundamental role in determining this flexibility, as these interactions can be effectively modulated by covalent modifications, protein levels and competition between proteins, and they provide a conceptual basis for the exquisite control of specific gene expression.

Furthermore, many coregulators share functions and enzymatic activities with other, related coregulators that can exert partly redundant functions in certain biological settings, but that have unique functions in others. An example is provided by the mammalian homologues of SWI/SNF2, Brahma and BRG1, which cannot genetically compensate for each other, and which associate with different BRG1-associated factor (BAF) chromatin remodelling complexes^{34–37} that are required by different nuclear receptors^{14,38,39}.

FIGURE 2 shows a generic nuclear-receptor-regulated transcription unit with a subset of the cohorts of coactivator and corepressor complexes that can participate in its control — the transition from repression to activation results from ligand binding to the receptor and from the exchange of corepressors for coactivators. Although this representation is useful to illustrate the multiplicity of coregulatory complexes, this type of model has turned out to be erroneously static and oversimplified. The switch between gene repression and gene activation, for example, can hardly be defined by the simple alternative recruitment of two different regulatory complexes. Rather, it is achieved by a series of sequential events that are mediated by multiple enzymatic activities that

ACTIN
An abundant protein that forms filaments that are the main constituents of the cytoskeleton of all eukaryotic cells: the monomeric form is known as G-actin and the polymeric, filamentous form as F-actin.

can be factor, promoter and cell-type specific, as will be discussed in more detail below^{17,32,40,41}.

The idea of transcriptional regulation as a highly dynamic event also raises the interesting issue of whether transcription complexes are temporarily recruited to each active gene for cofactor exchange to occur, or whether a crucial component of transcriptional activation might involve the migration of the promoters themselves to physiological 'stations' of preassembled transcription 'factories'^{42–44}.

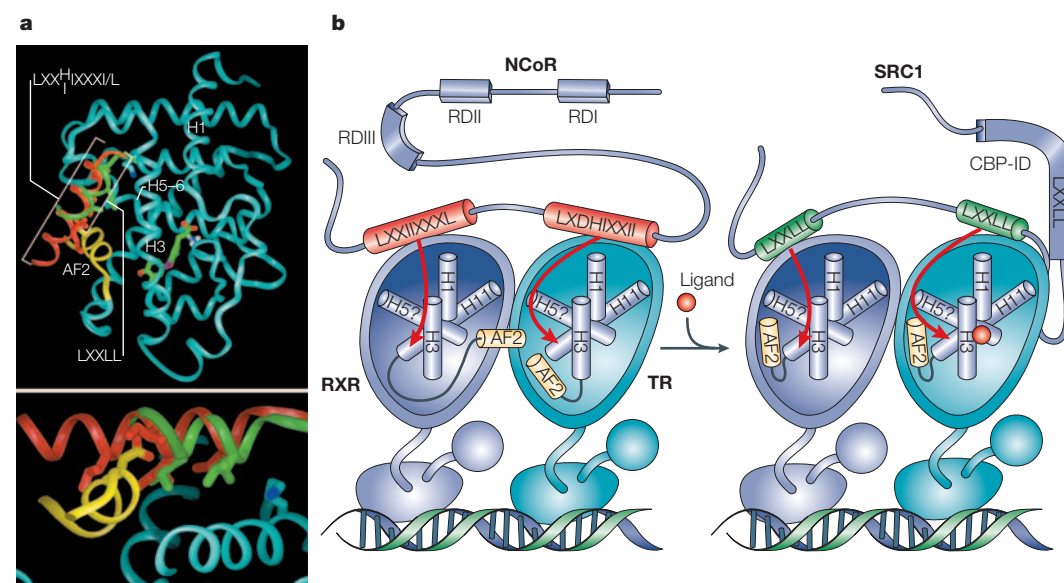
Interestingly, many studies have indicated a role for nuclear actin in regulating gene transcription^{45,46}, and therefore it is intriguing to consider that actin-binding proteins and actin itself, as detected in various biochemically purified cofactors complexes, might be important for mediating these nuclear relocalization events. For more detail on the functional architecture of the nucleus and the spatial localization of active domains of transcription, see REFS 47–49.

Box 1 | Nuclear-receptor-coregulator interaction motifs

Crystal structures of the ligand-binding domains (LBDs) of several nuclear receptors have revealed a three-layered, anti-parallel α -HELICAL sandwich, with a central core layer of three helices packed between two additional layers of helices to form a ligand-binding cavity. In the unliganded retinoic X receptor (RXR) structure, helix 12, which contains the transactivation domain AF2, extends away from the LBD¹³¹, whereas in the agonist-bound retinoic acid receptor (RAR) α , thyroid hormone receptor (TR) β , oestrogen receptor (ER) and peroxisome proliferator activated receptor (PPAR) γ structures, the AF2 helix (shown in yellow in part a of the figure) is tightly packed against the LBD and makes direct contact with the ligand^{132–137}. This spatial organization indicated that ligand-dependent changes in the conformation of the AF2 helix could form a novel surface that might facilitate coactivator interactions. In the presence of ligand, numerous coactivators, such as steroid receptor coregulator (SRC1 or NCoA1) interact with nuclear receptors through helical motifs that contain an LXXLL consensus sequence (see figure, part b)^{138,139}. Interestingly, co-crystal structures of nuclear receptors with LXXLL-interacting motifs from different coactivators indicate that the LXXLL helix fits into a defined pocket (indicated by red arrow), and that two conserved crucial residues of the receptor, a Glu in the AF2 helix and a Lys in the binding pocket, form a 'charge clamp' that stabilizes the interaction^{133,136,137}.

Similarly, corepressor proteins, such as nuclear-receptor corepressor (NCoR) and SMRT, interact with unliganded RAR and TR and with antagonist-bound steroid hormone receptors using two nuclear-receptor-interaction domains that share a core consensus motif: LXX I/H IXXX I/L or corepressor–nuclear-receptor (CoRNR) box (see figure, part b)^{140–142}. Interestingly, this motif is highly related to the coactivator-interacting motif, and can be visualized as an N-terminally extended helix (shown in red in part a of the figure) when compared to the shorter, LXXLL-containing helix (shown in green in part a of the figure). As the crucial determinants of corepressor binding reside in the same pocket that is required for coactivator binding, it was proposed that ligand-dependent exchange between corepressors and coactivators is caused by the difference in length of the interacting motifs that can be accommodated in the binding pocket in the two conformations¹⁴¹.

Screening of a phage-display library of CoRNR box motifs allowed the identification of several peptides that specifically bind to the antagonist-bound ER, which confirms that the receptor uses overlapping surfaces for binding to corepressors and coactivators. It also showed that it is possible to identify mutations that differentiate between the two classes of regulators¹⁴³. CBP-ID, CREB-binding protein (CBP)-interaction domain; RDI, RDII, RDIII, repressor domains 1–3; H1–H12, α helices 1–12.



HISTONE CODE

The post-translational modifications of histone tails usually in characteristic clusters, which include acetylation, phosphorylation, ubiquitylation, methylation and ADP-ribosylation. The modified histone tails combine to create an epigenetic code for the regulation of gene expression.

A HELIX

An element of protein secondary structure in which hydrogen bonds along the backbone of a single polypeptide cause the chain to form a right-handed helix.

CHROMATIN IMMUNOPRECIPITATION (ChIP). A technique that is used to specifically immunoprecipitate complexes of DNA with associated proteins. The use of antibodies specific for histone modifications, DNA-binding transcription factors or coregulators has allowed the study of promoter occupancy by different factors and the state of chromatin modification.

Ordered recruitment of coregulator complexes

Given the diversity of coregulatory complexes and their often mutually exclusive mode of interaction with transcription factors, it was important to establish whether these series of transcription-factor-coactivator interactions occur randomly or in a sequential and regulated fashion. Indeed, although the cell- and time-specific expression patterns of some cofactors can produce distinct modulations of nuclear receptor transcriptional activity owing to differences in the relative corepressor and coactivator protein levels⁵⁰, it is true that many other cofactors are coexpressed in the same cell type at relatively similar, high levels, which raises the possibility of their concomitant recruitment to a specific promoter.

In an elegant pioneering study, Cosma and colleagues⁵¹ presented the first unambiguous example of the ordered recruitment of different coregulatory complexes to a regulated transcription unit. Using CHROMATIN IMMUNOPRECIPITATION (ChIP) analysis to study the transcriptional activation of the *HO* gene in budding yeast, they found that the recruitment of the transcription factors *Swi5* and SBF, the SWI/SNF and the SAGA coactivator complexes, and the *Ash1* repressor followed a precise pattern in which every step was precisely timed and necessary for the subsequent event⁵¹.

This first observation presaged numerous studies in mammalian systems, often related to nuclear-receptor-regulated genes that have established a conceptually similar pattern of recruitment and release of cohorts of coregulatory complexes^{52–56}. A good example is provided by the examination of

ER-mediated transcriptional regulation of either the cathepsin D (*CATD*)⁵³ or the trefoil factor-1 (*TFF1/pS2*) promoter^{16,55} in breast cancer cells. These analyses revealed detailed and coordinated patterns of cofactor recruitment and preferential selectivity for factors that have similar enzymatic activities^{16,55}. In the case of the *pS2* promoter, for example, different classes of cofactors could be identified, which are each defined by distinct enzymatic activities and recruited to inducible promoters by the DNA-bound transcription factor in a factor-specific, precisely timed and sequential fashion^{16,32} (FIG. 3). Interestingly, in this experimental setting, some coregulators seemed to be redundant, and different family members were equally capable of being recruited alternatively to the promoter (that is, the nuclear-receptor coactivators *NCoA1*/SRC1 and *NCoA3*/pCIP or the coactivator-associated arginine methyltransferases *CARM1* and *PRMT1* never co-occupied the *pS2* promoter)¹⁶. By contrast, preferential interactions between a nuclear receptor and a specific histone acetyltransferase (HAT)-containing enzyme or a specific member of the NCoA family of coactivators have also been observed^{57–59}.

A similar scenario of coordinated recruitment of dedicated corepressor complexes is also emerging for transcriptional repression. For example, 4-hydroxy-tamoxifen (4-OHT)-bound ER α mediates the sequential recruitment of two different complexes, NCoR-TBL1-HDAC3 and the chromatin-remodelling complexes known as nucleosome remodelling and histone deacetylation (NURD), which each harbour distinct histone deacetylase (HDAC) activities⁶⁰. The N-terminal truncated ER α isoform (apo-ER α -46kD) also engages these two complexes, but in this case, unliganded ER recruits them in a combinatorial fashion and no sequential recruitment was observed⁶¹. Interestingly, the specific recruitment of diverse corepressor complexes could reflect a different specificity for the deacetylation of histone tail residues⁶², and this might be important to define a histone code for repression.

In conclusion, several recent studies have begun to depict a fascinating picture of the ordered recruitment of different complexes of cofactors to regulated transcription units. The challenge now is to extrapolate these single-factor-, single-gene-, single-cell-based observations to a more general level to understand the rules that govern the deposition of this cofactor code and the strategies for its modulation. An interesting aspect, for example, is that the promoter-specific DNA-binding sequence by itself could represent an allosteric regulator of receptor activity and cofactor recruitment^{63–65}. Similarly, the mode of interaction with DNA, either directly or through other transcription factors, could be an important regulator of specificity. Shang and Brown, for example, have reported differences in the regulation of the *Myc* and *CATD* genes by ER α that is bound to the antagonist 4-OHT, which might depend on the different organization of the two promoters analysed — which either contain or lack a classic oestrogen response element (ERE)⁵⁰. Furthermore, Rogatsky and colleagues adopted a genome-wide approach to

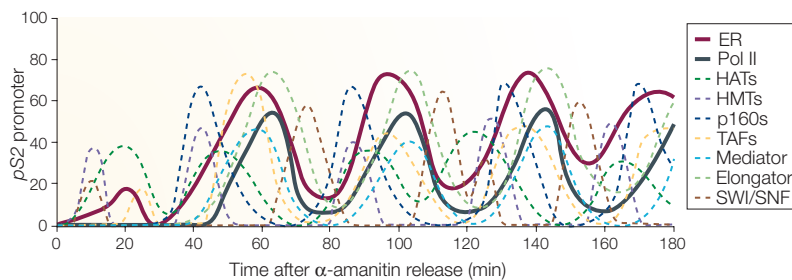


Figure 3 | Ordered cofactor recruitment by the ER. The diagram summarizes the observations of Metivier and colleagues on the kinetics of cofactor recruitment to the *pS2* promoter. In this kinetic ChIP analysis, the *pS2* promoter was stimulated with 10 nM oestradiol, and transcription by RNA polymerase II (pol II) was inhibited by treatment with α -amanitin¹⁶. For purposes of clarity, different factors that have the same enzymatic activities have been grouped together. However, this simplification might hide differences among proteins that belong to the same enzymatic class and that are represented as a single unit. For example, the histone acetyltransferase (HAT) GCN5 is always recruited slightly after the other family members, shown by the green curve. Also, in the first cycle, only p300, the HAT TIP60 and the histone methyltransferase (HMT) PRMT1 are engaged, whereas the other HATs and HMTs are not yet recruited. However, it is unclear whether this initial, non-productive cycle exists under physiological conditions, as the use of α -amanitin, although very important to distinguish the kinetics of ER-dependent recruitment from other basal activation events of the promoter, creates an artificial situation in which transcription has been completely shut off. The y axis represents the amount of immunoprecipitated *pS2* promoter expressed as a percentage of the input¹⁶. p160 proteins belong to the NCoA family of coactivators, components of the Mediator complex mediate pol II transcription, elongator factors are involved in transcription elongation and SWI/SNF are ATP-dependent chromatin remodelling factors. TAFs, TBP-associated factors.

identify GR targets and to determine differences in their regulation. This permitted the identification of several distinct patterns of use of specific GR domains, which indicates that a single transcription factor might perform differently at specific gene locations because diverse surfaces and different interacting cofactors can be engaged depending on the promoter context⁶⁶.

For further discussions about the models of cooperation between different complexes that regulate transcription and their ordered recruitment to specific gene promoters by transcription factors other than nuclear receptors, see REFS 32,67.

Kinetics of promoter occupancy

The dynamic recruitment of different coregulator complexes is associated with an equally dynamic binding of the nuclear receptor itself to the promoter. Indeed, in the past few years, the conventional view that receptors remain stably bound to their DNA response elements has been challenged. ChIP analyses of promoter occupancy by nuclear receptors have shown that the binding of the receptor to DNA is characterized by cycles of recruitment and release^{53–55} and, for example, in the case of ER α binding to the *pS2* promoter, the duration of each cycle is 15–20 minutes⁵⁵. Furthermore, photobleaching techniques, which were used to study GR turnover on synthetic promoters, have uncovered a much more rapid exchange of receptor molecules on DNA, which can be measured in seconds^{68,69}. Although these potential differences might have arisen from distinctions between the nuclear receptors analysed and from the comparison of artificial response elements with endogenous promoters, we suggest that the ChIP data accurately reflect the temporal differences of promoter occupancy and non-occupancy by the receptor. Superimposed on this, an ultra-rapid exchange of each individual receptor molecule on DNA, as identified by photobleaching analysis, might represent the ‘breathing’ of this highly dynamic system.

Interestingly, cycles of recruitment and release have been observed for different nuclear receptors and for nuclear factor- κ B (NF- κ B), which indicates that the ordered and cyclic recruitment of various components of transcription complexes might be a common feature of promoters that are activated by inducible transcription factors^{16,53–55,70}. AR-mediated transcriptional regulation, for example, involves a rapid and cyclic assembly of the AR transcription complex on the prostate-specific antigen (*PSA*) and kallikrein-2 promoters⁵⁴. This kinetic behaviour of inducible promoters is particularly interesting because the continual cycling of nuclear receptors and their associated complexes on inducible promoters might represent a way to monitor the environment, which allows a tighter control of the expression of genes that are under hormonal stimulation. Cycling of the receptor on the promoter would indeed allow regular clearance phases between each successive cycle. It is reasonable to think that these clearance phases are potential regulatory checkpoints during which gene activation can be rapidly reversed to a default repressed state unless the hormonal stimulation continues^{55,70,71}. It is

interesting that the SWI/SNF complexes are initially directly engaged on the *pS2* promoter after oestrogen activation, which induces chromatin remodelling and promotes an open chromatin conformation that is permissive for transcription initiation. But later, after the first cycle of nuclear receptor binding, the SWI/SNF complexes are again recruited to the promoter during each clearance phase, where they associate with HDACs and with the NURD complex¹⁶. This indicates that the cyclic recruitment of chromatin remodelling factors might play a part in resetting the permissiveness of the promoter for transcription.

Furthermore, nuclear receptor cycling might not be exclusively confined to the phase when the receptor is activated by ligand binding. Metivier and colleagues recently proposed the interesting hypothesis that the unliganded ER α can also cycle on the promoter, and that this unexpected nuclear role of the aporeceptor is important to keep the target gene ‘ready’ for activation when ligand stimulation occurs⁶¹. This challenges, or at least increases, the complexity of the classic model, in which steroid receptors are kept inactive in the cytoplasm until ligand stimulation triggers their dissociation from inhibitory complexes and their subsequent nuclear translocation.

Further studies, which include the investigation of nuclear receptors that bind constitutively to the promoter and are responsible for active repression, such as RAR or TR, will be required to understand more completely the rules that underlie the dynamics of nuclear receptor binding to DNA.

Nuclear receptor turnover and transcription

The cyclic turnover of nuclear receptors on regulated promoters seems to correlate with PROTEASOME-dependent degradation activity, chaperone activity and chromatin remodelling events^{55,69,72}. Accordingly, the degradation of transcriptional activators is often required for gene activation^{54,55,73–77}, and nuclear receptors, as well as many of their coregulators, can be ubiquitinated and regulated by protein degradation^{78–83}.

The significance of an association between transcriptional activation and proteolysis of the activator is not completely clear and is somehow counterintuitive, as one might expect the removal of activators to correlate with the negative control of gene transcription. However, as briefly discussed above, the cyclic clearance of nuclear receptors might be crucial, because it allows a continuous reassessment of the ‘state of the cell’ — each cycle would overcome the default programme of transcriptional repression only if the activating stimulus was still present. Furthermore, the site of ubiquitylation and the transcriptional activation domain overlap in many transcription factors. This raises the intriguing possibility that an ubiquitylation event is required to license the function of transcription factors, thereby linking their activity to their destruction⁷³. However, there are also cases in which proteasome inhibition has been reported to enhance transcriptional activation^{84–86}, which indicates that the role of protein degradation in transcriptional

PROTEASOME

A large protein complex that degrades intracellular proteins that have been tagged for destruction by the addition of ubiquitin.

regulation could be cell, nuclear-receptor and even promoter specific⁸⁶.

Finally, the inhibition of proteasome activity might affect many cellular processes and, therefore, the interpretation of the data obtained by using inhibitor reagents with respect to specific biochemical events can be misleading. More focused experiments will be crucial to examine the role of specific ubiquitylation events and of specific components of the ubiquitin–proteasome machinery.

From repression to activation

The possibility of switching gene expression from ‘off’ to ‘on’ and vice versa in living organisms emerged as a revolutionary concept in biology less than 50 years ago. Seminal work by Jacob and Monod showed that bacteria could respond to changes in the growth medium by the activation or repression of METABOLIC OPERONS that are important for using alternative carbon sources⁸⁷. The basic strategy that regulates this crucial switch has been conserved in higher eukaryotes, but the molecular mechanisms in mammalian systems have evolved further layers of complexity, which include several regulatory strategies that cooperate to impose the precise control of gene expression. These strategies include the use of different DNA-binding activators and repressors that compete for regulatory regions in promoters and/or ENHANCER regions; the modulation (inhibition or enhancement) of transcription factor activities; the regulation of the intracellular localization of transcription factors by external signals; and the allosteric regulation of the activity of transcription factors, such as the nuclear receptors, by ligands.

Corepressor–coactivator exchange. Ligand binding is the crucial molecular event that switches the function of nuclear receptors from active repression to transcriptional activation, at least in the case of heterodimeric receptors such as RAR or TR that are constitutively bound to DNA. A combination of elegant structural and molecular studies of the interactions between nuclear receptors and coregulators has provided the evidence that hormone binding induces a conformational change in the ligand-binding domain of the receptor, which results in reduced affinity for corepressors and, simultaneously, enhanced affinity for coactivators (see BOX 1 for references and details). Similarly, agonist binding to steroid receptors, such as ER, progesterone receptor (PR), GR or AR, also induces the adoption of a specific conformation that favours coactivator binding, whereas antagonist binding promotes the interaction with corepressors.

However, it recently became clear that there are additional and unexpected layers of regulation in the molecular events that modulate the nuclear receptor switch from repression to activation (FIG. 4). Indeed, recruitment of the ubiquitylation machinery and proteasome-dependent degradation of the repressors are required, at least in the case of the NCoR-containing corepressor complex, to fully promote the release of the corepressors in response to ligand binding⁷¹. Therefore,

the activation of the nuclear corepressor exchange (NCoEx) factors could represent a further control level, which is imposed to maintain more robust transcriptional repression and to avoid undesirable gene expression. This level of regulation would increase the amplitude of transcriptional activation events by imposing a repression checkpoint. Interestingly, **TBL1** and **TBLR1** — the NCoEx factors that are required as adaptors for this function — are components of the NCoR and silencing mediator of retinoic acid and thyroid hormone receptors (**SMRT**) corepressor complexes and are required for the repression of specific transcription units^{27,88,89}. This implies that, as a required component of this action, signals that promote gene induction must activate parallel pathways to activate the exchange machinery and release the repression checkpoint. Similar strategies are probably also used to regulate the release of the corepressor machinery by other transcription factors, as reported in the case of activator protein-1 (**API**) and NF- κ B^{70,71,90}.

Obviously, there are many common elements and similarities between this proteasomal-dependent degradation of corepressors and the cyclic degradation of nuclear receptors, which indicates that, although they are distinct events, they might also be tightly linked. Indeed, if the receptor periodic cycles are important to allow a continuous assessment of the hormonal state of the cell, the re-establishment of a repression checkpoint at each clearance phase could also provide a tighter control on activation. This means that the ubiquitylation and the release of the corepressors would be crucial, not only during the first activating step, but at each cycle of receptor assembly on the promoter.

Nuclear integration of signalling pathways

If we want to appreciate fully the fine regulation of the transcriptional activity of nuclear receptors, we have to consider the fact that they are not only able to respond to hormonal stimulation, but they can also integrate information derived from a large variety of external stimuli and interact on promoters with DNA-binding transcription factors under independent regulation. Several signalling pathways that are activated by various developmental or physiological signals have been reported to cross-talk with nuclear-receptor-mediated responses through both direct and indirect mechanisms. The transcriptional activity of nuclear receptors is modulated by the induction of post-translational modification of the receptor itself or of its coregulatory proteins. Phosphorylation, acetylation, SUMOYLATION, ubiquitylation and methylation are among the modifications that have been reported to modulate the functions of nuclear receptors and that potentially constitute an important cellular integration mechanism. It has been suggested that these modifications influence cellular localization, enzymatic activity and stability of targeted proteins, and could also be important in modulating the timing of the sequential recruitment of the different classes of coregulators to a single transcription unit.

METABOLIC OPERON

A group of contiguous bacterial genes that are required for a metabolic function, and which are transcribed together in a single mRNA molecule.

ENHANCER

A DNA regulatory sequence that modulates the rate of transcription of a specific gene from a distance.

SUMOYLATION

The enzymatic process of adding the small SUMO protein to Lys residues on target proteins.

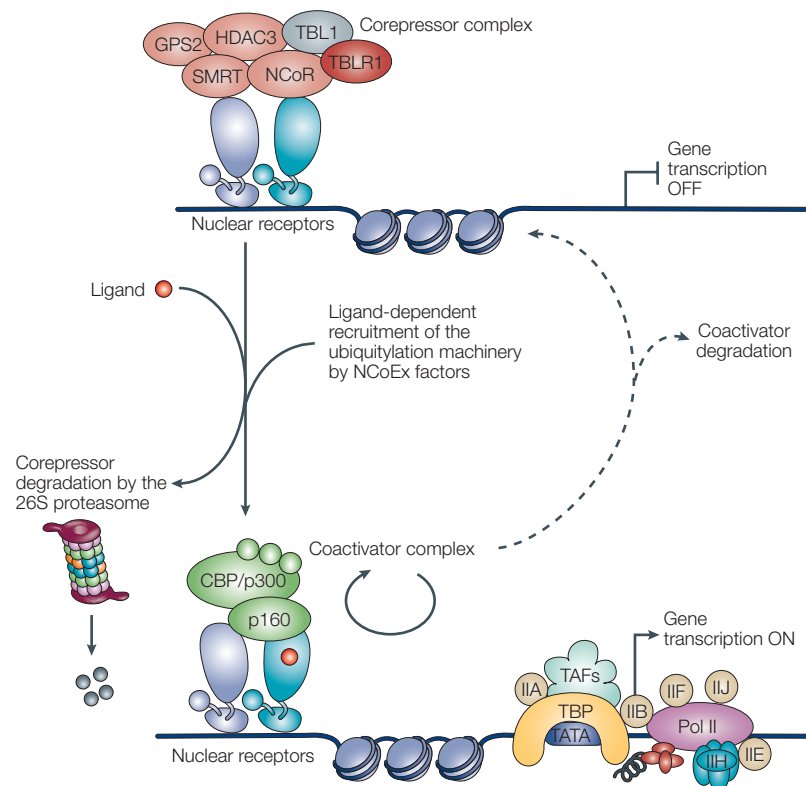


Figure 4 | Ubiquitin-dependent exchange of corepressors for coactivators. During the transition from gene repression to gene activation on ligand stimulation, there is a required exchange among cofactor complexes. This molecular switch is regulated by conformational changes in the nuclear receptor, which results in a different affinity for cofactor complexes, and by the recruitment of the ubiquitylation machinery, which has a fundamental role in the dismissal of the corepressor machinery. The diagram shows a generic, unliganded nuclear receptor heterodimer that recruits the NCoR and SMRT corepressor complexes to repress transcription. The nuclear corepressor exchange (NCoEx) factors TBL1 and TBLR1 are required to recruit the ubiquitylation machinery following ligand stimulation, thereby allowing the dismissal and degradation of the corepressor complex and the recruitment of coactivator complexes. Ubiquitin-dependent protein degradation events have also been associated with cycling of the receptor itself on the promoter and with coactivator turnover. IIA, IIB, IIE, IIF, IJ, IJ, general transcription factors A, B, E, F, H, J; CBP/p300; the co-activators CREB-binding protein and p300; GPS2, G-protein pathway suppressor-2; HDAC, histone deacetylase; NCoR, nuclear-receptor corepressor; p160, a co-activator of the NCoA family; pol II, RNA polymerase II; SMRT, silencing mediator of retinoic acid and thyroid hormone receptors; TAF, TBP-associated factor; TBP, TATA-binding protein.

A detailed description of all these modifications is beyond the scope of this review (for relevant reviews, see REFS 30, 91–94). Here, we will describe three examples — a nuclear receptor (RAR), a coactivator (cAMP-response-element-binding protein (CREB)-binding protein (CBP)) and a corepressor (NCoR) — to illustrate how signalling pathways can be integrated into specific transcriptional responses (FIG. 5).

Nuclear receptor modifications: RAR. Three RAR family members — α , β and γ — regulate the transcriptional activation and repression of target genes by functioning as the heterodimeric partners for retinoic X receptors (RXRs) in response to all-*trans*-RA or to its 9-*cis* isomer (9-*cis* RA). Their ability to integrate the input from multiple signalling pathways has been

studied extensively and multiple phosphorylation sites have been identified. Protein kinase A (PKA) activation, for example, exerts functional effects on the receptor because of the direct phosphorylation of a conserved Ser residue in the LBD⁹⁵, whereas the DBD can be phosphorylated by protein kinase C (PKC)⁹⁶, and both the N-terminal and the C-terminal RAR regions contain consensus phosphorylation sites for cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), and the Jun N-terminal kinases (JNKs)^{77,97–99}.

Phosphorylation of the N-terminus of RAR α by cyclin H–CDK7, which is associated with the basal transcription factor TFIIH, is required for the function of the transactivation domain, AF1 (REF. 99). This indicates that phosphorylation of this region might be important for its interaction with either important coregulators — to mediate the AF1 transactivation function — or for its direct interaction with the basal transcriptional machinery. Defects in the RAR α phosphorylation status due to mutations that affect the interaction between the receptor and TFIIH have been identified in patients with xeroderma pigmentosum¹⁰⁰, thereby providing genetic evidence of the importance of these regulatory mechanisms. RAR γ also shows similar regulation by CDK7 kinase, but in this case, phosphorylation of a nearby residue by p38MAPK is also required for full transcriptional activity^{77,98}. Interestingly, these phosphorylation events are important for the ubiquitylation and subsequent degradation of RAR γ , although they do not seem to play any part in RAR α ubiquitylation^{77,101}.

Coactivator modifications: CBP and p300. The transcriptional coactivators CBP and p300 are involved in numerous signal-regulated transcriptional events that are mediated by different DNA-binding transcription factors. CBP was originally identified as an interacting partner for the transcription factor CREB in response to cAMP signalling, and p300 was identified as a cellular-associated factor of the adenoviral protein E1A. Interestingly, CBP and p300 can promote transcriptional activation and integrate upstream signals through a series of different mechanisms, which includes not only direct chromatin remodelling activity by histone acetylation, but also acetylation of other factors, direct binding to components of the basal transcriptional machinery and recruitment of other coregulators, such as scaffold proteins^{102,103}.

Both CBP and p300 are well-known phosphoproteins¹⁰⁴. Interestingly, their phosphorylation status seems to be under cell-cycle control; p300, for example, was shown to be phosphorylated by CDC2 and CDK2 kinases in a physiological process that could be targeted and blocked by the adenoviral protein E1A¹⁰⁵. And the observation that the transcriptional function of p300 was negatively regulated by cyclin E–CDK2 allowed a further understanding of the functional implications of CBP and p300 phosphorylation by cell-cycle-dependent kinases¹⁰⁶. An interesting possibility is that the enzymatic activities of CBP and p300 are directly

G1/S TRANSITION

The cell-cycle checkpoint at the transition between the Gap phase-1 (G1) and the beginning of DNA replication.

modulated as a result of the phosphorylation events that occur during cell-cycle progression. This was suggested by Ait-Si-Ali *et al.*¹⁰⁷, who reported that general HAT activity peaked during the G1/S TRANSITION, and that the HAT activity of CBP was enhanced by the C-terminal phosphorylation mediated by cyclin E-CDK2 as well

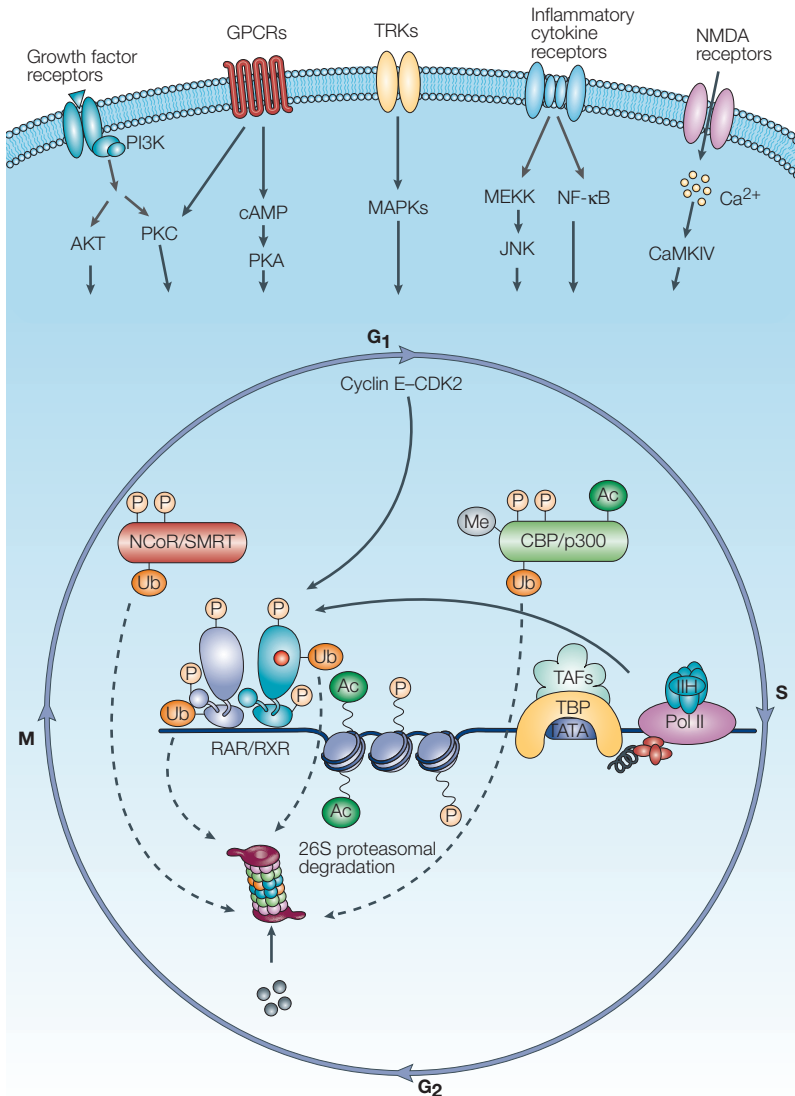


Figure 5 | Integration of signalling pathways on nuclear-receptor-mediated transcriptional regulation. Nuclear-receptor-mediated transcriptional regulation can be modulated by several signalling pathways that are activated by external or internal signals, including cell-cycle-dependent signals, growth factor stimulation, inflammatory signals and signals that activate G-protein-coupled receptors (GPCRs), tyrosine kinase receptors (TRKs) and ion channel receptors. The diagram shows retinoic acid receptor (RAR) as an example of nuclear receptors, the coactivator CREB-binding protein (CBP)/p300 and the corepressors NCoR and SMRT. For each, the activity and the ability to interact with other partners can be modulated by various mechanisms (see the main text for details and references), which include phosphorylation (P; yellow), methylation (Me; grey) and acetylation (Ac; green). Polyubiquitylation (Ub; orange) is often used to mark nuclear receptors and associated cofactors for proteasomal degradation. IiH, general transcription factor H; CaMKIV, Ca²⁺/calmodulin-dependent kinase IV; CDK2, cyclin-dependent kinase-2; JNK, Jun N-terminal kinase; MAPK; mitogen-activated protein kinase; MEKK, MAPK-kinase kinase; NCoR, nuclear-receptor corepressor; NF-κB, nuclear factor-κB; NMDA, N-methyl-D aspartate; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; pol II, RNA polymerase II; RAR, retinoic acid receptor; RXR, retinoic X receptor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptors; TAF, TBP-associated factor; TBP, TATA-binding protein.

as by the interaction with E1A. As E1A was reported to stimulate the phosphorylation of CBP and p300 (REF. 108), this suggests the interesting possibility that viral oncoproteins can mimic physiological signals that activate the HAT function of p300 and CBP. However, further studies are needed for a better understanding of the mechanisms of this regulation, as several reports have shown an opposite effect, in that the interaction with E1A inhibits the enzymatic activity of CBP^{109–111}.

Other kinases, which include PKA¹¹², Ca²⁺/calmodulin-dependent kinase IV (CaMKIV)¹¹³ and MAPK¹¹⁴, can phosphorylate different CBP residues, thereby enhancing its transcriptional-activation activity. For example, phosphorylation by p44 MAPK has been reported to have a positive effect on the enzymatic activity of CBP¹¹⁵. By contrast, p300 phosphorylation by PKC represses transcriptional activity¹¹⁶, perhaps consistent with the opposing activities of CBP and p300 on proliferation and the response to DNA damage.

Interestingly, detailed studies of the signalling pathways that are involved and exhaustive mapping analysis of the phosphorylation sites have uncovered a correlation between certain modifications and precise functional effects: for example, the phosphorylation of Ser301 of CBP by CaMKIV is required for the N-methyl-D aspartate (NMDA)-dependent activation of CREB-responsive genes in hippocampal neurons¹¹⁷.

Phosphorylation is clearly not the only modification that is used to integrate signalling pathways in the regulation of coactivator functions. For example, methylation by CARM1 of a specific domain of CBP (the KIX domain), which interacts with the kinase-interacting domain (KID) of CREB, has been shown to be important for inducing the dissociation of CBP from CREB, and for inhibiting CREB-dependent transcriptional activation¹¹⁸. Furthermore, p300 is also ubiquitylated and degraded by the ubiquitin–proteasome pathway during F9 embryonal carcinoma cell differentiation. Interestingly, p300 shows different phosphorylation patterns in undifferentiated versus differentiated cells, and the changes in phosphorylation status that are promoted by PKA affect its HAT activity only during differentiation^{119,120}.

Corepressor modifications: NCoR. Although the nuclear-receptor corepressors NCoR and SMRT do not harbour intrinsic enzymatic activity, each recruits several other corepressors, which include many histone deacetylases¹²¹. Indeed, there are numerous distinct NCoR and SMRT complexes that have been biochemically purified^{13,27,88,122,123}, but most components probably assemble to form a holoenzyme complex.

Several kinases, including MAPKs, AKT/protein kinase B (PKB) and casein kinase-2 (CK2), have been shown to modify the NCoR and SMRT corepressors and to induce their relocalization from the nucleus to the cytoplasm^{30, 124–126}. Interestingly, some of these modifications seem to be specific for either SMRT or NCoR, which introduces a fascinating level of specificity to their regulation. Jonas and Privalsky have recently shown that SMRT is negatively regulated by

CYTOKINE

A member of a large family of secreted proteins that interact with cellular receptors. Cytokine production results in the activation of an intracellular signalling cascade that commonly regulates processes such as immune function and inflammation.

SCF E3 LIGASE COMPLEX

A multisubunit E3 ubiquitin ligase complex that contains an F-box protein for specific substrate recognition.

MAPK signalling pathways in response to stress or growth factors, whereas NCoR is not affected by this regulation¹²⁷. Similarly, previous studies indicate that NCoR phosphorylation status and/or nuclear localization are modulated in response to CYTOKINES, such as interleukin (IL)1 β , or to differentiation factors, such as the ciliary neurotrophic factor (CNTF), whereas SMRT is not affected by them^{30,124}.

A recent paper by Hoberg and colleagues, who analysed the derepression of NF- κ B target genes, reports the direct phosphorylation of SMRT by inhibitor of NF- κ B (I κ B) kinase α (IKK α) and its subsequent ubiquitylation, release from repressed promoters and nuclear export⁷⁰. This is consistent with the observation that the ubiquitin-dependent dismissal and degradation of corepressors is required for the switch from gene repression to gene activation of nuclear receptors and other transcription factors⁷¹, and also with the observation that protein phosphorylation is commonly used to mark proteins to be ubiquitylated by SCF E3 LIGASE COMPLEXES¹²⁸. Furthermore, it is interesting to note that SMRT is recruited back to the promoter ~20–40 min after being released following phosphorylation and ubiquitylation, which indicates that ordered waves of corepressor recruitment also occur at NF- κ B-regulated promoters⁷⁰. This implies that, as discussed for nuclear receptors, there are transcriptional repression checkpoints that need to be periodically released by dedicated ubiquitylation-dependent strategies for each cycle of transcription to proceed. Interestingly, IKK α has also been shown to be important for the enhancement of the transcriptional activation of NF- κ B-regulated genes on cytokine treatment, because it can phosphorylate histone H3 on Ser10 and can modulate the subsequent CBP-dependent acetylation of Lys14 (REFS 129,130). This represents a fascinating example of a signalling pathway that simultaneously regulates both the dismissal of corepressors and the enhancement of coactivators, which indicates that similar strategies are also likely to operate for nuclear-receptor-mediated transcription.

Finally, the possibility of regulating the localization of corepressors in the cell by nuclear export in response to specific signals raises the interesting question of whether the degradation of NCoR and SMRT occurs directly at the promoter level, or whether its release from the promoter following phosphorylation/ubiquitylation is coupled with a relocalization event. This relocalization event might be associated with chaperones such as the 14-3-3 proteins, which confine the degradation of NCoR and SMRT to the cytoplasm, or at least to a nuclear location that is not closely associated with the promoter.

Concluding remarks

The nucleus is a cellular compartment that is crucial for coordinating the responses to diverse signals. At the level of transcriptional regulation, this coordination resides in numerous coactivators, corepressors and chromatin remodelling complexes that are recruited to their target genes by DNA-binding transcription factors. As these complexes have several functional domains, each of which is subject to modifications and interactions that can activate, nullify or switch their function, and as there are so many — literally hundreds — of components that can participate to form these complexes, the vast number of possible combinations allows for highly specific and flexible responses.

Therefore, in this review, we have tried to show how this complexity translates into an exquisitely precise sequence of events that cooperate to achieve the precise regulation of gene transcription, which depends on promoter context, cell-cycle stage and regulatory signals. Indeed, each transcriptional activation event comprises a series of changes, such as DNA demethylation, histone modification, cofactor modification and so on, each of which alone might not be sufficient for full activation, but together converge to define a specific ‘combinatorial code’. Furthermore, we have focused on the emergence of the rapid reprogramming of gene repression and activation as a major step in ligand-dependent gene regulatory events and have reviewed the most recent findings on the molecular mechanisms that mediate these events. Unfortunately, lack of space has not permitted a complete review of the phenotypic consequences of the deletion of single coregulators during development, as reported by several excellent *in vivo* analyses of mice models.

However, the more we learn about the regulation of nuclear receptor function, and as new and more powerful technologies become available to further study it, more novel, unexpected questions arise. For example, what distinguishes genes that respond to derepression alone from those requiring further ligand-dependent effects? How is the timing of the transcriptional response regulated? What are the molecular marks that permit the serial recruitment and release of distinct cohorts of coregulators? Which enzymatic components of coactivator and corepressor complexes function at transcriptional and post-transcriptional levels? How are these ‘rules’ of regulation applied on a genome-wide basis? Clearly, the stage is set for an exciting new season in which the secrets of nuclear receptor regulation will be further unravelled.

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