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# The Flip Side: Identifying Small Molecule Regulators of Nuclear Receptors

Review

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**Members of the nuclear hormone receptor superfamily function as ligand-activated transcription factors to regulate genetic networks controlling cell growth and differentiation, inflammatory responses, and metabolism. The ability to modulate nuclear receptor-dependent gene expression with small molecules has made the superfamily a favored target for drug discovery. Not surprisingly, small molecules that regulate receptor activity are currently used to treat a number of human disorders. Over the last 10 years, the availability of a common platform of functional assays suitable for any nuclear receptor has facilitated the identification of endogenous and synthetic ligands that have been used as tools to uncover previously unanticipated endocrine signaling pathways. Recent progress in understanding the molecular basis for ligand-dependent gene regulation suggests that a new era of "designer" ligands with tissue- and/or gene-selective activity will quickly be upon us.**

## The Nuclear Receptor Superfamily

Analysis of genome sequences has identified 48 human members of the steroid and thyroid nuclear hormone receptor superfamily [1]. Nuclear receptors (NRs) function as ligand-dependent transcription factors that regulate the activity of genetic networks essential for the proper control of development, differentiation, and homeostasis. The important roles played by NRs in human physiology and the ability to regulate their functional activity with synthetic small molecule mimics of natural ligands has long stimulated the interest of the pharmaceutical industry. Indeed, synthetic ligands for NRs are currently used to treat a host of human diseases including cancer [2], inflammation [3], and metabolic syndromes [4].

Admission into the NR superfamily requires an evolutionary conserved primary structure (Figure 1A) comprised of a variable amino-terminal domain, a highly conserved DNA binding domain (DBD) that includes two zinc-coordinating motifs, and a less well-conserved ligand binding domain (LBD) [5]. The LBD is functionally complex and mediates receptor dimerization, ligand binding, and ligand-dependent transcriptional regulation. While many NRs were identified based upon primary sequence homology to receptor DBDs, recent structural analysis of receptor LBDs has revealed a highly conserved 3-dimensional fold comprised of 12 $\alpha$ helices sandwiched around two $\beta$ sheets (Figure 1B) [6–8].

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The LBD crystal structures confirm molecular and biochemical studies indicating that ligand binding promotes a conformational change in NR structure (Figure 1B). What appears to be a relatively flexible conserved helix near the carboxyl terminus (helix 12) occupies unique positions when structures of unliganded, agonist-occupied, and antagonist-occupied LBDs are compared (Figure 2) [6–8]. Importantly, mutagenesis experiments indicate that helix 12 is necessary for ligand-dependent transactivation by nuclear receptors [9]. Recent work indicates that helix 12 contributes an essential surface to the formation of an agonist-dependent hydrophobic groove comprised of amino acids from helices 3, 4, 5, 6, and 12 that serves as a binding site for coactivators [10]. The alternative positions occupied by helix 12 in the unliganded or antagonist-occupied conformations preclude the formation of this binding groove and instead favor the binding of corepressors to a region that overlaps the coactivator binding site (Figure 2). As their name implies, corepressors function as inhibitors of transcription when complexed with unliganded or antagonist-occupied NRs [11]. Thus, by modulating protein-protein interactions, a conformational change induced by the binding of hormones or synthetic small molecule ligands is translated into a transcriptional response.

Numerous NR coactivators and corepressors have been identified using two-hybrid screens and in vitro protein-protein interaction assays [12]. Interestingly, as a group the ability of NR coactivators and corepressors (collectively referred to as coregulators) to interact with receptors in a ligand-dependent manner is mediated by relatively small hydrophobic $\alpha$ helical sequences. In particular, many coactivators utilize a common signature motif, LxxLL (L = leucine, x = any amino acid), present once or up to several times, to interact with NRs. These LxxLL motifs have also been referred to as NR boxes [13, 14]. When receptor-coregulator interactions have been examined, individual NRs exhibit distinct but overlapping preferences [15, 16]. As described below, the ability to measure ligand-dependent interactions between NRs and relatively small LxxLL containing peptides has been exploited for the identification of novel NR ligands.

## Identification of Nuclear Receptor Ligands

The structural and mechanistic conservation within the NR superfamily has allowed the development of a common platform of assays that have been used for ligand identification (Figures 3 and 4). Well-known signaling molecules such as steroids, thyroid hormone, vitamin D, and retinoic acid were shown to interact with specific NRs by direct binding assays with radiolabeled ligands. Additionally, these hormonal signaling molecules were used to regulate receptor transcriptional activity in functional assays that entail cotransfection of receptor expression plasmids and reporter plasmids linked to *cis*-acting DNA binding sites (hormone response elements,

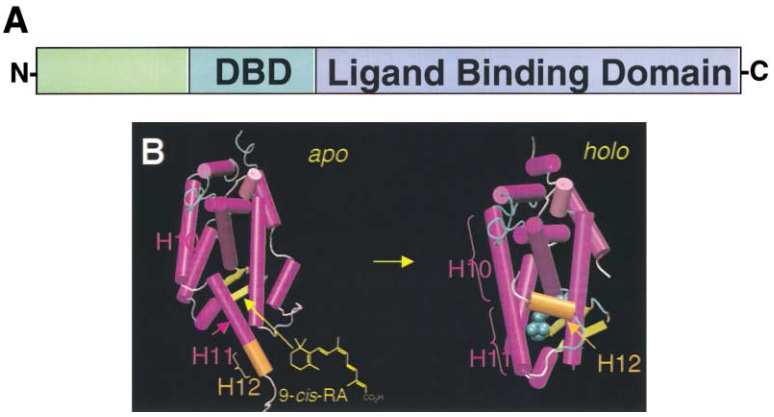


Figure 1. Conserved Structure of the Nuclear Receptor Superfamily

(A) Members of the nuclear receptor superfamily are characterized by a conserved structure that includes a variable amino-terminal domain, a highly conserved DNA binding domain that consists of two zinc binding domains, and the ligand binding domain (LBD). The LBD mediates ligand binding, receptor dimerization, transcriptional repression, and transcriptional activation.

(B) Structure of the RXR LBD in the absence (apo) [51] and presence (holo) [52] of 9-*cis* retinoic acid. Note the change in the position of helix 12 (orange) upon ligand binding.

HREs) into mammalian cells. The cotransfection assay reconstitutes a functional hormone response unit comprised of the receptor of interest bound to an HRE (usually multiple copies of an HRE sequence are arrayed in tandem; Figure 3A) upstream of a reporter gene that encodes an enzyme such as luciferase or β-galactosidase that can easily be quantitated [5]. Treatment of transfected cells with receptor-selective agonists increases the transcriptional activity of the receptor, resulting in increased levels of the mRNA encoding the reporter enzyme, and the elevated levels of mRNA of the reporter enzyme lead to increased enzymatic activity that can be detected in whole-cell lysates. Such transfection assays can easily be automated and carried out in a high throughput fashion, allowing the analysis of large numbers of compounds and facilitating the generation of detailed dose-response curves. Antagonist activity can also be measured by activating the receptor of interest with an agonist and quantitating decreases in reporter activity upon addition of increasing concentrations of antagonists.

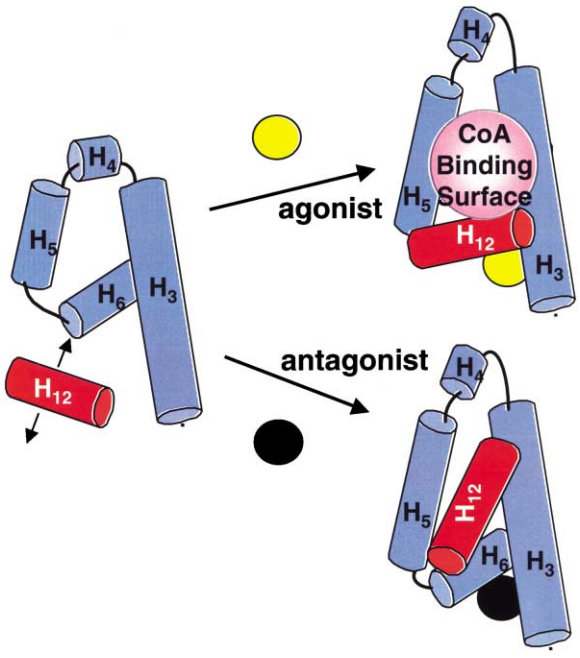


Figure 2. Agonists and Antagonists Promote Different Receptor Conformations

Agonist binding results in a repositioning of helix 12 near amino acid residues from helices 3, 4, 5, and 6, creating a groove for coactivator binding. Antagonist binding sterically inhibits the ability of helix 12 to form the active conformation. Instead, helix 12 occupies a position that covers coactivator binding.

In recent years the traditional cotransfection assay has been extended to include the use of fusions between NR LBDs and the DNA binding domain of the yeast transcription factor GAL4 (Figure 3B) and two-hybrid assays that detect ligand-dependent NR-coregulator interactions (Figure 3C). The GAL4 fusion assay is similar to the traditional assay described above; however, in this system the LBD of a receptor is fused to a DNA binding domain of a yeast transcription factor that does not have a ortholog in mammalian genomes. The GAL4 fusion is then transfected into cells along with a reporter gene containing three to five GAL4 binding sites. Taking advantage of the "foreign" yeast DNA binding domain limits interference from endogenous DNA binding proteins and other nuclear receptors that can bind to mammalian HRE sequences and many times results in assays with a larger dynamic range of sensitivity.

Like the GAL4 assay, two-hybrid assays also utilize artificially created fusion proteins. In this case the receptor interacting domain(s) of coregulators are fused to the GAL4 DNA binding domain while receptor LBDs are fused to a strong constitutively active transcriptional activation domain such as the well-characterized activation domain of the viral transcription factor VP16. The GAL4-coregulator fusion binds to the reporter gene but by itself does not activate transcription. Similarly, the receptor VP16-LBD fusion does not bind to the reporter, which only contains GAL4 binding sites. If the GAL4 fusion protein is derived from a coactivator, addition of agonists promotes a conformational change in the receptor LBD, allowing interaction with the DNA bound coactivator. This protein-protein interaction brings the strong VP16 activation domain to the reporter, resulting in increased transcription of the reporter gene that is read out as increased enzymatic activity. In contrast, if the GAL4 fusion is derived from a corepressor that interacts with unliganded receptors, reporter activity is high in the absence of ligands and low when the receptor-corepressor interaction is disrupted by agonist treatment. Along with the two-hybrid assays described

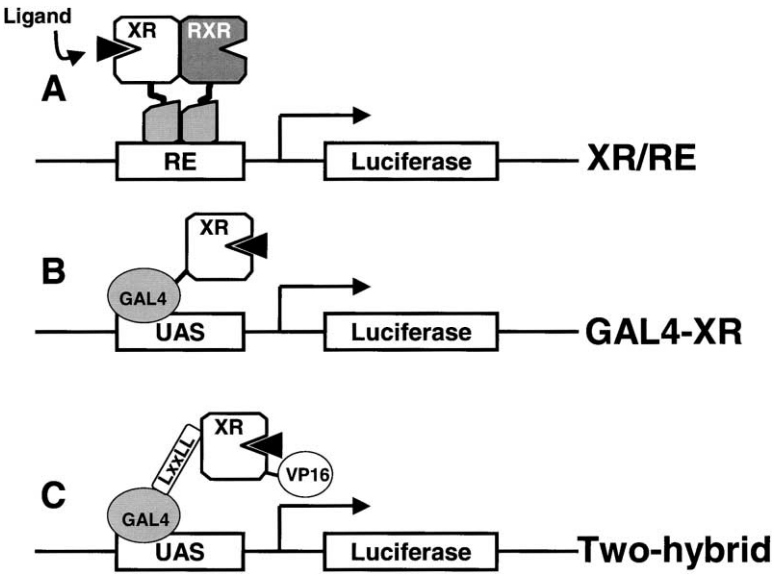


Figure 3. Cell-Based Assays for Nuclear Receptor Function

(A) Classic cotransfection assays that measure activity of nuclear receptors on hormone response elements. Receptors can function as heterodimers with RXR (shown), as homodimers, or as monomers.

(B) GAL4-receptor LBD assay utilizes the DNA binding domain of the yeast transcription factor GAL4 to target the LBD to a reporter with GAL4 binding sites.

(C) Two-hybrid assays comprising coregulator receptor-interacting domains fused to the GAL4 DNA binding domain, and receptors (LBDs or full-length) fused to the strong transactivation domain of the viral protein VP16. Ligand-dependent interactions between receptor and coregulator tether the VP16 activation domain to the promoter, resulting in activation of the reporter gene.

above, the ability to detect ligand-dependent interactions between NRs and short sequence motifs derived from coregulator boxes has allowed the development of biochemical-based time-resolved fluorescence energy transfer [17, 18] and fluorescence polarization assays [19] (Figure 4) that utilize recombinant receptors (usually LBDs) and synthetic peptides (7 to 25 amino acids in length). Like the two-hybrid assays, these biochemical assays monitor the ability of ligands to modulate protein-protein interactions (see Figure 4 for more details).

### Orphan Receptor Ligands: The Early Years

The linkage of classically defined signaling molecules such as steroid and thyroid hormones, vitamin A, and vitamin D to cDNA sequences encoding NRs during the late 1980s and early 1990s revealed a new regulatory paradigm by which hormones and small molecules could directly regulate gene expression. Nevertheless, the nuclear receptor field was left with a perplexing question. What are the ligands and physiological functions for the remaining 75% of the superfamily that were identified by DNA sequence homology? These receptors with no known ligand or function were classified as "orphan" receptors. Over the last ten years a combination of molecular, genetic, and biochemical approaches along with the nuclear receptor platform assays described in Figures 3 and 4 have been used to identify ligands for orphan receptors and uncover new signaling pathways [20]. Three examples of the approaches used are illustrated by the retinoid X receptors (RXR), the peroxisome proliferator activated receptor $\gamma$ (PPAR$\gamma$), and the liver X receptors (LXRs).

RXR$\alpha$ was originally cloned via homology to the DNA binding domain of the retinoic acid receptor $\alpha$ (RAR$\alpha$).

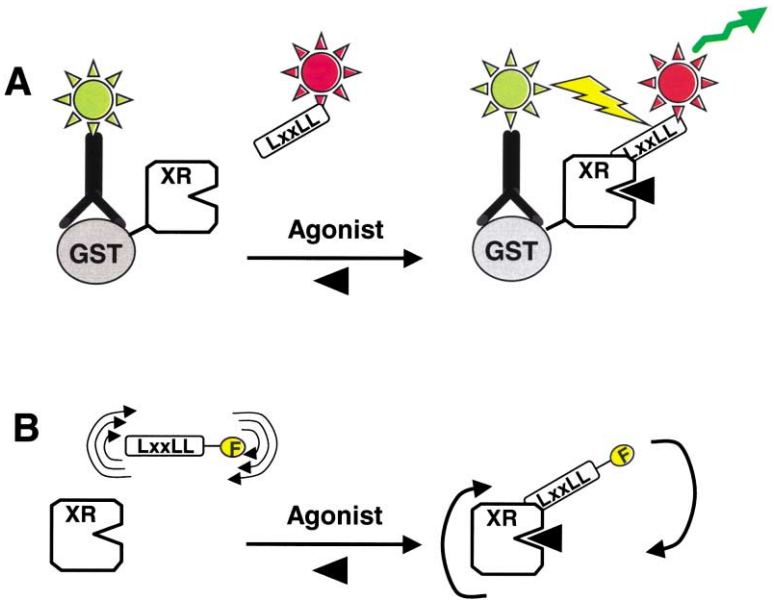


Figure 4. Biochemical Assays for Nuclear Receptor Function

(A) Time-resolved fluorescence resonance energy transfer. Interaction between labeled recombinant receptor (fluorescently tagged via an antibody to GST) and an LxxLL containing peptide labeled with a second fluorescent tag results in energy transfer from receptor to the peptide upon excitation with appropriate wavelength of light when the two tags are in close proximity. The tag on the peptide only releases light when excited by energy released from the tag on the receptor.

(B) Fluorescence polarization. Interaction between recombinant receptor and fluorescently tagged peptide results in a decrease in the rate of rotation of the peptide and a change in polarization.

A ligand hunt utilizing a cotransfection assay in mammalian tissue culture cells led to the surprising finding that all-*trans* retinoic acid, a vitamin A derivative already defined as a high-affinity ligand for the RARs, increased the transcriptional activity of RXR$\alpha$ in a dose-dependent manner. Direct binding of all-*trans* retinoic acid to RXR$\alpha$, however, could not be detected [21]. Subsequent biochemical fractionation of extracts from cells treated with all-*trans* retinoic acid led to the identification of 9-*cis* retinoic acid as a high-affinity ligand for both RXR and the RARs [22, 23]. Further characterization of the RXRs ($\beta$ and $\gamma$ iso-types were also discovered) identified these receptors as common heterodimeric binding partners of a large percentage of the receptor superfamily including many of the orphans [24].

Biochemical and functional studies defined the orphan receptor PPAR$\gamma$ as a master regulator of the genetic program leading to adipocyte differentiation [25]. The discovery of a biological role for this orphan receptor dovetailed nicely with a successful ligand hunt for PPAR$\gamma$, again using transient transfection assays that identified 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 as a low-affinity ligand (micromolar $EC_{50}$) [26]. These authors also showed that activation of PPAR$\gamma$ with 15-deoxy-$\Delta^{12,14}$-PDJ2 promotes adipogenesis in cultured cells. Interestingly, previous studies had demonstrated that the thiazolidinediones (TZDs), a class of potent synthetic insulin-sensitizing agents currently in use for the treatment of type II diabetes, also promote adipogenesis. These two observations dramatically came together when it was demonstrated that TZDs are ligands for PPAR$\gamma$ with affinities in the 30 to several hundred nanomolar range, thus connecting PPAR$\gamma$ and adipose tissue to insulin sensitivity and type II diabetes [26].

Like many orphan NRs, LXR$\alpha$ was cloned on the basis of DNA sequence homology, and subsequent expression profiling identified the liver, kidney, and intestine as sites of significant expression [27]. Genetic ablation of LXR$\alpha$ produces mice that appear normal; however, when challenged with a cholesterol-rich diet, LXR$\alpha^{-/-}$ mice accumulate massive amounts of cholesterol in the liver. This disturbance in cholesterol homeostasis arises from a failure to upregulate the catabolism of cholesterol to bile acids and the secretion of cholesterol from the liver to the intestine [28]. The characterization of LXR$\alpha^{-/-}$ mice led to the suggestion that this receptor functions as a sensor of cholesterol that insures homeostasis is maintained when cholesterol levels are elevated. This hypothesis was further supported by the identification of oxidized forms of cholesterol (oxy-sterols) as LXR ligands in extracts from bovine liver using a transfection assay that measures the functional activity of a GAL4 fusion containing the LXR$\alpha$ LBD [29]. More recently, the ability to regulate cholesterol transport and metabolism via LXR has been exploited to develop synthetic LXR ligands that display anti-atherogenic activity in animal models [30, 31].

Over the last ten years, similar approaches based upon the identification of ligands that activate individual receptors have resulted in the "adoption" of a significant percentage of the orphans. In many cases, ligand identification has uncovered previously unexpected signaling pathways including cholesterol transport and catabolism via the LXRs, control of bile acid and triglyceride metabolism via the farnesoid X receptor (FXR) [32, 33], and control of xenobiotic metabolism via the pregnane X receptor (PXR/SXR) [34] and constitutive active receptor (CAR) [35]. This process of using the cloned NR as an entryway into uncharacterized signaling systems has been termed reverse endocrinology [36]. Interestingly, many of the putative endogenous ligands identified are relatively low-affinity (micromolar) and this has led to the suggestion that improved and more sensitive assays are needed for the identification of naturally occurring high-affinity ligands that may be present at low concentrations or exist only transiently. Nevertheless, several of these low-affinity endogenous ligands such as fatty acids (PPAR ligands) and bile acids (FXR ligands) are present at concentrations that approximate their kd for receptor binding. The concordance between receptor kd and ligand concentration supports the hypothesis that such receptors are poised to alter gene expression in response to small changes in ligand concentration and thus function as sensors that respond to changes in food intake and metabolic status. Clearly, further definition of the signaling pathways regulated by the adopted orphans will be needed in order to determine if endogenous ligands that approach the affinities of the classically defined NR hormones exist.

Along with the identification of endogenous ligands, the ability to configure the NR platform assays in a high throughput format has allowed the screening of large libraries of structurally diverse synthetic compounds for nuclear receptor ligands. Following optimization using traditional medicinal and automated chemistry techniques, such synthetic ligands have subsequently been used as tools to decipher receptor function. A recent example of this process is the use of the synthetic PPAR$\delta$ ligand GW501516 to uncover a role for this receptor in the regulation of $\beta$-oxidation and inflammation [37]. At the time of writing, using the methods described in this review, natural or synthetic ligands have been identified for approximately 60% of the mammalian nuclear receptor family.

## Nuclear Receptor Ligands: The Next Generation

Over the next 5–10 years, we expect that recent developments in understanding NR signaling at the molecular level will usher in a new era in ligand identification. In particular, we anticipate progress along two fronts: the discovery of endogenous ligands for orphan receptors and the identification of synthetic ligands with tissue- and/or gene-selective activity.

To identify endogenous nuclear receptor ligands, the sites of synthesis and/or action need to be determined and sensitive methods of detection need to be available. The accessibility of highly sensitive mass spectrometers that can detect nanogram quantities of small molecules provides an answer to the detection problem. Cell or animal tissue extracts can be incubated with recombinant receptors, the receptor-ligand complex is than isolated via an affinity tag on the receptor, and the bound ligand can be detected and identified using a mass spectrometer. Once identified, the specifically bound components can be tested for functional activity using

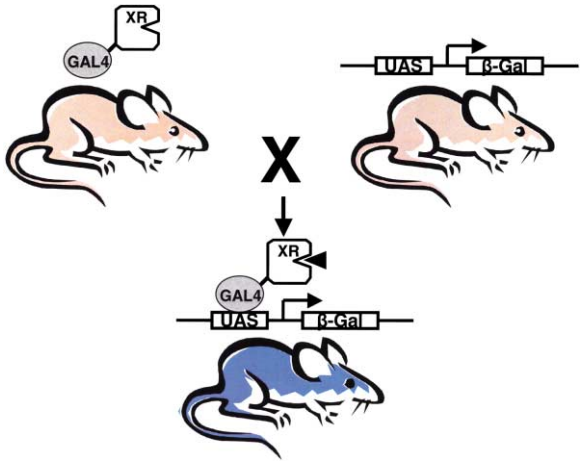


Figure 5. Transgenic System for Identifying Sites of Receptor Activity

Crossing transgenic mice expressing a GAL4-receptor LBD fusion protein with mice carrying a β-galactosidase reporter with GAL4 binding sites results in transactivation (detected by blue color) at sites where endogenous ligands are present. See Solomin et al. [43] for details.

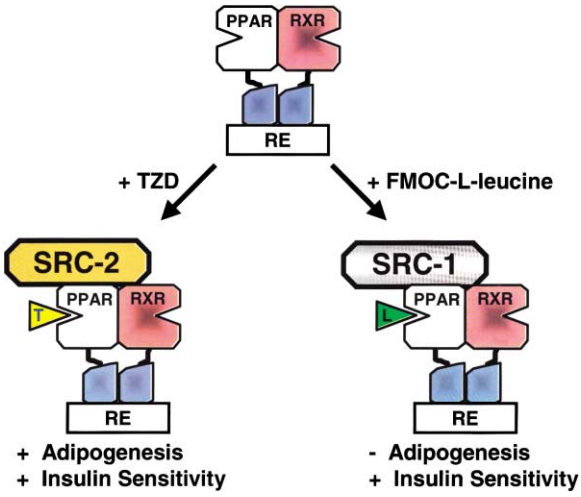


Figure 6. Coregulator-Selective Recruitment by Different PPARγ Ligands

Recruitment of SRC-2 by TZDs results in insulin sensitivity and adipogenesis. Recruitment of SRC-1 by FMOC-L-leucine preferentially influences insulin activity. See Rocchi et al. [47] for details.

the standard NR assays. Interestingly, several potential NR ligands have copurified along with recombinant LBDs expressed in bacteria for crystallization studies [38–40]. The copurification of bound ligands with orphan NRs previously considered to be constitutively active has led to the suggestion that some NRs may irreversibly bind endogenous agonists that lock the receptor in an active conformation [40]. An extreme example of this irreversible ligand hypothesis is the constitutively active receptor Nurr1. Structural analysis of this receptor does not detect a small molecule in the binding pocket; instead, several bulky hydrophobic amino acid residues in the region normally occupied by ligands [41, 42] extend into the putative binding pocket. Thus, in the case of Nurr1, side chains function as a pseudo-intramolecular agonist to lock the receptor in an active conformation. Clearly, additional structural studies and molecular modeling will be needed to determine if other orphan NRs have binding pockets available for small molecules.

To complement the identification of endogenous ligands using mass spectrometry, transgenic approaches have recently been exploited to uncover the sites of NR activity in vivo. Solomin et al. [43] have described the creation of reporter mice engineered with β-galactosidase reporter gene linked to a minimal promoter containing binding sites for the yeast transcription factor GAL4. Crossing these reporter mice with transgenics expressing GAL4 DNA binding domain-NR LBD fusions allows activation of the reporter in tissues where the receptor of interest is activated by endogenous ligands (Figure 5).

## Tissue- and Gene-Selective Ligands: The Holy Grail

Characterization of selective estrogen receptor modulators (SERMS) has convincingly illustrated the concept of tissue/gene-selective NR ligands. SERMS such as tamoxifen and roloxifene behave as ER antagonists in breast and as agonists in bone [44, 45]. Crystal structures of ER bound to these different ligands indicate that agonists such as estradiol and SERMS such as tamoxifen promote unique conformational changes in the ER LBD [7, 46]. A consequence of these unique conformations is that the individual receptor-ligand complexes exhibit differential affinities for the numerous coactivators and corepressors that translate the 3-dimesional structure of the receptor-ligand complex into a transcriptional response. These observations have led to the hypothesis that unique receptor conformations recognize distinct coregulators and that the ligand-selective NR-coregulator complexes consequently regulate unique sets or networks of genes. The characterization of the PPARγ ligand FMOC-L-leucine supports this proposed link between receptor conformation, coregulator interactions, and biological response. Like the well-studied TZDs, FMOC-L-leucine functions as an insulin-sensitizing agent in animal models of type II diabetes. In contrast to the TZDs, however, FMOC-L-leucine does not strongly promote adipogenesis. Strikingly, binding of FMOC-L-leucine promotes interaction between PPARγ and the coactivator SRC-1 while TZD binding favors interaction between PPARγ and SRC-2 (Figure 6) [47]. The ligand-selective coactivator recruitment correlates nicely with the observation that SRC-2$^{-/-}$ mice are resistant to diet-induced obesity, thus genetically linking this coactivator to adipogenesis [48]. Taken together, these studies suggest that interactions between PPARγ and SRC-1 are sufficient to promote insulin sensitivity while interaction with SRC-2 is necessary for the adipogenic effects of PPARγ ligands. Since increases in adipose mass may restrict the clinical utility of the TZDs currently on the market for diabetes, the ability to limit this unwanted side effect via coactivator-selective ligands appears to be an attractive alternative.

In the future, the combination of coregulator knockout

mice, RNA interference technologies, and gene expression profiling should simplify the ability to link individual coregulators to particular NR-regulated biological pathways. This knowledge base will allow targeted assays based upon NR-coregulator interactions to be developed in order to identify ligands that promote coregulator-selective interactions. The ability to multiplex such assays, for instance using two or more coregulator-derived receptor interacting domains with different fluorescent tags, should allow high throughput approaches to be used to identify ligands that promote differential coregulator interactions. Additionally, the use of combinatorial chemistry methods along with crystal structures of receptor-ligand-coregulator complexes will be needed to rapidly expand and optimize initial lead chemical series that demonstrate significant selectivity.

## The Coregulator Binding Groove: An Opportunity for Nontraditional Ligands

The definition of the coregulator binding region as a shallow hydrophobic groove on the surface of receptor LBDs [7, 8, 10] raises the possibility of identifying small molecules that bind in this groove and competitively inhibit coregulator interactions. Interestingly, not only would such coregulator inhibiting ligands bind to a different site than traditional ligands, but since formation of the coactivator binding surface requires an agonist-dependent conformational change, such inhibiting ligands may only bind to agonist-occupied receptors. Thus, coregulator-inhibiting ligands would be predicted to function as dominant-negative antagonists that block the activity of agonist-occupied receptors. This novel type of antagonist activity may be particularly useful for the treatment of androgen-independent prostate cancer. Many prostate cancer patients develop resistance to anti-androgen treatment by selection for androgen receptor (AR) mutants that recognize pocket binding antagonists as agonists. This promiscuous activity appears to arise when mutations altering the interaction between receptor and anti-androgen allosterically influence the position of helix 12 favoring the active conformation [49]. Interestingly, there is also evidence for overexpression of coactivators in specimens from recurrent prostate cancers [49]. In either case, blocking the ability of AR to interact with coactivators via occupation of the coactivator groove with a small molecule should inhibit AR transcriptional activity and inhibit cell proliferation.

Since the coactivator receptor interacting domain is defined by a relatively short leucine-rich $\alpha$ helix, the ability to find small molecules that mimic this motif while also demonstrating selectivity for individual NRs may be called into question. Nevertheless, receptor-selective peptide sequences as small as seven amino acids in length have been identified that function as receptor-specific antagonists in transient transfection experiments [16], suggesting that specificity can be achieved. The identification of synthetic small molecules that disrupt protein-protein interactions is not a new idea, although for the most part attempts in this arena have not met with much success and the few compounds identified have affinities in the high micromolar range [50]. The general failure to identify small molecules that interfere with protein-protein interactions arises partly from the use of chemical libraries developed and refined for targets with relatively deep hydrophobic pockets such as enzymes, proteases, and receptors. Thus, perhaps the greatest challenge to the identification of coactivator inhibiting ligands will be the creation of novel chemical scaffolds that mimic or resemble $\alpha$ helices and fit tightly into the shallow groove of the coactivator binding surface. Clearly, structural analysis will be required to allow development of small molecule peptide mimetics that can subsequently be optimized via traditional and automated chemistry methods. Although the development of coactivator inhibiting ligands is wrought with many challenges, success in this endeavor will not only provide a new class of NR ligands but will also provide a paradigm for targeting other therapeutically relevant protein-protein interactions.

The completion of fly, worm, mouse, and human genome projects over the last five years has served to define the NR family, and now more than ever the attention of the field has shifted toward functional characterization. Excitingly, the ability to exploit a common technology platform has allowed the rapid identification of natural and synthetic receptor-specific ligands that have uncovered a number of new and unexpected endocrine signaling pathways. More are likely to follow. Given the ability of nuclear receptors to positively impact a number of pathological conditions including cancer, inflammation, and metabolic disease, we anticipate in the coming years that our expanding molecular understanding of nuclear receptor function will allow the development of designer drugs with built-in tissue- or gene-selective activity that provide improved treatments for human disease.

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