Review Article

Ligand-Induced Allosteric Effects Governing SR Signaling

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Abstract. Steroid receptors (SRs) are a class of ligand-regulated transcription factors that regulate gene expression in response to the binding of steroid hormones. Ligand binding drives conformational changes within the SR ligand binding domain that alters the receptors' affinity for coregulator proteins that in turn modulate chromatin state and either promote or block the recruitment of transcriptional machinery to a gene. Structural characterizations of SRs have provided insight into how these conformational rearrangements modulate receptor function, including signaling between the ligand binding pocket and the site of coregulator binding. Here, we review some of the proposed structural mechanisms put forward to explain the ability of ligands to modulate SR function. We also provide a discussion on computational methods that have contributed to the elucidation of SR allosteric regulation. Finally, we consider broader discussions of allostery within the SR family, such as receptor-induced reverse allostery and allosteric binding sites located outside of the canonical ligand interaction site.

Keywords: Allostery, Steroid Receptors, transcription factor, ligand, structural mechanism.

1. Introduction

The steroid hormone receptor (SR) subfamily within nuclear receptors (NR) controls a diverse array of biological processes, including growth, development, immune responses, and various disease states [1]. Upon binding to endogenous cholesterol-derived steroid hormones, SRs translocate from the cytoplasm to the nucleus to interact with specific DNA sequences and regulate downstream gene transcription. The ability of these receptors to control diverse, tissue-specific processes in response to ligand binding has established them as high-value pharmaceutical targets [2]. The SR family includes the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), androgen receptor (AR), progesterone receptor (PR) and the estrogen receptor (ER) (Table 1).

SRs are comprised of a modular domain architecture, consisting of the N-terminal domain (NTD), the DNA binding domain (DBD), a variable hinge region, and the C-terminal ligand binding domain (LBD) (Figure 1). Ligand binding to the ligand binding pocket (within the LBD) initiates the allosteric transmission of information that drives a cascade of processes to regulate gene transcription. This cascade includes the release of bound chaperone proteins [3], nuclear translocation [4], homo- or hetero-dimerization [5], association with response elements within DNA promoters [6], and binding of coregulatory proteins [7].

The LBD consists of 12 α-helices and four β-strands, enclosing a hydrophobic ligand binding pocket (LBP) comprised of residues on helices 3, 5, 11, 12 (H3, H5, H11, H12) and beta strands 1 & 2 (S1, S2) (Figure 1). The primarily hydrophobic LBP contains a small subset of polar residues that make stabilizing hydrogen bond interactions with ligands, conferring specificity. The LBD also contains the activation function 2 surface (AF-2) at the confluence

Table 1: Steroid Receptors and their biological roles.

| SR	Endogenous (Human) Hormone	Biology
AR	Testosterone; 5-alpha dihydrotestosterone	AR plays an important role in development and manifestation of the male phenotype [8]. Alterations in AR function are associated with diseases such as androgen insensitivity syndrome [9] and prostate cancer [10, 11].
GR	Cortisol	In response to the binding of glucocorticoids, GR directly induces or represses the transcription of genes that govern a diversity of cellspecific processes ranging from stress response to metabolism [12, 13]. Glucocorticoids possess powerful anti-inflammatory and immunosuppressive properties, making them clinically desirable for the treatment of a plethora of diseases and cancers [14, 15].
ER	Estradiol; 17β-estradiol	Estrogen Receptors exist as two major sub-types, ERα and ERβ, which both bind estrogens to mediate a spectrum of biological effects in the CNS, immune, and cardiovascular systems [16, 17]. Estrogens play important roles in developing and regulating normal sexual and reproductive function.
PR	Progesterone	PR plays important roles in female reproductive tissue development, differentiation and maintenance [18, 19].
MR	Aldosterone	MR primarily functions as an electrolyte balancer [20, 21]. Widely expressed in the cardiovascular system, MR plays roles in endothelial function, fibrosis, vascular oxidative stress and blood pressure [22]. In addition to its cognate ligand, MR can also be activated by glucocorticoids and progestagens.
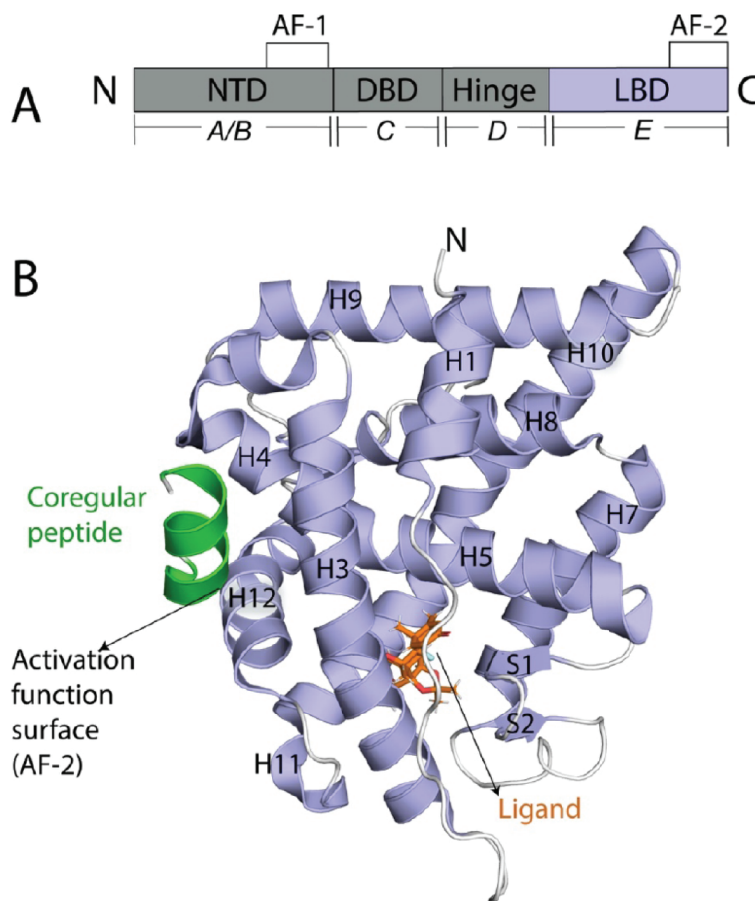
Figure 1: **Canonical domain structure of a steroid receptor.** A) SRs contain an amino-terminal domain (NTD), DNA-binding domain (DBD), hinge region and ligand binding domain (LBD). B) The LBD (light blue) is comprised of 12 helices, layered to form two functional sites: the ligand binding pocket, a hydrophobic cavity where the steroid ligand (orange) binds; and AF-2, the surface for coregulator (shown in green) interactions. The NTD contains a ligand-independent activation function 1 domain. (Crystal structure adapted from PDB 5UFS).
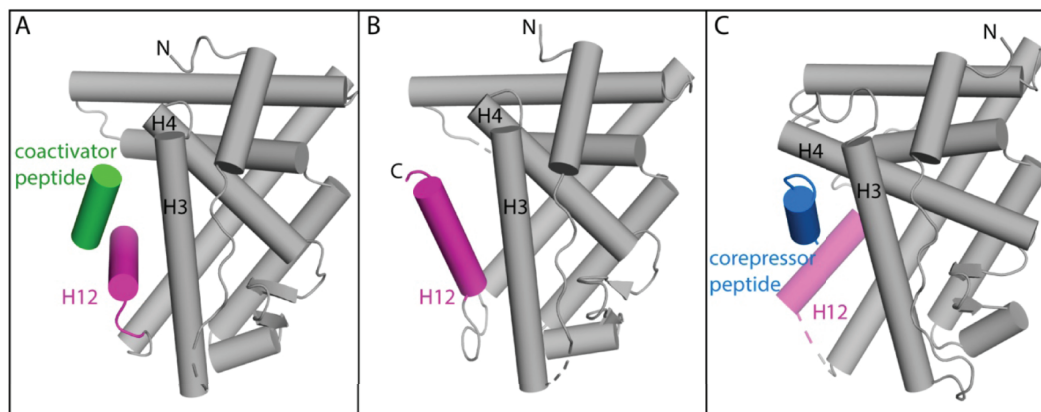
Figure 2: **Helix 12 is conformationally flexible.** A. In the agonist-bound state, H12 (magenta) is packed against the receptor by hydrophobic interactions, stabilizing the AF-2 surface for coactivator binding. This state is typically referred to as the 'agonist' conformation. B. An undocked H12 conformation (often observed in apo or antagonist-bound states) where H12 occupies the coactivator-binding groove. C. An undocked H12 conformation where the helix is displaced from the AF-2 surface and a corepressor peptide (blue) is bound.

of H12, H4 and H3, a critical binding site for coregulator proteins. The shape of the AF-2 surface, modulated by the conformationally-dynamic H12, is a critical determinant of the activation state of the LBD, dictating the selective recruitment of coregulator proteins and modulation of downstream transcriptional outcomes.

Initial structural studies suggested that H12 existed in one of two states: packed against the receptor, stabilized by hydrophobic interactions (Figure 2A), or flipped away from the body of the receptor (Figure 2B) [23–27]. Agonistic ligands are able to promote the condensed H12 state, creating a surface that can be bound by coregulator proteins known as coactivators. The binding of a coactivator protein to an SR serves to enhance transcriptional activation via recruitment of chromatin decondensing proteins and transcriptional machinery [28]. Conversely, apo and antagonist-bound SRs reveal H12 undocked from the receptor and repositioned, either spanning the coactivator binding groove (Figure 2B) or entirely shifted away from the AF-2 surface (Figure 2C). Labeled the 'antagonist' conformation, the displaced H12 conformation has been shown to favor the recruitment of corepressor proteins to AF-2 (Figure 2C). Corepressors, the second type of coregulator proteins, inhibit gene expression by recruiting histone deacetylases which drive chromatin compaction, reducing accessibility of DNA to transcription factors [29].

Upon further analyses, H12 was shown to have a highly dynamic nature [30, 31]. Several apo (unliganded) and antagonist-bound receptors were crystallized in the so-called 'agonist' conformation [31–33], demonstrating that H12 is conformationally variable and that the 'packed' state is not restricted to agonist complexes. Partial agonists, which display reduced activity compared to full agonists, are incapable of stabilizing the receptor exclusively in agonist or antagonist conformations [9, 34, 35]. Instead, structure and dynamics investigations suggest that SRs sample a variety

of states and ligand binding simply shifts the equilibrium between conformational states, generally restricting the conformational fluctuations [9, 27, 36, 37].

Furthermore, solution-state studies provide support for ligand-mediated conformational selection in LBD structures. Nuclear magnetic resonance (NMR) experiments on the nuclear receptors PPARγ, PPARα, and RXRα illustrated this phenomenon in a powerful way: in the absence of ligand, data showed missing resonances from LBP and AF-2, indicating fast exchange (on the order of microseconds to milliseconds) in those regions [9, 38]. The addition of a strong agonist stabilized the NR LBD conformation, allowing nearly all NMR resonances to be observed. These observations support a model where apo LBD samples an ensemble of conformations, a subset of which become stabilized upon ligand binding. Hydrogen-deuterium exchange coupled to mass spectrometry (HDX–MS) studies revealed similar conformational transitions; apo NR LBDs exhibited increased solvent accessibility and conformational dynamics in the LBP and AF-2 regions [39, 40]. The presence of full agonists introduced protection in those regions, indicating conformational stabilization upon ligand binding.

SR LBDs act as allosteric switches controlled by their cognate hormone; upon binding, ligands exert significant allosteric effects that propagate across SR domains. In this review, we present a brief discussion of well-established mechanisms of long-range allosteric communication in NRs, as well as the contributions of computational methods towards uncovering these mechanisms. However, our primary focus in this review rests on describing the local effects in SRs that accompany and/or permit ligand-regulated allostery, including structural reorganizations that facilitate shorter-range coupling between LBP and AF-2 or non-orthosteric binding surfaces on the LBD. We present examples of work that describe mechanisms involved in LBP-AF-2 coupling, antagonism and selective SR modulation. Finally,
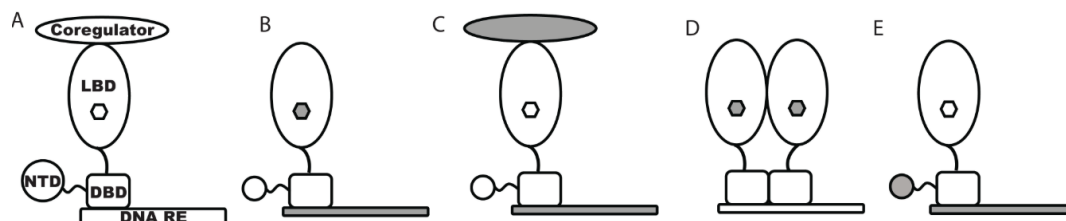
Figure 3: **Allosteric coupling in nuclear receptors.** A. Schematic representation of a NR-DNA-coregulator complex with individual domains, coregulator and DNA response element labeled. Allosteric coupling has been demonstrated between B. ligand-and-DNA (gray); C. DNA and coregulator (gray); D. ligand and ligand (gray) in a NR dimer, and E. DNA and the NTD (gray).

we provide brief discussions on i) receptor-induced reverse allostery and ii) allosteric sites that might be relevant across the SR subfamily.

2. Interdomain Allostery in NRs

Allostery plays fundamental regulatory roles in biological systems. In the NR superfamily, transcriptional activity is regulated by allosteric coupling of distinct sites on multiple domains (Figure 3A), whereby events at one site (e.g. ligand binding, mutations) induce effects at a distal site. Mechanisms of allostery in NRs have been heavily reviewed [41–47] and include:

(i) **Ligand/LBD-DNA allosteric coupling.** Through biochemical, structural and biophysical analyses, ligand binding and LBD amino acid mutations have been shown to allosterically regulate NR-DNA binding [48–51] (Figure 3B). For example, point mutations in the AR LBD have been shown to affect DNA binding and subsequent AR transactivation, with no change in ligand binding [52], suggesting key roles for the residues in facilitating LBD-DBD interdomain communication. Likewise, differential ligand binding to the GR LBD alters the electrophoretic mobility of GR-DNA complexes, indicating allosteric control of DNA binding [53] based on ligand identity.

(ii) **DNA-coregulator allosteric coupling.** NR DBD binding to DNA response elements propagates conformational changes across protein domains, extending to the AF-2 surface and allosterically regulating coregulator association [46, 54] (Figure 3C). Receptor binding to response elements has been shown to modulate coregulator binding preferences in ER [55], GR [56], and the vitamin D receptor (VDR) [57]. Similarly, the presence of thyroid receptor (TR) response elements alters TR binding affinity for various coregulator peptides [58]. Evidence also exists indicating that response element sequence can modulate NR coregulator preferences [41].

(iii) **LBD-LBD NR-dimer coupling.** Multiple studies have revealed that a ligand bound to one monomer of a dimerized NR complex can regulate the activity of the dimer partner (Figure 3D). In RXR heterodimers (e.g. VDR-RXR, RAR-RXR, CAR-RXR, LXR-RXR and TR-RXR), the presence of RXR agonist 9-*cis* retinoic acid results in altered transactivation by the cognate receptor [58–61]. Mechanisms underscoring the propagation of signal across NR dimer interfaces have been reported, providing explanations for related events such as coregulator recruitment to NR dimers.

(iv) **NTD-DNA coupling.** SR NTDs, known to be intrinsically disordered, have been observed to undergo conformational changes upon receptor-DNA binding in GR, PR, AR and TR among others [41, 42, 47, 62] (Figure 3E).

3. Computational Methods to Facilitate Investigation of NR Allostery

In silico methods have become increasingly important in providing structural and dynamic characterizations of NRs, circumventing experimental limitations and providing complementary information for X-ray crystallography, NMR, HDX-MS, and small angle X-ray scattering (SAXS). For example, protein-ligand docking is a powerful tool used to visualize possible ligand binding modes, which are important for structure-function analyses. Docking has been heavily used to obtain structures for SR-ligand complexes that have not been co-crystallized [9, 63–65]. Docking can also be used as a screening tool for different ligands as potential allosteric modulators [66–72].

Molecular dynamics (MD) simulations, a well-established method for studying dynamics of biomolecules, have provided insight into ligand-dependent conformational changes in various SRs [73–75]. Additionally, MD simulations can survey conformational distributions [73, 75, 76], reveal networks of amino acids connecting allosterically coupled sites [76–78], and identify atomic rearrangements that accompany ligand binding [79–84]. As powerful as these contributions have been, unbiased MD simulations are limited in their ability to probe larger conformational rearrangements associated with NR function, due to inability to capture slower molecular events that transpire over long timescales (e.g. helical movements). Steered MD and Monte Carlo methods, useful for overcoming some of these technical limitations,

have been employed to visualize larger scale protein movements and pathways associated with ligand binding [85–88]. Additionally, timescale limitations have been overcome by enhanced sampling techniques such as replica exchange dynamics (REMD) and bias-exchange metadynamics, which permit the simulation of larger conformational changes required to understand how ligand binding drives activity.

4. Structural Mechanisms of LBP-AF-2 Coupling

Signal transduction between the LBP and AF-2 surface has been widely studied, with the aim of identifying the conformational and structural transitions within the LBD that accompany ligand binding, modulating coregulator recruitment and downstream activity. While these mechanisms are still only partially understood, existing work has elucidated key structural features that may facilitate signaling within SR LBDs. Using specific SRs as case studies, we summarize findings on a) Allosteric coupling between LBP and AF-2, specifically via i) a proposed allosteric network in GR and ii) subtype-specific signaling in ER subtypes, b) Mechanisms of antagonism in AR, and c) selective steroid receptor modulation in PR.

4.1. LBP-AF-2 coupling

4.1.1. Allosteric network in GR. A proposed allosteric network in GR connects the LBP to AF-2, as well as other functional sites of the receptor [89]. A random mutation approach identified four residues, all located within this network, (M752I, F602S, Y598N and M604T, human GR numbering) that selectively stabilized agonist or antagonist GR conformations (Figure 4). M752 is on H12, forming part of the AF-2, while the remaining three are part of helices 5 and 6, and M604 interacts with the ligand within the pocket. The M752I mutation stabilizes the agonist conformation, enhancing the affinity of GR for coactivator peptides. The F602S and M604T mutations also stabilize the agonist conformation through direct ligand contact. The Y598N mutation, however, stabilizes the antagonist conformation, possibly because the loss of tyrosine inhibits a transcriptionally important tyrosine-phosphorylation event [90]. Furthermore, these mutations affect the regulation of GR function by the molecular chaperone HSP90. Altered receptor function resulting from mutation of network residues provides a platform to validate allosteric networks.

To assess allosteric coupling between the AF-2 and the LBP in GR, Pfaff et al investigated effects of coregulator-derived peptides on hormone binding and kinetics [91]. AF-2-bound peptides affected the kinetics of dexamethasone (dex) association/dissociation with GR, confirming previously-observed coupling between the two binding sites. Peptides derived from DAX-1, SRC-1, SRC-2 and PGC1α coactivators slowed both ligand binding and dissociation

from GR. Furthermore, Pfaff et al tested the previously identified M752I GR mutation for effects on dex binding. Similar to the effects observed with coregulator peptides, M752I slowed both ligand association and dissociation. The M752I substitution also increased GR affinity for coregulator peptides, identifying the 752 position as a two-way sensor and a key regulator of the network. The authors proposed a mechanism in which the insertion of the rigid, isoleucine sidechain alters the plasticity of the allosteric network, modulating both ligand and peptide binding.

4.1.2. Allosteric signaling in ER subtypes. The Estrogen Receptor consist of two major isoforms - ERalpha and ERbeta- which have 56% overall amino acid identity, but share a remarkably conserved binding pocket. Only two amino acids differ between the ERα/ERβ binding pockets - positions L384/M336 and M421/I373 (ERα/ERβ numbering) on H6 and H8, respectively. However, these combined differences introduce distinct shapes and properties into the ligand binding pocket, causing the same ligand to drive differential biological outcomes based on the receptor subtypes. For example, (R,R)-5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (THC) is a partial agonist for ERα but antagonist for ERβ [92, 93].

Nettles and co-workers used chimeric ERα-ERβ receptors to identify amino acids that are important for ligand selectivity in either receptor subtype [94]. A structural analysis of H11 in various ER-ligand complex structures spanning partial to full agonists revealed that a shift in the helix correlates with agonistic activity of ligand (Figure 5A-C). This ligand-specific modulation of H11 conformation in ERβ identified the helix as a key conduit of information between the LBP and the AF-2.

In a group of 50 ERα LBD structures, Nwachukwu et al presented a mechanism by which classes of synthetic ER ligands alter the shape of AF-2 to permit cell-specific signaling [95]. These ligands shifted H12 or perturbed H11 and/or H3 to modify the AF-2 surface, thus altering coregulator preferences. Additionally, the distance between H11 and H3 was shown to be predictive of proliferative effects in certain ER ligand classes. MD simulations of ERα identified an important role for H524 (located in the LBP on H10) in linking H3 and H11, ultimately influencing coupling between the LBP and AF-2 [74]. His524 forms hydrogen bonds with bound ligands but also participates in a hydrogen bond network between Glu339 (H3), Lys531 (H11) and Glu419 (H6-H7 loop). This network restricted the position of H12 (Figure 5D) and did not remain intact in antagonist simulations.

A set of diverse environmental ligands were investigated to gain insight into mechanisms involved in binding and activation of the ER subtypes [96]. These ligands included the plasticizers bisphenol A (BPA) and bisphenol C (BPC), the phytoestrogen ferutinine, and pesticides 2,2-bis(p-hydroxyphenyl)-1,1,1- trichloroethane (HPTE) and
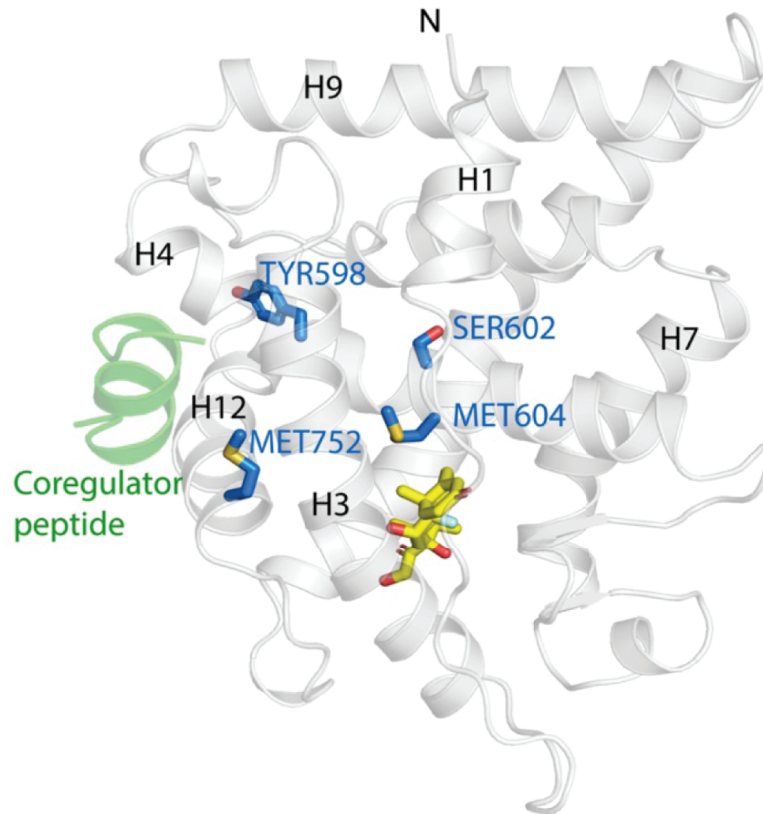
Figure 4: **Allosteric networks in GR regulate LBP-AF-2 communication and HSP90 interactions.** Four residue positions (752, 602, 598, 604) modulate GR conformational dynamics upon mutation (adapted from PDB 4UDC).
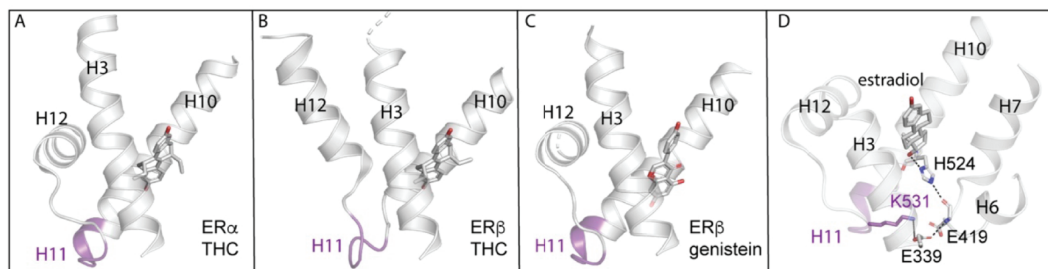

Figure 5: **Role of H11 in ERα-ERβ allosteric signaling.** Conformation of H11 (purple) is altered in a ligand-dependent manner to produce distinct responses. A) THC induces a suboptimal conformation in ERα, supporting partial agonism (ERα-THC, PDB 1L2I). B) H11 of ERβ-THC complex is shifted, forcing H12 in the antagonist conformation (ERβ-THC, PDB 1L2J). C) Genistein, an ERβ partial agonist, also induces a similar suboptimal conformation of H11 (ERβ-genistein, PDB 1X7J). D) H524 (H10) links LBP to AF-2 by coordinating a hydrogen bond network (dashed lines) between bound ligands and H3, H6-H7 loop and H11, ultimately restricting H12 positioning.

dichlorodiphenyldichloroethylene (DDE). Delfosse and co-workers show how the M421 (ERα) to I373 (ERβ) substitution modulates ligand interactions in the two subtypes [96]. Located on H8, M421 in ERα is flexible, able to reorient to accommodate bulky ligand groups (Figure 6A). The equivalent residue I373 in ERβ is more rigid, as observed in an overlay of multiple x-ray structures [96]. A bulkier environmental ligand (e.g. ferutinine) would induce a shift of adjacent helices towards H12, ultimately altering the position of H12 and the conformation at AF-2 (Figure 6B).

Alterations in coactivator-ERα interactions have also been observed by computational investigations. When ERα is activated by bisphenols, MD revealed destabilized dynamics in H12 and the H11-H12 loop, while energy calculations showed reduced coregulator affinity vs estradiol [97].

4.2. Mechanisms of antagonism in AR. Antagonists that compete with endogenous ligands to block receptor function act as valuable therapeutics for a wide range of diseases. [17, 98–100]. The primary mechanism for antagonist action is to
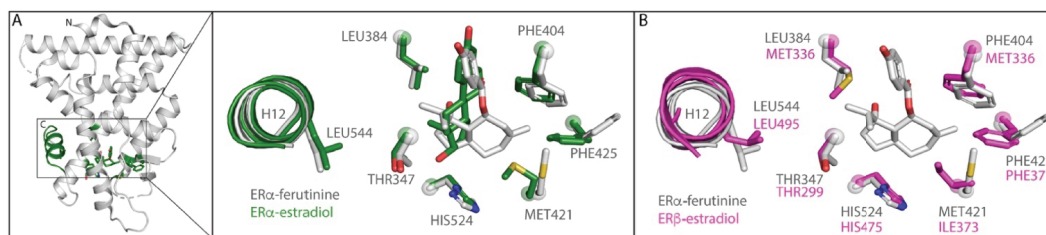
Figure 6: **ER ligands modify AF-2 surface conformations in subtype-specific manners.** A) M421 (ERα, H8) is flexible and reorients to accommodate the bulky ligand ferutinine (gray). H12 conformation remains unperturbed compared to estradiol-bound ERα (green). B) ERβ substituent I373 is less flexible than M421 of ERα, restricting the ability of the LBP to accommodate bulky ligands. This would potentially result in a shift of the ligand towards H12 and alteration of H12 conformation in ERβ (magenta) compared to ERα (gray), as observed.
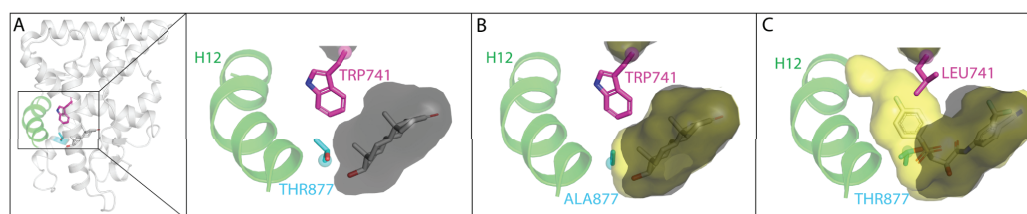

Figure 7: **AR mechanism of antagonism is disrupted by disease mutations.** Many clinical mutations widen the pocket, allowing large antagonist ligands to bind without perturbing H12, ultimately converting antagonists to agonists. A) Wild-type AR with DHT ligand (PDB 1I37) with cavity shown in black. B) T877A mutation (PDB 1I38) introduces the smaller alanine sidechain, increasing cavity volume (yellow) compared to wild-type AR (black). C) W741L mutation widens cavity (yellow) by replacing bulky tryptophan sidechain with leucine.

destabilize the AF-2 surface, preventing coactivator binding and inhibiting transcriptional activation of SR-responsive genes [101].

Androgens are critical for both normal prostate function and the unchecked cell growth observed in prostate cancer. A common strategy for prostate cancer treatment is to develop antiandrogens that compete with endogenous androgens to block AR function and reduce the production of androgens [102–104]. X-ray crystallography in combination with computational simulations has been beneficial for elucidating the structural mechanisms by which AR-bound antiandrogens allosterically disrupt AF-2 and H12.

While crystal structures of the inactive, antagonist conformation (i.e. with H12 displaced from AF-2) have been obtained for other SRs, a structure of AR in this conformation has not yet been obtained. In order to perform MD analyses on antagonist-bound AR, a structure was first needed. AR-antiandrogen complexes for *in silico* investigations were built by docking ligands into an apo AR model [105]. Replica exchange MD simulations on these complexes revealed that while the main body of the LBD remained in place, H12 was repositioned, sampling conformations similar to those seen in antagonistic models of ER and GR [105].

Clinical mutations in AR that arise in prostate cancer patients can often alter ligand responses, converting AR antagonists into agonists [106, 107], and ultimately fueling tumor growth. W741L/C and T877A AR mutations achieve an antagonist-to-agonist effect by increasing the size of the binding pocket, allowing certain antagonists, which would normally contact H12, to bind without perturbing the helix [76, 108–110] (Figure 7A-C). This permits AR to maintain an active conformation, despite being bound to an antagonist. Another clinical mutation, F876L, located on H11 (not shown) ablates contacts between the antagonist enzalutamide and H11/H11-H12 loop that would normally prevent H12 from adopting the agonist conformation. When AR F876L is bound to the antagonist MDV3100, the smaller leucine sidechain permits ligand binding without a H12 clash, abolishing antagonistic activity [107].

These mutations have been leveraged to obtain crystal structures of AR with antagonists, as they yield complexes that are more stable and favor crystallization [111]. Mutant AR complexes have offered insight into the structural mechanism of AR activation and antagonism, and identified the residues important for LBP-AF-2 communication [10, 112, 113]. For example, Duan et al reverted T877A and W741L-antagonist complexes to wildtype via *in silico* mutagenesis for MD studies. Using one microsecond simulations, they observed that upon equilibration, H12 is both destabilized and displaced, oriented away from the binding pocket. An allosteric network connecting the bound antagonist with H12 was proposed, consisting of W741 (H5), I899 (H12) and H874 (H10) [76] (Figure 8). W741 is stabilized by interacting with a bound ligand. In the absence of a ligand capable of sustaining this interaction (e.g. HFT antagonist, modeled in Figure 8B), it was proposed that W741 would rotate towards H874, repositioning the histidine sidechain to within hydrogen bonding distance of H12, potentially disrupting the
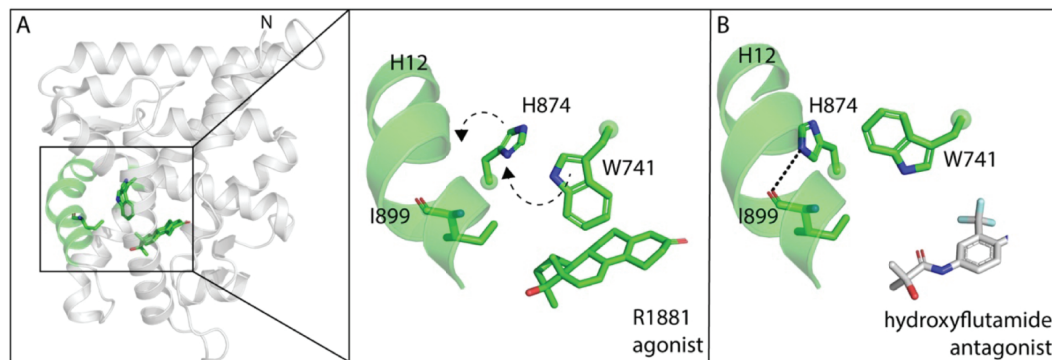
Figure 8: **Allosteric network in AR antagonism.** A) AR-1881 complex (PDB 1E3G), showing how agonists (such as R1881) interact stably with W741. Dashed lines indicate the proposed movement of W741 and H874 in the absence of a stable interaction. B) Antagonist HFT is modeled in wildtype AR pocket. With no ligand interaction, Duan et al propose that W741 would swing over, pushing H874 to hydrogen bond with backbone of I899. This interaction disrupts the H12 conformation, in a proposed mechanism of antagonism [76].
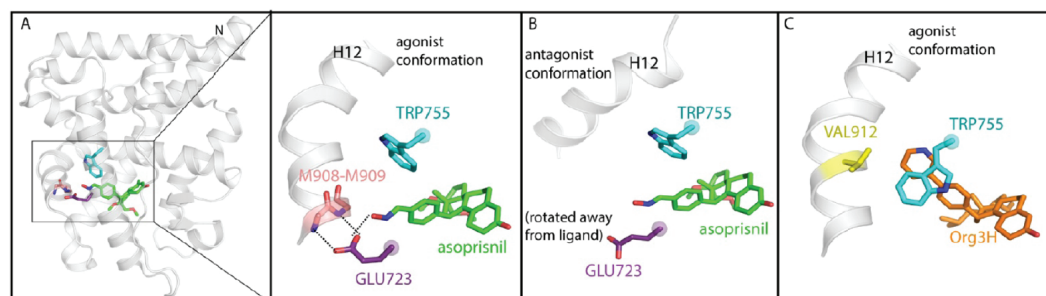

Figure 9: **Proposed mechanisms for partial agonism of PR.** Asoprisnil can switch between agonist and antagonist PR conformations, mediated by Glu723 positioning. A) PR-Asoprisnil in the agonist conformation (PDB 4A2J); the drug interacts with a hydrogen-bond network between Glu723 and Met908/Met909 backbone atoms, strengthening the network. B) PR-Asoprisnil in the antagonist conformation (PDB 2OVH); Glu723 is pointed away from the ligand, with no hydrogen bonds formed. C) In second mechanism, ligand activity is modulated by plasticity of Trp755. In the PR-Org3H agonist conformation (PDB 4APU), Trp755 is turned toward the ligand, different than conformation in PR-Asoprisnil complexes. The plasticity of this residue suggests that a larger pendant group could cause it to swing towards Val912, destabilizing H12.

helical conformation. This disruption of H12 was proposed as a mechanism of AR antagonism by HFT.

4.3. Selective SR modulation in PR. Selective steroid receptor modulators (SSRMs or SRMs), steroid receptor ligands that can display cell and tissue-specific agonist or antagonist activity, are a large focus of SR drug discovery efforts [115]. These ligands have the potential to provide desirable modulation in certain tissues while avoiding undesirable off-target effects in other cells, ultimately giving rise to improved therapeutic profiles compared to full agonists or antagonists [116–121]. SRMs display transcriptional responses that depart from the typical agonist or antagonist profile, including graded receptor activity. Responses are also dependent on the expression level of coregulator proteins in each cell-type.

Some selective progesterone receptor modulators (SPRMs), which possess great therapeutic potential for women's health conditions [122, 123], are partial agonists, able to recruit both coactivators and corepressors [124].

These mixed-profile ligands display decreased transcriptional activity compared to full agonists and increased activity compared to full antagonists. The SPRMs therefore provide a valuable tool for probing mechanisms that facilitate LBP-AF-2 communication in PR, permitting these mixed profiles.

Crystal structures were obtained for two mixed-profile PR ligands, Asoprisnil and 17β- cycloproplycarbonyl-16α-ethenyl-11β-[4-(3-pyridinyl)-phenyl]-estra-4,9-dien-3-one (Org-3H) (Figure 9), both 11β-substituted steroids, in both agonist and antagonist PR conformations. From these two ligands, two potential mechanisms explaining their action were proposed [115]. The first putative mechanism involves the rotameric conformation of Glu723 (H3), which either allows (Figure 9A) or disrupts (Figure 9B) stabilization of H12 via hydrogen bonding to Met909. A similar mechanism has been observed in selective estrogen receptor modulators (SERMs) [25, 26, 35, 125]. In the second mechanism, ligand pendant groups induce movement of Trp755 (H5), which can push against Val912 in H12, destabilizing its helical
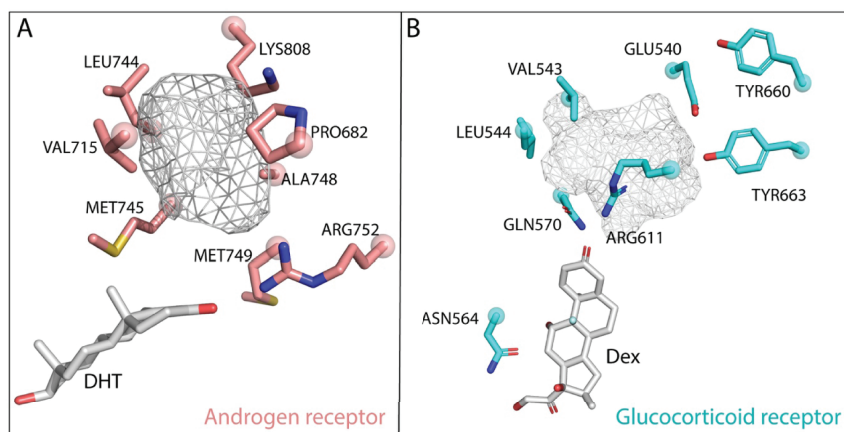
Figure 10: **Proposed alternate binding cavities in SRs.** A) PBS1 cavity (gray) in AR identified by docking, showing adjacency to the LBP with DHT bound [107]. B) Proposed binding cavity (gray) for DBT in GR, also adjacent to the dex-bound LBP. DBT co-binds with dex, but rearrangement of ASN564 in LBP potentially destabilizes bound dex [114].

conformation (Figure 9C). This plasticity is observed in the equivalent residue of AR (i.e. TRP741), which swings out of the LBP and towards H12, to accommodate bulky ligands.

5. Computationally-Proposed Allosteric Binding Pockets Distinct from the LBP

AR was proposed to have a novel binding site distinct from its LBP; this site was a putative binding site of organic pollutants, which are antagonistic for AR. Dichlorodiphenyldichloroethylene (4,4'-DDE), a potent environmental antagonist, bears structural resemblance to natural ligands of nuclear receptors, which might explain the ability of this ligand to disrupt AR signaling [107]. Molecular docking, MD simulations and free energy calculations identified both the LBP and an adjacent hydrophobic cavity formed by H1, H3, H5 and H8 (labeled PBS1) as energetically favorable binding sites for 4,4'-DDE (Figure 10A). Binding free energies calculated for the antagonist in both sites were comparable to the energy of LBP-bound DHT, identifying the allosteric site as a plausible binding site for 4,4'-DDE. However, no experimental studies have yet confirmed the existence of this binding pocket.

Another potential binding cavity was identified in GR as the putative binding site of Dibutyltin (DBT), a toxic organotin. DBT interferes with GR function by inhibiting both ligand binding and GR transcriptional activity [114]. Inhibitory activity of DBT (in reporter assays and NF-κB repression assays) was observed in the presence of saturating amounts of cortisol hormone, suggesting that DBT binds to an allosteric site on GR. Ligand docking revealed the LBP as the most likely binding site with an adjacent, allosteric pocket identified as a plausible, secondary site (Figure 10B). DBT binding to this secondary site would rearrange H3 residues (including ASN564), leading to the loss of important LBP contacts for dexamethasone. The loss of these crucial

contacts would explain the effects of DBT binding on dex-mediated GR activation.

6. Receptor-Induced Reverse Allostery

Reverse allostery refers to observations where the direction of allosteric regulation between two sites appears to be reversed. In SRs, this includes instances where interactions at AF-2 (or elsewhere) that alter the overall conformation of the receptor influence dynamics, conformations and binding of ligands in the LBP. Strategically placed mutations in H12 of ERα stabilized certain conformations of the receptor and improved ease of crystallization [126]. The Y537S (H12) mutation stabilizes the agonist ERα conformation while L536S (H12) stabilized the antagonist conformation (Figure 11). The Y537S substitution alters hydrogen bonding capabilities of H12, removing an interaction with N348 (H3) in favor of a new hydrogen bond between S537 (H12) and D350 (H3). L536 (H11-H12 loop) is buried, this buried hydrophobic residue stabilizes the H11-H12 loop and "locks in" the agonist conformation. The mechanism by which L536S stabilizes the antagonistic conformation is less clear, but the loss of a hydrophobic residue at this position was shown to lead to an inactive ligand conformation [127]. It was proposed that the L536S mutation promotes a stabilizing interaction between E380 (H3) and H12 [126]. Both mutations are on the surface of the LBD and do not make direct contacts with ligands.

Bruning et al. demonstrated that ligand orientation is modulated by receptor conformations [34]. WAY-169916, a synthetic partial agonist of ERα that eluded crystallization, was crystallized after introduction of the Y537S and L536S substitutions to ERα. The ligand adopted a range of orientations when bound to distinct receptor conformations, seen in co-crystal structures of the two ERα variants. Targeted modifications to the structure of WAY-169916 to
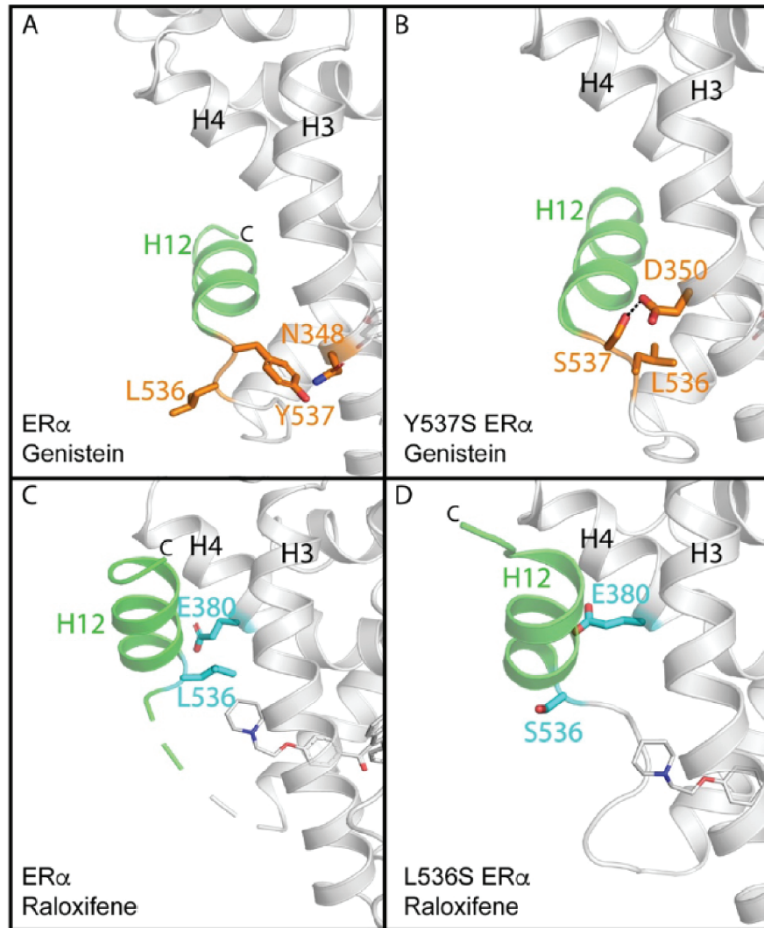
Figure 11: **ERα in agonist or antagonist conformations.** Helix 12 (green) is docked against AF-2 or displaced in agonist or antagonist structure, respectively. A) WT-ERα with genistein in pocket (PDB 2QA8). B) Y537S locks ERα in agonist conformation (PDB 1X7R) through altered hydrogen-bonding, burial of L536 sidechain and stabilization of H11-H12 loop by S537-D351 interaction. C) WT-ERα with raloxifene in the antagonist conformation (PDB 1ERR). D) L536S locks ERα in antagonist conformation (PDB 2QXS).

favor specific binding orientations in the LBP gave rise to transcriptional activation profiles that were improved or reduced as predicted, indicating that a ligand's biological activity is directly related to the ensemble of ligand binding orientations. Additionally, intermediate transcriptional activity observed in WAY-169916, in the form of partial agonist activity and intermediate levels of coactivator recruitment, may also result from the ligand binding differently to agonist and antagonist conformations.

7. Allosteric Sites Common to all SRs

BF3: Binding function 3, or BF-3, a binding surface on AR formed by residues in H1, H9 and the H3-H4 loop, was shown to be an allosteric regulator of the receptor [128] (Figure 12A). BF-3 has been widely studied and is implicated in coregulator recruitment and AR regulation [67, 129]. Estebanez-Perpina et al first identified novel antagonists that bound at BF-3 to inhibit SRC-3 coactivator peptide binding at AF-2 [128]. Accompanying structural analyses suggested

a functional link between the two binding surfaces. Conversely, coregulator motifs were also shown to bind at BF-3 and modulate AR activity [130]. To characterize allosteric communication between BF-3 and AF-2, Grosdidier et al introduced mutations on the BF-3 surface and evaluated the transcriptional activities of WT and mutant AR constructs in a luciferase reporter assay [131]. Mutations had a range of effects on DHT-induced transactivation, from moderate reduction to super-enhancement. Mammalian two-hybrid assays showed that mutations at the BF-3 surface affected interactions with the AR NTD, as well as with corepressors NCoR and SMRT. MD simulations suggested a proposed allosteric path between the two surfaces going through the H3- H4/5 loop. Conformational changes resulting from loop movements may introduce sub-pockets in AF-2 that alter coregulator binding [131].

While the BF-3 site (Figure 12A) has been validated in AR, Buzon et al. demonstrated that the pocket is conserved among SRs, constituting a potentially druggable site to modulate SR function [132]. Mutations in the BF-3 region
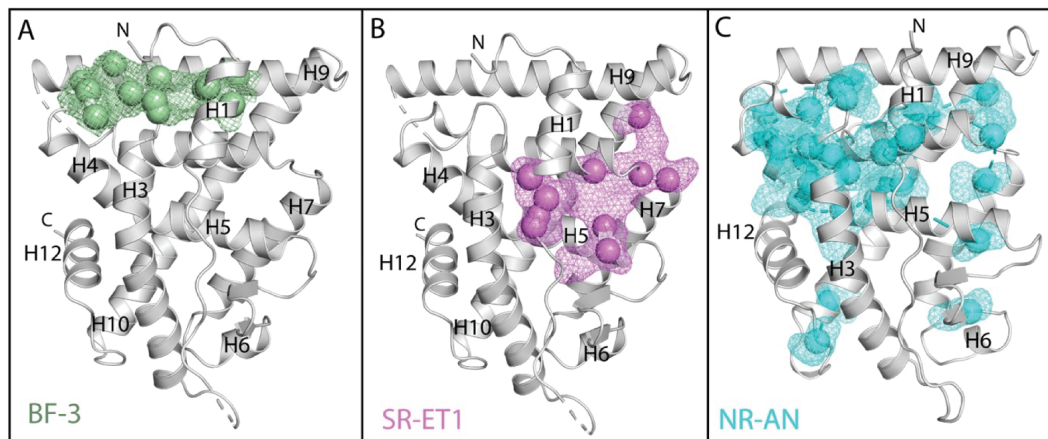
Figure 12: **Allosteric surfaces and networks in SRs.** A) The BF-3 surface on AR, formed by residues (green) on H1, H9 and the H3-H4 loop, B) ET1, a possible allosteric site in SRs, identified by the evolutionary trace method. ET1 is composed of 9 residues located on H1,H5,H8,H7 and H10. C) NR allosteric network (NR-AN) identified by statistical coupling analysis (SCA). The network is composed of 27 residues and links all the functional surfaces located on the LBD.

are associated with disease or altered SR function in AR and ERα [132], but a concrete role for important protein-protein interactions involving BF-3 has not been identified or established in any receptors other than AR.

ET1: The evolutionary trace method identified a group of amino acids (named ET1) on a novel surface that are likely to be biologically significant for nuclear receptors, particularly SRs (Figure 12B). The ET1 residues, located on H1, H5, H7, H8 and H10 were found to be non-overlapping with the dimerization interface, coactivator binding surface and ligand binding pocket. Mutations to these residues in ERα impact function of the receptor by reducing transcriptional activity and preventing binding of ligand, coactivator, and Hsp90 [133]. Of the 9 residues that make up the surface, 5 are disease-associated in AR and/or GR. Additional work on ERα has implicated these residues in SERM binding [36] and as a binding site for an antagonist peptide [134].

SCA allosteric network: Shulman et al used statistical coupling analysis (SCA) to identify a network of energetically coupled residues that govern allosteric communication in the nuclear receptor LBD, particularly by linking functional surfaces [60] (Figure 12C). The SCA method is based on a hypothesis that functional interaction of two residues within a protein drives coevolution. Through constraint of residue identity at one position in a sequence alignment of a protein family, a statistical analysis of the amino acids at all other positions can permit an identification of residues that are functionally coupled. Remarkably, the identified allosteric network connected residues on all of the functional LBD surfaces: LBP, AF-2/H12, and dimerization interface. Residues in this network were tested by mutagenesis in RXR heterodimers and were shown to affect transcriptional activation by ligand binding to either partner [60]. Though there are significant differences in dimerization between

Type I and Type II NRs, this analysis could potentially identify similar sites important for allosteric signaling in SRs.

8. Summary and Future Perspectives

SR-ligand binding drives an allosteric switch that triggers a host of transcriptional events [47, 135]. In this review, our particular focus has been on local, LBD-specific effects that result from ligand binding, communicating coregulator preferences via induced structural and dynamics changes. Our increased understanding of the allosteric coupling between LBP and AF-2 sites has implications for understanding other aspects of functional regulation in SRs, including interdomain coupling, dimerization, and the effects of various binding partners on the SR dynamic structure. In addition to small molecule ligands and coregulator proteins that interact with the LBD, SR binding partners include DNA response element sequences that bind the DBD and cofactor proteins that associate with the NTD. The ensemble perspective of allostery in NRs (and other proteins), described by Hilser et al. [42], suggests that NRs exist in a dynamic conformational ensemble wherein each domain can occupy multiple states. As each domain associates with various binding partners, the unique binding events associated with a single domain can lead to a redistribution of the entire ensemble. This redistribution would modulate conformational states across all domains, resulting in allosteric effects that may differ between domains. Therefore, the ensemble framework suggests that allosteric couplings within SRs is a response of the protein ensemble to perturbations, providing important considerations for future research geared at SR allostery.

The existence of allosteric networks in SRs is supported by the continued implication of amino acid residues in signal transduction that are outside the LBP. H11 appears to be a

key structural player in LBP-AF-2 coupling across multiple SRs. Conserved residues may also play a role e.g. PR-TRP755/AR-TRP741, which contribute to destabilization of H12 in AR and the response of PR to bound antagonist ligands, respectively. Published work suggests the existence of a network of residues that allosterically links all functional surfaces in NR LBDs [60]. In the SR subfamily, this allosteric network has only been experimentally tested in GR, where activity of the receptor is modulated by mutations to residues in the network. Future investigations will be tasked with assessing the applicability of this residue network to other members of the SR subfamily.

This review highlights the remarkable contribution of X-ray crystallography and computation to elucidating structural aspects of SR allostery. While crystal structures reveal the subtle structural reorganizations in the LBD induced by ligand binding, computational simulations have permitted a visualization of the motions that potentially contribute to signal propagation. Emerging techniques such as high-resolution cryo-EM will allow for characterization of full-length SRs sampling a range of conformations. These methods will illuminate crucial details of how allosteric control in SRs is linked to conformational dynamics of individual domains. Advanced *in silico* sampling methods, combined with powerful GPU-based algorithms, will allow simulations to approach biologically relevant (millisecond) timescales. These advances will increase the applicability of MD simulations as a powerful, complementary method for probing dynamics of allosteric transitions occurring in SRs along varying timescales.

Competing Interests

The authors declare no competing interests.

Acknowledgments

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